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FOREIGN TECHNOLOGY DIVISION

HANDBOOK OF MICROBIOLOGICAL DIAGNOSIS OF INFECTIOUS DISEASES

By

K. I. Matveyeva and M. I. Sokolov

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BY: K. I. Matveyeva and M. I. Sokolov

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FOREWORD

The specific laboratory diagnosis of infectious diseases has been substantially enriched during the past 10 years by new research methods. In this connection it has become necessary to publish a handbook of modern microbiological and serological diagnostic methods suitable for working laboratories.

The publication of such a handbook has been made possible by the participation of a group of authors recruited for this purpose.

In preparing this volume we have attempted to describe both the methods commonly employed and those used for rapid diagnosis and for detecting pathogenic microorganisms in the environment. It should, however, be noted that procedures of the latter type have not yet been fully worked out.

This handbook is intended for medical microbiologists working in clinical laboratories, sanitary-epidemiological stations, and other institutions concerned with the laboratory diagnosis of infectious diseases. It will also be of interest to epidemiologists and specialists in infectious diseases.

We hope that this volume will aid medical personnel in more successfully accomplishing the tasks set by the 22nd Congress of the CPSU regarding the prevention of infectious diseases in the Soviet Union.

Bearing in mind the responsibility and labor involved in preparing a handbook for publication, we will gratefully receive any criticisms or advice and will make use of them in our subsequent work.

The editors

GENERAL RESEARCH METHODS

VARIATIONS OF THE LIGHT MICROSCOPE

M.A. Prshkov

MECHANICAL PORTION OF THE MICROSCOPE

The mechanical portion of a modern light microscope consists of a massive horseshoe-shaped foot (Fig. 1) equipped with a bracket bearing t.. micromechanism housing.

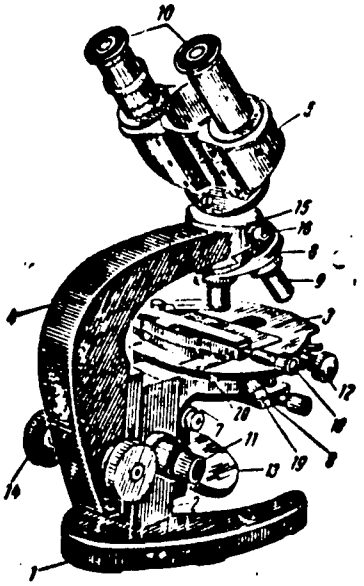


Fig. 1. General view of the MBI-3 biological microscope. 1) Foot or shoe; 2) housing and micromechanism; 3) object stage; 4) tube support; 5) binocular headpiece; 6) nosepiece revolving on slides; 7) condenser bracket; 8) analytic condenser for direct and indirect illumination; 9) objectives; 10) eyepieces; 11) micromechanism knob; 12) knob for longitudinal movement of slide holder; 13) nearer; 14) knob and gear for coarse adjustment of tube; 15) block for attachment of nosepiece, with locating socket for replacing tube; 16) screw holding binocular headpiece; 17) screw holding tube in proper position; 18) knob for transverse movement of slide holder; 19) stage centering screws; 20) head of screw holding rotating portion of stage.

This housing has a bracket for the condenser on one side and a

bracket for the tube support on the other. A special bracket on its upper side holds the rotating centering stage.

The lower portion of the arched tube support has a rack and pinion with two knobs, the so-called macroscrew, which is used for coarse adjustment of the tube. The lower side of the upper portion bears a block for attachment of the nosepiece, while its upper side has a special locating socket for attachment of the replaceable tubes, a binocular headpiece for visual examination and a straight monocular tube for photographic work (Fig. 2).

The microscope stage bears a device for two-dimensional movement of the object. The distance through which the object has been moved in one direction or the other can be determined from the scales, which have verniers reading to 0.1 mm.

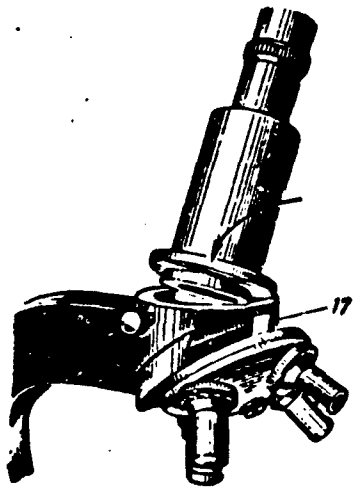


Fig. 2. Vertical headpiece with telescoping tube for MBI-3 microscope. The arrow points to the conically-ground annular flange, which is used to fasten the tube into the tube-support head.

The upper portion of the object stage can be turned after loosening a special screw.

The condenser-sleeve bracket is mounted on the micromechanism housing and can be moved along it with a rack and pinion.

The nearer yoke is attached to the micromechanism housing below the condenser sleeve.

The nosepiece has sockets for mounting the objectives. Rotating it switches objectives.

OPTICAL PORTIONS OF THE MICROSCOPE

Abbe Illuminating System

This device consists of a nearer, a rotating iris diaphragm, and a condenser composed of several lenses.

Mirror (see Fig. 1, 13). The mirror housing is mounted in a rotating semicircular yoke, whose pin is inserted into a special housing below the condenser sleeve, and carries two silvered mirrors, one plane and the other concave.

Both mirrors are silvered internally, so that a point light source produces numerous reflections from the glass and silver surfaces, which make it difficult for an inexperienced microscopist to adjust the light and lead to light losses. The mirror can be turned in two mutually perpendicular directions.

Condenser (Fig. 3). The condenser consists of several lenses mounted in a metallic housing, which is fastened into a socket in the condenser holder by a special screw. The housing consists of two detachable halves: one is conical and contains one or more short-focus lenses, while the other is cylindrical and carries a single long-focus lens.

Between the mirror and the condenser is the iris diaphragm, the so-called aperture diaphragm, since the extent to which it is opened regulates the aperture (effective opening) of the condenser, which should always be somewhat less than that of the objective used. Figure 3 shows the OI-14 analytic condenser of a MBI-3 microscope, which is equipped with a replaceable optical system for apertures of 1.4 and 0.4.

The OI-14 analytic condenser is quite convenient, since it rotates about its vertical (optical) axis in its sleeve. The diaphragm stage also rotates. The diaphragm can be moved away from the optical axis with the knob 4, which achieves the effect of indirect illumination.

Objectives. The objectives are a system of mutually centered lenses which produce an accurate image of the object.

There are two types of objectives, achromatic and apochromatic. Apochromatic lenses are distinguished by the fact that residual chromatism is more completely eliminated and that they achieve a more uniform

image definition and size in light of varying wavelength. Apochromats are used exclusively with specially computed compensating eyepieces, which eliminate the remaining defects of the objective. Powerful achromatic objectives are also employed in combination with compensating eyepieces. Huygens eyepieces are used in working with weak achromatic objectives.

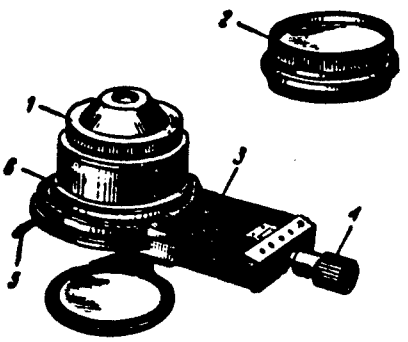


Fig. 3. OI-14 analytic condenser with aperture of 1.4. 1) Twin-lens condenser with aperture of 1.4; 2) detachable single-lens condenser with aperture of 0.4; 3) rotating condenser sleeve; 4) rotating iris-diaphragm stage; 5) diaphragm lever; 6) geared knob for shifting diaphragm to obtain indirect illumination.

Regardless of whether it is achromatic or apochromatic, each objective is characterized by its magnification, focal length, numerical aperture, and certain other constants.

The true magnification depends on the anterior focal length of the objective. Using focal length as a basis, objectives are classified as powerful (with focal lengths f = 1.5-3 mm), moderately powerful (f = 3-5 mm), medium (f = 5-12 mm), weak (f = 12-25 mm), and very weak (f > > 25 mm).

The focal lengths of foreign apochromatic objectives, which is customarily used to distinguish them, is engraved on the housing. The focal length is not indicated on Soviet objectives. The true magnification, numerical aperture, and occasionally the cover-glass thickness

for which the objective is intended are indicated on the housings of both achromats and apochromats.

The numerical aperture of objectives and condensers is determined by multiplying the sign of half the aperture angle at which the entrance pupils of the objective and the front lens of the condenser "see" the object by the index of refraction of the medium between these two optical systems. If the medium consists of layers of air alternating with the glass slide bearing the object, the aperture of the "dry" system almost never exceeds 0.95, since the index of refraction of air is one. In order to increase the aperture, the objective and condenser are immersed in a medium whose index of refraction is greater than that of air (water, glycerine, or immersion oil). Achromatic immersion objectives have been made with various focal lengths, which were formerly expressed in inches (e.g., 1/7 or 1/12 inch). Apochromatic immersion objectives have focal lengths, expressed in millimeters, of 3, 2, and 1.5 mm; their true magnifications are 60, 90, and 120 respectively. Only the true magnification and numerical aperture are indicated on Soviet-produced objectives.

Soviet apochromatic objectives also bear the letters "apokhr."

Both achromats and apochromats are fabricated as planochromats, which yield a flat image field.

Eyepieces (see Fig. 1, 10). The term eyepiece refers to the optical magnifying system through which the image of the object formed by the objective is viewed; just as when any object is observed visually or through a magnifying glass, an enlarged virtual image is obtained.

PATH OF LIGHT RAYS IN THE MICROSCOPE

The light rays emitted by the light source (the sky, sunlit clouds, an illuminating lamp) and reflected by the microscope mirror, are directed to the condenser, where they are collected at its focus, produc-

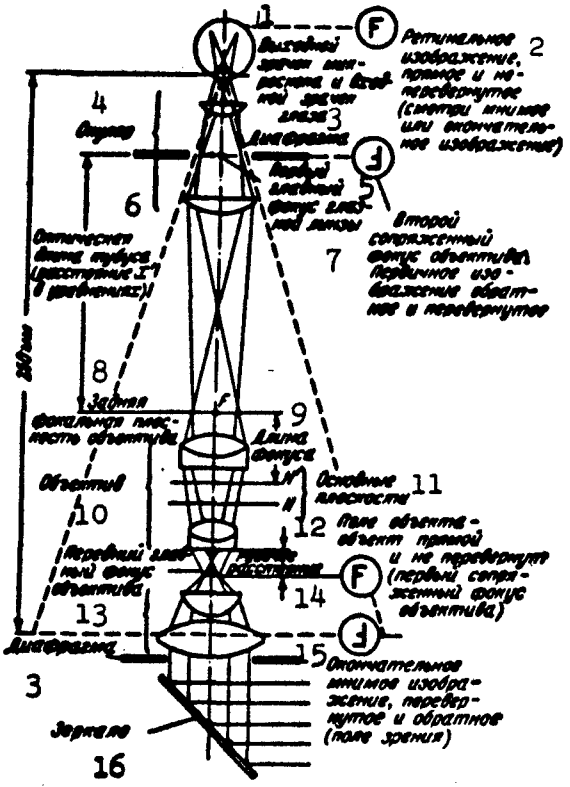


Fig. 4. Diagram of the path of light rays in a compound microscope. 1) Exit pupil of microscope and entrance pupil of eye; 2) retinal image, erect and unreversed (virtual or final image); 3) diaphragm; 4) eyepiece; 5) first primary focus of eye lens; 6) optical length of tube (distance x' in equations); 7) second conjugate focus of objective, primary image reversed and inverted; 8) posterior focal plane of objective; 9) focal length; 10) objective; 11) principal planes; 12) object field, object erect and unreversed (first conjugate focus of objective); 13) anterior principal focus of objective; 14) working distance; 15) final virtual image, reversed and inverted (field of view); 16) mirror.

ing a real, greatly reduced image of the source. When the height of the condenser is correctly adjusted with the rack, this real image should fall in the object plane. Since slides vary in thickness, the condenser should always be raised or lowered when a specimen is examined, so that the coincidence of the condenser focus and the upper surface of the slide bearing the object (the object plane) is not disrupted. The light rays collected by the condenser strike the specimen (object) in the form of a cone, whose apex faces the object and coincides with the

condenser focus. After passing through the specimen, the rays again diverge to form a cone geometrically similar to the initial cone, but with its base facing the objective (Fig. 4).

The objective, focused on the object illuminated by the image of the light source, forms a real image of the source in its posterior focal plane (which usually coincides with or lies quite near its posterior lens) and a real image of the object in the upper portion of the tube, at the level of the eyepiece diaphragm, which lies in the first focal plane of its eye lens. The distance to the second (posterior) focal plane of the eyepiece is known as the optical length of the tube.

During visual examination of the microscopic picture the eyepiece converts the convergent rays into parallel or nearly parallel bundles, which enter the eye through the pupil. The optical media of the eye (corneal membrane, crystalline lens, and vitreous humor) refract the parallel bundles leaving the exit pupil of the eyepiece and form a magnified image of the object on the retina (the retinal image), which is perceived by the brain as the microscopic picture; the eye is thus an extension of the microscope's optical system. As has already been pointed out, the microscopic pattern seen by the eye is a virtual image and is analogous to that seen through a magnifying glass. The object seen in the microscope lies in the same direction with respect to the observer as the real object represented by the rays which form its image on the retina. The distance which appears to separate the real image of the object from the observer's eye is 25 cm, which corresponds to the distance at which the normal unaided eye reads printed text.

In microscopic work we distinguish the object field and the visual field. The object field is the surface area of the slide bearing the specimen whose real image can be formed in the plane of the ocular diaphragm. It is constant in size for a given objective. The true size of

the object field can be determined by the following method. With the specimen properly illuminated and the microscope focused, the eyepiece or the entire tube is removed and a ground glass is placed in the usual plane of the ocular diaphragm. An image of the object field is then formed on the ground glass. When the specimen is properly illuminated it is seen that the object field is larger in diameter than the ocular diaphragm. While the size of the object field is constant for a given objective, the area visible through the eyepiece depends on the diameter of its diaphragm. With a given objective, the object field decreases in size as the eyepiece becomes more powerful.

The visual field is the illuminated circle which we see when we look into the microscope. It is bounded by the ocular diaphragm and lies in the plane of the object field. The visual field is usually convex, so that it is impossible to focus simultaneously on its center and its periphery; this can be corrected for photographic purposes only by using special negative lenses (homals). The curvature of the visual field is unimportant in visual observation, since the observer corrects it by adjusting the micrometer screw.

ILLUMINATION OF THE SPECIMEN IN THE MICROSCOPE

All microscopes are designed for illumination with parallel rays. The most suitable source of such rays is light from a cloudy sky, but its intensity is quite inadequate for working at high magnifications, of the order of 2000-3000 times.

Correct use of the intense sources of artificial light employed for microscope illumination has enabled us to achieve high magnifications (up to 5000 times) without loss of image definition.

The classical method of illuminating an object under the microscope was developed by Keller in 1898 (Fig. 5). In view of the importance of this technique in fine cytological work, we will discuss below

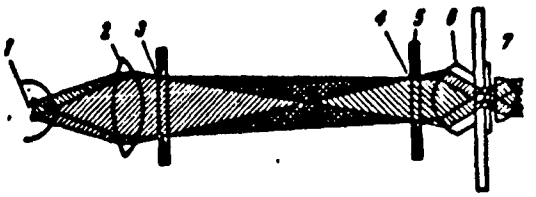


Fig. 5. Diagram of the path of light rays during illumination of a specimen by Keller's method in a compound microscope. 1) Lamp filament; 2) collector; 3) field diaphragm; 4) image of lamp filament in plane of aperture diaphragm; 5) aperture diaphragm; 6) condenser; 7) image of edges of field diaphragm in specimen plane.

the method of obtaining such precision illumination. As has already been pointed out, electric light is now used almost exclusively for microscope illumination, a low-voltage bulb with a thick filament usually serving as the source. The light must be high in intensity, but the area of the filament must be small, so that the source approximates a point.

All microscope lamps must have a collector lens, which makes it possible to focus the image of the incandescent filament in the diaphragm plane of the microscope condenser, which lies at its anterior focus (in order to produce parallel bundles of light rays). The collector must have a so-called field diaphragm, which is usually located in front of the lens. In contrast to the iris diaphragm of the microscope condenser, which regulates the aperture of the condenser and objective and thus the quantity of light which passes through them, the field diaphragm, mounted in front of the collector lens, limits only the size of the object field or visual field of the microscope, since its edge usually falls optically within the visual field, which is bounded by the eyepiece field diaphragm. In many modern microscopes (the MBI-2, for example) this principle is realized in such fashion that there is no need for a mirror: the bulb, collector, and field diaphragm are mounted in the microscope base, the real image of the filament being formed directly in the anterior focal plane of the condenser, which focuses the

image of the field diaphragm and specimen plane.

In order to make full use of the optical capacities of a microscope, one should not employ unsuitable light sources: powerful incandescent bulbs whose light is used directly, without an intermediate collector, or various types of imperfect lamps, where the collector is a water-filled flask or an ordinary long-focus lens of low illuminating power. Properly designed commercial lamps (the OI-7, OI-19, or OI-20; Fig. 6) or home made lamps with more or less point light sources (a very short arc between two tungsten spheres or a low-voltage incandescent bulb with a short, thick filament) whose intensity (filament incandescence) can be regulated with a rheostat, thus ensuring a smoothly varying current from the time the lamp is switched on until it reaches full incandescence, are satisfactory for this purpose. The bulb should be enclosed in a case with ventilation holes, which protects the observer's eyes from its light. The lamp is equipped with a collector, which should be corrected for spherical and chromatic aberration. The higher the illuminating power of the collector, the better will the lamp operate and the more light will enter the microscope at a given bulb incandescence. The collector should have a device which permits it to be focused with respect to the aperture diaphragm of the microscope condenser. It must also have an iris diaphragm whose diameter when completely open should equal that of the exit pupil of the collector and whose minimum diameter when completely closed should be of the order of 0.5 mm. The lamp should be mounted in a yoke attached to a massive base. Yoke-mounting ensures rigidity, but still permits the movement about the horizontal axis necessary to center the lamp with respect to the microscope optics. The lamp pivot rests in the yoke and is attached to its ends with nuts, which control the freedom with which the lamp turns. It must be added that the bulb socket should have a centering device,

no matter how simple (at best there are 3 leveling screws, while at worst the socket moves upward and downward, rotates about its axis, and has some sort of fastening device of the lock-nut type, which ensures maintenance of centering).

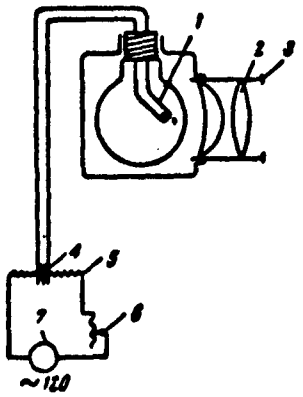


Fig. 6. Diagram of microscope lamp. 1) Low-voltage incandescent bulb; 2) collector lenses; 3) iris diaphragm; 4) secondary winding of transformer; 5) primary winding of transformer; 6) rheostat; 7) line current.

In working with a microscope, just as any optical instrument, the best results can be obtained only by centering all its optical components, including the lamp system, which, as we have already pointed out, constitutes a single unit together with the microscope. Adjustment of the illumination consequently begins by centering the light source, which is done in the following manner.

1. The lamp is set up opposite a white screen (a sheet of white paper) and switched to full incandescence, care being taken that the longitudinal axis of the bulb is perpendicular to the screen, which is 25-30 cm from the collector. The enlarged image of the lamp filament, which is seen as a broad, brightly shining band approximately 2 cm long, is focused on the screen by moving the collector toward or away from it. As a result of irradiation and uncorrected aberrations in the collector optics, the image of the filament is not sharp and should be surrounded

by a rather broad uniform bluish aureole (spherical and chromatic aberration).

An aureole of uniform width surrounding the image of the filament indicates that correct centering has been achieved. When the bulb filament has been oriented with respect to the optical axis of the collector we can move on to the next stage - centering of the light source and the Abbe illuminating apparatus of the microscope relative to one another.

2. The lamp is set up opposite the microscope, 30-40 cm away, and the beam of light is directed at the flat mirror, projecting an enlarged image of the filament on the underside of the completely closed aperture diaphragm.

The image of the filament must be focused as sharply as possible on the closed diaphragm, which is done by slightly advancing or withdrawing the collector or by moving it a little longitudinally with the field diaphragm completely open. Focusing of the real enlarged image of the filament in the plane of the aperture diaphragm is of great importance, since, as we know, the latter is in the anterior principal focal plane of the condenser (the entrance pupil of the microscope). The light from any object located at one of the principal foci of a lens is converted into parallel rays as it passes through it. Lenses intended to produce parallel bundles of rays are called collimators. By producing an enlarged real image of the filament covering the entire entrance pupil of the condenser and thus the posterior focal plane of the microscope, we can use the condenser as a collimator, since the rays forming the image are refracted in the condenser lenses and leave it as parallel rays, which illuminate the specimen in the requisite manner.

3. Having focused the image of the filament in the anterior focal plane of the condenser, the aperture diaphragm (the iris diaphragm of

the microscope) must be completely closed. Reducing the light to reddish-yellow incandescence (which may occasionally damage the eyes) and focusing a weak objective (10x) in combination with a medium eyepiece (10x) on any available specimen, the mirror is turned slightly to bring the image of the collector lens and field diaphragm into the microscope's field of view. Since the objective is focused on the specimen, the condenser must be raised or lowered to focus the image of the collector in the specimen plane. If all this is done correctly, the front lens of the collector, which can be seen together with the specimen in the field of view, should appear to be uniformly flooded with light (taking the form of a uniformly illuminated circle). If it is only partially illuminated or dark and covered with luminous iridescent dots, the lamp must be slightly turned about its horizontal axis in the yoke. The requisite position, in which the front lens of the collector is intirely flooded with light, is easily found by this method. It is occasional necessary to turn the lamp to the right or left about its vertical axis to obtain complete centering.

The light is precisely adjusted only when the field of view includes both the specimen and the image of the surface of the collector lens. Closing the field diaphragm (near the collector), we see a sharp image of its edges, which are touching. Keeping in mind the fact that the specimen is, as it were, at the focus of two objectives facing one another (the condenser and the objective), we finally focus the condenser on the specimen in such fashion that the image of the closed field diaphragm is surrounded by a red or blue corona and is rather sharp. A final adjustment of the mirror brings the luminous circle (whose size is determined by the opening of the field diaphragm) into the center of the field of view. This completes the adjustment of the light (by Keller's method, 1893) and we can now observe or photograph the subject

under a given magnification (with a given combination of objective and eyepiece).

In working with low magnifications (weak objectives), it is easily seen that the size of the collector-lens image, which is visible in the field of view even with a weak eyepiece, is insufficient to illuminate the visual field and thus the object field. When using an objective with a true magnification of 10x it is necessary to remove the condenser from the housing of its support, unscrew its upper portion, and replace the remaining cylindrical portion, which carries a single large lens, inserting the condenser mount into the housing to the flange, as usual. In contrast to the objectives, which must never be unscrewed separately, this procedure need not be avoided with the condenser, since it is intended to be separated into two halves.

The OI-14 analytic condenser, which can be used with the MBI-3 microscope, is equipped with an additional illuminating lens with an aperture of 0.4. In working at low magnifications the large-aperture portion of the condenser (see Fig. 3, 1) is replaced by a small-aperture condenser of the spectacle type (see Fig. 3, 2). Having inserted the lower half of the condenser, whose aperture has been deliberately reduced and its focal length increased by removing the upper lens, into the condenser-support housing, the rack is lowered until the opening in the closed field diaphragm can be seen in the object plane. Even though the specimen is the same, the image of the diaphragm opening appears far larger in the eyepiece than when the condenser is fully assembled. The images of the object and the field-diaphragm opening are then brought into precise optical correspondence (by focusing the objective on the specimen and raising or lowering the condenser) with the aperture diaphragm completely open; looking through the microscope, the field diaphragm is then opened so that its edge is just barely beyond that of

the eyepiece diaphragm (the second field diaphragm of the microscope). The field diaphragm should not be opened any further, since this does not intensify the illumination of the specimen, but only causes scattering of the light, greatly reducing image contrast. The disassembled condenser (its lower half) is used for visual and, particularly, microphotographic work, in combination with objectives of 10× and 20×, although when a 20× objective is employed it is better to reassemble the condenser and readjust the light as described above. When using the intact condenser, which has a large angular aperture (approximately 45°), it must be adjusted to conform to the objective employed, which is best done by observing the exit pupil of the objective with an auxiliary microscope mounted on the phase-contrast attachment.

Further closing of the aperture diaphragm is not necessary, since it automatically leads to a detrimental decrease in the aperture and thus the resolving power of the objective. When the opening of the aperture diaphragm is greatly reduced the image takes on a coarse, mottled appearance and loses most of its fine detail. When the objective is replaced by a more powerful one with a larger numerical aperture the condenser aperture must be adjusted to that of the objective.

Only when the condenser aperture has been adjusted to that of the objective can the field diaphragm be opened beyond the eyepiece diaphragm (which limits the field of view). If the observer then removes the eyepiece and looks into the microscope tube he sees a luminous circle, which corresponds to the posterior lens of the objective (and roughly to its posterior focal plane) and is uniformly flooded with light. This circle is surrounded by a gray ring, whose width (and thus the diameter of the circle) can be varied by adjusting the aperture diaphragm. It is theoretically assumed that the opening of the aperture diaphragm, whose image appears as a gray ring in the posterior focal

plane of the objective, should be such that it covers 1/10 of the field of this focal plane. This means that the width of the cone of light leaving the condenser should be 9/10 of the width of that which can enter the objective (i.e., the angular aperture of the condenser should be 9/10 of that of the objective). In practice, defects in the objective make it necessary to close the aperture diaphragm somewhat further, to 3/4-2/3, since with dry systems opening it to 9/10 does not completely eliminate the scattered light, which enters the objective and causes severe deterioration of the image of the object (reducing its contrast and destroying detail).

When the microscopist does not feel it necessary to employ illumination by Keller's method, a sheet of frosted or very fine-grained ground glass can be inserted between the front lens of the collector and the field diaphragm, 30 cm from the microscope mirror.

When using daylight it is easy to obtain the best illumination by aiming the mirror at white clouds. Working on a sunny day with brightly lit white clouds against a blue sky provides the most ideal conditions. It is, however, necessary to focus the light source (the white clouds) precisely on the object in order to keep from cutting down the condenser aperture. This is done by viewing the specimen under low magnification and raising or lowering the condenser (depending on the thickness of the slide) to obtain a sharp image of the cloud in the object plane. Under higher magnifications the image of the cloud then serves as a source of parallel light rays. The blue sky usually yields very little light. When the sky is filled with clouds the condenser must be focused by bringing the image of some distant object (a building, tree, or hill on the horizon or the molding of the window frame) into the object plane and then aiming the mirror at the brightest part of the sky. In contrast to work with artificial light sources, where the slightest

change in the inclination of the mirror disrupts centering, daylight, which furnishes parallel rays (diffuse light) from any point in the sky, makes it possible to turn the mirror (plane side) in any direction and catch the reflection of any cloud, whether it is on the horizon or at the zenith; the illumination remains centered so long as the optical axes of the condenser and the objective coincide.

PRINCIPAL ERRORS IN MICROSCOPIC WORK

The microscopist is often advised to use the convex mirror, which directly contravenes the principles of illumination. Use of the flat mirror in daylight and the convex mirror under artificial light is frequently recommended.

The convex mirror should never be used in working with the Abbe condenser, since the latter is intended only for use with the plane mirror, regardless of the character of the light source (a lamp, a cloud, etc.).

Another common gross violation of the rules for microscopic work is arbitrarily raising or lowering the lamp condenser without taking into account the thickness of the slide, paying no attention whatsoever to the height of the condenser, or varying its position to regulate the illumination, lowering it considerably to darken the field of view. A number of handbooks contain recommendations of this sort and, unfortunately, the majority of microscopists often employ these incorrect methods, thus distorting the picture which they see in the microscope's visual field.

The most common violation of the rules for microscopic work, and a very important one, is arbitrary handling of the lamp aperture diaphragm. The majority of microscopists do not understand the purpose of this diaphragm, using it not for reducing the working aperture of the condenser to 9/10 or 2/3 of the objective aperture, thus eliminating

scattered light and providing the necessary image contrast, but for incorrect regulation of field illumination. As is well known, field brightness should be controlled either by inserting a neutral filter into the optical pathway and thus reducing the intensity of the light from the source or, more practically (but less desirably, since it changes the color temperature of the source), by regulating the incandescence of the bulb with an appropriate rheostat for various sections of a transformer.

An even more common violation of the rules is ignoring the thickness of the slides used. In some cases, when working with weak or even powerful dry systems and a fluid specimen containing microorganisms and covered with a cover glass, extreme slide thickness is not very important, since the working aperture of the optical system must be greatly reduced in studying such specimens. However, when studying blood smears, microorganism smears, etc., which are always examined by oil immersion at the full numerical aperture of the system, a thick slide, which hampers correct focusing of the condenser, greatly reduces the clarity of the microscopic image and thus the precision of the result obtained. In cytological work slides of varying thickness are often used in preparing uniform serial specimens. During the subsequent investigation of individual specimens prepared on over-thick slides this is an unsurmountable obstacle to examination of important specimens with large-aperture systems. Use of too thick a cover glass makes it impossible to examine the specimen with a powerful short-focus immersion objective, whose working distance would be less than the thickness of the unsuitable cover glass. In this case the over-thick glass is a physical obstacle to use of the immersion objective, which cannot be brought into focus, since the housing of its front lens strikes the surface of the glass. This circumstance has often meant the destruction of large-aper-

ture immersion objectives, whose front lenses are not firmly held in their housings and are usually displaced after attempts to focus the objective under such conditions. Use of a thick slide disrupts only the focusing of the condenser, having no effect on the focusing of the objective. The fact that the working aperture of the microscope equals half the sum of the working apertures of the objective and the condenser makes it possible to obtain a more or less acceptable, although crude final result. Consequently, when there is no condenser (and its aperture is thus zero) the working aperture of a microscope equipped with an immersion objective with a numerical aperture of 1.3 is reduced to 0.75, which is not too small to permit resolution of the coarser details of the subject. However, gross diffraction phenomena develop when the condenser is removed or lowered: the edge of the specimen appears to be surrounded by a border of fine parallel lines, while thread-like dust particles in the eyepiece take on the form of characteristic round spots consisting of a system of concentric circles.

THE ULTRAMICROSCOPE OR THE DARK-FIELD METHOD

There are 3 basic methods for obtaining a dark-field effect: the first was proposed by Gigmondi and Zidentopf and consists in illuminating the medium or object to be examined with an intense lateral beam of light perpendicular to the optical axis of the objective (the slit ultramicroscope). This method is now employed primarily in studying colloidal systems. The second technique is based on obscuration in the objective, where a narrow beam of light formed by a special condenser passes through the object and enters the front lens of the objective, striking its flat-ground, engraved central portion, where it is absorbed. The light thus does not pass straight through the microscope; the luminous image of the specimen against a dark field is seen solely as a result of the diffracted rays.

The third method is the one most commonly employed in microbiology: it involves central obscuration in the condenser, the subject being illuminated solely by large-aperture (very indirect) rays, which is achieved by obscuring the central portion of the condenser. Any large-aperture Abbe condenser can be converted to a dark-field condenser, by disassembling it and covering its central portion (no less than 2/3 of its area) with a circle of black paper, whose final diameter is determined empirically. Small-aperture objectives must be used when the dark-field effect is produced by central obscuration in the condenser (with a paraboloid or cardioid condenser), since the indirect rays illuminating the subject would otherwise enter the objective and cause a decrease in contrast or general fogging of the image. With these condensers no special precautions are required to obtain a dark field with any dry system or with a 1/7 homogeneous immersion lens with a numerical aperture of 0.75. The latter system is to be specially recommended, since it produces an exceptionally high-contrast dark field. Special immersion objectives with a variable numerical aperture, which is achieved by including a special Davis diaphragm in the objective, are employed in some cases; the opening of the diaphragm is regulated by turning a ring on the housing. Such systems, whose numerical apertures range from 1.25 to 0.85, can be used equally successfully for light- and dark-field observations.

In dark-field work the light is adjusted and centered by the light-field method and the Abbe condenser is then replaced by the appropriate central-obscuration system, the aperture diaphragm of the lamp being opened as far as possible.

The specimen is prepared by the drop-dispersion method and is made as thin as possible. The slide should not exceed 1-1.1 mm in thickness, since the condenser focus (the point where the large-aperture rays in-

tersect) will otherwise lie within the side and, in checking for correct adjustment of the weak system, the observer will see a dark circle rather than a circle within which the object shines like a bright star against a black sky.

This pattern appears to be an optical section through a tent-like array of large-aperture rays surrounding a cone of complete darkness (the shadow of the central screen). Since the large-aperture rays intersect at their focus and diverge to form a funnel of light containing a second cone of darkness with its base facing upward, the observer may see a glowing ring surrounding a dark circle when the slide is very thin or extremely thick. In the first case we correct the situation by lowering the condenser and shifting its focus into the specimen, while in the second case the specimen must be reprepared on a thinner slide. Dark-field observations must be made with an immersion condenser. Cedar oil should be used as the immersion fluid, although other liquids with indices of refraction approximating that of glass ($n = 1.56$), such as vaseline oil, can be employed for large-scale work. Having obtained the dark-field effect, the point at which the condenser rays intersect is made to coincide with the optical axis of the objective by turning the condenser sleeve and the handle of the eccentric ring on its housing in opposite directions. Uncentered illumination is indicated by one-sided lighting of air bubbles or erythrocytes, which gives them a sickle-like appearance. When the light is centered air bubbles appear as dazzling rings. The lamp rheostat is intentionally disconnected in dark-field work; this ensures a maximum light intensity and increases the visibility of such fine details as bacterial flagella.

THE PHASE-CONTRAST METHOD

One technique for increasing the contrast of transparent and thus difficult-to-distinguish living subjects is the phase-contrast method.

Microscopic subjects can be either colored (naturally or artificially) or colorless. Colored specimens are easily seen under the microscope, since they stand out in contrast to the background formed by the visual field. Study of the structural details of such subjects is facilitated by use of special light filters, which not only increase the contrast of the subject or its details, but reduce extreme coloration, which often masks various structures (for example, a blue-green filter is employed in studying the nuclear structures of microorganisms stained by the Romanowsky-Giemsa method).

Colorless specimens are easily seen when they are sufficiently opaque and, absorbing light, appear dark against the light background of the visual field. Since such specimens alter the amplitude of the light waves passing through them, uniformly or nonuniformly attenuating them, they are referred to as amplitude-modifying.

In addition to amplitude-modifying objects there are those which are completely transparent. In passing through such objects light either retains its full intensity or is very slightly attenuated; however, if different portions of the specimen have different indices of refraction or the specimen is nonuniform in thickness, the phase of a light wave leaving it is altered. Such objects are referred to as phase-modifying, to contrast them with amplitude-modifying specimens. The majority of living cells and microorganisms are phase-modifying. The phase change produced by the passage of light waves through such specimens cannot be detected with the eye or on a photographic plate.

The phase-contrast method, which was proposed by Cernik in 1934, is used to detect the phase shift caused by transparent objects.

The phase-contrast method is based on the Abbe diffraction theory for image formation in the microscope.

Any object placed in the path of the rays passing from the conden-

ser to the objective serves as a diffraction grating, regardless of whether it has a periodic (the shells of diatom algae, the scales of butterfly wings) for an aperiodic (leucocytes) structure. In passing through the specimen the light rays undergo diffraction at different points, so that diffraction spectra of the light source develop around any object illuminated by direct or incident light from the condenser. Entering the objective, both the direct and diffracted rays are collected in its posterior focal plane, where a multiple image of the light source is formed; the central image is colorless and is called the zero maximum, while iridescent images are arrayed about the zero maximum and are referred to as first-order, second-order, etc., maxima. The distribution of the first-order and other maxima is determined solely by the character of the specimen. When it is periodic in structure the maxima take the form of parallel lines, the first-order maxima lying in a straight line, but on either side of the zero maximum.

Since the image of the annular diaphragm below the condenser is observed in the exit pupil of the objective, which is focused on the specimen, in the form of a zero maximum and first-order maxima that only slightly overlap or are entirely separate, it is possible to arbitrarily influence the character of the secondary (final) image by altering the quality of the light at the zero maximum by one means or another. Cernik (1934) suggested that the intensity of the light at the zero maximum be substantially reduced and its phase simultaneously change by 1/4 wavelength by inserting a so-called annular phase plate in the exit pupil of the objective. This plate is precisely equal in size to the projection of the annular diaphragm below the condenser. The light rays forming the first-order maxima interfere with the qualitatively and quantitatively altered rays at the zero maximum, producing an extremely contrasty image of transparent phase-modifying objects in

which slight differences in coefficient of refraction or thickness cause no attenuation of the light passing through them, altering only the phase of its waves. A graphic survey of the processes which occur in phase-contrast microscopy is given in the description supplied with each set of phase-contrast optics (KF 1) and they require no further explanation.

The method of using the KF 4 phase-contrast attachment reduces to the following.

1. The condenser is removed from the microscope and replaced by the special condenser from the set (which is equipped with a diaphragm turret and a centering device); the dial of the latter is set so that the numeral zero appears in the window.

2. The usual microscope optics are replaced by those from the KF 4 set, which consists of 10, 20, 40, and 90× achromatic objectives (the latter being for oil immersion).

3. The specimen is placed on the stage and the illumination adjusted by Keller's method under low magnification.

4. Having focused the required objective on the specimen, the eyepiece is replaced with the MIR-4 auxiliary microscope included in the set. The tube of the auxiliary microscope is withdrawn until the exit puiil of the objective, which takes the form of a light circle containing the darker concentric annular phase plate, is clearly in focus.

5. The rotating dial (turret) of the condenser is turned so that the figure corresponding to the true magnification of the phase objective to be employed shows in its window. The annular diaphragm is then seen by looking through the auxiliary microscope; the centering screws must be used to align the phaseplate so that it falls precisely within the light ring produced by the diaphragm.

6. This completes the adjustment. The auxiliary microscope is re-

moved and the eyepiece is replaced to examine the specimen. The procedures outlined in Paragraphs 4, 5 and 6 must be repeated each time the objective is changed.

Depending on the character of the phaseplate, phase-contrast outfits can produce dark images on a light background (positive phase contrast) or light images on a dark background (negative phase contrast). The type of apparatus is usually indicated in the instructions.

ANOPTRAL MICROSCOPY

In addition to the phase-contrast microscope, we must also recommend the so-called anoptral microscope developed by the Finnish physiologist Wilska.

During the development of the phase-contrast microscope Keller and Loos (1941) often attempted to improve on this method.

The method of light microscopy proposed by Wilska in 1953 was an interesting innovation in the field of optical contrasting. Applying an annular layer of carbon black to one lens of the objective (analogous to the phase ring of phase-contrast objectives) and using an annular diaphragm of appropriate aperture mounted in the condenser, he constructed a device with a negative phase-contrast effect which yields a more contrasty image of very thin phase-modifying objects than the phase-contrast microscope.

In 1954 M.A. Peshkov repeated Wilska's experiments and fabricated a set of anoptral objectives; these enabled him to conduct a series of scientific investigations which accelerated the commercial production of a Soviet anoptral apparatus under the trademark MFA-2.

In 1955, while investigating amplitude-contrast microscopy and diffraction phenomena in the exit pupil of a home-made anoptral objective, M.A. Peshkov concluded that the peripheral light diaphragm is detrimental, causing troublesome aureoles and emphasizing traumatic er-

rors in the optics of both home-made and commercial instruments. These observations made it necessary to extend the ring of carbon black to the edge of the lens.

The rather broad central opening in the layer of carbon black is a "gate" through which diffracted light from the lens is directed toward the main mask, while the broad layer of carbon black covering the remainder of the lens surface serves as a trap for undesirable peripheral diffracted light; this furnished the grounds for referring to the anoptral objectives designed by M.A. Peshkov as gate objectives.

In addition to the annular diaphragm, a central diaphragm was also installed in the condenser. The outer margin of the annular zero maximum was thus the condenser circle or the edge of the aperture diaphragm, operation of which made it possible to vary the width of the zero maximum.

The absorbing layer of the gate objective can be prepared from metal (copper) by Stefanov's method, as well as from carbon black. The absorbing layer should pass no more than 10% of the light, since maximum contrast can be ensured only under such conditions. In contrast to phase-contrast objectives, gate objectives are apparently based on amplitude contrast, in which a lesser role is played by interference of the diffracted light with the direct light, which has undergone 10-fold attenuation and been somewhat refracted during its passage through the layer of carbon black or other light filter.

Comparison of microphotographs taken with classical Wilska anoptral objectives and with M.A. Peshkov's gate objectives indicates that there is a sharp decrease in aureole intensity and better resolution of intracellular details in various cytological specimens, including bacteria, in the latter case.

LUMINESCENCE MICROSCOPY

The term "photoluminescence" refers to the light produced by a specimen as a result of absorbed radiant energy. For a number of reasons, this light has a longer wavelength than the absorbed light. It is consequently convenient to excite luminescence either with 30-400 mμ ultraviolet rays or with blue-violet light. In either case, the luminescence produced covers all or a large part of the visible spectrum, coloring the luminous image.

When the specimen does not exhibit luminescence it is stained with special fluorochrome dyes and its nonfluorescent components then begin to luminesce.

The setup for luminescence microscopy under visible light consists of a bright light source and a biological microscope. A blue-violet filter is placed between the light source and the microscope nearer and a yellow filter is slipped over the eyepiece. The blue-violet light striking the specimen excites it to luminesce. In order to detect the luminescence all the blue-violet rays must be eliminated, which is done with the yellow filter mounted on the eyepiece, so that only the long-wave fluorescent light enters the observer's eye.

A number of substances, such as vitamins A and B_2, many of the pigments and certain of the fats and antibiotics found in living organisms, and certain products of normal and pathological metabolism display marked fluorescence. Such fluorescence is referred to as primary.

The induced, or secondary, fluorescence produced by fluorochrome dyes is of far greater importance than primary luminescence.

Of the synthetic fluorochromes, acridine orange, coryphosphin, and chemotherapeutic preparations such as rivanol, acrichine, and trypaflavin yield the best results. They are usually employed in the form of extremely weak aqueous solutions.

Luminescence microscopy has the following characteristics (M.N. Meysel', 1955):

1) a colored image;

2) a high contrast between the luminescent specimen and the black background;

3) both transparent and opaque living specimens can be examined;

4) various vital processes can be investigated under dynamic conditions;

5) individual microbes and viruses can be detected and their localizations established;

6) extremely precise cytochemical and histochemical methods and techniques for rapid cytodiagnosis have been developed.

The OI-18 luminescence lamp with a SVD-120A mercury-quartz bulb is employed for luminescence work; it can be used with ordinary biological microscopes or with the special ML-2 luminescence microscope. A discussion of the use of luminescent Sera for diagnosing the causitive agents of infectious diseases is presented on page 74.

MICROSCOPIC INVESTIGATION OF LIVING MICROORGANISMS

Prof. M.A. Peshkov

The so-called hanging drop method has long been used for observing living bacteria under physiological conditions. A cover glass is grasped in a Cornet forceps and passed through a flame; after it has cooled a drop of the material to be studied is centered on it. The inoculated cover glass is quickly placed on a sterile slide with a concavity whose edge has been preliminarily smeared with vaseline. A hollow-ground slide can also be placed over the cover glass carrying the drop of culture. The chamber is made completely hermetic and the specimen is observed over a period of many days. One drawback of this method is the fact that the specimen (multiplying bacteria, growing spores, etc.) is in a liquid medium. Brownian or natural movement of the microorganisms is often undesirable; it can be prevented by suspending them in a drop of molten agar cooled to 45°. Compatible results can always be obtained in observing microorganisms in a classical hanging-drop chamber, particularly if the observer fixes his attention on individuals near the edge of the drop, where they are somewhat immobilized by surface tension. In working with such chambers the illumination must be adjusted in accordance with all the aforementioned rules, although the curvature of the concavity and the water drops which condense on the bottom of the chamber do not always make it possible to obtain a clear image of the field diaphragm. These optical defects keep us from obtaining perfect photographs, particularly when using large-aperture immersion systems.

Methods of Observing and Photographing Living Microorganisms Under Various Conditions

Observation through the bottom of a Petri dish. When it is necessary to study the development of microorganisms on dense, transparent

nutritive substrates (agar or gelatin) in Petri dishes under normal conditicns a long-focus water-immersion objective must be used, since it has a very long working distance (approximately 2 mm) and so permits observation of the developing specimen through the bottom of the Petri dish and the layer of agar if the two have a total thickness of no more than 1.8 mm. When using a water-immersion objective the thin-walled Petri dish containing the culture (with its cover removed) is placed on the gelatin side of a fixed, washed, and dried photographic plate (the gelatin layer, which absorbs moisture uniformly, prevents condensation on the bottom of the chamber). The edge is smeared with several drops of wax and the slide is placed on the microscope stage and positioned under low magnification. A drop of water and glycerine is placed on the slide, the objective is immersed, and the specimen is located and examined.

Peshkov's Ш-shaped chamber. Until recently the best type of chamber was the so-called Ш-shaped chamber, which permits use of a numerical working aperture of no less than 1.2 (M.A. Peshkov, 1940; Fig. 7). A slide no more than 0.5 mm thick is flamed and set up so as to be absolutely horizontal (in a Koch device for pouring gelatin plates). A layer of molten transparent agar no more than 0.2 mm thick is applied to the surface of the slide. The medium is permitted to set and six cuts are then made with a sterile scalpel, removing the unneeded strips 1 and 2 from the grooves thus produced.

We thus obtain a Ш-shaped layer of medium; only the medial strip, which is bounded by air spaces on the right and left, is used for the culture. The two outer strips and the base of the letter Ш are used to mount the cover of the chamber, a cover glass.

The medial strip is inoculated by one method or another (even if only the running-drop method). The inoculated chamber is quickly cov-

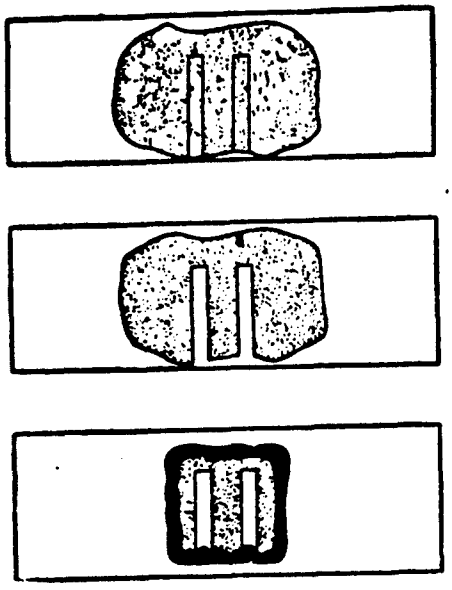


Fig. 7. M.A. Peshkov's Ш-shaped chamber.

ered with a thin sterile cover glass. The medium protruding beyond the cover glass is cut away and the edge of the preparation is sealed hermetically with melted paraffin (as described below), using a tweezers.

The strip of medium bounded by air spaces on either side guarantees normal development of highly aerophilic and facultatively anaerobic microbes, since a decreasing partial oxygen pressure is set up in it, the oxygen diffusing from the grooves toward the center of the inoculated strip; in addition, the hermetic sealing and optical homogeneity of the chamber prevents evaporation of moisture and the consequent disfocusing of the specimen inevitable with other types of microchambers, which greatly hampers Zeitraffer microcinematography.

The homogeneity of the various media (glass, agar, and glass), which have similar indices of refraction, makes it possible to use the Ш-shaped chamber with an immersion condenser (water type) and to study the specimen with oil-immersion objectives with numerical apertures of 1.3; although this reduces the contrast of the intracellular details of living bacteria or other cells, it permits the observer to see far more than any other method. Such chambers have been used to study the extremely fine changes in the cellular structures of dividing bacteria (M.A. Peshkov, 1939-1955).

The air-layer chamber. Another type of microchamber is intended for working with powerful dry or immersion systems, although it reduces

the working aperture of the objective to one. A drop of hot meat-infusion agar is applied to a flamed cover glass 0.09 mm thick and then quickly drained off. The cover glass is held vertically in a Cornet forceps until it is certain that the agar, which is distributed in a very thin layer over the glass, has dried (the total thickness of the glass and agar should not exceed 0.1 mm). A small drop of the inoculation material is applied to the surface of the agar, near the upper edge of the cover glass, with Pasteur forceps. It runs down, leaving behind a track containing the microorganisms to be cultured. The running drop is drawn off with a fine capillary tube or a piece of sterile filter paper. The culture is permitted to dry for no more than 2-4 min and the cover glass is then mounted on a very thin slide (no more than 0.5 mm thick), resting with the culture down on two fine capillary tubes previously attached to the slide with wax; a thin layer of 1% agar is then either smeared or poured over the slide. This precaution is necessary to prevent condensation on the bottom of the chamber.

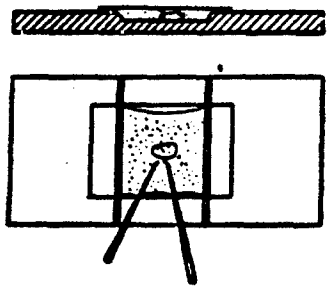


Fig. 8. Von Brun oil chamber.

The capillary tubes should reach to the edges of the cover glass, which are then smeared with hot wax or paraffin on all four sides. This is best done with a heated anatomic forceps, which is immersed in the paraffin and, having picked some up, is drawn like a pen, with its catch slightly loosened, along the very edge of the cover glass, thus ensuring complete hermeticity and precise preparation of the chamber. The total thickness of the latter (slide, air layer, agar layer, and cover glass) should not exceed 1.5-1.8 mm, so that an image of the field diaphragm can be obtained in the specimen plane. As may be seen from this description of the chamber, the air layer between the slide and the agar

film does not permit a numerical aperture of more than one for the condenser and objective and this forces us to use the chamber without an immersion condenser, since the latter adds nothing of value. The chamber can be employed for very precise observation of bacterial multiplication, development, and spore formation. The fact that the numerical aperture must be greater than one enables us to study many details and structures within the bacterial cell.

The Von Brun oil chamber. The so-called Von Brun oil chamber has come into wide use in recent years, having yielded invaluable results in a number of microbiological investigations (Fig. 8).

The oil chamber can be built-up (Fig. 8) or cut into a slide. A slide 0.5 mm thick is used to prepare a built-up chamber. Two glass strips of equal thickness are positioned 10 mm apart in the center of the slide, parallel to its short side, and glued down with a thick alcoholic solution of shellac. The strips are smeared with the mixture, heated in a Bunsen-burner flame to drive off the alcohol, and laid on a strongly heated slide. The shellac does not give off volatile products and, in contrast to Canadian balsam, is absolutely harmless to biological specimens.

The culture is prepared in the manner described above for the air-layer chamber. A cover glass bearing a strip of agar with the culture is placed on the glass flanges and fastened to them with drops of a molten mixture of wax and rosin (1-3 parts of wax and 1 part of rosin). Paraffin or pure wax cannot be used, since they dissolve in the vaseline oil with which the chamber is filled.

The preparation is quickly examined to be certain that all its components are immobile, air is blown through the chamber with a rubber bulb to evaporate the condensed water on its bottom, which is usually opposite the agar strip, and the chamber is rapidly filled with vase-

line oil through one of its open edges, using gravity flow from a Pasteur pipette. The oil used is pharmaceutical grade and need not be sterilized. Filling continues until the entire agar layer has been covered. The oil drop should not reach to the edges of the chamber, always leaving a gap (meniscus).

The very edge of the culture should be used for observation, since experience has shown that there is insufficient oxygen in the central portion and even at only a short distance from the edge, which distorts the development of the specimens. The chamber cannot be prepared in a warm dry room, since this would cause the agar layer to set too rapidly.

The total thickness of the chamber, including the slide, oil layer, agar layer, and cover glass, should not exceed 1.2-1.5 mm, since it will otherwise be impossible to focus the image of the field diaphragm in the specimen plane and obtain illumination by Keller's method.

MICROSCOPIC EXAMINATION OF STAINED MICROORGANISMS

Prof. M.A. Peshkov

Before staining a microorganism, smears of the material to be studied must be prepared on slides, which, because of the optical properties of the Abbe condenser, should not be thicker than 1-1.2 mm. The slides must be degreased, which is done by immersing them in concentrated sulfuric acid for 24 hr or by boiling them in a mixture of 6% potassium dichromate and 6% sulfuric acid, washing them carefully with water, and storing them in a jar containing 96° alcohol until they are needed. They are removed from the alcohol with a clean surgical forceps or tweezers. The degreased, alcohol-treated slides are wiped dry with a degreased linen rag and stored dry in a glass jar with a ground stopper.

A drop of water placed on a cold degreased slide should spread without collecting into droplets. A loop is used to transfer a small

drop of sterile tap water to the slide and then to pick up the material to be examined and carefully mix it into the drop of water, making circular movements and enlarging the area occupied by the drop. A broth culture is best picked up with a Pasteur pipette and a drop deposited on the slide. Difficult-to-crush substrates from fluid or solid media are mixed with a drop of water or applied directly to the slide (caseous lumps from tuberculous sputum), near one edge. Another slide is placed over the material in such fashion that the free edges of the two pieces of glass face in opposite directions; these edges are grasped with the middle and index fingers of both hands and, spreading the material between them, are slid apart in opposite directions. When necessary, if the material is wet or poorly crushed, the slides are again juxtaposed and slid apart. A third specimen-preparation method is the one usually employed for obtaining blood smears. A drop of blood (or an aqueous bacterial suspension) is applied near one of the short edges of a slide; a cover glass with a smooth edge or, even better, a slide with polished edges is inclined at an angle of 45° to the first slide in the direction of movement and slid along it. As a result of capillary action, the material is picked up by the upper slide, carried along as it moves, and smeared over the lower slide.

As soon as the smear has dried it is fixed, i.e., the bacteria in it are killed and simultaneously made to adhere to the slide. The most common method of bacterial fixation is heat treatment, which is carried out by slowly passing the smear upward three times through the flame of an alcohol or gas burner. The entire operation should take no longer than 5-6 sec and the specimen should be in the flame for a total of about 2 sec. The smear should not be overheated, since this will cause it to stain poorly.

Heat fixation is obligatory for checking microbial staining by the

Gram method.

Bacteria retain their body shape satisfactorily during heat fixation, but their dimensions are altered by shriveling. As for cellular inclusions and other structures, volutin grains and even endogenous spores can be fixed by heat (for gross examination), but this procedure cannot be employed for flagella, protoplasm, the nucleus, blood specimens, or organ smears.

In such cases the smears must be fixed in methyl alcohol for 5 min, ethyl alcohol for 20 min, Nikiforov's fluid (a mixture of equal volumes of ethyl alcohol and ether) for 20 min, and a formol-alcohol mixture (5 ml of undiluted commercial formalin and 95 ml of 96° ethyl alcohol) for 15 min.

The prestaining fixative, particularly in studying fine bacterial structure, is Carnoy's solution (60 ml of 96° ethyl alcohol, 30 ml of chloroform, and 10 ml of glacial* acetic acid), in which the specimen is fixed for 15 min. This solution cannot be used to fix blood smears or smears of internal organs, since it causes complete erythrocytic hemolysis.

Microbiological Staining Methods

The following are the most satisfactory stains. Reds: basic fuchsin (diamond fuchsin, rosanaline sulfate, or pararosanaline), acid fuchsin (rubin), neutral red, safranine, and Congo red. Violets: gentian violet, methyl violet, crystal violet, and dahlia (Lauth's violet). Blues: methylene blue, toluidine blue, opal blue, and analine blue. Greens: malachite green, brilliant green, and light green. Yellow-browns: chrysoidin, vesuvin, and certain others. In some cases (methylene blue, gentian violet, and crystal violet) saturated alcoholic solutions are prepared first (1 g of dye in 10 ml of 96° alcohol) and alcohol-aqueous solutions are then made up from these, mixing 1 ml of stain with 10 ml

of distilled water. The basic laboratory stains, which must always be at hand, are Ziehl's fuchsin (from which Pfeiffer's fuchsin is prepared by dilution), Loeffler's methylene blue, a set of reagents for Gram staining, and Romanowsky's stain. The dye solutions are stored in pipettes, which are kept in a refrigerator equipped with slots to hold them. In staining a specimen the slide is placed on a glass base. When heating is involved the slide is grasped in a Cornet forceps or an improvised wire clamp.

Ziehl's carbol fuchsin:

Basic fuchsin – 1 g

96° alcohol – 10 mg

Crystalline carbolic acid (Acidum carbolicum liquefactum can be used) – 5 g.

The fuchsin is ground in a mortar, the carbolic acid and alcohol are stirred in, and 100 ml of distilled water is added in small portions. This solution keeps very well and is used to stain spores and acid-fast microorganisms. Pfeiffer's fuchsin is used for simple staining of smears.

Pfeiffer's fuchsin:

Ziehl's fuchsin – 1 ml

Distilled water – 9 ml

Staining is rapid (10-30 sec). The solution deteriorates on standing; it is prepared ex tempore and should be used no later than the day following preparation.

Loeffler's methylene blue:

Methylene blue in saturated alcoholic solution – 30 ml

Distilled water – 100 ml

1% aqueous potassium hydroxide – 1 ml

This solution is stable. Its staining power and its capacity for

metachromatic staining of nucleic compounds increase during storage, as a result of azure formation.

For simple staining of smears (fixed as described above) Pfeiffer's fuchsin or Loeffler's methylene blue is poured over the specimen and permitted to act for no more than 1 min in the first case (restaining is possible) and for 3-10 min in the second case (restaining is not possible). The dye is poured off, the smear is rinsed with distilled water and dried (by thorough blotting with a piece of filter paper, taking care not to slide it sideways, which damages the smear), and the specimen is examined with an immersion system.

Special and Differential Stains

Staining by Gram's method – see page 60.

Staining of acid-fast microorganisms. Staining by Ziehl-Neelson's method. 1. A piece of filter paper is placed on a heat-fixed smear prepared from suppurative lumps taken from the sputum and crushed between two slides (see page 36); Ziehl's fuchsin is then poured onto the filter paper and heated until vapor is given off. The preparation is cooled, the paper is removed with a tweezers, and the smear is washed with water.

2. The specimen is bleached with 5% sulfuric acid until it has a yellowish tint.

3. The smear is carefully washed with water.

4. The specimen is rinsed with 96° ethyl alcohol (this stage is often omitted in the laboratory).

5. The smear is again rinsed with water and stained with Loeffler's methylene blue for 3-5 min.

Mycobacterium tuberculosis is stained ruby red, while the remaining acid-susceptible microflora is stained blue.

It is convenient to use Sinev's modification: the filter paper is

impregnated with an alcoholic solution of the dye (2% alcohol fuchsin), dried, cut into strips of the same size as the slides, and stored until needed. Several drops of distilled water are applied to the fixed specimen and the stained filter paper is placed over it and heated until vapor is given off; one then proceeds as in the method described above, beginning at the end of stage 1.

Staining bacterial spores. In observing unstained living spores the light is greatly refracted by the oval structures located centrally or terminally in bacilliform or distended individuals. In staining a spore-culture smear with Pfeiffer's fuchsin or Loeffler's methylene blue or by Gram's method the spore rudiments, or so-called prospores, readily absorb basic stains. As recent research has shown, the resistance of fixed spores to staining apparently results solely from the impermeability of their membranes; however, this resistance is greatly altered by brief treatment with hydrochloric acid heated to 60°. This property of dilute hydrochloric acid was noted by Auyeshkiy, who proposed a method for staining bacterial spores.

Auyeshkiy's method. 1. A smear of the sporulating culture is air-dried.

2. A 0.5% hydrochloric acid solution is poured over the unfixed specimen and heated for 2 min (until vapor is given off); when the preparation has cooled the acid is poured off and the smear is washed with water, dried, and heat-fixed.

3. As in the identification of Mycobacterium tuberculosis, the specimen is stained for one min with Ziehl's carbol fuchsin under gentle heating and then permitted to cool, without removing the dye.

4. The specimen is immersed in 5% sulfuric acid for several seconds (in order to decolorize the vegetative bodies) and rinsed with water.

5. Staining is carried out with Loeffler's methylene blue or 1% aqueous malachite green for 2 min; the smear is rinsed with water, dried, and examined with an immersion system. The spores are stained red and the bacterial bodies blue. In many cases it is sufficient to treat the specimen by Ziehl-Neelson's method to stain the spores.

A simple spore-staining technique which requires no chemical mordants or differentiating agents is Peshkov's method (1919-1924).

1. The smear is fixed by flaming or with alcohol formal.

2. Loeffler's methylene blue is applied to the fixed smear and heated to boiling by periodically introducing the slide into a Bunsen-burner flame for 15-30 sec.

3. The specimen is rinsed with water and stained for 30 sec with 0.5% aqueous neutral red. It is then washed with distilled water, dried, and examined with an oil-immersion objective. The spores are blue or dark blue, while young spores are blue-black and prospores violet-blue. The protoplasm of vegetative forms appears reddish, while its chromatin elements are violet.

Differential staining of the chromatin (nuclear) and metachromatin (volutin) elements of bacterial cells by Meyer's method. Metachromatin, or volutin, occupies a special place among the storage inclusions observed in bacteria (fats, glycogen, or pigments), since the bacterioscopic diagnosis of diphtheria is based on its presence and special location in Loeffler's bacillus.

Two smears are prepared from the material to be examined (by the method used for blood smears). These are fixed with heat or Carnoy's solution (for 5-15 min), the latter technique being better for preservation of other cellular structures. They are then stained for 10 min with Loeffler's methylene blue; after staining one smear is immersed for 5 min in 1% aqueous sulfuric acid (H_2SO_4) and the other is immersed

for the same time in 4% aqueous potassium carbonate (K_2CO_3). After the smears have been differentiated by labeling them A (acid) and AL (alkali) with a grease pencil they are dried with filter paper (without rinsing) and the acid-treated specimen is stained with 0.25% aqueous chrysoidin. When both specimens are examined with an oil-immersion objective it is seen that if volutin is present it has a cherry red color in preparation A, while the bacterial somae are light brown. The alkali treatment (preparation AL) stains the volutin even if it is partially dissolved, but the basophilic sections of the cytoplasm are not decolorized. Preparation AL consequently exhibits weakly stained somae and hollows at the sites of the volutin granules. In all investigations of volutin one should make use of its low solubility in boiling water (for 5 min) or in one N hydrochloric acid at 60° (for 6-7 min). Hollows are seen at the sites of the volutin granules in preparations treated by either of these methods; these areas are never stained, since the volutin has dissolved.

The nuclear elements and volutin granules of bacteria can be stained by Romanowsky-Giemsa's method after fixation with methyl alcohol, alcoholic formal, or Carnoy's solution (in the latter case the specimen must be washed with alcohol and carefully dried in air to remove all traces of the acetic acid). A slightly more alkaline solution (pH = 7.2) should be used for Romanowsky-Giemsa staining than for the staining of blood smears (pH = 6.8-7). The aqueous reaction for Romanowsky-Giemsa staining is set up in the following manner. A chemical beaker with a capacity of 250 ml is placed on a sheet of white paper near a window. A total of 200 ml of distilled water is poured into it and a small drop of 0.5% aqueous neutral red is added, so that the liquid appears to be tinted light red when viewed from above. (If the water is not tinted its reaction is above pH = 7.0; adding more water or

a negligible quantity of acetic acid then causes a reddish hue to appear.) A 1% aqueous solution of sodium carbonate (Na_2CO_3) is prepared separately and added drop by drop to the colored water, which is stirred with a glass rod after each drop of soda solution. No changes occur at first. After several drops of soda have been added the solution is decolorized, but not for long; the reddish tint returns after it is stirred. As many additional drops of soda are then added as are necessary to cause the reddish tint to disappear and not return within 3 min. The alkalized water, which contains neutral red as an indicator, is used to dilute the Romanowsky-Giemsa stain or Laveran's azure-eosin (see below). The traces of neutral red which remain in the water do not affect the stain. For staining by Romanowsky-Giemsa's method, 2 drops of the commercial stain are added to 1 ml of water. The preparation is laid smear down in a Petri dish, matches or pieces of broken slides being inserted beneath its edges. The stain is poured in from the side so that it wholly covers the smear, without bubbles. Staining lasts for from 1 to 24 hr. It is recommended that each new batch of water or stain be checked on a blood smear, which should take on a reddish-violet hue. The preparation is removed from the stain, rinsed with water at pH = 7.2, and examined with an immersion objective. The erythrocytic protoplasm should be reddish and the leucocytic nuclei reddish-violet; depending on the type of cell, the leucocytic protoplasm should be blue (lymphocytes), gray-blue (monocytes), or reddish (neutrophils). The oxyphilic granularity of the eosinophils should be clearly visible.

If a smear of a 18-hr culture of bacteria of the colon-typhoid-dysentery group is subjected to such treatment and examined microscopically, its nuclear elements, which are stained a reddish-violet, are clearly seen embedded in the cytoplasm, which is stained a weak reddish color. These elements are spherical or elongate in shape and are ar-

rayed along the length of the cell (M.A. Peshkov, 1945-1948).

If a younger culture is used the cytoplasm is stained a blue-violet (basophilia); the intensity of the staining increases as the age of the culture decreases and the size of its individuals increases (the physiological giantism and basophilia of young forms). As a result of the accumulation of ribonucleotides, the affinity of the cytoplasm of young forms for basic stains is so large that its deep violet color masks the stained nuclear elements, which can be seen only by using a special blue-green filter (M.A. Peshkov and A.N. Belozerskiy, 1948).

Romanowsky-Giemsa's method stains both the nuclear elements (a darker reddish-violet) and volutin (a cherry red, as in Meyer's method, since staining is with azures in both cases) of Corynebacterium diphtheriae (M.A. Peshkov, 1947-1948).

M.A. Peshkov (1943) suggested that the cytoplasm of bacteria which stain poorly by Romanowsky-Giemsa's method in "mature" cultures (18-24 hr) be treated by Giemsa's method and stained with an acidified solution of light green (30 sec in 0.25% aqueous light green acidified with 1-2 drops of acetic acid per 100 ml of stain).

This procedure gives the soma a bright green or blue-green color and the nuclear elements a reddish-violet color.

Pekarskiy proposed another method of treating bacteria to show their nuclear elements. Smears fixed by Carnoy's method are subjected to weak hydrolysis for 7 min in 1 N hydrochloric acid at 60° (as in the first stage of the Feulgen reaction) and then rinsed to remove all traces of the acid and stained by Romanowsky-Giemsa's method (1 hr at 37° or 24 hr at room temperature). During restaining the protoplasm is differentiated with 96° alcohol or with acetone-xylol mixtures:* 1 sec in acetone, 10 sec in a mixture of 14 parts of acetone to 6 parts of xylol, 10 sec in a mixture of 6 parts of acetone to 14 parts of xylol, and

1 sec in pure xylol. This procedure, which is intended to hydrolyze and remove the ribonucleotides soluble in dilute hydrochloric acid, which mask the color of the nuclear elements, gives the chromatin elements a dark-red color and the protoplasm a reddish color. Just as in the Giemsa light-green method, the specimen can be stained with light green to produce a very effective pattern (green cytoplasm and polar caps and red nucleus), although, for photographic purposes, it is better not to use a green stain (since it is difficult to obtain a clear image of the green cytoplasm and red nucleus in a monochromatic photograph), but to leave the cytoplasm reddish or to stain the soma with eosin or acid and then with basic fuchsin, in order to obtain a clear contour in photographs taken through a green filter.

Investigation of Mobility and Observation and Staining of Flagella and Capsules in Living Bacteria

Mobility is investigated by the classical technique, using the hanging-drop method. Hanging-drop observations always begin with dry systems: the edge of the drop is found and brought into the center of the field and the objective is then replaced by a more powerful one. An immersion objective is used when necessary, although active mobility is easily seen under low magnification. Bacterial mobility is distinguished from the molecular Brownian movement always observed by the fact that individuals swim through the field of vision, making rotary and circular movements, which cease if the culture under study is killed by heat, a drop of formalin, or formalin vapor.

In large-scale investigations of the mobility of various bacterial strains preparation of dozens and sometimes even larger numbers of specimens by the hanging-drop method is very tedious and unnecessary, since the active mobility of a given broth or agar culture can be determined with equal success by applying a drop of broth or condensation

water to a slide and examining it under moderate magnification, without placing a cover glass over it. A semifluid medium can be used for macroscopic determination of mobility in diagnostic laboraties occupied in analyzing feces for members of the family Enterobacteriaceae (page 238).

If it becomes necessary to study the flagellar apparatus of living bacteria this is done by dark-field examination of the culture, using Neumann's method (1925).

As is well known, the flagella of different bacteria vary greatly in thickness. Certain monotrichates have very thick flagella (0.1 μ). In such lophotrichates as the Spirilleae (the contents of the rectum and mid-gut of the oriental cockroach Periplaneta orientalis should be used to demonstrate Spirilleae) the locomotor apparatus consists of numerous (up to 30) individual flagella combined into a powerful sword-shaped organ, which is easily visible in both light and dark fields. The extremely powerful complex flagella, or flagellar "plates," of specimens of Proteus taken from a swarming area are also easily seen against a light field.

Another method, involving dark-field observation, was proposed by Neumann (1925). This method is based on the tendency of individual fine flagella to combine into complex "plates" under the influence of a viscous medium and a somewhat elevated electrolyte content.

Method 1. A total of 10% powdered pure gum arabic is added to a meat-infusion broth with an increased (1%) sodium chloride content. The mixture is heated to dissolve the gum and then clarified, like gelatin, with hens egg albumin. A drop of the clarified medium is checked in a dark field to be sure that there are no granules. It must be completely pure and contain no luminous grains. If such grains are present, the clarified broth is centrifuged to remove them. Once it is certain that all the granules have been removed, the broth is divided into small

portions, poured into ampules (containing no more than 0.25-0.5 ml), sealed up, and sterilized in a Koch apparatus or under pressure. The broth can be stored for years and is always ready for use.

In investigating Proteus it is necessary to take material from the swarming zone or, if the culture is made in the middle third of an agar slant, to take a drop of condensation water as soon as the swarming zone reaches it. For other bacteria 18-hr agar cultures are used. Observing all the rules for preparation of dark-field specimens (page 23), a small drop of the broth is applied to a slide and a loop bearing the material to be examined is immersed in it several times (care must be taken not to tear off the flagella). Having made a suspension of the bacteria (it is not superfluous to be sure beforehand that they will exhibit, active movement) and examined them against a light field in a hanging drop of the same broth, a thin cover glass is placed over the drop in such fashion that the latter spreads to the edge of the glass and, entering the capillary space between the slide and the cover glass, forms a very thin liquid layer; if this is not properly done it will be impossible to see the flagella. Many flagella are torn away, becoming tangled into thick threads and plates, when the cover glass is applied.

Staining of flagella

The staining of bacterial flagella is one of the most precise of microscopic operations. Despite the multiplicity of methods proposed at various times, one of the oldest, Loeffler's method (which is described here in slightly modified form), is especially reliable.

1. A 18-hr agar culture grown at the temperature optimum for the species to be investigated is used. A small quantity of material is taken with a loop or Pasteur forceps from the lower portion of the culture, but not from the condensation water (the test-tube is not framed and a cold loop is used).

A total of 5-6 ml of tap water at the temperature at which the microbes were grown is added to the test-tube. Physiological sodium chloride solution cannot be used. The loop or forceps bearing the fragment of material is carefully immersed in the water and left there until the bacterial mass breaks up. A drop of this suspension is examined microscopically to check the mobility of the bacteria. If the culture is immobile it should not be stained, although flagellar staining may succeed in temporarily immobile cultures. A thick suspension cannot be used.

2. A drop of the suspension is then applied to a clean watch glass and a drop of 2% osmic acid is added to it. After careful mixing a second watch glass is placed over the first and a cover glass is prepared for the specimen. (If no osmic acid is available step 2 can be omitted, although the results obtained are not as good).

3. A clean cover glass is taken in a Cornet forceps and slowly passed through the hot part of an alcohol or Bunsen-burner flame 3 or 4 times. It is then permitted to cool. A small quantity of the suspension is taken from the hourglass with a Pasteur pipette equipped with a very fine tip (it is taken directly from the test-tube if step 2 is omitted) and 5 small drops are applied to the flamed side of the cover glass. One drop is placed in the center of the glass and the four others near its corners; they should be spread over its surface. They will not spread if the glass has been poorly degreased by flaming: it is useless to stain such a preparation. The drops are permitted to dry and the glass is taken in a forceps and passed rapidly once through a Bunsen-burner flame (if the flagella cannot be stained this mild fixation must be omitted).

4. A mordant containing the following solutions is prepared:

1. Saturated alcoholic basic fuchsin. This is prepared by adding

1-2 large crystals of fuchsin to 10 ml of 96° ethyl alcohol; the test-tube is left to stand in a heater or water bath at 50° for 1-2 hr.

II. A 20% aqueous solution of tannin

III. A cold saturated aqueous solution of Mohr's salt (ferrous ammonium sulfate). The mordant is prepared by mixing 1 ml of solution I (fuchsin) with 10 ml of solution II (tannin) and adding 5.5 ml of solution III (Mohr's salt).

The best results are obtained with a mordant which has been permitted to age for a day or more.

Not all types of tannin can be used to prepare the mordant. The necessary type is selected on the basis of experience. A mordant which works poorly can be corrected by adding 0.1-0.2 ml of 1 N potassium hydroxide to each 10 ml.

The staining of flagella consists of: 1) preparation for staining, during which the diameter of the flagella increases as a result of precipitation of the mordant; 2) staining of the mordant-treated specimen with one of the basic stains.

The first procedure is carried out as follows. An excess of filtered mordant is poured over the fixed specimen and left on the cover glass for 10-15 min. Care must be taken that the mordant does not flow off the glass, beneath the forceps, or onto the lower side of the glass; it should not be permitted to dry. When the necessary time has elapsed the mordant is washed off with a strong stream of distilled water and the glass is rinsed until all traces of the mordant have been removed (approximately 30 sec). The reddish border of dried mordant is removed from the edge of the cover glass with a thin rag. The specimen is then again washed with distilled water.

The second procedure, staining, is carried out by placing the mordant-treated specimen smear down in a watch glass, to which Ziehl's car-

bol fuchsin diluted half-and-half with water (filter the stain!) is added. Staining lasts 2-5 min and the specimen is then washed with water, dried, and embedded in Canadian balsam. All 5 drops must be examined, since it is quite often possible to stain the flagella in only one area of the preparation.

Observation of the capsules of living bacteria. A drop of the bacterial suspension is applied to a slide and a drop of saturated aqueous Congo red (3%; boiled and cooled before use) or 10% freshly boiled aqueous opal blue (prepared by Epstein's method) is added to it. The suspension and stain are combined and a small drop of the mixture is applied to a clean slide; a cover glass is dropped over it and the excess fluid is blotted up with a piece of filter paper, which is pressed against the glass with the index finger. The paper absorbs the excess fluid and a capillary layer of stain, in which the bacteria lie in a single layer, is formed in the center of the compressed drop. It is necessary to be certain that the convex side of the cover glass (which is always slightly curved) is toward the fluid, since it would otherwise be impossible to press it down and obtain a thin liquid layer.

If the specimen is correctly prepared and the microscope objective is focused on the thin, almost colorless area where the cover glass is most tightly pressed down (held by suction), live bacteria and, when capsules are formed, the light aureoles around their somae may be clearly seen. A clearer capsule pattern is obtained when opal blue is used. It should be added that, using the method described above, Congo red or 1% eosin in physiological sodium chloride solution (yellowish eosin, eosin sodium, or eosin potassium) can permit rough evaluation of the relative number of live and dead bacteria in a given population. Enrici's method (1928) is based on the fact that dead bacteria are slowly stained by Congo red and eosin, while live bacteria are not stained,

appearing as light, well-outlined spots, and are often optically tinted in a color complimentary to the background hue (if the backgrouns is red, live bacteria appear slightly greenish).

Negative methods of investigating capsules and bacteria. Burri's method. The best liquid natural ink is diluted 10 times with distilled water (dry India ink can be ground to the same consistency) and centrifuged for an extended period. The supernatant layer is then drawn off and examined with an immersion objective to make sure that no coarse granules are present. If the ink is good 0.1 ml portions are poured into capillary tubes, which are sealed and sterilized in an autoclave. The specimen is prepared by emptying the contents of a capillary tube near one of the short edges of a slide and adding a drop of the material to be investigated (biopsy material from a hard chancre, material containing encapsulated bacteria, or cultures of Leptospira). The material is carefully mixed with the ink and a thin smear of the blood-smear type is prepared, using a cover glass or a polished slide with truncated corners. The specimen is examined as soon as it is dry, without fixation. The bacteria or spirochetes are white and clearly defined against a gray background. In addition to ink, 10% opal blue (Epstein), 3% Congo red, 10% nigrosin, or 2% collargol can be used for negative preparations of the Burri type. Acid substrates cannot be treated by negative methods; in order to avoid precipitation they must first be carefully neutralized, even if only with ammonia. With any negative preparation method it must be taken into account that the bacteria have not been killed and can still be a source of infection (as can those in preparations carefully heat-fixed).

Staining of bacterial capsules

Anthony's method

1. The smear is air-dried and stained, without fixation.

2. Staining is in cold 1% aqueous crystal violet.

3. The stain is washed off with 20% aqueous copper sulfate ($CuSO_4$).

4. The specimen is dried, but not rinsed with water.

Hiss' method

1. The specimen is fixed with alcoholic formal or Nikiforov's solution, but not with heat.

2. Staining is with 5% aqueous gentian violet and is continued until vapor appears.

3. The stain is washed off with 20% aqueous copper fulfate.

4. The specimen is dried, but not rinsed with water.

Treatment of Specimens in the Presence of Nuclear Nucleoproteins

The nuclear reaction to thymonucleic acid (one of the components of the nuclear nucleoprotein chromatin), suggested by Feulgen and Rossenback in 1924, occupies a special place among the various methods for staining the nuclear substance.

As is well known, chromatin, which was until recently a purely morphological or tinctorial concept, has now acquired a precise chemical representation, being a deoxyribonucleoprotein whose prosthetic group is deoxyribonucleic (thymonucleic) acid and whose protein is amphoteric, containing mono-α-amino acids.

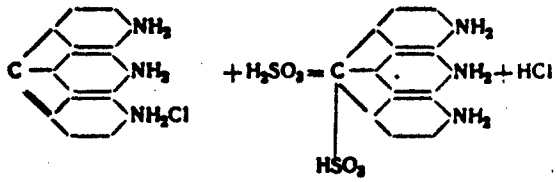
Deoxyribonucleic acid contains thymine among its nitrogenous bases, in contrast to ribonucleic acid, which is characterized by uracil; more important, the latter contains four residues of the pentatomic sugar deoxyribose (ribodesose), while the former contains four ribose residues. As the investigations of A.N. Belozerskiy (1939-1948) showed, the nuclear substance of saprophytic and pathogenic bacteria in no way differs in chemical composition from that of the plant or animal cells of multicellular organisms.

In order to identify the nuclear structures in animal, plant, or

bacterial cells it is thus very important to have a technique for carrying out Feulgen's reaction and to understand its mechanism.

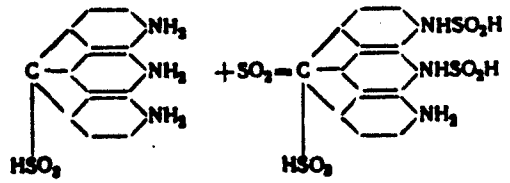
Weak acid hydrolysis of nuclear nucleoproteins causes cleavage of their purine and pyrimidine bases, which exposes the deoxyribose molecules. As the investigations of Stacey et al. (1946) showed, hydrolysis of deoxyribose converts it to w-hydroxylevulinic aldehyde, which causes a specific reddish-violet tint to appear when colorless fuchsin sulfurous acid (Schiff's reagent) is added.

Schiff's reagent, which has been used since 1861 for detecting aldehydes, with which it yields a typical reddish-violet color, is obtained by saturating aqueous basic fuchsin (I) with sulfur dioxide (SO_2). Combining with the fuchsin, the sulfurous acid formed converts it to leucosulfurous acid (II):



I II

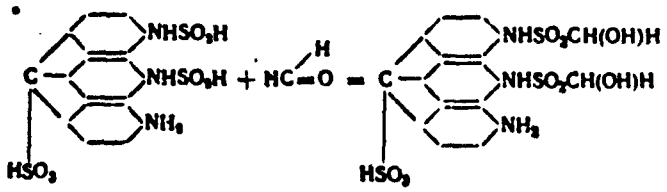
The further action of the sulfur dioxide on the leucosulfurous acid (II) forms fuchsin sulfurous acid (III):



II III

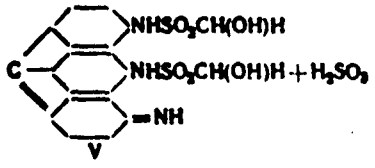
Combining with aldehydes (e.g., formaldehyde

) in the manner of aldehyde bisulfites



III IV

the fuchsin sulfurous acid (III) forms an unstable colorless product (IV), which loses the sulfur-containing group bonded to its central carbon, acquires a p-quinone structure, and becomes a red-violet chromogen (V), which also causes the typical "Feulgen" color to appear:



V

The specificity of the Feulgen reaction is confirmed by the still more specific Dische reaction (1930), which is based on the appearance of an intense blue color when deoxyribonucleic acid is boiled with diphenylamine.

Since both reactions (Feulgen's and Dische's) result from the hydrolytic formation of w-hydroxylevulinic aldehyde, these color reactions alone are not sufficient to demonstrate the presence of deoxyribonucleic acid, but should always be accompanied by use of other chemical indices (even if only identification of thymine and other data), such as those developed for bacteria by A.N. Belozerskiy.

Despite the seeming complexity of Feulgen's reaction, it is practicable and, when the rules discussed below are observed, is an irreplaceable method for detecting cellular deoxyribonucleoproteins (chromatin); the only procedures which are comparable (within certain limits) are those staining methods based on use of azure eosinates and methylene blue.

Despite the fact that Feulgen warned against the use of fixatives

containing aldehydes or oxidizing agents, recommending alcohol, alcohol and acetic acid, or sodium ethylate, Bauer has pointed out that the Feulgen reaction can be obtained after application of any "nuclear" fixative except picroformal and Bouin-Hermann's fluid. Each fixative has its own characteristic hydrolysis time.

Fixative	Hydrolysis time, min
Carnoy's (alcohol, chloroform, and acetic acid)	7
Shaudin's (alcohol, mercuric chloride, and acetic acid)	10
Flemming's (osmium chromacetate)	14
Navashin's (chromacetoformol)	15-20

After fixation with Carnoy's solution the smear is washed with 80° alcohol and immediately stained. After fixation with Shaudin's solution the specimen is treated with iodized alcohol (70° alcohol tinted with an iodine solution to the color of port wine or strong tea) to remove the mercuric chloride and then, when it has acquired a yellowish hue, is transferred to 70° alcohol until the yellow color caused by the iodine is no longer noted; only then is Feulgen's reaction carried out. After fixation with Flemming's or Navashin's solution the specimen must be carefully washed in running water (for from 1 to 24 hr).

It is recommended that the specimen be soaked for 24 hr after fixation in 96° alcohol in order to dissolve the special lipid substance known as plasmal, which Feulgen found in the plasma of living cells (although not in bacteria) and which is capable of producing a false reaction.

From the 96° alcohol the specimen is transferred through 70° alcohol to pure water, immersed for 30 sec in cold 1N hydrochloric acid (prepared by adding 82.5 ml of concentrated hydrochloric acid with a

specific gravity of 1.19 to 1 liter of distilled water), and then transferred to a hydrochloric acid solution heated to 60°, in which it is kept for a period that depends on the fixation method (see page 56). From the hot 1N hydrochloric acid the smear is moved to a cold hydrochloric acid solution for 10 sec in order to arrest the accelerated hydrolysis and then placed in Schiff's reagent. Hydrolysis is most conveniently carried out in a chemical beaker on a Bunsen stand. The beaker is placed on an asbestos grid and kept sufficiently high above the burner that the temperature of the 1N hydrochloric acid does not rise above 60°. A thermometer mounted on the stand is immersed in the beaker. Its mercury bulb should never tough the bottom of the beaker. The slide is inserted vertically and rests against the wall of the beaker. The acid should not be permitted to come into contact with the metal forceps. Distilled water is added to the beaker as the liquid evaporates.

It is recommended that bacterial smears be kept in Schiff's reagent for 4 hr. The Schiff reagent is poured into a vessel with a hermetically fitting cover (a large flat-bottomed biological test-tube or histological beaker fitted with a rubber stopper or a deep, rather broad box with a ground lid to keep the sulfur dioxide from escaping). The bottle containing the Schiff reagent must be stored under refrigeration, even while the specimen is being stained. The preparation is then removed from the fuchsin sulfurous acid (Schiff's reagent) and washed in three successive portions of an aqueous sulfur dioxide solution (the latter is serviceable until it gives off a sharp odor of sulfur dioxide and one of the portions, usually the first, shows traces of a reddish tint), followed by rinsing in running water for 10-60 min. It is then transferred to distilled water, stained with light green or analine blue, and embedded in Canadian balsam or dried and examined with an immersion objective, in the same manner as ordinary smears. The entire prepara-

tion should never be stained, since when the Feulgen reaction is weak it can clearly be seen without staining; intensive staining might obscure a positive reaction and it is therefore recommended that an unstained portion of the specimen be examined and careful staining be undertaken to show the outline and cytoplasm of the bacterium only when the requisite effect is obtained. In cases of overstaining with light green or analine blue (which are used in 0.1-0.25% aqueous solutions) the excess dye is easily removed from the specimen with water (this is true of all acid stains). The reddish-violet coloration of Feulgen-positive structures is so strong that it is not attenuated even when the specimen is kept in 96° alcohol for 24 hr.

Preparation of Schiff's reagent. Large crystals of basic fuchsin (1 g) are ground in a mortar and poured into an Ehrlenmeyer flask and 200 ml of boiling distilled water is added. The fuchsin is permitted to dissolve, which requires 5 min. It is occasionally necessary to heat the solution. The liquid is cooled to 50° and filtered into a bottle equipped with a ground (or rubber) stopper and 20 ml of 1N hydrochloric acid is added. The cooling of the solution to 50° before addition of the acid is intended to keep it from evaporating. As soon as the acid has been added the ruby red liquid becomes cloudy and takes on the appearance of cranberry or blueberry juice (part of the fuchsin coagulates). It is then cooled to 25°, 1 g of anhydrous sodium bisulfite ($NaHSO_3$) is added, and the vessel is stoppered. Evolution of sulfur dioxide begins (the solution is cooled to 25° to forestall excessively violent liberation of this gas) and within 24-48 hr the fuchsin has been converted to colorless or slightly brownish fuchsin sulfurous acid. The reagent should give off a sharp odor of sulfur dioxide. Appearance of traces of a reddish tint in even a thick layer of reagent means that it is unsuitable. Since sodium bisulfite is not always available,

Schiff's reagent can also be prepared with photographic sodium sulfite (Na_2SO_3). In this case 0.5 g of basic fuchsin is dissolved in 90 ml of boiling distilled water, the solution is cooled to 50° and filtered, and 2 ml of concentrated hydrochloric acid with a specific gravity of 1.19 is added. A separate portion of 2 g of anhydrous or 4 g of crystalline sodium sulfite (Na_2SO_3 or $Na_2SO_3\ 7H_2O$), chemically pure or photographic grade, is dissolved in 10 ml of cold distilled water. The sulfite solution is then added to the acidified fuchsin solution, which has been cooled to 25°, the bottle is stoppered, and the preparation is placed under refrigeration for clarification, storage, and future use.

Preparation of aqueous sulfur dioxide solutions. A tightly stoppered vessel is filled with 200 ml of distilled water and 20 ml of hydrochloric acid and 1 g of anhydrous sodium bisulfite is added. The resultant solution, which is saturated with sulfur dioxide, is poured into three well-stoppered flasks labeled I, II, and III. The specimen is washed first with the water from flask I and then with that from flasks II and III, returning the solutions to the appropriate flasks after using them. The contents of flask I are usually discarded after prolonged use and this flask is filled with the contents of flask II; the contents of flask III are poured into flask II and a fresh solution is prepared for the former.

Preparation of aqueous sulfur dioxide solutions with sodium sulfite. A flask is filled with 200 ml of distilled water and 4 ml of concentrated hydrochloric acid (specific gravity 1.19); 4 g of anhydrous or 8 g of crystalline sodium sulfite (Na_2SO_3 or $Na_2SO_3\ 7H_2O$) is dissolved in 25 ml of water and this solution is added to the acidified water. Sulfur dioxide is evolved slowly, forming sulfurous acid with the water and thus going into solution. This reaction takes the form

$NaHSO_3 + HCl = NaCl + H_2SC_3$ or, for sulfite, $Na_2SO_3 + 2HCl = 2NaCl +$ $+ H_2SO_3$.

BACTERIAL STAINING BY THE GRAM METHOD

Prof. G.P. Kalina

Staining by the Gram method makes it possible to divide all bacteria into two groups – gram-positive and gram-negative. This is of great importance in the classification and taxonomy of this class of organisms. Determination of Gram staining is now obligatory in establishing the species, genus, and family to which a given microbe belongs.

Gram, who proposed his method for staining bacteria in organ sections and prints in 1884, did not appreciate its great differential-diagnostic value, although he pointed out that some microorganisms, such as typhoid bacillae, are decolorized and take on a complimentary color. Roux, in 1886, was the first to show the value of Gram staining in differentiating bacteria.

Principles of the Gram method. The bacterium is stained with a basic analine dye belonging to the triphenylmethane group (which is usually violet, but sometimes green or blue) and then treated with a mordant (iodine, picric acid, potassium permanganate, etc.; see below), which results in the formation of a triple compound of the dye, the mordant, and the cellular component involved. This compound is very stable in gram-positive and very unstable in gram-negative bacteria. Subsequent treatment with alcohol, acetone, xylol, or certain other substances removes the stain from Gram-negative specimens (decolorizes them). The decolorizer is washed off with water and the specimen is stained with a contrast dye such as fuchsin or neutral red. Gram-positive bacteria are stained a dark violet or blue, while gram-negative bacteria acquire a red or reddish color.

As Henry and Stacey have shown, the cellular constituent which en-

ters into the triple compound with the stain and the mordant is the magnesium salt of ribonucleic acid. Treatment of a suspension of gram-positive bacteria with bile salts extracts the magnesium ribonucleate and the cells become gram-negative. Addition of this ribonucleate to such gram-negative cells causes them to revert to gram-positive.

Preparation of Specimens for Gram Staining

The procedure currently employed in preparing specimens for Gram staining usually ignores all the methodological details formerly assigned considerable importance; in many cases this leads to failure or to clearly erroneous results.

1. Concentration of bacteria. In order to obtain reliable staining results the specimen should be as thin as possible. The dye is often precipitated in thick smears containing aggregates ("clumps") of cells and it is difficult to decolorize gram-negative organisms. Conversely, aggregated gram-positive cells are decolorized substantially more rapidly than isolated cells. The bacteria in the specimen should consequently be as far apart as possible.

2. Drying the specimen. It was pointed out above that positive Gram staining results from the presence of magnesium ribonucleate in the cell. Extraction of this compound causes the microbe to become gram-negative. Physiological saline solution is among the agents capable of extracting the nucleic acids and their salts from the cell. Preparation of a bacterial suspension in saline solution from cultures grown on solid media and prolonged drying may cause partial extraction of the magnesium ribonucleate and make some of the cells gram-negative. Prolonged storage of a bacterial suspension in physiological solution, distilled water, or slightly alkalized water converts some or all of the gram-positive cells to gram-negative. Even extended drying of a drop of physiological-solution suspension on a slide may lead to par-

tial loss of the ability to withstand decolorization. A minimal quantity of a liquid culture or a suspension prepared from a solid culture must thus be applied to the slide with a loop and spread into as thin a layer as possible, in order to accelerate drying. Flaming to speed up drying is not recommended, since it promotes extraction of the magnesium ribonucleate.

3. Spreading the suspension on the slide. Traumatization of gram-positive bacteria may lead to loss of their ability to withstand decolorization. Over-vigorous rubbing of the suspension against the slide with the loop during preparation of the smear can cause a partial distortion of Gram staining.

4. Fixation. Specimens for Gram staining can be fixed only by flaming. Other fixation methods, particularly those involving methyl alcohol, mixtures of ethyl alcohol and ether, etc., may yield a distorted pattern. However, in heat fixation there is a direct relationship between the heating of the smear and the decolorization of gram-positive bacteria: the greater the heat to which the gram-positive cells are subjected, the larger is the number of gram-negative individuals found among them. Flaming must consequently be carried out carefully and sparingly.

5. Staining should begin only after the specimen has cooled completely. Pouring of the dye solution over a specimen still hot from fixation is not recommended.

Technology of Individual Stages in Gram Staining

Basic stain. Gentian violet was proposed as the initial Gram stain. It was later found that crystal violet and many other dyes belonging to the same group (Victoria blue, methyl violet, and brilliant green) may yield clearer results. The aqueous analine solution initially recommended for dissolving the stain was soon replaced by a 2-2.5% phenol

solution, which is stabler and simpler to prepare.

In addition to the analine or phenol solution, ammonium oxalate, ammonium sulfate, glycerine, boric acid, oxalic acid, or sodium bicarbonate was added to increase the staining power of the dye. However, it was found that the stain can be dissolved in distilled water quite as well as in an analine or phenol solution. In the method most commonly employed in the USSR, Sinev's technique (impregnation of pieces of filter paper with the stain for future use), the dye is extracted from the paper by rinsing it with distilled or tap water.

Mordant. The mordant initially recommended, iodine, is still an essential ingredient in all variants of Gram staining. However, under the influence of light and heat the iodine in Lugol's reagent is rapidly oxidized, regardless of its concentration, forming periodic acid (HIO_4); the presence of the latter makes the reagent completely unsuitable for Gram's method. In order to prevent this phenomenon Lugol's reagent should be stored in a yellow-glass bottle in a cool place; the reaction should be periodically checked and the reagent neutralized with sodium bicarbonate when an acid reaction appears. A still better solution is to replace the potassium iodide in this reagent with ten-normal sodium hydroxide.

The iodine used in Gram staining can be replaced by many substances - picric acid, bromine, ammonium picrate, alum, sodium chloride, ammonium sulfate, potassium permanganate, hydrogen peroxide, and mixtures of these compounds with iodine. All of them except picric acid are more effective than iodine in the Gram staining reaction.

Decolorization of gram-negative bacteria. Gram initially suggested absolute alcohol for this purpose. It was thought that the virtue of absolute alcohol lay in the fact that it would not decolorize gram-positive cells, even during prolonged exposure. However, absolute al-

cohol was gradually replaced in general practice by ordinary 96° alcohol, which is an equally effective decolorizer. Further dilution of the alcohol with water (to 40%) increases its decolorizing capacity. Hence it is clear that the change in alcohol concentration associated with certain techniques (e.g., immersion of a specimen carrying traces of Lugol's solution or rinse water in a beaker containing alcohol, the latter not being replaced for an extended period) has a strong influence on the results obtained. It is recommended that the preparation be thoroughly dried before decolorization in order to avoid this.

All alcohols can be used as decolorizers, but their decolorizing activity varies as a function of their molecular weight (methyl alcohol being the most active and amyl alcohol the least active). Acetone, either alone or mixed with alcohol, must be regarded as the most suitable decolorizer; it decolorizes gram-negative bacteria more rapidly and gram-positive bacteria more slowly than other agents.

Addition of iodine to the decolorizer (ethyl or methyl alcohol) makes it possible to avoid "over-decolorization" of gram-positive cells, even on prolonged exposure (Kaplan and Kaplan, 1933). Preston and Morrell (1962) detected a similar effect when iodine was added to acetone used as a decolorizer and suggested a modified staining method (see page 72) which they felt to be quite reliable for determining the true Gram-staining characteristics of bacteria. If gram-positive microbes stained a complimentary color are observed in using this modification they have actually lost their ability to stain gram-positive as a result of culture aging or for other reasons.

Complimentary staining of gram-negative bacteria. Gram originally suggested vesuvin as a contrast stain for the tissue and background. A wide variety of dyes, predominantly red, were later employed, although red does not contrast with violet. All such stains should be used in

0.5-1% solutions. Dilute Ziehl's carbol fuchsin is at present the most common stain, even though pyronine, vesuvin, and safranine (which stains gram-negative bacteria orange) have indisputable advantages (Hucker and Cohn, 1927).

Reagent-action time. The majority of authors have in the past recommended that treatment with the basic stain and iodine last for from 1 to 2-3 min, decolorization for approximately 30 sec, and complimentary staining for 5-10 sec. The "accelerated" variant developed by Klopshtok and Kovarskiy, in which each stage is reduced to 30 sec, is now quite common.

Rinsing the specimen. At first, almost no water was used in any stage except rinsing to remove the decolorizer and the final rinse after contrast staining. This was due to the fact that dye dissolved in an analine solution was precipitated by rinsing with water. However, many authors later avoided rinsing after application of the violet stain and iodine. As a result of the use of aqueous solutions of the basic stain or of paper-carried reagents prepared by Sinev's method, rinsing after each stage is now no longer contraindicated, but is to be recommended.

Factors affecting the results of Gram staining. Exposure of a culture to various substances or raising it on media containing impurities of these substances may cause gram-negative microbes to become gram-positive and vice versa. Variations in Gram staining may occur under physiological conditions, as well as when abnormal conditions are created. For each species of bacterium there is a definite maximum culture age at which the characteristic reaction of the species to Gram staining is most pronounced (Lasser and Schmidt, 1927). Young cultures of gram-positive bacteria are more resistant to decolorization than old cultures, although there are indications that a 48-hr culture is more

resistant than one of 24 hr. In especially important instances, particularly when determining the Gram staining of a newly described microbe, it is recommended that specimens for staining be prepared from cultures of three ages – 6-8 hr, 12-24 hr, and 48 hr.

Even the sight on the agar slant from which the specimen is taken may influence the results of Gram staining. A specimen prepared from material taken from the upper, already dry portion of the agar may yield completely different results from one taken from the lower, moister area.

Formulas for Stains and Reagents and Some Modifications of Gram Staining

The staining method proposed by Gram in 1884 is now never used in its original form, although some of its details were incorporated into the variant suggested by Weigert (1887); despite its imperfections and complexity, the latter method is still recommended by some. A modification of the Gram staining technique developed by many generations of practical workers has now come into general use.

Standard modification

Reagents:

A. Basic stain:

Crystal violet – 2 g

96° ethyl alcohol – 10 ml

1% aqueous phenol – 100 ml

B. Lugol's reagent:

Crystalline iodine – 1 g

Potassium iodide - 2 g

Distilled water – 300 (or 100) ml

C. Complimentary stain:

Ziehl's carbol fuchsin – 5 g

Distilled water – 95 ml

or:

Neutral red – 1 g

Distilled water – 100 ml

Steps in staining:

1. A piece of filter paper is placed over the fixed specimen and flooded with an excess of reagent A for 1-2 min.

2. This reagent is poured off and the specimen is rinsed with water and flooded with reagent B for 1-2 min.

3. Reagent B is poured off and the preparation is placed in 96° ethyl alcohol (still better, the alcohol is poured over the smear) for 30-60 sec.

4. The specimen is carefully rinsed with distilled water.

5. Complimentary staining with reagent C is carried out for 1-2 min and the specimen is rinsed and dried.

Hucker's modification

Reagents:

A. Basic stain:

10% alcoholic crystal violet – 20 ml

Ammonium oxalate – 0.8 g

Distilled water – 80 ml

B. Lugol's reagent (as in preceding modification)

C. Complimentary stain:

2.5% alcoholic safranine O – 10 ml

Distilled water – 100 ml

Steps in staining:

1. The specimen is stained with reagent A for 1 min.

2. It is rinsed with tap water for no more than 2 sec.

3. The preparation is placed in reagent B for 1 min.

4. It is rinsed with tap water and thoroughly dried.

5. The specimen is decolorized in 96° ethyl alcohol, being gently swished about, and thoroughly dried.

6. Staining with reagent C is carried out for 10 sec.

7. The specimen is rinsed, dried, and examined under the microscope.

Burke's modification

Reagents:

A. Basic stain:

Solution No. 1. Crystal or methyl violet – 1 g

Distilled water – 100 ml

Solution No. 2. Sodium bicarbonate – 1g

Distilled water – 20 ml

B. Lugol's reagent (with proportions of 1:2:100)

C. An acetone-ether mixture (in proportions of 1:1 or 3:1)

D. Complimentary stain:

Safranine O – 2 g

Distilled water – 100 ml

Steps in staining:

1. The smear is air-dried without heating.

2. The specimen is treated with reagent A: solution No. 1 is poured over the slide and 2-3 drops (or more, depending on the size of the area to be stained) of solution No. 2 is added, the two being permitted to act for 2-3 min.

3. The smear is rinsed with reagent B.

4. The preparation is flooded with reagent B for 2 min or more.

5. The smear is rinsed with tap water, which is then removed with filter paper, although the preparation is not dried. In the author's opinion, the extent to which the water is removed is of great impor-

tance: it is necessary to eliminate the free water from the surface of the smear without permitting the cells to dry out.

6. The specimen is immediately decolorized with reagent C, which is applied drop by drop until the stain stops coming off, which usually requires less than 10 sec.

7. The smear is air-dried.

8. Staining with reagent D is carried out for 5-10 sec.

9. The specimen is rinsed with tap water, dried, and examined under the microscope.

Kopeloff and Beerman's modification

Reagents:

A. Basic stain (as in preceding modification)

B. Mordant:

Iodine - 2 g

One normal sodium hydroxide (40.1 g in 1 liter) - 10 ml

Distilled water to make 100 ml is added after the iodine has dissolved.

C. Decolorizer (as in preceding modification).

D. Complimentary stain:

Basic fuchsin - 0.1 g

Distilled water - 100 ml

Steps in staining:

1. As in preceding modification.

2. Solutions Nos. 1 and 2 are first mixed in a ratio of 1.5:0.4 and the specimen is treated with reagent A for 5 min or longer.

3. The smear is rinsed with reagent B.

4. The specimen is treated with reagent B for 2 min or longer.

5. In contrast to the preceding modification, the preparation is not washed after exposure to the iodine; the excess reagent B is re-

moved and decolorization is immediately begun.

6. As in preceding modification.

7. As in preceding modification.

8. The specimen is stained with reagent D for 1-2 min.

9. The smear is rinsed, dried, and examined under the microscope.

A. Sinev's modification

A. Sinev suggested that, instead of using the basic stain in solution form, pieces of filter paper be impregnated with a 1% alcoholic solution of the stain, dried, and cut into strips somewhat narrower than a standard slide. During staining distilled water is poured over the slide and the dye-impregnated paper is immersed in it. The water extracts the stain from the paper. After 1-2 min the paper is removed, the specimen is rinsed with water, and subsequent treatment is carried out as outlined in the standard modification. Stain-bearing papers can also be prepared for the complimentary stain, impregnating filter paper with 1% alcoholic fuchsin or safranine.

G.P. Kalina's modification

The basic principle of this method is combination of several stages of the Gram method; the possibility of "over-decolorization" is eliminated.

Reagents:

A. Basic stain: 0.5% alcoholic crystal violet or brilliant green - 5 ml (stored in a test-tube with a rubber stopper).

B. A reagent combining the mordant, decolorizer, and complimentary stain for gram-negative bacteria:

0.5% alcoholic potassium iodide - 96 ml

5% alcoholic basic fuchsin - 2 ml

5% alcoholic iodine - 2 ml

The potassium iodide is first dissolved by heating to the boiling

point in a water bath with constant agitation. The fuchsin and iodine solutions are then added. The reagent is stored in a yellow-glass bottle with a ground or rubber stopper.

Steps in staining:

1. (A combination of preparation of the specimen and basic staining.) A small drop of distilled water (not physiological solution!) is applied to a cleaned, flamed, and cooled slide with a loop and a small quantity of the agar culture is added to it, but not mixed with it. In preparing a smear from a liquid culture a small drop is applied to a slide with a loop. The basic stain (reagent A) is then applied with the same loop and the mixture is spread over an area of approximately 1 cm^2. The preparation is dried at room temperature and slowly fixed by a single pass through a Bunsen-burner flame. As many as 8 smears can be prepared on the same slide, but they must be separated by lines drawn with a grease pencil on the face of the slide.

Preparation of a specimen from a liquid culture or liquid pathological material requires an additional step: after the smear is fixed it is immersed for several seconds in a beaker containing distilled water in order to remove the salts remaining on the slide and is then carefully dried with filter paper.

2. After a specimen from an agar culture has cooled or one from a broth culture has dried an excess of reagent B is applied in such fashion that it covers the entire surface of the slide. The optimum reagent-action time is 1/2-1 min, but it can be prolonged to 3-5 min without distorting the final results. After staining the preparation is washed with running (preferably distilled) water from a wash bottle, directing the stream at the slide and gradually turning it from the horizontal to the vertical position while continuing the rinsing process. The specimen is dried by any available method (with filter paper, in air or a

heater, or over a Bunsen-burner flame) and examined under the microscope. Gram-positive microorganisms appear dark violet or green and gram-negative microorganisms light red.

Preston and Morrell's modification

Reagents:

A. Basic stain:

Crystal violet - 2 g

Alcohol - 20 ml

1% aqueous ammonium oxalate - 80 ml

B. Fortis' iodine reagent:

Crystalline iodine - 10 g

Potassium iodide - 6 g

Alcohol - 90 ml

Distilled water - 10 ml

C. Decolorizer (iodine-acetone):

Fortis' iodine reagent - 3.5 ml

Acetone - 96.5 ml

D. Complimentary stain:

Ziehl-Neelson's carbol fuchsin - 5 ml

Distilled water - 95 ml

Steps in staining:

1. The specimen is stained with reagent A for 30 sec.
2. The stain is washed off with Lugol's solution.
3. The specimen is treated with Lugol's solution for 30 sec.
4. The Lugol's solution is washed off with the iodine-acetone mixture.
5. The specimen is decolorized with the iodine-acetone mixture for 30 sec.
6. The smear is rinsed with water.

7. Staining with the complimentary stain is carried out for 30 sec.

8. The specimen is rinsed, dried, and examined under the microscope.

The time for which each reagent is permitted to act can be increased to 1 min without distorting the results. All the reagents are poured over the slide to as to cover its entire surface.

Bartholomew's modification

Reagents:

A. Basic stain (see Hucker's modification, page 67).

B. Lugol's solution (in proportions of 1:2:100).

C. Decolorizer – n-propyl alcohol.

D. Complimentary stain – 0.25% aqueous safranine.

Steps in staining:

1. The specimen is stained with reagent A for 1 min.

2. The smear is rinsed for 5 sec by immersion in a 250 ml graduate containing running water (30 ml per second).

3. The excess water is removed with Lugol's solution.

4. The preparation is flooded with Lugol's solution for 1 min.

5. The smear is rinsed for 5 sec, using the same procedure as in step 2.

6. The moist specimen is decolorized in n-propyl alcohol, being successively passed through three dishes of alcohol; it is left in each dish for 1 min (this time can be reduced to 30 sec). After 10 smears have been decolorized the first dish is replaced by the second and the second dish by the third, while the first dish is filled with fresh alcohol and placed in the third position.

7. After decolorization the smear is washed for 5 sec, using the same procedure as in step 2.

8. The excess water is removed with the complimentary stain D.

9. Staining with the complimentary stain D is carried out for 1 min.

10. The specimen is rinsed, dried, and examined under the microscope.

USE OF LUMINESCENT SERA FOR DIAGNOSING AND INDICATING THE CAUSITIVE AGENTS OF INFECTIOUS DISEASES

T.I. Bulatova, Candidate of Medical Sciences, and M.Ya. Korn, Candidate of Medical Sciences

The serum-luminescence method is based on the fact that a fluorescent dye attached to the antigen molecule makes the antigen-antibody reaction visible in the luminescence microscope. In contrast to other serological reactions, in which the combining of the antigen with the antibody is evaluated from some secondary phenomenon which it evokes (agglutination, precipitation, etc.), the serum-luminescence method makes it possible to observe the reaction directly and thus to establish the presence and localization of the antigen. Using this technique, we can observe microscopically the attachment of luminescent antibodies to the homologous superficial antigens of individual bacterial cells, even in mixed cultures.

Although the serum-luminescence method makes it possible to detect isolated microbes in the visual field of the luminescence microscope, the concentration of the causitive agent in the material to be investigated should be rather high. This is due to the fact that we see in the visual field only a small portion of the material applied to the slide as a smear, print, or section.

Various luminescent dyes are used for attachment to the antibody molecules, the most common being fluorescein, rhodamine, and 5-dimethyl-1-naphthylene sulfochloride. Since these dyes will not react directly with a protein, their isocyanates, isothiocyanates, and sulfochlorides are used.

Attachment occurs at the carbamide bonds of the protein molecules. Antibody molecules treated in this manner retain their serological specificity.

The negligible quantity of fluorochrome bonded to the protein molecule, and thus the latter itself, can easily be detected from its bright luminosity in the luminescence microscope.

Preparation of Luminescent Sera

The technique of preparing luminescent sera is based on the method developed by Kuhns and Kaplan (1950), as modified by Soviet researchers.

The preparation of luminescent sera consists of four stages:

a) extraction of the globulin fraction from native sera, a step omitted if whole serum is used for tagging;

b) combination of the globulin fraction with the fluorochrome;

c) removal of the unbonded dye from the tagged globulins;

d) adsorption (specific and nonspecific) of the fluorescent serum.

Extraction of the globulin fraction. An equal volume of a saturated ammonium sulfate solution is added to serum diluted 2:1 with distilled water, stirring constantly. After being left to stand in a refrigerator at 4° for 30 min the mixture is centrifuged at 3000 rpm for 20 min. The supernatant is poured off and the residue is dissolved in distilled water to the original serum volume and reprecipitated with saturated ammonium sulfate. The latter is taken in a volume equal to that of the distilled water used to dissolve the residue. A third precipitation is then carried out in the same manner, the residue being the globulin fraction.

The final precipitate is dissolved in a small quantity of distilled water and transferred to a cellophane bag for removal of the ammonium sulfate. Dialysis is carried out against 0.15 M sodium chloride, with a 0.1 M phosphate buffer at pH = 7.4, and lasts 4-5 days at temp-

eratures of 0-4°. The point at which dialysis should be terminated is determined by an ammonia test with Nessler's reagent.

This procedure is best carried out under constant agitation with an electromagnetic or other mixture, changing the solution twice a day, in which case it lasts only 2-3 days.

When dialysis is complete the protein content of the globulin solution is determined by the usual Kjeldahl micromethod or by the Folin-Chiocalt method. This content should be no less than 20 mg/ml and if such is not the case the solution is concentrated by hanging the cellophane bag containing it in a refrigerator and blowing air past it with a table fan. After the solution volume has been reduced the protein concentration can be recalculated by taking into account the decrease in volume and assuming that the total quantity of protein has remained constant. As a check, the protein concentration can again be determined by the aforementioned methods.

After dialysis the serological properties of the serum are checked and compared with the titre of the original serum (agglutination titre, precipitation titre, or number of antitoxin units [AU] per ml). Under normal conditions, the titre should not be lower than that of the initial serum.

Combination (Conjugation) of the Protein and Fluorochrome

Combination (conjugation) of the protein and the fluorochrome (most frequently fluorescene isocyanate or isothiocyanate) is carried out by Kuhns' method, in a reaction mixture with the following composition:

a) the globulin, calculated in terms of dry weight, should constitute 1% of the total mixture;

b) a carbonate buffer solution at pH = 9.0 - 15% of the total mixture volume;

c) dioxane - 15%;

d) acetone – 7%;

e) fluorescene isocyanate – 5 mg for each 100 mg of globulin;

f) sufficient 0.15 M sodium chloride to bring the reaction-mixture volume to 100%.

For example, 500 mg of globulin in 20 ml of solution is to be tagged. The total volume of a reaction mixture with a 1% globulin content should be 50 ml (the specific gravity of the mixture is assumed to be 1). The fluorescene isocyanate (calculated in terms of dry weight) should constitute 5%, i.e.,

$$\frac{500 \cdot 5}{100} = 25 \text{ mg}.$$

If 12.5 mg/ml of fluorescene isocyanate is present in the dioxane-acetone solution, the volume of the latter is 25/12.5 = 2 ml. The volume of carbonate-bicarbonate buffer should be

$$\frac{50 \cdot 15}{100} = 7{,}5 \text{ ml},$$

The amount of dioxane present should be 7.5 ml (15%), but 0.66 ml (the dioxane constitutes 1/3 of the volume of the stain solution) must be subtracted from this, so that 7.5 – 0.66 = 6.84 ml of pure dioxane is required. The quantity of acetone needed is

$$\frac{50 \cdot 7}{10} = 3{,}5 \text{ ml},$$

from which 1.34 ml (2/3 of the volume of the dioxane-acetone mixer is acetone) must be subtracted, so that the volume of acetone required is 3.5 – 1.34 = 2.06 ml.

The volumes of the reaction-mixture components listed above are as follows:

Globulin – 20 ml

Fluorescene isocyanate – 2 ml

Carbonate-bicarbonate buffer - 7.5 ml

Dioxane - 6.84 ml

Acetone - 2.06 ml

Total 38.40 ml

To make the total reaction-mixture volume, which was calculated above to be 50 ml, 50 - 38.4 = 11.6 ml of 0.15 M sodium chloride must be added. All the components of the mixture must be combined cold in the following order.

The sodium chloride solution, buffer, dioxane, and acetone are poured into a flask. The globulin solution and stain are then added drop by drop to the cooled mixture. The dye should be added especially slowly, over a period of 20-30 min. A precipitate may be formed after the dye is added to the solution.

The mixture is kept in a refrigerator for 18-20 hr, being continuously agitated by an electromagnetic or other mixer. If a precipitate formed in the solution during preparation, it disappears after 18 hr of agitation.

Separation of unbonded stain from the tagged globulins

Unbonded fluorescene isocyanate is usually present in the luminescent serum. In order to remove it the serum is dialyzed for 8-12 days against 0.15 M sodium chloride with a 0.01 M carbonate buffer at pH = = 8.6-9.0; the solution is changed no less than twice a day.

The extent to which the unbonded fluorochrome has been removed can be checked by fluorometry or determined qualitatively in ultraviolet light: 10 ml of the dialysate to be studied is poured into one test-tube, while a 1:20,000,000 solution of sodium fluorescene is poured into a second (as the control).

Both test-tubes are examined in ultraviolet light. If the greenish luminescence of the dialysate is no stronger than that of the control

solution dialysis is complete.

Some researchers recommend that the tagged globulin be reprecipitated 2-4 times with saturated ammonium sulfate after 2-3 days of dialysis. This process is repeated until the supernatant liquid no longer fluoresces. After the last reprecipitation dialysis is carried out for 4-5 days against a buffer solution at pH = 9.0 in order to remove the ammonium sulfate.

Reprecipitation can also be conducted with ethyl alcohol or acetone.

Dialysis at pH = 9.0 prevents precipitation of part of the gamma-globulins, which gradually go out of solution if dialysis is conducted at pH = 7.4-7.6. However, at pH = 9.0 the fluorescent sera produce a powerful background luminosity, which reduces the intensity of the microbial luminescence. In addition, nonspecific microbial luminescence is also observed. It is consequently recommended that dialysis be terminated at pH = 7.4 with 2 or 3 solution changes. This pH is within the optimum limits for immunochemical reactions. After dialysis the serum is concentrated to a given protein content (2-5%) by blowing air past the cellophane bag at a temperature of 4°.

Adsorption of Fluorescent Sera

The dialyzed luminescent serum may still contain certain fluorescent substances which induce nonspecific luminescence in microorganisms, tissues, or other subjects investigated by this method.

It is recommended that the luminsecent serum be adsorbed with powdered mouse liver or activated charcoal in order to eliminate this type of nonspecific luminosity.

Methods have now been developed for the rapid production and purification of small quantities of luminescent serum, using molecular filters (Sefadex) and DEAE cellulose. Mouse liver is most often used for

adsorption.

After removal of the gall bladder the livers of white mice (or other animals) known to be healthy are repeatedly washed with physiological solution, ground to a pasty consistency in a mortar, and rinsed 3-4 times with 6-8 volumes of physiological solution by centrifuging or resuspension to remove the hemoglobin and other soluble proteins.

Two volumes of physiological solution and four volumes of acetone are poured over the liver mass and the mixture is agitated for 20-30 min and then left to stand or centrifuged.

The centrifugate is poured off and the residue is again rinsed 2-3 times with 6-8 volumes of physiological solution and then treated once more with acetone and physiological solution.

The residue is diluted with 4 volumes of acetone, agitated for 10-15 min, and filtered through a double paper filter in a Buchner funnel. The liver mass is dried on the filter in a heater and ground to a fine powder.

A total of 60-80 mg of powder per ml of luminescent serum is used for adsorption. The powder is a strong adsorbant, so that addition of large quantities extracts both the nonspecific components and specific antibodies of the serum.

After a thorough shaking the mixture is kept at room temperature for one hour and then centrifuged or filtered. Filtering can be carried out through a Zeitz filter or dense vegetable-pulp paper. During treatment with the powdered liver the serum loses 30-50% of its volume. The filtered serum is poured into ampules under sterile conditions. Before filtration 1% twice redistilled boric acid or 0.01% merthiolate is added as a preservative.

In addition to species-specific antibodies, some sera contain group antibodies to related species of pathogenic and nonpathogenic

bacteria. For example, anthrax serum may "specifically" stain strains of Bacillus anthracoides and Bacillus pseudoanthracis, while types A and B botulin sera can stain certain strains of Cl. sporogenes.

In order to remove these group antibodies luminescent sera are adsorbed by Castellani's method with those microbes which produce group luminescence. To 1 ml of luminescent serum with a protein content of 10 mg/ml is added 50-60 billion live bacteria or bacteria killed by heat or some other method. The procedure used to prepare the bacteria for serum adsorption does not alter their antigenic composition.

The serum is added to the bacterial residue and the two are carefully mixed and kept for 3-4 hr in a heater at 37° with constant agitation. The mixture is then held at 4° for 20 hr and the serum is centrifuged or filtered. After filtration the quality of the luminescent serum is checked by treating homologous and heterologous strains having related antigens with various serum dilutions.

It is recommended that strains morphologically similar to the microbe corresponding to the luminescent serum and bacteria from environmental objects usually subjected to laboratory examination for diagnostic and indicative purposes (fecal matter, soil, food stuffs, oral smears, air) be used to check the serum.

Native sera may be adsorbed with certain microbes before tagging in order to remove group antibodies. The adsorption method is the same as for luminescent sera. Adsorption with dry powders prepared from the bacteria by precipitation with acetone is the simplest and most convenient method.

Serum adsorption is a rather tedious process and requires great care; the volume of the initial serum, its protein content, the number of bacteria used, etc. must be taken into account. The adsorption principles discussed above are observed for all types of sera, but the in-

dividual stages of the process and the ratio of the quantity of serum to the number of bacteria may vary.

Preparation of Specimens

Preparation and fixation of smears. A drop of the material to be studied is applied to a thoroughly degreased slide. In investigating water or other liquids smears are best prepared from the residue left after centrifuging or from material washed from a membrane filter through which the liquid has been passed. The surface through which the liquid was filtered is pressed tightly against the slide. The bacteria from the filter can also be transferred to a nutritive medium to increase the culture size. This raises the sensitivity of the serum-luminescence method, but prolongs the investigation. Material washed from various environmental objects is best centrifuged and the specimen then prepared from the residue. Smears can be made from the pads with which the material is washed off. Soil, foodstuffs, and other solids are suspended and the suspension is then filtered through a cotton-gauze filter or filter paper in order to remove any large particles. Specimens are prepared directly from the filtrate or from the residue left after centrifuging. In order to facilitate subsequent manipulations (fixation and washing) the smears should be made near one end of the slide. The dried specimen is fixed in ethyl alcohol (10-15 min), Carnoy's solution (10-15 min), methyl alcohol (3-5 min), or certain other fixatives. It can also be fixed by flaming, but this method must be used with care, since overheating can lead to nonspecific results.

Treatment of smears with the fluorescent serum. There are two methods for employing fluorescent antibodies: a direct procedure and several types of indirect procedures.

In the direct method a smear of the material to be studied is treated with a fluorescent serum prepared from specific homologous im-

mune serum.

In the simplest variant of the indirect method (two-step) the smear is first treated with ordinary nonfluorescent diagnostic serum. If this serum corresponds to the antigen present in the smear it combines with it. In order to show this reaction the smear is treated with fluorescent serum obtained from specific immune antiserum for the species of animal used to produce the nonfluorescent diagnostic serum.

For example, if the smear is treated with nonfluorescent typhoid serum obtained from a rabbit, fluorescent antirabbit ser hould be used for the subsequent treatment. Thus, in the indirect method the antigen is detected by means of the antibodies of diagnostic sera which enter into an immunochemical bond with it. Several species of animals (rabbits, horses, cattle) are used to obtain diagnostic sera and the indirect method consequently involves several (2-3) luminescent antisera.

Goldwasser and Shepard proposed a three-step variant of the indirect method which requires only one (anticomplementary) luminescent serum. This method is a luminescence-microscopic modification of the complement-fixation reaction and consists in the following: the specimen is treated successively or simultaneously with specific immune serum and complement (guinea-pig serum). If an antigen-antibody reaction takes place the complex adsorbs the complement, which is then labeled with the luminescent serum containing the antibody to it. This serum is obtained by immunization of animals with guinea-pig serum.

In still another variant of the indirect method the preparation containing the bacterial cells is treated with a specific bacteriophage. After the bacteriophage is adsorbed on the cells it is labeled with luminescent antiphage serum.

The specimen is treated in the following manner: the fixed smear

is outlined with a grease pencil, since it usually is difficult to see after it is reacted with the serum and washed.

In the direct method a drop of fluorescent serum diluted with physiological solution is applied to the smear with a Pasteur pipette. The working (optimum) solution dilution is generally indicated in the instructions or selected by the experimenter in preliminary tests for each new series of serum.

The specimen, with the drop of fluorescent serum, is placed in a moist chamber (Petri dish), whose bottom is flooded with water. The slide rests on cross-pieces fashioned from glass tubing; each dish can hold as many as 4 slides. The staining time is usually 15-20 min at room temperature or 5-10 min at 37° in a heater and is generally indicated in the instructions for using the fluorescent serum. The serum cannot be permitted to dry on the slide, since this leads to nonspecific adsorption and may distort the results of the investigation.

Washing the smear. In order to remove the excess fluorescent serum the smear is washed in physiological solution or in a carbonate-bicarbonate buffer at pH 9.0 for 15 min at room temperature, changing the solution 1-2 times.

It is substantially more rapid to wash the smear in running tap water for 5-7 min. This is done quite conveniently in an apparatus specially designed for this purpose (Fig. 9). As many as 20 smears can be simultaneously washed in it.

The washed specimens are dried in air or in a heater and examined under the luminescence microscope.

Treatment of specimens by the indirect method proceeds as follows: 2-3 drops of ordinary untagged diagnostic serum diluted to diagnostic titre are applied to the dried fixed smear, which is then kept in a moist chamber for 15-20 min at room temperature or 37°. The excess un-

tagged serum is washed off by the method described above. After drying, the luminescent antiserum is applied to the smear. Subsequent treatment is conducted in the same manner as for the direct method.

Fig. 9. Bath for washing smears.

The dried smears, whether treated by the direct or indirect method, are covered with a cover glass in physiological solution or, still better, in a mixture of neutral glycerine (9 parts) and physiological solution (1 part). The excess liquid must be carefully removed with filter paper. They are then examined under a luminescence microscope or an ordinary microscope with a luminescence attachment.

Technique of Luminescence Microscopy in Working with Luminescent Sera

The finished specimen is examined under the luminescence microscope with an immersion objective, in incident blue or ultraviolet light. An ordinary biological microscope can also be used in conjunction with a type OI-17 or OSL-1 special luminescence attachment. The MLD commercial luminescence microscope provides simplicity and convenience in working with luminescent sera; its principal advantage is the fact that it furnishes a substantially brighter image than the ML-1 or ML-2 and is portable.

In luminescence microscopy the light source should have sufficient

power and a high surface intensity and radiate maximum energy in the blue-violet and near ultraviolet regions of the spectrum. These requirements are satisfied to some extent by carbon-arc lamps, iron-electrode arc lamps, low-voltage incandescent bulbs operating at red heat, and, especially, ultrahigh-pressure mercury-quartz lamps.

The majority of Soviet researchers use SVD-120A and SVDSh-250-3 (DRSh-250) ultrahigh-pressure mercury-quartz bulbs in conjunction with Soviet-produced OI-18 lamps (Fig. 10) and ML-1, ML-2, and MLD luminescence microscopes.

Fig. 11. Bacillus anthracis (vaccine strain STI) treated with luminescent serum, × 900.

TABLE 1

Relative Spectral Distribution of the Energy Radiated by Ultrahigh-Pressure Bulbs

1 Длина волны, mμ	Относительная энергия, % 2	1 Длина волны, mμ	Относительная энергия, % 2
218,2	28,1	365,0	100,0
289,5	9,1	373,0	16,2
296,0	25,2	405,5	64,1
301,0	31,2	436,5	73,1
302,0	38,3	546,5	77,3
313,8	68,1	578,0	86,2
334,5	19,9	605,0	7,3

1) Wavelength, mμ; 2) relative energy, %.

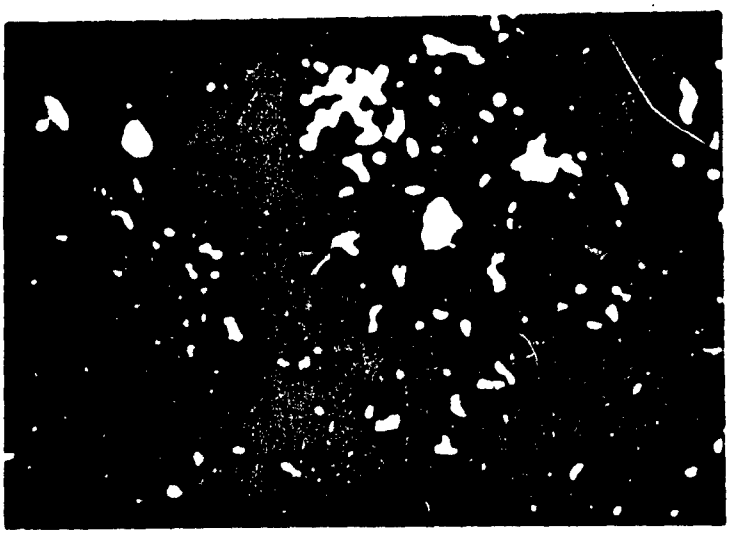


Fig. 12. Rickettsia prowazeki treated with luminescent serum, × 900.

Fig. 13. Cl. botulinum treated with luminescent serum, × 900.

Table 1 contains guideline data on the relative spectral distribution of the energy radiated by ultrahigh-pressure bulbs (the energy radiated at 365 mμ is taken as 100%). It is reproduced from the 1959 catalog of the Moscow Electric-Bulb Plant.

It must be noted that the bulbs mentioned above are not equivalent.

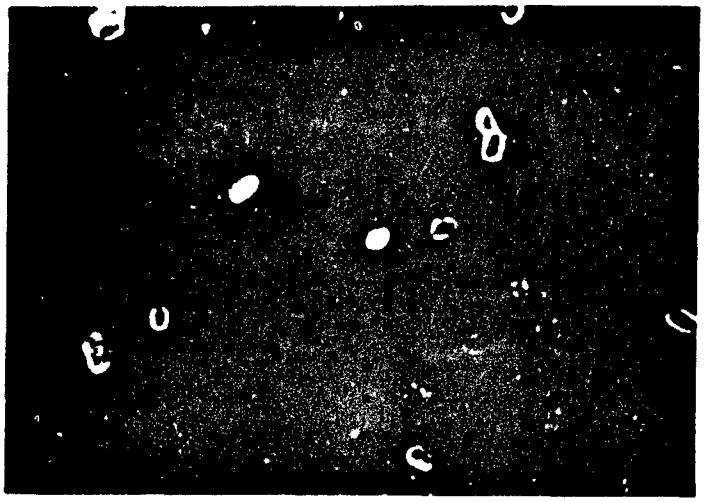


Fig. 14. Shigella dysenteriae treated with luminescent serum, × 900.

This is especially true of surface intensity, that of the SVD-120A clearly being rather low for working with luminescent sera.

Judging from its description (1959 catalog, 1st Moscow Electric-Bulb Plant), the DRSh-100 ultrahigh-pressure mercury-quartz bulb may be more suitable than the SVD-120A. However, they have a drawback in that they must be plugged into dc mains, with the obvious result that they have not come into wide practical use.

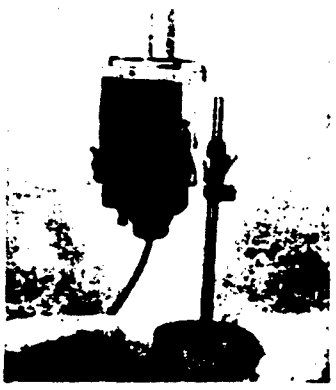


Fig. 10. OI-18 lamp re-equipped to take a SVDSh-250-3 bulb.

It is best to replace the SVD-120A bulb with the SVDSh-250-3 (DRSh-250), for which the OI-18 lamp can be modified. This lamp is completely suitable for working with luminescent sera.

The near ultraviolet or blue-violet region of the spectrum is used to excite fluorescence in luminescence microscopy. Both methods have their advantages

and drawbacks. Ultraviolet excitation may evoke the natural fluorescence of biological specimens; correct color transmission is maintained, but this is not important in working with luminescent sera.

The principal advantage of blue-violet light for exciting luminescence when using fluorescent sera lies in the brighter luminescence produced. This is due both to the fact that far more light is passed by blue-violet than ultraviolet filters and that, in accordance with S.I. Vavilov's law, fluorescence is intensified as the wavelength of the exciting light increases, since the absorption maximum of fluorescein lies at 490 mμ and that of luminescein at 585 mμ.

Glass filters are usually used to isolate the required portion of the spectrum. In order to absorb the excess light excited by luminescence, which passes through the microscope optics and alters the luminescence observed, a yellow filter is inserted into the eyepiece, absorbing the excess and passing the luminescent light. Thus, the cross-filter method is generally used in luminescence microscopy.

Various Soviet-produced filters, alone or in combination (FS-1, SS-4 + SS + 8, etc.), can be used to isolate the blue-violet portion of the spectrum.

In addition to filters which isolate the appropriate portion of the spectrum, filters which absorb the infrared and red radiation and keep the optics and specimen from overheating and a filter which absorbs short-wave ultraviolet radiation and so reduces the discoloration of the specimen are necessary.

The most acceptable results are obtained with a combination of a SZS-7 heat-protective filter, a FS-1 violet filter, a BS-8 short-ultraviolet-absorbent filter, and a T-2N yellow ocular tartracine filter. The T-2N has now been replaced by a filter formed by combining ZhS-18 and ZhS-19 filters, which is more stable with respect to ultraviolet and

blue-violet light.

Light-field and dark-field illumination with transmitted or incident light can be used to excite luminescence in the luminescence microscope.

The majority of Soviet researchers illuminate the specimen with incident light, using an opaque lamp with an interference light-separating plate. This illumination system has a number of advantages, including lower losses in the light produced by the source, an improved spectral composition, and only slight direct incidence of the light on the observer's eye. In addition, since the objective also serves as the condenser, the illumination of the specimen increases with its aperture and thus with its magnification. This is of especially great importance in studying very small microorganisms (Rickettsia etc.), where powerful immersion objectives must be used.

Ordinary glass microscope objectives can be employed for observing luminescence excited by the near-ultraviolet or blue-violet regions of the spectrum. Achromats are preferable to chromatic lenses, since the latter may exhibit natural fluorescence (Richards). The best results can be obtained by using special Soviet-produced luminescent objectives with no natural luminescence (as indicated by the letter L on their housings).

Luminescence microscopy can easily be conjoined with the phase-contrast method.

Simultaneous observation of specimens by phase-contrast and luminescence microscopy is of especially great value in working with luminescent sera, since it permits demonstration of the presence of the causitive agent in cases where it has not fixed the fluorescent antibody.

One of the signs which distinguishes the specific bonding of lum-

inescent antibodies to the corresponding antigen at the cell surface from autofluorescence or simple adsorption of the dye is the presence of a luminous ring around the cell. This luminescence is referred to as specific (Leptospirae are an exception, since when they are stained with homologous luminescent serum there is no luminous ring, the entire cell being stained; Figs. 11, 12, 13 and 14).

In addition to a sufficiently high objective aperture and magnification, the eyepiece magnification is of great importance in determining the specific character of the luminescence of extremely small microorganisms (Rickettsia, Brucella, and the causitive agents of tularemia and whooping cough). When the elimination is sufficient it is best to use a 7-10 power eyepiece. Low eyepiece magnifications can lead to inaccurate evaluation of the specimen's luminescence.

In the luminescence microscopy of microorganisms treated with luminescent sera a sufficiently clear image can be obtained only by using a high-quality nonfluorescent immersion oil. In addition to having proper indices of refraction and dispersion, the oil must be chemically inert and not affect the microscope optics. It should be noted that the dispersion of the oil is evidently of no great significance in working with specimens treated with serum tagged with fluorescein isocyanate, since the luminescence is more or less monochromatic.

A number of substitutes for nonfluorescent immersion oil have been suggested, but they do not all satisfy the aforementioned requirements. Specifically, dimethyl phthalate, which is recommended by many researchers, quickly dissolves Canadian balsam and can consequently cause deterioration of the specimen.

Use of ordinary cedar oil may lead to erroneous conclusions regarding the character of the specimen's fluorescence, since it alters the character of the luminescence observed when employed as a substi-

tute for nonfluorescent oil.

Water-immersion objectives, particularly those included with the ML-1 and ML-2 microscopes, can be used instead of oil-immersion lenses for luminescence microscopy. It must be noted that these microscopes are the most suitable for luminescence microscopy, permitting observation of fluorescence in transmitted and incident light, phase-contrast and luminescence studies, and microphotography of luminescent specimens.

The most important factor in the production of comparable results by different authors in working with luminescent sera is objective evaluation of the intensity of the exciting light and of the specimen's luminescence. Both these factors are now usually appraised visually. The specific luminescence of bacteria is currently evaluated on the 4-plus scale:

++++) bright-green radiant luminescence at the periphery of the cell, resembling a luminous capsule.

+++) bright-green luminescence at the periphery of the cell.

++) moderate yellowish-green luminescence at the periphery of the cell.

+) weak yellowish-green luminescence at the periphery of the cell.

−) cells seen as barely detectable shadows.

If the entire cell exhibits uniform bright-green or yellowish-green luminescence, this is regarded as a sign of nonspecific adsorption of the fluorescent serum. The further development of the luminescent-antibody method must include the divising of attachments for the luminescence microscope which will permit objective evaluation of these factors.

The advantage of the serum-luminescence method over other serological reactions lies in the fact that we can use it to evaluate the pre-

sence, concentration, and localization of the antigen in question in cells and tissues and simultaneously study the morphology of the causative agent under investigation. The presence of other antigens (naturally within the limits of serum specificity) presents no obstacle to its employment.

At the same time, use of this method for accelerated diagnosis of infectious diseases is not always effective: its high sensitivity and the conditions under which the specimens (sera and stains) are prepared lead to a substantial number of nonspecific reactions.

Such reactions may develop for the following reasons:

a) nonspecific adsorption of a tagged protein which does not have antibody properties on a fixation-altered antigen;

b) the presence of "normal" or heterogeneous antibodies in the tagged immune serum;

c) an antigenic similarity between different microorganisms not detectable by the usual serological methods because of their low sensitivity.

Nonspecific reactions arise under the following conditions: during treatment of the serum with the fluorescent stain the latter also attaches to nonantibody protein molecules, this being true even for the globulin fraction, which contains a large number of antibody molecules. Any high-molecular protein compound can easily be adsorbed on various substances, regardless of their antigenic structure. For example, the interaction between the tagged fluorescent protein and the specimen may depend on the difference in charge. Thus, Meyersbach showed that the acid proteins of the antibody and the alkaline proteins of the tissue under investigation interact. The capacity of various tissues for nonspecific protein adsorption has been used to differentiate normal and malignant tissues, which have been found to differ in their adsorption

of fluorescent albumin.

Similar nonspecific adsorption of tagged serum on various particles evidently also occurs in attempts to detect different causative agents in the feces themselves, although in this case the luminescence of a large number of different particles is also attributable to the presence of brightly fluorescing porphyrin compounds.

The fact that smears and prints prepared on slides and fixed by various methods are usually used to detect a causitive agent by the serum-luminescence method is of great importance in the development of nonspecific fluorescence associated with adsorption.

Crude or incomplete fixation can lead to denaturation, destruction, or elution of the antigen, which in turn causes a substantial increase in the number of nonspecific reactions. For example, such reactions are often observed when bacterial smears are overheated during flame fixation.

Another factor which causes nonspecific staining is the presence of so-called normal, or natural antibodies in the serum. In particular, antibodies of this type to Staphylococcus occur widely in immune sera. Consequently, erroneous results can easily be obtained when Staphylococcus is present in the material under investigation and is morphologically similar to the causative agent sought (as in investigating oral material in the presence of the causative agent of pertussis).

Errors may also arise in using the serum-luminescence method (when the luminescent serum has a low specificity) to detect infectious agents with an antigenic similarity to saprophytes which may also be present in the material under investigation.

Nonspecific adsorption of various fluorescent components of tagged immune serum can also be attributed to technical errors committed in combining the serum and the fluorescent dye and in treating the speci-

men with the serum. Nonspecific staining is observed: a) when the serum is insufficiently purified of free (unbonded) fluorochrome or when the latter is present in the serum as a result of dissociation of the protein-dye complex; b) when insufficiently diluted luminescent serum is used; c) when the specimen is treated too long with the luminescent serum; d) when the luminescent serum is not fully washed from the specimen.

A number of controls must be set up in order to be certain of the specific character of the staining observed. These include:

a) treatment of specimens known to contain the causative agent sought in pure culture with the serum in question;

b) treatment of the specimens under investigation with luminescent sera heterologous to causative agents not having antigens in common with that sought;

c) treatment of specimens containing a heterologous causative agent with the serum in question.

As an additional control specimens prepared from cultures from environmental objects or excreta usually subjected to examination but known not to contain the causative agent sought can be treated with the serum.

Some authors have proposed sequential or simultaneous treatment of specimens with homologous nonfluorescent and fluorescent sera, but this does not produce the expected decrease in luminescence and so cannot be recommended as a control.

One important factor which current complicates objective evaluation of the results obtained is the lack of standard methods for comparing the intensity of the bacterial fluorescence observed in the luminescence microscope. As was pointed out above, this comparison is made visually, on the 4-plus scale, which requires a certain skill.

Comparison of the results obtained by different researchers is hampered by the fact that the intensity of the exciting light varies from microscope to microscope and is not yet accessible to objective evaluation.

The foregoing enables us to assign the serum-luminescence method to a purely guideline role among other bacteriological-diagnosis techniques, permitting quite rapid establishment of the primary direction for further investigation.

The results obtained also make it possible to take prompt antiepidemic measures which do not require large expenditures or disrupt the normal course of everyday life before a final diagnosis is made.

The serum-luminescence method can now be successfully used for diagnostic purposes only in infections whose laboratory diagnosis is based solely on serological reactions (e.g., coloenteritis) and for the causative agents of infections accompanied by symptoms of bacteriemia, where the bacteria in question can be detected in the blood, organs, and tissues of the sick person, animal, etc. (e.g., in typhoid fever). In some cases it has been found necessary to combine the luminescent-antibody method with brief culturing on selected nutritive media in order to increase its sensitivity and specificity.

The clearest indications of the presence of an infectious agent in the environment are obtained for Bacillus anthracis and the causative agents of plague, cholera, tularemia, and brucellosis.

Use of the luminescent-antibody method for detecting microbes with a close antigenic similarity to the bacteria of the associated microflora has proved rather unsuccessful. Causative agents of this type can at present be identified only by studying the aggregate of cultural, biochemical, serological, and biological indications (Shigella dysenteriae, Bacterium typhosum in fecal matter, and Cl. botulinum). This

method is unsuitable for detecting the causative agents of pertussis and parapertussis, despite the fact that there are certain serological criteria for differentiating them from the persistent nasopharyngeal microflora.

INCREASING PHAGE-TITRE REACTION

Prof. D.M. Gol'dfarb

Determination of Bacteriophage Activity (Titre)

The activity of a bacteriophage is usually evaluated from its capacity to cause lysis of a bacterial culture in a liquid or solid medium and is expressed by the maximum dilution at which the phage exhibits a lytic action. Determination of the number of phage corpuscles per unit volume is a more precise evaluation method.

Regardless of which of these indices is used to appraise the activity of a given phage, it must be pointed out that it is relative, i.e., phage activity depends on various conditions. Prime among these are the biological characteristics of the bacterial cell whose activity is being determined, the physical properties and chemical composition of the medium, the ambient temperature, etc. It is obvious from the foregoing that specific standard conditions must always be taken into account in characterizing phage activity. Testing the same phage on the same culture in different media or on different strains of the same species of bacterium in the same medium may yield sharply variant indices of lytic capacity in liquid media and different numbers of colonies on solid media. The physiological state of the bacteria on which the phage is tested is reflected in its activity indices. This is best seen in studying the activity of the phage in cultures at different stages of multiplication. The phase of logarithmic growth, during which the majority of individuals are vitally active, is the most favorable for interaction between phage and cell. As the number of inactive bacteria in the popu-

lation increases phage activity is reduced.

Phage activity is usually tested on meat-infusion or Hottinger's agar. 1.5% meat-infusion and peptone agar are used in tests on dense media. The low agar concentration reduces medium viscosity, thus promoting better phage diffusion and colony formation. In working on solid media in Petri dishes the agar layer must be rather thick and 25-30 ml of agar is consequently poured into the dish. In studying the activity of dysentery, typhoid, and colon phages on agar by the dish method 0.1 ml of 4% gentian violet is added to each liter of medium. This almost completely prevents contamination of the medium with gram-positive airborne flora and so facilitates the work. The standard cultures on which the activity of the phage is determined should be raised on optimum nutritive media and used during their logarithmic growth phase. If this is difficult under specific experimental conditions it is necessary to provide for constant culturing conditions and for using cultures of the same age.

Appelman's method. A number of neutral-glass test-tubes of identical diameter are filled with precisely 4.5 ml of broth. A total of 0.5 ml of the phage under study is added to the first test-tube. Successive dilutions are then prepared by transferring 0.5 ml of phage from test-tube to test-tube with separate pipettes. Under normal conditions 10 test-tubes are used. The excess 0.5 ml of broth in the last test-tube is poured off and one drop of an 18-hr broth culture is added to all the test-tubes. The 11th and 12th test-tubes serve as controls. Broth and culture (but no phage) are added to the former and broth alone to the latter (sterility control). All 12 test-tubes are placed in a heater at 37° for 18 hr. The phage titre is determined from the last test-tube which remains clear when shaken and is expressed as the phage dilution. If the last test-tube which stays completely clear is the 7th, the

phage titre is 10^{-7}.

Furth's method, as modified by M.N. Fisher. A total of 2 ml of bacteriophage is mixed with agar melted and cooled to 45° and poured into a Petri dish. The dish is dried in a heater and then divided into 30 sectors, to each of which a drop of the broth culture under investigation is added. The culture is incubated at 37° for 18-20 hr and its lyzability is calculated.

Determination of phage titre by D'Herelle's method. A total of 0.00002 ($2 \cdot 10^{-5}$) ml of phage is added to 10 ml of a broth suspension containing 250 million bacteria per ml, the mixture is shaken, and 0.01 ml is spread over an agar surface. The number of phage colonies is counted after 18 hr in a heater.

Agar-layer method. The agar-layer method, proposed by Grazia in 1936, is the most convenient and precise technique for quantitative determination of a given phage. This procedure creates the best conditions for contact between the phage and the bacteria and for formation of individual phage colonies. It can successfully be used to study the mechanism of phage-colony formation and the morphological characteristics of various phages.

The technique employed is essentially as follows: 25-30 ml of 1.5% meat-infusion agar containing gentian violet is poured into dishes on the day before the experiment. The dishes are covered with sterile paper, dried under a bactericidal lamp, covered with lids, and left to stand overnight in an inverted position at room temperature. This is necessary to dry the agar, since the slightest moisture can distort the results of quantitative study of the phage.

At this point 2.5 ml of 0.7% agar is poured into a test-tube, melted, and cooled to 46-47°. To it is added 1 ml of the phage under investigation, followed by 0.1 ml of a standard culture, turning the

test-tube all the while; the contents are rapidly mixed, poured over the surface of the 1.5% agar, and distributed with careful motions. The dish is left to stand for 30 min in order to permit the agar to harden and is then incubated in a heater at 37°.

A count can be made after 5-6 hr of incubation in the heater. In order to obtain good results the dishes should be kept on a horizontal surface to prevent the agar from running to one side. It must also be kept in mind that melted 0.7% agar cooled to 46° cannot be stored for more than an hour, since it acquires a gelatinous consistency. After incubation in the heater the number of phage colonies is determined and multiplied by the dilution factor.

Jones and Kruger's method. These authors used their method for investigating Staphylococcus phage. Slides are washed with a chromate mixture, rinsed with tap water, acetone, and distilled water, dried at 55° between strips of paper, and placed in rows on a horizontal glass surface. A total of 2.5 ml of a suspension containing $6 \cdot 10^7$ bacteria per ml is placed in a test-tube, 0.5 ml of appropriately diluted phage is added, and the two are thoroughly mixed and left to stand at room temperature for 5-10 min. Now 2 ml of 0.75% agar melted in a boiling-water bath is added to the phage-culture mixture along the wall of the test-tube. The final mixture temperature should not exceed 45°. The contents of the test-tube are thoroughly stirred and 0.5 ml of the mixture is applied to the surface of a slide and permitted to dry for 5 min. The preparation is placed on glass rods in a Petri dish with a piece of moist filter paper on the bottom and incubated in a heater at 37° for 4 hr.

Increasing Phage-Titre reaction

The data which has now been amassed on the mechanism and phases of the interaction between phages and bacteria has created a basis for

TABLE 2

Sequence of Phases in the Quantitative Changes in a Phage Population in Contact with Bacteria

Адсорбция фага на бактериях 1	Латентный период роста 2	3 Лизис бактерий
Уменьшение числа частиц свободного фага 4	Число частиц 5 свободного фага без изменений	6 Увеличение числа частиц свободного фага

1) Adsorption of phage on bacteria; 2) latent period; 3) bacterial lysis; 4) decrease in number of free phage particles; 5) number of free phage particles unchanged; 6) increase in number of free phage particles.

developing a method for specific identification of causative agents in the material to be investigated, without isolation of a pure culture. The principle involved was derived from analysis of the quantitative changes which occur in a phage population in contact with bacteria.

The sequence of phases involved in these changes can be represented by the scheme shown in Table 2.

It is best to use the last phase of the interaction between phage and bacteria, i.e., the increase in the number of phage particles after the latent period of intracellular growth, as an indicator. Consequently, if the material under investigation contains the infectious agent sought homologous phage added to it reacts with the bacteria and multiplies, the subsequent increase in the intracellular free-phage concentration demonstrating the presence of the agent.

Proceeding from these premises, V.D. Timakov and D.M. Gol'dfarb developed a diagnostic reaction called the increasing phage-titre reaction, which consists in the following:

1. The material tobe investigated is transferred to the optimum medium for interaction of the phage and bacteria.

2. A definite number of indicator-phage particles is added to the

substrate.

3. The mixture (material and phage) is incubated at 37° in order to facilitate contact between the phage and bacteria and subsequent multiplication of the former in the latter.

4. The multiplying phage is separated from the foreign microflora.

5. The phage concentration is determined by Grazia's method.

Which phages can be used as indicators? On the basis of results obtained in studying the individual stages of the interaction of bacteria and phages, V.D. Timakov and D.M. Gol'dfarb (1960) formulated the concept of the "indicator phage," which has the following characteristics:

1. An indicator phage is a virulent phage characterized by a precisely delimited sphere of action.

2. It should have a rather high adsorption activity, a brief latent period of intracellular growth, and a high productivity.

3. It should have a rather high effective-reproduction index.

Experimental study of the effectiveness of the IPR as a function of the quantitative relationship between the phage and the bacteria has shown that the calculated increase in titre occurs only when this ratio does not exceed a level characteristic of the phage and bacterium in question.

In setting up the IPR it is necessary to take into account the possibility that the material to be investigated contains native phage. This phage, summing with the added indicator phage, can distort the results of the reaction and simulate an increase in titre. Two factors enable us to avoid this difficulty:

1. The difference in the morphology of negative colonies of the indicator phage and of the free phage in the material.

2. Checking the material for free phage.

All these data furnished a basis for the development and practical verification of a method for indicating various pathogenic bacteria in diverse materials.

Simplified increasing phage-titre reaction (after V.N. Kuznetsova)

This variant is precisely the same as the basic method to the point where the number of phage particles in the experimental and control mixtures is determined. It differs in the fact that the phage concentration in the mixtures is determined on slides or in depressions in a block of paraffin rather than in dishes. The modified IPR involving use of slides is a variant of the phage-titration method proposed by Jones and Kruger.

Preparation of slides. Melted paraffin is applied to carefully washed and degreased slides. One edge of the slide (1.5 cm) is left clear for the analysis number.

After the paraffin has dried a frame-like notch is cut into it, smoothing off the edges with a scalpel. A total of 2-3 ml of 1.5% agar is poured into the area bounded by the frame.

Preparation of depressions in paraffin block. Melted paraffin in portions of 25-30 ml is poured into Petri dishes. After it has cooled 4-5 2-2.5 cm circles are made with a compass or the pointed end of a large-diameter test-tube and the paraffin which they encompass is removed. The depressions thus formed are filled with 2-3 ml of 1.5% agar.

The paraffin dishes and slides with frames can be prepared beforehand, but the agar is poured into them immediately before the investigation. The agar-bearing dishes and slides can be used as soon as the agar is dry (5-10 min). A total of 0.2 ml of a mixture consisting of 1 ml of the sample to be investigated, 2 ml of 0.7% agar, and 0.1 ml of a standard bacterial culture is then applied to the surface of the 1.5% agar within the paraffin frame on a slide or in one of the depressions in a dish.

Each sample is applied to a single slide or a single depression in the paraffin block, while the phage-titre control is applied to 2 slides or 2 depressions. Thus, 4-5 samples can be accommodated by one Petri dish. As soon as the top layer of agar has dried (5-10 min) the slide is placed in a heater (at 37°). Slides are first placed in a desiccator or a Petri dish (on glass rods) with a piece of moist filter paper or cotton on the bottom and then incubated for 4 hr at 37°. The results are determined by counting the phage corpuscles.

Similar data were obtained in experiments involving parallel titration of Flexner's phage on slides, paraffin-block depressions (experiment), and Petri dishes (control). The number of negative colonies varied from 24 to 63 in the experimental samples and from 13 to 30 in the control samples. This indicates that it is possible to use slides and paraffin-block depressions instead of dishes to determine the number of phage corpuscles in the increasing-titre reaction.

This variant of the IPR is sufficiently precise and permits a substantial reduction in the consumption of nutritive media, Petri dishes, and pipettes, which makes it suitable for practical use. It proved important that negative colonies clearly appeared in the agar on the slides and in the depressions after 2-2 1/2 hr at 37°. This has made it possible to reduce the duration of the reaction from 11-12 hr to 9 hr.

Modification of the increasing phage-titre reaction for investigating specimens containing free phage

Evaluation of the results of the increasing phage-titre reaction reduces to counting the negative colonies in dishes containing cultures of the material under investigation after incubation with an indicator phage. A sharp increase in the number of negative colonies in comparison with the control (phage and broth) indicates that the causative agent sought is present in the material. However, natural substrates

(river or reservoir water, sewage, fecal matter) may contain free phage as well as bacteria; this phage sums with the added indicator phage and may in some cases simulate an increase in phage titre, thus distorting the results. In the overwhelming majority of cases, however, the titre of the phage present in the material is relatively low, so that the dilution of the experimental and control mixtures provided for in the reaction partially eliminates the difficulty. Nevertheless, the reaction also provides for a check on the presence of free phage. The increasing phage-titre reaction would obviously be substantially simpler and its results more precise if it were possible to free the material of phage beforehand without detrimentally affecting the infectious agent which it contained. The difficulty of solving this problem lies in the fact that many phages, particularly those of the colon type, are most resistant to the action of physical and chemical factors than the corresponding bacteria.

Its solution was made possible by the work of D.M. Gol'dfarb and Brocker, who studied the antiphagic and antibacterial action of a number of alkylating compounds, including embrychine and certain of its derivatives, which have a marked phagocidal action but no influence on bacteria. The insoluble preparation dimesol-14, whose antiphagic effect was discovered by D.M. Gol'dfarb and F.I.Yershov, proved to be the most suitable of the compounds studied.

The modified increasing phage-titre reaction using dimesol-14 is conducted in the following manner: a nutritive medium is added to the substrate under investigation, 200-300 γ/ml of dimesol-14 is introduced, and the mixture is shaken for 30 min at room temperature and precipitated by centrifuging at 500 rpm for 5 min or by being left to stand; the residue is removed and the indicator phage is added to the supernatant. The subsequent analysis is conducted in the same fashion as in

the ordinary increasing phage-titre reaction.

Use of dimesol-14 in the increasing phage-titre reaction led to destruction of 99.9-100% of the phage and made it possible to detect 100 bacteria in 1 ml of aqueous phage-containg substrates and 20 million bacteria in 1 g of fecal matter. Control bacteriological experiments conducted with the mixtures demonstrated the lower sensitivity of the usual investigation method.

USE OF IMMUNE REACTIONS FOR DIAGNOSING INFECTIOUS DISEASES AND INDICATING MICROORGANISMS

Prof. K.I. Matveyev, T.I. Bulatova, Candidate of Medical Sciences, B.D. Bychenko, Candidate of Medical Sciences, and T.I. Sergeyeva, Candidate of Medical Sciences

Various immune reactions are widely used for diagnosing infectious diseases in man and for determining the species of microbes isolated from patients, carriers, and the environment.

Diagnostic sera consisting of suspensions of killed bacteria in physiological solution are quite widely used in the agglutination reaction for diagnosing typhoid fever, typhus, brucellosis, tularemia, leptospirosis, and other infections. Well-studied strains of microbes which do not give group reactions must be used to prepare the sera. Their great advantage over live microbial cultures for immune reactions lies in the fact that they are prepared from specific special strains of microbes highly sensitive to the patient's antibodies. Microbes in diagnostic sera retain their antigenic properties for a considerable period, can be standardized beforehand, and are safe to work with. Bacteria killed with various chemicals retain their antigenic properties better than those killed by heat. Formalin has proved to be the best preservative for diagnostic sera. Madsen's investigations showed that use of killed bacteria yields more precise and consistent results than Widal's reaction with live Bacterium typhosum.

Selection of microbial strains for preparation of diagnostic sera is a tedious and important task, since they must not only be specific but must also be able to combine rapidly with the antibodies and yield clear immune reactions.

Specific diagnostic sera are employed for determining the species of pathogenic bacteria isolated from patients, carriers, or the environment. In the majority of cases the final and decisive stage of the investigation is an immune reaction with a pure microbial culture. The importance of the specificity and quality of the diagnostic serum in determining the species of a microbe is consequently extremely great. Carefully selected and thoroughly studied microbial strains must be used in preparing such sera. In addition to specific species-related or typological properties, the species must have good antigenic properties.

Bacteria are complex organisms with respect to antigenic composition, containing various chemical compounds, many of which have antigenic properties and cause antibody formation on immunization. When live or killed bacteria are introduced into the body several antibodies are formed, which may enter into an immune reaction with individual antigens of the microbial cell.

In using bacteria to produce diagnostic sera their variability must be kept in mind. Certain generations may have an altered chemical composition, undergo substantial loss of specific antigens, and acquire new antigens now always characteristic of the species of microbe in question. Individual antigens can be decomposed by heating the bacteria or can be isolated in pure form by chemical means. Sufficiently specific diagnostic sera can be obtained by immunizing animals with certain antigens.

Some sera with a broad immune-reaction spectrum must be adsorbed on antigens by Castellani's method, which removes a portion of their

antibodies and makes them more specific.

Selection of the producer animal is important in preparing a specific diagnostic serum. Some animals have normal antibodies to various species of bacteria and their toxins. If an animal has normal antibodies only to the microbe (toxin) which is to be used for immunization it is quite satisfactory for preparation of diagnostic serum. An animal whose blood contains normal antibodies to various species (types) of bacteria with similar antigenic and biological properties is unsuitable for production of specific diagnostic serum.

An important factor in correctly setting up an immune reaction for diagnosing an infection or determining the species of a microbe is observation of all the basic rules and conditions which promote an optimum reaction course and yield reliable results. A great deal of attention must be paid to proper preparation of the 0.85% sodium chloride solution used to dilute the antigens and sera. Solutions with a lower sodium chloride concentration must be used for the reaction in some cases. The pH of the antigen and serum dilutions must be determined in setting up immune reactions, especially precipitation and complement-fixation reactions. All immune reactions proceed best at a medium pH of 7.0-7.4.

Agglutination Reaction

Agglutination (from the Latin agglutinatio, meaning gluing) refers to the aggregation and precipitation (from a homogeneous suspension) of bacteria, yeasts, erythrocytes, and other cellular elements.

We distinguish specific agglutination, which is observed in a mixture of a dilute immune serum and a microbial suspension in physiological solution (immunoagglutination) and chemical, or acid agglutination, which occurs when a microbial suspension is introduced into a solution of lactic, acetic, or other acids or salts. Spontaneous agglutination

may take place when the colloid-chemical properties of a cellular antigen are altered. Acid and spontaneous agglutination are types of nonspecific agglutination.

Mechanism of the agglutination reaction. The agglutination reaction is based on the interaction between an antigen and an antibody. Bacteria, erythrocytes, or other cellular elements can be employed as antigens in the agglutination reaction. The antibodies are furnished by serum from animals immunized with the appropriate antigen or from sick persons.

The agglutination reaction is manifested in the fact that when immune serum is added to a uniform bacterial suspension the cells clump to form fine granules or flakes, which gradually settle to the bottom and leave the supernatant clear.

In order to demonstrate the agglutination reaction it is necessary that the interaction of the antigen and antibody take place in a medium containing small quantities of erythrocytes. This is permitted by use of physiological solution (0.85% sodium chloride) to dilute the reagents.

The manifestation of the bacterial agglutination reaction depends on the properties of the antigen: atrichous bacteria, which have only one somatic or O antigen, exhibit clumping of the cells themselves, so that the agglutinates have the form of small, compact granules. Such agglutination is referred to as fine-grained and takes place slowly, over a period of 18-24 hr. In flagellate bacteria the flagella adhere to one another and large porous flakes are formed. This type of agglutination is referred to as large-grained and takes place rapidly, within 2-4 hr at 37°.

The nature of the agglutination reaction observed in vitro is at present unclear. There is no doubt that it is a physicochemical process analogous to that which occurs when colloids precipitate in the presence

of electrolytes.

It has been established (Borde, 1939) that the agglutination reaction takes place in two stages: during the first stage the agglutinins are adsorbed on the cell surface, i.e., the antigen combines with the antibody. This is the specific phase of the reaction, a manifestation of the immunological relationship between the antigen and the antibody; it yields no apparent effect and proceeds undetectably.

During the second stage, which occurs only in the presence of electrolytes, the bacterial cells aggregate and precipitate by a nonspecific process similar to the precipitation of colloids.

The aggregation and precipitation of bacteria in the presence of electrolytes is due to a decrease in the number of electric charges on the cell, so that like-charged cells begin to attract rather than repel one another.

The agglutination reaction serves two basic functions:

a) diagnosis of diseases by detection of antibodies to a known species of bacterium (antigen) in the patient's blood serum;

b) determination of the species (type) of bacteria in various specimens with the aid of agglutinative sera containing known agglutinins.

Agglutination reaction with a patient's serum

The patient's serum, an antigen (bacterial suspension), a 0.85% sodium chloride solution in distilled water, and test-tubes and pipettes for diluting the serum and antigen are necessary to set up the agglutination reaction.

Patient's serum. In order to obtain serum from the patient 3-5 ml of blood is taken by venipuncture or from a finger under aseptic conditions. The blood is collected in a small sterile test-tube and left to stand for 1 hr at room temperature or for 20 min at 37° in a heater

to permit a clot to form. The clot is then removed from the wall of the test-tube with the closed end of a Pasteur pipette or a platinum loop and the test-tube is placed in a refrigerator for clarification of the serum. The serum is drawn off with a Pasteur pipette and transferred to a clean test-tube. If it contains erythrocytes it must be centrifuged and the clear serum is then again drawn off.

To preserve the serum 2% chemically pure boric acid is added and completely dissolved or the serum is diluted with physiological solution containing 0.5% carbolic acid.

The serum is shipped in sealed ampules or pipettes or in dry form. In the latter case 2 drops of blood or serum are applied to a piece of filter or parchment paper, which is carefully folded and shipped to the laboratory after drying (L.I. Shershacheva and L.V. Nikolskaya).

A piece is cut from the filter or parchment paper with scissors, flooded with 1 ml of water if carrying dry blood or 2 ml of water if carrying dry serum, and placed in a heater for 1 hr to permit extraction. Clear serum in a dilution of 1:20 (since the volume of 2 drops of blood is approximately 0.1 ml and the serum constitutes 50% of the blood volume, i.e., 0.05 ml) is obtained after standing or centrifuging.

The 1:20 serum dilution obtained serves as the starting point for the further dilution necessary for the reaction.

Blood or serum intended for analysis is furnished with a packing slip, which indicates the patient's surname, given name, patronymic, age, case-history number, and proposed diagnosis.

Antigen. A suspension of live or killed bacteria in physiological solution is used for the agglutination reaction with the patient's serum. In order to prepare the antigen the laboratory should have available cultures with smooth S-shaped colonies carefully selected for other properties and giving an agglutination reaction with specific homo-

logous serum when diluted to maximum titre.

In connection with the possible variability of the bacterial cultures used to prepare the antigen they are systematically recultured on the same medium (no less than once every 5-7 days) and their biochemical and other properties and agglutinability with specific serum are checked. Cultures which have been stored in the laboratory for extended periods occasionally exhibit spontaneous agglutination, which implies dissociation and appearance of bacteria producing coarse R-shaped colonies; on the other hand, the bacteria may produce generations with a reduced agglutinability. Cultures whose specific properties have changed are unsuitable for preparation of the antigen used in the agglutination reaction.

The activity and specificity of the bacterial antigen depends on the composition and structure of its complexes. Bacteria contain antigens of three types, which play a large role in the agglutination reaction: a thermostable somatic, or O antigen, a thermolabile flagellel, or H antigen, and a virulent, or Vi antigen. The thermolabile H antigen decomposes at a temperature of 100° or under the action of alcohol; it bonds with the flagella of the microbial cell. When reacted with specific serum it forms large porous flakes on the bottom of the test-tube; these appear after 2 hr at 37°. Bacteria adhere to one another by their flagella during H-agglutination.

The thermostable somatic O antigen withstands heating at 100° for 2 hr with its properties almost unchanged; it is found in the cytoplasm of the cell. When reacted with immune serum the O antigen forms fine grannular aggregates, which settle to the bottom of the test-tube as a compact, dense residue over a period of 18-24 hr at 37°. This process is known as O agglutination and involves adhesion of the cells themselves.

In 1934 Felix and Pitt discovered the Vi antigen in Bacterium typhosum; this antigen is found in freshly isolated virulent strains or in such strains cultured on complete nutritive media at optimum temperatures. It is relatively thermolabile: in microbial suspensions heated to 60-62° its agglutinability with Vi serum is greatly reduced, while at higher temperatures it decomposes. The Vi antigen is somatic, but lies closer to the cell surface than the O antigen, so that it prevents interaction between this antigen and O serum; this explains the O non-agglutinability of strains with a Vi antigen. When agglutinated with immune Vi-serum the Vi antigen forms a fine-grained agglutinate. It can be precipitated from mixtures with the O antigen by potash alum or uranium and lanthanum salts.

Diagnostic sera prepared from well-studied specific strains with good agglutinability for sera from patients are most often employed in setting up the agglutination reaction for diagnostic purposes.

The simplest method of preparing the somatic O antigen is to heat the bacterial suspension in a boiling-water bath for 1 1/2-2hr. The O antigen thus obtained over a prolonged period.

The H antigen is prepared by treating a 6-8 hr flagellate broth culture with 0.5-0.7% formalin, taking commercial formalin as 100%.

A suspension of live Bacterium typhosum, which contains the Vi antigen, is used to detect Vi antibodies in serum from patients.

Since certain strains of Bacterium typhosum contain O, H, and Vi antigens, the O and H antigens must be removed from the serum in order to obtain agglutination of the Vi agglutinins alone. This is done by adsorbing the O and H antigens by Castellani's method. The Ty_2 strain of Bacterium typhosum is usually used as the Vi diagnostic serum. The Ty 901-H and Ty 901-O strains of bacteria typhi abdominalis are used to adsorb the O and H antigens. The hemagglutination reaction has success-

fully been employed to detect the Vi antigen in cultures, vaccines, and diagnostic sera and Vi antibodies in sera.

TABLE 3

Serum-Dilution Scheme No. 1

Ингредиенты 1	№ пробирки 2					
	1	2	3	4	5	6 и т. д. 3
Физиологический раствор, мл . . . 4	—	1	2	3	4	5
Основное разведение сыворотки 1:100, мл . 5 . .	10	1	1	1	1	1
Полученные разведения сыворотки . . . . . 6 .	1:100	1:200	1:300	1:400	1:500	1:600

1) Ingredients; 2) test-tube No.; 3) etc.; 4) physiological solution, ml; 5) basic serum dilution of 1:100, ml; 6) serum dilutions obtained.

In order to prepare the antigen from a live culture the bacteria are cultured for 20-24 hr on a solid nutritive medium and then washed off with physiological solution, establishing a concentration of 1-2 billion bacteria per ml. Diagnostic sera are prepared by various methods, but even a carefully prepared serum can change during improper or prolonged storage. Flakes or granules may appear, the serum may lose its sensitivity to agglutinins, or a substantial portion of the bacterial cells may be lyzed. The service life of the majority of diagnostic sera is no more than 12 months. The quality of such sera must be regularly checked. In order to determine the sensitivity and specificity of diagnostic sera a laboratory must have a set of dried agglutinative sera or sera preserved chemically with sterile glycerine in a dilution of 1:1. Both the standard and diagnostic sera should be stored at temperatures of 5-10°.

There are several methods for setting up the agglutination reaction;

a) macroscopic (visible) agglutination in test-tubes;

b) accelerated methods of conducting the agglutination reaction;

c) rough agglutination in a drop of concentrated agglutinative serum on a slide;

d) microscopic agglutination.

Macroscopic agglutination. Either large-volume or drop methods can be used for the reaction, the former being more precise and the latter more economical.

Conducting the reaction by the large-volume method requires agglutinative serum, an antigen (bacterial culture or diagnostic serum), physiological solution, graduated pipettes with capacities of 1, 5, and 10 ml, Pasteur pipettes, and agglutinative test-tubes 10 x 0.8-1 cm in size.

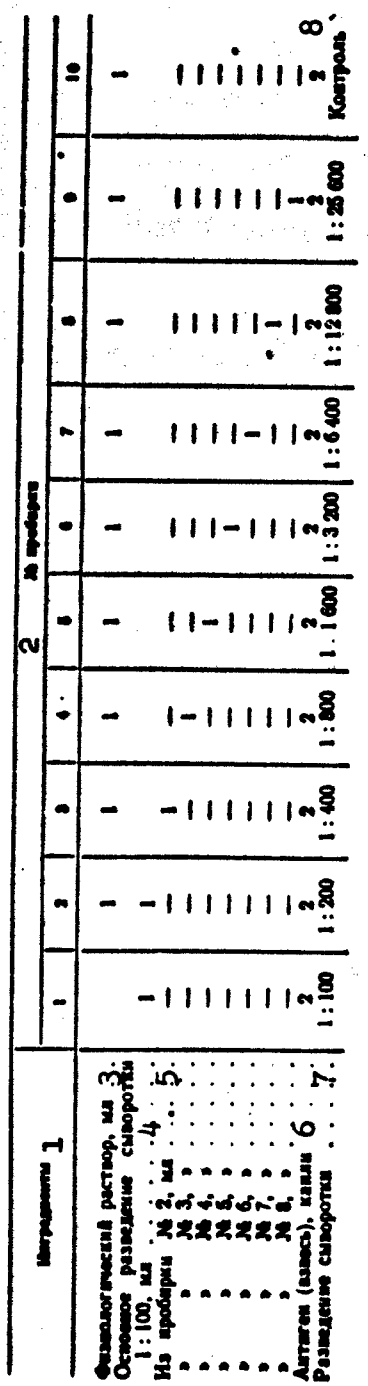
In setting up an agglutination reaction it is important to establish whether the patient's serum contains antibodies and to determine the quantity present, i.e., the agglutinin titre. The serum must be diluted with physiological solution for determination of the antibody titre. The maximum dilution depends on the quantity of agglutinins in the serum, which varies in different infections. As a result of the possible presence of normal or postvaccination agglutinins in the patient's serum, the agglutination reaction is of diagnostic value only at high serum dilutions.

In setting up the reaction initial dilutions of 1:10, 1:50, and 1:100 are prepared and further dilutions are made from them. The serum is diluted in bacterial test-tubes or in the agglutinative test-tubes themselves. In the first case the bacterial test-tubes are labeled with the serum dilutions and dilution is carried out in accordance with scheme No. 1 (Table 3).

The basic serum dilution is prepared by adding 0.1 ml of serum to 9.9 ml of physiological solution. It can be seen from the table that the sum of the number of milliliters of physiological solution and of

TABLE 4

Serum-Dilution Scheme No. 2



1) Ingredients; 2) test-tube No.; 3) physiological solution, ml; 4) basic serum dilution (1:100), ml; 5) from test-tube No. 2, ml; 6) antigen (suspension), ml; 7) serum dilution; 8) control.

the basic serum dilution indicates how many times greater the final dilution is than the initial dilution (1:100). The final dilutions are poured into agglutinative test-tubes in portions of 1 ml, using a single graduated pipette and working from low to high dilutions. Instead of the serum 1 ml of physiological solution is poured into the last test-tube as a control, using a fresh, absolutely clean pipette. To each test-tube, beginning with the last, 2 drops of antigen are added and the tubes are gently shaken.

If the dilutions are prepared in the agglutinative test-tubes themselves scheme No. 2 (Table 4) is followed.

In order to maintain a constant volume 1 ml of dilute serum is removed from the last test-tube, which contains the maximum dilution, before the antigen is added.

Drop method for serum dilution. A single Pasteur pipette is used to fill a series of test-tubes (scheme No. 3, Table 5) with 38,

12, 16, 18, 19 and 20 drop portions of physiological solution. It is then cleaned of any residual solution and employed to introduce 2 drops of the patient's serum into test-tube No. 1; the contents of the tubes are carefully mixed by drawing the liquid into a pipette and ejecting it. From test-tube No. 1, which contains the basic serum dilution of 1:20, 8 drops are transferred to test-tube No. 2, 4 drops to test-tube No. 3, 2 drops to test-tube No. 4, and 1 drop to test-tube No. 5; no serum is added to test-tube No. 6, which serves as the antigen control.

TABLE 5

Serum-Dilution Scheme No. 3 (Drop Method; after V.S. Kalinin)

	1 / № пробирки					
	1	2	3	4	5	6
Физиологический раствор, капли . . . 2	38	12	16	18	19	20
Сыворотка больного, капли 3	2	—	—	—	—	—
Число капель, перенесенных из пробирки № 1 4	—	8	4	2	1	—
Антиген-микробная взвесь, капли 5	2	2	2	2	2	2 7
Разведения сыворотки 6	1 : 20	1 : 50	1 : 100	1 : 200	1 : 400	Контроль антигена

1) Test-tube No.; 2) physiological solution, drops; 3) patient's serum, drops; 4) number of drops transferred from test-tube No. 1; 5) antigen (bacterial suspension), drops; 6) serum dilution; 7) antigen control.

After the antigen is added to serum diluted by any of the aforementioned methods the test-tubes are thoroughly shaken and placed in a heater for 2 hr and a preliminary evaluation of the reaction is made. The final evaluation is made after the preparation has been left to stand at room temperature for 18-20 hr. The O-antigen reaction is best carried out by holding the test-tubes in a water bath at 55° for 2-3 hr or in a heater at 37° for 18-24 hr.

During positive large-grained H agglutination a residue of aggregated cells is formed on the bottom of the test-tube. The liquid in the

tube is clarified after the residue has settled. The result of the reaction is regarded as specific if no agglutination takes place in the control test-tube, the fluid retaining its original cloudy appearance; there should be no bacterial residue, but if a small quantity appears it should be easy to break up. Neither flakes nor granules should be present after the test-tubes have been gently shaken.

The results of the reaction are designated by the following 4-plus scale:

++++) complete agglutination, liquid clear, very large quantity of residue;

+++) almost complete agglutination, liquid not fully clear, considerable residue;

++) slight agglutination, liquid not clear, very little residue;

±) doubtful reaction;

−) negative reaction, suspension uniformly cloudy, no residue.

The agglutination reaction can be set up for simultaneous determination of several bacteria.

In the majority of cases the specificity and intensity of the agglutination reaction is determined with the unaided eye, for which purpose the test-tube is shielded from the light with black paper; the extent and character (fine- or large-grained) of the agglutination are easily seen against the black background. Evaluation of the reaction begins with the control and the results can be cosidered reliable only when it is clear. In some cases, where the reaction is not sufficiently distinct, a magnifying glass or agglutinoscope is used to evaluate the results or the image of the bottom of the test-tube in a mirror is examined.

Accelerated methods of conducting the agglutination reaction: Minkevich's reaction. A drop of distilled water is applied to a slide

and a drop of the patient's blood is added to it. A drop of thick diagnostic serum or bacterial suspension is added to the hemolyzed blood and the two are thoroughly mixed with a platinum loop or glass rod. The agglutination reaction begins slowly and is manifested in the formation of flakes along the margin of the drop and clarification in its center. When the result is negative the drop remains uniformly cloudy.

Minkevich's reaction is used in various modifications for diagnosing tularemia, typhus, brucellosis, and other infections.

Rough agglutination. Rough agglutination is carried out on slides: a drop of serum from a patient or an immunized animal used as a producer is applied to a slide with a Pasteur pipette. The serum is preliminarily diluted to 1:10, 1:20, 1:30, or more with physiological solution. A drop of physiological solution is applied to the slide near the drop of serum as a control. One drop of diagnostic serum or one loopful of culture taken from a colony raised on a Petri dish is then added to each drop on the slide and mixed until a homogeneous suspension is obtained. After a few minutes the serum-containing drop exhibits marked clumping of the bacteria (provided that the antibody has made contact with the antigen); this process takes place more rapidly if the slide is gently tilted back and forth. The control drop remains uniformly cloudy.

This reaction is employed for rapid preliminary determination of the species to which bacteria grown on solid media in dishes belong and for rough evaluation of the antibodies in patients and immunized animals.

Noble's method is based on the use of preserved ingredients (bacterial suspension, patient's serum) and a mechanical factor which accelerates agglutination (vigorous shaking). This makes it possible to obtain results 2-5 min after the reaction is begun.

Agglutination is accelerated by having the initial contact with the

antigen made in a small serum volume and further speeded up by shaking.

The patient's serum is taken in dilutions of 1:10, 1:20, and 1:40. The bacterial suspension is used in concentrations of 3.6 billion bacteria per ml of physiological solution. Stable thick diagnostic sera preserved with 0.2% formalin can be employed.

Portions of 0.1 ml of the diluted serum and undiluted antigen are measured out into the agglutinative test-tubes. The resultant mixture is vigorously shaken for 3-5 min and 0.8 ml of physiological solution is added to facilitate evaluation of the result. The reaction must be accompanied by an antigen control in physiological solution. When the serum is mixed with the antigen its volume is doubled, so that the reaction takes place at dilutions of 1:20, 1:40 and 1:80, which must be taken into account in recording the results. The addition of 0.8 ml of physiological solution does not change the serum dilution, since it is carried out after agglutination has been completed. A heater is not required for the reaction. Results can be obtained 20-30 min after the reaction is begun.

Microscopic agglutination. In microscopic agglutination the results of the reaction are evaluated with the aid of a microscope. In setting up the reaction a drop of serum diluted to 1:20, 1:40, or 1:80 is applied to a cover glass, a drop of bacterial suspension is added (doubling the serum volume), the two are mixed with an intact Pasteur pipette, and the glass is covered with a slide with a well whose edges have been smeared with vaseline, pressing the two pieces of glass tightly together. The slide is then quickly and carefully inverted and placed in a heater at 37° for 1-2 hr. The size of the drop of antigen-serum mixture should be such that it hangs without touching the wall of the well when the slide is inverted. After 30 min in the heater the results are evaluated microscopically under high magnification and at a small dia-

phragm aperture or with a phase-contrast microscope. At first the bacteria are distributed over the entire visual field, but they then begin to clump and form conglomerates, mobile bacteria losing their mobility. Observations of this type can also be made in a compressed drop.

In order to obtain more readily apparent results a number of techniques have been proposed for staining the antigen or antigen-antibody complexes with azure-eosine, gentian violet, and other dyes.

Heddleson developed an accelerated agglutination reaction for diagnosing brucellosis; in order to make his method more graphic he suggested that the brucellosis antigen be stained with gentian violet added in the form of 0.01 ml of a saturated aqueous solution per 100 ml of antigen.

V.V. Gavrilov proposed that Heddleson's antigen-staining method be used for the serological diagnosis of typhoid fever and paratyphoid A and B. T.V. Bocharov employed staining of the antigen-antibody complex to produce higher contrast in the agglutination reaction of rickettsiosis antigen. In this method the mixture of dilute serum and rickettsiosis antigen is kept in a heater at 37° for 2 hr and in a refrigerator at 4° for one day. After the latter period has elapsed smears with an area of approximately 1 cm^2 are made on slides from each dilution and the preparations are dried, fixed with Nikiforov's mixture (alcohol and ether) and stained with azure-ecsin, carbol fuchsin, or gentian violet by the usual procedure. They are then examined microscopically with an immersion system.

Luminescent microagglutination of bacteria can yield very clear results (see page 74).

The microscopic agglutination reaction is used for diagnosing tularemia, brucellosis, typhus, and other diseases.

Determination of the species of microbes isolated from the body or the environment

The specific properties of bacteria isolated from the body or the environment are studied by culturing on selected nutritive media, staining, microscopy, determination of enzymatic characteristics, determination of pathogenic characteristics by animal inoculation, and other methods. Conclusive establishment of species rather frequently involves the agglutination reaction, which is of great value in identifying bacteria of the coliform group and those responsible for tularemia, brucellosis, cholera, etc.

Special attention must be paid to selection of a good strain for preparing the antigen and of the animal to be immunized for production of the agglutinative serum.

Use of a strain not typical of the species of bacterium in question or preparation of the antigen from dissociative variants of the bacterium can lead to production of a nonspecific agglutinative serum. The sera of some animals contain normal agglutinins to several similar species of bacteria and such animals are consequently unsuitable for producing specific agglutinative sera. Every animal to be used in the production of a certain type of agglutinative serum must be checked before immunization for its content of normal agglutinins to the microbe with which it is to be inoculated and for its content of normal agglutinins to other microbes with antigenic properties similar to those of the immunizing bacteria. Selection of the producer animal is a very important part of the job. Rabbits are most often used for production of agglutinative sera, since they are easy to keep, immunize, and draw blood from.

The antigen is prepared from one- or two-day cultures raised on liquid or solid media. In the latter case the microbial growth is washed

off with physiological solution and the bacterial concentration per ml is determined by comparison with a bacteriological standard. In the majority of cases cultures grown on liquid media must be centrifuged and the bacterial residue is then diluted with physiological solution to the requisite concentration per ml. The suspension is heat-killed at 58-60° for 1 hr and 0.2-0.75% formalin or phenol is added as a preservative. The bacteria can also be killed by adding 0.2-0.75% formalin or phenol to the antigen and holding it at 37° in a heater for 1-2 days.

Animals are usually immunized intravenously to produce agglutinins, but the antigen can be administered by a combination of intravenous, subcutaneous, and intraperitoneal injection. Immunization begins with 200 million microbes, followed by 500 million, 1 billion, 1.5 billion, and 2 billion or more at intervals of 2-5 days. The animal's reaction is observed, it is weighed, and its temperature is taken. A blood sample is taken 7-8 days after the last injection of antigen and the agglutination titre is checked. If it is satisfactory phlebotomy or exsanguination is performed.

In determining the species of a microbe it must be kept in mind that such bacteria as Salm. aertrycke, Salm. suipestifer, Salm. paratyphi B, etc., form colonies with specific and group phases. These colonies are identical in form and cannot be distinguished by visual inspection. In the specific phase the colony contains bacteria which agglutinate well with their specific serum, but in the nonspecific phase it contains microbes quite insensitive to their specific agglutinins and easily agglutinated by group antibodies to produce large granules.

The conditions under which bacteria are cultured significantly influences their agglutinability, which is affected by the quality, temperature, and pH of the medium.

Setting up the agglutination reaction to determine the species of a microbe. A macroscopic agglutination reaction with immune serum diluted to titre (here the term titre refers to the maximum serum dilution at which agglutination with the corresponding microbe occurs) is employed for conclusive identification of bacteria.

This reaction requires: 1) specific agglutinative serum of high titre; 2) a pure culture of the microbe with a concentration of 1 billion cells per ml; 3) physiological solution; 4) graduated pipettes with capacities of 1, 5, and 10 ml; 5) agglutinative test-tubes in a small rack.

The basic serum dilution of 1:100 is prepared by the large-volume or drop method, as outlined above. The number of dilutions used in the reaction depends on the serum titre. The bacterial suspension under investigation is added to each of the serum dilutions in portions of 1-2 drops. The rack containing the test-tubes is placed in a heater at 37° for 1.5-2 hr and a preliminary evaluation of the reaction is made. The final evaluation is made after the preparations have stood at room temperature for 18-20 hr. When the maximum serum dilution at which a positive agglutination reaction is obtained coincides with or is half that of the serum taken the culture under study is considered to be homolo- to it.

Specificity of the agglutination reaction and methods of suppressing group reactions. The agglutination reaction is a valuable diagnostic technique, being very sensitive and simple to set up. It does, nevertheless, have a number of drawbacks. One of its principal deficiencies lies in the fact that it does not completely satisfy the requirements for early diagnosis of a disease. This reaction is an immunity phenomenon which records the appearance of antibodies in the body 7-10 days after the onset of illness. In a number of cases the agglutination

reaction becomes positive still later and in a certain percentage of cases it remains negative until the disease has run its course.

Another drawback lies in the fact that in some cases highly dilute serum from patients or animals yields an agglutination reaction with several bacteria having similar antigenic structures. This phenomenon hampers serological diagnosis and can alter the identification of a microbe from the environment for a patient. When the patient's serum contains group agglutinins it is diluted to maximum titre and it is determined which of the bacteria gives a positive agglutination reaction at the nighest serum dilution. Once found, this microbe can be regarded as the causative agent of the infection, taking into account the symptomatology of the disease for which it is responsible.

This procedure is also followed in determining the species of a microbe. The specific diagnostic agglutinative serum is diluted to maximum titre and if the bacterium agglutinates it at high dilutions the former can be assumed to belong to the species (type) for which the serum is intended.

In these cases the action of the group agglutinins is suppressed with the aid of high dilution of the patient's serum or the diagnostic agglutinative serum. In the majority of instances the group antibodies are present in lower titre than the specific antibodies and lose their agglutinative effect at high serum dilutions, while the specific agglutinins still cause agglutination of the corresponding bacterial species.

Observation of the dynamics of the agglutination reaction is of great importance in diagnosing a disease. An increase in agglutination titre when the reaction is repeated is a serious indication that the disease is present.

Castellani's method. In 1902 Castellani suggested analyzing the antigenic structure of bacteria with the aid of sera subjected to bac-

terial adsorption. When an agglutinative serum is saturated with homologous bacteria they adsorb both the specific and group agglutinins. If heterologous bacteria are used to adsorb the antibodies they adsorb only the group agglutinins, leaving the specific agglutinins in the serum.

Bacteria cultured for 24 hr on agar slants in test-tubes are used to adsorb the agglutinins. In order to completely remove the antibodies from 1 ml of serum diluted to 1:100 it is necessary to wash the bacteria from 5-6 test-tubes with physiological solution. Before being added to the serum the bacterial suspension is centrifuged, the supernatant is drawn off, and the residue is then mixed with the serum. The mixture is placed in a heater at 37° for 2 hr and then kept in a refrigerator for 18-20 hr. The suspension of bacteria in the serum is centrifuged and the adsorbed serum is drawn off and employed to set up an agglutination reaction with the bacteria used to adsorb the agglutinins. A negative reaction indicates complete adsorption of the antibodies.

All experiments involving Castellani's method should be performed under sterile conditions.

A suspension of killed bacteria can be used to adsorb the agglutinins. If they are heat-killed they adsorb O agglutinins, while if they are formalin-killed they adsorb H agglutinins.

Castellani's method is used to prepare monovalent specific agglutinative sera for bacteria, Rickettsiae, and other microbes.

Precipitation Reaction

The term "precipitation reaction" was proposed by Kraus, who observed precipitation when clear filtrates of cultures of typhoid, plague, and cholera bacteria were mixed with homologous immune sera.

The antibodies which participate in the reaction are called precipitins, while the antigens are called precipitinogens.

Because slight traces of specific protein (1:1,000,000) can be de-

tected with the precipitation reaction it is of great importance in forensic medicine for identifying blood and protein spots, for detecting various impurities in foodstuffs, etc.

The precipitation reaction of an antibody-antigen system is distinguished from agglutination principally by the size of the antigen particles. While whole cells or cellular detritus is used as the antigen for agglutination, in the precipitation reaction the antigen is in a more or less molecular state, in the form of clear, occasionally opalescent solutions.

Calculations made by some researchers have shown that if a given bacterial suspension is broken down to protein molecules the total surface of the latter is 10,000 times that of the original bacteria, so that 10,000 times as much antigen is required to cover the active surface as to cover the bacteria. For this reason the antigen rather than the serum is diluted in the precipitation reaction.

When the antigen and antibody are mixed, visible aggregates, or flakes, are formed (the solution becomes cloudy) and gradually settle to the bottom of the test-tube. When the antigen solution is carefully overlayered on the serum, maintaining the boundary between the liquids, the precipitate forms at the boundary (Ascoli's annular precipitation reaction).

Just as in the agglutination reaction, the precipitation reaction can be weakly positive or even negative when the antigen concentration is high with respect to the antibody concentration. It must be noted that this phenomenon occurs in experiments with horse sera to a protein antigen, but not with rabbit sera or polysaccharide antigens.

As the antigen added to the serum is diluted to a certain point the rate of the precipitation reaction and the quantity of precipitate increase, but when the antigen dilution becomes sufficiently high they

decrease and tend to zero. The precipitation reaction thus has a zonal character and depends on the antibody-antigen ratio; this can be expressed by appropriate curves for each antigen-antibody system.

It is best to combine the antibody with the antigen in a medium with a certain electrolyte (sodium chloride) concentration at a neutral pH and a temperature of 15-40°; this process is reminescent of reversible chemical reactions. Its rate is such that 80-90% of the precipitate is formed within 3-5 sec after the liquid reagents are mixed; this releases heat energy, measurement of which has shown that almost complete bonding occurs within 3 min (Boyd). Such rapid bonding is especially characteristic of polysaccharide-antibody systems.

The stability of the antigen-antibody complex is least at the beginning of precipitate formation and increases with time; however, the two reagents can be dissociated by various methods (heating, filtration, acid treatment, etc.), even after several months.

Certain substances (urea, guanidine, sodium benzoate or salicylate, etc.) are capable of suppressing the precipitation reaction to some extent. Conversely, trivalent aluminum ions raise the sensitivity threshold of the reaction.

All these effects are based on purely chemical processes (denaturation, salting-out, etc.).

The precipitation reaction is far more specific than other chemical and physical methods, particularly electrophoresis, since it makes it possible to differentiate proteins from very similar species of animals, such as chickens and turkeys. The keratin of human hair can easily be distinguished from that of animal hair with this reaction.

The specificity of the reaction depends on the chemical structure of the antigen and the corresponding antibody and is a property of the smallest molecular components, which cannot be reversibly split off.

Artificial alteration of the chemical structure of the antigen can lead to a change in or disruption of its specificity (denaturation, powerful enzymatic digestion, treatment with strong acids or alkalies, esterification, treatment with phenol isocyanate, etc.).

Preparation of antigens for the precipitation reaction

In producing antigens for the precipitation reaction it is important to keep in mind the possibility of a nonspecific reaction caused by the proteins of the medium on which the microorganism to be studied is raised. In order to avoid such erroneous results the antigen should contain no traces of protein from the medium on which the bacterial suspension used for immunization was prepared. This is accomplished by preparing the antigen for the reaction and for serum production on media consisting of meat from different species of animals or by preparing it on synthetic media. There are also various methods for purifying the antigen of traces of protein (fractional precipitation, electrophoresis, etc.).

Physical methods

Pulverization of the cells. A thick paste of bacteria in physiological solution is carefully ground in a sterile mortar with sand or powdered glass. It is then emulsified in neutral physiological solution and centrifuged or filtered to obtain a clear liquid, which is used as the antigen.

The bacteria can also be broken down in special ball mills or in flasks containing glass beads, which are shaken in an agitator for a given period. An optimum exposure time must be selected for each species of bacterium, since the strength of the cell membrane varies for different microorganisms. The extent to which the cells are broken down is determined by microscopy.

Freezing followed by slow thawing (repeated several times) can

cause the cell wall to break down, so that the protoplasm escapes into the solution. The antigen is ready for use after centrifuging.

Destruction of the cells by ultrasound. Bacteria washed several times by centrifuging with physiological solution are suspended in fresh physiological solution at concentrations of 2 to 20 billion per ml, depending on the species of bacterium; the concentration is determined by comparison with an optical or other standard.

The ultrasonic apparatus is plugged in and permitted to warm up for 30 min to 1 hr. The metal rod of the apparatus is first cooled in the freezing compartment of a refrigerator. A 20 ml portion of the microbial suspension is poured into a special glass tumbler first carefully washed with distilled water. The end of the rod is immersed to approximately 0.5 cm in the liquid and the apparatus is set for 20,000-21,000 cps.

When the setup is properly adjusted the liquid in the tumbler begins to boil. The tumbler is immersed in an ice bath. The bacteria are broken down by exposures of 1, 2, 5, 10, and 20 min, taking care that the rod and liquid do not become too hot.

The resultant solution is freed of suspended particles by centrifuging at 6000 rpm for 10-15 min. The clear supernatant is used in the precipitation reaction. The residue is examined microscopically to determine the extent to which the cells have been broken down.

The results of the reaction may vary as a function of the time for which the ultrasound is permitted to act. The breakdown period must be shorter (1-2 min) to obtain active enzymes than to isolate polysaccharides, whose antigenic properties remain almost unchanged even after treatment for 20 min.

Rather prolonged exposure (20-30 min) is necessary to obtain satisfactory antigens from sporogenous Bacilli and Clostridia or even from

acid-fast bacteria and Actinomycetes.

The principal drawback of this method lies in the fact that it decomposes certain labile enzymes and proteins (e.g., deoxyribonucleic acid).

Destruction of cells by sound waves. Special devices of the "Mickel" type, in which a buzzer is immersed in the liquid and breaks down the cells in a manner similar to ultrasound, is occasionally used instead of ultrasonic devices for studying the antigens of the cell membrane. Their action is, however, gentler and they make it possible to isolate the cell wall almost intact.

High-pressure method. In 1921 a press was devised in Sheffield (England) for breaking down bacteria. Two metal blocks are polished and matched to one another. A thick pasty bacterial suspension is placed in a special receptacle and compressed under high pressure (with a piston-screw arrangement). The entire block should be cooled to low temperature, since heat may be liberated when the screw is turned, altering certain enzymes and proteins.

The broken-down bacteria and their contents drip into a channel in the lower portion of the block. Approximately 20 sec is required to crush the cells. This method is of great value in isolating labile antigens (certain enzymes and proteins).

Chemical method

Autolysis of bacteria. Cultures raised on a liquid medium are kept in a heater for several days. In some cases almost complete cellular lysis can be achieved by the bacterial enzymes (possibly as a result of the action of bacteriophages).

Clear culture centrifugates, purified or native, are used as antigens.

Enzymatic destruction of bacteria. A suspension of washed vegeta-

tive bacteria in a definite concentration is treated with trypsin or other proteolytic enzymes under optimum activity conditions for a given time (from 30 min to 6 hr, depending on the species of microbe) in order to induce lysis.

The liquid is clarified by centrifuging and used as the antigen. In some cases antitryptic serum is added to the antigen to neutralize the residual trypsin.

Drying in acetone. A thick bacterial suspension is mixed with 5 volumes of glacial acetone. The bacteria settle to the bottom and are collected on a piece of ordinary filter paper moistened with glacial acetone and then with ether. The dry powder remaining after the ether has evaporated can be used to extract the intracellular enzymes, since the acetone dissolves the lipids in the cell wall and makes it more permeable to liquids.

Boiling. Boiling a bacterial suspension for 1-2 hr causes coagulation of its protein components, but yields rather good results in extracting the polysaccharide fractions of certain species of microorganisms (Streptococci, Clostridia, Bacilli, et al.). Antigens obtained from Clostridia exhibit high specificity (K.I. Matveyev, 1941; B.D. Bychenko, 1961).

This method has come into use for diagnosing anthrax (the so-called Ascoli thermal- or annular-precipitation reaction). In this case both the polysaccharide and the specific capsular polypeptide are extracted.

Excessive exposure to high temperature and pressure (autoclaving at 110-120° for 30 min) leads to profound changes and loss of specificity in the antigens of Bacillus anthracis (T.I. Sergeyeva). Such altered antigens may yield a precipitation reaction with "normal" sera. On the other hand, antigens obtained from a mixture of saprophytic bacteria isolated from water react with specific precipitative anthrax se-

rum in 15.2% of all cases.

The specific antigens of certain microorganisms (Streptococci) are best extracted by boiling the cells in an acid medium (Porges-Lensfield's method, 1928).

The following procedure is generally accepted for extracting antigens from bacteria of the coliform group: the bacterial colonies are suspended in 1-2 ml of 1% acetic acid. The suspension is boiled for 45-60 min in a water bath, filtered through an asbestos pad, and neutralized to pH 7.2-7.4 with an indicator, bromthymol blue, added. The precipitate is removed by centrifuging and the clear supernatant is used as the antigen.

Fuller's method (1938) is very convenient for working with a small quantity of bacterial suspension in order to extract polysaccharide antigens: 5 ml of a 18-hr culture of Streptococcus on glucose broth is centrifuged and the supernatant is removed as completely as possible. A total of 0.1 ml of formalin is added to the residue and the test-tube is shaken, placed in an oil or paraffin bath at 150° for 15 min, and cooled to room temperature; 0.25 ml of acidified alcohol (a mixture of 95 parts anhydrous alcohol and 5 parts 2 N hydrochloric acid) is then added. The liquid is stirred and the precipitate formed is removed by centrifuging. The supernatant is drawn off with a pipette and drained into a small test-tube containing 0.5 ml of acetone. The test-tube is shaken, the residue is centrifuged, and the supernatant is carefully removed. The acetone precipitate can be dried and used experimentally, its quantity being expressed in terms of weight. Usually 1 ml of physiological solution and a drop of indicator (phenol red) are added to the residue and the liquid is neutralized with a weak sodium acetate solution (0.25%). If a precipitate forms it is removed by centrifuging. The clear liquid serves as the antigen.

There is also a simpler method, in which the bacterial residue is dissolved in formalin at 150° for 15 min, 1 ml of physiological solution is added, the preparation is neutralized, the precipitate is discarded, and the supernatant is employed as the antigen.

Landy's method, as modified by V.D. Gekker, A.P. Konikov, and Ya. N. Ivanova, is used to extract the Vi antigen of Bacterium typhosum. The culture under investigation is raised on meat-infusion agar and washed off with distilled water after 16-18 hr. The suspension thus obtained is mixed with 3 volumes of chemically pure acetone, shaken, and centrifuged. The residue is treated with a fresh portion of acetone and left to stand for one day at 37°. The bacterial suspension is precipitated by centrifuging, the acetone is removed, and the residue is vacuum-dried. The dry powder thus produced is treated with 0.90% sodium chloride (1 g of powder in 100 ml of solution) for 30 min with constant agitation. The suspension is then centrifuged at 6000 rpm and the supernatant is dialyzed in running water for 2 days. The dialysate is again centrifuged, the supernatant is saturated with sodium chloride, and the Vi antigen is precipitated with trichloracetic acid, which is added until a pH of 3.0 is reached (in which case a flaky residue appears), or with 1.5-2 volumes of alcohol. The precipitate is dissolved in distilled water and dialyzed for 2-3 days in running water or one day in distilled water. The residue is removed by centrifuging and the clear supernatant is then subjected to lyophilic drying and used as the antigen.

There are many other methods of preparing various bacterial antigens, such as Westphal's water-phenol method, Belozerskiy's nucleoprotein-extraction method, etc., which are described in the specialized literature.

The antigen is standardized in accordance with the weight of protein or polysaccharide per ml, depending on its nature. Protein or pep-

tide antigens can also be standardized in accordance with their nitrogen content in milligram-percent.

Preparation of precipitative sera

The procedure for preparing precipitative sera differs little from the methods used for producing agglutinative sera.

Use of intact bacterial cells for immunizing animals makes it possible to obtain antibodies to both the superficial and somatic antigens of the microbe, since the cells undergo lysis when they enter the animal's body and produce the same immunizational stimulation as cellular extracts.

Live or killed suspensions of bacteria washed from the culture medium are used as the antigens for preparing precipitative sera in animals. The microbes are usually rendered harmless with 0.4-1% formalin or by exposure to high temperature (boiling at 100°).

In many cases precipitative sera are obtained to protein antigens (toxins, toxoids, proteins, etc.) by inoculating animals with them.

Among the important conditions for obtaining a good specific precipitative serum are the following:

1) selection of the immune antigen; 2) selection of appropriate animals; 3) preliminary checking for the presence of normal antibodies; 4) checking the specificity of the final sera in cross reactions with various antigens.

It is not always possible to obtain precipitative sera of high titre when animals are immunized with certain bacterial antigens (even protein antigens), using various administration schemes. This is because the antigen has very weak immunogenic properties, i.e., is a poor stimulant to antibody production. Such antigens are most frequently obtained from R-type bacteria.

When sera to such bacteria cannot be prepared an attempt must be

made to obtain them in the S form or to check them experimentally in several (3-5 animals) rather than just one. The varying individual sensitivity of the subjects to different antigens occasionally makes it possible to obtain satisfactory results.

In order to prolong the immunological stimulation, especially at the beginning of immunization, the antigen is often administered in combination with a deposition agent (lanolin, mineral oil, potash alum, small doses of calcium chloride, etc.).

Domestic rabbits, in which normal antibodies are very rarely encountered, are the best producers of precipitative sera. Good sera are also obtained from 5-day-old chicks, owls, pheasants, and partridges.

The final precipitative sera must always be checked in precipitation reactions with homologous and heterologous bacterial antigens, as well as with the sterile medium on which the antigen for immunization was prepared. Sera which react only with homologous antigens can be regarded as specific. If a precipitative serum reacts with the homologous antigen and the proteins of the medium on which the antigen was prepared the possibility of a nonspecific reaction must be eliminated before it can be used. This is done by preparing the antigen on a medium containing protein from another species of animal, by careful preliminary purification of the antigen, etc.

There are various satisfactory schemes for preparing precipitative sera. The first immunization cycle usually involves doubling doses of bacteria, beginning with 100 million per ml and terminating with 10-20 billion per ml, or protein, beginning with 10 mg and terminating with 100-200 mg.

In order to obtain sera to Cl. perfringens types A, B, C, D and E immunization begins with 100 million bacteria per ml, 8-9 injections being given at intervals of 3-4 days (B.D. Bychenko).

There is also another immunization scheme, involving injection of equal doses over the first 3 days of each week followed by a 4-day interval, the cycle lasting 3-6 weeks. The first injection for each week is given intraperitoneally or subcutaneously, while the others are given intravenously. The manner in which the antigen is administered plays a large role. Thus, for example, a precipitative serum can be produced by subcutaneous injection of horses with rabbit-serum albumin, but almost no precipitin is formed if the same albumin is administered intravenously.

An economical method which yields good results in globulin immunization consists in injecting increasing doses of antigen beneath the conjunctiva on 3 successive days followed by a two-day interval, repeating this pattern for 4 weeks (Yu.N. Zubzhitskiy, 1960).

Precipitin formation begins 4 days after administration of the antigen and reaches its maximum after 8-9 days. Blood is consequently taken from the immunized animal 8-9 days after the final injection.

The blood is processed and the precipitative serum prepared in essentially the same manner as in the processing of agglutinative sera. Sodium merthiolate (1:10,000) is used as a preservative. Addition of 0.5% phenol may cause changes in the serum leading to nonspecific results in the annular precipitation reaction. If the titre of the prepared sera is not high enough immunization must be prolonged through 2-3 or more cycles. Beginning with the second cycle the number of injections can be reduced to 3 at intervals of 4 days. The injections start with doses half that of the final dose of the last cycle and terminate with a dose twice as large.

As the number of cycles increases the titre of the precipitative serum rises and its action spectrum, i.e., the equivalence zone, becomes broader.

Sera obtained after numerous immunization cycles occasionally lose their specificity. Such sera can be used experimentally after 20-fold dilution in physiological solution, which ensures a specific precipitation reaction.

Methods for conducting the precipitation reaction

Reaction with dilute antigen

Mixing antigen and serum. This procedure involves 10 test-tubes about 7 cm long with an inside diameter of 0.5 cm. To 9 of them we add 0.2 ml of undiluted precipitative serum. Consecutive 10-fold dilutions in physiological solution of the antigen under investigation, from 1:10 to $1:10^7$, are prepared separately and 0.2 ml of each dilution is added to one of 7 of the test-tubes containing immune serum. The other 2 serum-containing test-tubes are used as a control: 0.2 ml of physiological solution is added to one of them, while 0.2 ml of any heterologous antigen or of the sterile medium on which the antigen was prepared is added to the other. The tenth test-tube is filled with 0.2 ml of normal serum and 0.2 ml of 10-fold diluted antigen. All the test-tubes are shaken until the liquids are completely mixed and then placed in a heater for 2 hr.

A preliminary evaluation of the reaction is made after the tubes are shaken, since a precipitate may settle to the bottom. The antigen titre is assumed to be equal to the last dilution which becomes visibly cloudy when mixed with the serum, the controls remaining negative. All the test-tubes are placed in a refrigerator at 0-5° and the reaction is again evaluated after 24 hr.

If there is any suspicion that the serum is very weak the reaction must be carried out under sterile conditions, in test-tubes sealed with cotton plugs. After being left to stand in a heater all the test-tubes are kept in a refrigerator for up to 7 days. The sterility of the pre-

cipitate is checked microscopically, since the possibility of bacterial contamination cannot be excluded.

If the antigen titre must be determined more precisely it is prepared in dilutions of 1:2, 1:4, 1:8, etc., i.e., having the concentration double from one solution to the next.

Annular precipitation. Portions of 0.1 ml of undiluted serum are poured into a series of test-tubes 4 cm long and 0.3-0.4 cm in inside diameter. Consecutive dilutions involving successive 10-fold decreases in the concentration of the antigen under investigation are prepared separately. Portions of 0.1 ml of each dilution are carefully discharged through the drawn-out tip of a Pasteur pipette onto the surface of the serum in the test-tubes in such fashion that the boundary between the two liquids remains quite distinct (they do not mix). The specificity of the reaction is checked with the following controls.

Serum control. Separate portions of 0.1 ml of physiological solution, 0.1 ml of the sterile medium on which the antigen was prepared, and 0.1 ml of 1 or 2 heterologous antigens are overlayered on immune serum in individual test-tubes.

Antigen control. Separate portions of 2-3 "normal" sera from the species of animal in which the precipitative serum was produced are poured into individual test-tubes and overlayered with 0.1 ml of antigen.

All the test-tubes are placed in a rack at room temperature (20°) and the results are evaluated after 5 min, 15 min, and then after an hour.

The reaction is considered positive if: 1) the disk of precipitate appears soon after the antigen is added (within a few minutes, never more than an hour); 2) the precipitate formed is deposited gradually and has no tendency to break up or create so-called double rings; 3)

the reaction in the control test-tubes is negative. The antigen titre is assumed to be equal to the last dilution at which a distinct precipitate disk clearly visible against a black background is formed within 30 min.

This method should be regarded as qualitative and only very roughly quantitative; it is not particularly suitable for precise quantitative determinations. Nevertheless, it has a number of material advantages: only small quantities of antigen and antibody are required, so that the reaction can be carried out in capillary tubes when necessary, and the presence of a boundary between the liquids prevents formation of a zone in which the reaction is suppressed, since the reagents gradually diffuse into one another.

TABLE 6

Titration of Precipitative Serum by Dilution Method (after Ivens)

Титр сыворотки 1	2 Раствор антигена					
	1:10	1:100	1:1000	1:10000	1:100000	1:1000000
1:20	—	+	+	+	+	—
1:40	—	—	+	+	+	—
1:80	—	—	—	+	+	—
1:160	—	—	—	—	+	—
1:320	—	—	—	—	—	—

1) Serum titre; 2) antigen solution.

Serum-dilution method. Progressive double dilutions of the serum under study are prepared from 1:2, 1:4, etc., to 1:40,1:4096. A 0.2 ml portion of each dilution is poured into a test-tube (0.5 cm in diameter and 7 cm long) and 0.2 ml of a solution of the antigen to be investigated is added. The control consists of the following mixtures: 1) 0.2 ml of normal serum and 0.2 ml of homologous antigen; 2) 0.2 ml of the immune serum to be studied and 0.2 ml of physiological solution; 3) 0.2 ml of the immune serum and 0.2 ml of the sterile medium on which the

antigen was prepared or 0.2 ml of a heterologous antigen.

The least antigen dilution (titre) for a constant serum concentration is determined beforehand for antigen-antibody systems in which the reaction is suppressed in the zone of excess antigen (see above).

This antigen dose is used for titrating the serum (Table 6), since the serum titre may otherwise be too low.

By way of example, Table 6 shows the results of titration of immune horse serum with a protein antigen. As this table indicates, there is no reaction when concentrated antigen (1:10) is used.

The highest serum titre, 1:160, is obtained with antigen diluted to the limit of reaction sensitivity ($1:10^5$).

Optimum-proportion method. This method can be employed to determine the rate of a precipitation reaction at the optimum antigen-antibody concentration ratio. In practice, this ratio often coincides with the equivalence point or zone.

There are two modifications of this procedure, the alpha and beta methods.

Dian and Webb's alpha optimum-proportion method (dilution of the antigen) is used principally for antibodies obtained from rabbits. A series of test-tubes are filled with 0.2 ml of immune serum and 0.2 ml portions of antigen in progressive 2-fold or 10-fold dilutions are added. The tubes are shaken to mix the liquids and placed in a water bath at 37° under constant observation. The time required for the contents of the tubes to become cloudy is noted. The antigen dilution at which the reaction first appears is determined.

It has been established that the optimum antigen-antibody ratio is almost independent of the antibody concentration employed and is consequently a characteristic of the serum.

Ramon's beta optimum-proportion method (addition of a constant an-

tigen volume to varying quantities of serum) is used primarily for antibodies obtained by immunization of horses. The classic technique for using this method is the flocculation test for toxins and toxoids. Progressively increasing quantities of antitoxic serum are introduced into a series of test-tubes (0.5 cm in diameter and 7 cm long) with micropipettes graduated for from 0.2 to 0.001 ml. Portions of from 0.01 to 0.045 ml of antitoxin at intervals of 0.005 ml are added to the test-tubes (Table 7). A total of 2 ml of the toxin or toxoid under investigation is then added to each tube. The tubes are shaken, placed in a water bath at 50°, and kept under constant observation. Those which become cloudy (exhibit a flocculation reaction) soonest contain the maximum toxin-antitoxin ratio. Table 7 shows a reaction of this type between diphtheria toxin and antitoxin.

TABLE 7

Beta Method Involving Optimum Ratio of Diphtheria Toxin to Antitoxin (Ramon's Flocculation Method)

№ пробирки 1	Антитоксин, мл 2	Токсин, мл 3	Время реакции преципитации (флокуляции), минуты 4
1	0,010	2	—
2	0,015	2	—
3	0,020	2	—
4	0,025	2	60
5	0,03	2	40
6	0,035	2	35
7	0,04	2	40
8	0,045	2	55

Note: A dash indicates a time of more than 60 min.

1) Test-tube No.; 2) antitoxin, ml; 3) toxin, ml; 4) time of precipitation (flocculation) reaction, min.

In this case the optimum ratio of antibody to toxin occurs in test-tube No. 6. More precise titration can be carried out by reducing the antitoxin dose from 0.03-0.04 ml to 0.001 ml.

The following computation is used to determine the flocculation

unit. Let us assume that 1 ml of antitoxin contains 500 units, i.e., one unit amounts to 1 ml/500 = 0.002 ml. If 0.035 ml of antitoxin reacts most rapidly with 2 ml of toxin in the experiment, 2·0.002/0.035 = = 0.115 ml, or 1 ml of toxin contains 1/0.115 = 8.7 flocculation units.

Determination of excess antigen in the supernatant is employed when it is necessary to establish the purity of the antigen or the correspondence of the optimum antigen-antibody ratio to the equivalence point (the two do not coincide in some systems).

The reaction is carried out by the antigen-dilution method, as described above. After determination of the optimum antigen-antibody ratio the test-tubes are held at 0-4° for 2-3 days to permit complete precipitation. The tube containing the optimum antigen and antibody concentrations and the 2 or 3 closest to it are then centrifuged for 30 min at 3000-6000 rpm to obtain a clear supernatant.

When the experiment is properly set up the test-tubes should not be heated during centrifuging.

The supernatants to be studied are poured into 2 series of clean test-tubes, 0.2 ml in each. Portions of 0.2 ml of fresh undiluted precipitated serum are added to the first series, while 0.2 ml of antigen diluted to titre in physiological solution (in order not to suppress the reaction with an excess of antigen) is added to each of the tubes in the second series. All the tubes are shaken, incubated in a heater for 2 hr, and then held in a refrigerator at 0-4° for 24 hr. The appearance of a specific precipitate in the tubes of the first series indicates an excess of antigen in the antibody supernatant. Absence of a precipitate in the supernatant indicates the equivalence point. The narrower the equivalence zone, the purer is the antigen and vice versa.

The volumetric semiquantitative method yields good results when the antigen molecules are not very large. The precipitation reaction is

carried out in special small graduated test-tubes. The experimental conditions are the same as for the antigen-dilution method. After being kept in a refrigerator for 24 hr the tubes are centrifuged for 15 min at 3000 rpm.

The volume of precipitate is determined with the scale on the tubes. The smallest volume usually corresponds to the equivalence zone.

Heidelberger's quantitative method permits extremely precise quantitative measurement of the reagents. It is now widely used in studying various antigens, including those of bacteria and is especially important in analyzing antigens which yield cross reactions or contain impurities of other antigens; it is also employed for accurate titration of antibodies.

The general procedure for ordinary quantitative analysis consists in the following: increasing quantites of antigen are added to a series of test-tubes containing equal volumes of precipitative serum. The contents of the test-tubes are mixed and the tubes are placed in a refrigerator at 0-4°. After 48 hr they are centrifuged, being cooled during the process. The precipitates are washed twice with physiological solution under refrigeration and analyzed for nitrogen content by Kjeldahl's micromethod. For convenience, the figures are expressed in units of nitrogen rather than protein. These reactions are usually repeated and the mean values calculated.

Each supernatant is tested by addition of antigen and precipitative serum, as described in the section headed "Determination of excess antigen or antibody in the supernatant."

If analysis of the supernatant shows that it contains no antigen, it is assumed that all the antigen is in the precipitate (provided that it is a simple homogeneous substance) and the antibody nitrogen is calculated by subtracting the nitrogen in the antigen added from the total

precipitate nitrogen. If the antigen is a polysaccharide the precipitate contains only antibody nitrogen and this operation need not be performed. The quantity of antibody which reacts with a given antigen (in units per mg of nitrogen) and, conversely, the quantity of antigen fixed by a given quantity of antibody can easily be calculated from Heidelberger and Kendall's theoretical quadratic equation.

A detailed description of the various modifications of this method for different antigen-antibody systems is given in an article by Kabat and Meyer.

There are also other methods for the quantitative and semiquantitative determination of serum-precipitin titre, but these are employed less frequently and are therefore only mentioned here.

Titration of precipitative sera by the agglutination-reaction method. A suitable adsorbent, colloidal particles of a low-activity substance (pumice, dermatol, talc, ion-exchange resins, suspension of killed bacteria, etc.), is selected for the antigen to be investigated. These particles should be adsorb the antigen molecules actively, but should have no great influence on the character of their chemical (antigenic) activity.

The adsorbent particles must satisfy the same requirements with respect to physical state as any bacterial suspension used for the agglutination reaction (see the section headed "Agglutination reaction").

Progressive double dilutions of the serum under study are prepared and adsorbent particles preliminarily soaked in the antigen solution are added to each dilution in experimentally-determined optimum constant concentrations (N.M. Shklyar).

Technically, this method is the same as the ordinary agglutination reaction. One modification is the highly sensitive Hudner method, in which the colloidal particles are added to the solution after the anti-

gen and antibody are mixed.

General notes on methods for the precipitation reaction. The precipitation reaction requires observation of certain conditions, violation of which may lead to erroneous results. A very large excess of antigen suppresses the reaction. A similar phenomenon may occur in certain (toxin-antitoxin) systems when an excess of antibody is present (flocculation reaction). Maintenance of standard pptimum experimental conditions plays an important role in precise quantitatice determinations. It has been established that several hours are sufficient for complete precipitation of an antigen-antibody system when strong sera containing more than 10 mg/ml of antibody nitrogen are used, while approximately a week is required when weak sera (e.g., from patients recovering from pneumonia) are used. Analysis of sera containing less than 0.1 mg/ml of antibody nitrogen usually requires 2 days or more.

The reaction proceeds most rapidly and completely at the equivalence point and most slowly in the suppression zone (sometimes requiring more than a week).

Precipitation is maximal at low temperatures (0-4°). The difference in the reactions at 0 and 37° is not very great for rabbit sera, while for horse sera it may reach 13%.

The salt (sodium chloride) concentration is of great importance. It has been established that rabbit and horse sera produce the largest amount of precipitate at a salt content of 0.9-1%, while the maximum for avian sera lies at 8%. Absence of salt occasionally suppresses the reaction completely (e.g., in the flocculation reaction between diphtheria toxin and antitoxin).

The pH has a definite influence on the precipitation reaction, although many antigen-antibody systems yield almost uniform results at any pH between 6.4 and 7.8. In the annular precipitation reaction a

more acidic medium promotes the appearance of so-called nonspecific double (split) rings, which have a tendency to be displaced in opposite directions (into the serum and antigen zones).

In conducting experiments over a prolonged period of time (days or weeks) it is necessary to maintain sterile conditions to avoid contamination of the system with common microorganisms; the results cannot be regarded as reliable if this is not done. Sterility is achieved by conducting the experiment under aseptic conditions or by using bacterial inhibitors such as merthiolate (0.01%). Phenol, which is often employed in the diffusion method on agar, cannot be used as an inhibitor when the reaction is carried out in the liquid phase, since it produces erroneous results.

Attention must be called to the fact that some immune sera are nonspecific, reacting with the homologous antigen and many others and sometimes with physiological solution. In such cases the validity of the results depends wholly on the preliminary check of serum specificity and the setting up of appropriate controls.

The presence of rather complex common antigens in many species of bacteria (e.g., the coliform group) often makes it difficult to use the ordinary precipitation reaction to study their fine structures. Even the serum-depletion method with common antigens does not always yield satisfactory results.

Precipitative sera obtained to various bacteria usually consist of broad-spectrum groups of antibodies; in practice, these are difficult to separate or purify, even by electrophoresis.

Methods involving diffusion in agar can be used to detect individual antigens or antibodies present in sufficient concentrations in various mixtures.

Precipitation reaction in agar

As a result of the varying dispersion of the antigen and antibody particles, methods involving diffusion in agar permit some separation of individual antigen-antibody systems in mixtures. Over a given period the antigen and antibody molecules in the agar move through different distances, which depend on the dispersion, so that the point where they meet (precipitation zone) is easily distinguished visually.

Agar-diffusion methods have been developed by Oudin, Ouchterloni, Elek, Oakley, and Fulsorp. We distinguish two procedures: 1) simple diffusion, in which one reagent diffuses into agar containing a uniform concentration of the other; 2) double diffusion, in which the two reagents move toward one another and meet in a neutral layer of the agar.

It has been demonstrated that the specific precipitation of an antigen-antibody system has nothing in common with the nonspecific phenomenon known as Lisegang's ring. It is assumed that a single antigen reacting with a homologous antibody forms only one precipitation zone (line).

If several antigen-antibody systems are moving through the agar they are independent of one another. The number of precipitation zones (lines) corresponds to the minimum number of antigen-antibody systems present in the fluids under investigation.

Simple-diffusion methods

Oudin's method. A good brand of agar which does not yield a large amount of residue is selected, washed several times with redistilled water, ground fine, dissolved in a fresh portion of redistilled water to produce a 4% solution, and mixed with normal rabbit serum (10%). The mixture is sterilized in an autoclave at 115° for 15 min and centrifuged at 7000 rpm while hot until a clear supernatant is obtained. Distilled water is added to the latter to produce a 2.4% agar solution, to which 0.85% sodium chloride is added. The prepared agar is poured into

test-tubes and twice sterilized at 100° for 30 min at a 24-hr interval.

Before setting up the precipitation reaction the agar is melted in a water bath, cooled to 50°, and mixed half and half with the serum to be studied. Portions of 1 or 2 ml of the mixture are poured into sterile narrow test-tubes (0.7 cm in inside diameter and 7 cm long) sealed with cotton plugs.

After the agar has solidified 1 or 2 ml of the antigen solution under investigation is poured over it and the test-tubes are sealed with sterile rubber stoppers and placed in a refrigerator at 0-4°. The reaction is checked daily, recording the appearance time, number, and movement of the precipitation zones.

When the antigen concentration is high weak precipitation zones develop within a few hours or days, having the form of uniform disks perpendicular to the axis of the test-tube and exhibiting a tendency to move after several days.

When several antigen-antibody systems participate in the reaction many precipitation zones which appear homogeneous when they develop may split into several new zones after 3-7 days.

If the antigen concentration is low precipitation zones may not develop until several (5-7) days have elapsed, often having a meniscoid form and virtually no tendency to move. A sharply delineated reaction zone indicates that the antigen is homogeneous, while a split zone without clear boundaries indicates a polydispersed antigen.

Petri and Stiben's method was proposed for diagnosing the causative agent of gas gangrene (Cl. perfringens type A, Cl. oedematiens, Cl. septicum).

Antitoxic serum tto Cl. septicum, Cl. perfringens, or Cl. oedematiens is added to the 2% beat-infusion agar used for culturing anaerobic Clostridia in a dose of 8 units per ml. The species of clostridia

to be studied is transferred to different points on 3 dishes by the injection or streak method. The dishes are incubated at 37° for 24 hr under anaerobiotic conditions. If the bacterium produces a homologous toxin weak concentric precipitation lines (zones) appear around the sites of culture growth and become more pronounced after 48-72 hr.

Ouchterloni's method. One part of agar is dissolved in 30 parts of hot water and precipitated with 0.5% calcium chloride. The residue is removed by filtration. The solidified agar is ground and dialyzed in water for 72 hr. It is then remelted and 1 liter is mixed with 1.6 g of sodium chloride; merthiolate (1:10,000) or methyl orange (3:100,000) is then added to suppress growth of common microorganisms. The prepared agar is stored in a refrigerator in sterile flasks.

Before use the agar is melted and cooled to 50°, 50% precipitative serum is added to it (e.g., 50 ml of agar and 50 ml of serum), and a thin layer (0.2 cm) is poured into a sterile Petri dish with a smooth, clean bottom and permitted to solidify.

Triangular or tubular metal molds set up no less than 1 cm apart and filled with a 0.3 cm layer of fresh agar.

After the agar has cooled the molds are carefully removed and the wells thus formed are filled with the antigen to be studied. All these procedures must be carried out under relatively sterile conditions.

The dishes, with their wells filled, are left on a level surface for 2-5 hr and then placed in a refrigerator at 0-4°. The precipitation zones take the form of concentric circles around the antigen-containing wells. The reaction is observed for 14 days.

The wells can also be formed with special metal punches, keeping a constant distance between the centers of the tubes. Such punches can be produced in any machine shop. This method is somewhat more sensitive than the test-tube technique.

The time required for the precipitation zones to appear depends to some extent on the thickness of the agar layer; the thicker the layer, the more rapidly the antigen diffuses.

Double-diffusion methods

Elek's method. A meat-infusion agar is prepared, based on a medium suitable for the exotoxin-producing microorganism in question. A thin transparent layer (0.3-0.4 cm) of sterile agar is poured into a Petri dish with a smooth, clean bottom. A strip of sterile filter paper moistened in antitoxic serum is placed on the surface of the solidifed agar, along the diameter of the dish.

The cultures under investigation are applied in streaks perpendicular to the paper strip; the streaks should be no less than 1 cm apart. The inoculated dishes are incubated under conditions optimum for the bacterium in question. Precipitation zones appear around cultures treated with homologous toxin after 24-72 hr.

Various indicators, such as washed erythrocytes, clear lecithovitellin solution, powdered skin, dentine, etc., can be added to the medium to permit simultaneous detection of the enzymatic activity of certain toxins (lecithinase, collagenase, etc.).

Oakley and Fulsorp's method. Ouchterloni's method is used to prepare 1% neutral agar. A 0.5% phenol solution is added to the agar in order to inhibit common microorganisms; the agar is poured into ordinary bacteriological test-tubes and stored in a refrigerator. Before use it is melted and cooled to 50°.

A total of 1 ml of 1% agar is mixed with different volumes of serum and 0.4 ml portions of the mixture are carefully poured into two narrow test-tubes (0.7 cm in diameter and 7 cm long) and permitted to solidify (lower layer). At this point an additional 1 ml of hot 1% agar is carefully mixed with an equal volume of physiological solution and

added to the 0.4 ml in the two experimental test-tubes (middle layer). Finally, a new portion of melted agar (1 ml) is mixed with an equal volume of the antigen under investigation and poured over the 0.4 ml in the experimental tubes (upper layer); each layer is approximately 1 cm deep. Two test-tubes are employed because the experiment must be duplicated in order to avoid incorrect results. The experimental tubes are sealed with sterile rubber stoppers and placed in a refrigerator at 0-4°. The antigen and antibody, moving to meet one another in the middle agar layer, form precipitation zones. This procedure is more sensitive and less affected by temperature fluctuations than Wilson's simple-diffusion method.

Many modifications of the double-diffusion method have been proposed. Oakley developed a convenient technique for double diffusion in agar in Petri dishes.

A 0.3-0.4 cm layer of neutral agar prepared with 0.5% phenol by Ouchterloni's method is poured into a Petri dish with a smooth, clean bottom and permitted to solidify. A special metal tube with an outside diameter of 0.5 cm and a sharp edge is used to make wells, following a predrawn pattern placed under the bottom of the dish.

The tube can be connected to a water-jet pump through a glass bulb, so that the wells are immediately cleaned out by aspiration of the agar disks as they are cut away.

A total of 31 wells is prepared, their margins being separated by 0.6 cm. This makes it possible to conduct various combined experiments with one or more sera and one or more antigens, which is especially important in setting up cross-precipitation reactions with antigens from one or more species of bacteria.

G.I. Abelev suggested a modification of the precipitation reaction in agar for studying antigens and antibodies with common and different

components. In essence, this method consists in cutting wells in the corners of a square, in such fashion that the distance between their margins along the diagonal is 1 cm. The antigen and corresponding antibody are poured into diagonally opposite wells.

A.I. Gusev and V.S. Tsvelkov's micromodification of the agar-diffusion method (1961) can be used to obtain good photographs of the precipitation zones. A thin layer of agar is applied to clean, degreased 9 x 12 photographic plates separated by glass strips at 2 or 3 different points. Wells are cut into the agar with special standard metal punches attached to the tube of a microscope.

The punch tubes have an outside diameter of 2-5 mm; each punch has from 4 to 7 tubes with a distance of 5.5-10 mm between their centers.

The use of punches makes it possible to maintain a constant distance between the wells and to fill them with equal volumes of reagents. The plates, with the wells filled, are placed in a moist chamber (a desicator with water on the bottom) in order to keep them from drying out and the results are evaluated after 1-2 days. Good photographs of the precipitation zones can be taken by illuminating the plates indirectly.

Densitography of the precipitation lines on agar plates approximately 3 mm thick, using a selenium photocell, can be employed for quantitative evaluation of precipitation reactions carried out in agar by the double-diffusion method.

The precipitation zones are treated with special stains in order to show up olysaccharides or monoproteins in the precipitates. If the researcher has only negligible quantities of antigen and antibody available, he can use the capillary micromethod for conducting the precipitation reaction in agar (O.Ye. Vyazov et al., 1959), which permits analysis of 0.001-0.004 ml of the substance under study. Purified 1% neutral agar dissolved in physiological solution containing 0.1% phenol is

poured into test-tubes, autoclaved, and stored in a refrigerator. Before use the agar is melted, centrifuged at 3000 rpm in order to remove suspended particles, and then poured into capillary Pasteur pipettes with an inside diameter of 0.6-0.8 mm. The agar column has an optimum height of 1-1.5 cm. It is kept 3 cm from the end of the pipette and permitted to solidify and the capillary is then cut f om the remainder of the pipette, 1.5-2 cm from the edge of the agar, with a carborundum disk.

Tuberculin syringes with fine needles are used to inoculate one side of the agar with the antigen and the other side with the antibody (taking care to avoid air bubbles). The capillary is placed on a side, its ends are sealed with paraffin, and it is incubated at 37° for 1 day and then kept in a refrigerator for 17 days.

Precipitation zones appear after 2-48 hr, but the clearest results are observed between the 7th and 17th days. The density of the zones is evaluated with a microscope optical system connected to a photocell and galvanometer.

Recording is carried out by compilation of appropriate curves and microphotometry.

The advantage of the various methods for conducting the precipitation in agar lies in the fact that they permit analysis of mixed antigens and antibodies and use of relatively cloudy antigens and are technically simple.

Their drawbacks lie in the imperfection of the quantitative analysis, the rather long duration of the reaction, and their low sensitivity to certain bacterial toxins (tetanus, botulin, Cl. perfringens) (B.D. Bychenko, 1960; T.I. Bulatova, 1961).

Complement-Fixation Reaction

In the complement-fixation reaction (CFR) the antigen and antibody

combine to form a complex capable of adsorbing complement. An antigen-antibody complex is formed if the two are homologous. If they are not homologous no complex is formed and the complement remains free. Complement-fixing immune antibodies are present only in immune serum and are specific to the corresponding antigen.

The antigen-antibody-complement complex is invisible when produced. A hemolytic system is introduced into the reaction in order to determine whether the complement has been adsorbed or remains free. This system is an indicator for the first phase of the reaction. It consists of an antigen (sheep erythrocytes) and hemolytic serum specific to the erythrocytes. Hemolysis takes place in the presence of complement. If the antigen and antibody used in the experiment are homologous the complement is adsorbed on the antigen-antibody complex and the erythrocytes are not hemolyzed when the hemolytic system is added. If the antigen and antibody are heterologous the complement remains free and hemolysis occurs when the hemolytic mixture is added.

The hemolysis is very graphic and can be observed as soon as hemoglobin is released from the stroma of the erythrocytes, staining the liquid red.

Consequently, if there is no hemolysis the complement-fixation reaction is diagnostically positive and the serum under investigation corresponds to the antigen used. Conversely, if hemolysis does take place the complement-fixation reaction is diagnostically negative and the serum is not homologous to the antigen.

The complement-fixation reaction is thus a combination of two reactions, the first being adsorption of the complement by the specific antigen-antibody complex (the basic specific phase) and the second the hemolytic reaction (which indicates whether or not complement fixation has occurred).

Of the hypotheses which have been advanced regarding the mechanism of the lytic reaction, the most acceptable is that which regards the immune antibody as a sensitizing agent that prepares the cell to receive the complement. Adsorption of the latter by the antigen-antibody complex leads to cellular lysis.

The complement-fixation reaction can be used: 1) to determine the properties of an unknown serum from its interaction with a known antigenic diagnostic serum; 2) to detect and determine the species of an unknown antigen from its interaction with a known diagnostic serum. The CFR is the only practicable immunological reaction for diagnosing infectious diseases such as glanders, gonorrhea, syphilis (the Bordet-Wassermann reaction), tuberculosis, pertussis, rhinoscleroma, and echinococcosis. It is also a valuable research technique in other infections (tularemia, brucellosis, rickettsiosis, and various viruses) and in solving a number of scientific and theoretical problems in the research laboratory.

In addition to these advantages, the complement-fixation reaction has a number of characteristics which present a material obstacle to wide utilization. It involves 5 components, which must be used in strictly quantitative proportions, and this creates certain technical difficulties. Some of these components are very labile and must consequently be prepared immediately before the reaction is set up (the complement) or several days beforehand (the sheep erythrocytes). The need for complement and sheep erythrocytes forces the laboratory to keep the appropriate animals (guinea pigs and sheep) before they are used, which is not always possible under practical conditions.

The difficulty of setting up the complement-fixation reaction is also due to the fact that the serum and antigen often have anticomplementary properties, which can distort the results. In order to avoid in-

correct results the reaction is based on a quantitative method, which requires preliminary titration of the ingredients to determine the working dose, i.e., the quantity of a given component necessary. These preliminary titrations complicate the procedure.

The complexity of the reaction explains the fact that it is often carried out over 2 days. The necessary preliminary work is conducted during the first day, while the basic experiment is performed on the second.

Vessel and apparatus. Ordinary bacteriological or, still better, Wassermann test-tubes (10-12 x 80-100 mm), racks to hold them (6-10 holes), graduated 1.5 and 10 ml pipettes, graduates, and flasks are necessary to set up the complement-fixation reaction.

All the vessels should be washed clean and sterilized. A new vessel must be cleaned especially carefully in hot water with a brush, but without using acids or alkalies. If a dirty vessel is washed with a chromate mixture it should then be carefully rinsed in running tap water. Vessels used for the CFR are not employed for other work. It is necessary to have a 3000 rpm centrifuge, a water bath for the racks and test-tubes, and a heater.

Medium conditions for the complement-fixation reaction. The medium used to set up the reaction has a substantial influence on its course and results. The antigen-antibody complex may dissociate at a very acid or alkaline pH, which distorts the results of the reaction. The optimum pH for complement fixation is 7.0-8.0.

Hypertonic sodium chloride, solutions of calcium and lithium salts, and isotonic physiological solution have an inhibitory effect on formation of the specific complex.

The function of the complement varies with the ion concentration in the medium. K, Na, Ca, Ba, and NH_4 cations and NO_3, Y, SO_4, F, Cl,

and other anions have a strong inhibitory action. In this connection the physiological solution must be prepared with chemically pure sodium chloride whose content of potassium and calcium and magnesium sulfates should not exceed thousandths of a percent and of calcium and magnesium chlorides should not exceed hundredths of a percent (S.I. Ginzburg and V.S. Kalinin).

Redistilled water must usually be used in preparing the physiological solution.

Reaction components

A total of 5 components participate in the complement-fixation: hemolytic serum, sheep erythrocytes, complement, antigens (diagnostic or unknown), and serum (unknown or diagnostic).

The total reaction volume is usually 2.5 ml, 0.5 ml of each component. It is permissible to double or halve the total volume, which then amounts to 5 ml in the first case and 1.25 ml in the second. A mixture volume of 5 ml is most favorable for a correct complement-fixation reaction, but the usual volume is 2.5 ml because of a lack of some components.

In setting up the reaction it is first necessary to prepare the hemolytic serum.

Hemolytic serum

The hemolytic serum is prepared by immunizing rabbits. The animals are repeatedly (3-4 times) given 2-5 ml of a 50% suspension of washed sheep erythrocytes at intervals of 2-3 days. Blood is taken from the rabbits 6-8 days after the last injection if preliminary titration (after 6-7 days) shows a serum titre of no less than 1:1000.

After 2 or 3 weeks of rest the animals are revaccinated and the next immunization cycle begins. The serum obtained must be inactivated in order to remove the complement (at 56° for 30 min). For preservation

TABLE 8

Scheme for Preparation of Hemolytic-Serum Dilutions From a Basic Dilution of 1:100

1 № пробирки	2 Необходимое разведение сыворотки	Гемолитическая сыворотка, мл 3	4 Физиологический раствор, мл
1	1:1000	1,0	9
2	1:1300	1,0	12
3	1:1500	1,0	14
4	1:1700	1,0	16
5	1:2000	1,0	19
6	1:2500	1,0	24
7	1:3000	1,0	29

1) Test-tube No.; 2) serum dilution required; 3) hemolytic serum, ml; 4) physiological solution, ml.

1% dry boric acid is added to the serum. A mixed series is prepared from sera obtained from several rabbits, titrated, poured into ampules, and stored in a refrigerator.

Serum with a titre of no less than 1:1200-1:1500 must be used in the complement-fixation reaction. The hemolytic serum is titrated in accordance with the following scheme: it is diluted with physiological solution in a ratio of 0.1 ml of serum to 9.9 ml of solution and the basic dilution of 1:100 is then further diluted (Table 8).

TABLE 9

Titration Scheme for Hemolytic Serum (after V.S. Kalinin)

1 № пробирки	Объем гемолитической сыворотки (мл) и ее разведение 2	3 3% взвесь эритроцитов, мл	4 Комплемент в разведении 1:10, мл	5 Физиологический раствор, мл	6 Режим	7 Результат
1	0,5 (1:1000)	0,5	0,5	1,0	8 Термостат при 37° в течение 1 часа	9 Полный гемолиз
2	0,5 (1:1300)	0,5	0,5	1,0		» »
3	0,5 (1:1500)	0,5	0,5	1,0		10 » »
4	0,5 (1:1700)	0,5	0,5	1,0		Частичная задержка 11 гемолиза
5	0,5 (1:2000)	0,5	0,5	1,0		Полная задержка 12 гемолиза
6	0,5 (1:2500)	0,5	0,5	1,0		То же
7	0,5 (1:3000)	0,5	0,5	1,0		» »
8	Без сыворотки 13	0,5	0,5	2,0		» »

1) Test-tube No.; 2) volume (ml) and dilution of hemolytic serum; 3) 3% erythrocyte suspension, ml; 4) complement diluted to 1:10, ml; 5) physiological solution, ml; 6) regime; 7) result; 8) heater at 37° for 1 hr; 9) complete hemolysis; 10) partial arrest of hemolysis; 11) complete arrest of hemolysis; 12) the same; 13) no serum.

In order to titrate the serum it is necessary to prepare a 3% suspension of sheep erythrocytes and complement in a dilution of 1:10 (see below).

Portions of 0.5 ml of the consecutive serum dilutions are poured into test-tubes. The serum is diluted with a graduated (1 ml divisions) pipette, beginning with the highest dilution. 0.5 ml of the 3% sheep-erythrocyte suspension, 0.5 ml of complement diluted to 1:10, and 1 ml of physiological solution are then added to each test-tube. The control is a test-tube containing 2 ml of physiological solution and 0.5 ml of the 3% sheep-erythrocyte suspension, i.e., an erythrocyte-resistance control (Table 9). The test-tubes are placed in a heater at 37° for 1 hr.

Hemolysis is usually observed at low dilutions. The titre of the hemolytic serum is assumed to be equal to the maximum dilution in a 0.5 ml volume at which complete hemolysis of 0.5 ml of the 3% erythrocyte suspension occurs in the presence of 0.5 ml of complement diluted to 1:10 when the tubes are kept in a heater at 37° for 1 hr.

In Table 9 the titre of the hemolytic serum is 1:1500, since this is the maximum dilution which produces complete hemolysis. This titre is reduced by a factor of 3 in setting up the complement-fixation reaction.

For example, serum with a titre of 1:1500 is taken in a dilution of 1:500 (1 ml of the basic 1:100 dilution and 4 ml of physiological solution or 0.1 ml of undiluted serum and 49.9 ml of physiological solution) for the CFR. Each series of hemolytic serum is titrated when it is prepared. If it is stored for an extended period its titre is checked periodically (every 3-4 weeks).

Sheep erythrocytes

Sheep erythrocytes serve as the antigen in the hemolysis reaction.

The erythrocyte producers are usually healthy one-year-old sheep, which are replaced by fresh animals after 1-1 1/2 years. Portions of 100-150 ml of blood are taken from the jugular vein no more often than once every 10-15 days. When phlebotomy is performed more frequently the resistance of the erythrocytes is lowered: such erythrocytes cause attenuation of positive CFR results.

In taking blood the wool on the neck is clipped and the site where the needle will be inserted is washed with alcohol. An assistant applies a tourniquet below the site of the puncture. The vein is located with the index finger of the left hand, slightly compressed, and punctured with the needle. A rubber tube should be slipped over the needle, emptying into a flask containing glass beads. The blood is collected, defibrinated by shaking the flask for 10-15 min, and aspirated or filtered through 2 or 3 layers of gause to free it from fibrin clots. The erythrocytes are then washed with 3-4 portions of physiological solution by centrifuging and resuspension. After the supernatant, which should be colorless, has been removed the erythrocytes are stored in a refrigerator for 5-6 days. A 2.5-3% erythrocyte suspension is usually used in the reaction; it is prepared by adding 39-33 ml of physiological solution to 1 ml of the residue of washed erythrocytes.

For more prolonged storage of the erythrocytes it is recommended that they be preserved with formalin or calcium chloride. A total of 1 ml of a 2% solution of formalin in physiological solution is added to 10 ml of the erythrocyte residue. The preserved erythrocytes are stored for 2-3 weeks. However, there are indications that such formalized erythrocytes have anticomplementary properties. In addition, they are often unsuitable for the CFR because of the rapid conversion of their hemoglobin to methemoglobin.

Preservation with calcium chloride has not come into wide use,

since it causes nonspecific arrest of hemolysis.

Preservation with Ringer-Locke's solution containing boric acid is more commonly employed. A solution consisting of 1 liter of distilled water, 9 g of sodium chloride, 0.2 g of potassium chloride, and 1 g of sodium diacetate is sterilized in a steam flow for 30 min and mixed with 10 g of dry chemically pure boric acid. The sheep erythrocytes are twice washed in this solution and a 3% suspension of the erythrocytes in it is prepared after the supernatant has been removed. When kept under refrigeration the 3% erythrocyte suspension in Ringer-Locke's solution can be used for 2-3 weeks. If the erythrocytes acquire a dark hue and the supernatant is discolored their use in the complement-fixation reaction is contraindicated.

A number of observations have shown that it is possible to use unwashed defibrinated blood in the CFR, which eliminates the tedious process of washing the erythrocytes by centrifuging. The defibrinated blood is filtered through gauze and diluted with Ringer-Locke's or physiological solution in a ratio of 1 ml of blood to 19 ml of solution.

The density of the erythrocyte suspension is of material importance in the CFR, since it has a considerable influence on the complement titre.

There are various methods for standardizing sheep erythrocytes. Standardization from the hemoglobin content of the sheep's blood (N.M. Nikolayev) is not sufficiently precise.

Standardization of the suspension in a photoelectrocolorimeter is the most accurate method (A.I. Vorob'yev and Ye.M. Bessonova). A 3% suspension of washed sheep erythrocytes is prepared, 9 ml of distilled water is added to 1 ml of the suspension, and the hemolyzed blood is poured into the colorimeter cell. Another cell (the control) is filled with 1 ml of physiological solution and 9 ml of distilled water. The

observations are made through a green filter. If the suspension has a 3% erythrocyte content the colorimeter reading is 36 extinction units. If the reading is above or below 36 the erythrocyte content is correspondingly greater or less than 3%. The calculation is made with the usual formula. For example, if the colorimeter reading is 45, X = = 45·3/36 = 3.75%.

This means that a 3.75 rather than a 3% erythrocyte suspension has been prepared. The appropriate quantity of physiological solution must be added to obtain a 3% solution.

Complement

Complement (alexin) is present in the serum of any species of animal, but the complementary function is most pronounced in mammalian sera, especially that of guinea pigs. It can be detected in the blood serum, exudates and transudates, the lymph, and the cerebrospinal fluid. A characteristic property of complement is its sensitivity to heating and other physical agents: heating at 56° for 30 min completely destroys it. It is also broken down when the serum is stored for an extended period or shaken, when acids are added to it, and under the action of other physical and chemical agents.

The properties of complement are similar to those of an enzyme, but its nature has not yet been determined. It is an intricate complex of normal serum proteins, some of which are often very labile, so that the function of complement is disrupted by various physicochemical factors (ultraviolet irradiation, shaking, and addition of acids, alkalies, alcohol, and ether).

When complement is dialyzed it separates into two fractions, a globulin fraction which precipitates and an albumin fraction which remains in solution. Both components are thermolabile.

A third (thermostable) component was discovered in inactivated se-

rum; it is broken down by yeasts, cobra venom, and insulin. The fourth component of complement proved to be thermostable and is broken down by ammonium salts, ether, chloroform, cadmium chloride, and other substances. The fifth component is not inactivated even when heated to 100° and is decomposed only by benzol. The sixth component is broken down by histamine and ethylene diamine. The components of complement are designated by C_1', C_2', C_3', C_4', etc.

The role of the fifth and sixth components in the CFR is still unknown. During the first phase of the CFR the antigen-antibody complex inactivates the C_2' and C_4' completely and the C_1' partially. The C_3' plays an important role in hemolysis and acts on sensitized erythrocytes.

Clear, fibrin-clot-free guinea pig serum taken on the day before the experiment is usually used as the complement in the CFR. The blood is generally drawn from the heart (see page 208).

Methods of preserving complement and obtaining dry complement which retains its activity for several months have now been developed. For preservative purposes 4% boric acid and 5% sodium sulfate in dry form are added to the guinea pig serum. After these substances have dissolved the complement is stored in a refrigerator. The preserved complement retains its activity for 1-3 months.

Dry complement is usually dissolved in physiological solution before use, as indicated on the packing slip accompanying the ampule.

Selection of the optimum complement dosage required for the reaction is a necessary condition for setting up the CFR properly and obtaining reliable results. There is an inverse relationship between the complement and hemolytic serum in the reaction; the larger the complement dose, the less hemolytic serum is required and vice versa. An excess of complement distorts the result of the reaction, since it causes substantial attenuation of a positive reaction and may lead to complete

loss of a weak positive reaction. A deficiency of complement causes retardation of hemolysis in negative reactions and in all controls, so that it is impossible to evaluate the reaction.

TABLE 10

Scheme for Titration of Complement Without Antigen

1 № пробирок	2 Комплемент в разведении 1:10, мл	3 Физиологический раствор, мл	4 Режим	5 Гемолитическая система, мл	Режим
			6		7
1	0,05	1,45	Термостат при 37° в течение 45 минут	1,0	Термостат при 37° в течение 30 минут
2	0,1	1,4		1,0	
3	0,15	1,35		1,0	
4	0,2	1,3		1,0	
5	0,25	1,25		1,0	
6	0,3	1,2		1,0	
7	0,35	1,15		1,0	
8	0,4	1,1		1,0	
9	0,45	1,05		1,0	
10	0,5	1,0		1,0	
11K	0,5	1,5		0,5*	
12K	—	1,5		1,0	

*Only 0.5 ml of the 3% etythrocyte suspension and no hemolytic serum is added to test-tube No. 11K.

1) Test-tube No.; 2) complement diluted to 1:10, ml; 3) physiological solution, ml; 4) regime; 5) hemolytic system, ml; 6) heater at 37° for 45 min; 7) heater at 37° for 30 min.

Only the optimum, i.e., correctly selected, complement dose ensures maximum adsorption on the antigen-antibody complex and complete hemolysis in the control when the reaction is positive.

The complement is titrated, i.e., the working dose is determined, before each experiment and a basic complement dilution of 1:10 is prepared. When high-activity complement, which guinea pig serum contains during the summer, is employed the basic dilution is raised to 1:20: this gives a more precise complement titre. The complement is titrated in accordance with the scheme shown in Table 10.

Portions of 0.05-0.5 ml of the basic complement dilution are poured into a series of test-tubes and sufficient physiological solution to bring the total volume to 1.5 ml is added to each tube. The rack holding

the tubes is placed in a heater at 37° for 45 min. The hemolytic mixture is prepared 30 min before the rack is removed from the heater: an equal volume of hemolytic serum (in a dilution equal to one-third of its titre!) is added to the 3% sheep-erythrocyte suspension. The two are thoroughly mixed and left in the heater at 37° for 30 min. The rack holding the complement-containing test-tubes and the hemolytic mixture are removed from the heater at the same time and 1 ml of the latter is added to each tube; the tubes are thoroughly shaken and placed in the heater at 37° for 30 min. The controls for complement titration are as follows: 1) 0.5 ml of 1:10 complement, 0.5 ml of the 3% sheep-erythrocyte suspension, and 1.5 ml of physiological solution (test-tube No. 11); 2) 1 ml of the hemolytic system and 1.5 ml of physiological solution (test-tube No. 12). Hemolysis should not occur in either control, since the first contains no hemolytic serum and the second no complement. Hemolysis in the controls may result from a number of factors: firstly, the erythrocytes may undergo hemolysis in physiological solution when the sheep in question has undergone frequent phlebotomy or when they have been stored for a prolonged period and, secondly, the hemolytic serum or complement may dissolve the erythrocytes.

The complement titre is assumed to be the minimum quantity in whose presence 0.5 ml of hemolytic serum in triple titre lyses 0.5 ml of the 3% sheep-erythrocyte suspension within 30 min at 37°. Complete dissolution of the erythrocytes occurs when large doses of complement are present, while small doses usually retard hemolysis.

When the complement is titrated it may be either too strong (guinea pig serum during the summer) or too weak. In the former case it causes hemolysis at almost all dosages used for titration. Hemolysis may be observed at low complement dosages when erythrocyte resistance is reduced. When the complement is weak hemolysis occurs only at large doses.

TABLE 11

Scheme for Complement Titration in the Presence of Antigen (after V.S. Kalinin)

1 Номер пробирки	2 Антиген, разведенный по титру, мл	3 Комплемент в разведении 1:10, мл	4 Физиологический раствор, мл	5 Режим	6 Гемолитическая смесь, мл	Режим
1	0,5	0,05	0,95	7 Термостат при 37° в течение 45 минут	1,0	8 Термостат при 37° в течение 30 минут
2	0,5	0,1	0,9		1,0	
3	0,5	0,15	0,85		1,0	
4	0,5	0,2	0,8		1,0	
5	0,5	0,25	0,75		1,0	
6	0,5	0,3	0,7		1,0	
7	0,5	0,35	0,65		1,0	
8	0,5	0,4	0,6		1,0	
9	0,5	0,45	0,55		1,0	
10	0,5	0,5	0,5		1,0	

Note: The hemolytic mixture is kept in a heater at 37° for 30 min before being added to the mixture of the first 3 reaction components.

1) Test-tube No.; 2) antigen diluted to titre, ml; 3) complement diluted to 1:10, ml; 4) physiological solution, ml; 5) regime; 6) hemolytic mixture, ml; 7) heater at 37° for 45 min; 8) heater at 37° for 30 min.

Reduced complement activity often results from a decrease in the hemolyzing power of the serum.

In order to obtain greater precision in determining the complement titre it is necessary to reduce the dosage more gradually; it is recommended that the basic dilution be 1:20 rather than 1:10 and that more highly fractionated doses be used.

A complement dose 20-35% larger than titre is used in the complement-fixation reaction, depending on the extent to which hemolysis was retarded in the preceding test-tube during titration. This dose is called the working dose. In practice, it is that quantity of complement which would produce complete hemolysis in the second test-tube.

If an antigen whose anticomplementary properties are unknown is employed in the complement-fixation reaction the complement is then titrated in its presence (Table 11).

The results obtained are used to compare the complement titres ob-

served with and without the antigen. If the antigen does not have anticomplementary properties the results of the two titrations are identical. An antigen with strong anticomplementary properties greatly increases the complement titre and cannot be used in the complement-fixation reaction. If the antigen has weak anticomplementary properties the working dose is determined by titrating the complement in its presence.

TABLE 12

Scheme for Factorial Titration of the Complement Working Dose

1 № пробирки	2 Сыворотка, мл	3 Физиологический раствор, мл	4 Рабочая доза комплемента, мл	5 Гемолитическая смесь, мл	6 Термостат 7 10 минут	6 Термостат 20 минут	6 Термостат 30 минут	8 Результат
1	0,5 (I)	0,5	0,5	1,0	++++	—	—	9 Доза достаточна
2	0,5 (II)	0,5	0,5	1,0	+++	—	—	
3	0,5 (III)	0,5	0,5	1,0	++++	—		
•								

1) Test-tube No.; 2) serum, ml; 3) physiological solution, ml; 4) complement working dose, ml; 5) hemolytic mixture, ml; 6) heater; 7) min; 8) result; 9) dose sufficient.

It is permissible to use human complement for the Wasserman reaction when guinea pigs are not available. Serum from 4 or more donors (Wasserman negative) is mixed together no more than 24 hr after the blood is taken and preserved with 4% boric acid and 5% sodium sulfate. The complement is titrated from a basic dilution of 1:1 in accordance with the scheme described above, using a 2% sheep-erythrocyte emulsion.

Since determination of the complement working dose is of great importance for a proper reaction, a number of authors have proposed more accurate methods for this purpose.

Cope's modification. In addition to titration of the complement without antigen, Cope suggested that it be titrated in the presence of a working dose of antigen and normal serum in accordance with the scheme described above. For highly fractionated complement doses (0.02, 0.04,

0.06, 0.08, 0.1, 0.12, etc.) are also employed, which makes it possible to titrate the complement working dose more accurately.

In setting up the complement-fixation reaction it is necessary to determine whether the complement working dose selected corresponds to the sheep-erythrocyte suspension prepared for the reaction.

For evaluation of the extent of this correspondence V.S. Kalinin and S.I. Ginzburg proposed a method of factorial titration of the complement working dose, in which the time factor is determinative (Table 12). Portions of 0.5 ml of three different sera are poured into three test-tubes. If a patient's serum is to be investigated it is diluted to 1:5, while if the species of an antigen is to be determined the test is made in the presence of specific sera diluted to 1:20. A working dose of complement, 0.5 ml of physiological solution, and 1 ml of hemolytic serum previously kept in a heater for 30 min are then added to each of the test-tubes. After thorough shaking the test-tubes are placed in a heater for 30 min and the results are recorded after 10, 20, and 30 min.

If retardation of hemolysis is observed after 10 min and complete hemolysis after 20 min the complement working dose is sufficient. If slightly retarded (+ or ++) or complete hemolysis is noted after 10 min the dose is too large and must be reduced by 0.02-0.04 ml. If hemolysis is still retarded after 20 min, even though in only one of the 3 test-tubes, the working dose is insufficient and must be increased by 0.02-0.04 ml.

Antigen

The complement-fixation reaction is carried out both with intact bacteria and cells and with dissolved antigens. There are various methods of preparing the antigen, using either fresh or dried initial material. A suspension of heat-killed bacteria in physiological solution is the most frequently employed. The bacteria can be heated at 100° for

TABLE 13

Scheme for Determination of Antigen Working Dose

1 № пробирки	2 Антиген, мл	3 Физиологический раствор, мл	4 Рабочая доза комплемента, мл	5 Режим	6 Гемолитическая система, мл	Режим	7 Результат
1	0,05	0,95	0,5	8 Термостат при 37° в течение 1 часа	1,0	8 Термостат при 37° в течение 1 часа	9 Гемолиз
2	0,1	0,9	0,5		1,0		»
3	0,15	0,85	0,5		1,0		»
4	0,2	0,8	0,5		1,0		»
5	0,25	0,75	0,5		1,0		»
6	0,3	0,7	0,5		1,0		Гемолиз
7	0,35	0,65	0,5		1,0		10 Задержка гемолиза
8	0,4	0,6	0,5		1,0		
9	0,45	0,55	0,5		1,0		11 То же
10	0,5	0,5	0,5		1,0		» »

1) Test-tube No.; 2) antigen, ml; 3) physiological solution, ml; 4) complement working dose, ml; 5) regime; 6) hemolytic system, ml; 7) result; 8) heater at 37° for 1 hr; 9) hemolysis; 10) retarded hemolysis; 11) the same.

15-20 min or at 60° for 1 hr.

A bacterial suspension treated with formalin or alcohol can be used as the antigen. In the latter case alcohol is added to the residue of washed bacteria in a ratio of 1:5 and the mixture is left to stand at room temperature for 2-3 days. After the excess alcohol has been removed the bacteria are flooded with physiological solution to a suspension density of 1 billion cells per ml and 1% dry boric acid is added as a preservative. Since the suspension is cloudy it is best to use clear bacterial extracts, which can be obtained by various methods (antiformin gonococcus, alcoholic tuberculosis antigen), as the antigen. Such extracts are distinguished by a higher sensitivity in the complement-fixation reaction than bacterial suspensions. They are more stable than suspensions, their titre remaining unchanged during prolonged storage. In order to obtain an extract the bacterial suspension is heated and then centrifuged. The clear supernatant serves as the antigen in the reaction. Complete antigens prepared by Buaven's method, polysaccharides, and alcoholic tissue extracts are often employed in the reac-

tion.

Many antigens have anticomplementary properties, i.e., are capable of adsorbing the complement directly in the absence of immune serum. In this connection the complement titre must be determined in the presence of the antigen before the basic experiment is set up. The titration scheme has already been described. The antigen should not suppress complement activity by more than 30%.

It is also necessary to determine the antigen working dose in accordance with the scheme shown in Table 13 before setting up the basic experiment. The complement, hemolytic serum, and erythrocytes are used in titrating the antigen, each being taken in a volume of 0.5 ml. The reaction volume is thus 2.5 ml. Physiological solution is added instead of the serum under investigation.

Different antigen doses (from 0.1 to 0.5 ml) are poured into a series of test-tubes and sufficient physiological solution is added to make a total volume of 0.5 ml. After thorough shaking the tubes are placed in a heater for 1 hr. After this period has elapsed 1 ml of hemolytic serum previously kept in a heater for 30 min is added to each tube. After shaking the test-tubes they are again placed in the heater for 1 hr.

The greatest quantity of antigen at which hemolysis takes place is the antigen titre and is called the solvent dose.

The antigen working dose necessary to carry out the basic CFR is usually 2/3 of the solvent dose (if the antigen is aqueous). In the example given in Table 13 the antigen working dose is 2/3 of 0.3 ml, i.e., 0.2 ml.

When an alcoholic antigen is titrated the working dose is generally half the largest dose which causes hemolysis (0.15 ml in the example in question).

Since the antigen may have hemotoxic as well as anticomplementary

properties, i.e., may dissolve erythrocytes, its hemolytic characteristics must be checked. A total of 0.5 ml of the 3% erythrocyte suspension and 1 ml of physiological solution are added to 1 ml of antigen diluted to titre. The mixture is shaken and held at 37° for 1 hr. Hemolysis should not take place and if it does the antigen is unsuitable.

Serum

A known (diagnostic) serum can be used to determine an unknown antigen in the complement-fixation reaction. More often, however, a known (diagnostic) antigen is used to detect the presence of homologous antibodies in the serum under investigation. Serum from patients is the usual object of study. For this purpose 5-10 ml of blood is taken from a vein with the patient's stomach empty, at least 6 hr after his last meal, and the serum is extracted. The latter must be inactivated for 30 min at 56° on the day before the CFR. It is mixed with 2% dry boric acid and stored under refrigeration. After prolonged storage the serum is again inactivated for 10-15 min on the day before the reaction. In addition to breaking down the complement, inactivation stabilizes the serum colloids necessary for the reaction.

In view of its low specific activity, the patient's serum is generally used in a dilution of 1:5-1:10 in the complement-fixation reaction.

Diagnostic sera or the complement-fixation reaction are usually obtained by immunization of rabbits. Equine sera are rather inactive in this reaction. The diagnostic sera are employed in dilutions of 1:50, 1:100, and 1: 1000, since lower dilutions (1:5-1:10) yield nonspecific results. They are inactivated before the CFR is set up.

Setting up the basic experiment

All the components of the complement-fixation reaction are diluted with physiological solution prepared from chemically pure sodium chlor-

TABLE 14

Scheme of Basic Experiment for Investigating an Unknown Serum (after V.S. Kalinin)

1 Что исследуется	2 № прибора	3 Сыворотка (мл) и ее разведение	4 Антиген, разведенный до титра, мл	5 Физиологический раствор, мл	6 Комплемент, мл	7 Режим	8 Гемолитическая смесь, мл	Режим	9 Результат
						15		16	
10 Исследуемая сыворотка	1	0,5 (1 : 5)	0,5 A_1	—	0,5	Термостат при 37° В течение 1 часа	1,0	Термостат при 37° В течение 40 минут — 1 часа	++++
	2	0,5 (1 : 5)	0,5 A_2	—	0,5		1,0		++++
	3	0,5 (1 : 5)	0,5 A	—	0,5		1,0		++++
	4	0,5 (1 : 5)	—	0,5	0,5		1,0		—
11 Нормальная сыворотка	5	0,5 (1 : 5)	0,5 A_1	—	0,5		1,0		—
	6	0,5 (1 : 5)	0,5 A_2	—	0,5		1,0		—
	7	0,5 (1 : 5)	0,5 A_3	—	0,5		1,0		—
	8	0,5 (1 : 5)	—	0,5	0,5		1,0		—
12 Положительная сыворотка	9	0,5 (1 : 5)	0,5 A_1	—	0,5		1,0		++++
	10	0,5 (1 : 5)	0,5 A_2	—	0,5		1,0		++++
	11	0,5 (1 : 5)	0,5 A_3	—	0,5		1,0		++++
	12	0,5 (1 : 5)	—	0,5	0,5		1,0		—
13 Контроль антигена на антикомплементарность	13	—	1,0 A_1	—	0,5		1,0		—
	14	—	1,0 A_2	—	0,5		1,0		—
	15	—	1,0 A_3	—	0,5		1,0		—
14 Контроль антигена на гемотоксичность	16	—	1,0 A_1	0,5	—		1,0		++++
	17	—	1,0 A_2	0,5	—		1,0		++++
	18	—	1,0 A	0,5	—		1,0		++++

In positive reactions the extent of the hemolysis is designated by the following 4-plus scale:

++++) complete arrest of hemolysis (erythrocytes settle to bottom, supernatant colorless);

+++) marked retardation (substantial precipitation of erythrocytes, supernatant slightly reddish);

++) partial retardation (erythrocyte residue on bottom, supernatant highly colored);

+) slight retardation (slight precipitation of erythrocytes, supernatant highly colored);

−) complete hemolysis (no residue, supernatant highly colored).

1) Subject; 2) test-tube No.; 3) serum (ml) and dilution; 4) antigen diluted to titre, ml; 5) physiological solution, ml; 6) complement, ml; 7) regime; 8) hemolytic mixture, ml; 9) result; 10) serum under investigation; 11) normal serum; 12) positive serum; 13) antigen control for anticomplementary properties; 14) antigen control for hemotoxic properties; 15) heater at 37° for 1 hr; 16) heater at 37° for 40 min to 1 hr.

ide and distilled water. The preliminary work (preparation of the complement, washing of the erytnrocytes, inactivation of the sera to be investigated) is carried out on the day before the reaction. The necessary dilutions of all the components are made up and the complement

working dose is determined on the day of the basic experiment (Table 13).

When an unknown serum is to be studied the CFR is usually conducted with three different series of antigen prepared by the same or different methods (A_1, A_2, and A_3). The serum, antigen, and complement are first combined in the doses shown in Table 14. After thorough shaking the rack is placed in a heater at 37° for 1 hr. The hemolytic mixture is then prepared from different volumes of the 3% erythrocyte suspension diluted to triple titre with the hemolytic serum. The mixture is placed in the heater 30 min before the rack holding the tubes for the basic experiment is removed. A total of 1 ml of the mixture is added to each of the test-tubes. The contents of the tubes are mixed and they are again left in the heater for 40 min to 1 hr, depending on the time required for hemolysis to develop in the control tubes. The results of the experiment are recorded after the tubes are removed from the heater and on the following day, the rack holding the tubes being kept in a refrigerator for 15-18 hr.

The experiment is accompanied by the following controls;

1) normal serum, i.e., serum known to be negative (tubes Nos. 5, 6, and 7), should yield complete hemolysis;

2) positive serum, which is usually kept from prior experiments (tubes Nos. 9, 10, and 11), should produce complete arrest of hemolysis;

3) all the test-tubes containing experimental serum but no antigen (tubes Nos. 4, 8, and 12) should exhibit complete hemolysis;

4) test-tubes Nos. 13, 14, and 15 (the antigen control for anti-complementary properties) should yield complete hemolysis;

5) test-tubes Nos. 16, 17, and 18 (the antigen control for hemotoxic properties) should exhibit complete arrest of hemolysis.

The reaction is recorded only when the controls react correctly.

Retardation of hemolysis in the serum control may result from too small a complement dosage. In this case it is necessary to titrate the complement working dose more precisely and repeat the experiment. In addition, the serum under investigation may have a capacity for autoretardation or, quite commonly, an anticomplementary action. The autoretardation properties of a serum are manifested as a result of changes in the dispersion of the serum colloids caused by prolonged storage or overheating during inactivation. All new serum portions or samples must be tested.

When the CFR yields a positive diagnostic result in the first three test-tubes containing the serum under investigation (tubes Nos. 1, 2, and 3) and in those containing serum known to be positive (tubes Nos. 9, 10, and 11) hemolysis is arrested (++++).

When a negative diagnostic result is obtained in test-tubes Nos. 1, 2, and 3 hemolysis must be complete, as in the test-tubes containing normal serum (tubes Nos. 5, 6, and 7).

The complement-fixation reaction is occasionally employed to identify an antigen. Cerebospinal fluid (in meningitis), bacterial extracts, the patient's excreta, serum containing bacteria or their decomposition products, etc., can be used as the antigen.

Diagnostic serum in a dilution of 1:20 is usually used as the antibody.

Normal serum, an antigen known to be positive, and an antigen known to be negative are taken as the controls. The CFR is most frequently carried out under refrigeration to identify antigens.

Complement-fixation reaction under refrigeration

A number of researchers (V.I. Ioffe, K.M. Rozental', and S.L. Shirvindt, I.V. Veynerov and R.B. Kalinina, L.S. Reznikova and O.A. Stoyanova) have shown the value of prolonged complement fixation under re-

frigeration at 0-4°. A decrease in temperature causes an intensification of complement adsorption.

The complement-fixation reaction under refrigeration requires the same preliminary work as the reaction under normal conditions. The complement working dose is 50% larger than that titrated in the presence of the antigen. The remaining volumes and quantitative relationships of the reagents are the same as in the usual set-up.

After the components which participate in the first phase of the reaction (antigen, serum, and complement) have been mixed the test-tubes are kept in a refrigerator at 0-4° for 18-20 hr. The hemolytic system, which has also been stored under refrigeration for 18-20 hr, is then added to all the tubes and they are placed in a heater until hemolysis appears in all the controls.

Conducting the complement-fixation reaction under refrigeration increases its sensitivity. This method is especially effective when small quantities of antigen or antibody are present in the material under investigation. Serum from patients with primary (serologically negative) syphilis yields a positive result when the reaction is carried out under refrigeration.

A modification of the complement-fixation reaction (A.I. Konikov) in which the back-titration method widely employed in analytic chemistry is used for the serological reaction has been proposed for quantitative determination of antigens and antibodies. This procedure differs from the usual method in the fact that the quantity of complement is sufficiently great that it is not completely fixed in either the control or experimental tubes. The residual free complement is titrated and the extent of fixation is determined from the difference in titres.

This variant has the normal sensitivity of the complement-fixation reaction. Its advantages are manifest when the reagents are highly an-

ticomplementary or when more precise quantitative serological analysis is necessary. It is rarely used.

A complement-fixation suppression reaction (CFSR) has also been proposed. It is used to determine antibodies which inhibit complement fixation. Such antibodies are not active in the complement-fixation reaction, since the complex which they form with homologous antigens does not fix complement.

A total of 6 components participate in the complement-fixation suppression reaction; in addition to the 5 components of the ordinary complement-fixation reaction, standard immune serum homologous to the antigen used and active in this reaction is added.

The complement-fixation reaction is suppressed in the following manner: the serum under investigation and the antigen are mixed and, after a predetermined interval, the complement and standard immune serum active in the complement-fixation reaction are added. The hemolytic system is added after a given period has elapsed.

If hemolysis is arrested after the mixture has been held in a heater the result is diagnostically negative: the serum under study has not aided the antigen in combining with the standard immune serum, the complement has been fixed on the antigen-antibody complex, and hemolysis has thus not occurred. The immune serum thus contains no antibodies homologous to the antigen used.

Occurrence of hemolysis indicates a positive diagnostic result: the serum under investigation contains antibodies homologous to the antigen and has formed a complex which inhibits combination of the antigen with the standard serum active in the complement-fixation reaction (a complement-fixation suppression reaction has occurred). The complement remains free and hemolysis takes place.

In view of its complexity and a number of unsolved problems, the

complement-fixation suppression reaction has not come into wide use. It is not known how widely complement-fixation-inhibiting antibodies are encountered, whether they occur in all species of animals, and in which human diseases they are formed. This reaction is used for diagnosing certain virus and rickettsial diseases of man.

The Hemagglutination Reaction

Hemagglutination refers to the aggregation of erythrocytes under the action of various agents. We distinguish active (direct) and passive (indirect) hemagglutination.

Active hemagglutination is the ability of erythrocytes to aggregate under the action of different types of substances. It is observed under the action of viruses, bacteria, bacterial metabolic products, plant and seed extract, snake venoms, etc., and is nonspecific.

Adsorption of an antigen on erythrocytes need not always be accompanied by aggregation. Such erythrocytes agglutinate only if they are mixed with a specific immune serum antigen. This reaction is called passive (indirect) hemagglutination.

In 1946 A.T. Kravchenko and M.I. Sokolov established that human erythrocytes are capable of adsorbing specific bacterial polysaccharides, which can then be detected on the erythrocytes with the aid of the agglutination reaction with specific serum. We have used this principle as a basis for developing a method for detecting antigens in the environment and for diagnosing infectious diseases.

The data obtained by A.T. Kravchenko and M.I. Sokolov have been confirmed by many Soviet and foreign researchers and are used for diagnosing typhus, intestinal infections, diphtheria, plague, tularemia, brucellosis, streptococcal infections, leprosy, pertussis, glanders, gonorrhea, and other infectious diseases.

The mechanism of the indirect hemagglutination reaction has not

been sufficiently well studied. A.T. Kravchenko and M.I. Sokolov (1946) showed that the attachment of an antigen to erythrocytes is an adsorptive process. However, a number of authors have established that sensitization is an active process at a temperature of 37° but ceases at temperatures below 5°, which does not correspond to normal physical adsorption, in which the extent of the adsorption is inversely proportional to the temperature.

It has been proposed that the receptors of erythrocytes which adsorb antigens are lipid in character. Erythrocytes are note sensitized by polysaccharide antigens in the presence of cholesterol and lecithin, substances which adsorb bacterial polysaccharides well (Neter, 1953). Even high polysaccharide concentrations, however, are not able to block all the erythrocyte receptors. This is shown by the fact that it is possible to sensitize erythrocytes with several successive antigens and by the fact that sensitized erythrocytes retain their specific and group characteristics. Boyden (1951) distinguished two phases in the indirect hemagglutination reaction: 1) a change in the surface properties of the erythrocytes as a result of adsorption of the antigen; 2) subsequent adsorption of antibodies on the sensitized erythrocytes and formation of conglomerates.

Setting up the indirect hemagglutination reaction

1. Preparation of erythrocytes. Human or sheep erythrocytes are used in the indirect hemagglutination reaction. Blood is taken from a sheep and defibrinated and its erythrocytes are drawn off. Freshly prepared erythrocytes or cells preserved with Alsever's solution can be used in the reaction.

Alsever's solution consists of 0.42 g of sodium chloride, 0.8 g of sodium citrate, 2.05 g of dextrose, and distilled water to make 100 ml.

The pH is brought to 6.1 with 10% citric acid. The solution is

sterilized by autoclaving. A total of 1.2 volumes of sterile Alsever's solution is added to one volume of blood taken under sterile conditions and the two are left to stand for 3 days. Such erythrocytes are suitable for the hemagglutination reaction for 2-3 weeks if they are stored at 4°.

Freshly prepared erythrocytes or cells preserved with Alsever's solution are washed 3-4 times with physiological solution in a centrifuge at 2000-3000 rpm. The washed erythrocytes can be kept for 3 days at 4°.

2. Sensitization of erythrocytes with antigen. The methods of preparing the antigens used for the indirect hemagglutination reaction will not be discussed here, since they differ for different bacteria and are described in the appropriate sections of the article devoted to this topic.

A predetermined volume of washed sheep erythrocytes (from 0.1 to 1 ml) is mixed with a given quantity of antigen. For example, in diagnosing tuberculosis 10 ml of an extract prepared from tuberculosis bacteria is mixed with 0.5 ml of washed sheep erythrocytes (after Middlebrook and Dubois); in diagnosing typhus 0.1 ml of washed sheep erythrocytes is added to 4 ml of antigen (V.A. Yablonskaya, 1959).

The erythrocyte-antigen mixture is usually kept in a heater for 1-2 hr. The mixture is periodically agitated to improve adsorption of the antigen by the erythrocytes. It is then centrifuged, being washed as many as 2-3 times with physiological solution in some cases, the supernatant is discarded, and a 0.5-1% suspension is made up from the residue of sensitized erythrocytes. Erythrocytes prepared in this manner are used in setting up the indirect hemagglutination reaction. They can be stored at 4° so long as hemolysis does not occur.

3. Preparation of sera to be studied. The patient's serum is di-

luted by a factor of 2-10; 1-2 drops of washed sheep erythrocytes are then added to each milliliter of dilute serum to remove nonspecific antibodies. The mixture is left to stand at room temperature for 15-20 min and then centrifuged. This adsorption procedure is repeated 2 or 3 times. The supernatant, which is dilute serum free of nonspecific hemagglutinins, is drawn off and inactivated at 56° for 30 min.

This serum is then diluted, taking into account the dilution made before the erythrocytes were added. In diagnosing tuberculosis the serum is diluted by factors of 2, 4, 8, 16, etc., while in diagnosing typhus it is diluted by factors of 100, 200, 400, 800, etc.

Portions of 0.4 ml of the various serum dilutions are poured into test-tubes. In diagnosing tuberculosis 0.4 ml of a 0.5% sensitized-erythrocyte suspension is added to each tube, while in diagnosing typhus 0.1 ml of a 1% suspension is added. The result of the reaction is evaluated after 2 hr at 35° in diagnosing tuberculosis, 1:8 being taken as the diagnostic titre.

In diagnosing typhus the tubes are placed in a heater at 30° for 16-18 hr, 1:1000 being taken as the diagnostic titre, although lower serum dilutions (1:250-1:500) cannot be regarded as nonspecific; the blood tests must be repeated in the latter case (V.A. Yablonskaya, 1959).

The reaction is accompanied by the following controls:

1) sensitized-erythrocyte control; 0.4 ml of physiological solution and sensitized erythrocytes in the volume used in the basic experiment;

2) 0.4 ml of physiological solution and unsensitized erythrocytes;

3) 0.4 ml of the serum under investigation and unsensitized erythrocytes. The reactions in all the controls should be negative.

It is thought that the indirect hemagglutination reaction is more sensitive to antibodies than the agglutination reaction and is in no

way inferior to the complement-fixation reaction. It is a valuable laboratory diagnostic method for nonpulmonary forms of tuberculosis (Berlinger and Brodhage, 1953). There is no unanimity of opinion regarding its diagnostic value in pulmonary tuberculosis.

In a number of other infectious diseases (typhus) the hemagglutination reaction is equal in its diagnostic value to the complement-fixation reaction.

Since only polysaccharides of various types can be used as the antigen in the reaction described above, its scope is limited. Protein antigens are adsorbed poorly on erythrocytes and thus do not produce a hemagglutination reaction. It has recently been discovered that treatment of erythrocytes with certain substances enables them to adsorb proteins from solution.

Thus, erythrocytes treated with tannin are capable of adsorbing protein antigens and agglutinating with corresponding immune sera (Boyden). Bidiazotized benzidine is also used to treat erythrocytes.

There are two variants of the indirect hemagglutination reaction with tannin-treated erythrocytes: 1) to detect antibodies; 2) to detect antigens.

Very little research has been done on the mechanism of the indirect hemagglutination reaction with erythrocytes treated with tannin or bidiazotized benzidine.

It is thought (Stavitskiy and Arquil) that tannin alters the physicochemical properties and structure (anionic permeability, sedimentation rate, osmotic resistance, resistance to various chemical compounds, shape, and surface structure) of erythrocytes. Dehydration reduces their stability in physiological solution (Boyden) and a solution of normal rabbit serum is consequently used as the stabilizer.

Tannin-treated erythrocytes are sensitized with homologous antigen

in order to detect antibodies. This modification of the indirect hemagglutination reaction is known in the literature as Boyden's reaction. It is used to detect antitoxins in human serum during immunization with diphtheria toxoid (Landy et al., Fisher), antibodies in the serum of plague patients (Landy and Trapani, Neil and Balthazar), and antibodies to certain viruses.

Conduct of Boyden's reaction

1. The sheep erythrocytes are prepared in the same manner as for the Middlebrook-Dubois reaction. A 2.5% suspension is prepared from the residue of washed erythrocytes (1 ml of the erythrocyte residue and 39 ml of physiological solution).

2. Tannin solution. A fresh tannin solution is usually prepared in a dilution of 1:1000, i.e., 10 mg in 10 ml of physiological solution at pH = 7.2. The dilutions necessary for the experiment (1:20,000 and 1:100,000) are then prepared from this basic dilution.

3. Buffer solutions. In order to prepare a 0.15 M Na_2HPO_4 solution 11.876 g of the salt is added to 1 liter of physiological solution; in order to prepare a 0.15 M KH_2PO_4 solution 9.078 g of the salt is added to 1 liter of physiological solution.

In order to obtain buffered physiological solution at pH = 7.0 61.1 ml of the 0.15 M Na_2PO_4 solution and 38.9 ml of the 0.15 M KH_2PO_4 solution are mixed, while to obtain a pH of 6.4 53.4 ml of the Na_2PO_4 solution and 186.6 ml of the KH_2PO_4 solution are mixed.

4. Solution of normal rabbit serum. Serum from fresh rabbits is inactivated for 30 min at 56° and nonspecific antibodies are removed by adding one volume of the washed-erythrocyte residue to 2 volumes of the inactivated serum. The mixture is left to stand at room temperature for 30 min and then centrifuged; the serum is drawn off and diluted to 1:100 with physiological solution before use.

5. Sensitization of erythrocytes with antigen. The erythrocyte suspension (2.5%) is mixed with an equal volume of tannin in a dilution of 1:20,000 and held for 10 min at 37°; the tannin is then carefully washed off and the erythrocytes are resuspended in the solution of normal rabbit serum to produce a 2.5% suspension.

One volume of the erythrocytes is combined with the antigen and 4 volumes of the buffered physiological solution (pH = 6.4) and left to stand at room temperature for 30 min. The erythrocytes are then washed with the solution of rabbit serum and resuspended in it to produce a 2.5% suspension.

6. Conduct of basic experiment. Before use the serum to be investigated is inactivated at 56° for 30 min and adsorbed on the sheep erythrocytes (one volume of the 2.5% erythrocyte suspension is added to one volume of serum) at room temperature for 10 min.

Various serum dilutions (usually progressing in twofold steps) are prepared in the solution of normal rabbit serum and 0.5 ml portions are ured into test-tubes. A drop of the 2.5% suspension of antigen-sensitized erythrocytes is added to each tube. The mixture is kept at room temperature and the results are evaluated after 2 1/2-3 hr.

The following controls are set up:

1) the least dilution of the serum to be studied and the tannin-treated erythrocytes;

2) the solution of rabbit serum and the sensitized erythrocytes;

3) the least serum dilution and erythrocytes treated with antigen but not with tannin.

In a second variant of the indirect hemagglutination reaction the tannin-treated erythrocytes are sensitized with immune serum. This reaction was first employed by T. Rytsaya to detect botulin toxin under experimental conditions and is called Rytsaya's modification of the in-

direct hemagglutination reaction.

Conduct of Rytsaya's reaction

1. Sheep erythrocytes prepared by the method described above are washed with physiological solution and a 2.5% suspension is prepared from the residue.

2. Tannin. A 1:20,000 tannin solution in physiological solution is prepared immediately before the experiment from the initial 0.1% solution, which can be made up beforehand (10 mg in 10 ml) and stored in a refrigerator for several days.

3. Antibody. Antitoxic sera purified by the "diatherm-3" method or by combined dialysis are suitable for this reaction, regardless of their antitoxic titre. Since not all series of serum yield a positive reaction with toxin, suitable series must be selected in preliminary experiments with known toxins. Native antitoxic sera usually give a negative result, even in high titre, and are consequently unsuitable for the indirect agglutination reaction. This is apparently the result of the presence of albumin, which (according to Neter et al.) has an inhibitory effect on the indirect agglutination reaction.

4. Solution of normal rabbit serum. Fresh normal rabbit serum is inactivated at 56° for 30 min. The inactivated serum is adsorbed on sheep erythrocytes for 30 min at room temperature. A total of 0.5 ml of the erythrocytes is added to 1 ml of serum and the mixture is left to stand at room temperature for 30 min. After centrifuging the serum is diluted to 1:100 in phosphate-buffered physiological solution at pH 7.0.

The solution of normal rabbit serum prevents agglutination of the erythrocytes after they are treated with tannin.

5. Antigens. If the presence of an antigen in blood serum is to be established the serum is first inactivated at 56° for 30 min and then diluted with a solution of normal rabbit serum prepared as described

above. Antigens capable of agglutinating on sensitized sheep erythrocytes (certain toxins and plant proteins) are adsorbed on erythrocytes at room temperature for 10 min (3 drops of the washed-erythrocyte residue are added to 1 ml of extract).

A total of 1 ml of a 1:20,000 tannin solution is added to a test-tube containing 1 ml of a 2.5% sheep-erythrocyte suspension prepared as described in Paragraph 1. The tube is shaken and placed in a water bath at 37° for 10 min. The erythrocytes are then centrifuged, washed once with physiological solution at pH = 7.2, and suspended in 1 ml of physiological solution.

In order to sensitize the tannin-treated erythrocytes to antibodies 3 ml of immune serum diluted to 1:10 with physiological solution at pH = 6.4 is added to a test-tube containing 0.1 ml of the tanninized erythrocytes. The tube is shaken and held at a temperature of 37° for 1 hr. The erythrocytes are then centrifuged, washed twice with a solution of normal rabbit serum (1:100), and suspended in 5 ml of the same solution.

6. Conduct of the basic experiment. Successive two-fold dilutions of the antigen are prepared in phosphate-buffered physiological solution (pH = 7.0) containing normal rabbit serum (1:250).

Portions of 0.1 ml of the antibody-sensitized erythrocytes are added to test-tubes containing 0.5 ml of various dilutions of the antigen under investigation. The following controls are set up:

1) the antigen and tannin-treated unsensitized erythrocytes;

2) the solution of normal rabbit serum (1:250) and the tannin-treated sensitized erythrocytes;

3) the solution of normal rabbit serum (1:250) and tannin-treated unsensitized erythrocytes.

After the erythrocytes have been added all the test-tubes are sha-

ken and left to stand at room temperature or kept in a heater at 37° for 1 hr. The results are evaluated after 2-3 hr.

The reaction is evaluated on the following three-plus scale:

+++) distinct unbrella-like aggregation of erythrocytes, partially displaced;

++) umbrella-like aggregation near bottom of test-tube;

+) red ring around edge of umbrella-like aggregation;

−) compact red disk or distinct red ring.

Rytsaya's hemagglutination reaction is recommended by certain researchers for detecting botulin toxins, but it has not come into wide practical use. According to the data of T.I. Bulatova, it has a number of drawbacks in the detection of botulin toxins (see the section entitled "Botulism").

Neutralization Reaction

It is well known that a toxin mixed with homologous antitoxic serum does not have a toxic action on the body. It was previously thought that when an antitoxin was mixed with homologous toxin the latter was decomposed. However, data have now been amassed which indicate that a toxin mixed with antitoxin is not broken down, but only neutralized (rendered harmless).

The compound formed by the toxin and antitoxin may dissociate. Toxin-antitoxin mixtures can be separated by filtration: since the toxin molecules are smaller than those of the antitoxin, gelatin filters, which pass only the toxin, can be used. The antitoxin is thus separated from the toxin.

When a mixture of snake venom and the corresponding antitoxin is heated to 68° the thermolabile antitoxin is decomposed, while the thermostable toxin remains intact. A neutral or even hyperneutral mixture of snake venom and homologous antitoxin can be separated by adding hydro-

chloric acid.

It is also known that a mixture of tetanus toxin and antitoxin neutral for healthy guinea pigs kills animals whose resistance has been reduced by certain infections and that a mixture neutral for mice is toxic for guinea pigs. If the toxin has been broken down a mixture neutral for one animal cannot be toxic for another.

A toxin-antitoxin mixture may be either toxic or antitoxic, depending on which component is present in excess.

A neutral toxin-antitoxin compound is obtained only when the reagents are mixed in definite proportions. If toxin is added to an antitoxin in a quantity sufficient to produce a neutral mixture neutralization occurs only if all the toxin is added at once. If the same quantity of toxin is added in individual portions, with an interval between one portion and the next, the mixture remains toxic (Danish's phenomenon). Danish felt that his observations proved that a toxin can combine with its antitoxin in varying proportions.

The toxicity of the mixture formed during gradual addition of toxin to an antitoxin in equivalent proportions does not remain constant. When the mixture is permitted to stand the toxin-antitoxin complex dissociates, its toxicity thus gradually decreases, and the mixture becomes neutral, since dissociation liberates antitoxin, which combines with the remaining free toxin groups.

The toxin-antitoxin reaction is strictly specific, with rare exceptions (e.g., as a result of the common antigens of botulin toxins types C and D). Tetanus toxin is neutralized only by antitetanus serum, botulin type A toxin is neutralized only by antibotulin type A serum, and diphtheria toxin is neutralized only by antidiphtheria serum. This enables us to use the neutralization reaction for diagnostic purposes and for identifying the toxins of the causative agents of various in-

fectious diseases.

The neutralization reaction requires a typologically specific diagnostic antitoxic serum for each species and type of toxin. Therapeutic sera may often contain natural antibodies and the process by which they are prepared does not provide for strict typological specificity. For example, botulin therapeutic serum type A often contains, albeit in low titre, type B antitoxin.

The following basic principle is observed in setting up the neutralization reaction: two animals are injected with identical quantities of the material suspected of containing toxin, but one is given material to which antitoxic serum has been added to neutralize the toxin, while the other receives material mixed with physiological solution instead of serum.

In order to neutralize the toxin an excess of antitoxin is always added to the extract or filtrate under investigation, so that one always has a mixture known to be neutral in comparison with the control, to which physiological solution instead of serum is added. For example, when botulism is suspected 0.8 ml of extract and 0.2 ml of diagnostic serum containing 1000 units per ml are used in the neutralization reaction. Consequently, 0.2 ml of the serum contains 200 units, which neutralize 20,000 toxin doses or more in mice; dosages of this size are virtually never encountered in the laboratory examination of small quantities of extracts from various foodstuffs, cadaver organs, or human excreta suspected of containing botulin toxin. When carrying out the neutralization reaction with highly toxic filtrates (of pure cultures), in which the toxin content per ml of culture fluid may be very high, the filtrates must be diluted by factors of 10, 100, 1000, or more before the reaction is set up.

Setting up the neutralization reaction

Portions of 2.4 ml of the material to be studied (extracts of foodstuffs, organs, or human excreta or culture fluids) are poured into test-tubes, calculating 0.8 ml per mouse. When sufficient material is not available the portions can be reduced to 1.8 or 1.5 ml. The number of tubes corresponds to the number of diagnostic sera with which the material is to be tested, one tube being used as a control; physiological solution is added to the latter instead of serum.

Diagnostic serum is then added to the material in a volume of 0.6 ml (0.2 ml for each mouse). A sterile pipette must be used for each serum, in order to avoid transferring the antitoxin. A total of 0.6 ml of physiological solution is added to the control tube. The mixture is thoroughly shaken, held at room temperature for 30-45 min, and administered intravenously, subcutaneously, or intraperitoneally to the mice in doses of 1 ml, depending on the nature of the material. When tetanus is suspected the material is administered subcutaneously, while when botulism is suspected it is administered intravenously, intraperitoneally, or subcutaneously. Individual sterial syringes are used for taking samples from each test-tube, in order to avoid carrying toxin and serum from one tube to another.

Other ratios of material to serum, e.g., 0.5 ml of extract and 0.5 ml of serum, etc., can be used in the experiment. However, the following principle must always be observed: all the animals received the same quantity of material, both mixed with serum and with physiological solution as a control.

The neutralization reaction is evaluated as soon as the control mice die. Mice which survive after the controls have died indicate that the sample contains toxin corresponding to the serum mixed with the material under study and administered to these mice. The reaction is not

evaluated when the control mice survive. If all the mice die it can be assumed that the sample contains a mixture of several (at least two) toxins. In this case it is necessary to dilute the material by factors of 2, 5, 10, and 100 in order to dilute one of the toxins, assuming that it will not cause death at these dilutions, while the other toxin retains its toxicity. Such phenomena are usually encountered in investigating culture fluids and cultures prepared from the patient's intestinal contents or excreta, the soil, sewage, etc.

When the material contains several toxins neutralized by polyvalent botulin serum types A, B, C, and E but not neutralizable separately 2-3 or more sera can be mixed for use in the neutralization reaction. In this case a mixture of two botulin toxins is probably present in the extract. For example, if the extract contains A and C toxins the neutralization reaction is negative for each individual botulin serum. The toxins present in the material can be determined only by conducting the reaction with bivalent sera (A + B, A + C, A + E, B + C, B + E, and C + E).

The neutralization reaction can also be used in investigating certain environmental objects for pathogenic bacteria capable of producing toxins (in testing soil for Cl. tetani and Cl. perfringens). The antitoxic serum is mixed with the soil extract and administered to mice. The serum is neutralized by the toxin formed in the animal's body when the soil contains the bacteria in question. In this case control mice given soil extract and physiological solution die as a result of toxin formation.

The neutralization reaction is a reliable method for laboratory diagnosis. Despite the fact that it is inferior to some reactions with respect to sensitivity and the rapidity with which results are obtained, it is still a reliable method and is consequently used as a control for

other reactions (the precipitation reaction, phagocytosis-suppression reaction, indirect hemagglutination reaction in botulism, etc.).

USE OF LABORATORY ANIMALS FOR INDICATING PATHOGENIC BACTERIA AND DIAGNOSING INFECTIOUS DISEASES

Prof. K.I. Matveyev and T.I. Bulatova, Candidate of Medical Sciences

Various laboratory animals are widely used for indicating and diagnosing infectious diseases. Bacterial can be permitted to accumulate in the bodies of such animals in order to accelerate the investigation.

Environmental material suspected of containing pathogenic bacteria and their toxins is shaken in physiological solution and then filtered through membrane or talc filters in order to concentrate the bacteria and toxins. Sensitive animals are inoculated with the material washed from the filter. Such investigations are conducted with mice to detect botulin toxins, the causative agents of plague, tularemia, and anthrax, and other bacteria, yielding good results.

Biological testing in animals is a very valuable and reliable diagnostic method for isolating pathogenic bacteria from the body, especially when accompanied by a control involving immune sera (the neutralization reaction); it sometimes yields a characteristic clinical pattern in the inoculated animal (tetanus).

Only certain species of animals are, however, suitable for these purposes. According to A. Metelkin, rabbits are used in staphylococcal, streptococcal, pneumococcal, and meningococcal (of rabbits) infections, tuberculosis (bovine type), anthrax, tetanus, gas gangrene, botulism, and other diseases, guinea pigs in tuberculosis (human type), diphtheria, glanders, plague, brucellosis, pseudotuberculosis, tularemia, cholera, salmonellosis, listerellosis, anthrax, melioidosis, tetanus, gas gangrene, botulism, etc., mice in pneumococcal and diplococcal infections, anthrax, pasteurellosis, listerellosis, tularemia, melioidosis,

botulism, tetanus, gas gangrene, pertussis, etc., white rats in tuberculosis (bovine type), melioidosis, and other infections, monkeys in dysentery, tuberculosis, listerellosis, melioidosis, cerebrospinal meningitis, pertussis, typhoid fever, etc., cats in amebic dysentery, staphylococcal infections, glanders, pertussis, etc., and birds in tuberculosis (avian type), avian pasteurellosis, rhinoscleroma (of chickens), etc.

Rabbits are used for producing immune diagnostic sera, most commonly agglutinative and precipitative. The safety of vaccines, sera, and other preparations is tested on laboratory animals. Animals are also used for testing the protective properties of sera and the effectiveness of vaccines, as donors of blood used for preparing nutritive media (blood agar), and for producing complement (guinea pigs).

Preparation of Animals for Experimentation

It is recommended that the animals be obtained from a single breeding farm, which is kept under constant veterinary observation. Animals of the same strain should be used for experimentation. The animals must be quarantined in a special room before the experiment is performed.

Animals of the requisite breed, of approximately identical weight, and of the same sex are selected for experimentation. They are kept in cages, one (mature monkeys, dog, rabbits), 2-3 (younger animals of these species), 5-6 (guinea pigs), 8-10 (rats), or 15-20 (mice) individuals to a cage. Each cage should contain animals of the same age and sex, which is important for proper care and for preventing pregnancy.

Before receiving animals the cages are washed and disinfected and a slip indicating the number of the experiment, the date on which it was begun, the researcher's surname, and other necessary information is placed in each cage.

The following rules must be observed in conducting experiments:

animals cannot be moved from one cage to another, they should be transported from the vivarium to the laboratory and back in special disinfected boxes, and only animals from the same cage should be placed in the same box.

If the experimental animals are to be inoculated with especially dangerous bacteria they are kept in an isolated room. The inoculation department should have separate rooms for the researchers to don and remove their special protective clothing, for inoculation and dissection of animals, for keeping inoculated animals, and for rendering infectious material harmless. No work with infectious materials should be carried out in the dressing room. This room is intended solely for donning protective clothing for working with infectious materials. After such work has been completed the protective clothing is removed in the undressing room and washed in a disinfectant solution.

Animals inoculated with especially dangerous bacteria are kept in metal cages, while mice are kept in glass jars with metal cases. The jars containing the inoculated animals are placed on shelves in a room intended solely for this purpose. The shelves are covered with gauze wetted in a disinfectant solution. Litter from the inoculated animals and cadavers are burnt in a special furnace.

The laboratory cages in each vivarium are regularly (approximately once a week) disinfected or autoclaved. The water dishes and feeders are washed and boiled daily.

Proper care of the experimental animals in the vivarium is of great importance in the outcome of the experiment. Incorrect feeding or failure to observe hygienic measures in caring for the animals causes them to weaken and increases their sensitivity to various spontaneous infections. Such infections develop during experimentation and may distort the results.

Weighing. Ordinary scales can be used to weigh the animals. The subject is placed in a plywood box or cardboard carton. Mice are best weighed on postal or pharmaceutical scales.

Measurement of temperature. In the majority of cases the body temperature of the experimental animals must be measured. A small mercury thermometer or a medical maximum thermometer is usually used for this purpose. For precise temperature measurement the thermometer must be inserted into the rectal lumen to a depth constant for the species of animal in question. It is inserted to 3.5 cm for guinea pigs and rabbits and to 3-4 cm for dogs. To aid in observing this rule an index mark consisting of a ring cut from a piece of rubber tube is placed over the glass stem above the mercury bulb. Before and after use the thermometer must be disinfected with 10% lysol or denatured alcohol, dried with cotton, and shaken and the mercury bulb should be smeared with vaseline. The animal's temperature must always be taken under identical conditions, with the same thermometer and for a predetermined period (3-5 min).

In order to take a guinea pig's temperature it is held in the left hand, belly up, and slight pressure is exerted on the inguinal region with the index finger. The thermometer is then inserted, being held almost vertically at first and then moved to a horizontal position. If the thermometer strikes an obstacle it should be carefully rotated to move it further into the rectum, but force should not be used, since this might damage the intestine.

To take a rabbit's temperature the animal is immobilized with a towel fastened around its knees, so that its head rests on the wrist of one hand. This hand is used to raise the tail, while the other inserts the thermometer into the rectum.

Marking the animals. Numbered metal plates fastened to the ears

are used for rabbits and guinea pigs. The outer surface of the ear is quickly pierced with the sharp ends of the clip and they are bent inward over the inner surface. Such plates can be cut from thin tin plate and numbered with special punches or by writing on them with a slender rod and ink consisting of 10 ml of saturated copper sulfate and 2 ml of concentrated hydrochloric acid. The ink is immediately blotted with filter paper.

The ears of rabbits and guinea pigs are occasionally numbered with special tatooing forceps. There is also a quite simple, convenient method for numbering guinea pigs, which has come into rather wide use. The outline of the guinea pig is drawn in a notebook with the aid of a previously prepared template (usually a silhouette cut from cardboard) and spots of various colors are placed on it to correspond to those present on the animal's coat, as seen in top view.

Mice and rats are marked by staining the coat with various dyes. A 0.5% carbol fuchsin solution, aqueous picric acid, alcoholic brilliant green, etc., are most commonly employed. The animals must be marked with dyes of different colors, which represent different numbers (for example, fuchsin arbitrarily represents units and picric acid tens), in accordance with a predetermined scheme. The site of the fuchsin spot represents the following arbitrary numbers: on the left front paw – No. 1, on the left side – No. 2, on the left hind paw – No. 3, on the head – No. 4, on the back – No. 5, at the base of the tail – No. 6, on the right front paw – No. 7, on the right side – No. 8, and on the right hind paw – No. 9. Staining the same site yellow with picric acid represents Nos. 10, 20, 30, 40, 50, 60, 70, 80, and 90 respectively. Thus, for example, if the left side is stained yellow and the head red, the animal is No. 24, etc.

For individual marking of birds (chickens, pigeons, or ducks) an

aluminum ring bearing the sequential number is applied to the right foot or a metal number is attached by piercing the wing membrane.

Dogs are usually numbered by attaching a metal tag to the collar.

In monkeys the sequential number is tatooed on the inside of the upper third of the thigh.

Removal of hair. When an animal is to be given infectious material it is sometimes necessary to remove the coat from its skin. For laboratory animals this is often done with a razor. A depilatory consisting of 7 parts of talc, 7 parts of white flour, 1 part of soap powder, and 5 parts of sodium sulfite paste is sometimes used. It is mixed with water to obtain an ointment before application. The animal's coat is clipped, the skin is smeared with a thick layer of the depilatory, and it is gently rubbed in (rubber gloves must be worn). After a few minutes the hair is cleaned away from the skin and the latter is washed with warm water, dried, and smeared with vaseline. Injections can be given or other manipulations performed in this area after 1-2 days.

Immobilization of animals. Before administration of the material under investigation the animal must be immobilized, so that it cannot move and the experimentor can conveniently give injections or perform other manipulations.

Rabbits, guinea pigs, rats, and mice can be fastened to boards or tables with short legs. Special boards and restraining devices of this type are usually employed when prolonged immobilization is necessary.

Simpler techniques are generally used. For intravenous injections rabbits are conveniently wrapped in a towel or an old set of coveralls, first flexing the legs on the abdomen; the animal's head should remain free. A rabbit can also be immobilized by sitting it on a table and holding the skin of its back. In taking blood from a vein or injecting material into a vein or the eyes it is convenient to use the special

rabbit boxes designed by O.G. Birger.

These boxes are made of wood and are 40 cm long, 15 cm wide, and 15 cm deep. They consist of two sections, lower and upper, the latter being a lid; the lower section is 9 cm deep and the upper section 7 cm deep. A round cut-out 2.5-3 cm in radius is made in the square side wall of the box for the rabbit's neck. Within the box, on the side opposite the cut-out, is a movable plywood wall, which makes it possible to vary the length of the box to correspond to the rabbit's size. Blocks of different thicknesses are occasionally used instead of the movable plywood wall. The box is opened, the rabbit is placed in it with its head toward the cut-out, the movable plywood wall (or block) is moved to a point determined by the rabbit's size, the lid is closed, and the catch is fastened, the animal's head remaining outside.

Guinea pigs are usually immobilized in an assistant's hands. It should be kept in mind that the front paws must be held carefully, so as not to suffocate the animal.

Mice are immobilized by various methods. An assistant usually takes the mouse by the tail in his right hand, brings it to the table, and, still holding it by the tail, permits it to move forward. When the mouse is stretched out the skin on the back of the head is grasped with the middle and index fingers of the left hand and the animal is raised, slightly extending it. For intravenous injections an assistant grasps the mouse by the skin of the back of the head and the base of the tail, the experimentor holding the tail. For intraperitoneal injections the assistant holds the mouse with its head down, by the tip of the tail and the skin of the back of the head, turning its abdomen towards the experimentor.

Intraperitoneal inoculation of mice can easily be carried out by one person if the following immobilization method is used. The mouse's

tail is grasped in the right hand, while the left hand holds the skin on the back of the head in the manner described above. The left hand is then turned palm up, the mouse is extended, and its tail and left hind paw are grasped between the palm and the 3rd, 4th, and 5th fingers. This leaves the right hand free to give the injection.

Mice can also be given intravenous injections without an assistant. The mouse is placed in a box with a hole for its tail or the skin on the back of the head is grasped with a Pean forceps and the animal is then placed in a glass jar head down.

Rats are usually immobilized with surgical forceps, stretching them out on a table; the skin on the back of the head is grasped with one pair of forceps and the base of the tail with another.

Various Methods for Administering Material to Animals

Subcutaneous injection. Subcutaneous injections of infectious material are most frequently given beneath the skin of the back or some other portion of the body for rabbits, beneath the skin of the abdomen or side for guinea pigs, and beneath the skin of the back or the back of the head for rats and mice. In subcutaneous injections the skin is lifted with two fingers of the left hand and the right hand inserts the needle into the fold thus formed. In order to keep the material from escaping once it has been administered the needle is moved to the side after puncturing the skin and inserted half-way and the material is injected slowly. After the injection has been completed the fold of skin is released, a cotton pad wetted with disinfectant is applied to the needle, and the latter is rapidly withdrawn. If the injected fluid begins to seep out through the puncture in the skin it is necessary to press a piece of sterile cotton against the puncture or to seal it with collodion.

For intracutaneous injections it is necessary to remove all the

hair from the injection site and rub the skin with alcohol. The injection is given with a fine needle (No. 18-20), which is introduced into the skin at a very acute angle. The tip of the needle should be visible through the epidermis and the epidermal layer should be raised to form a blister at the surface ("lemon pip") when the fluid is injected (usually in a volume of 0.1 ml). Intracutaneous administration is also carried out by the scarification method. An area of skin on the back as inaccessible to the animal as possible is freed of hair, thoroughly rubbed with alcohol, and wiped dry. A row of parallel scratches is then made with a smallpox-vaccination needle, the tip of a sharp scalpel, or a surgical needle and a drop of material is applied to them and thoroughly rubbed in with a glass rod or spatula.

A protective collar of heavy cardboard is usually placed about the neck of an animal given an intracutaneous injection or subjected to scarification, to keep it from licking or scratching the injection site. A cardboard circle 10-20 cm in diameter (for a guinea pig) is cut out and an oval hole somewhat larger than the circumference of the animal's neck is made in its center. The ring thus obtained is wrapped in bandage or gauze with a thin underlayer of cotton, so that its edge does not bear directly on the neck. The collar is cut radially and placed on the animal and the cut is sewn up with thread.

Intramuscular injections are usually administered in the muscle of a hind leg, most frequently its outer side, for rabbits, guinea pigs, and rats; birds receive such injections in the pectoral muscles.

Intraperitoneal injections are given in the following manner: a section of skin on the left side of the posterior third of the abdomen is freed of hair and treated with alcohol and iodine. During injection of the material the animal is held head down, so that the intestine is displaced downward from the injection site. A short needle whose tip

has been sharpened with a small file should be used.

For rabbits and guinea pigs a small incision is made in the superficial cutaneous layer and a blunt needle is inserted horizontally between the skin and muscles, brought gently into a vertical position, and advanced with careful motions. Penetration of the needle into the abdominal cavity can be felt in the disappearance of resistance. When intraperitoneal injections are given to white mice the hair is generally not removed and no preliminary incision is made in the skin.

Intraperitoneal inoculation cannot be employed when the material under investigation contains many foreign bacteria (soil, intestinal contents, etc.), since it might cause fatal peritonitis.

Two persons are required to give intraperitoneal injections to rabbits, guinea pigs, and other large animals, one holding the animal and the other administering the infectious material. Intraperitoneal inoculation of mice can easily be carried out by one person, using the immobilization procedure described above.

The maximum doses for intraperitoneal, intramuscular, and subcutaneous injections are as follows: mice - 1-2 ml, rats - 3 ml, guinea pigs - 5-10 ml, and rabbits - 5-20 ml (depending on the animal's weight).

Intravenous injection. Different veins are used for intravenous injections in different animals. Rabbits are given such injections in the marginal vein of the ear, an assistant holding the animal immobile. The hair along the margin of the ear is cliped. For better filling of the vein the helix must first be washed with certain alkalies or xylol at the injection site and the vein is then compressed near the root of the ear. The ear is rubbed with alcohol and taken in the left hand, lying on the middle and index fingers, while the fourth finger holds it at the the top, near the injection site. The needle is inserted into the vein in the direction of blood flow and almost parallel to the sur-

face of the ear, so that it does not pierce the opposite wall of the vein. The venipuncture is painful and the animal usually jerks its head at the instant of penetration. In order to be sure that the needle has entered the vein the plunger should be slightly depressed. If it moves freely and the skin around the needle is not distended the fluid has gone into the vein. If a swelling forms after a small quantity of material has been injected the needle has entered beneath the skin. The needle is withdrawn and reinserted at another point nearer the base of the ear in this case. After the injection the vein is pinched below the puncture, the needle is withdrawn, and a piece of sterile cotton is pressed against the injection site to prevent bleeding.

In guinea pigs and mature rats the material is injected into the jugular vein in the neck: it is recommended that the vein first be exposed. The animal should be immobilized on a special small table. After the injection the wound is closed with sutures or surgical staples.

Guinea pigs can also be given injections in the vein on the inside of the lower leg, but this vein can be easily detected only in white animals. Xylol or mechanical stimulation must also be used to provide better filling of the vein.

Guinea pigs are often given intracardiac injections by the same method employed to take blood from the heart (see below).

Mice and young rats receive injections in the veins on either side of the tail, using tuberculin-test needles with short points. For better filling of the veins the mouse's tail is preliminarily immersed in hot water (approximately 50°) or wetted with a cotton pad first soaked in boiling water. If a large number of mice must be given intravenous injections at the same time they are closely packed in a jar or cage, where they become overheated; this makes their veins clearly visible. An assistant holds the mouse and pinches the base of the tail, while the

experimenter inserts the needle into the vein in the lower third of the tail, almost parallel to its surface. Once the needle has been introduced into the vein the assistant stops pressing on the root of the tail, but continues to hold the mouse in this area. If the needle has entered the vein the syringe plunger moves freely. If the tail becomes swollen and white around the needle and the plunger is difficult to move the needle has entered beneath the skin. It is then withdrawn and reinserted nearer the base of the tail.

Mice can also be given intravenous injections without an assistant, using one of the immobilization methods described above.

In birds (chickens or pigeons) it is convenient to use the vein located on the inner surface of the wing, which is clearly visible if the feathers are clipped.

During intravenous injections care must be taken that air bubbles are not injected with the fluid, since they cause instant death from air embolism when they enter the blood; small particles of various solids or small drops of fat also produce embolisms. The maximum doses for intravenous injection are as follows: mice - 0.5-1 ml, rats - 1-2 ml, guinea pigs 1-2 ml, and rabbits - 5-10 ml (depending on the animal's weight).

Intraocular administration. In this technique the material is injected into the anterior chamber of the eye or dropped onto the conjunctiva. Injection into the anterior chamber is easiest in large animals (rabbits and dogs) and substantially more difficult in guinea pigs. The eye is first cocainized by application of several drops of a 3% cocaine solution beneath the eyelid. After 1-2 min, when the eye is anesthetized, a fold of the conjunctiva is grasped outside the upper margin of the cornea with a slender ocular forceps, in order to immobilize the eyeball. A fine needle (without the syringe) is dipped in the material,

inserted into the cornea at a very acute angle, and introduced into the anterior chamber to a depth of no less than 1 mm. After the eyeball has been immobilized and the cornea punctured one usually waits for some of the aqueous humor to emerge from the needle. Several drops of this fluid are permitted to escape, the syringe is fitted to the needle, and no more than 0.05 ml of the material is injected; the needle is then quickly withdrawn. The small wound heals of itself. If the needle has been properly inserted none of the aqueous humor escapes. The eye is rinsed with sterile water or a weak (3%) boric acid solution.

Injections into the central nervous system are given dogs, rabbits, and guinea pigs through a trepanned opening in the skull; the material is injected beneath the dura mater. The operative zone is freed of hair over an area extending from the eyes to the ears. The operation is performed under anesthesia with the animal immobilized on a table. An assistant holds its head behind the ears, pulling it back. The operative field is thorough smeared with iodine, the skin is moved somewhat to one side, and an incision 2-3 cm long is made with a scalpel parallel to the longitudinal cranial axis and slightly above the posterior ocular line. The edges of the wound are separated, a cruciform incision is made in the periostium, and the latter is drawn apart. A special tool, a trepan, is used to make a circular hole in the bone. This aperture should lie outside the median line, so as not to damage the longitudinal sinus. A slightly blunted Francke needle can be used in operations on rabbits and guinea pigs when no trepan is available. The bone is punctured three times with the needle, at the apices of a small triangle, which can then be easily cut free with fine blunt ocular scissors. The glossy surface of the dura mater is visible through the window in the bone; it is punctured with a needle bent through a right angle or with an ordinary needle. In the latter case the syringe is dropped to a

horizontal position after the puncture has been made, the needle is moved laterally and outward beneath the membrane, and the material is injected. The needle is then withdrawn and the wound sutured.

Material can be injected directly into the brain through such a trepanned opening. Trepanning is not, however, obligatory in this administration technique. It is sufficient to pierce the skull with a sharp pin or Francke needle and insert the syringe needle into the hole thus formed. In intracerebral injections it is necessary to use a needle with a sleeve stop, so that the material is injected to a uniform depth (2-3 mm) in all animals.

Intracerebral inoculation of smaller animals (rats and mice) is carried out with a tuberculin-test syringe and a fine, sleeve-equipped needle. The material is injected to a depth of 1.5-2 mm. The needle should have a bluntly truncated point. The experimenter holds the mouse firmly in his left hand, as in intraperitoneal injection, grasping its tail between the little and fourth fingers and the skin on the back of the head between the middle and index fingers. The animal is pressed against the operating table. The right hand is used to smear the coat at the injection site with iodine and insert the sleeve-equipped needle in the middle third of the line joining the inner corner of the eye and the base of the ear. The material is injected slowly, so as not to cause an increase in intracranial pressure.

Injection into the cerebellomedullary cistern by suboccipital puncture is employed only for large animals (rabbits or dogs). The subject is firmly fastened to the operating table, stomach down; the pectoral girdle should lie at the front edge of the table. An assistant holds the animal's head in both hands, bending it forward in such fashion that the planes of the trunk and the neck form a right angle. With the head in this position the distance between the first cervical ver-

tebra and the skull is increased and the ligaments between them are stretched. The coat is clipped in the vicinity of the occipital protuberance and on the neck; the skin is rubbed with alcohol and smeared with iodine. A needle with a bluntly truncated point is selected. It must be taken into account that the distance between the dura mater and the brain is 1-2 mm. A puncture is made between the occipital protuberance and the spinous process of the 2nd cervical vertebra, precisely on the median line. The needle (equipped with a mandrin) must be inserted carefully. When the dura mater has been pierced, as indicated by a slight crackling sound, movement of the needle is halted and the mandrin is removed. A drop of clear liquid, the cerebrospinal fluid, usually emerges from the needle after a short interval and the syringe is then attached and no more than 0.5 ml of the material injected. The needle is withdrawn and the puncture site is smeared with iodine.

There is also a simpler method of immobilizing rabbits for suboccipital injections, which requires a minimum of time and permits mass experiments to be conducted within a short period. This technique can easily be mastered with only a little practice on the part of the experimenter.

The rabbit's fore and hind legs are tied tightly together and the animal is wrapped in an old pair of coveralls and placed on its side near the edge of the table, its head to the experimenter's left and its torso to his right. An assistant stands on the experimenter's right and pulls the animal's head forcefully by the ears with his right hand, while his left holds its trunk. The operative field is prepared in the manner described above. The experimenter locates the occipital protuberance and the spinus process of the 2nd vertebra with the index or middle finger of his left hand and, holding the finger against the protuberance, uses his right hand to insert the mandrin-equipped needle di-

rectly behind it, precisely on the median line and anterior to the spinus process. The mandrin is removed and, as is generally the case, fluid immediately begins to flow from the needle. The remaining manipulations are carried out in the manner described above.

Injections into a peripheral nerve are given through the outside of the thigh; the skin is freed of hair and disinfected and an incision 3-4 cm long is made. The thigh muscles are separated under local anesthesia and the sciatic nerve is exposed. The nerve is raised from the bottom of the wound, inserting a gauze pad beneath it, and punctured with a fine needle at a very acute angle; as small a quantity of material as possible is then injected and the wound is sutured.

In addition to these artificial methods for introducing infectious material into an animal's body, there are techniques which approximate natural infection modes. These include inoculation through the digestive and respiratory tracts.

Administration through the digestive tract is carried out by mixing the material with food or by introducing it orally, either directly into the stomach with a probe or into the esophagus with a cannula. It is difficult to determine the quantity of material administered with sufficient precision in the first method, procedures of the latter type being more accurate.

When the material is administered orally with a hypodermic needle the animal is held vertically, its mouth is opened with forceps or by pressing against the neck with the fingers, and a drop of material is introduced. When the animal has swallowed it the next drop is introduced and in this manner, drop by drop, the entire quantity of material is gradually administered. It is necessary to avoid dropping large quantities of fluid into the mouth all at once, so that it does not enter the respiratory passages.

When the material is to be introduced directly into the stomach a very slender elastic probe (7.5 cm or more long and 0.5 cm in diameter) is smeared with vaseline and inserted through the nose. As soon as it reaches the nasopharynx water is dropped into the mouth and the prove is moved forward when the animal swallows; no difficulty should be encountered in advancing the tube. When it has been introduced into the stomach the material under investigation is injected into its outer end with a syringe but no needle.

In rabbits, guinea pigs, and mice material is introduced into the esophagus with a syringe fitted with a metal capsule or a curved needle with an olive-shaped thickening at its tip.

Administration through the respiratory passages. There are various methods of inoculation through the respiratory tract. The simplest procedure consists in dropping the material into the animal's nose under light ether anesthesia. When the anesthetic is properly administered the animal deeply inhales the material, which then enters the lungs. When the anesthesia is too deep breathing becomes shallow and the material is not inhaled. The maximum doses for this type of administration are as follows: 0.03-0.05 ml for mice, 0.05-0.1 ml for rats, and up to 2 ml for guinea pigs and rabbits.

Material can also be introduced into the trachea or bronchi with a probe.

Methods of Taking Blood from Laboratory Animals

Guinea pigs and rabbits are the laboratory animals most frequently used for taking blood. Blood drawn from the hearts of guinea pigs is used to produce complement, while that taken from rabbits is, in the majority of cases, added to nutritive media; blood from both animals is used to test the properties of immune sera during immunization. When the experimenter has a certain skill this operation usually causes no

harm to the animal and can be performed repeatedly. In order to take blood from the heart the animal is strapped to the table on its back, in a horizontal position. The coat is shaved in the cardiac region and the skin is smeared with tincture of iodine. The syringe and needle to be used for taking blood are thoroughly sterilized; the needle should not be too sharp.

The animal is strapped to the table in a horizontal position, abdomen up, or simply stretched out, an assistant holding its fore and hind legs (this technique is usually employed for guinea pigs). The coat is shaved from the chest and the cardiac region is smeared with iodine. It is recommended that the blood be drawn with a sterile glass Luer syringe. The pulse is located with the tip of a finger and the needle is inserted at this point, near the left margin of the thorax, directing it slightly inward and introducing it to a depth of 1.5-2 cm (in guinea pigs). The needle must first overcome the resistance of the thoracic muscles and myocardium and then enters the left ventricle. If the needle has been properly inserted blood begins to flow into the syringe in spurts, raising the plunger.

In rabbits it is convenient to insert the needle in the 3rd intercostal space, 3 mm from the left thoracic margin.

No more than 10 ml of blood is usually taken from guinea pigs weighing 500 g or more, while 25-30 ml is taken from mature rabbits. As soon as the blood has been drawn the animal is given a subcutaneous injection of physiological solution heated to body temperature in a quantity twice that of the blood taken.

In mice the tip of the tail is cut off and the blood which flows out is collected in a test-tube or drawn off with a Pasteur pipette.

Exsanguination is used to obtain the maximum quantity of blood from rabbits. A longitudinal incision is made in the skin of the neck,

a short distance from the median line, and the carotid artery is exposed. The artery is separated from the vein and nerve and two silk ligatures are applied to it: it is then severed between the ligatures. The central end of the artery is grasped with forceps and inserted into a sterile test-tube. The arterial wall is slit behind the ligature and a strong stream of blood begins to flow into the test-tube. When the blood flow slows down the heart is massaged to collect the last portion of blood.

G.M. Fedorov (1961) has proposed a simple device which makes it possible to obtain several milliliters of blood from a guinea pig. It consists of a thick-walled test-tube of the type used in certain chemical and toxicological investigations; these tubes are 11 cm long, 1.8 cm across, and 3 cm in diameter at their bulbous bottoms. The lower portion of the test-tube can be removed when necessary. The opening of the tube is closed with a cuff or a piece cut from the rubber nipple of an infant's bottle. Blood is taken in the following manner. An incision is made in the animal's front paw (near the ankle) with a razor blade. The paw is inserted through the cup into the tube, whose side-arm is connected to an electric vacuum pump. From 1 to 4 ml of blood collects in the tube within 1-2 min. This method is simpler and safer than cardiac puncture, but does not completely ensure sterility of the collected blood.

Lutsenko has proposed a method for massive bleeding of laboratory animals (rabbits and guinea pigs).

Cardiopuncture under anesthesia. The principal drawback of the completely sterile bleeding method usually used to obtain large quantities of blood, as described above, is the fact that the animal dies, which means that it cannot be used again. Cardiopuncture under anesthesia makes it possible to obtain 60-70 ml of blood from rabbits, but

does not cause shock as a result of the large blood loss.

Method. Several flasks with capacities of 100-200 ml are prepared. Volumes of 50, 75, and 100 ml are indicated on the flask wall. The flasks are hermetically sealed with a rubber stopper through which two large-diameter phlebotomy needles are passed. A rubber tube is attached to one needle for evacuation of the air from the flask; the other needle bears a rubber tube up to 35 cm long, with a No. 17M mandrin-equipped needle tightly fitted into its open end for the cardiopuncture. The No. 17M needle is placed in a thick-walled test-tube to keep it sterile after the flask has been autoclaved. Before the puncture is made the flask is connected to a vacuum pump through a rubber tube with a cotton filter, in order to exhaust as much air as possible from it. As a last resort, this can be done with a water-jet pump. When the air has been sufficiently exhausted the walls of the rubber tubes collapse and Pean forceps are applied to them to hold the vacuum in the flask. It is necessary to check the retention of the vacuum.

The animal is prepared for cardiopuncture in the usual manner: it is immobilized on its back, its coat is shaved, the experimenter uses his hands to rub alcohol into the skin, and the puncture area is copiously smeared with tincture of iodine. A funnel-shaped paper mask containing a cotton pad wetted with ether is placed over the animal's nose. Once the animal is anesthetized the pulse is located with the index finger, which is smeared with tincture of iodine, and the tip of the sterile needle is introduced into the pleural cavity with a sharp blow of the right hand. Only then does an assistant remove the forceps on the rubber tube connected to the needle and check for the appearance of an inflow of blood to the flask. If such a flow does not appear the position of the needle is changed in an attempt to insert it into the heart. In rabbits the needle is most conveniently inserted in the 3rd

intercostal space, 3 mm from the outer thoracic margin, while monitoring the pulse. The needles used for cardiopuncture should have tips which are not very beveled or sharp, in order to prevent pericardial hemorrhaging.

After 60-70 ml of blood has been taken the animal is given a subcutaneous injection of 30-40 ml of physiological solution heated to 37° and is kept in a warm room until the following morning.

A.P. Okuntsov's cardiopuncture method for obtaining blood from chickens. Methods for taking blood from the peripheral vessels of birds have a number of material drawbacks. They do not ensure that the blood obtained will be sterile, since it is in prolonged contact with the ordinary atmospheric microflora and is contaminated with microorganisms from the feathers and hands, which leads to rapid hemolysis, especially during the summer. It is consequently best to draw blood from the heart.

The bird is immobilized on a table. An assistant holds it on its back and extends its legs with his right hand, straightening them at the joints; the fingers of the left hand grip the wings near their base, the middle finger simultaneously exerting gentle pressure on the crop region. The line joining the carina of the breastbone and the point where the clavicle diverges is divided into two equal segments. A perpendicular is dropped from the point of bisection to 1 cm from the horizontal line joining the scapular joint and the apex of the median sternal process. The terminus of this perpendicular is the point at which the needle should be inserted to puncture the heart. The feathers at the puncture site are clipped and the skin is rubbed with alcohol or ether. Blood is best taken from the heart with a sterile 5-gram Luer syringe. The needle should be no less than 6 cm long and 1-1.5 mm in diameter (a finer needle can be used). The operation should be performed with a sharp needle: this has certain advantages in giving the

operator greater tactile sensitivity and does not cause any great tissue trauma. The needle and syringe should be perpendicular to the skin. Having pierced the skin and muscle, the tip of the needle is used to locate the caudal margins of the 1st or 2nd sternal area of the ribs and the needle is introduced into the body cavity. Rhythmic movement of the syringe shows that the needle has entered the ventricular cavity; the plunger is raised by the blood entering the syringe. The depth to which the needle must be inserted into the thoracic cavity depends on the species of the bird and the size and development of its thorax and musculature, usually being 3.5-5 cm. A frothy reddish fluid appears in the syringe when the needle has not been properly inserted. In this case the needle must be quickly withdrawn and the syringe is washed several times with physiological solution through it, until the solution in the syringe appears clear.

Cardiopuncture is carried out from the left side of the thorax, as described above, to obtain arterial blood. Venous blood is obtained by cardiopuncture on the right side of the thorax.

In adding blood for bacteriological investigations it is necessary to keep it from being contaminated with common microorganisms; for this purpose it is transferred from a sterile syringe to a sterile cylinder through a special needle.

Chickens begin to scratch for food immediately after cardiopuncture.

Dissection and Bacteriological Investigation of the Organs of Dead Animals

Animals which have died or which have been killed at a certain interval after administration of the material under investigation are dissected. The cadaver material (the animal's organs) is investigated bacterioscopically (by microscopic examination of smears) and bacteriologi-

cally (by culturing). Extracts for immunological reactions are also prepared from the organs.

Certain rules must be observed in dissecting an infected corpse. Both dissection and bacteriological investigation should be carried out as soon after death as possible. The cadaver should be kept under refrigeration until the autopsy is performed.

The dissection must be carried out on a separate table, observing all the conditions for preventing contamination of the cadaver with foreign bacteria and contamination of various objects with infectious material from the cadaver.

The autopsy must be performed with sterile instruments, following the rules of asepsis. The material removed should not come into contact with disinfectants before culturing. All the instruments and materials necessary for dissecting the animal should be at hand on the table: these include a beaker half full of alcohol for holding the instruments (scissors, scalpels, and anatomic and surgical forceps), cotton pads (sterile and unsterile), platinum loops, nutritive media, sterile Petri dishes, a mortar, Pasteur pipettes, slides, matches, and physiological solution.

The dead animal is prepared for dissection in the following manner: it is grasped in a pair of forceps and laid stomach up on a specially prepared table covered with wrapping paper or on a small table in an enameled or zinc-lined pan. All four of its paws are fastened to the table with sharp tacks, pins, or special clamps. Mice can be dissected on a sheet of cork or the wax plate formed in a pan after molten wax has been permitted to solidify.

Before dissection the animal's coat is washed with a cotton pad wetted with 2% lysol or 5% carbolic acid, so as not to scatter bacteria-bearing hair during the autopsy. It is quite convenient to burn off the

coat with a Bunsen-burner flame.

Before dissecting an animal which was inoculated with an especially dangerous infection it is grasped in a pair of forceps, immersed in a bucket containing kerosene (in order to kill blood-sucking parasites which might be infectious for man), and placed on a piece of screening to permit the excess kerosene to drain off.

The dissection and examination of the cadaver are conducted in three stages:

1) dissection and examination of the skin;

2) dissection and examination of the thoracic cavity;

3) dissection and examination of the abdominal cavity.

A sharp scalpel is used to slit the skin in a straight line from the lower jaw to the symphysis, without damaging the underlying tissues. The same scalpel and a pair of forceps are employed to separate the skin on either side, exposing the entire anterior abdominal wall; lateral incisions are then made in the skin along the paws. The skin is separated to either side of the incision. Any changes in the subcutaneous cellular tissue (hemorrhages, edema, vasodilitation, the state of the lymph glands, etc.) are noted. Smears are made from the glands if they are enlarged or suppurative.

The exposed muscle surface of the anterior thoracic wall is rubbed with a pad wetted in alcohol and the thoracic cavity is opened with other sterile instruments (a scissors and forceps). The xiphoid process is grasped with a pair of forceps, a cut is made beneath it, and, inserting the scissors through this incision, the ribs are transected on both sides, at the points where they join the cartilages. The sternum is tilted upward and the thoracic cavity is examined, noting the presence or absence of an exudate or blood and the color and external appearance of the lungs, heart, and mediastinal glands. When fluid is present in

the thoracic cavity cultures and smears on slides are made from it.

Culturing of the blood from the heart is obligatory; several methods are recommended for making such cultures.

The surface of the right ventricle is cauterized with a red-hot scalpel until a white deposit is formed and a Pasteur pipette is introduced into the ventricular cavity. A drop of blood from the pipette is applied to an agar surface or placed in broth. An incision can be made with a red-hot scalpel or scissors at the cauterization site and blood is drawn through it with a Pasteur pipette and transferred to a nutritive medium.

Fragments are sometimes cut from the heart with sterile scissors and used to make prints on the surface of an agar dish, smears then being made from the blood. After these cultures have been prepared the thoracic organs are examined in greater detail.

The abdominal cavity is dissected and investigated after the thorax has been examined. The abdominal wall is lifted with forceps and cut with scissors from the diaphragm to the mons. The incision must be made carefully, so as not to damage the intestine. Smears and cultures are made from the exudate. The spleen is examined with special care, cutting away fragments and preparing cultures and prints. Cultures and smears are then made from the other adbominal organs where necessary.

In making cultures of the intestinal contents a loop of the intestine is grasped in an anatomic forceps and squeezed. A platinum loop is heated to red heat, permitted to cool, inserted into the intestinal lumen, and used to collect its contents and transfer them to a nutritive medium.

Smears are prepared from organs either by drawing a cut surface along a slide or by printing (a small fragment is cut from the organ and taken in a forceps and the cut surface is pressed several times

against a slide). The smears are fixed by flaming or, if it is desired to preserve the structure of the cellular elements, with a liquid fixative.

During dissection and culturing care must be taken that the infectious material does not come into contact with the table or surrounding objects. After each manipulation all the instruments used must be carefully wiped with a cotton pad wetted in water or alcohol and placed in a beaker containing alcohol.

The working area must be carefully cleaned after the autopsy has been completed. The instruments are placed in a sterilizer or pot and boiled for 45-60 min. Any remnants of infectious material are immersed in a pot containing 5% lysol. The cadaver is burnt in a special furnace or autoclaved for 1 hr at 120°. The table is treated with lysol. The pan is filled with lysol, which is permitted to stand for 24 hr. The cultures are carefully labeled and placed in a heater.

NUTRITIVE MEDIA FOR CULTURING BACTERIA

Prof. M.A. Peshkov

It is impossible to prepare a medium with an identical nutritive value for all pathogenic bacteria, because of the extremely diverse individual requirements of different species. Nevertheless, bacteriology has purely empirically established so-called general, or universal nutritive media suitable for culturing the majority of bacteria. There are many diverse media for isolating and raising more demanding microbes and for investigating their biochemical properties. Nutritive media can consequently be divided into three types:

1. Ordinary, or universal media, which are used for culturing the majority of pathogenic bacteria;

2. Special media, which are used for isolating and raising certain pathogenic bacteria which grow poorly or not at all in ordinary media;

3. Cumulative, or "selective" media, in which some species of bacteria (which must be isolated) develop more rapidly than others or, conversely, develop more slowly than others which can be isolated.

Simple Media

Nutritive media are best prepared in enameled, aluminized, or steel (stainless steel) pots or enameled cans. Vessels used for cooking nutritive media must not be employed for other purposes, especially for diluting disinfectants, since even the slightest trace of such substances makes the medium unusable.

Preparation of glass vessels for media. The vessel selected is washed with water, using a brush, and dried on a rack. If a whitish deposit remains in a vessel which has already been used it is immersed in a 5-10% solution of technical hydrochloric acid and then carefully rinsed with water. A new vessel must be boiled in 1-2% hydrochloric acid.

After acid treatment the vessel is thoroughly rinsed with water and dried. Vessels to be used for diluting ordinary media are not sterilized. Media which cannot be pressure-sterilized are best diluted in sterile vessels.

Reaction of nutritive media. In order to maintain bacterial growth and viability nutritive media should contain sufficient quantities of the nutritive substance and salts and have a definite reaction. Bacteria adapt to some fluctuation in this reaction, but there is a pH limit for each microbe.

Determination of hydrogen-ion concentration. The colorimetric method, which is comparatively simple, yields sufficiently precise results in determining hydrogen-ion concentration under laboratory conditions.

Colorimetric determination of pH is based on the ability of a num-

ber of substances (so-called indicators) to change color when the pH of the solution changes.

There are two variants of the colorimetric method, buffered and unbuffered, each of which has its advantages and is described below.

In the majority of bacteriological investigations it is necessary to determine a hydrogen-ion concentration near the neutral point, usually at pH = 6.8-7.7 Michaelis suggested metanitrophenol and paranitrophenol as indicators for this pH region. Paranitrophenol is suitable for determining reactions between pH = 5.4 and pH = 7.0, while metanitrophenol can be used between pH = 6.8 and pH = 8.4. Both indicators are light yellow in color and only their intensity changes with pH. It is desirable to use blue glass for such determinations, since it makes it easier to see the change in tone. A set of standards and an encased comparator block are available commercially. The standards range from pH = 5.4 to pH = 8.4. Ten of the standard test-tubes contain the indicator metanitrophenol and correspond to pH's of 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8, 8.2, and 8.4. The other 9 test-tubes contain paranitrophenol and correspond to pH's of 5.4, 5.6, 5.8, 6, 6.2, 6.4, 6.6, 6.8, and 7.0. A 0.1% paranitrophenol solution and a 0.3% metanitrophenol solution in redistilled water are used as indicators.

In determining the reaction 1 ml of the medium under investigation, 4 ml of distilled water, and 1 ml of the indicator are poured into a test-tube with the same diameter as those containing the standard solutions; this tube is placed in the second hole of the comparator and tubes containing 2 ml of the medium and 5 ml of distilled water are placed to the right and left of it (i.e., in the first and third holes). A tube containing distilled water is placed in the fifth hole and standards in the 4th and 6th holes. The light passing through the standard test-tubes, Nos. 4 and 6, also passes through tubes Nos. 1 and 3, which

contain medium but no indicator, while the tube containing both medium and indicator is backed by the tube containing water; the illumination conditions are consequently identical for all the test-tubes. By changing the standards the one corresponding to the hue of the medium is selected.

In setting up the necessary reaction the tubes are left in the same positions, but standard test-tubes exhibiting reactions on either side of that to which it is desired to bring the medium (e.g., 7.2 and 7.4 if a reaction of 7.3 is desired) are placed in holes 4 and 6. A 20 N solution of acid or alkali is added to the tube containing the medium until a hue corresponding to the standard with the desired pH is obtained. The quantity of alkali or acid added to the tube is used to calculate how much of one or the other must be added to the entire medium to bring it to the required pH.

Technique for setting up the nutritive-medium reaction. In order to facilitate and accelerate determination of its pH, the medium is first tested with corallin paper (or with phenol red). This paper has an orange-yellow color and becomes slightly reddish at a pH somewhat greater than 7.0 and bright red at pH = 7.7. If the paper remains unchanged in color or becomes yellowish after being immersed in the medium and placed on a piece of dry filter paper small portions of a 10-normal solution of sodium hydroxide or sodium bicarbonate are added to the medium until the indicator paper takes on a slightly reddish hue. A reaction corresponding to approximately pH = 7.1-7.2 is thus established first and then determined precisely with the comparator.

Let us assume that a medium with a volume of 2 liters has been found to be slightly alkaline with corallin paper. We must obtain a medium with a pH of 7.4. Portions of the medium are poured into 3 test-tubes (2 ml in each for Michaelis's method and 5 ml in each for Gils-

pay's method) and 4 ml of distilled water and 1 ml of metanitrophenol or Michaelis' method or 10 drops of bromthymol blue for Gilspay's method are added to one of them; the tubes are then placed in the first row of the comparator, with the tube containing both medium and indicator in the middle and those containing medium but no indicator on either side of it. Standard test-tubes at pH = 7.3-7.5 are placed in the end holes of the second row, i.e., behind the tubes containing medium but no indicator, while the tube with medium and indicator is backed by a tube (or 2 tubes in Gilspay's method) containing distilled water. It is then easily determined whether the medium is more acid or more alkaline than desired by comparing the hues of the standards and the indicator-containing medium. Let us assume that the color of the standard at pH = =.7.2 is the same as that of the medium; the reaction is consequently more acid than required. Small portions of 20 N sodium hydroxide are then added from a microburette until the color of this tube corresponds to that of the standard at pH = 7.4 or is intermediate between those of the standards at pH = 7.3 and pH = 7.5. After each small portion of alkali is added the contents of the tube are well mixed before comparing it. For greater precision the same quantity of alkali should be added to the tubes containing the pure medium, in order to compensate for the change in the color of the natural medium pigment or the appearance of cloudiness.

Let us assume that 0.32 ml of 20 N alkali is necessary to bring the color of the tube containing 2 ml of medium to a hue corresponding to pH = 7.4; we then obtain the proportion 2:0.32 = 2000:x;

$$X = \frac{0,32 \cdot 2000}{2} = 320,$$

i.e., 320 ml of 20 N alkali, 160 ml of 10-normal alkali, or 16 ml of one-normal alkali must be added to 2 liters of the medium. Titration

of the small portions of medium in the test-tubes must be carried out very carefully, using an accurate apparatus (pipette). A small error is inevitable even when all the rules are observed and it is naturally multiplied in calculating the total quantity of alkali or acid which must be added to the medium as a whole. In the example given, it is thus necessary to add somewhat less than 16 ml of one-normal alkali and check the reaction in the comparator once more.

Experience has shown that it is convenient to dilute concentrated (approximately 10%) acid or alkali by a factor of 50 or 100 times ex tempore (using a small 50-ml graduated flask). The reaction is set up in the tube under study, employing a microburette or, as a last resort, a graduated pipette with 0.01 ml divisions; then, having made the calculation, a corresponding volume of the initial concentrated acid or alkali from which the dilution was prepared is added to the medium. In this technique the dilute solution used for titration should not be stored, always employing a fresh dilution. This method is more reliable than use of a precisely calculated 20 N sodium hydroxide solution and subsequent conversion for a solution of a different strength, which must be prepared as carefully as the 20 N solution to avoid errors; it is also more convenient, since it does not require titrated solutions, but only precise dilution of the initial concentrated solution in a graduated flask, without any need for special accuracy.

The procedure described above pertains to liquid media; for solid media (agar and gelatin) we use a technique based on the principle that dilution of well-buffered solutions alters the hydrogen-ion concentration so slightly that the change can be detected only by very precise methods. The majority of nutritive media, which contain various proteins, amino acids, phosphates, etc., are well-buffered solutions. The liquified agar or gelatin medium is diluted in 7 parts of hot distilled

water or physiological solution. The diluted medium remains liquid for some time, which makes it easy to conduct colorimetric investigations.

It must be kept in mind that a medium reaction carried out at room temperature is more acid at 37°. The pH at this temperature is 0.3-0.4 lower, depending on the medium composition.

Media become more acid after sterilization. It is consequently difficult to obtain a medium with a definite hydrogen-ion concentration after sterilization. This difficulty is even greater when the medium is sterilized in test-tubes. In order to avoid to some extent errors resulting from this change during sterilization, it has been suggested that 5 ml of the medium be thoroughly boiled for 30 sec and then quickly cooled before the reaction is set up. The medium reaction may also change during storage, as a result of adsorption of carbon dioxide from the air. This change is small, but occasionally significant.

Filtration and clarification of media. After the required reaction has been established liquid media, broth, peptone water, etc., are filtered through a folded double layer of filter paper preliminarily wetted with distilled water. Gelatin is filtered through the same type of filter, but it is best that the funnel and the flask containing the filtered medium be placed in a heated Koch apparatus or autoclaved to accelerate filtration and prevent solidification.

It is very difficult to filter agar through paper and many different methods and devices for accelerating filtration have consequently been proposed.

Instead of being filtered, melted agar can be poured into a deep cylinder or a large conical graduate, placed in a hot autoclave, and then permitted to solidify slowly (by standing overnight). All the sediment settles into the lower portion of the agar. The vessel should be heated in hot water, the agar permitted to solidify, and its lower por-

tion out away with a knife. The agar is then cut into chunks, placed in a pot, and melted in water bath or a Koch apparatus.

The medium is sometimes clarified before filtration. For this purpose the white of a hen's egg (one egg per liter of medium), which should be shaken (in a jar with a ground stopper) with twice its volume of cold water, is added to the nutritive medium, which is cooled to below 50°. The eggs must always be fresh. After the albumin has been added the medium is thoroughly mixed and boiled over a flame for 10 min or heated in an autoclave at 105° for 30 min. The albumin coagulates and takes with it all the particles suspended in the medium. The latter is then filtered. Instead of egg white one can use 20-30 ml of blood serum from horses or other animals per liter of medium. Occasionally, however, addition of albumin causes the medium to become even more cloudy. It is consequently best to make a preliminary clarification test in a test-tube before adding the albumin, mixing 0.3 ml of the dissolved albumin or serum with 10 ml of the nutritive medium. Coagulation of agar with meat gives very good results.

Pouring nutritive media. A glass funnel whose end is fitted with a rubber tube bearing a Moore clamp and an elongated glass tip is used for pouring finished nutritive media. The entire system is placed in a rack and the medium is poured through a funnel into the vessel intended to hold it.

A simple, easily-assembled device can be used when it is necessary to pour out definite quantities of medium.

A paper cap is placed over the cotton plug in each flask or bottle and fastened with fine binder twine or, far more convenient, a rubber band.

Special aluminum caps standardized for the standard test-tube sizes and equipped with internal springs have recently come into general

use abroad. These caps are very convenient and ensure complete sterility in working with and storing the test-tubes.

Methods of preparing basic media

Meat infusions and peptone account for the majority of media used to raise bacteria.

Meat infusion is obtained by extracting sausage meat with water for 12-24 hr. The meat is then pressed and the cloudy liquid obtained is brought to the boiling point, which causes the proteins which it contains to coagulate. The coagulate is filtered; the weakly acid (pH = = 6.2) clear yellowish liquid thus produced (the meat infusion) lacks proteins, but contains small quantities of amino acid salts, carbohydrates, growth hormones, and extractives.

Peptone is the product of comparatively mild protein decomposition caused by weakly acidic or alkaline hydrolysis or by enzymatic cleavage of the proteins of pepsin. It was introduced into bacteriological practice in 1882. Peptone is highly soluble in water, does not coagulate when boiled, and does not precipitate under the action of acids. A clear 1% peptone solution to which sodium chloride and sodium nitrate have been added is called peptone water and is used as an independent nutritive medium for raising Vibrio comma. Peptone water containing glucose and sodium chloride is used for determining the titre of coliform bacteria by Eichmann's method, while peptone water to which sodium chloride has been added is used as the basis for colored media containing carbohydrates.

A knowledge of the principal chemical indices of the basic solutions from which nutritive media are made up makes it possible to follow their preparation with a high degree of certainty and to compile formulas for various media.

Meat-infusion broth

Meat from a steer, horse, or other animal is freed of fat, tendons, and bones, passed through a meat grinder, and weighed. Distilled water is then added (2 ml of water g of meat) and the meat is thoroughly mixed and left to stand in a cool place for 12-24 hr. The mixture is passed through a piece of linen and squeezed with the hands or a press; the liquid thus obtained should be equal in volume to the water originally used. The filtered meat infusion can also be poured into a bottle and 1% chloroform added. A total of 1% powdered peptone and 0.5% [redacted] chloride is added to the infusion. Since the latter exhibits an acid reaction, a saturated solution of sodium carbonate (Na_2CO_3) is used to make it slightly alkaline, as shown by litmus paper. To precipitate its proteins the liquid is then boiled for 2 hr with continuous agitation or sterilized in an autoclave for 15-20 min (at a pressure of 1.25 atm and a temperature of 115°). The hot liquid and precipitated proteins are filtered through folded filter paper preliminarily wetted with distilled water; the filtered liquid should be completely clear and straw-yellow in color. It s reaction (usually at pH = 7.1-7.2) is precisely determined with a comparator. It is necessary to warn against making the reaction too alkaline, since the broth then becomes cloudy during sterilization. The same thing will happen if frozen meat is used to prepare the broth. After its reaction has been determined the broth is poured into a clean unsterile vessel or into test-tubes, filling them no more than 1/3 full. The tubes are then sealed with cotton plugs and sterilized in an autoclave at 120° for 15-20 min (the final filtered broth can be stored for many months without sterilization if chloroform is added, just as meat infusion; see Table 15).

Liebig's meat extract can be used instead of meat infusion; in this case 10 g of extract, 0.5 g of sodium chloride, and 10 g of pep-

tone are usually added to 1 liter of distilled water and the mixture is heated until the additives dissolve, filtered, poured into tubes, and sterilized. Another formula calls for 3 g of meat extract, 5 g of peptone, and 1 liter of distilled water.

TABLE 15

Chemical Indices of Ingredients and Meat-Infusion Broth used as the Basis for Common Laboratory Media

	Пептон, % 1	Аминный азот, мг % 2	Триптофан, мг % 3
Мясная вода 4	0	58	0
1% раствор сухого пептона 5	1	35	0
Мясо-пептонный бульон (мясной воды 100 мл, пептона 1 г, хлористого натрия 0,5 г) 6	1	91	0

1) Peptone, %; 2) amine nitrogen, mg-%; 3) tryptophan, mg-%; 4) meat infusion; 5) 1% dry peptone solution; 6) meat-infusion broth (100 ml of meat infusion, 1 g of peptone, and 0.5 g of sodium chloride).

Meat-infusion agar

Meat infusion is prepared in the same manner as for broth and 1% peptone and 0.5% sodium chloride are added; the mixture is boiled over a flame until all its proteins have precipitated and then filtered, its reaction is determined, and 1.5-2% (15-20 g per liter) of agar-agar is added. The liquid is then boiled in a pot until the agar has completely dissolved or kept in an autoclave at 115-120° for 3/4 hr or at 100° for 1-1 1/2 hr. The reaction is then checked once more and adjusted if necessary. If the liquid is cloudy it is clarified with protein by the method described above and filtered. The filtered agar is poured into test-tubes in approximately 5 ml portions or into 0.5-liter flasks or bottles in 100-200 ml portions and sterilized in an autoclave at 120°. Strong heating has no influence on the solidification of the agar, but repeated heating and cooling reduces its density. The agar becomes soft

and exhibits an acidic reaction. So-called agar slants are prepared in order to obtain the maximum possible surface area for bacterial growth. The agar is sterilized in the tubes and the latter are placed on a table to solidify, propped up by glass tubes or rods or folded towels in such fashion that the upper portion of each tube is only slightly higher than the lower portion.

Agar-agar is produced in the USSR, Japan, China, India, and the United States from certain red marine algae of the family Flondeae. It contains no nitrogen, swells in water, and produces a gel which liquifies on heating. Different brands of commercial agar produce gels which liquify at temperatures of from 70 to 100° and solidify between 40 and 50°.

Media prepared from hydrolyzates of the liquid-tryptone type.

General notes. Hydrolyzates diluted with water are used as the basis for Hiss' and Levin's media.

In preparing the broth the hydrolyzate is diluted with a phosphate mixture containing 1000 ml of tap water, 7 g of purified sodium chloride, and 1 g of Na_2HPO_4 or K_2HPO_4.

Tryptone broth (TB) consists of a hydrolyzate (1 part by volume) and the aforementioned phosphate mixture (4 parts by volume). The pH is adjusted to 7.4, the mixture is autoclaved at 0.5 atm for 5 min and filtered, its reaction is checked, and it is poured into a bottle; chloroform is added in a dose of 10-20 mg per liter and the bottle is closed with a hermetically-fitting stopper. The broth can be stored without sterilization indefinitely. When needed some of the broth is poured into test-tubes or flasks and sterilized at 1 atm for 15 min.

Tryptone agar (TA) consists of a hydrolyzate (1 liter), the phosphate mixture (4 liters), and dry agar (100 g). The mixture is placed in an autoclave, the pressure is raised to 1 atm, and the autoclave is

then shut off and left to stand overnight, without being opened. In the morning the pot containing the agar is removed from the autoclave and the agar is cut into pieces and emptied ontc a piece of paper.

The residue is cut away and the clear agar is sliced and placed in a wide-mouth bottle; a little chloroform (30-40 ml) is added. The bottle is hermetically stoppered. When needed the requisite quantity of agar is poured out and melted, its reaction is adjusted, and it is poured into test-tubes and sterilized at 120° and 1 atm for 15 min.

Broth for rapid determination of indol and hydrogen sulfide formation

This broth contains 200 ml of egg hydrolyzate and 800 ml of the aforementioned phosphate mixture and has a pH of 7.4. Sterilization is carried out at a pressure of 0.5 atm for 5-10 min (for clarification). The mixture is filtered, its reaction is checked, and it is stored with chloroform. When needed 1-5 ml portions are poured into test-tubes, which are then sterilized at 120° and 1 atm for 15 min.

Indol formation is checked after 18-14 hr, pouring Ehrlich's reagent over the surface of the medium (without ether extraction). The presence of hydrogen sulfide is indicated by lead foil wetted with lead acetate, which is introduced into the test-tube immediately after culturing (the foil is held between the stopper and the wall of the tube).

Mixing equal quantities of egg and casein hydrolyzates yields very good results. Media prepared from eggs and casein are completely colorless, resembling distilled water.

Nutritive media prepared from yeast

In order to reduce the cost of preparing nutritive media attempts have long been made (Shardinger, 1896) to replace animal-protein derivatives (peptone) with infusions, extracts, and hydrolyzates of the seeds and embryos of various plants and of yeasts.

Ordinary brewer's yeast or yeast fungus contains up to 60% nitro-

genous substances, including the albumin cerevisin, the protein zymocasein, which is similar to the casein of milk and the legumin of the pea, globulins, nucleoproteins, nucleic acids of the ribose (cytoplasm) and deoxyribose (nuclear chromatin) types, and lecithin. Yeasts are rich in glutathione. Ergosterol, glycogen, mannan, dextran, and various pentosans are among the nonnitrogenous organic compounds, which make up 2-5% of the total. Yeasts also contain proteolytic enzymes of the papain polypeptidase, and dipeptidase types. Kutcher (1901) showed that washed yeast cells kept at 37° undergo autolysis and form products characteristic of the tryptic digestion of proteins, although the process occurs in the presence of an acid reaction (which is typical of papains). This property of yeasts is utilized in preparing media.

Preparation of a yeast autolyzate. Pressed yeast fungus is diluted with water, 1 kg of yeast in 3-5 liters, and heated to 55-58°. The mixture is thoroughly stirred to obtain a uniform suspension and placed in a heater (usually in glass bottles) at 50-55° for 18-24 hr. When autodigestion is complete the bottles containing the autolyzate are autoclaved at 120° for 15 min. After 24 hr the autolyzate has separated into a clear light-colored layer and a residue of incompletely digested yeast cells. The autoclaved autolyzate can be stored for future use. The light-colored layer is usually used in preparing media, but the residue can also be employed for mass production.

Representative chemical indices of yeast autolyzates include: peptone – approximately 1%, amine nitrogen – 280-300 mg-%, and free tryptophan – 150 mg-%.

The medium is prepared by mixing the yeast autolyzate with 0.5% of sodium chloride, 0.1% of Na_2HPO_4, and 0.5% sucrose (granulated or refined sugar) to activate bacterial metabolic processes associated with nicotinamide or nicotinic acid.

Differential-Diagnostic Media

Since bacteria contain enzymes, some of which decompose proteins and carbohydrates to varying degrees while others produce oxidation and reduction reactions, various chemical processes occur during bacterial growth on differential-diagnostic media. Either the changes which take place in the medium or the corresponding reactions are used for diagnostic purposes.

All differential-diagnostic media can be divided into four groups.

First group. Media containing proteins in which bacterial enzymes produce characteristic changes: blood, milk, gelatin, coagulated serum, etc. They are used for determining proteolytic or hemolytic activity, etc.

Second group. Media containing sugars or high-molecular-weight alcohols which are decomposed by the appropriate enzymes of certain microorganisms and alter the medium's reaction, thus serving as indicators.

Third group. Media containing substances which are altered by the oxidative or reductive action of bacteria, e.g., dyes such as neutral red, methylene blue, etc., salts of telluric acid, etc. Media containing indifferent substances which can serve as a nutritive source for some species of bacteria but cannot be assimilated by others. For example, media containing salts of citric acid are used to differentiate Escherichia coli, which cannot assimilate such compounds, from other species of coliform bacteria, which decompose citrates.

Media for determining proteolytic activity

Nutritive media to be used for detecting proteolytic activity should not contain glucose or glycerine.

The simplest method of determining proteolytic activity is puncture culturing in a column of nutritive gelatin.

Nutritive gelatin. This nutritive medium is prepared with the high-quality gelatin generally used in food or with that employed in the manufacture of photographic plates. We distinguish hard, moderately hard, and soft gelatin, depending on its melting point when swollen in water. Poorly-solidifying brands with very low melting points are often encountered. The melting point of hard gelatin (10% solution) should be between 32 and 34°, while its solidification point should not be below 22-26°. Poor gelatin often only partially solidifies at 17-18°. Good brands of gelatin usually exhibit a weakly acid reaction (as determined with litmus paper); such gelatin yields clear solutions, in contrast to alkaline gelatine, which very often is cloudy or opalescent in solution. It must be kept in mind that the density of the solidified gelatin depends to a large extent on its reaction; thus, at pH = 6.3 it solidifies poorly and at pH = 7.7 it often does not solidify at all.

Preparation method. A single sheet containing 200 g of foodstuff gelatin (150 g in winter) is melted under constant agitation in 1000 ml of meat-infusion broth (at pH = 8.0) and brought to the boiling point. The liquid is permitted to cool to 30° and two egg whites beaten until they are white are added. The mixture is vigorously stirred for 5 min and placed in a Koch flowing-steam apparatus for 1 hr. The albumin coagulates, taking with it all the turbidity. The solution is filtered through cotton (in a Koch apparatus if the room temperature is below 20°). The nutritive gelatin thus obtained should be absolutely clear. Portions of 5 ml are poured into test-tubes and sterilized in a Koch apparatus for 1 hr 3 times, on alternate days.

There is also another method for preparing nutritive gelatin: a 10% solution of chunked gelatin is added to meat-infusion broth or Hottinger's broth, permitted to swell, and heated in a water bath at 40-50°. The reaction becomes acid after the gelatin has dissolved; it is

corrected by adding approximately 2 ml of 10% crystalline sodium carbonate (Na_2CO_3) per 100 ml of medium and then adjusted to pH = 7.0-7.1 with a comparator. After the reaction has been adjusted the gelatin is filtered through a double folded filter and a little of the filtrate is poured off into a test-tube; the liquid should be completely clear and should not become cloudy when boiled. The filtered gelatin is poured into test-tube and sterilized at 100° for up to 20 min on 3 consecutive days.

Prolonged heating must be avoided, since high temperatures cause the gelatin to lose its ability to solidify. It is consequently helpful to cool the gelatin after each sterilization, immersing it in cold water. The test-tubes are cooled in a vertical position, taking care that the upper surface of the gelatin column is level. The gelatin must be stored in a cool place. During the hottest part of the summer nutritive gelatin is usually prepared as a 15% solution.

The meat-infusion gelatin "column" is inoculated by puncture with a platinum needle bearing the culture. The puncture must be made in the center of the column and the needle should reach to the bottom of the tube. The cultures are kept in a vertical position at a temperature of 20-22°.

The results are evaluated 3 times, after 1, 10, and 20 days. If the bacteria under investigation does not grow at 20° it can be cultured in gelatin at 37° or whatever temperature is optimum for it and the test-tube is then placed in a refrigerator; the experimenter notes how much longer the gelatin takes to solidify than uninoculated gelatin. In order to speed up the process the inoculated gelatin can be placed in a heater at 37°, removed after 1-2 days, and cooled under running cold water or in a refrigerator, checking to see whether the gelatin has solidified.

Coagulated horse serum. Proteolysis is manifested by solidification and formation of pits on the surface of the medium.

Milk (see page 240). In cultures raised on milk proteolysis is manifested in homogenization, slight clarification of the milk, and dissolving of the casein coagulate formed during the first few days of bacterial growth.

Eichmann's milk agar. A total of 1-2 ml of sterile skimmed milk is added to 10 ml of melted sterile agar in a test-tube, observing all aseptic precautions, and the mixture is poured into Petri dishes (clumps of casein are formed when the milk and agar are sterilized together). The surface of the medium is inoculated with the culture under investigation in such fashion as to produce isolated colonies. If the bacterium has a proteolytic action the casein is peptonized and light aureoles are formed around the colonies, against a cloudy background.

Media for determination of hemolytic activity

The most graphic method for detecting hemolytic activity is to inoculate the surface of a special blood agar (see below) poured into Petri dishes with the culture under investigation (by the streak or spatula method). Greatest activity is obtained at 37°. Zones of clarification are formed around colonies which produce hemolysis, as a result of dissolution of the erythrocytes; we distinguish α-hemolysis, in which the colony is surrounded by a greenish zone, and β-hemolysis, in which the colony is surrounded by a colorless transparent zone.

The blood agar consists of 95 ml of 2% meat-infusion agar and 5 ml of defibrinated horse, sheep, or rabbit blood.

Preparation method. A given quantity of agar is melted and cooled to 45° (the temperature should be no higher than this, since the blood will otherwise become darker in color).

The agar, in a test-tube or flask, is placed in hot water, the

blood is added with a heated pipette, and the mixture is thoroughly stirred. The vessel containing the finished agar is placed in warm water.

In order to prepare agar slants the medium is dropped into sterile, preliminarily heated test-tubes with a heated pipette and the tubes are immediately placed in an inclined position.

Dishes. The prepared medium is first dropped into sterile, preliminarily heated test-tubes in 10-15 ml portions with a pipette, the tubes then being placed in hot water. The contents of the tubes are emptied into sterile Petri dishes preliminarily warmed in a heater, permitted to solidify, and dried in a heater. The agar layer should be cloudy and have a uniform reddish color.

In order to investigate a broth culture decreasing doses are dropped into a series of test-tubes with a graduated pipette and the liquid volume in each tube is brought to 2 ml by adding physiological solution. One drop of defribinated blood is then added to each of the tubes. It is necessary to set up a control consisting of 2 ml of physiological solution and a drop of blood. The contents of the tubes are mixed by shaking and the rack holding the tubes is then placed in a heater at 37° for 2 hr. When a hemotoxin is present the erythrocytes dissolve and the cloudy suspension becomes a clear red solution.

Media for determining carbohydrate fermentation

A great many pathogenic microorganisms have the ability to ferment, i.e., decompose, various carbohydrates and high-molecular-weight alcohols to form aldehydes, acids, and gaseous products (CO_2, H_2, and CH_4). Even within the same genus there is a great diversity with respect to given carbohydrates: some bacteria ferment glucose and not lactose, while others ferment glucose and lactose but not sucrose; some bacteria ferment carbohydrates to form acids and gases, while others produce

acids but not gases, etc. This specificity of the action of different microbes with various carbohydrates and high-molecular-weight alcohols or, as they are customarily termed, sugars is widely used in bacteriological practice for differentiating different genera of bacteria. A color series, i.e., a series of test-tubes with nutritive media containing various carbohydrates and indicators, is used to detect the decomposition of these sugars; for clearer observation of gases tubes sealed at one end (floats) are introduced into the test-tubes or the medium is made semifluid by adding 0.2-0.5% of agar-agar. The reaction is evaluated from the change in the color of the indicator. In ordinary practical work the color series consists of test-tubes containing sugar media (glucose, lactose, and mannose), which can be supplemented with sucrose, maltose, milk and lactic litmus serum. Other carbohydrates can also be tested in studying and determining atypical strains.

The different types of sugars and alcohols used for this purpose should be chemically pure.

In order to prevent possible errors a preparation of unknown origin must be checked first, inoculating the medium containing the carbohydrate to be tested with bacteria known not to decompose it.

Colored media containing various carbohydrates. A medium of the Hiss type* containing 1 g of peptone giving a red biuret reaction, 0.5g of chemically pure sodium chloride, and 100 ml of water is preferable for microbes which grow well on artificial nutritive medium. The peptone and salt are dissolved in not water for several minutes and filtered through filter paper; the solution should be absolutely clear. It is also possible to use Hottinger's broth (100 ml of the basic hydrolyzate and 900 ml of Hottinger's phosphate mixture) at pH = 7.0. A total of 0.5-1% of any of the carbohydrates to be used or 0.2% of glucoside is dissolved in the medium and the float and indicator are added.

Tincture of litmus (see page 242), added in a quantity of 5 ml per 100 ml of medium, or azolitmin, added in a quantity of 0.25 g per liter, are occasionally used for this purpose. Bromthymol blue (1 ml of a 1.6% alcoholic solution in 1 liter of medium) is quite often used instead of litmus, which is reduced and decolorized during the growth of certain bacteria. All Clark and Lebs indicators persist well in this solution. If it is undesirable to add alcohol to the medium saturated aqueous solutions of the indicator are prepared (see page 242) and used in quantities of 2 ml per liter of medium.

A medium containing bromthymol blue should be grass-green when its reaction is neutral, yellow when its reaction is acid, and blue when its reaction is alkaline.

In addition to the aforementioned indicators, Bronfenbrenner proposed a mixture of analine blue and corallin (or, better, its sodium salt, since corallin is toxic for gram-positive microorganisms), the so-called CR indicator. This mixture consists of equal volumes of 2% corallin in 50% alcohol and 1% aqueous analine blue. A total of 0.25 ml of the indicator is added to 100 ml of the nutritive medium and the medium reaction is preliminarily adjusted to pH = 6.8-7.0. At this pH the medium is yellowish after sterilization and its color changes are the opposite of those of litmus, i.e., it becomes red when alkaline and blue when acid. The Dry-Media Laboratory of the TsIEM [Central Institute of Epidemiology and Microbiology] produces dry media containing the indicator VR, which also has the properties described above, for the color series.

Hiss' solid media containing carbohydrates and Andrade's indicator. The total of 1000 ml of distilled water, 20 g of dry peptone, and 5 g of sodium chloride are mixed, boiled, adjusted to pH = 7.6, poured into bottles, and sealed with cotton plugs. The bottles are sterilized

at 120° for 15 min, cooled to 37° after sterilization, and inoculated with a 18-hr culture of a coliform bacterium (Esch. communior is best) in order to destroy all traces of carbohydrates; the medium is then kept in a heater at 37° for 24 hr. (this precaution destroys the carbohydrates which certain brands of peptone contain). The bottles are then sterilized in a Koch apparatus for 30 min. Some of the liquid is poured off from each of the bottles and the reaction is adjusted to pH = 7.0. Finely chopped agar is added in quantities of 5 g per liter of liquid. A total of 10 ml of Andrade's indicator* is then added for each liter of the basic medium. The solution is stirred and poured into bottles in 200 ml portions. Portions of 1 g of glucose, lactose, mannose, sucrose, and maltose are weighed out and introduced into the appropriate bottles. The latter are placed in a water bath for 10 min and then shaken and 4 ml portions of the medium are poured into test-tubes (each carbohydrate-containing medium is poured through a separate funnel, in order to avoid mixing the carbohydrates). The tubes are sterilized for 20 min at 115° and the medium is permitted to solidify into columns, keeping the tubes vertical. Each tube is immediately labeled with glass-marking crayons or ink to show the type of sugar (using abbreviations). The finished Hiss media containing Andrade's indicator are colorless or slightly yellowish. They become straw-colored when acids are formed. The media should be stored in a cold, dark place.

Sterilization of carbohydrate-containing media. Media to which carbohydrates have been added usually cannot be sterilized under pressure. They are poured into sterile test-tubes after the indicator has been added and are sterilized at 100° for 20 min on 3 consecutive days.' However, in order to speed up the process the tubes can in some cases be sterilized in an autoclave for 20 min at 115°. If the reaction of the carbohydrate-containing medium is above pH = 7.0 it often becomes

so acid after sterilization that its pH reaches 6.0, especially when it contains glucose or lactose. Attempts to prevent or correct this by adding an excess of alkali before sterilization do not work, since the increase in the number of hydroxyl ions from the sugar causes formation of large quantities of acid products. Exposure of the sugar to an alkali produces many different substances, including acids ranging from saccharic to lactic. The reactions of such media should consequently be adjusted as carefully as possible, never lying outside pH = 7.1-7.2.

If it is necessary to investigate the enzymatic activity of bacteria which grow poorly or not at all on peptone media Hiss' original medium is used: 25 ml of water in which 1 g of the carbohydrate necessary for the investigation (glucose, lactose, etc.) has been dissolved is added to 75 ml of sterile bovine (not equine) serum. The sugar is dissolved in distilled water, boiled, cooled, and then mixed with the serum.After the sugar has been added an equal amount of a sterile aqueous solution of the indicator is run in. The mixture is poured into sterile test-tubes, which are kept in a heater at 37° for 24 hr.

Peshkov's medium for macroscopic determination of mobility and of acid and gas formation from glucose.

I. Hottinger's hydrolyzate - 100 ml, tap water - 900 ml, glucose - 10 g. The mixture is adjusted to pH = 7.2.

II. Hydrolyzate diluted by a factor of 10 and adjusted to pH = 7.2 is used to prepare 1.2% agar. The agar is clarified with egg white and filtered through filter paper (in an autoclave) and 100 ml portions are poured into flasks, which are sterilized at 120° and 1 atm for 15 min. A total of 100 ml of the 1.1-1.2% filtered agar is added to each 900 ml of the diluted glucose-containing hydrolyzate.

At this point 4 ml of a 1% alcoholic bromthymol blue solution or 10 ml of Andrade's indicator is added to 1000 ml of the medium. The

mixture is poured into a bottle, 20-30 ml of chloroform per liter is added, and the bottle is hermetically sealed. When needed the requisite quantity of medium is poured into test-tubes in deep columns (6-7 cm) and sterilized at 112° and 0.5 atm for 20 min. The medium should be absolutely clear, having a greenish color when it contains bromthymol blue and being colorless when it contains Andrade's indicator. The culture is made within the column. Immobile bacteria grow as stalagtites or finger-like branches through the medium. The space between the branches should remain crystal clear. Labile bacteria grow uniformly throughout the entire medium, as shown by its uniform clouding. A change in the color of the indicator (to yellow with bromthymol blue or red with Andrade's indicator) shows that acids have been formed from the glucose. Alkalization of the medium is indicated when it turns blue (if the indicator is bromthymol blue).

Formation of gases is indicated by a persistent froth on the surface of the medium.

Highly aerobic bacteria (e.g., Pseudomonas aeruginosa, B. fluorescens, Alcaligenes faecalis) form only a surface film. This medium furnishes a reliable mobility control when used as a hanging drop with a microscope.

Milk. The milk is first freed of cream (by standing and skimming, but better by drawing it from a separator) and its reaction is tested with litmus: it should be slightly alkaline. The milk is poured into test-tubes and sterilized at 100° for 30 min on 3 consecutive days. Milk sterilized at 115° often spoils, since its casein is peptonized and its lactose caramelized (turned yellow) at this temperature.

Milk mixed with water and litmus is Minkevich's medium. A total of 0.5 liter of fresh milk is used (a meat-red color should be obtained when a drop of 1% aqueous neutral red is added to 5 ml of the milk). A

straw color indicates an acid reaction and such milk is not suitable. The milk is boiled, mixed with 0.5 ml of distilled water and 30 ml of tincture of litmus (see page 242), and poured into a bottle and 50 ml of chloroform is added. The bottle is closed with a tight-fitting stopper, shaken, and left in a dark place for 2-3 days. The chloroform, which is heavier than water, settles to the bottom and takes with it the cream which it has dissolved. The defatted Minkevich's medium is carefully drawn off, leaving the cream, and 5 ml portions are poured into test-tubes, which are sterilized at 115° for 20 min. When taken from the autoclave the medium is yellow in color as a result of reduction of the litmus; its natural violet color returns only after a few hours.

Chloroforming the milk is an extremely convenient method of defatting and simultaneously preserving the medium. Preliminary boiling is obligatory, since the natural proteases of the milk would otherwise cause sterile peptonization.

Petrushki's litmus serum. Equal quantities of milk and distilled water are heated to 40-45° and 5% sulfuric acid is carefully added until all the casein has precipitated (checking the filtrate). A slight excess of acid is added and the solution is filtered. The filtrate is usually cloudy and this is no cause for concern. The filtrate is neutralized with a saturated sodium carbonate (Na_2CO_3) solution and heated in a flask in a pot containing boiling water for 1 hr; the reaction is checked from time to time and should become acid, then being neutralized with 1% sodium carbonate. The greenish liquid containing a copious amorphous residue thus obtained is permitted to cool and filtered through a double layer of filter paper without agitation. A completely clear greenish filtrate which yields a neutral or amphoteric (the latter being better) reaction with litmus should be obtained. A total of

5% of tincture of litmus is added and the mixture is poured into sterile test-tubes and sterilized in flowing steam for 30 min on 3 consecutive days.

Tincture of litmus. Preparation begins with pulverization of 80 g of granular litmus in a mortar and addition of 150 ml portions of water. The resultant mixture is poured into a flask and gently heated in a water bath. The liquid is decanted and 300 ml of 40% alcohol (125 ml of 95% alcohol diluted to 300 ml with water) is added to the residue; the mixture is then heated for 1 min. The alcoholic extract is decanted, mixed with the first aqueous extract, permitted to settle (overnight), decanted or filtered through paper, and mixed with 300 ml of 40% alcohol. One-normal hydrochloric acid is added drop by drop, shaking occasionally, until the solution is no longer violet. A total of 1-2 ml of acid for each 100 ml of extract is usually required. When its reaction is correctly adjusted the solution should be blue in tap water and violet in boiling distilled water. Tincture of litmus prepared in this manner remains unchanged for a very long period.

Zeitz's artificial litmus serum. Preparation of litmus serum from milk requires a great deal of time and accuracy, so that it is simpler to use Zeitz's artificial litmus serum, which gives the same results as natural serum. The following ingredients are dissolved in 1000 ml of distilled water: 20 g of chemically pure lactose, 0.4 g of chemically pure dextrose, 0.5 g of sodium hypophosphate, 1 g of ammonium sulfate, 2 g of sodium citrate, 5 g of chemically pure sodium chloride, 0.05 g of Witte peptone, and 0.25 g of azolitmin.

The solution is poured into sterile test-tubes and sterilized at 100° for 15 min on 3 consecutive days.

The Witte peptone can be replaced by Hottinger hydrolyzate, adding the latter in a quantity of 50 ml per liter of medium.

Media for determining the rate of acid formation from glucose

1. Reaction with methyl red in Clark's medium.

Clark's medium is made up by mixing 0.5 g of peptone, 0.5 g of glucose, and 0.5 g of K_2HPO_4 in a mortar and dissolving them in 80 ml of distilled water under constant agitation; the solution is then heated in a water bath for 20 min, filtered through filter paper, cooled to 20°, and brought to a total volume of 100 ml with distilled water. Portions of 5 ml are poured into test-tubes, which are sterilized at 100° on 3 consecutive days. A total of 5 drops of a 0.04% methyl red solution (1 ml of 1.6% alcoholic methyl red and 39 ml of 95° alcohol; the solution can be stored for an indefinite period) are added to 5 ml of a 4-day culture. When acid formation is intense (pH < 5.0) the culture becomes red, while when formation is weak (pH > 5.0) it becomes yellow. The reaction is designated as MR+ for red and MR- for yellow.

2. Foges-Proskauer's reaction. Formation of acetylmethylcarbinol from glucose.

For this reaction the culture is transferred to Clark's medium (see methyl-red reaction) and incubated for 5 days at 30°. At this point 1 ml of 10% aqueous potassium hydroxide is added to 5 ml of the culture and it is placed in a heater at 37°. Staining is gradual and the reaction is evaluated after 18-24 hr. A reddish color with yellow fluorescence (characteristic of alcoholic eosin solutions) indicates a positive result. If the medium remains unchanged in color (colorless) the result is negative. This reaction is usually obtained in bacteria which ferment glucose.

The reaction is apparently absolutely clear in cultures of bacteria which give a negative reaction with methyl red (pH > 5.0).

Media for determining starch hydrolysis (amylolytic activity).

Starch agar is made up as follows: 0.2 g of dissolved starch boiled

in 5 ml of distilled water is added to 100 ml of ordinary melted nutrient agar and the mixture is poured into dishes, permitted to solidify, dried, and inoculated.

In Debout's method for preparing dissolved starch ordinary potato, wheat, or rice starch is treated with 1-2% hydrochloric acid at 53° for 18-20 hr. The acid should be taken in a quantity sufficient that a consistency equivalent to that of thickened milk is obtained when it is mixed with the starch and shaken. Heating continues until the starch sample is wholly dissolved in the hot water. The starch is permitted to stand and the acid is added; the latter is then washed from the starch by rinsing it several times with cold water, permitting it to stand each time, and decanting the remaining liquid. Washing continues until a neutral reaction is obtained with litmus: the last portion of water is then poured off and the starch is left to dry at room temperature.

Many pathogenic bacteria produce a diastatic enzyme which converts starch to sugar. In order to detect this type of activity the bacterium under investigation is cultured on a dish containing starch agar and placed in a heater at 37°. The culture is examined after 24 hr if the bacterium grows well and after 7 days if it grows slowly. The surface of the dish is flooded with a saturated and filtered solution of iodine in 50° alcohol; this solution is permitted to stand until the medium acquires a dark blue color and the dish is then examined in transmitted light. The extent of the clear colorless zones around the colonies indicates how intensively the starch has been hydrolyzed.

Media for determining reductive activity

Media containing dyes. The ability of bacteria to decolorize (oxidize or reduce) certain organic dyes and convert them to colorless leucobases, which regain their basic color when a copious oxygen supply is present, is called their reductive capacity, using Ehrlich's terminol-

ogy. Among the dyes suitable for determining reductive capacity are methylene blue, thionine, litmus, indigo carmine, neutral red, etc. Many dyes, such as methyl violet, are unsuitable for the reduction test, since they retard bacterial growth and can themselves alter the medium.

The aforementioned dyes are added to ordinary broth or agar to determine the reductive properties of microorganisms.

One drop of 1% aqueous methylene blue or 3-4 drops of this solution diluted to 0.5:1000 (1 ml of 0.5% aqueous methylene blue and 9 ml of water) are added to the test-tube containing the broth (5 ml).

The reduction of the methylene blue is evaluated after incubation at 37° for 24 hr (complete decolorization indicates a positive reaction, a green tint a weakly positive reaction, and no decolorization a negative reaction).

Tincture of litmus is added in a quantity of one drop for each 5 ml of medium. The advantage of this dye is the fact that it can be used to determine both the reduction (from its decolorization) and the change in pH (its violet hue changing to red or blue).

Indigo carmine (sodium indigo sulfate) is added in a quantity of 1 ml of a 5% solution for each 100 ml of sterile medium or one drop for each 5 ml of broth. The dye solution is sterilized separately. It must be kept in mind that this dye is also decolorized as a result of oxidation. It is easy to determine whether we are dealing with reduction or oxidation: if the dye regains its color when brought into contact with air (by shaking the medium) decolorization resulted from reduction.

Neutral red is used in a 2% aqueous solution (1-2 drops in a test-tube full of medium).

Reduction caused by bacteria with strong reducing properties can be detected very quickly in broth; with weakly reductive bacteria it is

necessary to pour a layer of sterile liquid paraffin or vaseline oil over the surface of the medium, so that the gradual is not halted by oxidation where the medium comes into contact with the air.

Stab cultures in agar are recommended for this purpose. Such cultures permit observation of the gradual reduction (decolorization) within the puncture. It is also necessary to have a control tube containing the same medium, to be sure that the medium has not undergone autoreduction.

Media containing nitrates. In microbiology the term nitrate reduction, or denitrification, is used to refer to the ability of bacteria to reduce salts of nitric acid (nitrates) to salts of nitrous acid (nitrites) and then to ammonia or free nitrogen. Many pathogenic bacteria are capable of reducing nitrates to nitrites.

In view of the wide occurrence of nitrites in nature it is necessary to use especially carefully washed vessels and chemically pure initial ingredients.

A total of 0.2% of nitrite-free potassium nitrate (KNO_3) is added to nitrite-free meat-infusion broth (checked with the reagent described below), the mixture is again checked for nitrites, and 5 ml portions are poured into test-tubes. The latter must be washed 5 times with tap water. They are then sterilized for 15 min at 120° and the results are evaluated 3 days after culturing.

Preparation of reagent: solution I: 1 g of dissolved starch, 0.5 g of potassium iodide, and 100 ml of distilled water. The starch is dissolved in boiling water, permitted to cool, and mixed with the potassium iodide.

Solution II: 10% chemically pure sulfuric acid. Equal parts of solutions I and II are mixed before the reaction is carried out. The reagent is usable for 15 min.

Portions of 1 ml of the reagent are added to each of the tubes containing the inoculated nitrate broth. A dark blue color is obtained if the nitrate is reduced to nitrite, since metallic iodine is liberated from the potassium iodide and interacts with the starch.

Some bacteria completely decompose both nitrates and nitrites. In order to check this a pinch of powdered or granulated zinc is added to the test-tubes containing cultures which yielded negative reactions. The fluid should immediately turn blue. If the broth does not turn blue after the zinc is added all the nitrate and nitrite in the culture under investigation has been completely decomposed (M.A. Peshkov). This reaction is based on the fact that zinc liberates hydrogen from sulfuric acid. This hydrogen reduces the nitrate to nitrite, which then reacts with the potassium iodide. Griss's reagent is often used in place of the starch-iodine reagent.

Two solutions are prepared separately: 1. A total of 0.2 g of naphthylamine ($C_{10}H_7N_2$) and 20 ml of distilled water are heated in a porcelain dish and the resultant solution is carefully poured into 150 ml of 12% acetic acid. Violet drops of undissolved naphthylamine should remain in the dish.

2. A total of 0.5 g of sulfanilic acid ($C_6H_4NH_2 \cdot SO_3H$, sulfamidobenzoic acid) is dissolved in 150 ml of 12% acetic acid. The two solutions are mixed and stored in brown bottles with tightly-fitting stoppers. Griss' reagent should be colorless and it must consequently be prepared rapidly and in a carefully washed vessel. If it becomes reddish immediately after preparation or sometime later it cannot be used.

Media containing substances assimilated by only certain bacteria

Simmons' citrate agar. This agar is made up of 5 g of sodium chloride, 0.2 g of magnesium sulfate, 1 g of monobasic ammonium, 2.77 g of sodium citrate, 1 liter of distilled water, 20 g of agar-agar, and 10

ml of 1.5% alcoholic bromthymol blue; it is brought to pH 7.2 and sterilized for 15 min at 120°. Escherichia coli does not grow on this medium and does not change its olive color. Aerobacter aerogenes grows well, alkalizing the medium and causing it to turn a deep blue (when bromthymol blue is used as the indicator).

DRY NUTRITIVE MEDIA

N.V. Ploskirev, Candidate of Biological Sciences

Dry media are loose grayish or reddish powders and have a number of advantages over media prepared in the usual manner. They can be stored for up to 5 years in a cool, dry, dark place in hermetically sealed containers (glass jars with resin-sealed screw caps)* Dry preparations can be shipped, are convenient to use, and make it easy to obtain comparable results in bacteriological investigations.

Solid media for isolating bacteria consist of a nutritive base, agar-agar, indicators, and other organic and mineral compounds which accelerate the growth of some bacteria and retard the development of others.

Meat-infusion broth and tryptic hydrolyzates of beef, grain and leguminous crops (wheat, corn, soy beans, peas, etc.), technical protein products (whale and fish meal, casein, yeasts, etc.), noncommercial fish, and other waste products are used as the basis of media for raising bacteria of the coliform, typhoid, and dysentery groups. Because of the nonstandard character of these products it is difficult to use them to produce uniform nutritive media.

Synthetic nutritive media are more standardized. However, they often contain glucose and other carbohydrates, which reduce their differential properties. In addition, synthetic media containing amino acids, vitamins, and other organic compounds in definite proportions are economically infeasible. Protein products consequently continue to

be employed in the production of nutritive media for isolating and differentiating bacteria.

Tryptic hydrolyzates from fish and whale meal, noncommercial fish, and other wastes from commercial fish are used as the nutritive basis for the dry media produced in the USSR.

Dry nutritive broth is prepared by drying tryptic hydrolyzates of fish or whale meal or waste products from commercial fish. It contains approximately 3% amine nitrogen, 10% total nitrogen, 5-8% moisture, 30% ash, and 25% chlorides.

In order to prepare a nutritive broth 2-3 g of the dry preparation is added to 100 ml of distilled water and sterilized for 20 min at 120°. This liquid medium is suitable for culturing bacteria of the colon group and other microbes. After 24 hr of incubation at 37° cultures grown in tubes containing this broth produce approximately 2 billion Escherichia coli, 1 billion S. typhi, 0.5 billion Shig. dysenteriae, or 0.5 billion Sch. flexneri per ml of medium. In order to prepare a solid medium 1.5-2.5% of agar-agar is added to the broth.

Dry nutritive agar is produced in the USSR by the method developed by N.V. Ploskirev and A.N. Bitkova (1947). In this method agar-agar is impregnated with a concentrated nutritive base, dried, and powdered. A tryptic hydrolyzate of fish or whale meal is concentrated to a dry-residue content of 50% by evaporation and mixed with highest-quality frozen cake agar-agar in a ratio of 4:1. Autolyzates of sprats and fish entrails are also used for preparing dry nutritive agar (N.A. Likhvar', P.F. Chanpalov, S.F. Bubes, V.G. Migayev, et al., 1956).

Dry nutritive agar contains approximately 5% total nitrogen, 2% amine nitrogen, 4% moisture, 20% ash, and 18% chlorides and is a loose yellowish hydroscopic powder. In making up the medium 5 g of the dry preparation is added to 100 ml of distilled water and the mixture is

boiled until the agar melts, poured into test-tubes, and sterilized for 20 min at 120°. The solid medium has a gel strength of up to 500 g on the Valenti apparatus and a pH of 7.3-7.5. It is suitable for culturing coliform and other bacteria. Up to 3 billion S. typhi or 2 billion Schig. flexneri per ml of medium can be grown in test-tubes. Dry nutritive agar is also used as a base for preparing dry media for isolation and differentiation of coliform bacteria.

Dry media for isolating and differentiating bacteria are produced in the Soviet Union by the rather simple method proposed by N.V. Ploskirev (1942); it consists in thoroughly mixing powdered dry ingredients. The preliminarily tested powdered ingredients for a given medium are poured into a ball mill in definite quantities and thoroughly mixed to yield a uniform final powder.

Dry bismuth sulfite agar is used for isolating the causative agents of typhoid and paratyphoid from infectious material. The composition of this dry preparation is shown in Table 15a.

In preparing the solid medium 6 g of the dry preparation is added to 100 ml of distilled water and the mixture is heated until the agar dissolves, cooled to 50°, shaken in order to distribute the residue uniformly, and poured into Petri dishes. The latter are left open to permit the medium to solidify and then dried in a heater for 1 hr. This solid bismuth sulfite medium greatly retards the growth of Escherichia coli and other bacteria of the human intestinal microflora, which makes it possible to culture large quantities of material. When 100,000 Escherichia coli and 100 S. typhi in 0.05 ml of physiological solution are cultured on a dish and permitted to dry uniformly over the surface of the medium during a 24-hr period at 37°, isolated colorless colonies of E. coli and black colonies of S. typhi surrounded by shining silver aureoles are produced. The blackening is caused by the bismuth, which

TABLE 15a

Composition of Dry Media for Isolating and Differentiating Coliform Bacteria

1 Наименование сухого препарата	2 Содержится в сухом препарате, %																		
	3 сухого питательного агара	4 основного фуксина	5 эозина	6 метиленового синего	7 розоловой кислоты	8 водного голубого	9 бриллиантового зеленого	10 сульфита	11 фосфорнокислого натрия (двузамещенного)	12 кальцинированной соды	13 соли Мора	14 лактозы	15 сахарной пудры	16 лимоннокислого висмута	17 глюкозы	18 сахарозы	19 мальтозы	20 маннита	21 хлористого натрия
22 Сухой агар Эндо	76	0,9	—	—	—	—	—	2,6	1	0,5	—	19	—	—	—	—	—	—	
23 Сухой агар с эозином и метиленовым синим	65	—	0,75	0,15	—	—	—	—	1,3	0,2	—	14,5	18,1	—	—	—	—	—	
24 Сухой висмут-сульфит агар	73	—	—	—	—	—	0,04	8,4	6,2	0,5	1,5	—	—	3,7	6,7	—	—	—	
25 Сухой препарат с индикатором ВР и глюкозой	59,4	—	—	—	0,1	0,4	—	—	6	0,1	—	—	—	—	16	—	—	—	18
26 То же и лактозой	59,4	—	—	—	0,1	0,4	—	—	6	0,1	—	16	—	—	—	—	—	—	18
27 » » и сахарозой	55,4	—	—	—	0,1	0,4	—	—	6	0,1	—	—	—	—	—	22	—	—	16
28 » » и мальтозой	59,4	—	—	—	0,1	0,4	—	—	6	0,1	—	—	—	—	—	—	16	—	18
29 » » и маннитом	59,4	—	—	—	0,1	0,4	—	—	6	0,1	—	—	—	—	—	—	—	16	18

1) Dry preparation; 2) content in dry preparation, %; 3) dry nutritive agar; 4) basic fuchsin; 5) eosin; 6) methylene blue; 7) coralin; 8) analine blue; 9) brilliant green; 10) sulfite; 11) sodium phosphate (double-substituted); 12) roasted sodium carbonate; 13) Mohr's salt; 14) lactose; 15) powdered sugar; 16) bismuth citrate; 17) glucose; 18) sucrose; 19) maltose; 20) mannose; 21) sodium chloride; 22) dry Endo agar; 23) dry agar with eosin and methylene blue; 24) dry bismuth sulfite agar; 25) dry preparation containing VR indicator and glucose; 26) the same with lactose; 27) with sucrose; 28) with maltose; 29) with mannose.

is converted from bismuth citrate to bismuth sulfide under the action of the metabolic products of typhoid-paratyphoid bacteria. The growth of the latter is not retarded, but the development of the E. coli is suppressed. The brilliant green and sodium sulfite in the medium also severely suppress E. coli and other coliform bacteria.

Dry Engo agar is used for isolating coliform bacteria from water, foodstuffs, etc; its composition is shown in Table 15a. In order to prepare a solid medium 5 g of the powdered preparation is added to 100 ml of distilled water and the mixture is heated until the agar melts, boiled for 5 min, and poured into sterile Petri dishes. After the medium has solidified it is dried in a heater. Typhoid-paratyphoid and dysentery bacteria produce colorless colonies on this medium, while colonies of bacteria which form acid metabolic products are red in color.

Dry agar containing eosin and methylene blue is used for the same purposes as dry Endo agar. A solid medium is prepared from this dry agar in the same manner as from dry Endo agar. When the medium is dissolved the eosin and methylene blue form a complex compound, which precipitates at pH = 4.7 and stains colonies of acid-forming bacteria a dark color.

Endo agar and agar containing eosin and methylene blue do not suppress the growth of coliform bacteria cultured on them. They are consequently ineffective in isolating pathogenic bacteria from feces. Ploskirev's dry bacterial agar is more effective for this purpose.

Ploskirev's dry bacterial agar (1955) contains approximately 53.6% nutritive agar and bile salts, 11% hyposulfite, 0.03-0.06% neutral red, 0.002% brilliant green, 0.4% iodine, 12% lactose, 14.4% sodium bicitrate, 3.7% sodium hypophosphate, 3.7% sodium chloride, and 1.2% roasted sodium bicarbonate.

In order to obtain a solid medium 6 g of the dry preparation is added to 100 ml of distilled water and the mixture is heated until the agar dissolves and poured into Petri dishes. The dishes are left open for 1 hr to permit solidification and drying of the medium and are then covered with lids; the medium is now ready for culturing and has a pH of 7.2-6.8.

Despite the unsterile conditions under which the medium is prepared, atmospheric microorganisms will not grow on it during the first few days. When 0.05 ml of physiological solution containing 100 Shigella paradysenteriae Flexneri and 10,000 E. coli is cultured on a dish and incubated for 24 hr at 37° separate reddish colonies of E. coli and isolated clear, colorless colonies of Sh. paradysenteriae Flexneri are produced. Coliform bacteria are severely inhibited on this medium. Sh. paradysenteriae Grigoryevi-Shigae does not grow at all. However, all these bacteria begin to grow if the medium is alkalized to pH = 7.8-8.0.

Dry preparations containing VR indicator and carbohydrates (glucose, lactose, sucrose, maltose, and mannose) are used in semiliquid color series for differentiating coliform bacteria. Their composition is shown in Table 15a. In order to prepare a semiliquid medium 2 g of powdered preparation (one of the 5 shown in the table) is heated in 100 ml of distilled water until it dissolves, poured into test-tubes, and sterilized for 20 min at 120°.

Under the action of bacterial metabolic products the reddish color of a semiliquid medium containing VR indicator and a carbohydrate changes in a manner opposite to that of litmus, i.e., becomes blue when acid and red when alkaline. VR indicator is a mixture of analine blue and corallin.

Dry nutritive agar D (N.V. Ploskirev, N.S. Semcheva, O.S. Yemel'yanova, et al.) is used as the basis for media for culturing the caus-

ative agents of brucellosis and tularemia. The nutritive base of this preparation is a tryptic hydrolyzate of fish meal and brewer's yeast (D is the first letter of the word yeast in Russian). Dry nutritive agar D contains approximately 8% total nitrogen, 1.8% amine nitrogen, 4% moisture, 25% ash, and 15% chlorides.

In order to prepare a solid medium 5 g of the powdered agar is added to 100 ml of distilled water, the mixture is heated until the agar dissolves, the pH of the medium is adjusted if necessary, and the solution is filtered and sterilized for 30 min at 110°. When 1% of glucose and 2% of glycerine are added the medium can be used for isolating Brucella from the organs of animals.

Nutritive agar D is used for checking the viability of bacteria in dry live brucellosis vaccine. When 1% of glucose and 0.1% of cysteine are added it can be employed for culturing the causative agent of tularemia.

SAZh dry nutritive medium is used to detect the causative agent of gas gangrene (N.V. Ploskirev, O.A. Komkova, et al.). It consists of 72.5% dry sprat autolyzate or dry nutritive broth, 7.5% agar-agar, 7% gelatin, 3% glucose, and 10% sodium chloride.

In order to obtain the dry sprat autolyzate 1 kg of waste sprat is mixed with 1 liter of water and held at 50° for 7 hr. The mixture is then heated to the boiling point and the residue is filtered out. The filtrate is alkalized to pH = 8.0, heated to the boiling point, filtered, and dried in a water bath or diffusion drier. The yellowish powder thus obtained is the dry sprat autolyzate. It contains up to 11% total nitrogen, 3.4% amine nitrogen, 40% oxidized substances, 6% moisture, and 30% ash.

In order to prepare a semiliquid medium 5 g of the dry nutritive medium is added to 100 ml of distilled water and sterilized for 20 min

at 120°. This medium is suitable for rapid detection of the causative agent of gas gangrene by a new method. This technique is based on the development of specific changes when the bacterium is cultured in a semiliquid medium in the presence of specific antitoxic sera. Under these conditions the causative agent grows in isolated colonies and forms streptobacillary aggregations, in contrast to the diffuse growth of bacillary aggregations on the medium without serum.

SM dry medium is intended for rapid detection of Cl. perfringens. It is similar to Wilson and Blair's iron sulfite medium, but differs from it in the fact that it contains Mohr's salt instead of very hydroscopic ferric chloride and a different nutritive base (V.F. Runova and N.V. Ploskirev).

SM dry medium contains 79% dry nutritive broth or dry sprat autolyzate, 2% Mohr's salt, 7% anhydrous sodium sulfite, 9% sodium chloride, and 3% powdered agar-agar.

In order to prepare a semiliquid medium 5 g of the dry preparation is added to 100 ml of distilled water and sterilized for 20 min at 120°. This medium is suitable for detecting Cl. perfringens. The medium, heated to 37°, is inoculated with massive doses of the material under investigation. The test-tubes are placed in a heater at 37°; if Cl. perfringens is present the inoculation site turns black within 2-2 1/2 hr.

Dry semisynthetic whale-yeast agar containing charcoal (PKDAU) (N.V. Ploskirev and Ye.K. Matkovskaya). This medium contains peptone-free acid whale-meal hydrolyzate, yeast extract, mineral salts, starch, cysteine, and activated charcoal. The dry preparation is a loose black powder.

Dry PKDAU contains approximately 2.7% amine nitrogen, 3-5% moisture, and 20% chlorides. In order to prepare a solid medium 5.5 g of

powder is heated in 100 ml of distilled water until it dissolves and sterilized for 30 min at 110°. The solid medium has a gel strength of 500 g. It is suitable for isolating and culturing the causative agent of pertussis.

AKZh and KPD dry media (L.G. Ivanova, T.I. Sergeyeva, N.V. Ploskirev, and N.N. Sitnikova) are used for diagnosing food poisonings caused by Cl. botulinum types A, B, C, and E and Cl. perfringens types A, B, C, D, E, and F.

AKZh dry medium consists of sprat autolyzate, gelatin, and inorganic salts. In order to prepare the medium 5 g of the powder is mixed with water and heated until it dissolves and the pH of the solution is adjusted to 7.8; 70 ml portions are poured into bottles with cotton plugs, 3.5 ml of vaseline oil is added, and the bottles are sterilized for 30 min at 110°.

KPD dry medium consists of dried tryptic hydrolyzate of whale meal and liver, yeast extract, and mineral salts. In order to prepare the medium 7 g of the powder is mixed with 100 ml of distilled water and brought to the boiling point, the pH of the solution is adjusted to 7.8, 0.5 g of glucose is added, 70 ml portions are poured into stoppered bottles, 3.5 ml of vaseline oil is added, and the bottles are sterilized for 30 min at 110°.

A total of 1 ml of a culture of a given bacterial strain or fragments of an organ (liver, large or small intestine, etc.) from a human or animal corpse is added to the bottles containing the broth prepared from the AKZh or KPD dry medium. The cultures are incubated at 37° (28° for Cl. botulinum type E). The toxins are titrated in white mice after 18 hr for Cl. perfringens and after 5-6 days for Cl. botulinum. Macroscopic smears are made from the cultures at the same time and, if necessary, a pure culture is isolated.

Toxin formation is as follows: Cl. perfringens type A – 100, type B – 500, type C – 2000, type D – 1000, type E – 20, and type F – 300 mld per ml of culture fluid; Cl. botulinum type A – 100,000, type B – 100,000, type C – 10,000, and type E – 1000 mld per ml of culture fluid.

METHODS OF CONCENTRATING PATHOGENIC BACTERIA ISOLATED FROM THE ENVIRONMENT

Prof. K.I. Matveyev

Methods of Concentrating Bacteria Isolated from Water

Taking water samples. Proper taking of water samples for detection of pathogenic bacteria and their toxins is a very important aspect of investigations of bodies of water. This task should be entrusted to reliable, skilled persons, who should observe personal safety rules while taking the samples.

Water samples to be used for detecting pathogenic bacteria and their toxins must be substantially larger than those used for investigating the titre of coliform bacteria. Portions of 3-5 liters of water must be collected in sterile bottles with rubber stoppers; larger portions are sometimes collected when repeated investigations are intended. Before sterilization the vessels are closed with cotton plugs or tied-down paper covers; a carefully selected rubber stopper, wrapped in paper, is tied to each bottle. After the water sample has been taken the bottle is tightly sealed with the stopper.

For open bodies of water samples are taken at points of use (from the surface, from a depth of 10-15 cm, and from a depth of 0.5-1 m), 5-10 m from the shore, and roughly in the center of the body. For bodies of stagnant water samples must be taken from the bottom and from the adjoining water layer. For rivers samples are taken from the upper, middle, and lower reaches of the stretch assumed to be contaminated.

To take samples from a water-supply system water is drawn from a

tap. The latter is preliminarily flamed with an alcohol torch or blow-torch; the first sample is drawn, 30 liters of water are drained off, and the remaining bottles are filled, taking care not to splash the water. Weighted bottles or special devices are used for taking samples from deep wells.

After the sample has been taken the outside of the bottle is wiped off and the collector washes his hands with a disinfectant.

A label pasted on the bottle or a tag tied to its neck gives the time, date, sampling number, name and location of the body of water, suspected contaminant, and point of sampling. All these data are also recorded on an accompanying packing slip.

The samples must be quickly delivered for laboratory examination (within 30 min). When necessary they can be stored in a cold dark place, at a temperature no higher than 10°, but for no longer than 2-3 hr. In exceptional cases they can be kept on ice for up to 6 hr. The samples must be protected against freezing in winter and overheating in summer; this requires special containers, boxes, etc., which can be sealed when the samples are shipped.

Investigation of water. Despite the normally low concentration of pathogenic bacteria in water, samples must still be cultured directly on solid and liquid nutritive media. It is best to use blood agar containing glucose, semiliquid agar, and other media, the selection depending on the pathogenic bacterium suspected to be present. Positive results are obtained only after massive inoculation. Direct cultures on nutritive media exhibit vigorous growth of the saprophytic aqueous microflora, which suppresses the development of pathogenic bacteria. Some authors consequently base their investigations on culturing in media to which various inhibitors which retard the growth of the saprophytic microflora have been added. All these methods are based on the one used

for coliform bacteria (Ficker-Hoffman's medium containing caffeine and crystal violet, Shantemess, Vensan, and Paet's medium containing phenol in various concentrations, Lamcke's medium containing malachite green and hydrochloric acid, the medium containing gentian violet and sodium sulfite used for the causative agent of plague, etc.). These substances, which retard the growth of the saprophytic microflora, are not indifferent for pathogenic microorganisms, whose growth they suppress to some extent. This furnished the impetus for seeking methods of concentrating pathogenic bacteria found in water.

One of the methods proposed for concentrating pathogenic bacteria from large volumes of water is coagulation: the water to be studied is mixed with coagulants, chemicals which clump and precipitate when they enter into a chemical reaction, extracting the pathogenic bacteria from the water. The precipitate is then removed from the water and cultured on nutritive media. Various chemicals have been suggested as coagulants, including ferric sulfate, ferrous chloride, lead nitrate and hyposulfite, potash alum, kaolin, etc.

Precipitation with specific agglutinative serum. Ye. A. Sheipilevskiy proposed precipitation with specific agglutinative serum for isolation of pathogenic bacteria from water. Water contaminated with pathogenic microorganisms is added to meat-infusion broth, in a ratio of one part of broth to five parts of water. When a bacterial growth is present after one day the culture is filtered through a layer of sterile cotton. Agglutinative serum is added to 100-200 ml of the filtrate in a quantity such that its final dilution is 1:1000-1:1500. The mixture is shaken and placed in a heater for 2 hr. When agglutination takes place the bacteria settle to the bottom. The upper portion of the fluid is poured off or aspirated. The residue thus obtained is diluted in a small volume of physiological solution and cultured on solid and

liquid nutritive media. This method was proposed for isolating the causative agents of typhoid, cholera, and dysentery and other pathogenic bacteria from water, air, and feces.

Physical methods of concentrating bacteria. Pathogenic bacteria and other microorganisms can be most effectively concentrated during isolation by filtering the water through various filters.

At the end of the past and the beginning of the present century Hesse used Berkefeld filters covered with diatomaceous earth and Shantemess used Chamberland porcelain filters for concentrating bacteria. Filtration through porcelain made it possible to concentrate bacteria on a small surface, but this technique never came into practical use, since the adsorptive properties of the filters themselves made it impossible to remove the trapped bacteria from the filters with a backflow of water or by scraping off the residue.

Since 1932 membrane filters have been used in the bacteriological investigation of water for concentrating bacteria from different sample volumes.

A.S. Razumov and then Ye.V. Dianova and A.A. Voroshilova suggested that membrane filters be used to determine the bacteria count; at the same time, K.K. Barsov proposed that the coliform index be determined with membrane filters. This method has since been used for concentrating many pathogenic microorganisms and isolating them from water.

Membrane filters are porous nitrocellulose plates, which are permeable to water and have the form of flat disks 35 mm in diameter. Membrane filters for bacteriological research are produced by the Mytishchi Experimental Ultrafilter Plant. There are 5 filter grades, which indicate different pore diameters and water-filtration rates. Filter No. 1 has a mean pore diameter of 0.35 μ and a water-filtration rate of 500 ml in 9 min. Filter No. 3 has a mean pore diameter of 0.70 μ and

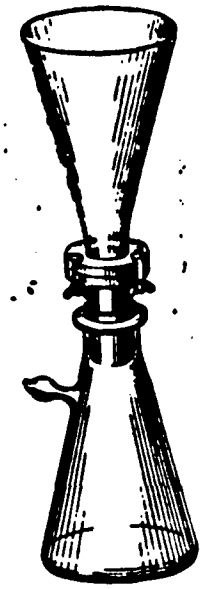


Fig. 15. Rublevskiy tap-water station

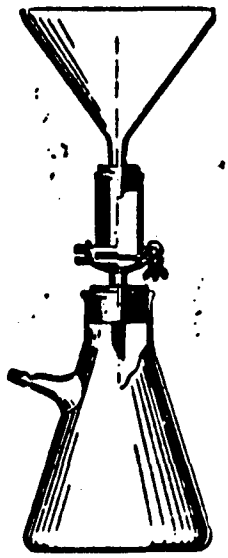


Fig. 16. Zeitz apparatus with funnel.

a water-filtration rate of 500 ml in 2 1/2 min, while filter No. 5 has a pore size of 1.20 μ and a filtration time of 35 sec for 500 ml of water. As a result of its large pore size, the latter filter retains bacteria poorly.

Membrane filters Nos. 1, 2, and 3 are most suitable for checking water for pathogenic bacteria.

In addition to these filters, there are also so-called preliminary or Plankton filters, which are intended to trap large suspended particles. Such filters are completely permeable to bacteria.

The dull side of the filter is termed the air side and should face upward during filtration. It is occasionally difficult to distinguish the dull and shiny sides of a wet filter and a dot should consequently be made at the very edge of the dull side with a lead pencil before sterilization.

Membrane filters withstand prolonged storage (a year or more)

without any change in their filtration properties.

A Rublevskiy tap-water station or a Zeitz device (Figs. 15 and 16)* is most frequently used for filtering the water under investigation. The vacuum necessary for filtration can be set up with a Komovskiy vacuum pump, an oil vacuum pump, or a water-jet pump.

The working sections of a filtration apparatus can be sterilized by boiling or by rubbing them with alcohol and then flaming them.

Membrane filters are sterilized by boiling for 30 min in distilled water to which 4-5 drops of formalin has been added. After 15 min the water is poured off and replaced with fresh distilled water without formalin, the filter then being boiled twice for 15 min.

After sterilization the filter is left in the same water until needed. Membrane filters can also be sterilized by boiling without formalin.

When the apparatus has cooled the sterile filter is placed on the screen inside the device. Membrane filters may tear when a high vacuum is created in the receiving vessel. In order to avoid this a sterilized piece of filter paper is placed on the screen and the membrane filter is laid on it. The filter is then adjusted and the upper portion of the device is set in place; the upper portion is filled with the water under investigation and a vacuum is created in the receiving vessel. When filtration has been completed the upper part of the apparatus is removed and the membrane filter is transferred with sterile forceps to the surface of a solid nutritive medium to permit culturing of the trapped microorganisms (which can also be cultured in liquid media) or to a sterile Petri dish to permit the bacteria to be washed from it and preliminarily identified.

As a result of their high retentive power, membrane filters have become indispensable for various bacteriological investigations of wa-

ter. Membrane filters are used primarily in sanitary-bacteriological investigations of various types of water, for determining the coliform titre and coliform index, and secondarily for investigating water suspected (on the basis of epidemiological indications) of containing various pathogenic agents (bacteria, fungi, Leptospirae, etc.).

The membrane-filter method is recommended primarily for investigating water suspected of containing the causative agents of typhoid, paratyphoid, dysentery, anthrax, plague, cholera, brucellosis, tularemia, glanders, melioidosis, and other infections.

The bacteria are washed from the membrane filter with a small quantity of physiological solution and examined to get an idea of the species to which they belong. This can be done by the serum-luminescence method, using highly specific luminescent sera, by the microagglutination reaction on slides, using phase-contrast microscopy, by the precipitation reaction with hapten, by Gram staining, etc. The material washed from the filter is also cultured in Petri dishes with selected nutritive media and, if necessary, animals are inoculated with it.

Detection of sporagenous bacteria in water by direct microscopy of specimens prepared from material washed from a membrane filter after bacterial concentration (T.I. Sergeyeva and N.B. Chernetskaya). In this technique the water is filtered through a No. 3 membrane filter in a Zeitz apparatus. After filtration the filter is transferred to a sterile Petri dish with a sterile pair of forceps and the bacteria on its surface are washed off with 0.5 ml of physiological solution, using a glass spatula made from a Pasteur pipette. All the washings are picked up from the Petri dish with a Pasteur capillary pipette and placed on a slide. After the specimen has dried it is flame-fixed and stained by Gram's method. This technique makes it possible to detect 50,000 sporagenous aerobic of anaerobic bacteria in 250 ml of water.

Some authors (Richards and Krabek) recommend that the bacteria be examined under a phast-contrast microscope while still on the filter, in order to determine their morphological characteristics. For this purpose the air-dried filter is placed on a slide, flooded with immersion oil (which makes it transparent), covered with a cover glass, and examined microscopically.

Biological methods of concentrating bacteria. It is possible that no pathogenic bacteria will be detected by the methods described above after large quantities of water have been filtered through membrane filters. Biological concentration methods are consequently used to isolate the bacteria from the water in subsequent investigations.

The most commonly employed technique involves culturing for 3-8 hr or more on the filter, which is placed on a selected solid nutritive medium or immersed in a liquid medium. The filter should be laid carefully on a solid medium, taking care that no air bubbles are formed between it and the surface of the agar. However, the growth of pathogenic microorganisms is retarded when more than 2500 saprophytic colonies are present, so that in investigating impure water culturing is best carried out at a temperature of 43°, to retard saprophyte growth; in addition, bacteria are transferred from the surface of the filter to one or more Petri dishes, the number being determined by the extent of the suspected contamination. The nutritive medium should not be dried before culturing and the medium layer should be thicker than usual, to prevent rapid drying. This ensures more satisfactory diffusion of nutritive substances from the medium to the bacteria through the filter.

When the material on the filters is cultured at 43° on bismuth sulfite agar this method makes it possible to detect the causative agents of typhoid and paratyphoid in artesian-well and tap water in quantities of 4 cells per liter of water. Under the same culturing con-

ditions, these bacteria can be detected in quantities of 20 cells per liter of water from dug wells and 100 cells per liter of river water (V.S. Rossovskaya).

A biological concentration method involving culturing of the filter-trapped bacteria on Levin's medium for 18 hr at 37° can be used instead of filtration to detect small quantities of pathogenic bacteria in water samples (N.N. Sitnikova). After 18 hr the material is washed from the filter with 3 ml of 1% acetic acid. In order to prepare a hapten the washings are boiled for 40-60 min, filtered through an asbestos pad, and alkalized until a neutral or slightly alkaline reaction is shown by bromthymol blue. A precipitation reaction is set up with the hapten, overlayering it on specific precipitative serum.

The precipitation reaction is positive in 100% of all cases involving 100 ml of water containing 10, 100, or 500 Bacterium typhosum. A positive precipitation reaction is obtained for 91-98% of all 250 ml water samples containing 10-100 bacteria. Water-suspended Escherichia coli from typhoid patients does not give a positive reaction. The precipitation reaction is positive with haptens from bacteria having antigens in common with Bacterium typhosum, i.e., S.enteritidis and S. typhi murium. The precipitation reaction is negative with the haptens of other coliform and typhoid bacteria.

Examination of a large number of water samples from the Moscow River has shown that Escherichia coli and other bacteria always found in open waterways do not give nonspecific precipitation reactions with typhoid serum after filtration and culturing on the filter.

Culturing for 6 hr on solid nutritive media, in semiliquid 0.75% agar (for Bac. anthracis and Bac. anthracoides), or in semiliquid alkaline agar (V. Metschnicovii) makes it possible to detect 100,000-200,000 V. Metschnicovii or 5000 Bac. anthracoides or Bac. anthracis in

250 ml of water (T.I. Sergeyeva and N.B. Chernetskaya). Sporagenous aerobic bacteria can be cultured in test-tubes containing Hottinger's broth and chunks of meat. The membrane filter is placed in the test-tube containing the nutritive medium and covered with vaseline oil. Sporagenous anaerobes can be detected after 6 hr in samples initially containing 2500 cells in 250 ml of water.

In addition to culturing on a filter placed on a nutritive medium, all investigations of water for pathogenic bacteria must include inoculation of infection-sensitive animals in order to permit the bacteria to accumulate. Several animals are injected with material washed from the filter. The species of animal and the administration method depend on the suspected contaminant. Inoculation of animals with material washed from filters is obligatory in investigating water for the causative agents of plague, tularemia, anthrax, brucellosis, glanders, melioidosis, etc. In some cases pathogenic bacteria can be detected in water only by this method.

If it is not possible to inoculate animals with material washed from filters they are injected with the unfiltered water. Several animals are usually inoculated, so that they can be killed at different intervals and dissected to permit detection of bacteria in their organs (see the chapter entitled "Use of Laboratory Animals for Indicating and diagnosing infectious diseases").

Methods of Concentrating Bacteria Isolated from Air

Precipitation of bacterial aerosols by the percussive action of an air jet

Rough indication with the aid of impact devices. Devices have now been developed which permit rapid detection of the appearance of large quantities of bacteria or dust particles in the air. They include impact devices of various designs. An apparatus of this type makes it

possible to deposit the aerosol particles mechanically on metal plates or slides and to examine them microscopically. In order to hold the bacteria and aerosol particles, the slides used in the apparatus are coated with a very thin layer of castor oil, resin, or other substances. The air is forced through the device with any feasible pump.

The bacteria deposited on the slide can be stained by various methods before microscopy, which makes it possible to determine their morphological and tinctorial characteristics. In addition, slides bearing deposited microorganisms can be treated with luminescent sera containing antibodies to individual bacterial species, which permits rapid determination of the species to which air-borne bacteria belong. This is especially important for the causative agents of particularly dangerous infections.

Bacteria deposited on plates or slides can be washed off with physiological solution and used for culturing on selective media or for animal inoculation. In addition, slides can be covered with agar and the deposited bacteria cultured on them in a moist atmosphere.

In order to conduct investigations with impact devices the laboratory should have a set of special dyes for simple or complex staining of the causative agents of especially dangerous infections and other microorganisms. It is also necessary to have a set of high-specificity luminescent sera for microorganisms responsible for especially dangerous and other infections, as well as selective media and animals for further investigation of the bacteria deposited in the device.

The first single-stage impact device was proposed by Heigemann (1936). May (1945) devised a four-stage cascade impact apparatus for collecting and determining the size of aerosol particles containing droplets more than 1 μ in diameter. This apparatus subsequently served as the basis for development of impact devices for studying bacterial

aerosols. It consists of 4 rectangular apertures, one behind the other. Opposite each aperture is a collector plate or slide. The apertures gradually decrease in size; when air-borne particles or bacteria enter the device they are accelerated as they pass through each aperture in turn. Larger particles adhere to the second and third slides and very fine particles to the last slide.

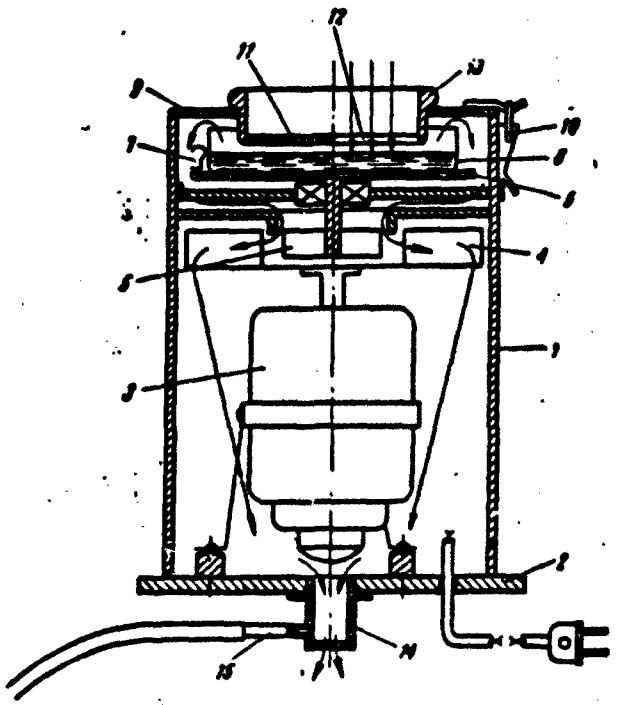


Fig. 17. Krotov's apparatus. 1) Cylindrical housing; 2) base; 3) electric motor; 4) central fan; 5) eight-bladed fan; 6) disk; 7) springs; 8) Petri dish; 9) lid; 10) catch; 11) plexiglass disks; 12) wedge-shaped slit; 13) split ring; 14) connecting pipe with diaphragm; 15) outlet tube.

Four- and five-stage impact devices with narrower slits were later designed for removing particles 0.2-1 μ in diameter from dry aerosols. These devices operate at the same air-flow rate as the May apparatus, approximately 17.5 liters per min. The four-stage cascade apparatus is convenient and can be used for bacteriological investigation of air under field conditions.

Investigation of bacterial aerosols with the cascade impact device has shown that it removes up to 90% of the bacteria in the air. It is quite suitable for rough determination of the concentration and distribution of bacteria in different volumes of air (Sonkin, 1950, and other authors).

Bacterial aerosols in the dust and droplet phases can be collected with an impact device. The number and size of the bacteria collected depend to a considerable extent on the velocity of the through-flowing air. The best results are obtained if the air flows at 18-25 liters per min. Devices of this type are suitable for investigating air at low temperatures. A rough result can be obtained 40-50 min after air samples are taken.

It has been suggested that the biuret reaction be used for nonspecific determination of the number of bacteria in air (Strickland, 1951). The bacteria in the air are concentrated with an impact device or membrane filters or by some other method, a suspension of given quantities of bacteria is prepared in distilled water or physiological solution, and sodium hydroxide and copper sulfate are added. The excess copper hydroxide is removed by centrifuging until a clear solution is obtained. The intensity of the purple hue of the solution is directly proportional to the number of bacteria which it contains and is determined with a photoelectric absorption meter. According to Strickland's data, this method has proved to be the most suitable for determining small quantities of bacteria in air.

Krotov's apparatus (Fig. 17) consists of a cylinder covered with a removable lid. Beneath the lid is a stage bearing a Petri dish fitted with attachments to hold it in place and containing agar or some other solid medium. Air is taken into the device through a wedge-shaped slit in the lid, using an electric motor (127 and 200 v). The motor also

turns the stage bearing the Petri dish. At the bottom of the cylinder is an aperture through which the air passes into the outlet tube, which is connected to a fluid manometer that shows the quantity of air passing through the apparatus per min. Before conducting the investigation Petri dishes 10 cm in diameter must be filled with selective nutritive media for the causative agents of especially dangerous and other infections. Use of selective media for different bacteria is obligatory, forming the basis for rapid culturing. The medium must be poured so as to produce a horizontal surface and permitted to dry.

Before samples are taken the motor is plugged in and the air-flow rate is adjusted to 25 liters per min with the rheostat. The motor is then shut off, the lid is removed, and an open Petri dish is fastened to the stage with the springs; the lid is then closed and locked and the motor is plugged in again.

The microorganisms which enter the apparatus with the air are distributed uniformly over the Petri dish. After culturing in a heater for 10-15 hr or, in some cases, less, smears can be prepared from the bacterial colonies and stained by various methods, including the luminescent-serum technique. Bacteria from mature colonies can be used for the microagglutination reaction on slides, for preparation of haptens for the precipitation reaction, and for animal inoculation. Further identification of the bacteria depends on the preliminary data obtained.

Determination of bacterial contamination of air with membrane filters

(Temporary methodological instructions for determination of the dust content and bacterialological contamination of atmospheric air in cities. Ministry of Public Health USSR, 1955)

A Zeitz-type filter device and membrane filters Nos. 2, 3 and 4 are used for analyzing atmospheric microflora.

Preparation of membrane filters and apparatus. The membrane filters are checked for defects (cracks, holes, etc.). The filters selected are

placed in distilled water, heated to 50-60° (in order to avoid warping) boiled for 5 min, and then, changing the water, boiled twice for 15 min. The filters are removed with a flamed forceps and 3 or 4 are placed in a sterile Petri dish to dry, interlayering them with three layers of sterile filter paper. Circular pieces of filter paper are cut to conform to the size of the dish, with 4 small slits on their four sides (to permit more convenient removal of the filters) and sterilized beforehand with dry heat in Petri dishes.

The dishes containing the filters are placed in a heater and held at 43-45° for 24 hr or 37° for 48 hr to permit complete drying. The filters must be completely dry (their reticular structure should not be visible), since the air will not otherwise pass through them. The sterile filters can be prepared beforehand, maintaining conditions to prevent contamination during storage. Before samples are taken the filter device is sterilized in an alcohol- or Bunsen-burner flame. Care must be taken that the water which condenses on the surface of the apparatus (the upper cylinder and funnel) is completely dried, since the membrane filter will otherwise adhere to the surface of the device after air is aspirated and be difficult to remove. The sterilized and dried cylinder is covered with a sterile tumbler or glass stopper (from a bottle). The membrane filter is placed on the sterile, dried surface of the stage with sterile forceps and fastened in place. The cylinder should fit tightly around the funnel, so that the joint is hermetic.

Investigative technique. In order to draw air through the membrane filter a rubber tube is attached to the fitting on the stage and connected to a blower through a rheometer. The outlet for drawing off air is hermetically sealed with a rubber tube fitted around a glass rod. A blower of the type designed by the Petroleum Institute imeni Gubkin, equipped with an electric motor, can be used for drawing air through the

device. A water aspirator can be employed if electric power is not available, setting up a difference in level of 2.5 m. The apparatus is mounted in a stand in an inclined position, its open end facing into the air flow; the glass stopper or tumbler is removed from the cylinder and the motor is switched on. Air is drawn through at a rate of 10 liters per min. A total of 100-200 liters of air is drawn through each membrane filter at the highest elevation, while 50-75 liters is drawn through 1.5 m above the ground. The quantity of air investigated can be varied in accordance with its bacterial contamination. When filtration is finished the membrane filter is placed on the surface of a solid nutritive medium with sterile forceps.

In taking samples of anaerobic bacteria the membrane filter is placed culture down on a ferrous sulfite medium (see page 973) and flooded with 10-15 ml of ordinary meat-infusion or nutritive agar. It is necessary to keep air bubbles from forming between the membrane filter and the surface of the nutritive medium. A total of four membrane filters can be placed on each dish containing nutritive medium. The cultures are incubated and examined in the same manner as in Krotov's method for determining the bacterial contamination of air.

Example of calculation. Air is passed through a membrane filter for 5 min at a rate of 10 liters per min, for a total of 50 liters. The membrane filter is placed on a nutritive medium and 240 bacterial colonies grow.

The number of bacteria per m^3 of air equals $240 \cdot 1000/50 = 4800$.

In investigating air under special conditions the filters to be used for culturing are placed on solid selective nutritive media developed for the bacteria responsible for especially dangerous infections. The material is also washed from the filters with a small quantity of physiological solution and used to inoculate animals sensitive to es-

pecially dangerous infections.

Filters of nitrocellulose and other materials have been suggested for investigating air. The data obtained in evaluating their suitability for this purpose are contradictory. Some authors believe them to be very suitable for investigating air, while others hold that they yield unsatisfactory results.

Determination of bacterial contamination of air with soluble filters. The idea of using soluble filters is very attractive. Attempts have been made to pass air through sodium chloride, sugar, sodium biphosphate, and magnesium sulfate. The adsorbants were then dissolved and cultured on a solid nutritive medium. It was, however, found that many adsorbants suppress bacterial growth. Mitchell, Timmons and Dorris (1950) developed a method for preparing water-soluble filters from a dry gelatin froth. A total of 40 g of gelatin and 4 ml of glycerine are added to 100 liters of distilled water. The mixture is held in a water bath at 52° until the gelatin dissolves and shaken to obtain a uniform gelatin solution. It is best to use a mixture to stir the suspension. The solution is poured into rectangular wax-paper molds and placed in a vacuum desiccator containing calcium chloride. The air is evacuated from the desiccator until all the air bubbles have disappeared and a layer of froth is formed. Vacuum drying is continued for 3-4 days. The dry gelatin froth is porous and has a high tensile strength. The size of the pores can be regulated by varying the thickness of the gelatin layer, a thin layer having finer pores. Filters of the requisite size are cut from the blocks of gelatin, using a special template and a razor blade or knife. They are placed in sterile paper envelopes and sterilized with carboxide, which is a mixture of 10% ethylene oxide and 90% carbon dioxide by volume. Since the solubility of the filters is substantially reduced when the ethylene oxide is sterilized, some authors feel that unsterile filters can be

used for collecting pathogenic bacteria, neglecting the small number of saprophytes which may be retained during their preparation.

In order to aspirate air the gelatin filters are placed in a Zeitz apparatus or in the special device designed by A.Ye. Vershigora (1957), which is a small metal funnel in which the filter is fixed with a metal washer. After aspiration the filters are placed in sterile envelopes of cellophane or some other heavy material and forwarded to the laboratory for examination. At the laboratiry the filters are dissolved in 100 ml of physiological solution containing glass beads, heating them in a water bath at 37° for 30 min and periodically shaking them.

A.Ye. Vershigora (1959) somewhat modified Mitchell's method. The requisite number of gelatin filters is obtained by the following method: 50 g of nutritive gelatin, 140 ml of distilled water, and 9 ml of glycerin are poured into a flask with a capacity of 500-700 ml. After the gelatin has swollen it is dissolved by placing the flask in a water bath at 60-65° for 15-20 min. The mixture is then shaken to produce a uniform mass. The gelatin solution is poured into paraffin-covered boxed with the necessary diameter and 3 cm high. The boxes are placed in a vacuum desiccator containing 1.5 kg of crystalline calcium chloride. The air is evacuated from the desiccator with a vacuum pump, so that the gelatin mixture becomes a froth. This froth is vacuum-dried in the desiccator for 3 days. The dry froth becomes spongy and filters of the necessary size can be cut from it with a scalpel. Gelatin filters are equal in bacterial collecting capacity to the D'yakonov apparatus. They can be used for investigating the atmospheric microflora at low temperatures, where liquid media or media containing agar freeze and become unusable.

In working with gelatin filters it is necessary to avoid prolonged heating at elevated temperatures when dissolving them, since this sharply reduces the number of vegetative bacteria in the filter. In investi-

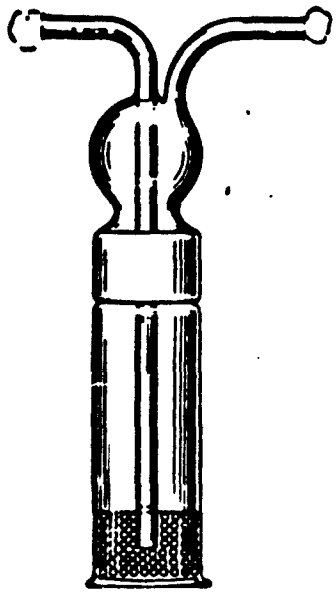


Fig. 18. D'yakonov apparatus.

gating air for pathogenic bacteria gelatin filters are used for culturing on selective nutritive media and for inoculating sensitive animals.

Sodium alginate, a fibrous material similar to cotton, can be employed for soluble filters. Experiments have shown that it retains all microorganisms when air is aspirated through it. It is highly soluble in water and withstands sterilization at 125°. Some authors recommend sodium gluconate for soluble filters.

Determination of microbiological contamination of air with liquid filters. Various liquids retain microorganisms when air is passed through them. The majority of the numerous devices which have been proposed for investigating air by this method are modifications of the well-known D'yakonov apparatus and differ from one another in the shape of the flask containing the collecting liquid and the size and shape of the outlets for the tubes through which the air is drawn. One advantage of such devices is the fact that they have a greater bacterial collecting capacity than devices using membrane or soluble filters. In addition, liquids retain viruses and Rickettsiae when air is aspirated through them.

The United States Public Health Service recommends a 12-flask air-sampling apparatus with a liquid bacteria collector and suggests that Civil Defense units be equipped with these devices for taking air samples. The apparatus permits continuous sampling over a period of 4 hr. Other air-sampling methods have also been proposed.

One drawback of devices employing liquids is the fact that they cannot be used at very low temperatures; in addition, when the air is

passed through the liquid in the form of very large bubbles only those bacteria at the surface of the bubbles are adsorbed.

P.P. D'yakonov's apparatus (Fig. 18). This apparatus consists of a glass vessel with two tubes - one for the air stream, which reaches almost to the bottom of the vessel, and the other for attachment of the pump. The tubes are plugged with sterile cotton when the device is in use. The bottom of the vessel is filled with glass beads and the collecting liquid (broth or physiological solution) is poured in. The apparatus is then sterilized. After air has been drawn through the device the liquid is poured and investigated by various bacteriological methods, making smears and staining them by simple and complex methods, including the luminescent-serum method, making cultures on selective media, and inoculating animals.

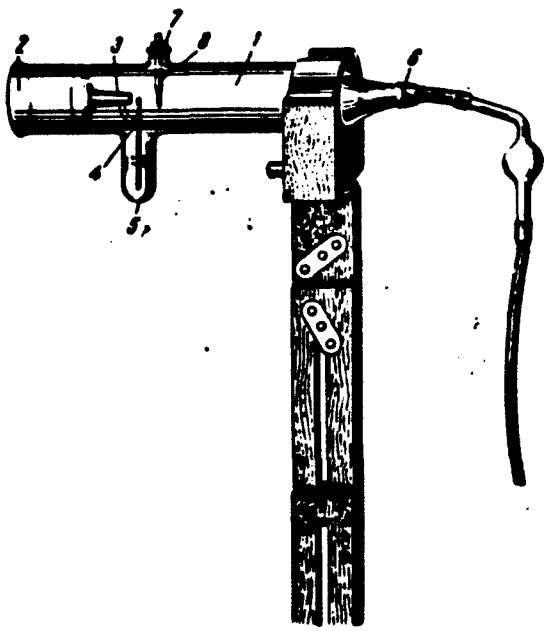


Fig. 19. Rechmenskiy apparatus.

S.S. Rechmenskiy's apparatus (Fig. 19). This apparatus is based on repeated atomization of a liquid, which traps microorganisms when air is

passed through the device. Researchers who have worked with the apparatus feel that it has good collecting properties. It consists of a glass cylinder (1, 2) containing a funnel which terminates in a capillary (3). The latter is approached at a right angle by a second capillary (4), which leads to a reservoir containing 3-3.5 ml of the collecting liquid (5). The capillaries form an atomizer. The upper wall of the cylinder has an aperture (7) into which a blade (8) is fitted to increase the dispersion of the liquid and the precipitation of bacteria when air is passed through the device. At the end of the cylinder is an aperture (6) for attachment of a rubber tube and rheometer, in order to determine the quantity of air pumped through the apparatus. After a test has been run the liquid is poured out through aperture No. 7 and used for various bacteriological and virological investigations (smears, cultures, inoculation of animals, etc.).

V.S. Kiktenko's apparatus (Fig. 20). This apparatus is very simple and can be set up in any laboratory. It requires a 250-ml bottle 14 cm deep and 6.5 cm in diameter, with a side-arm; a glass U-tube approximately 25 cm long and 1.5 cm thick is attached to the mouth of the bottle. The bottle us turned bottom up and a rubber tube 30-40 cm long is attached to the side-arm, which is located near the bottom. The U-tube and the lower portion of the bottle are filled with 225-230 g of glass beads (approximately 725-730 beads) 6 mm in diameter. The beads must be manufactured from neutral glass. A total of 40 ml of physiological solution or broth is then poured into the apparatus. When the experimenter inhales through the tube near the bottom air is aspirated from the bottle and new air enters it from the atmosphere. Any bacterial aerosols present in this air are collected when it passes through the liquid and beads. An intermittent pump or human breathing can be used to operate the device. In taking samples of contaminated air the apparatus is con-

nected to the respiratory valve of a gas-mask.

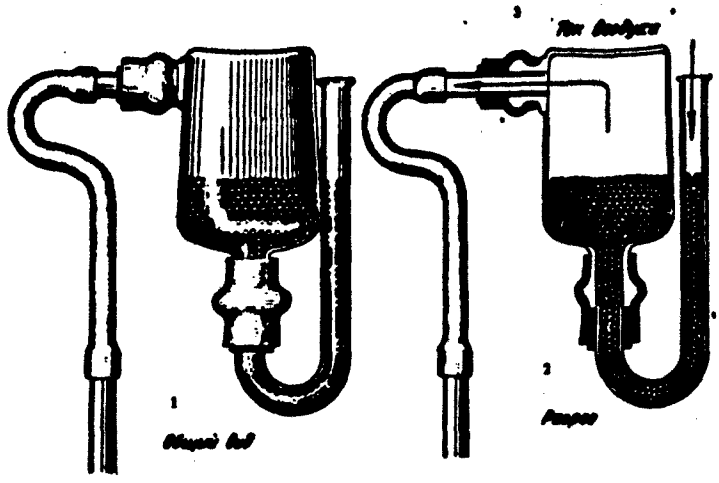


Fig. 20. Kiktenko apparatus. 1) General view; 2) cross-section; 3) air flow.

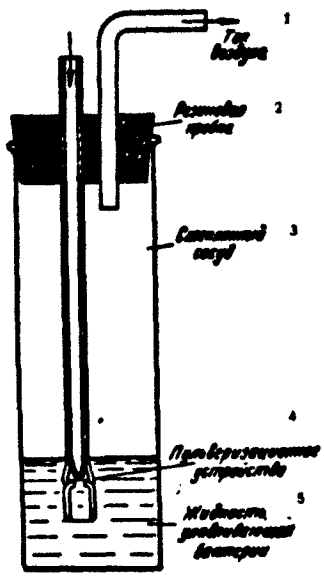


Fig. 21. Rudenko apparatus. 1) Air flow; 2) rubber stopper; 3) glass vessel; 4) atomizer; 5) bacteria-collecting liquid.

After the air samples have been taken 30 ml of the liquid is poured into a sterile test-tube or flask, using a sterile pipette to hold back the beads. The liquid is used for various bacteriological and

virological investigations.

N.M. Rudenko's apparatus (Fig. 21). In 1956 N.M. Rudenko proposed a very simple original device for collecting virasols, based on the atomization principle. His apparatus consists of a glass vessel sealed with a rubber stopper. A tube bearing an atomizer and a tube for the air outflow pass through holes in the stopper. In order to collect bacteria, viruses, and Rickettsiae physiological solution or broth is poured into the bottom of the vessel and then used for microbiological investigations and animal inoculation. When in use the device is connected through rubber tubing to a rheometer and then to a pump.

A liquid prepared by mixing 70 ml of physiological solution and 30 ml of neutral glycerine can be used for collecting microorganisms at low temperatures with the D'yakonov, Rechmenskiy, Kiktenko, and Rudenko devices. This mixture remains liquid down to -18°.

A comparative evaluation of methods for bacteriological investigation of air from enclosed areas has shown that, if the results obtained with the Krotov apparatus are taken as 100, the Rechmenskiy device yields a figure of 139%, membrane filters a figure of 82.1%, and the D'yakonov device a figure of 59.9% (V.V. Vlodavets, 1957). The Krotov and Rechmenskiy devices are consequently the most advanced and efficient devices for bacteriological investigation to determine the dust-phase bacterial content of air from enclosed areas, other methods and devices being less effective. The Rechmenskiy collector can be used to detect pathogenic bacteria, viruses, and Rickettsiae in air. The collecting efficiency of various devices is almost identical with respect to coarsely dispersed bacterial aerosols, but varies from device to device in the following manner for finely dispersed aerosols (A.Ya. Vershigora, 1959): Rechmenskiy syphon collector - 150%, bubbler collector - 121%, Krotov apparatus - 107%, D'yakonov apparatus - 100%, gelatin filters - 95%,

and dish method - 20%.

The following figures have been obtained for collection of bacteria from finely dispersed aerosols: Rechmenskiy syphon collector - 150%, Krotov apparatus - 130%, D'yakonov apparatus - 100%, gelatin filters - 93%, bubbler collector - 72%, and dish method - 18%.

These data, which describe the devices and their comparative efficiency, indicate that several of them must be used simultaneously for collecting bacterial aerosols. Some of them are only suitable for detecting pathogenic bacteria, while others can be used for detecting bacteria, viruses, and Rickettsiae in air. Only thorough investigation of air, involving the use of several devices, culturing on selective media, animal inoculation, and identification of the pathogenic bacteria found, yields satisfactory results.

Use of a well-designed impact device is extremely important in rough identification. Luminescent sera should be used to stain the precipitated aerosol on slides in the impact device. The material washed from the slides must be cultured on nutritive media and employed for animal inoculation. Bacterial aerosols must be simultaneously cultured on solid and liquid selective media, using the Krotov apparatus and membrane and gelatin filters.

The D'yakonov and Rechmenskiy or D'yakonov and Rudenko devices should be used in parallel for investigating aerosols, employing a liquid which collects bacteria, viruses, and Rickettsiae. As soon as the air samples have been taken the liquid from the devices is cultured on selective media and used to inoculate animals. Smears are also prepared and stained with luminescent sera.

Taking Soil Samples and Samples from the Surface of Objects

In taking soil samples for detection of bacterial contaminants special attention is paid to those areas which exhibit signs indicating con-

tamination. A sanitary-epidemiological survey should be conducted to indicate the areas from which samples should be taken.

The soil samples are taken in previously prepared sterile bacterial test-tubes, which should be sealed with tightly fitting rubber stoppers. The test-tube is opened, its upper end is used to loosen the surface layer of the soil to a depth of no more than 0.3 cm, the entire tube is filled with earth, taking 25-30 g, and it is then tightly restoppered.

Soil samples are taken every 30-50 m along a given street or field and more frequently in areas suspected of being contaminated.

Samples taken from a given locality are numbered and wrapped in heavy paper, which is labeled with the address (city, neighborhood, street, house number, etc.) and date (year, month, day, and hour). The paper-wrapped test-tubes are packed in tightly closed boxes, which are also labeled with the address where the samples were taken. The packing slip which accompanies the soil to the laboratory indicates the number of samples, the sampling site, and the date.

Individual vegetation samples can also be sent to a microbiological laboratory for examination; these are cut with sterile scissors and placed in sterile jars or large test-tubes or wrapped in sterile heavy paper or cellophane. The packing slip shows the sampling site and the date.

It is also necessary to take samples from various surfaces with cotton pads wetted in physiological solution. The pads are prepared beforehand, moistened, and placed in sealed sterile jars. A pad is removed from its jar with a tweezers or forceps, wiped across the surface of the object, and placed in another tightly sealed sterile jar. Such pads can be used for taking samples from the roofs and walls of houses, the surface of asphalt streets and sidewalks, highway vehicles, outer clothing, furniture, and other objects. A tag indicating the sampling site and the

date (year, month, day, and hour) is attached to the jar containing the used pads. The latter is then wrapped in paper, padded with cotton or paper, packed in tightly covered boxes, and sent to the laboratory for examination. The packing slip indicates the sampling site, the date, and the number of samples.

PACKING AND SHIPPING OF MATERIAL FOR LABORATORY EXAMINATION

Prof. K.I. Matveyev

Material for laboratory examination can be taken from a patient, a cadaver, or the environment. Material should be taken for examination in accordance with pathogenetic data on the disease. The appropriate specific chapters indicate what type of material should be taken from the patient in individual infections. It is important to follow these recommendations.

Material taken from patients should be placed in an appropriate well-sealed sterile container. Small jars of different sizes with ground or tightly fitted rubber stoppers can be used for this purpose. Flasks with rubber stoppers can be employed for liquid and semiliquid materials. During sterilization the jars and flasks are stoppered with cotton plugs and paper-wrapped rubber stoppers of the proper size are tied to them. Small, tightly sealed wide-mouth jars are used for solids taken from cadavers (pieces of organs, etc.).

The packing slip accompanying all material taken from patients or cadavers must indicate the patient's surname, given name, and patronymic, the date when the specimen was taken (month, day, and year), and a description of the material and the site from which it was taken, e.g., blood from vein, blood from placenta, pus from wound, pus from anthrax carbuncle, etc. When pieces are taken from a cadaver's organs the site (liver, lungs, small intestine, etc.) must be listed. The packing slips are pasted onto the containers and must be fastened down with twine or

thread, since they may drop off if they are merely glued on.

Since certain pathogenic microorganisms (the causative agents of dysentery, meningitis, etc.) soon die outside the human body, it is necessary to try to make the time between the taking of the material and the beginning of the examination as short as possible. In such cases cultures are made on nutritive media at the patient's bedside. In addition, the material taken from the patient can be shipped with a preservative (in investigating the feces for typhoid, paratyphoid, and dysentery bacteria, etc.).

The material taken from the patient (corpse) is sent to the laboratory with an accompanying packing slip, which indicates the patient's surname, given name, and patronymic, the date when the material was taken, the manner in which it was taken, and the number of jars, flasks, etc. A brief note is also made of clinical data, the data of the onset of the disease, and the preliminary or final diagnosis.

The same techniques are used for taking material from the environment and sending it to a laboratory as for material taken from patients. All samples must be shipped in sterile vessels or containers, as soon after they are taken as possible.

Water is shipped in large sterile bottles enclosed in appropriate containers, which should keep the water from freezing in winter and overheating in summer. Water samples to be examined should be shipped immediately after they are taken.

Air samples are taken under field conditions with mobile laboratories specially equipped for this purpose. Initial examination of air samples can also be carried out in these laboratories.

The basic examination of air samples must be conducted in permanent laboratories, to which all the initial material must be sent. Air shipped for examination should be treated in precisely the same manner

as infectious material taken from patients with especially dangerous infections. Special iron boxes should be fitted out for shipping fixed smears; the boxes are sterilized after use. Culture-bearing media are shipped in special containers, which keep them from freezing in winter and overheating in summer. Filters through which air has been passed are shipped in especially adapted iron boxes. Liquids are shipped in sterile flasks or test-tubes sealed with rubber stoppers, which are placed in containers that protect them from heating and freezing. The packing slip should indicate the sample number, the sampling site, the sampling date (year, month, and day), the apparatus used (the method of taking air samples, etc.), and the material taken for examination (air, water, soil, washings from surfaces, etc.).

Samples of solid foodstuffs which have caused or are suspected of causing food poisoning are carefully wrapped in parchment paper, cellophane, etc.; liquids are shipped in flasks and semiliquids in well-sealed jars, which are first sterilized or thoroughly boiled. Foodstuffs shipped to a laboratory for examination should be accompanied by a packing slip indicating the sampling site (address), the sampling date (year, month, and day), the number of samples, the name of the product, the type number, the series, the manufacturer, and who sold the material to the consumer. If there are patients whom the product has poisoned a brief description of their symptoms and diagnosis is appended.

Samples taken from the environment, a patient, or a cadaver for examination are sent to the laboratory by special messenger, the individual in question being given papers indicating that he is legally transporting the infectious material entrusted to him.

INTESTINAL INFECTIONS

TYPHOID FEVER

Ye.G. Mel'nik, Candidate of Medical Sciences

As a result of the wide recent use of antibiotics for treating intestinal infections, typhoid fever is now characterized by an extreme diversity of clinical manifestations, with an increase in the proportion of mild and nonsymptomatic forms, which has created serious difficulties in its clinical diagnosis. This has made laboratory diagnostic methods of decisive importance in a number of cases.

Regardless of whether the illness is caused by S. typhi (typhoid bacterium), S. paratyphi (paratyphoid A bacterium), or S. schcttmuelleri (paratyphoid B bacterium), it is usually accompanied by a unique clinical symptomatology and always has the same anatomic basis. Differential diagnosis of typhoid and paratyphoid can consequently be carried out only by laboratory methods – isolation of a culture of the causative agent from the patient's body or detection of specific antibodies in his blood serum.

The difficulties of laboratory diagnosis lie in the fact that typhoid-paratyphoid bacteria have many characteristics similar to those of other coliform bacteria.

The causative agents of typhoid fever fall into the extensive family Enterobacteriaceae, genus Salmonella, and differ in serological classification (see Table 24) in accordance with the composition of their somatic (O) antigens.

Morphology of the causative agents of typhoid fever

The bacteria responsible for typhoid and paratyphoid A and B do not differ morphologically from one another or from other coliform bacteria. They are usually short rods with rounded ends, 1-3 μ long and 0.5-0.8 μ in diameter. Typhoid and paratyphoid bacteria occasionally are coccal, spherical, or elongate in form. The rods are mobile and

have 8-14 flagella arrayed over the entire body. The causative agents of typhoid and paratyphoid form neither capsules nor spores; they stain well with all the basic analine dyes and are gram-negative.

Biology of the causative agents of typhoid fever; cultural properties

The optimum temperature for growth of typhoid and paratyphoid bacteria is 37°. They grow well on all nutritive media, both in the presence of a copious oxygen supply and under anaerobiotic conditions. They develop most actively when the medium has a weakly alkaline reaction (pH = 7.4-7.6). They grow well on simple nutritive media (meat-infusion agar, meat-infusion broth, gelatin, etc.). They yield a uniform cloudiness in broth, occasionally forming a small precipitate on the bottom of the test-tube, which rapidly disappears on shaking. Transparent or semi-transparent colonies, which are rounded or slightly convex and lustrous (the smooth S form), are formed on the surface of meat-infusion agar; the colonies can also be flat, with ragged edges (the rough R form). Colonies of paratyphoid B are larger and cloudy. Freshly isolated strains of paratyphoid B exhibit the ability to form spindles after incubation in a heater at 37° for 18-20 hr or at room temperature for 1-2 days; this is an important diagnostic characteristic for identifying cultures of S. schottmuelleri (Fig. 22).

During the first day of their development certain strains of paratyphoid B produce a continuous slimy growth, which resembles a mass of jelly.

The majority of the coliform bacteria exhibit a rather consistent biochemical activity, which can be used to identify the cultures isolated. In contrast to E. coli , typhoid-paratyphoid bacteria do not decompose lactose, sucrose, or salicin, but produce acids and gases in media containing manose, glucose, maltose, and other sugars. Bacteria of the typhoid-paratyphoid group do not form indol, do not liquefy gela-

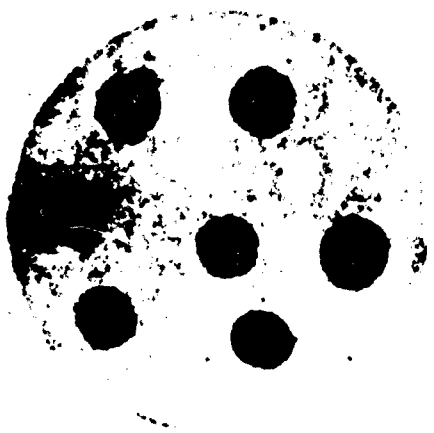


Fig. 22. Formation of spindles by paratyphoid B bacteria. ×20.

tin, and do not ferment or otherwise alter milk for quite some time. After 14 days the milk undergoes clarification and acquires a slightly yellowish-brown tint. These biochemical characteristics differ from those of E. coli and other nonpathogenic bacteria found in the human intestine. Typhoid cultures can be divided into three types in accordance with their ability to decompose xylose and arabinose. Type I comprises cultures which decompose xylose and not arabinose, type II includes strains which affect neither xylose nor arabinose, and type III comprises cultures which ferment both xylose and arabinose. The xylose-arabinose characteristics of S. typhi can be used for epidemiological purposes.

The first cultures of typhoid bacteria taken from patients often will develop only in liquid nutritive media. Culturing on solid media including blood agar, does not produce colonies. Growth of dwarf colonies (Fig. 23) is observed only after incubation for 3-4 days in a heater at 37°.

A high degree of bacterial polymorphism is usually detected in preparations made from dwarf colonies. These bacteria usually take on the dimensions normal for typhoid bacilli when subsequently cultured on liquid media (Ye.G. Mel'nik).

Colonies of typhoid and paratyphoid bacteria are colorless on dishes containing differential media (Ploskirev's, Levin's, Endo's, etc.) with milk sugar (lactose; see Table 16), since they do not ferment lactose. Colonies of E. coli decompose lactose to form an acid, so that they are stained different colors, depending on the indicator used.

TABLE 16

Color of Colonies in Nutritive Media

1 Наименование среды	2 Колонии тифозные и паратифозные	3 Колонии кишечной палочки
4 Плоскирева	Прозрачные, бесцветные 8	Кирпично-красные 10
5 Левина	» »	Темно-синие 11
6 Эндо	» »	Красные с металлическим 12 блеском
7 Вильсон Блера	9 Через 24 часа плоские, темно-грязноватого цвета; через 48 часов черные, иногда более светлые на периферии	Единичные коричневого оттенка 13

1) Medium; 2) typhoid and paratyphoid colonies; 3) E. coli colonies; 4) Ploskirev's; 5) Levin's; 6) Endo's; 7) Wilson-Blair's; 8) transparent, colorless; 9) flat, dark, dingy color after 24 hr; black after 48 hr, occasionally lighter at periphery; 10) brick red; 11) dark blue; 12) red, with metallic luster; 13) isolated colonies brownish.

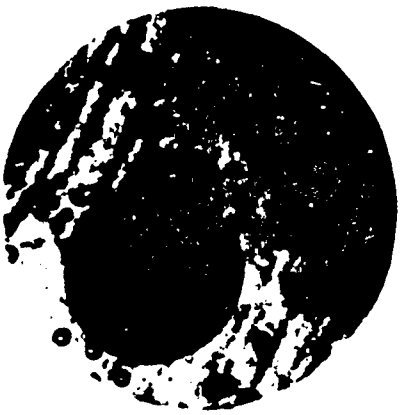


Fig. 23. Smooth and dwarf colonies of typhoid bacteria grown on agar for three days at 37°. ×20.

In making a differential diagnosis it is necessary to keep in mind the similarity of the cultural, biochemical, and antigenic characteristics of certain bacteria of the Salmonella group. Thus, for example, S. paratyphi and S. Sendai differ biochemically only with respect to xylose; S. Sendai ferments this compound to form an acid and gas, while S. paratyphi does not decompose it. The somatic and flagellar antigens must be analyzed in making a complete serological examination. In contrast to S. Sendai (1, 9, 12; a; 1.5), S. paratyphi (1, 2, 12; a; -) is

monophasic, so that the 2nd phase must be detected before a differential diagnosis can be made. Serum 9 must be used for analyzing the O antigens, since it agglutinates S. Sendai and not S. paratyphi.

In view of the diverse clinical forms of paratyphoid caused by S. schottmuelleri, it must be differentiated from S. java when cultures are isolated from the patient's body. Since they have the same antigenic structure (1, 4, 5, 12; b; 1, 2) they can be distinguished only by their enzymatic action on d-tartrate and the spindle formation of S. schottmuelleri. Thus, cultures of S. java ferment d-tartrate, while S. schottmuelleri does not.

As was pointed out above, S. schottmuelleri forms slimy spindles around its colonies (see page 288), while S. java does not.

This same characteristic makes it possible to differentiate S. schottmuelleri and S. typhimurium, which have similar bicchemical and serological properties. These two species also differ in: 1) the "creeping of the culture on a gelatin slant; 2) formation of daughter colonies on agar containing 1% raffinose; 3) enteric infection of mice (Table 17).

TABLE 17

1	2	3	4
	Влагообразование	Стекание на желатине	Дочерние колонии	Энтеральное заражение мышей
S. schottmuelleri	+	+	+	—
S. typhimurium	—	—	—	+

1) Spindle formation; 2) creeping on gelatin; 3) daughter colonies; 4) enteric infection of mice.

Certain characteristics of typhoid-paratyphoid cultures. Identification of the bacteria isolated presents no difficulty if they have typical cultural, biochemical, and serological properties. More detailed study is necessary when cultures which have lost one of their basic

Fig. 24. S. colonies of E. coli (dark) and R colonies of Salmonella (light, with festoon-like margins). ×20.

traits (atypical cultures) are isolated.

In recent years, since antibiotics have come into wide use in the treatment of typhoid-paratyphoid diseases, the first generation more and more frequently exhibits R colonies ranging in size from 0.5 to 1 cm, with festoon-like margins (Fig. 24); mucoid colonies are occasionally encountered. In some cases these colonies are hard to distinguish from R colonies of Shigella paradysenteriae. These R colonies acquire a smooth O form in subsequent cultures.

When bacteria lose their agglutinative properties in the first generation they must be cultured on 10% bile broth, repeatedly passed through this medium, and cultured on agar slants. Detection of agglutinative properties is aided by heating at 56° for 30 min or by boiling the cultures. The enzymatic properties of typhoid-paratyphoid bacteria are found to be altered in some cases: strains are encountered which are unable to ferment certain sugars. Paratyphoid A bacteria may acquire the ability to form hydrogen sulfide.

It must be kept in mind that paratyphoid cultures occasionally lose their capacity for gas formation. In such cases the fact that they are Salmonella can be established by a cross-agglutination reaction with monophasic agglutinative sera.

Phagolyzability can be used for studying atypical cultures.

Antigenic structure

Microorganisms of the genus Salmonella have a mosaic antigenic structure: we can distinguish the thermostable somatic O antigen and

the thermolabile flagellar H antigen.

Microscopic examination of a hanging drop will reveal a qualitative difference between H and O agglutination. H agglutination is characterized by an absence of bacterial mobility resulting from the adhesion of their flagella, which causes formation of a coarse conglomerate that can easily be broken up. In O, or polar, agglutination the bacterial somae adhere to one another, the flagella remaining free, and the fused microbes are capable of moving. The O agglutinate is granular and can be broken up with difficulty by shaking.

The O antigen is a glucide-lipid-polypeptide complex, which is easily extracted from the cell with a trichloracetic acid solution (Buaven). This method leads to denaturation and decomposition of a majority of the cellular proteins and causes the phospholipid-protein-polysaccharide complex to go into solution; this complex is toxic and has antigenic properties.

The flagellar H antigen is protein in nature, since heating or treatment with trypsin makes it unable to cause antibody formation or to react with specific antibodies.

The O and H antigens differ in sensitivity to chemicals and to temperature, which makes it possible to obtain either of them in pure form from the cell. Sera containing H or O antibodies are obtained when animals are immunized with the antigens.

In addition to these two basic antigens, Felix and Pitt (1934) found that the O-antigen contains an additional antigen, which is encountered in strains freshly isolated from the body. Felix and Pitt named this strain the Vi antigen, associating it with the virulence of the culture. The Vi antigen is extremely labile when heated or under the action of dilute acids, alcohol, phenol, etc. Discovery of the Vi antigen was a substantial aid in determining the serological properties of

S. typhi and solving the riddle of O inagglutinability. The Vi antigen is distributed over the surface of the cell and prevents it from agglutinating with O sera; it easily agglutinates with O and H sera after the Vi antigen has been destroyed. Serum containing Vi agglutinins was obtained by immunizing rabbits with live O inagglutinable strains of S. typhi. The presence of the Vi antigen was demonstrated by experiments involving immunization, agglutination, and absorption.

It was established that O inagglutinable strains are more virulent for mice; the virulence of Vi cultures is determined in terms of 50 million cells by intraperitoneal injection of mice. The Vi antigen has been detected both in S. typhi and in other bacteria of the Salmonella group and certain members of the coliform group.

According to the data of Kaufman and Vahlne (1945), the Salmonella have additional superficial K antigens. The K antigens are nonuniform, consisting of three antigens: antigen 5, the Vi antigen, and the M antigen. Antigen 5 bears no relationship to culture virulence. Kaufman detected the M antigen (mucoid antigen) in mucoid strains of S. schottmuelleri.

While the M antigen may not be identical for all types of Salmonella, it at least has a fraction common to all types.

The O and H antigens of typhoid bacteria are complex antigens consisting of several components. Study of the composition of these antigens enabled Kaufman and White to develop a serological classification of the genus Salmonella (see Table 24). The somatic O antigen of different bacteria consists of individual antigenic components, whose combinations characterize the individual groups of the genus Salmonella. Following Kaufman's suggestion (1954), the O antigens are now customarily designated by the arabic numerals 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc. They were previously designated by Roman numerals (I, II, II, etc.)

and this system is still encountered. The Salmonella are divided into Groups A, B, C, D, E, F, G, H, J, etc., in accordance with their common O antigen.

TABLE 18

Antigenic Structure of Typhoid Bacteria

1 Наименование	2 Группа	3 Антигены: 4 соматические	специфическое, фаза Н 5	неспецифическое, фаза Н 6
S. paratyphi	A	1, 2, 12	a	—
S. schottmuelleri	B	1, 4, 5, 12	b	1—2
S. typhi	D	9, 12, vi	d	—

1) Bacterium; 2) group; 3) antigens; 4) somatic; 5) specific, H phase; 6) nonspecific, H phase.

The flagellar H antigen is nonuniform in the majority of Salmonella, consisting of two phases - specific and nonspecific. The H antigen of the specific phase is customarily designated by Roman letters (a, b, c, d, e, etc.), while the nonspecific phase is designated by arabic numerals (1-2, 1-5, 1-6, 1-7, etc.). The specific phase of the H antigen furnishes a basis for dividing the members of each group into serological types, which plays a large role in diagnosing diseases caused by bacteria of the Salmonella group.

Table 18 shows the antigenic structure of typhoid bacteria, following the Kaufman-White scheme (see Table 22).

Specific adsorbed and nonadsorbed agglutinative sera and monoreceptor Salmonella sera are used for serological determination of bacteria of the Salmonella group.

Laboratory Diagnosis of Typhoid Fever

Material for examination

The presence of typhoid fever can be confirmed and its etiology determined by bacteriological investigation of blood, bile, bone marrow,

urine, feces, roseolar contents, cerebrospinal fluid, sputum, saliva, or pus from patients, milk from nursing mothers, or sectioned material from cadavers.

Bacteriological examination

Bacteriological examination of blood. Blood for examination is taken from the patient's ulnar vein with a sterile Luer syringe (the needle and syringe are sterilized in an autoclave or with dry steam), observing all the rules of asepsis. The quantity of blood needed for culturing depends on the phase of the illness. The sample to be cultured can be limited to 10 ml at the height of the fever, while a larger quantity, up to 20 ml, must be drawn once the fever has abated.

Study of the pathogenesis of typhoid fever has shown that typhoid bacteria appear in the blood at the end of the incubation period and are present during the first few days of fever; bacteriological examination of the blood is consequently a valuable diagnostic procedure, making it possible to recognize the disease at its very outset.

Blood can be used for culturing throughout the entire febrile period. Whether or not a positive result is obtained depends on a number of factors, undoubtedly including the time at which the blood is taken, the severity of the illness, the quantity of blood taken, and the quality of the nutritive medium used for the culture.

The media which can be used for concentration include Rapoport's medium, 20% bile broth (100 ml portions in flasks), bovine bile (5-10 ml portions in test-tubes), meat-infusion broth with 1% glucose added (100 ml portions in flasks), sterile distilled water (Klodnitskiy's method), tap water (Samsonov's method; 80-100 ml portions in flasks), and Kalin's citrate medium (5 ml portions in test-tubes).

The volume of the concentration medium should be 10 times that of the blood to be cultured, since blood in a lesser volume of nutritive

medium may have a bactericidal action on typhoid-paratyphoid bacteria. The only exception is G.P. Kalin's citrate medium (which can be used in a ratio of 1:1).

Blood clots prepared in the laboratory by Widal's reaction should be used for the hemocultures. The blood serum is left to stand and the resultant clot is pulverized with a sterile glass rod, placed in a flask with the concentration medium, and kept in a heater for 24-48 hr.

Bacteriological investigation of the culture then takes the following course: the flasks containing the concentration medium are examined on the second day. The medium becomes cloudy if bacterial growth has occurred. When no growth has taken place the flask is returned to the heater for 7 days and it is noted that there was no growth. Final determination of whether the culture is negative is made on the 7th day. If the medium is clear it is kept in the heater for 10-15 days and examined daily.

A growth of typhoid-paratyphoid bacteria in Rapoport's medium not only produces cloudiness, but stains the medium, since the sugar is broken down to form an acid, which in turn reduces the Andrade indicator and causes it to turn red. In addition to changing the color of the medium, a growth of paratyphoid bacteria causes gas formation, which results in displacement of material from the liquefied area. Special attention must be paid to any change in the color of the medium in this area, which results from growth of mobile bacteria. A growth of coccal bacteria causes the medium in the flask to become cloudy, while that in the liquefied area remains unchanged.

The affected medium is cultured on Petri dishes containing differential-diagnostic media (Levin's, Endo's, etc.) and kept in a heater for 18-20 hr. The dishes are examined 3 days after the blood is cultured and 2 or 3 suspected colorless colonies are isolated on Ressel's

medium containing urea or on a short compound series (agar slants and Hiss' medium containing glucose and lactose) and incubated in a heater at 37°.

The growth on the media is examined after 18-20 hr (i.e., on the 4th day of the investigation). S. typhi does not change the color of slants prepared from Ressel's medium, but stains columns red as a result of decomposition of the glucose. In addition to changing the color of columns, S. paratyphi and S. schottmuelleri fissure agar as a result of gas formation.

The morphological properties of the cultures grown on agar slants are studied (by staining smears by Gram's method). The lability of the bacterium is studied by the hanging- or crushed-drop method or in a semiliquid medium.

If labile gram-negative bacilli are detected in the preparations a rough agglutination reaction is set up on a slide, using specific Salmonella sera. When a positive reaction is obtained with these sera the pathological characteristics of the culture are determined with monophasic adsorbed sera and a preliminary report is made.

A more precise study of the culture is made by serial agglutination with specific sera and culturing on media containing various carbohydrates and alcohols (compound series; see Table 24).

The serial agglutination reaction and the biochemical activity of the culture in the completed compound series are evaluated on the 5th day of the investigation. The morphology and lability of the pure culture isolated are again studied at this time. The final report is made on the basis of the aggregate of the data obtained.

M.A. Rapoport suggested a method for determining the bacteriemia index, using broth containing glucose and acid fuchsin as an indicator. The patient's blood is cultured in 20 test-tubes containing this medium,

introducing fractionally increasing quantities of blood, from 0.1 to 2 ml. The bacterial growth is determined from the change in indicator color. If blood is taken from a patient in serious condition all the test-tubes will show bacterial growth, while in mild cases there will be no growth in any of them. In moderately severe cases growth will be observed in those test-tubes inoculated with large quantities of blood. The growth index depends on the severity and duration of the illness.

Bacteriological examination of bone marrow. The wide use of antibiotics has greatly reduced the possibility of culturing bacteria from the body. While severe cases will yield positive hemocultures in 100% of all cases during the first week, in 70% of all cases during the 2nd week, in 60.7% of all cases during the 3rd week, and in 45.3% of all cases during the 4th week before administration of antibiotics (Ye.G. Melnik, 1938), after treatment with levomycetin and synthomycetin cultures were obtained in 60% of the patients examined on the 1st and 2nd days and 8% of those examined on the 3rd day. The percentage of positive hemocultures was still lower at later times (V.I. Pokrovskiy and I.G. Bulkina, 1956).

The body is never, however, completely freed of bacteria and microbes can often be isolated by culturing the feces, urine, or bile, even during treatment or convalescence. A total of 5% of all patients discharged will be bacteria carriers. The frequency with which relapses occur also indicates that the body retains bacteria.

Typhoid bacteria may persist for a long time in the bone marrow, even during antibiotic treatment.

A comparative analysis of the culturability of bone-marrow puncture specimens and blood samples showed that myelocultures could be isolated in 85.4% of all cases, while hemocultures were positive in only 41.7% of all cases.

Bone-marrow cultures produce a more rapid and copious bacterial growth than blood cultures.

Sternal punctures are carried out with a Raskin needle, which is fitted with a movable sleeve that makes it possible to insert the needle to a predetermined depth and thus avoid piercing through the entire thickness of the sternum. The puncture is made in the manubrium or body of the sternum, a short distance from the midline. After the puncture has been made a tightly-fitting syringe is attached to the needle and used to extract from 0.5 to 0.75 ml of marrow. The specimen thus obtained is cultured in 3 ml of bovine bile or some other concentration medium and incubated in a heater at 37° for 18-20 hr; the investigation then proceeds in the same manner as for blood cultures.

Bacteriological examination of the duodenal contents. Bile cultures can be made either for purposes of early diagnosis or to detect bacteria carriers who have recovered from typhoid. Making such cultures during the early phase of the illness results in 100% culturability (G.Ya. Sinay, G.M. Kapnik, and Ye.G. Mel'nik).

A duodenal probe is inserted through the mouth or nose (in children), with the stomach empty. The first portion of duodenal juice, which is light in color, is withdrawn after 15-20 min. This is portion A. After this portion has been removed 30-50 ml of 30% warmed sterile magnesium sulfate solution is introduced into the duodenum through the probe; a dark brownish or greenish portion of stored bile, portion B, is then withdrawn. Light yellowish bile from the biliary ducts, portion C, appears soon after portion B is taken. Each portion is collected in an individual sterile test-tube.

The material thus obtained is kept in a heater for 18-20 hr and then cultured on Petri dishes containing differential media, which are incubated in the heater. The cultures are studied by the technique des-

cribed in the section headed "Bacteriological examination of feces."

It is unnecessary to culture the bile in concentration media, since it is itself a good nutritive medium for typhoid-paratyphoid bacteria. When a negative result is obtained for the first culture the bile is kept in the heater for 10 days and then cultured on solid differential media.

Bacteriological examination of feces. Fecal cultures for detection of typhoid-paratyphoid bacteria are made from the very first days of illness onward, the process being repeated until the patient recovers. Correct taking of samples from ill and healthy persons for detection of carriers is of great importance. Fecal samples can be collected in lined vessels (which should have no traces of disinfectant) or sterile enameled or cardboard trays, pans, etc.

A liquid stool facilitates detection of bacteria in patients and convalescents. Healthy persons to be examined to see whether they are bacteria carriers should preliminarily (2-3 hr before the sample is taken) be given a laxative (a mixture of magnesium sulfate and sodium sulfate) in a total dose of 25-30 g.

The action of the laxative intensifies bile secretion and the contents of the small intestine are rapidly evacuated into the large intestine and rectum, mucous "pouring" from the crypts; all this increases the possibility of a positive result.

The liquid fecal matter from the upper portions of the intestine is selected for the samples, using wooden spatulas or glass tubes kept in a preservative consisting of a glycerine mixture (30% neutral glycerine and 70% physiological solution), an alkaline solution, or 10-20% bile broth. The material can be cultured in Muller-Kaufman's concentration medium (see page 288).

The material and preservative can be stored in a refrigerator for

6-8 hr. At the laboratory it is immediately cultured on Petri dishes containing Ploskirev's, Endo's, Levin's, or Wilson-Blair's medium. Ploskirev's medium yields the best results. It should, however, be noted that use of this medium requires 10 times as much material as Levin's medium or the others.

The cultures are incubated in a heater for 18-20 hr. On the 2nd day the dishes are examined with the naked eye or a magnifying glass. Suspected colonies, transparent or cloudy, are cultured on a short compound series (see above) or Ressel's medium. They can also be cultured on agar slants, Peshkov's medium, or broth; pieces of indicator paper are inserted between the wall of the last test-tube and the sample, to permit detection of indol or hydrogen sulfide formation.

If no suspected colonies of pathogenic bacteria are detected on the dishes material is transferred from the concentration medium to dishes containing differential media and all the cultures are placed in a heater overnight.

The results of this culturing are examined on the 3rd day. Cultures which ferment lactose and sucrose are discarded and, if there are no suspected cultures on the concentration medium, a negative report is made.

Cultures which do not alter media containing lactose and urea are subjected to further study. Preparations are made and stained by Gram's method and their lability is determined. Labile gram-negative bacilli which do not decompose lactose and ferment glucose to produce an acid may be suspected of being typhoid cultures, while nonlabile colonies may be suspected of being dysentery bacteria. In the first case a rough agglutination reaction is set up on slides with typhoid serum; if a positive reaction is obtained the antigenic structure of the bacterium is determined with monoreceptor O, H, and Vi sera. A preliminary posi-

tive report is made if this reaction is sharply positive for all three sera. For investigation of nonlabile cultures see page 370.

Cultures which ferment glucose to form an acid and gas may be suspected of being paratyphoid bacteria (see page 339).

A detailed study of the culture isolated is made by culturing on an expanded compound series (see Table 24) and by setting up a serial agglutination reaction with washings from a culture raised on Ressel's medium or an agar slant. The serum is diluted with sterile physiological solution, beginning at 1:100 and continuing to the serum titre. A drop of culture is added to each test-tube and placed in a heater for 2 hr; the tubes are then permitted to stand at room temperature until the following day.

The enzymatic properties of the culture are studied on the 4th day. A negative report is made if the culture ferments sucrose and produces indol. The results of the serial agglutination reaction are noted, the biochemical activity of the bacteria is evaluated, the species to which tney belong is determined, and the final report is made.

Tests involving concentration media are made if the direct cultures yield a negative result.

Bacteriological examination of urine. Urine cultures are made for diagnostic purposes from the first day of illness until the patient is discharged from the hospital; they are also made to detect bacteria carriers among healthy individuals. Before taking material the orifice of the urethra is washed with sterile physiological solution (the first portion of urine being discarded) and 10-20 ml of urine is collected in a sterile test-tube or bath.

The urine to be cultured is centrifuged and the residue is drawn off with a Pasteur pipette and used to inoculate the concentration medium. At the same time 2 or 3 drops are cultured on Petri dishes con-

taining a differential medium. The cultures are placed in a heater overnight. The subsequent analysis is conducted in the same manner as for fecal matter.

Bacteriological examination of roseolar material. In order to obtain roseolar contents an area of the skin bearing pronounced roseolae is treated with alcohol, rinsed with physiological solution, and wiped with gauze and the roseolae are scarified. A total of 1 or 2 drops of bile broth is applied to the scarification site and the material to be examined is collected with a Pasteur pipette and cultured in a flask containing bile broth. The culture is incubated in a heater at 37° for 18-20 hr. Subsequent examination is carried out in the same manner as for blood cultures.

Bacteriological examination of cerebrospinal fluid. Typhoid fever is occasionally accompanied by a meningoencephalitic syndrome. Lumbar puncture is carried out in such cases for diagnostic purposes. If bacterioscopy reveals gram-negative bacilli in the cerebrospinal fluid it is cultured in a concentration medium. Subsequent bacteriological examination is conducted in the same manner as for blood cultures.

Bacteriological examination of pus. Typhoid fever is sometimes complicated by suppurative processes such as otitis, phlegmon, lymphangioitis, etc. In such cases typhoid or paratyphoid bacteria may be detected in cultures from the suppurative foci. Pus to be examined is collected in sterile test-tubes and sent to the laboratory. The pus is cultured on 2 Petri dishes containing Ploskirev's medium and blood agar; a parallel culture is made in a concentration medium. Subsequent examination is conducted in the same manner as for feces.

Bacteriological examination of sputum. Pulmonary complications of typhoid fever may take the form of specific typhoid pneumonia, which develops at the height of the illness (Yu.M. Lazovskiy and Ye.G. Mel'-

nik, 1947).

Sputum is collected in sterile Petri dishes. Suppurative lumps are collected and cultured on Petri dishes containing Ploskirev's medium and blood agar. The cultures are incubated in a heater for 18-20 hr and suspected colonies are isolated. Further identification of the culture is carried out in the same manner as for feces.

Bacteriological examination of milk. The milk of female patients can be examined during the lactation period. It is collected in a sterile vessel; the nipple and the surrounding skin must first be treated with alcohol and then washed with sterile physiological solution. The milk is cultured in a concentration medium and then on differential media. Subsequent examination is conducted in the same manner as for feces.

Bacteriological examination of sectioned material. The following types of material can be examined: blood from the heart, bile, fragments of the liver, spleen, lungs, kidneys, and brain, intestinal lymph nodes, the contents of the large and small intestines, tissues, etc. Material is taken from a cadaver on a dissecting table, observing all the rules of asepsis. Blood from the heart and the contents of the gall bladder are taken in quantities of 3-5 ml with a Pasteur pipette. The site at which the Pasteur pipette is to be inserted is first cauterized with a red-hot spatula. The material is cultured in a concentration medium.

Fragments are cut from organs with sterile scissors and placed in sterile Petri dishes or jars for shipment to the laboratory.

In culturing sectioned material the surface layers of the organ fragments are cut away with a sterile scalpel and the inner portions are placed in sterile mortars and crushed; cultures are then made in concentration media and in Petri dishes containing Ploskirev's medium and blood agar.

The cultures are incubated in a heater at 37° for 18-20 hr and subsequent examination is conducted in the same manner as for feces.

In making a bacteriological examination of material from a cadaver the time elapsed since the patient's death must be taken into account. Cultures should not be made more than 2 hr after death, since bacteria from the intestinal tract may otherwise invade the cadaver's organs and tissues.

Serological investigation

Serological research methods are among the auxiliary techniques for laboratory diagnosis. They can be used to determine the presence of both antibodies and antigens.

In typhoid-paratyphoid diseases diagnostic antibody titres appear toward the end of the first and the beginning of the second week of illness.

In order to obtain serum a blood sample of 0.5-1 ml is taken from a finger and collected in a sterile test-tube, which is then placed in a heater to permit clotting; the sample is then centrifuged, removed to a second test-tube with a Pasteur pipette, and used for the desired reaction.

Widal's agglutination reaction. Because of its simplicity, the Widal method is widely used in the laboratory.

The Widal reaction requires: 1) the patient's serum; 2) a set of diagnostic sera; 3) physiological solution (0.85% NaCl).

The reaction is set up with four antigens - typhoid O and OH and paratyphoid A and B. Each row of the rack holds 4 or 5 test-tubes. The number of rows corresponds to the number of antigens.

In view of the fact that a group agglutination reaction is often obtained in typhoid as a result of the similarity of the antigenic structures of bacteria of the Salmonella group, the serum must be taken

in dilutions of the order of 1:100, 1:200, 1:400, 1:800, etc.

The serum dilutions are prepared beforehand, beginning with 1:50. For this purpose 4.9 ml of physiological solution is added to 0.1 ml of serum. Portions of 1 ml of physiological solution are then poured into all the test-tubes. A total of 1 ml of the 1:50 serum solution is poured into the first test-tube, thus yielding a dilution of 1:100. The contents of the tube are carefully mixed and 1 ml of the first dilution (1:100) is transferred to the second test-tube, etc. A total of 1 ml is poured off from the fourth test-tube. We thus obtain a series of serum dilutions ranging from 1:100 to 1:800. Portions of 1 or 2 drops of the appropriate diagnostic serum are then added to each test-tube.

After the diagnostic serum has been added the tubes are shaken and placed in a heater at 37°. After being incubated in the heater for two hours the racks containing the test-tubes to which the OH diagnostic sera was added are removed and kept at room temperature overnight. The racks containing the tubes to which the O diagnostic sera was added are left in the heater overnight and the results of the reaction are then noted. If the reaction is sharply positive in the last dilution it is repeated with higher dilutions (from 1:800 to 1:12,800) to establish the maximum titre. Each Widal reaction is accompanied by serum and antigen controls. The result of the reaction can be evaluated with the naked eye or with a magnifying glass and agglutinoscope.

The agglutination reaction is regarded as positive if the solution becomes clear and a dome-shaped precipitate is formed on the bottom of the tube. The test-tubes containing the O diagnostic serum can be shaken; fine-grained agglutination should be observed. It is not recommended that the tubes containing the OH diagnostic serum be shaken, since the flaky precipitate is rapidly broken up. The results of the reaction are evaluated from the titre (the maximum serum dilution at which ag-

glutination is observed). The intensity of the reaction is graded on a four-plus scale: if the liquid is completely clarified and there is a great deal of precipitate the reaction is ++++. If the liquid is slightly cloudy and there is a great deal of residue the reaction is +++. When the liquid is still more cloudy and there is not too much residue the reaction is ++. When the liquid is markedly cloudy and agglutination is weak the reaction is +. Uniform cloudiness indicates a negative agglutination reaction.

The Widal reaction is regarded as positive when marked agglutination occurs at serum dilutions of 1:200 or more.

The Widal reaction may be positive in high titres (up to 1:800) in some individuals who have recovered from typhoid, who have been vaccinated, or who are suffering from other infectious diseases (tuberculosis, typhus, etc.). Such reactions may reduce the validity of serological diagnosis. However, the titre of anamnestic agglutinins does not increase. Setting up the Widal reaction under dynamic conditions consequently makes it possible to avoid errors in diagnosing the disease present. It is recommended that the serum be heated at 56° for 30 min to eliminate nonspecific group reactions. More highly concentrated sodium chloride solutions (2.9, 5.84, and 11.68%) can be used for this purpose, since they promote an intensification of the specific agglutination reaction (Fisher).

According to the instructions, in acute febrile conditions the Widal reaction must be accompanied by a parallel reaction with Rickettsiae prowazeki, in order to exclude typhus; the Bailey-Felix reaction can be used when the rickettsial reaction cannot be set up.

O and H monodiagnostic sera of the Salmonella group are now recommended for the Widal reaction. Comparative analysis of the results of the Widal reaction with ordinary diagnostic sera and monodiagnostic sera

has shown the advantage of the latter, especially O monodiagnostic sera.

A continuous course of treatment with moderate therapeutic doses of levomycetin or synthomycetin causes no pronounced decrease in the titres of the Widal reaction (K.V. Bunin).

In addition to the Widal agglutination reaction, in which the results can only be evaluated after 18-20 hr has elapsed, there are accelerated diagnostic methods which make it possible to determine the results of the reaction within 15-20 min (Noble's plate method, etc.). Concentrated antigens and antibodies and mechanical agitation are employed in setting up accelerated reactions. The accelerated methods are less specific than the classical method for the Widal reaction (see page 119).

Vi agglutination reaction. The reaction with Vi diagnostic serum is now widely employed as a serological diagnostic method.

Before the agglutination reaction is set up all the sera to be tested are heated for 30 min at 56° in order to destroy group agglutinins. The specific Vi agglutinins survive heating. The serum to be heated is taken whole or in a dilution of 1:10. A rough Vi agglutination reaction is first set up on slides with a heated serum diluted to 1:10. A drop of dilute serum is applied to a slide and a drop of Vi diagnostic serum is added; the slide is placed in a moist chamber at room temperature for 10 min and the results are evaluated. A parallel serial agglutination is carried out in test-tubes: the heated serum is prepared in dilutions from 1:10 to 1:320-1:640 etc., and 1 or 2 drops of Vi antigen are added to each test-tube. The experiment is accompanied by a serum control in a dilution of 1:10 and an antigen control. The test-tubes are shaken and incubated in a heater at 37° for 2 hr and then at room temperature for 18-20 hr. The reaction is fine-grained in character. The results of the reaction are evaluated with the aid of an

agglutinoscope, a magnifying glass, or a microscope mirror (which gives an enlarged reflected image).

All persons who yield positive Vi agglutination reactions in a dilution of 1:20 are subjected to repeated bacteriological examination; their bile, urine, and feces must undergo bacteriological investigation for detection of bacteria carriers.

It should be kept in mind that diagnostic titres of Vi agglutinins, beginning at a dilution of 1:20, may also be encountered in persons vaccinated against typhoid.

Vi hemagglutination reaction. This reaction is intended to detect Vi antibodies in the serum of persons who are suffering from or have recovered from typhoid. The Vi hemagglutination reaction is based on the fact that sheep and rooster erythrocytes and, still better, human erythrocytes (type O) have a marked capacity for surface adsorption of the Vi antigen of typhoid bacteria and for agglutination of serum containing Vi antibodies.

The advantage of the Vi hemagglutination method over bacterial Vi agglutination lies in the fact that it yields no nonspecific reactions; this is due to the fact that Vi antigens dissolve easily and are rapidly adsorbed on erythrocytes (Landy et al., 1953). The high sensitivity of this reaction facilitates early detection of Vi antibodies in typhoid patients.

The following ingredients are necessary for setting up the hemagglutination reaction: 1) purified Vi antigen or an extract of an erythrocyte-sensitization culture; 2) erythrocytes; 3) Vi and O antisera; 4) the sera to be tested.

The Vi antigen used for treating erythrocytes is a purified chemical preparation of Vi antigen made up from a culture of Ty_2 at the Moscow Scientific Research Institute of Epidemiology, Microbiology, and

Hygiene by Landy's method. It has standard properties, is stable when stored, does not give nonspecific reactions, and has a standard working dose.

Vi antigen solution does not lose its activity when stored in sealed ampules for extended periods; it is best to store the ampules at +4-8°C.

The erythrocytes are washed three times and then centrifuged with 10 volumes of physiological solution for 5 min at 1500 rpm. The supernatant is poured off after each centrifuging and replaced with fresh physiological solution. The washed erythrocytes can be stored in a refrigerator for 2-3 days, but it is best to preserve them with Olsver's solution (2.05% glucose, 0.42% sodium chloride, and 0.55% sodium citrate). Erythrocytes can be kept in this solution for 3-4 weeks.

Before the serum under investigation is titrated in the Vi nemagglutination reaction the working dose of Vi extract required for erythrocyte sensitization is determined; for this purpose 1-2 parts of washed erythrocytes are added to 9 parts of Vi extract in dilutions of from 1:8 to 1:1024. The mixture is held at 37° for 30 min. The erythrocytes are then washed twice with physiological solution (they are centrifuged for 3-5 min at a low centrifuge speed in order to avoid spontaneous agglutination).

The washed erythrocytes sensitized with each antigen dilution are made up into 0.5% suspensions, 0.5 ml portions of which are added to the Vi and O antisera diluted to 1:25-1:50; portions of 0.2 ml of these mixtures are then poured into agglutination test-tubes or clear plastic wells of the type customarily used in virology. The results are evaluated on a four-plus scale after the reaction has been permitted to proceed for 45-50 min at a heater temperature of 37°. The extract titre is assumed to be the last dilution at which +++ agglutination is noted in

the complete absence of hemagglutination in the control. The optimum sensitizing dose of Vi antigen is also established by this preliminary titre. A Vi antigen solution consisting of 10 micrograms of antigen per ml is added to the residue of thrice-washed erythrocytes in a dose of 2 ml of solution per 0.1 ml of residue. The mixture is incubated for 2 hr in a heater at 37°, being agitated every 30 min.

A 0.5% suspension in physiological solution is then prepared from the sensitized erythrocytes, from which the excess Vi antigen has been washed; the subsequent procedure is the same as in working with the extract.

In setting up the hemagglutination reaction with the serum under investigation the antigen is taken in four-fold titre; for example, if the last antigen dilution which yielded positive agglutination to +++ was 1:512, the working dilution is 1:128. This working dilution is used for sensitization of the requisite number of erythrocytes (as determined in terms of serum quantity) for the reaction with the serum under investigation, using the method described above.

The serum to be studied is made up in increasing double dilutions from 1:4 to 1:320. It is inactivated for 30 min at 56° before being diluted. Portions of 0.2 ml of each of the serum dilutions are poured into wells into plexiglass plates or into agglutination test-tubes. Then 0.5 mg of 0.5% suspension of erythrocytes sensitized with the antigen working dose is added to each serum dilution.

The results are evaluated after 2 hr in a heater and again after 24 hr at room temperature.

The following controls are set up for the specific reaction with the Vi extract or Vi antigen: 1) hemagglutination with known Vi or O antisera in dilutions of from 1:4 to 1:320-1:620; the Vi antiserum should yield marked hemagglutination, while the reaction with the O an-

tiserum should be negative; 2) a 0.5% suspension of unsensitized erythrocytes reacted with the least serum dilution; this reaction should be negative.

A suspension of sensitized erythrocytes in physiological solution is used as a control for the absence of spontaneous erythrocyte agglutination.

A positive hemagglutination reaction is characterized by the appearance of a red disk with serrated edges on the bottom of the test-tube. When the reaction is sharply positive (++++) the erythrocytes uniformly cover the entire bottom of the test-tube or the well, the remaining liquid being completely clear; flakes form when the tube is tilted.

When the reaction is negative and in the control the accumulation of erythrocytes on the bottom of the tube or well takes the form of a disk with smooth edges and a smooth surface; a uniform cloudiness is produced by tilting the tube.

Yu.M. Mikhaylova's modification of the indirect hemagglutination reaction for determination of antibodies in Salmonella infections. A total of 0.9 ml of antigen extract is added to 0.1 ml of thrice-washed rooster erythrocytes or group I human blood; the mixture is shaken and placed in a heater for 1 hr, the antigen is poured off, and the erythrocytes are washed once with physiological solution. After the supernatant has been removed a 1% suspension of the erythrocytes is prepared in physiological solution. The patient's serum is diluted in geometric progression, beginning with a dilution of 1:10, and 0.25 ml portions are poured into agglutinative test-tubes, to which 0.25 ml of the 1% erythrocyte solution is added; the mixture is shaken and left to stand at room temperature for 45 min. The results of the reaction are then evaluated.

Antigen extracts prepared by the following methods can be used: 1) acid hydrolyzate: a washed bacterial suspension is treated with 0.4 N acetic acid and then boiled for 2 hr; 2) alkaline hydrolyzate; a washed bacterial suspension is treated with 1N alkali and then heated at 60° for 1 hr.

A simpler method of preparing the antigen extract involves autoclaving of a 70-billion-cell microbial suspension at 1 atm for 20 min; the suspension is then centrifuged and the supernatant is used to set up the reaction. The antigen working dose is preliminarily titrated with standard rabbit serum. The experiment is accompanied by normal erythrocyte, immune and normal serum, and antigen controls.

Antigen-titration scheme. In order to determine the antigen working dose it must be titrated with standard immune rabbit serum. Whole antigen is used for this purpose, the dilutions starting at 1:40. Erythrocytes are then sensitized with the antigens (whole and dilute) and the remainder of the experiment is carried out by the method described above. That dose at which the indirect hemagglutination reaction occurs in highest titre is regarded as the working dose.

Complement-fixation reaction. The complement-fixation reaction has been used as a supplemental diagnostic method for typhoid and paratyphoid by many researchers (see page 154 for a description of the method).

Precipitation reaction. The annular precipitation reaction is a simple, rapid serological diagnostic method and requires no special equipment. Whole antigen from typhoid-paratyphoid bacteria is used (see page 126 for the method).

Opinion differs on the advantages of the precipitation reaction over the agglutination reaction with respect to both sensitivity and specificity and it has consequently not come into wide use in the laboratory diagnosis of typhoid-paratyphoid diseases.

Accelerated bacteriological examination methods

Blinkin's accelerated method for bacteriological examination of blood. Sucrose agar containing 1% glucose is slanted on one side of a flat 200 ml flask and agar containing 1% lactose is slanted on the opposite side; the pH of the medium is adjusted to 7.6 during preparation. Andrade's indicator is added to the sugar media. For culturing 50% bile broth or 50% bile water is added to the flask. No less than 10 ml of the patient's blood is added to the bile and the mixture is then placed in a heater for 12 hr. After removal from the heater the flask is tilted first to one side and then to the other, wetting the surface of the glucose- and lactose-containing agar with the bile-blood mixture. The results are evaluated after one day. If no bacterial growth is present the surface of the sugar media is again wetted and the flask is placed in a heater for an additional 12 hr.

The luminescence-microscopy method with luminescent sera is also an accelerated method (see page 74).

Nutritive Media

Bile broth. A total of 200 ml of native bile (pH 7.6) is added to 800 ml of meat-infusion broth. The mixture is poured into flasks and sterilized in an autoclave at 112° for 20 min.

Rapoport and Vayntrub's medium. A total of 100 ml of bile, 20 g of glucose or mannose, and sufficient acid fuchsin to produce a clear reddish color are added to 1 liter meat-infusion broth; portions of 50-100 ml are poured into flasks with floats. Tubes with a transverse diameter of 0.8-0.9 cm and a length sufficient that approximately 5-10 cm protrudes above the surface of the liquid after culturing are inserted into flasks or, still better, dishes containing 50 ml of this medium; the dishes are then sterilized at 112° for 20 min.

N.N. Klodnitskiy's starvation medium. Portions of 75-100 ml of tap

water are poured into 100 ml flasks and sterilized.

G.P. Kalin's citrate medium. This medium is prepared by mixing 2 g of peptone, 0.5 g of sodium chloride, 2 g of glucose, 4 g of sodium citrate, 10 ml of Sorensen's buffer solution, and sufficient distilled water to make 100 ml; 5 ml are poured into test-tubes and sterilized at 120° for 15 min. Blood is cultured directly in this medium at the patient's bedside, adding 5 ml to the test-tube.

Blinkin's medium for detection of hemocultures. Agar containing 1% glucose is slanted on one wall of small matrices (plane-parallel flasks) with a capacity of 200 ml and agar containing 1% lactose is slanted on the opposite side; the agar is adjusted to pH 7.6 during preparation. Portions of 12-15 ml of the glucose-containing agar are poured into clean flasks. Equal quantities of the lactose-containing medium are poured into separate test-tubes. After the medium in the flasks has been sterilized it is slanted and permitted to cool; the lactose-containing agar is then poured down the opposite side. After the agar in the flasks has solidified 50 ml of sterile bile broth is added. Andrade's indicator (2.5 g of acid fuchsin dissolved in 16 ml of one-normal sodium hydroxide) is added to the sugar media. After the indicator has dissolved sufficient distilled water to make 100 ml is added and the mixture is filtered through cotton. The indicator solution is kept in a brown-glass bottle. A total of 3 ml of the indicator is added to 1 liter of the agar medium.

Muller's medium. Portions of 5 g of chemically pure chalk are weighed out into sterile 200 gram flasks and sterilized with dry heat. A total of 90 ml of any nutritive broth at pH 7.0-7.1 is added to each flask; the mixture is sterilized at 120° for 30 min. The pH of the finished medium is 7.6-7.8.

Before culturing 10 ml of 50% sodium hyposulfite ($Na_2S_2O_3$) and 2

ml of Lugol's reagent are added to each flask and agitated to permit formation of sodium tetrathionite, the principal active component of Muller's medium.

Lugol's reagent. A total of 20 g of iodine and 25 g of potassium iodate are added to 100 ml of distilled water and the mixture is stored in a brown-glass bottle to protect it from light.

Sodium hyposulfite solution. Portions of 50 g of chemically pure sodium hyposulfite ($Na_2S_2O_3$) are weighed out and sufficient distilled water is added to make a volume of 100 ml; the mixture is then transferred to a flask and dissolved over heat, filtered through filter paper, poured into bottles, and sterilized in running steam for 30 min.

Kaufman's medium. This medium is Muller's medium to which is added: a) 0.1% brilliant green (1 ml per 100 ml of Muller's medium) in a quantity such that the final dye concentration is 1:100,000; b) 5% sterile bovine bile, 5 ml of bile per 100 ml of medium. To make up the brilliant green solution a basic 1% aqueous solution of the dye is prepared (by adding 1 g of brilliant green to 100 ml of distilled water) and left to stand for 24 hr in a heater; it is then filtered through filter paper and the working dilution of 1:1000 is prepared from this basic solution.

Shustova's medium. A: 2% meat-infusion agar (pH 7.4); B: 50% hyposulfite solution; C: Lugol's reagent (25 g of iodine, 20 g of potassium iodide, and 100 ml of distilled water).

A total of 10 ml of solution B and 2 ml of solution C are added to 100 ml of the agar (A), which is first melted and cooled to 50°; the mixture is shaken and poured into Petri dishes or slanted in wide test-tubes.

Stern's glycerine-fuchsin broth. A total of 5 or 6 drops of filtered saturated alcoholic basic fuchsin, 2 ml of a freshly prepared 10%

aqueous solution of nonefflorescent sodium sulfite, and 1 ml of glycerine are added to 100 ml of meat-infusion broth. The mixture is poured into flasks and sterilized at 100° for 10-15 min. The cold medium should have a greenish-yellow color. It must be stored in a dark place.

Bitter's rhamnose-containing medium.

1. This medium contains 0.5 g of dibasic sodium phosphate, 1 g of ammonium sulfate, 2 g of tribasic sodium citrate, 0.5 g of sodium chloride, 0.05 g of peptone, 5 g of rhamnose, and 1000 ml of distilled water. The solution is boiled, filtered, poured into flasks, and sterilized fractionally.

2. A total of 2-3 drops of 0.5% alcoholic methyl red is added to a 26-20 hr culture grown on this medium. A yellow color indicates a negative reaction and a red color a positive reaction. S. typhi murium (Breslau) gives a red hue, S. paratyphi B a yellow color in the majority of cases, and S. enteritidis (Gärtner) and S. suipestifer an orange or yellow hue.

Colored media containing various carbohydrates. These media are based on ordinary peptone water (although Hottinger's broth can also be used, in which case the basic solution must be diluted by a factor of 10), which is filtered and sterilized by autoclaving at 120% for 20 min. A total of 0.5-1 g of the appropriate carbohydrate and 1 ml of Andrade's indicator (or any other indicator) are then added to 100 ml of the peptone water; 10 N sodium hydroxide is added to bring the pH to 7.2-7.4 and the mixture is poured into test-tubes with floats (for glucose, lactose, and mannose) or flasks (for storage) and sterilized in flowing steam for 20-25 min on 3 consecutive days or in an autoclave under pressure at 112° for 15 min.

It is possible to avoid the use of floats by making up Hiss' medium from semiliquid agar. For this purpose 0.3-0.5% agar is added to

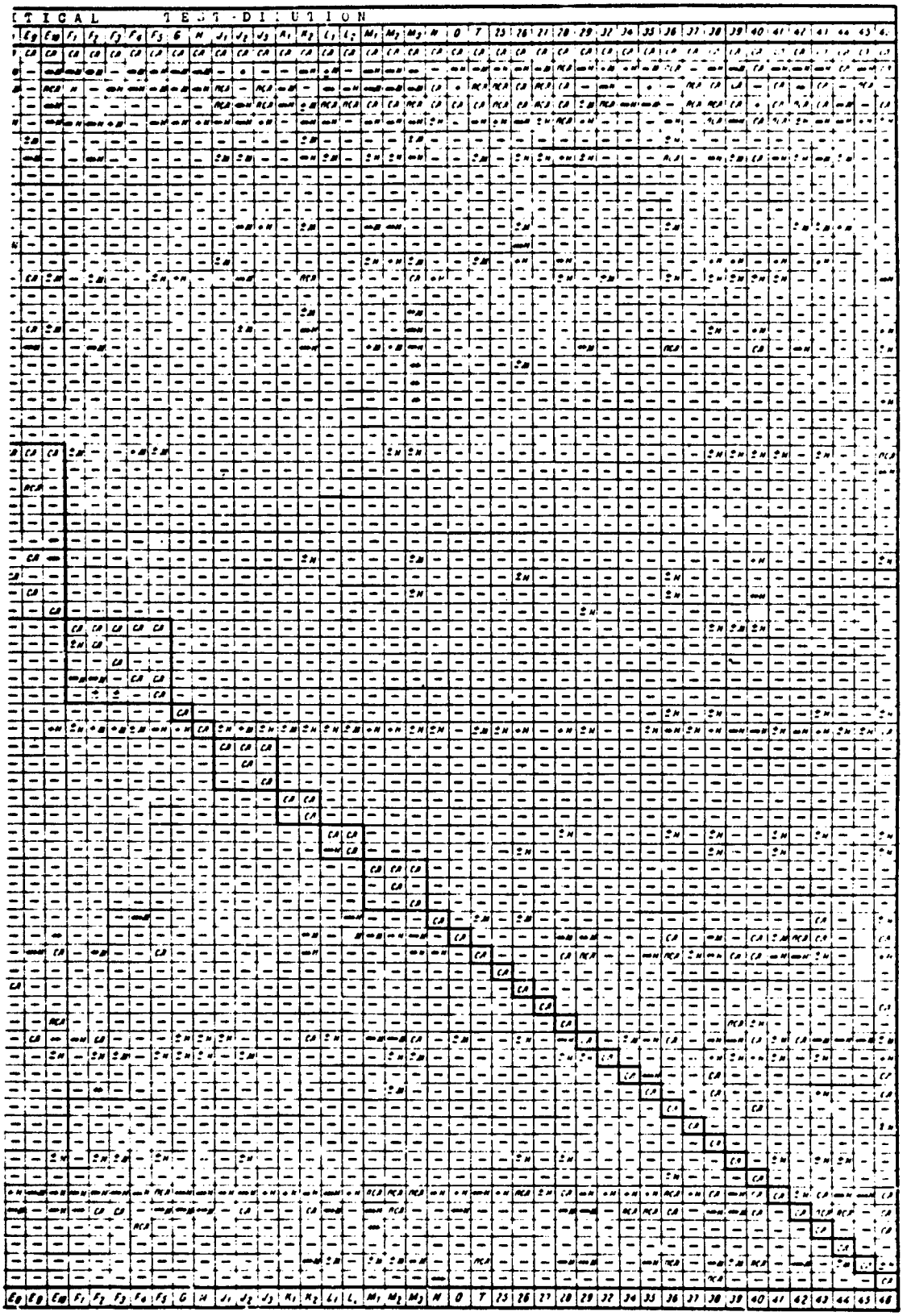
Vi type strain	TYPE PHAGES IN CRITICAL																																							
	A	B_1	B_2	B_3	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	D_1	D_2	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	D_{11}	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	E_{10}	F_1	F_2	F_3	F_4	F
A	CL	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	C
B_1	±N	CA	–	–N	–	–	+	–N	–	+N	–	–	–N	–N	–	–	–	–	–	–	–	–	+N	–	–	–	+N	–N	–	–N	–N	–	–N	–N	–	–	–	–	–
B_2	–N	–	CA	–N	–N	–	–	–	–	–	–	–	–N	–N	–	–N	–N	–	–N	+	–N	–	–N	–	–N	CA	–N	–N	–N	–N	–	RCA	N	–	–N	–N	–N	–	–
B_3	–	–	–	CA	–	–	–	–	–	–	–	–	CA	–N	–	–	–	–	RCA	–	RCA	–	–	–	–	–N	–	–	–	–	–	–N	–	–	–	–	–	–
C_1	+N	–N	–N	–N	CA	CA	CA	CA	CA	CA	CA	CA	CA	–N	–	+N	–N	–N	–N	+N	±N	+N	–	–N	–	±N	–N	±N	+N	+N	–N	–	–N	–N	+N	–	–	–	–
C_1-30	–	–	–	–	–	CA	–	–	–	–	–	–	–N	–	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	±N	–	–	–	–	–	–	–
C_2-30	–	–	–	–	+N	CA	CA	+N	–	+N	±N	+N	–N	–	–	–	–	–N	–	–	–	–	–	–	–	–	–	–N	–	–N	–N	–	–	–N	–	–	–	–
C_4	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C_5	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C_6	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C_7	–	–	–	–	+N	–	–	–N	–N	–N	CA	–N	±N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C_8	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±N	–	–	–	–	–	–	–
C_9	–	–	–	±N	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D_1	–	±N	–	–	±N	CA	–	–	–	–	–	–	–	CA	CA	CA	CA	CA	CA	CA	CA	CA	–	–	RCA	CA	–	–	±N	–	CA	±N	–	±N	–	–	–	±
D_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	±N	±N	–	–	–	–	–	–	–	–	–	+N	–	–	–	–	–	–	–	–	–	–	–
D_4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–N	+N	+N	+N	+N	–	–	–	–	+N	–	–	–	–	–	–	–	–	–	–	–
D_5	–	±N	–	–	±N	CA	–	–	–	–	–	–	–	±N	–	–	CA	CA	–	CA	CA	–	–	±N	–	RCA	–N	–	–	–	CA	±N	–	–	±N	–	–	±
D_6	–	–	–	–	–	RCA	–N	–	–	–	–	–	–	+N	–	–	–	CA	–	–	–	–N	–	–	–	–	–	–	–N	–	–N	–	–	–N	–	–	–	–
D_7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	RCA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D_8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	RCA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D_9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–N	–	–	CA	RCA	CA	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D_{10}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D_{11}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E_1	–	+N	–	–	±N	–	–	–	–	–	–	–	±N	–	–	–	–	–	–	–	–	–	–	CA	CA	CA	CA	CA	CA	CA	CA	CA	±N	–	–	+N	±
E_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E_3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	RCA	–	–	–	RCA	–	–	–	–	–	–	–	–
E_4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–	–
E_5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–
E_6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–
E_7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	CA	–	–	–	–	–	–	–
E_8-N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–	–
E_9	–	–	–	–	–	+N	–	–	–	–	–	–	–	–	–	–	–	–N	–	–	–	±N	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–	–
E_{10}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–	–	–	–
F_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	CA	CA	CA	C
F_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±N	CA	–	–	–	–
F_3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CA	–	–	–
F_4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–N	–N	–	CA	C	–
F_5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	±	–	–	C
G	–	–	–	–	±N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±N	–	–	–	–	–	–	–	–
H	–	+N	+N	+N	+N	–	+N	+N	+N	+N	–	–	+N	–	–	–	–	–	±N	–	–	±N	–	±N	–	–	+N	±N	+N	+N	–	–	+N	±N	+N	+N	±N	±	–
J_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
K_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
K_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M_3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
N	–	–	–	–	–	–	–	–	–	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O	–	–	–	–N	–	–	–	–N	–	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P	–	–N	–N	–	–	–	–N	–N	–	–	–	–	–	–N	–	±N	±N	–N	±N	–	–	–	–	–	–	–	–	–	–	–N	–	–	–	–N	–	–	–	C
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	RCA	–	–	–	–	–	–
29	–	+N	–N	–N	±N	CA	CA	+N	–	–	–	+N	–N	±N	–	–	–	CA	–	–	–	CA	–	–	–	–	–	–	CA	–	CA	–N	–N	CA	–	–	–	–
31	–	±N	±N	±N	+N	±N	±N	±N	±N	±N	±N	±N	±N	–	±N	–	–	–	–	–	–	–	±N	–	–	±N	CA	–	±N	–	–	±N	–	±N	±N	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	+N	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	–N	–	–	–	–
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
39	–	±N	–	–	–	–	–	–	–	–	–	–	±N	–	–	–	–	±N	–	–	–	–	±N	–	–	–	–	–	–	–	–	±N	–	±N	±N	–	–	–
40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
41	–	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	–N	+N	+N	–N	–	+N	–N	+N	+N	–N	+N	–N	–N	–N	–N	–N	–N	–N	–
42	–	–	–	RCA	–	–	–N	CA	–	RCA	RCA	CA	CA	–	–	–	–	CA	–	–	–	CA	–N	–	–	–	–N	–	CA	–N	–	–N	CA	CA	–	–	–	–
43	–	–	–	–N	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
45	–	–	–	–	–	–	–	CA	–	–	–	–	–N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–N	–	–	–	–	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	A	B_1	B_2	B_3	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	D_1	D_2	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	D_{11}	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	E_{10}	F_1	F_2	F_3	F_4	F

SL – continuous lysis; PSL – semi-continuous from ± to ±N-increasing numbers of plaques; N – plaques visible lysis;

A

To article by W. D. Krylova (page 328)

TABLE 18a



s; M – plaques visible to naked eye; M – plaques visible only through a magnifying glass.

B

the peptone water before sterilization, the mixture is boiled and sterilized as indicated above, and the carbohydrate and indicator are then added. The final medium is sterilized in the same manner as any liquid medium. This semiliquid medium retains gas bubbles, which are especially obvious during the first few days, when the gas has still not escaped into the atmosphere.

Christensen's citrate medium (to replace Molcke's litmus). This medium consists of 3 g of sodium citrate, 0.2 g of glucose, 0.5 g of yeast extract, 0.1 g of cysteine hydrochloride, 1 g of monosubstituted potassium phosphate, 5 g of sodium chloride, 0.012 g of phenol red, 15g of agar, and 1000 ml of distilled water. Its pH is not adjusted. It is sterilized for 15 min at 1 atm.

Preparation of medium. All the salts are first dissolved in small quantities of water and then mixed with the agar and left to stand until the latter has swollen. The resultant suspension is boiled until the agar dissolves, filtered, and mixed with 0.4% phenol red (3 ml per liter of medium).

Preparation of yeast extract. A total of 500 g of baker's yeast is dissolved in 1 liter of water, boiled for 1 hr, and then filtered through filter paper. The extract is sterilized for 30 min under a pressure of 0.5 atm. It is then added to the medium (20 ml per liter of medium).

Medium for observation of formation of mucoid spindle. A total of 3.5% of agar, 1% of peptone, and 1% of litmus are added to 100 ml of distilled water and the mixture is sterilized at 120° for 20 min; spindle formation is observed after 18 hr of incubation at 37° or after 3 days at room temperature.

Medium for observation of creep. A 20% meat-infusion gelatin is made up and poured into test-tubes; the tubes are sterilized in a flow-

ing-steam apparatus for 15 min on 3 consecutive days and slanted during cooling.

Phage Typing of Typhoid Bacteria

M.D. Krylova, Candidate of Medical Sciences

This method is based on the fact that cultures react differently to Vi phage of serotype II.

The Vi-II phage was isolated from a lyzogenic culture of Salm. typhi and has the ability to adapt easily to resistant individuals, losing its activity with respect to the original strain. Cregy and Yen obtained all their preparations of type phages by adaptation. They also used nonadapted Vi typhoid phage of serotypes I, III, IV, V, VI, and VII, as well as a number of O and Vi phages for additional subdivision of types A, E1, etc.

This classification system has now been perfected and standardized. The standardization is based on the type phages distributed by the International Typing Center in London. The typing technique has also been standardized.

The list of Vi type phages and strains is growing continuously. According to the latest reports, there are now 72 such strains. The phages and strains are designated by Roman letters and numerals. Type phages are used in critical test dilutions (CTD); this dilution is assumed to be the maximum tenfold phage dilution which produces continuous lysis of the homologous strain on a solid medium. The homologous strain is that culture in which a given phage reproduces; strains of other types are referred to as heterologous. The necessity of diluting the phage results from the fact that concentrated phage may nonspecifically (without reproduction) lyze heterologous phagotypes on a solid medium. The technique has been standardized by the International Association of Microbiologists.

Table 18a shows the activity of type phages in their critical test dilutions with respect to homologous and heterologous strains (data obtained by the International Center for phagotyping of coliform bacteria during October-November 1960).

The scheme shown is standard. All the phages are serologically identical and are variants of Vi-II phage type A. The differentiation of groups B, C, D, E, F, etc., into subtypes is based on the fact that strains bearing the number 1 (E1, D1, C1, etc.) are clearly dissolved (by continuous lysis) by the homologous phage and phages of all other subtypes of the group in question. Strains of the remaining subtypes are usually dissolved only by homologous phages.

Up to 80-100% of typhoid cultures have now been typed with standard phages in the majority of countries. Cultures whose types cannot be determined can be divided into 3 groups.

The first group comprises strains which lack the Vi antigen (are not lyzed by Vi phages and are not agglutinated by Vi serum). They are designated as the W form.

The second group comprises cultures having the Vi antigen which are resistant to all the preparations of Vi-II phage employed. They are referred to as untypable Vi strains, imperfect forms, or group Vi-I-Vi-IV, since they are sensitive to phages of serotypes I and IV. There may be new, as yet unknown phagotypes among the untyped cultures.

The third group comprises polylyzable or degraded Vi strains, which are dissolved by several type phages. Prolonged storage often converts these strains to type A and they can be lyzed by all type phages.

For successful typing of polylyzable cultures it is best to send strains isolated from no less than 10-12 colonies to the laboratory, since some of them may retain the traits of the initial phagotype.

Individual types (A, C1, E1) are quite often encountered only with-

TABLE 19

Subdivision of Phagotype A According to Nicol, Diverno, and Bro (1958)

	a Штаммы	b Фаги							
		1	2	3	4	5 (Vi-фаг) c	6	7	8 (фаг d Vi-IV Крейги)
I группа e	Коннльхетиль (Coquilhatville)	сл	сл	—	<сл	±	<сл	—	±
	Монреаль (Monreal)	сл	—	сл	—	сл	сл	сл	сл
II группа	Тананарив (Tananarive)	—	сл	сл	сл	сл	сл	±	сл
	Дуала (Douala)	—	сл	сл	сл	сл	сл	—	—
	Шамбли (Chamblee)	—	сл	—	сл	сл	сл	±	сл
	Вельшпул (Welshpool)	—	сл	сл	сл	сл	—	—	сл
III группа	Освестри (Oswestry)	—	—	сл	сл	сл	сл	сл	сл
	Леопольдвиль (Leopoldville)	—	—	сл	—	сл	сл	—	сл
IV группа	Маракайбо (Maracaibo)	—	—	—	±	сл	сл	—	сл

Note: sl indicates continuous lysis, ± indicates a nonuniform reaction, and − indicates no reaction.

a) Strains; b) phages; c) Vi phage; d) Cregy's Vi-IV phage; e) group.

in a single country. Some of these phagotypes can be subdivided in accordance with biochemical characteristics (reaction with xylose, arabinose, sodium citrate, and sodium and potassium d-tartrates).

Phagotypes can often be differentiated with bacteriophages. There are two proposed schemes for additional subdivision of type A. Deranlo and Martin divide type A into three subtypes − common, AУ, and AФ. The strains of each group react differently to unadapted Cregy Vi phages of types I, III, and IV and Deranlo and Martin Vi phages of types V and VI. The common type A reacts with all 5 phages, subtype AУ reacts only with

phages of serotypes I, IV, and V, and subtype AΦ reacts only with phages of serotypes III, IV, and VI.

TABLE 20

Nicol and Diverno's Scheme

a Подтип	b Бактериофаг							
	a	b	c	d	e	f	g	h
1	сл	сл	сл	++++++	сл	сл	+++	сл
2	»	»	»	++++++	»	—	»	»
3	»	»	—	++сл	—	сл	сл	»
4	++	—	—	»	—	сл	+	—
5	—	—	—	—	—	—	сл	++сл
6	—	—	++	—	—	—	—	»
7	—	—	—	—	—	сл	—	—
8	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—
13	—	—	—	—	—	—	—	сл

a) Subtype; b) bacteriophage.

A second scheme for the additional subdivision of type A has been proposed by Nicol, Pavlato, Diverno, and Bro. Using eight phages, these authors subdivided the phagotype in question into 9 subtypes, which they named after the areas where they were isolated (Table 19).

It may be seen from Table 19 that the subtypes are divided into 4 groups, in accordance with their reactions with phages, 1, 2, 3, and 5. There is a group phage for each of these categories. Thus, phage 1 is specific for group I (the Coquilhatville and Monreal subtypes), phage 2 is specific for group II (the Tananarive, Douala, Chamblee, and Welshpool subtypes), phage 3 is specific for group III (the Oswestry and Leopoldville subtypes), and phage 5 is specific for group IV, which includes only one subtype, Maracaibo. The subtypes within the groups differ in their reactions with 7 "indicator" phages.

Phagotype El can be divided into 2 subtypes, Ela and Elb, which are stable for all practical purposes. Brandis set up this classification with the aid of a phage designated as Vi-VII (originally 1 Ar),

which differs serologically from all other known Vi phages. The Vi-VII phage was isolated from sewage. In its critical test dilution it dissolves only some of the strains of phagotype E1 (subtype E1a), failing to react with the remainder (subtype E1b). Vi phages of serotypes I and IV also lyze only subtype E1a, while E1 and E2 phages lyze both subtypes.

Untyped Vi cultures (group Vi-I-Vi-IV) can be differentiated with the set of O and Vi phages used to subdivide type A.

A special typing scheme has recently been proposed. It utilizes killed phages isolated from cultures of group Vi-I-Vi-IV in A subtypes (Tananarive) or Salm. dublin. Using 8 such bacteriophages, Nicol and Diverno subdivided strains of group Vi-I-Vi-IV obtained from different areas and countries into 13 subtypes (Table 20).

Cultures of identical phagotypes, untyped Vi cultures, polylyzable cultures, and W cultures can also be subdivided in experiments to determine lyzogenecity. This test is based on the fact that epidemiologically related strains are not lyzogenic for one another; only a culture from another nidus can be used to demonstrate this property. The appearance of lyzogenicity in a pair of cultures of identical phagotypes shows that they are epidemiologically nonhomogeneous.

Preparation of type phages and determination of critical test dilution

Type phages reproduce in bacteria of homologous phagotypes. Any strain of typhoid bacterium which is rich in Vi antigen and capable of retaining it can be used for the reproduction of other Vi phages (I, III, IV, V, VI, VII). O phages multiply in strain O-901, which contains no Vi antigen.

The type phage to be prepared must be purified. For this purpose Petri dishes containing pure agar are inoculated with 6 or 7 drops of a day-old broth culture of the homologous strain. The culture is

spread over the surface of the agar in such fashion that it is continuous. When the culture has dried drops of several phage dilutions are applied to it to produce isolated sterile spots. The culture is placed in a heater at 37-38.5° for 16-18 hr. Negative phage colonies ("plaques") are transferred to broth-containing test-tubes together with the underlying agar and surrounding culture; the tubes are incubated for 3-4 hr and then heated at 57° for 40 min (or filtered) and the phage is retitrated on a solid medium with the corresponding type strain. This procedure is repeated 3 or 4 times (the negative phage colony isolated from the last dilution is recultured on broth, etc.) to obtain a pure strain of the phage.

The type-phage preparations are made up directly in the necessary stock volume. The highest phage titre is obtained when the phase and bacteria concentrations are equal. A total of 10^8 bacteria in the logarithmic-growth phase and approximately the same number of homologous phage particles are introduced into 100 ml of broth preliminarily heated to 37°. The mixture is then incubated at 38.5° until the broth has been completely clarified, with a maximum of $7\frac{1}{2}$ hr. The lyzate is heated at 57° for 40 min and centrifuged at 3000 rpm for 20-30 min to remove the killed bacteria. The preliminary critical test dilution is then determined on a solid medium: the phage is titrated in ten-fold dilutions with the homologous strain and with phagotype A.

If it proves impossible to obtain a satisfactory critical test dilution of the phase in the liquid medium, it is helpful to employ Grazia's agar-layer method. A total of $4.0 \cdot 10^8$ bacteria is mixed with type-phage particles in 3 ml of semiliquid 0.45% agar; the phage dosage is selected empirically and should equal the least number of particles which produces continuous lysis with the aforementioned quantity of bacteria. These conditions ensure a maximum number of phage-reproduction

cycles, leaving a minimum number of bacterial cells intact. The phage-bacteria mixture is poured onto a layer of ordinary agar and the culture is incubated at 38.5° for 16 hr. A total of 6 ml of broth is then added to each of the dishes, the surface layer of the agar is scraped off, and the mixture of broth and semiliquid agar is transferred to a 20 ml flask, which is heated at 57° for 40 min and centrifuged. This produces a phage suspension containing up to 10^{11} particles per ml.

After the phage has been obtained in sufficient strength a final determination is made of the critical test dilution. The preliminary critical dilution and the two following dilutions are tested on all known phagotypes.

There is some difficulty in determining the critical test dilutions of type phages which produce extremely small sterile spots in homologous phagotypes and large spots in certain heterologous phagotypes. As is well known, for continuous lysis a drop of phage should yield approximately 100 large negative colonies and more than 1000 small colonies (visible only under a magnifying glass). A small-spot-producing phage in a dilution sufficient to cause continuous lysis with a homologous culture will consequently similarly lyze heterologous phagotypes, yielding large spots. In order to avoid this, the critical test dilution of such phages is assumed to be that dilution which yields about 100 sterile spots with the homologous phagotype.

Typing method

Media. Ordinary nutritive media are used for the phage typing of coliform bacteria, changing only the concentrations of individual ingredients. It is easy to obtain comparable results on standard dry nutritive media. The following formula is recommended for liquid media: 20 g of dry nutritive broth (Difco), 8.5 g of sodium chloride, and 1000 ml of distilled water. The mixture is sterilized at 120° for 25

min and has a final pH of 6.8. Adjustment of the pH with alkalies is not recommended. In order to prepare a solid medium 1.3-1.5% of high-quality agar is added to the liquid medium. When Soviet-produced dry nutritive agar is used 4 rather than 5 g of powder is added to 100 ml of water, the remainder of the procedure being handled in the manner indicated on the packing slip.

This medium ensures standard typing results, although they are not always sufficiently clear. More intensive culture and phage reproduction can be achieved in trypsin-digested media prepared in the laboratory. In trypsin digestion by Hottinger's method no less than 500 g of fresh meat is added to 1 liter of water. It is necessary to avoid excessive use of sodium hydroxide in adjusting the pH, which should be 7.5. The residue is removed, since an excess of phosphates inhibits lysis. In order to prepare a solid medium 1.3-1.5% of agar is added to the broth. Each new batch of this medium must be compared with the preceding batch, typing phagotypes N, O, and T in it. The medium is ready for use if critical test dilutions of the type phages produce distinct small sterile spots in these phagotypes.

It is useful to add 0.2% of sodium sulfate to the agar for typing cultures which produce dwarf colonies, since they grow normally on this medium. Addition of 5% of glycerine to the medium increases the size of negative phage colonies, but simultaneously causes a rise in the number of cross-reactions with heterologous phagotypes and is consequently contraindicated.

Phages of the same type from different production series are invariant only with respect to continuous lysis. Weaker reactions (from $\pm$ to +++) are not as consistent and cannot be regarded as diagnostic; the strains with which the reactions appear vary from series to series.

The phages are stored at 4°. Undiluted preparations retain their

titre for 8-10 years. Critical test dilutions remain stable for several months at the aforementioned temperature.

Typing technique

For typing typhoid cultures a laboratory should have a set of Vi-II phages for all presently known phagotypes, Vi-I, Vi-III, Vi-IV, Vi-V, Vi-VI, and Vi-VII phages, and adsorbed typhoid Vi and O sera. It is also desirable to have the phages used for additional subdivision of phagotypes and standard type strains of the known phagotypes.

Portions of 20 ml of agar are poured into Petri dishes and dried uncovered in a heater. Squares or sectors corresponding to the number of phages used are drawn on the bottoms of the dishes and the designations of the phages are recorded. It is convenient to mark the dishes with a rubber stamp bearing a grid-like design, which is wetted and placed on a surface impregnated with glass-marking ink. With practice it is possible to avoid writing any information on the bottoms of the dishes, provided that the phages are used in a predetermined order previously noted on a sheet of paper. A counterclockwise spiral is drawn on the bottom of each dish; when viewed through the agar layer it appears to run clockwise. A straight line at the beginning of the spiral indicates the point where the first phage in the series is to be applied. It is impossible to apply one drop of phage on top of another, since the first phage is not permitted to dry before the second is applied and is thus clearly visible. In evaluating the results all the drops of phage are visible in relief against the velvety matte background of the growing culture.

Phages of rarely encountered phagotypes are best employed in mixtures of 4 or 5 phages. Separate typing is carried out when lysis occurs where the mixture has been applied. The following phage-application order is recommended: A, A in CTD × 20, B1 [B2, B3], C1 [C2, C3, C4,

C5], [C6, C7, C3, C9], D1, D2, D4, D5, D6, [D7, D8, D9, D10, D11], E1, E2, [E3, E4, E5, E6, E7, E8, E9, E10], F1, F2, [F3, F4, F5], [G, H, I1, I2, I3], [K1, K2], [L1, L2, M1, M2, M3], N, O, T, [25, 26, 27, 28], [29, 32, 34, 35, 36, 37], [38, 39, 40, 41, 42], [43, 44, 45, 46], Vi-I, Vi-IV, Vi-VII and O.

The phages indicated in brackets are mixed with one another, each phage being taken in its critical test dilution.

Phage A is the most specific, lyzing only phagotype A in its critical test dilution. Phagotypes which have lost their specificity acquire a sensitivity to this phage. In order to detect such degradation phage A is employed in its critical test dilution and in a concentration 20 times that of the critical dilution.

Vi phages of other serotypes and O phages in critical test dilutions are used in addition to Vi-II phages. Inclusion of these phages serves a three-fold purpose. Firstly, cultures which are not broken down by Vi-II phages may yield distinct reactions with Vi and O phages and can thus be differentiated. Secondly, lysis by Vi phages in critical test dilution is specific for S. typhi and shows its typhoid character. Thirdly, Vi and O phages aid in the further subdivision of phagotypes A, E1, C1, etc., which are dominant in the microflora of a given city or republic. It is also recommended that undiluted Vi-1 phage be used as an indicator for Vi antigen. For simplicity, the designations of the phages and mixtures in the dishes can be replaced by arbitrary numbers.

It is best to type young broth cultures, since they contain the greatest quantity of Vi antigen and consequently adsorb Vi phages intensively. The cultures to be studied is added to 2 ml of undiluted Hottinger's trypsin-digested medium or Martin's peptone until a barely visible cloudiness appears. The mixture is kept in a heater at 38.5° un-

til the cloudiness roughly corresponds to a content of $5 \cdot 10^8$ bacteria per ml, which requires 2-2½ hr. The surface of the agar is then inoculated with 7 or 8 drops of the culture to be typed, distributing them uniformly over the dish with gentle perpendicular movements of the spatula. The dishes are left uncovered to dry and drops of the diagnostic phages are placed on the dry culture surface. It is most convenient to employ Pasteur pipettes for this purpose, using a separate pipette for each phage. In order to keep the drop from spreading a small quantity of phage is drawn up into the capillary of the pipette and applied by gently touching the drop to the surface of the agar. After the drops have dried the covered dishes are turned bottom up and incubated at 38.5°, the optimum temperature for typing. The minimum optimum temperature is 33°, since there is a tendency toward an increased number of cross-reactions when the temperature is further reduced.

Evaluating typing results

The results are first evaluated after 6-7 hr of growth. In emergency cases this makes it possible to report the typing results on the same day that the cultures are taken. The second and final evaluation

TABLE 21

Anderson's Arbitrary Symbols for Recording Results

1 Размер негативных колоний	2 Число негативных колоний	3 Лизис
Б — большие 4	— 0 — 5 8	ПСЛ — полусливающийся лизис
Н — нормальные 5	± 6 — 20	9 СЛ — сливающийся лизис
М — мелкие 6	+ 21 — 40	
ОМ — видимые только с лупой 7	± ± 41 — 60	< ПСЛ / < СЛ промежуточные степени лизиса
	+ + 61 — 80 10	
	+ ± + 81 — 120	
	+ + + > 120	МЛ — сливающийся мутный лизис (мутность вызвана вторичным ростом) 11

1) Size of negative colonies; 2) number of negative colonies; 3) lysis; 4) large; 5) normal; 6) small; 7) visible only under magnifying glass; 8) semicontinuous lysis; 9) continuous lysis; 10) intermediate lysis; 11) continuous cloudy lysis (cloudiness caused by secondary growth).

is made after 18-24 hr.

The lysis reactions are read with the unaided eye through the bottom of the dish, using direct or indirect illumination and employing one hand as a slowly moving screen between the light source and the dish. It is best to evaluate the results in daylight. All doubtful or negative reactions are checked with a 7-10 power hand magnifying glass.

Anderson recommends the arbitrary symbols shown in Table 21 for recording the results.

Determination of phagotype presents no difficulties for a strain in normal condition, provided that the critical test dilutions of the phages are made up correctly and that their activity conforms to that shown in the table. Examples of characteristic positive typing reactions are shown in Fig. 25.

The following difficulties may be encountered in typing:

1. The culture is not lyzed by adapted Vi-II phages but is sensitive to other Vi phages. Figure 26 shows this type of reaction, which may be caused by either of the following factors:

a) The culture is a new type. In this case it must be tested with phages of the rarer types or Vi-II phage (type A) must be adapted to it. The new phage must be checked in its critical test dilution against all the standard phagotypes. Selective lysis with the culture to which the phage was passivated and lack of lysis with standard strains confirms that a new phagotype has been isolated;

b) the culture is an imperfect form and does not sustain reproduction of Vi-II phage. Some cultures of this variety can be typed from their lysis reactions with O phages and with unadapted Vi phages of serotypes I, III, IV, V, VI, and VII, as well as with the special set of phages proposed by Nicol and Diverno.

2. The culture undergoes continuous or semicontinuous lysis by

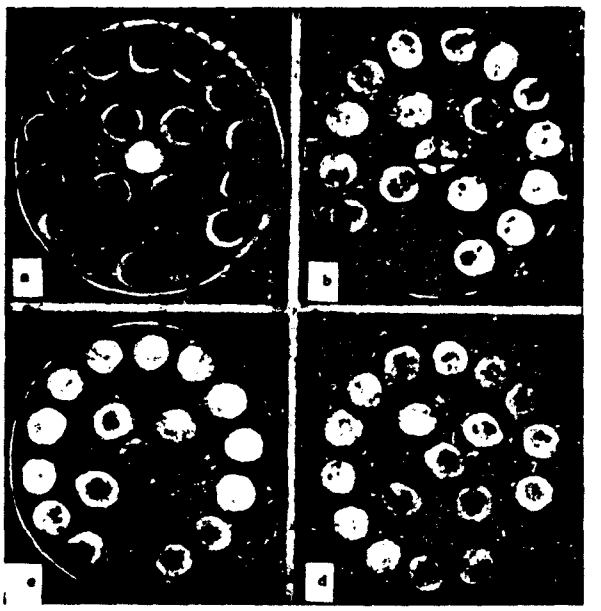


Fig. 25. Reactions of phagotypes A, F_1, F_2, and I of S. typhi with adapted Vi-II phages in critical test dilution. a) Type A; b) type F_1; c) type F_2; d) type I (after Anderson and Williams).

several adapted Vi-II phages. Such cultures are known as polylyzable or degraded Vi strains. Figure 25 illustrates the reactions of such a strain. The culture is clearly lyzed by phage type A, which shows that it is degraded. The reactions with Vi-II phages, which are usually constant, can be used to recognize and combine epidemiologically similar strains. It is permissible to refer to the aforementioned cultures as phagotype A, indicating the gaps in their lytic reactions. Among the polylyzable cultures special mention must be made of strains which are lyzed by phage N and individual phages of group D (the so-called D_3 or $N+D_1$ phagotype). Certain strains of phagotype N may yield this reaction when typing is carried out at a low temperature (36-37.5°). Incubation of the dishes at 38.5° reduces the number of nonspecific reactions with D phages and the strains are classified as normal type N.

3. The culture is weakly lyzed by several type phages, strongly

lyzed by unadapted Vi phages, and is insensitive to phage A. This may be caused by a decrease in the size of the phage colonies in an atypical phagotype variant. The homologous phage in critical test dilution yields continuous lysis when the sterile spots are of normal size. The decrease in plaque size causes continuous lysis to fail to appear at the critical test dilution, thus producing doubtful results. In such situations it is recommended that the typing be repeated with the phages in a concentration 10 times the critical test dilution.

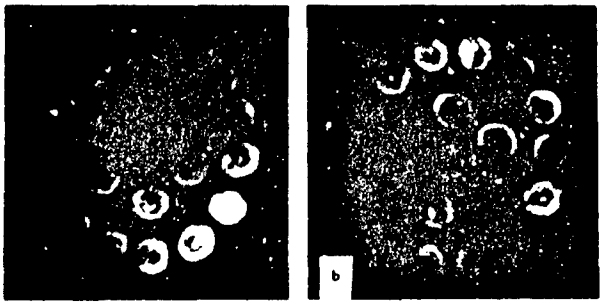


Fig. 26. a) Untypable Vi culture of S. typhi (lysis occurring with a mixture of Vi-I and Vi-IV phages); b) degraded Vi culture.

In extreme cases, where the strain cannot be clearly differentiated with any of the standard phages, it is possible to employ so-called indirect typing. Vi-II phage is adapted to the strain to be typed and the new phage is tested with all the standard type strains. The strain under investigation is regarded as identical to the phagotype which corresponds to the new phage preparation. If the new phage is lytically inert with respect to the standard phagotypes the type phage of the phagotype which has been isolated is missing from the laboratory's set of phages.

4. The culture is not lyzed by any of the Vi phages in critical test dilution, but is sensitive to concentrated Vi-I phage. This may be caused by poor development of the Vi antigen in the culture, which ham-

pers its adsorption of the Vi type phages. An attempt can be made to type the culture with more concentrated phages (one order of dilution less than the critical test dilution).

The following techniques can be employed to increase the Vi-antigen content of the culture:

a) culturing the strain on 1.3-1.5% agar, followed by selection of colonies which appear cloudy in transmitted light and agglutinate well on slides with commercial adsorbed typhoid Vi serum. A total of 40-50 isolated colonies from different subcultures must be checked.

b) Passage through broth containing 5% typhoid O serum for 6-18 hr, followed by transfer of the culture from the upper broth layer to dishes containing agar and selection of colonies with Vi serum.

c) Passage through white mice. A day-old culture raised on ordinary broth is diluted to 1:20 and 0.5 ml of this dilution is injected intraperitoneally. The mouse is killed with chloroform after 60 hr. Agar is inoculated with blood from the heart and the exudate from the abdominal cavity, which is washed out with physiological saline solution. No fewer than 20 colonies are checked for Vi antigen in rough agglutination tests.

5. The culture is not broken down by any of the Vi phages in critical test dilution, including undiluted Vi-I phage. This means either that the culture contains no Vi antigen at all or that it is not typhoid in character. Strains found in the pure W form usually do not regain their Vi antigen. They are typed with O phages or biochemically if necessary.

It must be kept in mind that clones of the same culture may differ from one another in their content of Vi antigen and the extent of their specificity. It is consequently helpful to repeat the typing with other clones when the reactions are unclear. It is desirable to send a mix-

ture of 10-12 colonies of the initial culture for typing, especially if they have been isolated from a concentration medium.

It is preferable to store cultures on Dors' egg medium in darkness at 4° after minimal incubation at 37-38°. Individual strains exhibit a tendency toward loss of some of their Vi antigen during storage, so that it is helpful to rejuvenate them by culturing them on agar and to select clones rich in Vi antigen by the method described above. Colonies which yield clear Vi agglutination are transferred to Dors' medium and stored in the usual manner.

Increasing Phage-Titre Reaction

Prof. D.M. Gol'dfarb

Indicator phage. D.M. Gol'dfarb and Z.S. Ostrovskaya obtained a typhoid indicator phage by intensive passivation.

Production method. The following ingredients are necessary to obtain the phage.

1. Polyvalent typhoid phage, which reacts with typhoid cultures containing Vi antigen.

2. A day-old culture of S. typhi strain Ty-2 raised on a meat-infusion agar slant. Ty-2 cultures are capable of retaining their Vi antigen, so that culturing and selection of colonies which agglutinate with Vi serum on slides is usually sufficient for selection of a full-valued subculture (if the Vi-antigen content is reduced).

3. Meat-infusion broth at pH 7.0.

A total of 0.5 ml of the polyvalent phage, which is preliminarily titrated by Grazia's two-layer method, and 0.1 ml of the culture, which is washed from the agar slant with 5 ml of physiological saline solution, are added to 4.5 ml of the meat-infusion broth. The mixture is kept in a heater at 37° for 1 hr. It is then heated at 58° for 30 min and 0.5 ml is poured from test-tube No. 1 into test-tube No 2 (second

passage), which contains 4.5 ml of sterile meat-infusion broth to which 0.1 ml of the culture has been added. Passages of this type are made over a period of 5 days (6 days for certain phages), with one-hour incubations in the heater, to produce a high-specificity typhoid phage (which has been arbitrarily designated BF5). The 5th culture is checked for specificity by the usual method, applying drops to a solid medium (meat-infusion agar). The specificity of the phage increases as it is passivated; the 5th and 6th passages usually reinforce the passivation.

The other indices of this phage also satisfy the requirements imposed on indicator phages. Its 5-min adsorption on strain Ty-2 amounts to 80-90%, while its latent period is 12-15 min. It is thermostable, not being inactivated by heating at 58° for 30 min.

The increasing phage-titre reaction is set up in the same manner for typhoid as for dysentery. Strain Ty-2 serves as the standard culture. The results of the reaction are evaluated on the following scale:

\+ rise in phage titre by factor of 2.5;
++ rise in phage titre by factor of 3-5;
+++ rise in phage titre by factor of more than 5;
++++ rise in phage titre by factor of more than 10.

SALMONELLOSIS

L.M. Margorina, Candidate of Medical Sciences

Morphology and biology of Salmonella; cultural characteristics

Salmonella (which cause food poisoning) are short, labile, gram-negative rods; they do not ferment lactose, usually decompose glucose, mannose, and maltose to form an acid and a gas, and produce hydrogen sulfide.

An expanded composite series containing a fuller set of carbohydrates and polyatomic alcohols is used for more careful differentiation of the individual members of the Salmonella group (see Table 24).

In the laboratory identification of Salmonella it is necessary to take into account possible changes in biochemical properties, which are produced principally by the action of antibiotics and other drugs. Thus, in addition to lactose-negative strains, there are variants which ferment lactose to form an acid and variants which do not ferment mannose and do not form a gas during fermentation of glucose and mannose, yet retain the other characteristics of bacteria of the Salmonella group.

In order to check the uniformity of their colonies, such strains are cultured on differential media and subcultures are then subjected to more thorough study by the method used for identification of Salmonella. It is sufficient to conduct a number of passes through 10% bile broth, Hottinger's broth, and agar slants to restore the initial biochemical properties of atypical cultures.

Antigenic structure; serotypes

The antigenic structure of Salmonella (see page 291) served as the basis for the classification compiled by White and Kaufman. This classification system utilizes the differences in the structure of the O antigens, which make it possible to divide the Salmonella into a number of serological groups (A, B, C, D, E, etc.). The differences in the H antigens, both specific and nonspecific, make it possible to differentiate types within the individual groups (see Table 24). More than 500 types and 14 serological groups of Salmonella differing in their O antigens have now been listed.

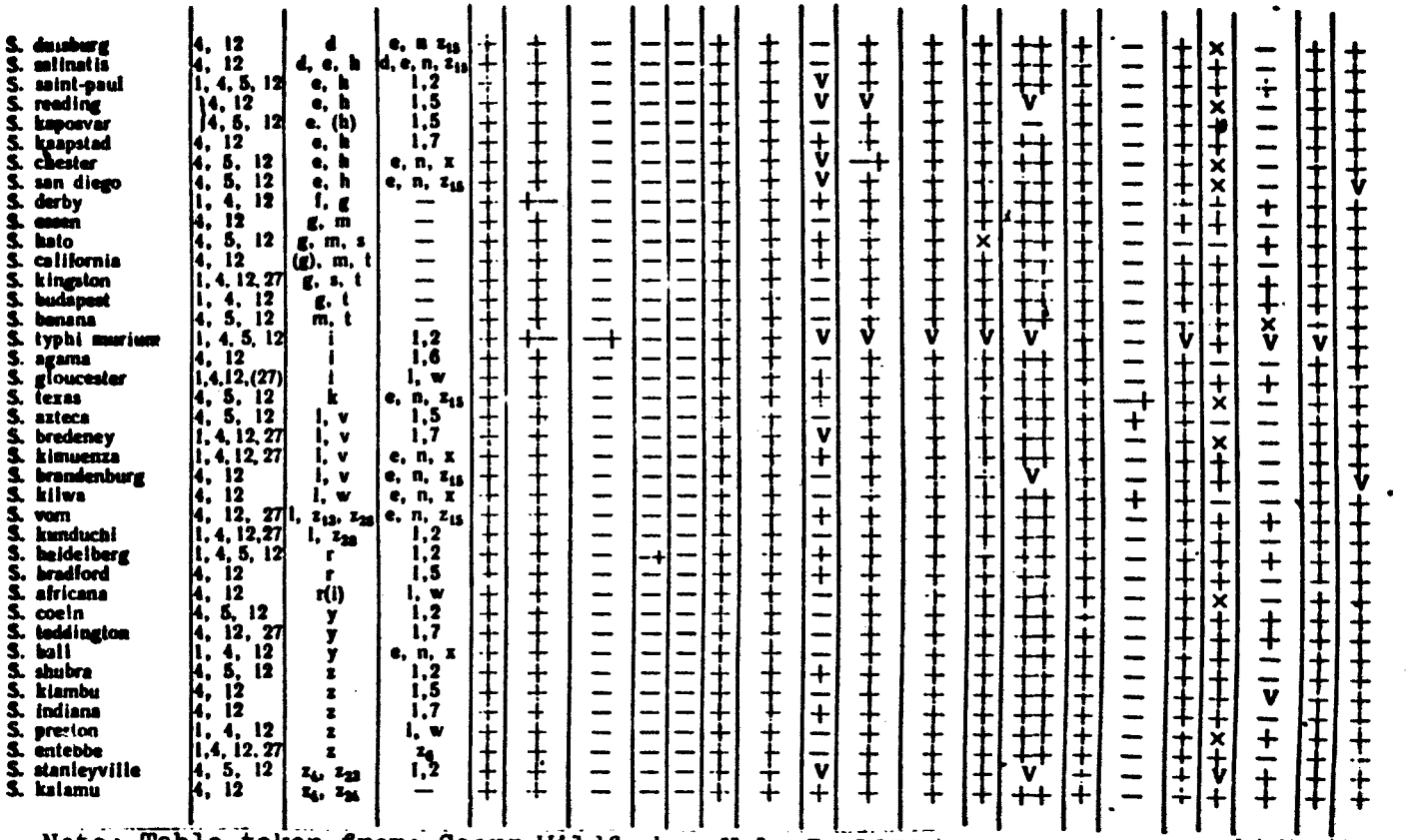
The continuous increase in the number of serological types of Salmonella is in all probability associated with the change in their antigenic structure produced by different living conditions (adaptation to the environment and to animals and man).

Unadsorbed specific OH sera (typhoid, paratyphoid A, paratyphoid B, Gertner paratyphoid enteritis, Breslau paratyphoid [typhi murium], and

paratyphoid Suipestifer [cholerae suis]) can be used for rough agglutination on slides. A mixture of these sera diluted to 1:20 can also be employed. When a positive agglutination reaction is obtained on slides it is necessary to set up an expanded agglutination reaction, which makes it possible to determine whether the culture under investigation belongs to the Salmonella group. In order to determine the group and type of Salmonella to which a given culture belongs it is necessary to conduct a receptor analysis, using O and H monoreceptor sera for agglutination on slides (see Table 24); a mixture of O monoreceptor sera can also be employed, subsequently differentiating the cultures into individual O serological groups and types.

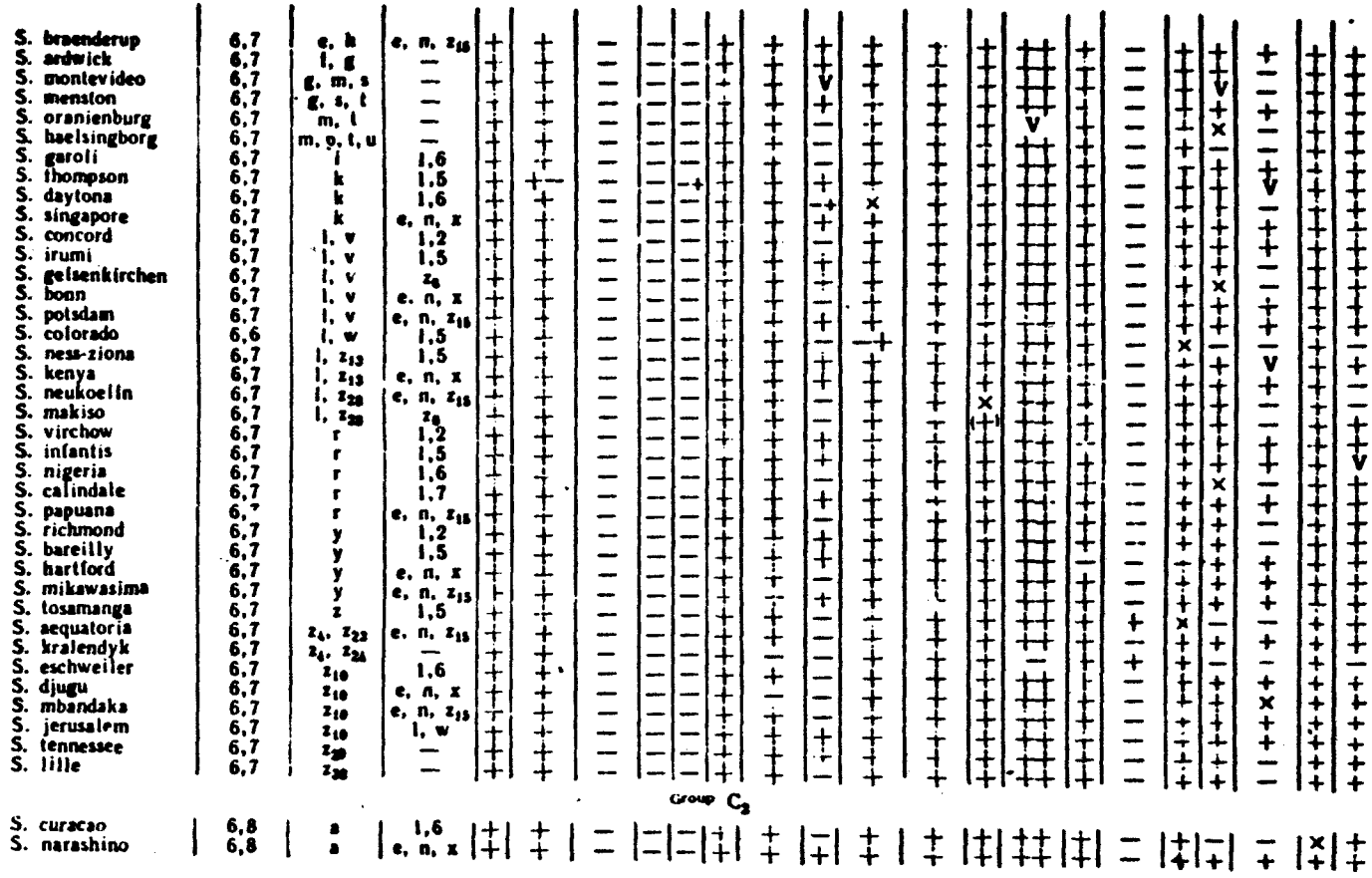
TABLE 24 Serological and Biochemical Properties of *Salmonella*[a]

Kaufmann–White antigenic structure				biochemical properties																						
	O-antigen	H-antigen		glucose		lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d-tartrate	l-tartrate	l-tartrate	citrate	mucous
		1st phase specific	2nd phase nonspecific	acid	gas																	
						Group A																
S. paratyphi A	1, 2, 12	a	1	–	+	+	–	–	+	+	–	+	+	–	–	V	–	–	–	–	–	–
						Group B																
S. kisangani	1, 4, 5, 12	a	1,2	+	+	–	–	–	+	+	V	+	+	+	+	+	–	+	+	+	+	+
S. hessarek	4, 12	a	1,5	+	+	–	–	–	–	+	–	+	+	×	–	–	–	+	×	–	+	–
S. fulica	4, 5, 12	a	1,5	+	+	–	–	–	+	×	–	+	+	+	+	–	–	+	+	+	+	+
S. arechavaleta	4, 5, 12	a	1,7	+	+	–	–	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+
S. bispebjerg	1, 4, 5, 12	a	e, n, x	+	+	–	–	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+
S. abortus equi	4, 12	–	e, n, x	+	+	–	–	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+
S. tinda	1, 4, 12, 27	a	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+
S. paratyphi B	1, 4, 5, 12	b	1,2	+	+	+	–	–	+	V	V	V	+	+	V	V	–	+	+	+	+	+
S. java	1, 4, 5, 12	b	1,2	+	+	–	–	–	+	V	V	V	+	+	V	V	–	+	+	+	+	+
S. limete	1, 4, 12, 27	b	1,5	+	+	–	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+
S. uppsala	4, 12, 27	b	1,7	+	+	–	–	–	+	+	–	+	+	×	+	+	–	+	+	+	+	+
S. abony	1, 4, 5, 12	b	e, n, x	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. abortus bovis	1, 4, 12, 27	b	e, n, x	+	+	–	–	–	+	+	–	+	+	–	+	+	–	+	+	+	+	+
S. wagenia	1, 4, 12, 27	b	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. schleissheim	4, 12, 27	b	–	+	+	–	–	–	+	–	–	+	–	V	+	+	–	+	+	+	+	+
S. wien	4, 12	b	1, w	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. abortus ovis	4, 12	c	1,6	+	+	–	–	–	×	+	–	×	–	+	+	×	–	+	+	+	+	+
S. altendorf	4, 12	c	1,7	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. womba	4, 12, 27	c	1,7	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. bury	4, 12, 27	c	z_6	+	+	–	–	–	+	+	–	+	+	+	V	+	–	+	+	+	+	+
S. stanley	4, 5, 12	d	1,2	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. cairo	1, 4, 12, 27	d	1,2	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. schwarzengrund	1, 4, 12, 27	d	1,7	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. anajana	4, 12, 27	d	e, n, x	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	×	–	+	+



Note: Table taken from: Georg Wildfuehr, Vol. I, 1959.

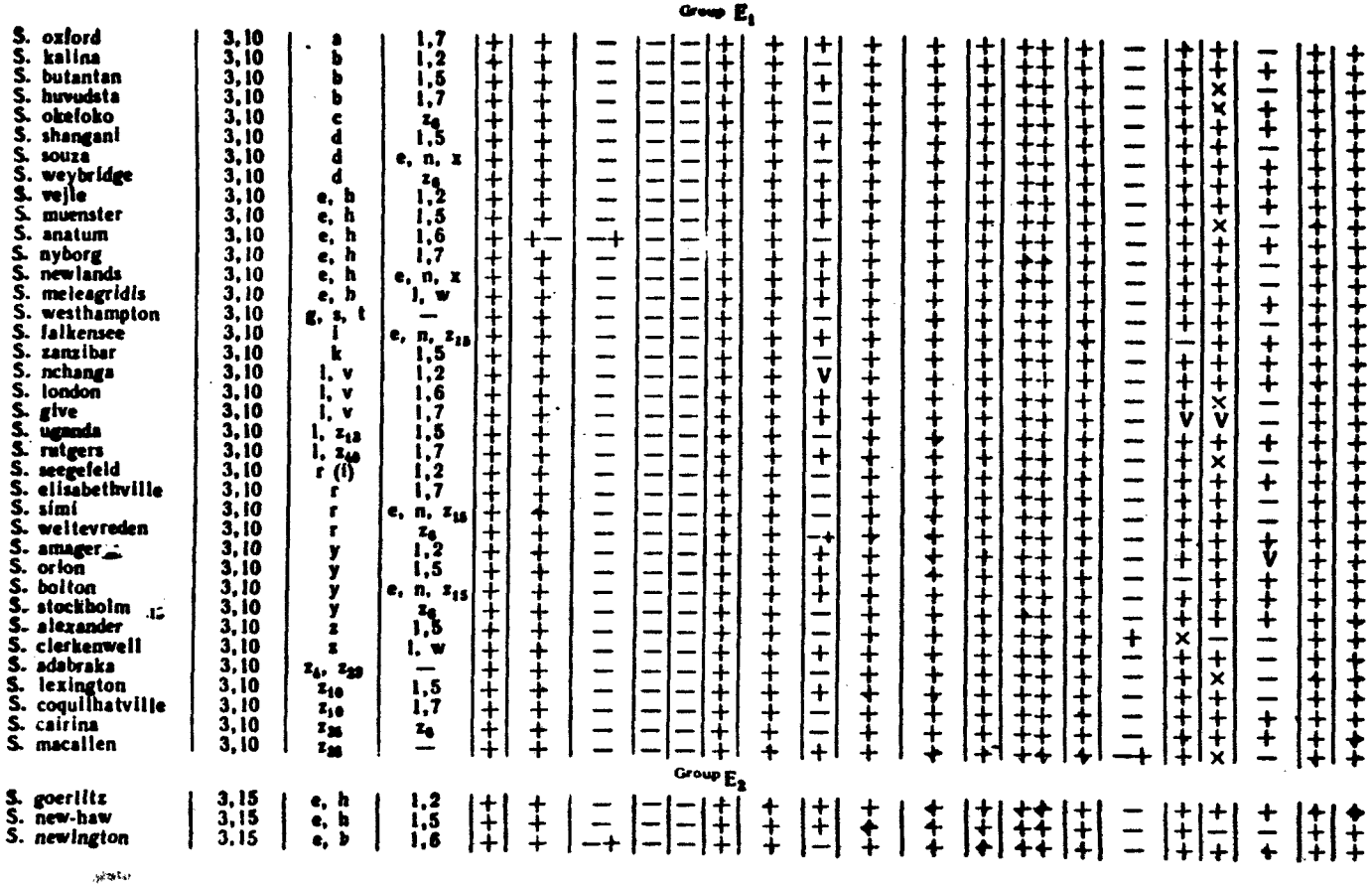
Kaufmann-White antigenic structure						biochemical properties																
	O-antigen	H-antigen 1st phase, specific	H-antigen 2nd phase, nonspecific	glucose acid	glucose gas	lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d-tartrate	l-tartrate	l-tartrate	citrate	mucate
S. haifa	1, 4, 5, 12	z_{10}	1,2	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. ituri	1, 4, 12	z_{10}	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. tudu	4, 12	z_{10}	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	+	+	+
S. fortune	4, 12, 27	z_{10}	z_6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	+	+	+
S. brancaster	1, 4, 12	z_{39}	−	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
						Group C_1																
S. san juan	6,7	a	1,5	+	+	−	−	−	+	+	V	+	+	+	++	+	−	+	V	−	+	+
S. umhlali	6,7	a	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. austin	6,7	a	1,7	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. oslo	6,7	a	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. denver	6,7	a	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. brazzaville	6,7	b	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. edinburg	6,7	b	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. georgia	6,7	b	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. leopoldville	6,7	b	z_6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. kotte	6,7	b	z_{35}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. paratyphi C	6,7 Vi	c	1,5	+	+	−	−	−	V	+	−	+	+	+	−	V	−	+	−	−	+	×
S. cholerae suis	6,7	c	1,5	+	+	−	−	−	−	×	−	−	−	+	−	+	−	V	×	×	×	×
S. typhisuis	6,7	c	1,5	+	+	−	−	−	+	−	−	+	+	+	−	+	−	+	+	+	+	+
S. cholerae suis var. kunzendorf	6,7	−	1,5	+	+	−	−	−	−	×	−	−	−	+	−	+	−	V	×	×	×	×
S. decatur	6,7	c	1,5	+	−	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. birkenhead	6,7	c	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	−	+	+
S. kaduna	6,7	c	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. mission	6,7	d	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. mission var. isangi	6,7	d	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. amersfoort	6,7	d	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. livingstone	6,7	d	l, w	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. lomita	6,7	e, h	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	−	+	+
S. norwich	6,7	e, h	1,6	+	+	−	−	−	+	+	−	V	+	+	V	+	−	+	V	V	V	+


S. braenderup 6,7 e, k e, n, z15
S. ardwick 6,7 l, g —
S. montevideo 6,7 g, m, s —
S. menston 6,7 g, s, t —
S. oranienburg 6,7 m, t —
S. haelsingborg 6,7 m, o, t, u —
S. garoli 6,7 i 1,6
S. thompson 6,7 k 1,5
S. daytona 6,7 k 1,6
S. singapore 6,7 k e, n, x
S. concord 6,7 l, v 1,2
S. irumi 6,7 l, v 1,5
S. gelsenkirchen 6,7 l, v z6
S. bonn 6,7 l, v e, n, x
S. potsdam 6,7 l, v e, n, z15
S. colorado 6,6 l, w 1,5
S. ness-ziona 6,7 l, z13 1,5
S. kenya 6,7 l, z13 e, n, x
S. neukoelln 6,7 l, z28 e, n, z15
S. makiso 6,7 l, z28 z6
S. virchow 6,7 r 1,2
S. infantis 6,7 r 1,5
S. nigeria 6,7 r 1,6
S. calindale 6,7 r 1,7
S. papuana 6,7 r e, n, z15
S. richmond 6,7 y 1,2
S. bareilly 6,7 y 1,5
S. hartford 6,7 y e, n, x
S. mikawasima 6,7 y e, n, z15
S. tosamanga 6,7 z 1,5
S. aequatoria 6,7 z4, z23 e, n, z15
S. kralendyk 6,7 z4, z24 —
S. eschweiler 6,7 z10 1,6
S. djugu 6,7 z10 e, n, x
S. mbandaka 6,7 z10 e, n, z15
S. jerusalem 6,7 z10 l, w
S. tennessee 6,7 z29 —
S. lille 6,7 z38 —
Group C2
S. curacao 6,8 a 1,6
S. narashino 6,8 a e, n, x

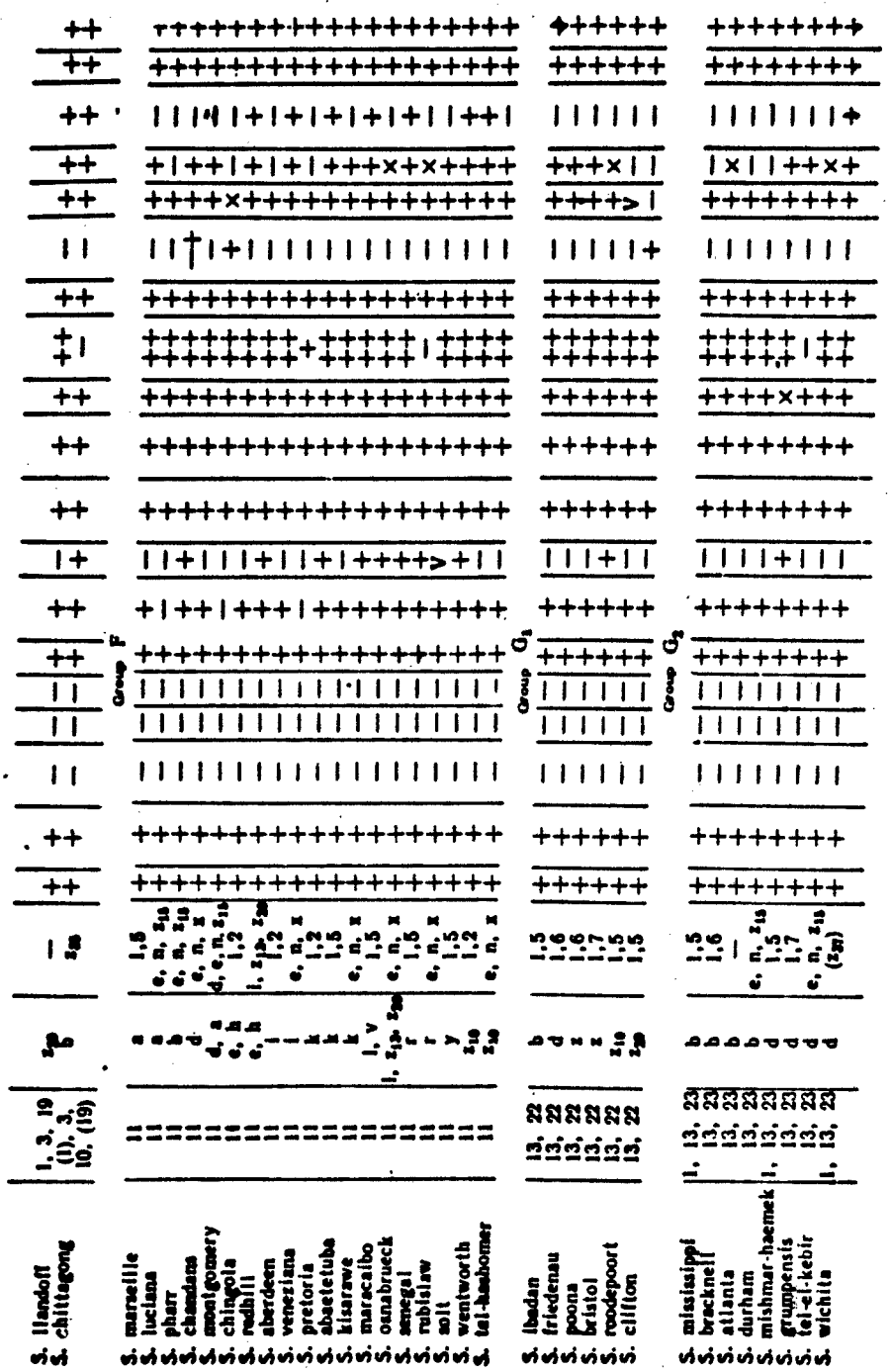
	Kaufman–White antigenic structure					biochemical properties																
	O–antigen	H–antigen: 1st phase, specific	H–antigen: 2nd phase, nonspecific	glucose: acid	glucose: gas	lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d–tartrate	l–tartrate	i–tartrate	citrate	mucate
S. nagoya	6,8	b	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. gatuni	6,8	b	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	−	−	+	+
S. banalia	6,8	b	z_6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. utah	6,8	c	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. bronx	6,8	c	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. belem	6,8	c	e, n, x	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	−	−	+	+
S. quiniela	6,8	c	e, n, z_{15}	+	+	−	−	−	+	+	+	+	−	+	−	+	−	+	x	−	+	+
S. muenchen	6,8	d	1,2	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. manhattan	6,8	d	1,5	+	+	−	−	−+	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. tabadi	6,8	d	z_6	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. newport	6,8	e, h	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. kottbus	6,8	e, h	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	V	+	+	+	+
S. tabionqwe	6,8	e, h	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. lindenburg	6,8	i	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. takoradi	6,8	i	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. bonariensis	6,8	i	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	−	−	+	+
S. aba	6,8	i	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. blockley	6,8	k	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	x	+	+
S. litchfield	6,8	l, v	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. manchester	6,8	l, v	1,7	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. edmonton	6,8	l, v	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. fayed	6,8	l, w	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	x	−	+	+
S. baragwanath	6,8	m, t	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	+	−	−	−	+	+
S. germiston	6,8	m, t	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	+	−	−	−	+	+
S. bovis morbificans	6,8	r	1,5	+	+	−	−	−	+	+	V	+	+	+	++	+	−	+	+	V	+	+
S. hidalgo	6,8	r	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. goldcoast	6,8	r	l, w	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. tananarive	6,8	y	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. alagbon	6,8	y	1,7	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	x	+	+	+
S. praha	6,8	y	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+

Group C_3
Group D_1
chailey
duesseldorf
tallahassee
hadar
glostrup
senga
shipley
virginia
emek
kentucky
amherstiana
hindmarsh
corvallis
albany
sendai
miami
wuerzburg
saarbruecken
loma-linda
durban
onarimon
irintrop
mjimwema
alabama
ridge
zuerich
typhi
adolo
zega
jaffna
eastbourne
israel
berta
enteritidis
blegdam
pensacola
dublin

Kaufmann–White antigenic structure						biochemical properties															
	O-antigen	H-antigen		glucose		lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d-tartrate	l-tartrate	l-malonate	citrate	mucous
		1st phase, specific	2nd phase, nonspecific	acid	gas																	
S. rostock	1, 9, 12	g, p, u	—	+	+	−	−	−	+	+	−	−	+	+	−	+	−	+	+	−	+	+
S. moscow	9, 12	g, q	—	+	+	−	−	−	+	+	−	−	+	+	+	+	−	+	−	−	+	+
S. neasden	9, 12	g, s, t	e, n, x	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+
S. hamburg	1, 9, 12	g, t	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	−	−	+	+
S. hannover	9, 12	(g), m, t	—	+	+	−	−	−	+	+	V	+	+	+	+	+	−	+	−	−	+	+
S. seremban	9, 12	i	1,5	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	+
S. claibornei	1, 9, 12	k	1,5	+	+	−	−	−	+	+	−	−	+	+	+	+	−	+	−	−	+	+
S. mendoza	9, 12	l, v	1,2	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	−	−	+	+
S. panama	1, 9, 12	l, v	1,5	+	+	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	+	+
S. kapemba	9, 12	l, v	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	×	−	+	+
S. goettingen	9, 12	l, v	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	V	+	−	+	+	−	+	+
S. victoria	1, 9, 12	l, w	1,5	+	+	−	−	−	+	+	−	+	+	+	−	+	−	+	−	−	+	+
S. dar-es-salaam	1, 9, 12	l, w	e, n	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	×	−	+	+
S. napoli	1, 9, 12	l, z_{13}	e, n, x	+	+	−	−	−	+	+	−	+	+	+	−	+	−	+	V	−	+	+
S. javiana	1, 9, 12	l, z_{28}	1,5	+	+	−	−	+	+	V	−	+	+	+	+	+	−	+	+	×	+	+
S. wangata	9, 12	z_4, z_{23}	—	+	+	−	−	−	+	+	−	+	+	+	V	+	−	+	+	×	+	+
S. portland	9, 12	z_{10}	1,5	+	+	−	−	−	+	−	−	+	+	+	+	+	−	+	+	−	+	+
S. canastel	9, 12	z_{29}	1,5	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	−	−	+	+
S. penarth	9, 12	z_{35}	z_6	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	−	×	+	+
S. gallinarum	1, 9, 12	—	—	+	−	−	−	−	+	+	−	+	+	+	−	+	−	+	+	×	+	+
S. pullorum	9, 12	—	—	+	+	−	−	−	+	−	−	+	+	+	−	+	−	+	×	−	+	+
Group D_2																						
S. strasbourg	(9), 46	d	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	×	+	+	+
S. marylebone	(9), 46	k	1,2	+	+	−	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
S. shoreditch	(9), 46	r	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	+
S. fresno	(9), 46	z_{38}	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. haarlem	(9), 46	z	e, n, x	+	+	−	−	−	+	+	−	+	+	+	+	+	+	−	−	−	+	+


Group E1
S. oxford 3,10 a 1,7
S. kalina 3,10 b 1,2
S. butantan 3,10 b 1,5
S. huvudsta 3,10 b 1,7
S. okefoko 3,10 c z6
S. shangani 3,10 d 1,5
S. souza 3,10 d e, n, x
S. weybridge 3,10 d z6
S. vejle 3,10 e, h 1,2
S. muenster 3,10 e, h 1,5
S. anatum 3,10 e, h 1,6
S. nyborg 3,10 e, h 1,7
S. newlands 3,10 e, h e, n, x
S. meleagridis 3,10 e, h l, w
S. westhampton 3,10 g, s, t —
S. falkensee 3,10 i e, n, z15
S. zanzibar 3,10 k 1,5
S. nchanga 3,10 l, v 1,2
S. london 3,10 l, v 1,6
S. give 3,10 l, v 1,7
S. uganda 3,10 l, z13 1,5
S. rutgers 3,10 l, z40 1,7
S. seegefeld 3,10 r (i) 1,2
S. elisabethville 3,10 r 1,7
S. simi 3,10 r e, n, z15
S. weltevreden 3,10 r z6
S. amager 3,10 y 1,2
S. orion 3,10 y 1,5
S. bolton 3,10 y e, n, z15
S. stockholm 3,10 y z6
S. alexander 3,10 z 1,5
S. clerkenwell 3,10 z l, w
S. adabraka 3,10 z4, z29 —
S. lexington 3,10 z10 1,5
S. coquilhatville 3,10 z10 1,7
S. cairina 3,10 z35 z6
S. macallen 3,10 z38 —
Group E2
S. goerlitz 3,15 e, h 1,2
S. new-haw 3,15 e, h 1,5
S. newington 3,15 e, h 1,6

Kaufmann–White antigenic structure						biochemical properties																
	O-antigen	H-antigen		glucose		lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d-tartrate	l-tartrate	dl-tartrate	citrate	mucous
		1st phase, specific	2nd phase, nonspecific	acid	gas																	
S. selandia	3,15	e, h	1,7	+	+	–	–	–	+	+	+	+	+	+	+	+	–	+	+	–	+	+
S. cambridge	3,15	e, h	1, w	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. new brunswick	3,15	l, v	1,7	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	×	+	+
S. kinshasa	3,15	l, z_{13}	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. lanka	3,15	r	z_6	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. tuebingen	3,15	y	1,2	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. birta	3,15	y	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. manila	3,15	z_{10}	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
Group E_3																						
S. minneapolis	(3), (15), 34	e, h	1,6	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	+	+	+
S. canoga	(3), (15), 34	g, s, t	–	+	+	–	–	–	+	+	–	+	+	+	–	–	–	+	+	–	+	+
S. thomasville	(3), (15), 34	y	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. illinois	(3), (15), 34	z_{10}	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. harrisonburg	(3), (15), 34	z_{10}	1,6	+	+	–	–	–	+	+	–	+	+	V	++	+	–	+	+	+	+	+
Group E_4																						
S. accra	1, 3, 19	b	z_6	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. niloese	1, 3, 19	d	z_6	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
S. liverpool	1, 3, 19	d	e, n, z_{15}	+	+	–	–	–	+	+	+	+	+	+	–	+	–	+	+	+	+	+
S. senftenberg	1, 3, 19	g, s, t	–	–	+	–	–	–	+	+	–	+	+	+	++	–	–	+	+	–	+	+
var. newcastle	1, 3, 19	g, s, t	–	–	+	–	–	–	+	+	–	+	+	+	–	–	–	+	+	+	+	+
S. taksony	1, 3, 19	l	z_6	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. krefeld	1, 3, 19	l, w	y	+	+	–	–	–	+	+	–	+	+	+	–	+	–	+	+	×	+	+
S. schoeneberg	1, 3, 19	z	e, n, z_{15}	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	+	+	+
S. simsbury	1, 3, 19	–	z_{22}	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+


Group F
Group G1
Group G2
S. llandoff
S. chittagong
S. marseille
S. luciana
S. pharr
S. chandana
S. montgomery
S. chingola
S. redhill
S. aberdeen
S. veneziana
S. pretoria
S. abaetetuba
S. kisarawe
S. maracaibo
S. oranabrueck
S. senegal
S. rubislaw
S. salt
S. wentworth
S. tel-hashomer
S. ibadan
S. friedenau
S. poona
S. bristol
S. roodepoort
S. clifton
S. mississippi
S. bracknell
S. atlanta
S. durham
S. mishmar-haemek
S. gruppensis
S. tel-el-kebir
S. wichita

Kaufmann–White antigenic structure						biochemical properties																		
	O-antigen	H-antigen		glucose		lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H_2S	gelatin	d-tartrate	l-tartrate	i-tartrate	citrate	mucate
		1st phase, specific	2nd phase, nonspecific	acid	gas																	
S. havana	1, 13, 23	f, g	—	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	V	+	+
S. luanshya	13, 23	g, s, (t)	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. okatie	13, 23	g, s, t	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. worcester	1, 13, 23	m, t	e, n, x	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. tunis	1, 13, 23	y	z_6	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	+
S. nachshonim	1, 13, 23	z	1,5	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. worthington	1, 13, 23	z	l, w	+	+	−	−	−	+	+	−	+	+	+	−	+	−	+	+	−	+	+
S. ajiobo	13, 23	z_4, z_{23}	—	+	+	−	−	-	+	×	−	+	+	+	+	+	−	+	+	−	+	+
S. cubana	1, 13, 23	z_{29}	—	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	+
S. fanti	13, 23	z_{28}	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	×	+	−	+	+
						Group H																
S. heves	6, 14, 24	d	1,5	+	+	−	−	−	+	−	−	+	+	×	−	+	−	+	+	−	+	+
S. florida	(1),6,14,25	d	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. clarity	6, 14	d	e, n, x	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. teko	(1),6,14,25	d	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	×	+	+
S. albuquerque	6, 14, 24	d	z_6	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	×	+	+
S. onderstepoort	(1),6,14,25	e, (h)	1,5	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. warragul	(1),6,14,25	g, m	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. caracas	(1),6,14,25	g, m, s	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. buzu	(1),6,14,25	i	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. boecker	6, 14	l, v	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. horsham	(1),6,14,25	l, v	e, n, x	+	+	−	+	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. carrau	6, 14, 24	y	1,7	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. madella	(1),6,14,25	y	1,7	+	+	−	−	−	+	+	−	+	+	+	V	+	−	+	+	−	+	+
S. homosassa	(1),6,14,25	z	1,5	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. sundsvall	(1),6,14,25	z	e, n, x	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
S. siegburg	6, 14, 18	z_4, z_{32}	—	+	+	−	−	−	+	+	V	+	+	+	+	+	−	+	+	−	+	−
S. uzaramo	(1),6,14,25	z_4, z_{24}	—	+	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+
						Group I																
S. brazil	16	a	1,5	+	+	−	−	−	+	−+	−	+	+	++	++	++	−	++	++	−	++	+
S. hull	16	b	1,2	+	+	−	−	−	+	+	+	+	+	++	++	++	+	++	++	+	++	+

Group J
Group K
Group L

	Kaufmann–White antigenic structure					biochemical properties																
		H–antigen		glucose																		
	O–antigen	1st phase, specific	2nd phase, nonspecific	acid	gas	lactose	sucrose	indol	ambinose	dulcitol	inositol	rannose	trehalose	xylose	glycerine	H_2S	gelatin	d–tartrate	l–tartrate	i–trate	citrate	mucous
S. ruiru	21	y	e, n, x	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	×	–	+	+
S. gwaai	21	z_6, z_{28}	–	+	+	–	–	–	+	+	–	+	+	+	++	+	+	–	–	–	+	+
						Group M																
S. solna	28	a	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. dakar	28	a	1,6	+	+	–	–	–	+	+	–	+	+	+	–	+	–	+	×	–	+	+
S. seattle	28	a	e, n, x	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+
S. langford	28	b	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	×	–	+	+
S. kaltenhausen	28	b	z_9	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	–	–	+	+
S. mundonobo	28	d	1,7	+	+	–	–	–	+	+	–	+	+	+	++	+	+	–	–	–	+	+
S. patience	28	d	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	×	–	+	+
S. ona	28	g, s, t	–	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. vinohrady	28	m, t	–	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	–	–	+	+
S. illala	28	k	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. taunton	28	k	e, n, x	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. leoben	28	l, v	1,5	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. nashua	28	l, v	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	++	–	–	+	×	–	+	+
S. chicago	28	r	1,5	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+
S. kibusi	28	r	e, n, x	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+
S. nima	28	y	1,5	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	×	–	+	+
S. pomona	28	y	1,7	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+
S. tel-aviv	28	y	e, n, z_{15}	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	×	–	+	+
S. ezra	28	z	1,7	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	+	–	+	+
S. umbilo	28	z_{10}	e, n, x	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	–	–	+	+
S. moroto	28	z_{10}	l, w	+	+	–	–	–	+	+	–	+	+	+	++	+	–	+	–	–	+	+
						Group N																
S. urbana	30	b	e, n, x	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	–	+	+
S. godesberg	30	g, m	–	+	+	–	–	–	+	+	+	+	+	+	++	+	–	+	+	–	+	+
S. landau	30	i	1,2	+	+	–	–	–	+	+	V	+	+	+	++	+	–	+	+	+	+	+

S. morehead	30	i	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. aqua	30	k	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	−	+	+
S. angoda	30	k	e, n, x	+	+	−	−	−	+	×	+	+	+	+	++	+	−	+	+	−	+	+
S. donna	30	l, v	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−+	+	−	−	+	+
S. matopeni	30	y	1,2	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. bodjonegoro	30	z_4, z_{24}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	−	+	+
Group O																						
S. djakarta	31	z_4, z_{24}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	−	−	+	+
Group P																						
S. sumhlatazana	35	a	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. yolo	35	c	−	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. adelaide	35	f, g	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. monschaui	35	m, t	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. gambia	35	i	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. alachua	35	z_4, z_{23}	−	+	+	−	−	−	+	+	+	+	+	+	++	+	−	−	−	+	+	+
Group Q																						
S. shefield	38	c	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. thiaroye	38	e, h	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. kasenyi	38	e, h	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. korovi	38	g, m, s	−	+	+	−	−	−	+	V	−	+	+	+	++	+	−	+	+	V	+	+
S. mgulani	38	i	1,2	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. inverness	38	k	1,6	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. roan	38	l, v	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. lindi	38	r	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
S. emmastad	38	r	1,6	+	+	−	−	−	+	+	−	+	+	+	++	−	−	+	+	+	+	+
S. freetown	38	y	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	−	+	+
S. colombo	38	y	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	×	+	+	+
Group R																						
S. wandsworth	39	b	1,2	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. champaign	39	k	1,5	+	+	−	−	−	+	+	+	+	+	+	−	+	−	+	+	−	+	+
Group S																						
S. springs	40	a	z_{39}	+	+	−	−	−	+	+	−	+	+	+	++	+	+	×	−	−	+	+
S. riogrande	40	b	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−+	+	×	−	+	+
S. johannesburg	1,40	b	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. duval	1,40	b	e, n, z_{15}	+	+	−	−	−	+	−	−	+	+	+	−	+	−	+	+	−	+	+
S. boksburg	40	g, s	e, n, z_{15}	+	+	−	−	−	+	+	+	+	+	+	++	+	+	−	−	−	+	+

Kauffmann–White antigenic structure						biochemical properties																
	O-antigen	H-antigen: 1st phase, specific	H-antigen: 2nd phase, nonspecific	glucose: acid	glucose: gas	lactose	sucrose	indol	arabinose	dulcitol	inositol	rhamnose	trehalose	xylose	glycerine	H$_2$S	gelatin	d-tartrate	l-tartrate	i-tartrate	citrate	mucate
S. allandale	1,40	k	1,6	+	+	−	−	−	+	+	+	−	+	+	++	+	−	+	+	−	−	−
S. degania	40	z_4, z_{24}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	+	x	−	−	+	+
S. karamoja	40	z_{41}	1,2	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	x	+	+
Group T																						
S. waycross	41	z_4, z_{23}	−	+/−	+	−	−/−	−/+	+	−/+	−/−	+	+	+/−	++	+/−	−	−/+	−/−	+	+/−	+
Group U																						
S. chinovum	42	b	1,5	+	+	−	−	−	+	+	−	+	+	+	−	+	+	−	−	−	+	+
S. uphill	42	b	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−/+	x	−	−	+	+
S. kampala	1,42	c	z_6	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. kaneshie	1,42	i	l, w	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. nairobi	42	r	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	x	−	−	+	+
S. rand	42	z	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	+	x	−	−	+	+
S. weslaco	42	z_{28}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−/+	+	−	−	+	+
Group V																						
S. berkeley	43	a	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	+	+	+
S. milwaukee	43	f, g	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−/+	+	+	+	+	+
S. ahuza	43	k	1,5	+	+	−	−	−	+	+	+	+	+	+	++	+	−	+	+	−	+	+
S. kingabwa	43	y	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	+	+	+
Group W																						
S. niarembe	44	a	l, w	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. christiansborg	44	z_4, z_{24}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. guinea	44	z_{10}	−	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+

				Group X																		
S. deversoir	45	c	e, n, x	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	−	+
S. dugbe	45	d	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. windhoek	45	g, t	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	+	−	−	−	+	+
				Group Y																		
S. bergen	47	i	e, n, z_{15}	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	+	−	+	+
S. kaolack	47	z	1,6	+	+	−	−	−	+	+	−	+	+	+	++	+	−	+	−	−	+	+
				Group Z																		
S. dahlem	48	k	e, n, z_{15}	+	+	−	−	−	+	−	−	+	+	+	++	+	−	+	−	−	+	+
				Group 49																		
S. bulawayo	(1), 49	z	1,5	+	+	−	−	−	+	+	−	+	+	+	++	+	+	+	−	−	+	+
				Group 50																		
S. greenside	50	z	e, n	+	+	−	−	−	+	+	−	+	+	+	++	+	+	+	−	−	+	+

Symbols for Table 24

Carbohydrates:	(−)	negative until 30th day;
	(−+)	negative, occasionally positive;
	(+)	positive result after 24 hr;
	(+−)	positive, rarely negative;
	(V)	various biochemical types;
	(x)	late or irregularly positive or negative result
Gelatin:	([x])	negative in the majority of cases. Gelatin;
	(+)	rapid liquefaction of gelatin;
	(+−)	slow liquefaction of gelatin;
	(−)	no liquefaction of gelatin. Stern's glycerine-fuchsin broth;
Stern's glycerine-fuchsin broth:	(++)	lilac;
	(+)	purple;
	(−)	same color as in control, negative result after 8 days of observation. Organic acids;
Organic acids:	(+)	positive after 1st or 2nd day;
	(−)	negative after 14th day.

For determination of the O antigen culture material is taken from the upper portion of an agar slant in a test-tube, while for determination of the H antigen it is taken from the lower portion of the slant.

Freshly-isolated cultures are sometimes incapable of agglutinating with either specific or monoreceptor sera. Such cultures may regain their agglutinative properties after repeated daily passivation (2-3 or more times) in semiliquid (0.4%) agar. Passivation in freshly-prepared weakly alkaline agar, bile, or bile broth repeated 3-5 times will also increase agglutinability; alternate passivation in agar and a bile medium can also be employed. Repeated processing can also be used in investigating freshly-isolated cultures which agglutinate in several heterogeneous sera (degenerative forms).

The specific phase of the flagellar H antigen can be isolated by Swan and Grad's dish-culturing method. This technique is based on the fact that when homologous H serum is added to agar the mobility of the bacteria is selectively suppressed in accordance with the phase present. The phase which is not affected by the serum increases in quantity and can be isolated from the margin of the colony.

Technique. Portions of 15 ml of 2% molten agar to which meat-infusion broth (or Hottinger's broth) has been added are poured into small flasks. After the agar has cooled a drop of the requisite serum is added to it and the mixture is agitated and poured into Petri dishes. The dishes are dried in a heater at 37°. A streak of a day-old culture is applied to the center of each Petri dish with a loop and the dishes are returned to the heater for 18-20 hr.

The culture material extending beyond the edges of the macrocolonies is tested on slides with adsorbed monoreceptor sera.

This method can also be used to determine the second H phase when monophasic cultures have been isolated.

Certain cultures, such as S. paratyphi C (which does not contain Vi antigen) and S. cholerae suis, cannot be distinguished by serological receptor analysis (Table 24); in such cases the cultures are identified from supplemental biochemical reactions. S. paratyphi C usually ferments arabinose and always ferments trehalose quite quickly, while S. cholerae suis does not decompose either of these sugars. S. paratyphi C soon ferments dulcitol, while S. cholerae suis gives a retarded or negative reaction in media containing this compound. The cultural characteristics which distinguish B. paratyphi schotmuelleri from the other Salmonella of group B include: 1) formation of a mucous spindle within a few days after a 24-hr 37° culture is shifted to room temperature (20-22°; it is best that the medium contain 2.5% of agar and an equal quantity of sodium chloride); 2) formation of daughter colonies in agar containing raffinose; 3) creep on gelatin slants over a period of 4-10 days.

Laboratory Diagnosis of Salmonellosis

Material for examination; taking of samples. The initial material for bacteriological examination may be vomitus, lavage water, blood, feces, or urine.

Remains of food eaten by the patients and washings from the vessels in which this food was cooked and stored and from the tables on which it was prepared are also subjected to bacteriological investigation. When the illness terminates in death the parenchymatous organs, blood from the heart, the contents of the stomach and small intestine, and the lymph nodes are examined. Cadaver material can be cultured on the spot or shipped to the laboratory in individual sterile vessels.

Whenever possible the material to be examined is collected before the patient receives antibiotics.

The patient's dejecta (fecal matter and vomitus) are collected in

quantities of 50-100 g, while lavage water is collected in quantities of 100-200 ml. If the vomitus and lavage water have an acid reaction they must be neutralized with 5-10% sodium bicarbonate before culturing. Impurities of disinfectants in the lavage water makes it unsuitable for bacteriological examination.

Vomitus with a thick consistency is thoroughly shaken in 10 parts of physiological solution before culturing and as much of the solid matter as possible is permitted to settle (liquid vomitus is cultured immediately).

A total of 1 or 2 drops of material from the upper layer of the settled suspension is applied to a nutritive medium and, using the same spatula, the material is streaked on dishes containing various color media (Ploskirev's agar, Levin's medium, and Endo's medium). In addition to direct inoculation of solid nutritive media, cultures are also made on concentration media (Muller's, Kaufman's, Rackiten-Retger's, etc., media).

Portions of 1 ml of the material to be examined are placed in test-tubes.

It is desirable that lavage water be centrifuged and the residue cultured both on solid media and concentration media. Blood for hemocultures, feces, and urine are cultured in the same manner as in typhoid (page 294).

In Investigating meat and meat products several chunks, weighing from 200 to 500 g if possible, are used; it is desirable to select meat containing lymph glands and a tubular bone (when large chunks are not available all remnants suspected of being infected are examined).

Before culturing solid foodstuffs (meat, meat products, sausages, cheese, etc.) they are sterilized with a red-hot metal spatula or knife and samples are taken from within the material. 5-g chunks of solid

foodstuffs, taken under sterile conditions, are crushed in a sterile mortar and transferred to a flask containing sterile water or physiological solution (1:5 or 1:10). When meat contains tubular bone the marrow is extracted, emulsified in heated physiological solution (40-45°), and cultured separately. Any lymph glands present are removed and cultured separately on differential media and in concentration media.

Samples of 100-200 g of semiliquid and liquid products (sour cream, melange, milk, etc.) are taken after careful mixing with sterile or flamed spoons; if the quantity of food is small it is all used. The material to be cultured is drawn off in graduated pipettes. Semiliquid products, such as oil and margarine, are first heated to 40-45°.

Fragments of food from containers and scrapings from tables where the suspected food has lain are collected with sterile pads wetted in physiological solution, broth, or bile broth.

The used pads are immersed in a test-tube containing bile broth or a concentration medium and placed in a heater at 37° for 3-4 hr.

After the first culturing the test-tubes containing the washings are again placed in the heater for 18-20 hr and the material is repeatedly cultures.

After appropriate treatment all material undergoing bacteriological examination is cultured on differential media and a concentration medium. Subsequent investigation of the cultures proceeds in the same manner as in identification of Salmonella or the causative agents of typhoid and paratyphoid A and B (page 300).

Bacteriological examination of the Salmonella cultures isolated ends with study of their serological properties.

A culture is identified as Salmonella only on the basis of its morphological, cultural, biochemical, and serological characteristics.

Widal's reaction can be used as a secondary method for the labora-

tory diagnosis of Salmonellosis (see the section headed "Typhoid and Paratyphoid A and B," page 305). An increase in the agglutinin content of the blood of Salmonellosis patients is noted on about the 5th day of illness.

Investigation of the blood for detection of agglutinins must be carried out under dynamic conditions. Diagnostic sera prepared from paratyphoid cultures, live (laboratory) cultures, autogenous strains, and O and H monodiagnostic sera (separately) are used in the Widal reaction. The H agglutinins appear in the blood later.

Accelerated methods of bacteriological examination

When it is necessary to obtain more rapid results the bacteriological examination can be conducted by an accelerated method. Suspicious colonies (which have a clear yellowish-red form on Ploskirev's agar, a semitransparent light-red form on Endo's medium, a semitransparent blue form on Levin's medium, and a black form with a metallic lustre on Wilson-Blair's medium) are cultured in a test-tube containing broth and then, (after 3-4 hr) transferred to agar slants and a short composite series (lactose, glucose, mannose, maltose, and sucrose); the media are preliminarily heated to 40-45°. The morphology of the bacteria is studied at the same time.

Moscow Institute imeni Mechnikov variant of method for accelerated bacteriological diagnosis. In essence, this method consists in utilization of selective nutritive media, a short composite series, and adsorbed sera, taking into account the lability and morphology of the culture. It reduces the duration of the analysis by one day.

Bacterial colonies suspected of belonging to the Salmonella group are transferred from Ploskirev's medium, bacterial agar Zh, or bismuth sulfite agar to a short composite series (Ressel's medium or lactose, glucose, and agar slants) and graduates containing weakly alkaline semi-

liquid (0.4%) agar for determination of their lability.

Cultures with the biochemical and morphological properties typical of Salmonella are subjected to rough agglutination on slides with a mixture of Salmonella sera. This mixture consists of the usual paratyphoid A, paratyphoid B, Breslau, Suipestifer, and Gertner agglutinative sera (1 ml of each serum in 20 ml of physiological solution). A total of 1% of twice-recrystallized boric acid is added to the mixture as a preservative.

Cultures which give a positive agglutination reaction with this mixture of paratyphoid sera are first tested with adsorbed O sera to determine their serological group (A, B, C, D, or E) and then with H sera to establish the species of bacterium.

If a labile culture with typical biochemical and morphological characteristics agglutinate well with adsorbed O sera, thus establishing its serological group, and with the H sera of a definite species, the result is positive.

If there is any ambiguity in the results yielded by the accelerated method the investigation is continued by the ordinary method described above.

This technique can be employed only for examining material from patients and in massive work during epidemics. Its use for examination of environmental material is not recommended, since atypical cultures requiring more thorough study are often encountered in such cases.

Increasing Phage-Titre Reaction

Prof. D.M. Gol'dfarb

Indicator phage. D.M. Gol'dfarb, Z.S. Ostrovskaya, and V.N. Kuznetsova isolated this phage from the fecal matter of monkeys in 1959. It is passivated in S. typhi murium strain No. 573. The host strain has the antigenic structure: O1, 4; H1, 2. The phage is thermostable and is

TABLE 25

Action Spectrum and Specificity of Salmonella Indicator Phage

Культура 1	2 Число испытывавшихся штаммов	Число лизированных штаммов 3	Число нелизированных штаммов 4	Процент лизированных культур 5
6 Кишечная палочка 7 Паракишечная палочка	423	21	402	4,9
8 Палочка Моргана 9 Дизентерийные бактерии разных видов	67	1	66	1,4
10 Сальмонеллы (кроме S. typhi murium)	161	122	39	75,1
S. typhi murium .	140	134	6	96,4

1) Culture; 2) number of strains tested; 3) number of strains lyzed; 4) number of strains not lyzed; 5) percentage of cultures lyzed; 6) E. coli; 7) paracolon bacteria; 8) Proteus morgani; 9) various species of dysentery bacteria; 10) Salmonella (except S. typhi murium).

not inactivated by heating at 58° for 30 min. Negative colonies are large, transparent, clearly delineated, and approximately 1 mm in diameter.

This phage is highly specific (only 4.9% of nonpathogenic cultures react with it on solid media; see Table 25).

Strain 465 is used as the standard culture for determination of the phage concentration in the final stage of the reaction.

The indicator phage is easily isolated in this strain; the free intestinal phages detected in the fecal matter of healthy persons do not react with it. The Salmonella indicator phage is consequently passivated in one strain, while another phage is used as the standard in the increasing phage-titre reaction.

BACTERIAL DYSENTERY

Prof. Ye.D. Ravich-Birger

Classification of Dysentery Bacteria

Bacterial dysentery has several causative agents, which can be distinguished by their biological, enzymatic, and serological proper-

ties. They belong to the family Enterobacteriaceae and the genus Shigella.

Bergey's classification of dysentery bacteria (1957):

I. Does not form acid from mannose.

A. Does not form acid from arabinose or rhamnose. Does not produce indol.

1. Shigella dysenteriae.

B. Forms acid from arabinose; acid + or − from rhamnose; indol+ or −.

1. Forms acid from rhamnose. Retarded, irregular formation of acid from arabinose. Produces indol.

2. Shigella schmitzii.

2. Does not form acid from rhamnose. Forms acid from arabinose slowly. Does not produce indol.

3. Shigella arabinotarda (Large-Sachs group).

II. Forms acid from mannose.

A. Does not form acid from lactose.

1. Does not form acid from rhamnose. Usually forms acid from zylose.

4. Shigella boydii.

2. Forms acid from rhamnose irregularly. Does not form acid from zylose or dulcitol. Does not produce trimethylamine from oxytrimethylamine.

5. Shigella flexneri.

3. Forms acid from dulcitol. Produces trimethylamine from oxytrimethylamine.

6. Shigella alkalescens.

B. Retarded formation of acid from lactose.

1. Does not produce indol.

7. Shigella sonnei.

2. Produces indol.

8. Shigella dispar.

The current classification of Shigella is based on a complex of biological and serological properties, as is reflected in the classification adopted by the International Commission on Bacterial Nomenclature (Table 26).

The temporary Soviet classification of 1953, set up by the Committee on the Nomenclature of Pathogenic Microorganisms of the Ministry of Public Health USSR, was accordingly reviewed in 1960 and it was decided to change over to the international classification. Certain points in the international system are, however, disputable. Thus, for example, we cannot agree with the fact that there is no differentiation among species in the first subgroup (A), which comprises bacteria that do not decompose mannose. Sh. grigoriewa-Shigae, Sh. stutzeri-schmitzii, and Sh. large-sachsii differ so widely in their biochemical and especially their serological properties that they cannot be designated by numbers alone, like the types of Sh. flexneri. It is best to retain the species designations of these bacteria.

Objections also arise when we consider subgroups B and C. Type 6 of subgroup B is nonuniform: it includes the Newcastle, Manchester, and Boyd 88 bacteria, which are biochemical variants of the same serological species, which is in turn closely related to Flexner's species. The new Soviet classification retains these variants as subspecies of the Newcastle bacterium.

The international classification also has an independent group C, Sh. boydii, which Soviet researchers feel to be closely related genetically to the Flexner bacteria and similar to them biologically and

TABLE 26

International Classification of Shigella

[1] Подгруппа	[2] Тип и подтипы	[3] Прежние обозначения
A. Не расщепляющие маннит	1	B. dysenteriae, B. Shigae.
	2	Sh. Ambigua, B. Schmitz.
	3	
	4	
Sh. dysenteriae	5	[4]
	6	Типы Large-Sachs
	7	
	8	
	9	
	10	
B. Расщепляющие маннит	1—a, b	[5]Типы Эндрюса и Иннеса
	2—a, b	
Sh. flexneri	3—a, b, c	
	4—a, b	[6]Тип 103 Бойда
	5—(x+), (x—)	[7]Тип P119 и P119X
	x —	[8]Бойда
	y —	
	6	[9]Типы Manchester, Newcastle, Boyd 88
C. Расщепляющие маннит	1	[10] Тип 170 Бойда
	2	» 288 »
	3	» [11] Д1 »
	4	» [12] P274 »
Sh. boydii	5	» [13] P148 »
	6	» [14] Д19 »
	7	Lavington, Sh. etouse Serotype 112
	8	» » » » 1296/7
	9	
	10	
	11	
	12	
	13	
	14	
	15	
D. Расщепляющие маннит и медленно лактозу		B. Sonne-Duval, B. Kruse-Sonne.
Sh. sonnei		B. dysenteriae E. B. ceylonensis

A) Does not decompose mannose; B) decomposes mannose; C) decomposes mannose; D) decomposes mannose and lactose, the latter slowly. 1) Subgroup; 2) type and subtypes; 3) prior designations; 4) Large-Sachs types; 5) Endrus and Inmen types; 6) Boyd type 103; 7) type R119 and R119Kh; 8) Boyd; 9) Manchester, Newcastle, and Boyd 88 types; 10) type 170 Boyd; 11) D1; 12) R274; 13) R148; 14) D19.

serologically. In the Soviet classification these bacteria are included as subspecies of the Flexner species (see Table 27).

For purposes of continuity and to permit an understanding of data, abstracts, and scientific papers using the temporary Soviet classification of 1953 we must compare the designations of the subspecies and

TABLE 27

Soviet Classification of Shigella, Adopted 16 January 1962 by the Committee on the Nomenclature of Pathogenic Bacteria

	1 Вид	2 Подвид	Типовой антиген 3	Подтип 4
Не расщепляющие маннит 5	Григорьева-Шига (Sh. grigorjewa-shigae)	—	1	—
	Штуцера-Шмитца (Sh. stutzeri-schmitzii)	—	2	—
	Лардже-Сакса (Sh. large-sachsii)	—	3	—
		—	4	—
Не расщепляющие маннит		—	5	—
		—	6	—
		—	7	—
	Провизорные[1]	—	8	—
	»	—	9	—
	»	—	10	—
Расщепляющие маннит 7	Флекснера (Sh. flexneri)	Флекснера (Sh. flexneri)	1	1a
				1b
			2	2a
				2b
		»	3	3a
				3b
				3c
		»	4	4a
				4b
		»	5	5(x+)
				5(x—)
		»		var. x[8]
				var. y[8]
	То же 8	Ньюкасл (Sh. newcastle)	6	
	То же	Бойда (Sh. boydii)	1	—
			2	—
			3	—
			4	—
		»	5	—
		»	6	—
		»	7	—
			8	—
		»	9	—
		»	10	—
			11	—
		»	12	—
			13	—
		»	14	—
			15	—
Медленно расщепляющие 9 лактозу	Зонне (Sh. sonnei)	—	—	—

1) It is unclear to which species these microorganisms belong.

2) Lacking type antigens, these are differentiated with group antigens.

1) Species; 2) subspecies; 3) type antigen; 4) subtype; 5) do not decompose mannose; 6) provisional; 7) decompose mannose; 8) the same; 9) decompose lactose slowly.

types of Shigella employed in the two classifications.

The following summary presents data obtained in a comparison of Shigella.

The types of Sh. large-Sachsii correspond to the following mannose-negative bacteria described by E.M. Novgorodskaya and O.A. Semenova:

Type 3 corresponds to the "Roman" type.

Type 4 corresponds to type 1618.

Type 5 corresponds to type 819.

Type 6 corresponds to the "Tyakht" type.

Type 7 corresponds to type 2435.

In the Flexner subspecies:

Type 1 corresponds to type f.

Type 2 corresponds to types c and b.

(2a and 2b)

Type 3 corresponds to types e and d.

(3b and 3var.)

Type 4 corresponds to type a (Boyd 103).

Type 5 corresponds to type g (Boyd 119).

The types of the Boyd subspecies correspond to the following Boyd-Novgorodskaya types:

Type 1 corresponds to type I.

Type 2 corresponds to type V.

Type 4 corresponds to type III.

Type 5 corresponds to type VII.

Type 7 corresponds to type II.

Type 9 corresponds to type IV.

Type 12 corresponds to type VI.

The following are the principal species of bacteria commonly recognized as the causative agents of bacterial dysentery:

1. Grigoriev-Shiga bacterium (Sh. dysenteriae). This microorganism was first described by Grigoriev in 1891 and was studied in detail by Shiga in 1898; it is a clearly defined species biochemically and antigenically. It possesses an exotoxin. The most severe forms of the disease are associated with this species, depending on the etiological factor.

2. The Stutzer-Schmitz bacterium (Sh. Schmitz, 1917) was isolated by Stutzer from the stools of dysentery patients in a prisoner-of-war camp in 1917, but was not described until his return to the USSR in 1920. Schmitz described it in 1917. It is encountered rather infrequently (in 3-17% of isolated cultures), usually during geographically limited epidemics.

3. The Large-Sachs bacterium, which exhibits delayed decomposition of arabinose, was isolated and described by Large, Sankara, and Sachs. Christensen and Gowan (1944) named it Sh. arabinotarda and divided it into two variaties, A and B. The international classification now recognizes 8 variants of this bacterium.

The biological properties of Sh. large-Sachsii are similar to those of Sh. dysenteriae, displaying only slight variations in its decompositions of carbohydrates.

4. Flexner bacteria (Sh. flexneri). These microorganisms are the principal representatives of the dysentery bacteria which decompose mannose. They do not form an exotoxin and are consequently referred to as the nontoxogenic group of dysentery bacteria. Sh. flexneri produces a dysentery with a course which ranges from severe to extremely mild. It is at present the most common causative agent of dysentery throughout the entire world.

The Soviet classification divides these bacteria into 3 subspecies - Flexner, Newcastle, and Boyd. The subspecies differ in enzymatic

TABLE 28

Antigenic Structure of Flexner Bacteria

A Тип	B Подтип	C Типоспецифический антиген	D Групповые антигены		
1	1a	I	3,4	—	—
	1b	I	3,4	6	—
2	2a	II	3,4	—	
	2b	II	—	—	7,8
3	3a	III	—	6	7,8
	3b	III	3,4	6	7,8
	3c	III	—	6	—
	3 var.	III	3,4	6	—
4[1]	4a	IV	3,4	—	—
	4b	IV	(3,4)[3]	6	—
5[2]	5 (x +)	V	—	—	7,8
	5 (x —)	V	(3,4)	—	—
	var. x	—			7,8
	var. y	—	3,4		—

1) Including strain Boyd 103.
2) Corresponds to strain Boyd R119.
3) Parentheses indicate "irregular."

A) Type; B) subtype; C) type antigen; D) group antigens.

characteristics and, especially, serologically.

The true Flexner subspecies decomposes maltose, mannose, glucose, and occasionally sucrose and sorbitol; it is inactive with respect to lactose, zylose, salicin, and adonitol and usually does not form hydrogen sulfide, although some strains produce indol. It is serologically nonuniform, consisting of several (5) serological types with different type antigens. The group antigens vary slightly. The complex of group antigens determines the subtype to which the bacterium belongs in the international classification (Table 28).

The Newcastle subspecies is divided into 3 varieties, in accordance with biochemical properties: the Newcastle bacterium, the Denton-Manchester bacterium, and strain Boyd 88. These varieties differ from one another in biochemical properties, but are serologically uniform (Table 29).

TABLE 29

Biochemical Properties of Cultures of Sh. Newcastle

1 Наименование типа	2 Расщепление углеводов			6 Образование газа
	3 глюкозы	4 маннита	5 дульцита	
7 Ньюкасл	+	—	± (на 5-й день)	±
8 Бойд 88	+	+	±	—
9 Дентон-Манчестер	+	+	±	+

10

1) Type; 2) decomposition of carbohydrates; 3) glucose; 4) mannose; 5) dulcitol; 6) formation of gas; 7) Newcastle; 8) Boyd 88; 9) Denton-Manchester; 10) on 5th day).

The Boyd subspecies was first described in the Soviet Union by E.M. Novgorodskaya and it is consequently referred to in the temporary Soviet classification (1953) as the "Boyd-Novgorodskaya subspecies."

By decision of the Nomenclature Committee on 15 January 1962, this variety was renamed the "Boyd subspecies."

5. Sonne bacteria (Sh. sonnei) were first described by Duval, Kruse, and Castellani under different names. In 1915 they were studied in detail and described by Sonne.

An unusual property of Sh. sonnei is its ability to grow in colonies of two types, round and flat (Fig. 27). Both forms are unstable and easily break down on reculturing (Table 30).

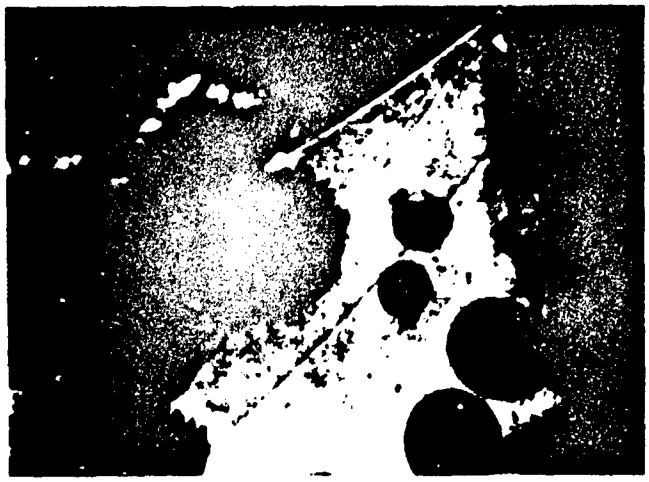


Fig. 27. Two types of Sh. sonnei colonies. x20.

Morphology and biology of dysentery bacteria; cultural characteristics

All dysentery bacteria are gram-negative, nonlabile rods 2-3 μ long and 0.6 μ wide. They multiply readily on ordinary nutritive media at an optimum temperature of 37°. They are all aerobes and facultative anaerobes, forming delicate, semitransparent colonies on agar. Except for Sh. sonnei, dysentery bacteria do not ferment lactose; except for

Sh. Newcastle, they do not produce gas in media containing carbohydrates (Table 30).

Laboratory Diagnosis of Dysentery

Bacteriological examination for detection of dysentery bacteria can be conducted for the following purposes:

1) diagnosis of disease;

2) epidemiological monitoring of convalescence to detect bacteria carriers;

3) detection of persons (especially in collectives) suspected of having chronic dysentery;

4) detection of persons suspected of being the source of infection in epidemics;

5) examination of workers in the food industry, the commercial distribution network, food-preparation units, pediatric institutions, etc.;

6) detection of dysentery bacteria in foodstuffs on special assignment.

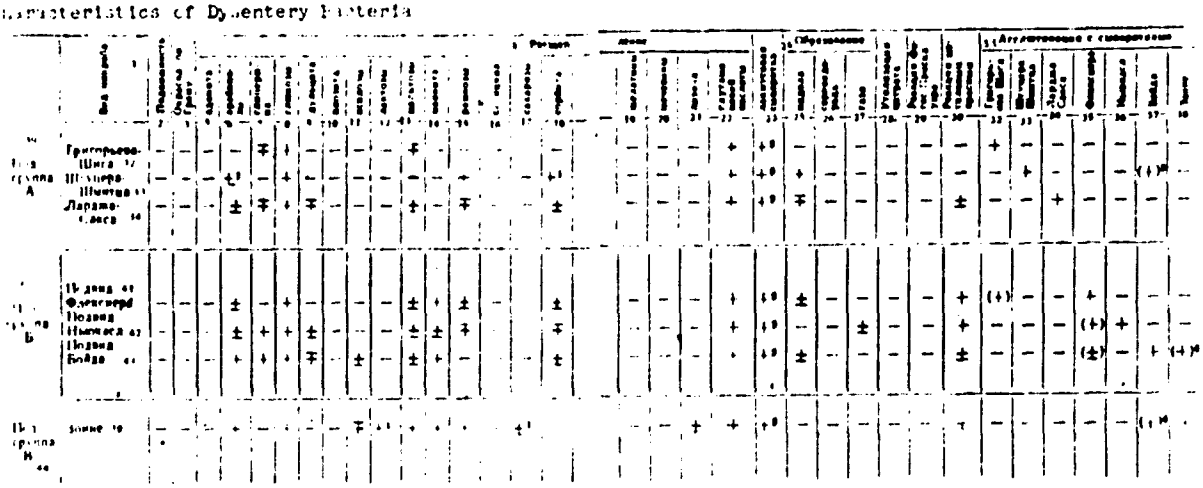
Each of these tasks has its specific conditions. Thus, a single diagnostic examination yields a satisfactory percentage of positive results only during the first few days of illness, provided that the samples are taken before treatment begins. Investigations carried out after the 3rd day of illness, especially when drugs have been taken, often yield negative results and it is only by repeated testing that the pathogenic bacteria can sometimes be isolated.

During epidemics the excreta of patients in therapeutic institutions must, in accordance with the instructions of public health groups, be repeated until negative results have been obtained on three occasions.

In accordance with paragraphs 4 and 5 of the epidemiological instructions, individual examinations must take into account the subject's

ABLE 30

Characteristics of Dysentery Bacteria



Notes: 1. More than 48 hr; 2) slightly reddish color; 3) type II; 4) type 6; ± indicates that not all types or cultures exhibit the characteristic in question; ∓ indicates that the majority of cultures do not decompose the substance in question.

[illegible]; 2) motility; 3) Gram staining; 4) decomposition; 5) dulcitol; 6) arabinose; 7) glycerine; 8) glucose; 9) dulcitol; 10) inositol; 11) xylose; 12) lactose; 13) maltose; 14) mannose; 15) rhamnose; 16) [illegible]; 17) sucrose; 18) sorbitol; 19) gelatin; 20) urea; 21) lysine; 22) glutamic acid; 23) litmus serum; 24) formation; 25) indol; 26) hydrogen sulfide; 27) gas; 28) citrate utilization; 29) Voge-Proskauer reaction; 30) methyl red reaction; 31) agglutination with sera; 32) Grigoriev-Shiga; 33) Stutzer-Schmitz; 34) Larin-Sachs; 35) Flexner; 36) Newcastle; 37) Boyd; 38) Sonne; 39) subgroup A; 40) subgroup B; 41) Flexner subspecies; 42) Newcastle subspecies; 43) Boyd subspecies; 44) subgroup C.

[illegible] clinical condition. A single examination is permissible only for absolutely healthy persons who have never had an acute gastrointestinal illness. A single examination is not sufficient when any intestinal symptoms (irregular stool, colitis, entercolitis, etc.) are present, even if it yields negative results. The number of bacteria isolated from [illegible] persons may at times be very small, but this is usually a variable

quantity. A single examination consequently cannot reveal persons suffering from chronic dysentery or bacteria carriers.

Material for examination; taking of samples

Since dysentery bacteria are localized primarily in the small intestine, the feces (the intestinal contents in studying corpses) are the principal material for examination for dysentery. In collecting feces it must be kept in mind that: 1) the bacteria are found principally in mucous and pus, so that it is necessary to select fragments containing mucous and pus (but never blood); 2) the first fecal portions are the plug from the ampulla recti and the bacteria in them may have died, so that it is necessary to collect the following portions, which come from the upper area of the rectum and the sygmoid colon. The last, fluid portions should not, however, be collected, especially after a laxative has been given, since they are the contents of the lower segment of the small intestine. They are of interest only when there is a suspicion of an ulcer in the upper portions of the large intestine or the lower region of the small intestine.

Feces can be collected without waiting for defecation by using a cotton pad or rectal tube. The latter is a glass tube ranging in diameter from 5 mm (for children) to 15 mm (for adults) and approximately 150 mm in length. Its lower end is hermetically sealed, like a test-tube. At a distance of 15-20 mm from the sealed end are two holes in opposite walls of the tube (one slightly below the other). The open end is sealed with cotton and the tube is inserted into a test-tube through a cotton plug and sterilized in this form. The tube is introduced as far as possible into the rectum; it is sufficiently wide that it touches the intestinal walls and, when it is withdrawn, material from the upper portion of the rectum collects in the blunt end.

It is also convenient to use ordinary open glass tubes with one

end sealed. A cotton plug is inserted into one end, while the other is inserted into a test-tube through a cotton stopper. The tube and test-tube are then sterilized together. Such tubes are very convenient for collecting material from chamber-pots, diapers, and the rectums of small children.

In large-scale examinations for dysentery collection of material is facilitated by using gauze pads held in forceps. Previously prepared gauze pads are sterilized in small boxes in an autoclave. Three pairs of forceps are oiled in a sterilizer; one is used to collect material, the second is left to cool, and the third is kept boiling. A gauze pad is picked up from the box with the sterile forceps, the catch of the latter is fastened, and the pad is inserted as far as possible into the rectum. It is then withdrawn, attempting to touch the intestinal wall with it and thus to collect mucous. Dishes can immediately be inoculated with material from the pad or it can be placed in preservative in a test-tube (or, still better, a penicillin flask) for culturing at the laboratory.

Fecal matter can be dried by applying drops of the material to a small (1.5-2 x 4-5 cm) piece of filter paper. The filter paper is then air-dried and wrapped in black paper for storage. Dysentery bacteria retain their viability for from 6 to 35 days under these conditions. For culturing the filter paper is inserted into a test-tube containing broth or a concentration medium and placed in a heater. After 18-20 hr differential media are inoculated with 2 or 3 drops of the resultant solution. It is also possible to flood the filter paper with 1-2 ml of physiological solution and then to culture 2 or 3 drops after 1-2 hr.

Examination of the patient by rectoromanoscopy makes it possible to collect cultural material directly from the affected areas of the mucosa. Mucous is gathered from the intestinal wall through the recto-

scope, using a cotton pad on a long wire. Cultures can be made immediately or the pad can be removed and placed in preservative.

The blood and urine are usually not cultured, since bacteria are rarely found in them.

The organs of cadavers (intestines, mesenteric glands, and parenchymatous organs) must be examined as soon after death as possible. The usual procedure is followed, first taking pieces from the liver, spleen, and glands and then dissecting the intestines.

Foodstuffs, especially milk products (pot cheese, sour cream), are examined when an epidemic occurs. Samples are taken in the usual manner; 25-30 ml of milk and several grams of other milk products are sufficient for bacteriological examination.

Products of this type are collected in heat-sterilized glass vessels, which are tightly stoppered and tied with string; the vessels should contain no traces of disinfectants.

Rectal tubes and pads should be inserted through cotton stoppers into test-tubes filled 1/3 full with a preservative.

The use of a preservative for feces from dysentery patients is dictated by the rapid death of dysentery bacteria in native fecal matter. The acid reaction of old feces and the presence of numerous other microorganisms, both symbiotic and occasionally antagonistic, have a destructive action on the rather unstable dysentery bacteria. The preservative should give the sample a neutral reaction and prevent multiplication of saprophytes. Nevertheless, direct culturing of feces yields better results than culturing from a preservative, provided that one basic rule is observed: the period between taking of the sample and culturing should be as short as possible. The greatest number of positive results is obtained when the material is processed at the patient's bedside. When feces are sent to the laboratory the time before culturing

should not exceed 2 hr. If this requirement cannot be met it is necessary to use preservatives. The feces of ambulatory patients must be stored in preservative.

Among the many preservatives for dysentery bacteria Tage's glycerine solution (see page 392) has received deserved recognition and come into wide use. Zack's modification of this glycerine mixture (page 392) can also be employed. Formalin in a dose such that its final concentration is 1:10,000 can be added to the glycerine mixture to inactivate any bacteriophage present in the feces.

In addition to the aforementioned glycerine mixture, a buffered solution of phosphate salts at pH 7.6 gives satisfactory results.

Test-tubes or other vessels are filled 2/3 full with the preservative, the material to be studied occupying the remaining 1/3.

If the material cannot be processed immediately it must be stored in preservative at 2-6° (in a refrigerator); culturing can be delayed for 12-24 hr under these conditions.

Bacteriological examination

The results of examinations for dysentery are determined both by correct taking of samples and by careful observation of the techniques for laboratory processing.

The patient's feces can be cultured directly in his room or in the hospital laboratory, without a preservative.

During the first day of the investigation a platinum loop is used to take pieces of mucous from the pussy, mucoid, or blood portions of the feces; these are carefully washed with physiological solution in two successive Petri dishes or 2-3 successive test-tubes. The mucous is then transferred to a medium in a Petri dish with the same platinum loop and spread with a glass spatula, which is then (without flaming) used to make cultures on a second and third dish.

Since the chance of a positive result increases with the quantity of initial material, many laboratories now make the initial culture by a method similar to that used for culturing water. A piece of mucous is rubbed into a limited area of the surface of the medium. The spatula is then removed from the agar and (without flaming) used to smear the remainder of the material over the rest of the dish. New material is taken for a second and, if possible, a third dish. When the experimenter acquires a certain skill and does not apply too large a drop to the dish, separate colonies grow over the entire surface of the medium, as well as in the area initially inoculated.

It is sometimes difficult for technical reasons to culture the material immediately on 2 or 3 dishes. A culture is then made on one dish on the first day and the remainder of the material is stored in preservative in a refrigerator until the following day. On the second day all samples which yielded negative results in their first culturing are recultured on one or 2 dishes.

Feces which contain no pieces of mucous or pus (small pisiform fragments) are mixed with 5-10 ml of physiological solution and this suspension is cultured on dishes in the manner described above.

For feces which have been stored in preservative any pieces of mucous or pus are removed and cultured by one of the methods described above, without washing. If there is no mucous or pus a drop of the preservative emulsion is applied to the surface of the medium.

Sectioned material and solid foodstuffs are ground in a sterile mortar, with sterile sand if necessary, and suspended in physiological solution in a ratio of 1:5 or 1:10. Dishes are inoculated with 0.1 ml portions of this suspension by the usual method. Milk and other liquid products are cultured undiluted.

The inoculated dishes are placed in a heater at 37° for 18-24 hr.

Various colored differential media can be used for detection of dysentery bacteria. The best of these are: 1) selective media containing bile salts and other reagents which suppress the growth of the concomitant microflora: Ploskirev's bacterial agar, cholic citrate medium, cholic biosulfate-citrate medium (following I.S. Ol'kenitskiy's formula), and other media of this type; 2) Levin's medium containing methylene blue and eosin; 3) Liberman and Assel's medium containing Congo red, in its original form or in Chandelet's modification; 4) bromcresol purple medium.

The existence of a large number of dysentery bacteria resistant to the most common antibiotics (synthomycetin and levomycetin) has led to the notion of adding synthomycetin to the nutritive media used for isolation of these bacteria (A.B. Chernomordik, P.S. Kobelev, and A.D. Kovalenko; Ye.V. Dianova). Such media increase the culturability of dysentery bacteria, promoting the growth of resistant forms, particularly those unaffected by synthomycetin and its derivatives.

Since Sh. Grigoriewa-shigae grows poorly or not at all on selective media, it is recommended that feces and other material be cultured on 2 dishes, one containing deoxycholate-citrate agar (Ploskirev) and the other containing Levin's medium, Liberman-Assel's medium, etc. The use of a pair of differential media is generally to be recommended, since every medium has its drawbacks, which are nullified by the use of two media.

As is customary for detection of Salmonella, use of a concentration medium or, as it is still called, a cumulative medium is to be recommended in examinations intended to detect persons carrying dysentery bacteria. The selection of a medium of this type for Shigella presents substantial difficulties, since these microorganisms have a substantially lower viability than Salmonella or other members of the co-

lon family. Bile or bile salts in combination with citrates are apparently obligatory components of such a medium. Satisfactory results are yielded by bile broth (20-40%) and by Kaufman's concentration medium with bile and brilliant green added. One advantage of these media is the fact that they are capable of concentrating Salmonella and Escherichia as well as Shigella. Shigella cultures on such media should be incubated in a heater for no more than 20 hr (16-18 hr is best) or kept at room temperature. Prolonged incubation promotes copious growth of E. coli and Proteus.

On the second day the Petri dishes are removed from the heater and the cultures are examined with the unaided eye and then with a magnifying glass (7 or 10 power), so as not to overlook any small colonies. Almost all pathogenic bacteria of the coliform group grow in round, colorless, transparent colonies on the majority of colored media; certain Salmonella produce cloudy colonies. Sh. sonnei can be recognized from the characteristic growth of two types of colonies, one of which is flat and has smooth margins, while the other is round, convex, and has a moist lustre (see Fig. 27). Some authors attribute this difference to dissociation and regard the flat colonies as rough and the round colonies as smooth. Bacteria from the smooth colonies do not, however, go out of suspension spontaneously, have antigenic and immunogenic properties, and are rather distinct serologically, i.e., do not have the basic characteristics of rough forms.

Rough colonies of any species of bacteria have the same characteristics: they are small and broad, have serrate margins and an irregular surface, and appear cloudy but are colorless. Bacteria isolated from such colonies precipitate when suspended in physiological solution, are agglutinated by trypaflavin solution (1:500 in 0.5% saline solution), and exhibit no specificity in agglutinative sera, being agglutinated by

heterologous sera (serological cosmopolitanism).

Suspected colonies are transferred to a short composite series consisting of agar slants and Hiss' medium containing lactose and glucose (with floats in the test-tubes). If facilities are available and the colonies have not all been used for culturing, rough agglutination can be carried out on slides with a mixture of Flexner and Shiga sera or of Stutzer-Schmitz and Boyd sera. The drops are then permitted to dry and the smears are fixed and stained by Gram's method.

Many authors successfully use Ressel's medium (see Appendix) instead of the short composite series.

More complex combinations are now employed instead of Ressel's medium for culturing colonies: a) slants (Ye.D. Ravich-Birger and V.N. Meshalova's medium); b) triple media (Chernomordik's medium, Roland's modification of Gain's medium) etc.

All these media are intended to combine indications of the various enzymatic systems of the bacterium in a single test-tube.

Several colonies (5-10) are transferred from each dish. Not all the suspected cultures isolated need be studied, one or two being retained until the end of the investigation, for use if the cultures investigated must be discarded.

The cultures are kept in a heater at 37° until the following day.

On the third day the data obtained makes it possible to get some idea of the results. If media containing lactose are greatly altered during the first few days as a result of fermentation the culture can be discarded as nonpathogenic. If all the cultures isolated ferment lactose during the first few days the examination is discontinued as having yielded a negative result. The same is true of cultures which decompose urea, a compound usually not broken down by the pathogenic members of the coliform family. Strains which do not affect media con-

taining lactose and do not decompose urea are studied morphologically in Gram-stained preparations and examined for mobility in hanging drops. If the morphology of the culture does not correspond to that of gram-negative bacteria the result is negative.

Cultures which do not decompose urea, ferment lactose to some extent, and do not form gas in glucose-containing media are subjected to further study (Sonne bacteria). Cultures which ferment glucose to produce a gas and alter lactose-containing media belong to the genus Escherichia, those which decompose urea belong to the genus Proteus, etc.

Mobile gram-negative rods which do not break down lactose but ferment glucose to form a gas are suspected of being Salmonella.

Mobile gram-negative rods which do not ferment lactose but decompose glucose without gas formation are suspected of being S. typhi.

Nonlabile gram-negative rods which do not decompose urea, do not ferment lactose, and break down glucose without gas formation are suspected of being Shigella.

All suspicious strains can be checked by rough agglutination on slides with the corresponding nonadsorbed specific sera in dilutions of 1:25 and 1:50. The occurrence or nonoccurrence of agglutination does not, however, resolve the question of the species to which the culture belongs and should be regarded as a guideline. The first generations of freshly isolated cultures often agglutinate poorly. At the same time, cultures in the transitional or rough phage may easily be agglutinated at high titres by heterologous sera.

Cultures selected as suspicious are transplanted to agar slants. Test-tubes containing mannose, maltose, and sucrose (as well as rhamnose, dulcitol, and arabinose if possible) are added to a short series of Hiss media. The tubes containing lactose and glucose are retained from the preceding day. Cultures are also made in tubes containing sim-

ple Hottinger's (tryptophan) broth and 1% peptone water for determination of indol and hydrogen sulfide formation (see page 393).

Investigation of the sucrolytic capacity of the culture can be carried out more rapidly and compactly in a Petri dish. Simple agar is inoculated with a heavy growth of the culture. The indicator-containing agar is divided into 4-6 segments and a disk of filter paper wetted in a 30% carbohydrate solution is applied to each of them (Luce, Petersen and Rustum).

Ordinary agar containing bromcresol purple can be used, impregnating the pieces of filter paper with 0.03-0.05 ml (one drop) of a 20% carbohydrate solution. After the bacterial growth has been transferred the pieces of filter paper are placed on the dish and a thin layer of simple agar is poured over it to remove all gases (B.V. Karal'nik and A.I. Kharitonova, A. Lentsner).

All the cultures are kept in a heater at 37° overnight.

On the third day of the investigation, i.e., 48 hr after culturing, a preliminary evaluation can be made on the basis of the morphology of the colonies and cells, the biochemical activity of the bacteria in the short composite series, and the results of the rough agglutination on slides.

On the fourth day the growths in all the test-tubes are examined. Utilizing the data yielded by the composite series and the rough agglutination, an agglutination reaction is set up with various dilutions of homologous serum (the expanded agglutination reaction). Since the agglutination of dysentery bacteria is somatic in character, the test-tubes are placed in a heater at 37° for 18-20 hr or in a water bath at 55° for 4 hr.

The sera used to identify the cultures isolated should be of sufficiently high quality. In normal work it is necessary to have avail-

able the following set of sera: 1) Flexner specific agglutinative serum, including all serological types and adsorbed Flexner type (monoreceptor) sera; 2) Newcastle serum; 3) Boyd serum and adsorbed Boyd type sera; 4) Shiga serum; 5) Stutzer-Schmitz serum; 6) Sonne serum to agglutinate both the round and flat types.

This set of sera makes it possible to determine the species and type of the cultures isolated. Type is determined for cultures of Sh. large-sachsii and the Flexner and Boyd subspecies. It is helpful to use mixtures of sera for different species (no more than 4 sera per mixture) for an initial orientation. The reaction is carried out on slides and a positive result is checked by expanded agglutination with each component of the mixture.

The type of the culture is determined with adsorbed sera on slides. Drops of the monoreceptor sera, which are diluted to the requisite titre when prepared, are applied to slides. Some of the culture is introduced into each drop with a platinum loop and the two are mixed until a uniform cloudiness is obtained. A fresh portion of culture is used for each serum and the loop flamed after each use.

A catalase test can be conducted on the same day; this test yields a positive result with all dysentery bacteria except Sh. grigoriewa-shigae. A drop of hydrogen peroxide is applied to a slide and a glass rod (sealed Pasteur pipette) is used to stir in a bit of a day-old agar culture. A positive reaction is indicated by the appearance of gas bubbles in the drop.

The fifth day is the day on which a final decision is reached regarding the nature of the culture and a report is made. The composite series is examined and delayed fermentation is noted. Indol formation is determined by Ehrlich's method in the two-day broth culture and hydrogen sulfide formation is noted.

The latter two tests are conclusive when a number of conditions are observed. Determination of indol formation with paper impregnated with oxalic acid is not a sufficiently sensitive method and is permissible when only a rough evaluation is needed. Ehrlich's method and Ehrlich's reagent or paper wetted in modified Ehrlich's reagent must be employed for more precise determination.

For determination of hydrogen sulfide formation cultures must be made in 1% peptone water or by the stab method in media containing iron salts. Such media include Chernomordik's iron-citrate medium and Christensen's medium.

Finally, the agglutination reaction is evaluated. Because of the fine-grained character of the agglutination, reactions involving dysentery bacteria must be evaluated with an agglutinoscope or a magnifying glass. Identification of the cultures isolated presents no difficulties if their biological and serological properties conform to the characteristics of one of the species of Shigella (see Table 30).

Freshly isolated cultures do not, however, always correspond to this scheme in all their properties. Cultures which differ in one given characteristic are often wholly pathogenic and epidemiologically dangerous. When cultures which are not wholly typical are isolated an attempt should consequently be made to determine their true nature. This is made even more important by the fact that widely employed antibiotics and sulfanilamides act on the intestinal flora in a manner which promotes variability of both pathogenic and saprophytic bacteria. No less significant a factor in this variability is the use of selective media, which undoubtedly suppress the vital activity of pathogenic members of the coliform group, although to a lesser extent than they suppress that of the concomitant microflora. These two factors, together with others previously known, have led to the appearance of large num-

bers of so-called atypical forms. The nature of these forms varies and they may occur with equal frequency among pathogenic bacteria and saprophytes; determination of their genetic affinities is consequently difficult at times.

The most common deviation from the typical pattern is exhibited by strains which have lost their agglutinability. These strains should not be lightly dismissed, even when their morphological and biochemical characteristics correspond to none of the species of Shigella. Their agglutinability can occasionally be restored by several (3-5) daily transplantations to fresh (moist) agar slants. These transplantations can be alternated with reculturing on broth containing glucose. It is also recommended that nonagglutinable cultures be transplanted to dishes, followed by selection of those mature colonies which agglutinate with the corresponding serum on slides. Finally, the best method of identifying its culture is study of its thermostable antigen. Agglutination with a boiled bacterial suspension is employed for this purpose. Material washed from the culture in 3-5 ml of physiological solution is heated in a boiling-water bath for 1-1½ hr. An expanded agglutination reaction is set up with the boiled emulsion in the usual manner. The serum dilutions begin at 1:20 and the results must be evaluated with an agglutinoscope or magnifying glass, since the agglutinate is fine-grained. The thermostable antigen can also be determined quite successfully by precipitation with a hapten obtained from the culture under investigation.

When the antigenic character of a bacterium can be determined only by reacting it with a boiled antigen or hapten, the strain is designated as nonagglutinative. Cultures which react at no less than half the serum titre are regarded as highly agglutinative. Cultures which agglutinate at less than half the serum titre are designated as poorly ag-

glutinative.

Atypical cultures may be highly agglutinative but exhibit a deviation in one of their biochemical characteristics. In such cases it is first necessary to be certain that the strain is pure. A double culture is sometimes produced when colonies are isolated, especially if they do not derive from a single bacterium, but are initially a symbiosis of two species. Inoculation of a differential medium with a liquid suspension of the culture makes it possible to isolate the strain in pure form, which is then found to be typical.

A culture will occasionally exhibit biochemical deviations but still be suspected of belonging to the Shigella group. It is then necessary to study its somatic antigen by one of the methods described above. If the agglutination reaction with boiled antigen or the precipitation reaction with a hapten yields negative results the culture must be discarded. It is necessary to regard the agglutinability of the culture as an acquired characteristic or to attribute it to the presence of common antigen fractions in different bacteria or to random convergence of components of the culture's antigenic structure. One example of this is the fact that strains of Sh. flexneri often agglutinate with typhoid serum. Such a culture sometimes agglutinates with identical titres of Flexner serum and typhoid serum. The investigator's conclusion then depends on the presence or absence of lability and hydrogen sulfide formation and sometimes on the results obtained by reacting the culture with a boiled bacterial suspension.

Use of bacteriophage may be of some aid in identifying dysentery cultures. Both a composite series and 2 test-tubes containing broth are inoculated with the culture. After 4 hr 2 drops of dysentery bacteriophage (polyvalent or monovalent) diluted to 1:10 are added to one of the test-tubes. An equal quantity of bacteriophage is added to a tube

containing sterile broth and all the tubes are placed in a heater. The intensity of the growth in the two tubes containing inoculated broth is compared on the following day. The tube containing broth and bacteriophage but no culture serves as a bacteriophage-sterility control.

It is possible to add the bacteriophage at the time the culture is transplanted. In this case the action of the bacteriophage is manifested not in lysis, but in retardation of culture growth; the results are evaluated in the same manner. Complete or partial clarification of the broth containing culture and bacteriophage, the broth containing culture alone remaining cloudy, indicates that the bacteriophage has had a specific action, i.e., that it is related to the culture. If the bacteriophage is polyvalent it may be assumed that the culture consists of dysentery bacteria. A precise identification is made by comparing other characteristics. When monovalent phages are used lysis or retardation of growth confirms the nature of the culture with high reliability.

M.A. Yelshina et al., have developed media which make it possible to differentiate Sh. flexneri and Sh. sonnei from Escherichia and Proteus. These consist of synthetic nutritive media with and without nicotinic acid added. All Escherichia grow on both media, Proteus grows on neither of them, and Sh. flexneri (except Sh. Newcastle) and Sh. sonnei grow only on the medium containing nicotinic acid (page).

Accelerated methods of bacteriological examination

Every experimenter has his own method of accelerating evaluation of the results of examinations for dysentery. Use of any of these methods is justified if it includes determination of all the characteristics of the bacterium. Accelerated techniques for bacteriological examination should not exclude study of the morphology and biochemical or serological properties of the microbe. It is permissible to omit determination of some of these characteristics only in making a rough evaluation.

Accelerated bacteriological examination for dysentery is conducted by the following method, which can serve as an example for practical work but is open to a number of modifications.

As in the usual procedure, the material (feces, foodstuffs, etc.) is cultured on colored differential media on the first day. After 18-20 hr suspicious colonies are isolated on composite media: slants (containing lactose, glucose, and urea) or 2 test-tubes containing media of the Ressel type (one with 1% lactose and 0.1% glucose and the other with 1% sucrose and 0.1% mannose). Both series include one test-tube containing peptone water* and one containing Hottinger's broth, with appropriate paper for determination of hydrogen sulfide and indol beneath their stoppers.

After 4 hr smears are made from the growth on the Ressel medium and Gram-stained; the bacteria are investigated for lability and a rough agglutination reaction is set up with a mixture of the most common sera. If these data indicate that a pathogenic microorganism may have been isolated a preliminary report can be made on the second day.

Transplants are simultaneously made from the broth to a composite series** and agar slants. Cultures are also made in 2 test-tubes containing broth, to one of which 2 drops of polyvalent dysentery bacteriophage in a dilution of 1:10 are added, while the other (without bacteriophage) serves as a growth control.

The results of the composite series and the phage test are evaluated at the end of the 2nd day and an expanded agglutination reaction is set up; the latter is evaluated after 4 hr in a water bath at 55°.

A final report can be made on the 3rd day after the examination begins.

Our knowledge of the antigenic structure of bacteria, especially of the antigenic composition specific to gram-negative bacteria, has

made it possible to develop diagnostic methods based on detection of primary antigens. Antigen complexes can be extracted from bacterial cells by chemical means and detection of such complexes in human excreta undoubtedly indicates the presence of the corresponding bacteria. At the same time, such antigens react with sera from persons infected by the corresponding species of bacterium. A complete antigen (the somatic antigen) and a hapten are used for this purpose.

Annular precipitation reaction with hapten. The specificity of somatic antigens and, especially, of haptens (which are polysaccharides) makes it possible to detect them in mixtures of different bacteria if sufficiently sensitive sera are employed. This reaction is sensitive and specific. It reveals the presence of given bacteria even when they are very few in number (40-60 million) or when they are inaccessible to ordinary bacteriological methods.

The hapten is obtained from washings made from an initial fecal culture in a dish containing any medium except medium Zh or Ploskirev's medium. Just as in bacteriological examination, pieces of mucous or pus or a drop of fecal matter (or any other material) is applied to the surface of the agar. There is no need to provide for growth of separate colonies, since the thicker the initial culture, the greater will be the chances that the dish contains dysentery bacteria. After 18-20 hr the growth is washed with 4-5 ml of 1% acetic acid and the washings are poured into a test-tube and boiled in a water bath for 30 min to 1 hr. The emulsion is then filtered through asbestos wool of the type used in the Ascoli reaction. A small piece of the asbestos is packed tightly (but not too tightly) into the neck of a small funnel, which is inserted into a test-tube. Filtration yields a clear colorless filtrate, which is brought to a neutral reaction with 20% sodium carbonate in the presence of an indicator - an alcoholic-aqueous solution of rosolic

acid (0.1 g of rosolic acid, 10 ml of alcohol, and 70 ml of distilled water) or bromthymol blue. If the solution becomes cloudy during alkalization the filtration is repeated. The clear filtrate (in 0.1-0.2 ml portions) is overlayered on an equal quantity of precipitative serum, either whole or diluted to 1:1, in narrow test-tubes. There should be a clearly defined boundary between the two liquids, at which a white ring appears when the reaction is positive. The observation period lasts 2 hr and if no ring forms during this time the result must be regarded as negative.

The precipitative serum must receive special attention. The higher its precipitative titre, the more sensitive will be the reaction. However, not all sera which precipitate with the complete antigen react with the hapten. Each series of serum must consequently be titrated with a known hapten obtained from a homologous culture. Washings from the culture are treated in the manner described above and overlayered on the serum in dilutions of 1:2, 1:4, 1:8, 1:16, etc. The higher the hapten dilution with which annular precipitation is obtained, the more suitable is the serum. Flexner serum should include all serological types.

Rapoport's accelerated method

In this method colonies taken from medium Zh are cultured on indicator-containing glycerine agar in Petri dishes rather than on a composite medium in test-tubes. The glycerine test is used to differentiate dysentery bacteria. Neither any of the serotypes of Sh. flexneri nor Sh. boydii type VI decompose glycerine, while Sh. grigoriewa-shigae, Sh. sonnei, Sh. newcastle, Sh. stutzeri-schmitzii, and the remaining types of Sh. boydii break down this compound. The capacity of the culture for gas formation can also be determined, since E. coli and certain Salmonella produce gases in glycerine media.

Serological investigation

Bacteria cannot be isolated from the feces in the later stages of dysentery and this technique consequently cannot be employed to establish a retrospective diagnosis. It also fails to give any indication of the character and outcome of the processes taking place in the body during the illness. The results of dynamic immunological study of the patient or convalescent's blood serum indicates whether he has previously incurred the disease and whether a chronic process has developed and furnishes other data on the body's reaction to the infectious agent.

The character of agglutination under dynamic conditions with diagnostic sera prepared from dysentery bacteria (Sh. flexneri, Sh. sonnei) varies in accordance with the form of the disease. In acute cyclic dysentery the agglutinin-titre curve has a normal trend, exhibiting a rise which begins during the 2nd week or later. In protracted dysentery, when the body reacts sluggishly to the infection, agglutinins in titres of more than 1:100 appear later, occasionally not until the 4th-5th week of illness, and increase slowly. Appropriate therapeutic intervention leading to recovery gives the agglutinin-titre curve the form described above for acute cyclic dysentery. When a chronic condition develops this curve has a wave-like form, dropping to a titre of 1:50 or less and increasing to a titre of 1:400-1:800.

While in acute dysentery the increase in titre indicates development of immunity, in the chronic form it results from activation of a latent focus. This hypothesis is borne out very well by simultaneous determination of the agglutinin titre and the phagocytic activity of the leucocytes. In chronic cases, especially before exacerbation, high agglutinin titres are conjoined with a reduced capacity for leucocytic phagocytosis of the causative agent. Both indices increase when the patient recovers.

The agglutination reaction is set up in the same manner as Widal's reaction for typhoid and paratyphoid. The serum dilutions range from 1:50 to 1:800-1:1600. However, the typological diversity and marked variability of dysentery bacteria requires that special attention be paid to the antigen. It is best to use diagnostic sera prepared from selected, tested cultures and fixed with 0.1-0.2% formalin in Widal's reaction for dysentery. Test-tubes containing controls for each diagnostic serum must be included in setting up the reaction. Diagnostic sera prepared from Sh. flexneri should incorporate the maximum number of type cultures or an individual serum should be made up from each type culture. In the latter case the type sera are used separately in the reaction.

The reaction is evaluated after 20-24 hr in a heater at 37° or after 4 hr in a water bath at 55°. Since the agglutinate is fine-grained, the results are determined with an agglutinoscope or magnifying glass. In using diagnostic sera the test-tubes should not be shaken vigorously while reading the results, since the bacteria do not adhere tightly and the granules are easily broken up.

The agglutination reaction is an auxiliary diagnostic method. The most reliable results are obtained by studying the reaction under dynamic conditions. Repeated investigations make it possible to detect agglutinins formed at later times, to exclude nonspecific group reactions, and to study the trend of the increase in agglutinin content.

The annular precipitation reaction with complete antigens is based on the sensitivity and specificity of preparations obtained from gram-negative bacteria by chemical treatment (complete antigens).

Fresh nonhemolyzed serum and complete antigens prepared by Buaven's method from Sh. flexneri and Sh. sonnei (a combination of serological type) are used for the serological diagnosis of dysentery.

The antigen is stored in dry form and diluted by a factor of 1000, 1 mg in 1 ml of physiological (0.85%) sodium chloride solution, before the reaction is set up (several hours beforehand or on the preceding day). This initial dilution can be used for 3 weeks when stored in a refrigerator.

Approximately 0.1 ml of the serum to be investigated, whole or diluted to 1:1, is poured into a narrow test-tube. The same quantity of antigen is overlayered on it with a fine Pasteur pipette. There should be a distinct boundary between the liquids, at which a clearly defined white ring appears if the reaction is positive.

The observation period lasts 1 hr at room temperature.

In the majority of cases of dysentery the annular precipitation reaction yields positive results between the 5th and 14th days of illness, i.e., when bacteria can rarely be isolated from the feces.

Nutritive Media; Preservatives

Tyge's glycerine mixture. A total of 0.5 liter of glycerine is added to 1 liter of physiological solution and the pH is adjusted to 8.0 by addition of 20% sodium phosphate (Na_2HPO_4). The mixture is sterilized at 112° for 15 min and then has a pH of 7.6-7.8. In Zack's modification 0.1% alcoholic methyl red is added to indicate any reaction during storage.

Phosphate buffer mixture. A total of 0.45 g of potassium hypophosphite (KH_2PO_4) and 5.34 g of efflorescent sodium phosphate (Na_2HPO_4) are added to 1 liter of distilled water. The mixture is poured into bottles and sterilized in an autoclave at 120°.

Bile broth. A total of 200 ml of native bile at pH 7.6 is added to 800 ml of broth and the mixture is sterilized in an autoclave at 120° for 30 min.

Preparation of indicator for hapten reaction. Rosolic acid. A to-

tal of 0.1 g of rosolic acid is dissolved in 10 ml of alcohol and 70 ml of distilled water is then added. The resultant solution is ready for use.

Bromthymol blue. A total of 0.1 g of indicator is ground in a mortar with 3.2 ml of 1/20N sodium hydroxide and the two are added to 25 ml of distilled water. This basic solution is diluted by a factor of 10 to make up the working solution.

Preparation of indicator paper: for indol.

Paradimethylamidobenzaldehyde - 5 g.

96° alcohol - 50 ml.

Purified concentrated phosphoric acid - 10 ml.

The ingredients are mixed until the powder dissolves. Strips of filter paper are wetted with the warm solution thus obtained, dried, and cut into pieces.

The paper is yellow in color. When indol is present in the broth the paper changes color to a lilac red or intense crimson.

Paper for hydrogen sulfide.

Sheets of filter paper are wetted in the following solution:

Distilled water - 100 ml.

Lead acetate - 20 g.

Sodium diacetate - 1 g.

The paper is dried and cut into strips. It is colorless when prepared and it turns black in the presence of hydrogen sulfide.

Gain's medium.

Roland's triple modification:

Distilled water - 1000 ml.

Dry agar - 13 g.

Peptone - 20 g.

Sodium chloride - 5 g.

Bring to pH = 7.5 and add:

Lactose – 10 g.

Glucose – 1 g.

Sodium hydrosulfite – 0.2 g.

Ferrous ammonium sulfate – 0.2 g.

pH = 7.4.

1% aqueous phenol red – 2.5 ml.

The medium is slanted to obtain a column.

Ressel's medium. This medium contains 0.1% glucose, 1% lactose, and an indicator and is slanted in such fashion that a column of agar is left 2-3 cm from the bottom. Stab cultures are made in the column and streak cultures on the slanted surface. This medium replaces 3 test-tubes: the slanted surface can be used instead of an agar slant; smears and cultures can be made from the medium and it can be used for rough agglutination reactions; the lactose which is contained can be reacted, the slanted surface being stained by an appropriate indicator when the lactose ferments (red when Andrade's indicator is used). The glucose in the column can be reacted, staining the column when it is decomposed; if gas is produced it collects in the bottom of the tube and forms bubbles in the agar.

Preparation of Ressel's medium from dry nutritive media. A total of 40 g of dry nutritive agar containing BP indicator and lactose and 5 g of dry nutritive agar are added to 950 ml of distilled water. The mixture is dissolved by heating to the boiling point. A total of 1 g of glucose is dissolved in 50 ml of distilled water and added to the mixture. Portions of 5-6 ml are poured into sterile test-tubes and twice sterilized in running steam for 30 min. The medium is slanted so as to leave a small column.

Ressel's medium containing mannose and sucrose is prepared in ac-

cordance with the formula given above: 40 g of dry nutritive agar containing BP indicator and sucrose is mixed with 950 ml of distilled water and 1 g of mannose dissolved in 50 ml of distilled water is then added.

The medium is grayish red in color and turns blue when an acid is formed.

"Slant column" medium.

It is recommended that the "slant column" medium be prepared in two stages: 1) 0.75-1% of agar-agar and 20 ml of a complex indicator consisting of 100 ml of Andrade's indicator and 0.4 g of thymol blue are added to 1000 ml of broth (or peptone water). This portion of the medium is sterilized in an autoclave at 120° for 30 min. 2) a total of 1 g of glucose, 10 g of lactose, and 10 g of urea are dissolved in 100 ml of sterile distilled water in a sterile vessel. This solution is added to the basic medium (1) and 5-6 ml portions of the resultant mixture are poured into sterile test-tubes and sterilized in running steam for 30 min. The medium is slanted like Ressel's medium, i.e., to leave a column. It is yellowish in color and turns red in the presence of an acid reaction and blue or violet in the presence of an alkaline reaction.

In addition to this method of preparing slant columns, there is a simpler method using dry Hiss' nutritive medium containing lactose and BP indicator.

A total of 4 g of dry Hiss' medium containing lactose and BP indicator, 0.1 g of glucose, and 1 g of urea are added to 100 ml of hot sterile distilled water; the resultant solution is poured into test-tubes and sterilized in running steam for 30 min. The medium is slanted like Ressel's medium.

The medium is reddish in color and turns blue in the presence of an acid reaction and orange in the presence of an alkaline reaction.

Rapoport's glycerine medium

A total of 2 ml of sterile bovine bile, 2 ml of chemically pure glycerine, and 2 ml of 2% aqueous acid fuchsin are added to 100 ml of unmelted agar. The agar is melted, cooled to 50°, thoroughly mixed, and poured into Petri dishes.

Chernomordik's ferrous citrate medium

Water – 1 liter.

Peptone – 15 g.

Ferrous ammonium citrate – 1 g.

Potassium hypophosphite – 1 g.

Hyposulfite – 0.15 g.

Agar-agar – 5 g.

After all the ingredients have been dissolved in boiling water the medium is poured into test-tubes and sterilized in an autoclave under a pressure of 1 atm. Cultures are made by the stab method; the column of medium turns black when hydrogen sulfide is formed.

Christensen's medium

A total of 100 ml of molten 1% Hottinger's agar at pH = 7.2 is mixed with 0.5 ml of 1% aqueous ferrous chloride. The mixture is poured into sterile test-tubes to form columns, which are inoculated by the stab method.

Nicotinic acid medium

See M.A. Yelshina, B.G. Zatulovskiy, Ye.G. Zaydenberg, Ye.T. Litovchenko, and V.V. Shubs (Laboratornoye delo [Laboratory News], 1957, No. 3).

Drobot'ko's synthetic medium, which consists of 1 g of $(NH_4)_3PO_4$, 1 g of K_2HPO_4, 0.2 g of Na_2SO_4, 0.1 g of $MgSO_4$, 5 g of NaCl, 5 g of

glucose, and 1000 ml of distilled water, is mixed with 4 µg of nicotinic acid per ml; the resultant medium is sterilized in running steam and 2.5 ml portions are poured into test-tubes. No fewer than 200,000 bacteria per ml are cultured from washings made from agar cultures. The observation period lasts 1-2 days.

Increasing Phage-Titre Reaction

Prof. L.M. Gol'dfarb

Indicator phages. a) Indicator phage for Sh. flexneri. This phage was obtained by V.D. timakov and D.M. Gol'dfarb from serum and polyvalent dysentery phage produced by the Tbilisi Vaccine Institute by prolonged passivation in a S-form culture of Sh. flexneri No. 170 (type C). The phage is passivated in meat-infusion broth at pH = 6.8-7.0; 0.1 ml of day-old washings from a culture on agar slants and 0.5 ml of phage are added to 4.5 ml of broth and incubated at 37° for 18-20 hr. This is followed by heating at 58° for 30 min. The action spectrum of the phage was studied by application to the culture under investigation on a solid medium. The results, which are shown in Table 30, indicate the high specificity of this phage, since coli and other members of the coliform group constituted no more than 4.5% of the total.

The action spectrum of the phage is not limited to Sh. flexneri alone, since it reacted with some of the Sonne cultures tested. This indicator phage must consequently be categorized as interspecific in its action.

The yield of this phage, determined by Ellis and Delbrook's method, varies from 100 to 250 particles. Its latent period averages 14-15 minutes and its adsorption amounts to 80%.

The phage is thermostable and is not inactivated at 58°, which enables us to use this temperature for heating the mixture after incuba-

tion.

b) Sonne indicator phage. This phage was obtained by D.M. Gol'dfarb and V.N. Kuznetsova from serum and polyvalent dysentery phage produced by the Tbilisi Vaccine Institute by prolonged passivation in Sh. dysenteriae Sonnei strain No. 714.

According to V.N. Kuznetsova's data, the phage has a sufficiently high specificity.

c) Indicator phage for Newcastle dysentery bacteria. This phage is a pure strain obtained from the monovalent Newcastle phage described by Solobay. It was isolated by F.I. Yershov and R.M. Chernyaskaya, who passed the pure strain through a culture of the "Sergeyev" strain of Sh. newcastle. It is characterized by high specificity, being limited to Sh. newcastle. This phage also has a high adsorptive capacity, being 90% adsorbed on the "Sergeyev" strain within 10 min. Its reproductive latent period is 30-35 min, its growth period 20 min, and its yield 550 particles per cell. It is thermostable, having a temperature threshold of 67°.

These phages have been used to conduct investigations for detection of dysentery bacteria in artificially inoculated substrates and in natural subjects. The manner in which the reaction is set up varies somewhat in accordance with the character of the subject.

Examination of feces. A total of 2-3 g of unpreserved infected feces is flooded (in a flask) with 10 ml of meat-infusion broth per g. The contents of the flask are shaken with glass beads for 5-10 min. Wide test-tubes are used for the remainder of the procedure. Portions of 9 ml of the fecal suspension under investigation are poured into test-tubes Nos. 1 and 2 and 9 ml of meat-infusion broth is poured into tube No. 3. A total of 1 ml of the indicator phage diluted with broth to a concentration of the order of 10^4 particles per ml is then added

to tube No. 1. The phage dilution used in setting up the increasing phage-titre reaction is referred to as the "working dilution." A total of 1 ml of meat-infusion broth is added to tube No. 2, which serves as the free-phage control. A total of 1 ml of the working phage dilution is added to tube No. 3, which is the indicator-phage titre control. This latter control becomes one of a group of simultaneous analyses. All three mixtures are incubated at 37° for 4½-5 hr. After incubation the contents of all the tubes are diluted with meat-infusion broth, the extent of the dilution being such that when 1 ml of the mixture from tube No. 3 (the titre control) is cultured several tens of negative phage colonies are obtained. The diluted mixtures are heated at 58° for 30 min. The phage compensation per ml is then determined by Grazia's agar-layer method. The standard bacterial cultures are Sh. flexneri No. 170 for the Flexner phage, Sh. sonnei No. 714 for the Sonne phage, and the "Sergeyev" strain of Sh. newcastle for the Newcastle phage. It is desirable to investigate the contents of the experimental tube (No. 1) and the phage-titre control (tube No. 3) on two dishes, while one dish is sufficient for the free-phage control.

Example of calculation of dilutions. Let us assume that the initial indicator phage contained $5 \cdot 10^8$ particles per ml. In order to obtain the working dilution the phage is diluted by a factor of 50,000 in meat-infusion broth, so that the phage concentration becomes 10,000 particles per ml. Since this phage dilution is added to the material and the control in a ratio of 1:10, its concentration is then 1000 particles per ml. The quantity of phage in tube No. 1 may increase after incubation, while that in tube No. 3 (the phage-titre control) remains unchanged. The subsequent dilution should be such that 1 ml of material from tube No. 3 cultured on a dish will yield several tens of colonies; the contents of the tube are consequently diluted by a factor of 200,

i.e., 50 negative colonies are expected in the control.

The results of the reaction are evaluated by counting the number of negative colonies growing on dishes inoculated with the appropriate bacterium by Grazia's method. The phage-inoculated dishes are incubated for 5 hr.

The reaction is evaluated on the following scale: increase in phage titre by factor of up to 3 – negative reaction (–); increase by factor of from 3 to 5 – weakly positive reaction (+); increase by factor of from 5 to 7 – positive reaction (++); increase by factor of from 7 to 10 – positive reaction (+++); increase by factor of more than 10 – strongly positive reaction. When the feces contain "free" phage the latter can be detected in the dish on which the material from tube No. 2 has been cultured. In this case the evaluation of the reaction is based on a count of both phages, free and indicator. When the reaction is positive the number of negative colonies in the dishes containing cultures from tube No. 1 should exceed the sum of the numbers of colonies obtained by culturing material from the titre control and the free-phage control.

Examination of water. Portions of 80 ml of the water to be investigated are poured into 2 sterile flasks (Nos. 1 and 2).

A total of 10 ml of a concentrated medium composed of 100 ml of meat-infusion, 10 g of peptone, and 5 g of sodium chloride (pH = 7.2-7.4) and 10 ml of the working indicator-phage dilution are added to flask No. 1 (experimental sample).

A total of 10 ml of the aforementioned concentrated medium and 10 ml of ordinary meat-infusion broth are added to flask No. 2 (which serves as a control for free phage). Flask No. 3 is filled with 90 ml of meat-infusion broth and 10 ml of the working phage dilution (phage-titre control). The latter control becomes one of a group of simultan-

tous analyses.

The flasks are kept in a heater at 37° for 4½-5 hr and are then examined by the technique described for feces.

In order to increase the sensitivity of the reaction the time for which the water is in contact with the phage can be prolonged to 16-18 hr.

The results are evaluated on the scale described above.

Detection of dysentery bacteria on environmental objects. Wipings are made from the surface to be investigated with a pad wetted in sterile tap water. The pad is placed in a test-tube to which 10 ml of meat-infusion broth has been added. The tube is thoroughly shaken and the pad is wrung out and removed; portions of 4.5 ml of the washings are poured into test-tubes Nos. 1 and 2. A total of 0.5 ml of the working indicator phage dilution is then added to tube No. 1 (experimental sample). A total of 0.5 ml of meat-infusion broth is added to tube No. 2 (free-phage control). A total of 4.5 ml of meat-infusion broth and 0.5 ml of the working phage dilution are added to tube No. 3 (phage-titre control).

The phage-titre control becomes one of a group of analyses. The test-tubes are incubated in a heater at 37° for 16-18 hr and then examined by the method described above.

When it is impossible to study each washing separately a mixture of washings from a group of different objects can be examined. In such cases each pad is washed with a small quantity (2-3 ml) of meat-infusion broth and, after being thoroughly shaken, the washings are poured into a flask. The remainder of the procedure is the same as usual.

Examination of milk. Portions of 0.5 ml of milk are poured into test-tubes Nos. 1 and 2.

A total of 8.5 ml of meat-infusion agar and 1 ml of the working phage dilution are added to test-tube No. 1 (experimental sample). A

total of 9.5 ml of meat-infusion broth is added to tube No. 2 (free-phage control). A total of 9 ml of meat-infusion broth and 1 ml of the working phage dilution are added to tube No. 3 (phage-titre control).

All the tubes are incubated in a heater at 37° for 16-18 hr and then examined in the usual manner.

Examination of kefir. Before examination the kefir is neutralized with 2N or 1N sodium hydroxide (the medium reaction should be slightly alkaline); the remainder of the procedure is the same as for milk.

Examination of bread. Pieces of bread with a total weight of 2-3 g are crushed in a mortar with 10 ml of meat-infusion broth and the suspension is left to stand.

Portions of 1 ml of the suspension are poured into tubes Nos. 1 and 2. A total of 8 ml of meat-infusion broth and 1 ml of the working phage dilution are added to tube No. 1 (experimental sample). A total of 9 ml of meat-infusion broth is added to tube No. 2 (free-phage control). A total of 9 ml of meat-infusion broth and 1 ml of the working phage dilution are added to tube No. 3 (phage-titre control).

The remainder of the examination is conducted in the usual manner.

COLIBACILLOSIS

N.A. Yakhina, Doctor of Medical Sciences

Colibacillosis is a disease caused by bacteria of the genus Escherichia. The principal representative of this genus is E. coli, a facultatively pathogenic microorganism found in humans and animals. While it is one of the major species inhabiting the intestines under normal conditions, E. coli is capable of inducing disease when it enters other regions of the intestinal tract (the small intestine) or other systems and organs (the urinary and biliary tracts). Under such conditions it often produces inflammatory processes (pyelocystitis, peritonitis, cholecystitis, enteritis, appendicitis, otitis, etc.) or

symptoms of general sepsis (colibacillary sepsis). In addition to colon bacteria with facultatively pathogenic properties, there are certain serological types of E. coli which are pathogenic. In young children these bacteria cause inflammation of the small intestine, or enteritis, which in a substantial percentage of cases is characterized by a severe course and a tendency to spread in groups of children (pediatric institutions and hospitals), thus producing epidemics.

TABLE 31

Enzymatic Characteristics of Enteropathogenic Types of E. coli (after E.M. Novgorodskaya, I.M. Ansheles, and A.G. Loseva)

1	2	3	4	5	6	7	8
O111 : B_4	−	+ (2—4)	+	−	+	+	+
O55 : B_5	−	+ (1—2)	− 24 hrs X	+ 1—2	+	+	+
O26 : B_6	−	+ (2—3)	+	+	+	+	±
O86 : B_7	−	+ (2—3)	−	+ (2—3)	+	+	+
O127 : B_8	−	+ (3)	−	+	+	+	+

Symbols: +) Fermentation during first day; −) no fermentation; figures in parentheses indicate fermentation time in days; ×) late, irregular negative or positive fermentation. *Culture O111: B_4H_{12}.

1) Serotypes; 2) adonitol; 3) dulcitol; 4) sorbitol; 5) sorbose; 6) arabinose; 7) zylose; 8) rhamnose; 9) maltose; 10) salicin; 11) inositol; 12) lactose; 13) sucrose; 14) mannose; 15) glucose; 16) indol; 17) hydrogen sulfide; 18) gelatin; 19) ammonium citrate; 20) potassium nitrate; 21) Foges-Proskauer test; 22) methyl red; 23) urea; 24) or.

The clinical data on diseases caused by E. coli do not always make it possible to differentiate them from diseases with other etiologies; microbiological investigation techniques are of decisive importance in establishing a diagnosis for such illnesses.

Morphology of E. coli

E. coli are short, rather thick rods with rounded ends and are 1-2 μ long and 0.4-0.6 μ wide; they occur singly and, less frequently, in

pairs. The rods vary widely in size and coccal and filamentous forms are encountered. E. coli is gram-negative, has peritrichial flagella, and moves sluggishly. Both labile and nonlabile variants are encountered.

Biology of E. coli; cultural characteristics

When grown in broth E. coli produces cloudiness and forms a residue on the bottom of the test-tube; it does not liquefy gelatin and clabbers milk without peptonization. It forms an acid and frequently a gas in Hiss' colored media.

On solid media E. coli forms flat or convex colonies, which are opalescent, cloudy, and rich in color and have a lustrous surface. Colonies on Endo's medium are fuchsin-colored with a metallic lustre or reddish, but are sometimes colorless with dark centers. Similar differences in coloration (ranging from dark to colorless) are observed on other colored media, such as Levin's medium. Colonies on agar range from 0.3 to 0.5 cm in diameter.

Bacteria belonging to the genus Escherichia actively ferment carbohydrates; the various species can be differentiated on the basis of differences in their fermentation of individual carbohydrates and Kaufman and Erskov et al., have established that there are definite serological types and biochemical subtypes. utilizing the lyzability of cultures of different phages, Nicol and LeMinor detected phagotypes within the serological types. In the view of these authors, determination of biochemical subtypes and phagotypes can be employed for epidemiological analysis of colienteritic diseases.

In view of the need to be guided by data on the resistance of the cultures isolated to antibiotics in selecting therapeutic drugs, the sensitivity of the causative agent to such compounds is tested in conjunction with determination of its serological and enzymatic properties. Under practical conditions this is done with disks impregnated with so-

lutions of biomycin, levomycetin, synthomycin, and streptomycin, which are placed on dishes inoculated with the culture; the zones of retarded growth are then measured.

Serial dilutions in test-tubes are used when it is necessary to determine sensitivity to antibiotics for which no disks are available or when a more precise determination of sensitivity must be made; this technique involves determination of the least quantity of the drug which produces a zone of retarded culture growth (Table 31).

Antigenic structure; serotypes

Even strains of the same species differ substantially in antigenic structure. During the past decade, however, detailed study of E. coli isolated from children with colienteritis, so-called enteropathogenic E. coli, has made it possible to assign them to definite serological types on the basis of antigenic differences and has thus facilitated development of bacteriological diagnostic methods.

Study of the antigenic structure of enteropathogenic types of E. coli has shown that they have 3 antigens: 1) a somatic O antigen; 2) a superficial (membrane, capsular) K antigen combining a number of different antigens; 3) a flageller H antigen. The K antigens include thermolabile L, B, and Vi antigens and thermostable A and M antigens. In contrast to the L, B, and Vi antigens, the A and M antigens retain their ability to combine with the corresponding antibodies when the culture is heated. The K antigens inhibit agglutination of E. coli in the corresponding O antiserum, so that detection of O antibodies requires that the culture be heated at 100° for 1½-2 hr.

Investigations conducted to study the antigenic structure of E. coli and its pathogenicity for man have shown that strains of O groups 1-125 (following Kaufman, Knipshildt, and Veln's classification) are isolated more frequently from pathological material than from the

feces of healthy persons. Serotypes 0111, 055, 026, 086, 0127, 018a, 018c, 0128, 0125, 0126, 044, 025, and 0119 and 4 strains isolated in the Soviet Union, Nos. 408, 145, 9, and 561, have been described as causative agents of choloenteritis.

Strains of E. coli isolated from pathological material are serologically more uniform than strains isolated from fecal matter. They are O-inagglutinable and a substantially higher percentage belongs to the first 25 O groups that is the case for strains from normal material. E. coli type 0111 has been isolated from the blood, pus from the ears, and the duodenal contents, while types 0111 and 055 have been isolated from the urine.

Pathogenicity, virulence, and toxin formation

Determination of the pathogenic properties of cultures isolated in colibacillosis. The virulence and dermatonecrotic and hemolytic characteristics of E. coli are studied to determine its pathogenic properties. A relationship has been noted between the hemolytic and dermatonecrotic properties and the toxicity of colon bacteria isolated in diseases of the biliary and urinary tracts and colibacillary sepsis.

Determination of virulence and dermatonecrotic and hemolytic properties is also employed to characterize enteropathogenic E. coli isolated in juvenile enteritis. No correlation has, however, been established between the pathogenicity of such cultures and the presence of the aforementioned properties. Thus, the type of E. coli most pathogenic for man, 0111:B_4, does not have pronounced hemolytic or dermatonecrotic characteristics. At the same time, type 026:B_6, which is less pathogenic for man and is capable of causing diarrhea in calves, has intensive hemolytic and dermatonecrotic properties. The pathogenic properties of enteropathogenic E. coli are apparently manifested predominantly in their capacity to survive and multiply in the human small intestine, an

ability whose extent cannot as yet be determined by laboratory techniques.

Determination of the virulence of E. coli

a) Oral inoculation of white mice. A total of 1-2 ml of a two-billion-cell suspension of a 24-hr culture washed from an agar slant is introduced into the esophagus of a white mouse with a slender probe consisting of a slightly curved Pasteur-pipette capillary connected to a syringe with a fine rubber tube. When the result is positive the animal develops symptoms of acute enteritis (convulsions, paralysis, and paresis) and dies.

b) Intraperitoneal inoculation of white mice. A suspension of the culture under investigation in physiological solution is injected intraperitoneally in doses of 100, 200, and 500 million and 1 and 1.5 billion cells, each dosage being administered to 3-5 mice. The observation period is 4 days. The virulence of the culture is determined from the dosage in millions of cells which produces a 50% mortality, the M (LD_{50}), and the least dosage which causes all the inoculated animals to die (1DCL).

Strains which contain a thermolabile antigen are more toxic for mice than strains which lack such antigens (Kaufman).

Dermatonecrotic properties are determined with the aid of Gross' intracutaneous test. A total of 0.1 ml of a two-billion-cell suspension of bacteria washed from an agar culture is injected intracutaneously into an area of shaved skin on a guinea pig's side or abdomen. An inflammatory infiltration of varying severity, which passes into necrosis of greater or lesser intensity, develops in positive cases.

The hemolytic properties of cultures are determined by the following methods:

a) Determination of hemolysis on a solid medium. Cultures are made on agar to which 1.5-2% of defibrinated sheep blood has been added. The

results are evaluated in the usual manner after 24-48 hr of growth at 37°.

b) Determination of hemolysis in a liquid medium by Slesarevskiy's method. A total of 2-3 drops of defrinitated sheep blood is added to a 24-hr culture in peptone water. The results are evaluated after the sample has been kept in a heater at 37° for 24-48 hr.

c) Widholm's method: portions of 1 ml of a culture grown in 1% peptone water containing 0.5% glucose for 24 hr are poured into test-tubes and diluted with physiological solution in ratios of from 1:2 to 1:128. The culture content accordingly ranges from 50% to 0.8%. To each test-tube is added 0.5 ml of a 2% suspension of erythrocytes washed twice with physiological solution. The erythrocyte control consists of 0.5 ml of the erythrocyte suspension and 1 ml of physiological solution, while the medium control consists of 0.5 ml of the erythrocyte suspension and 1 ml of the nutritive medium. The test-tubes are held at 37° for 1 hr and at room temperature for 1 hr and the results are then evaluated.

d) Kaufman's method: sterile horse blood is mixed with 10% sodium citrate in a proportion of 10:1. This mixture is centrifuged and the erythrocytes are washed three times in physiological solution. Sufficient physiological solution is then added to the tube containing the erythrocytes to make up the initial blood volume.

Portions of 0.2 ml of the erythrocyte suspension are added to portions of 2 ml of 1% peptone in slender test-tubes. The tip of a needle is then used to introduce a 20-hr agar culture into the mixture and the tubes are incubated at 37° for 48 hr. The results are noted after 24-48 hr, since hemolysis is retarded in some cases.

E. coli forms 2 toxins: one is an exotoxin and is neurotropic and thermolabile. It decomposes when exposed to air, a characteristic com-

mon to the various colon bacteria. Some authors attribute the hemolytic properties of E. coli to the functions of its exotoxins. The second toxin is an endotoxin and corresponds to the "complete antigen." It is thermostable and has an enterotropic action.

Laboratory Diagnosis of Colibacillosis

Material for examination; taking of samples

The materials used for examination include:

1) feces (reserved in Tyge's solution) in diseases accompanied by symptoms of intestinal dysfunction (simple and toxic dyspepsia, parenteral dyspepsia, etc.).

In addition to the feces, in severe cases of colienteritis vomitus and mucous from the mouth and nose are examined for pathogenic bacteria, following epidemiological indications.

The contents of the small intestine, blood from the heart, and pieces of organs (the liver, spleen, lungs, kidneys, and the intestinal lymph nodes) are examined in the case of patients suspected of having died of colienteritis. The usual technique is employed in taking material from cadavers;

2) the feces, vomitus, and suspected foodstuffs are examined in food poisoning;

3) the urine, which is collected in a sterile vessel through a sterile catheter, is examined in affections of the urinary bladder and ureters;

4) the duodenal contents, which are sampled with a duodenal probe, are examined in affections of the gall bladder and biliary tract;

5) the blood is examined when colibacillary sepsis is suspected.

Bacteriological examination

Investigations of this type must be conducted as soon as possible after the onset of the illness; the first analysis should be made be-

fore treatment begins. Since the causative agent can be cultured from all portions of the feces, use of rectal tubes for taking samples is not recommended. Fecal matter is taken directly from the patient's diaper or bedpan.

The material to be investigated is cultured on differential media (Endo's and Levin's media).

In view of the fact that it is in some cases difficult to make a differential diagnosis among colienteritis, salmonellosis, and dysentery, the feces are cultured on bacterial agar Zh or Ploskirev's medium, on Wilson-Blair's medium, and on a concentration medium (5% bile broth or Muller's medium) as well as on Endo's and Levin's media.

The colonies produced on the solid differential media are evaluated and studied 18-24 hr after inoculation.

Since the enteropathogenic types of E. coli differ from so-called normal colon bacteria in their antigenic structure, determination of the serological properties of the bacteria isolated is of basic importance in identifying them. Under laboratory conditions the subsequent course of the examination involves the following stages:

1. Agglutination of the colonies produced on slides with a series of OB sera and subsequent inoculation of agar slants with the colonies which agglutinate.

2. Agglutination of the cultures on slides and rough agglutination with live and boiled cultures.

3. Study of the enzymatic properties of the cultures isolated.

4. No fewer than 10 different types of colonies from each dish should be tested in the agglutination reaction on slides. A mixture of monovalent OB sera from different types of E. coli (0111, 055, 026, 086, etc.) is used for this purpose. Each serum being taken in a dilution of 1:10. The tests are first conducted with mixtures of 3-5 of the

available sera. When agglutination occurs 3-5 of the colonies which agglutinated are cultured on agar slants.

Day-old cultures on the agar slants are repeatedly checked for agglutination with the serum mixture and tested with each of the sera in the mixture when agglutination occurs. An expanded agglutination reaction is set up with any serum which gives a positive result. Live and boiled (for 1-2 hr in a water bath at 100°) culture washed from the agar with physiological solution is used as the antigen.

The agglutination reaction is begun at a dilution of 1:100 and continued to a dilution of 1:6400-1:12,800.

The test-tubes containing the suspension of live culture are incubated at 37° for 2 hr and the results are evaluated on the following day (the tubes are kept at room temperature or in a refrigerator at 5°). The tubes containing the heated culture are incubated at 37° (50° according to Kaufman) for 20 hr and the results are then evaluated.

It must be emphasized that the reaction with the live culture indicates only the presence of the corresponding K antigen (B_6, B_4, or B_5) and cannot be used as a basis for assigning the strain isolated to a definite O group. The final evaluation is made from the results of test-tube agglutination with the heated culture.

When the result is positive the live culture yields large-grained agglutination in a dilution of 1:200-1:400 and the heated culture produces fine-grained agglutination in a dilution of 1:800-1:1600 (which corresponds to 3/4 of the serum O-antigen titre). This confirms that the culture belongs to the type corresponding to the OB serum.

If agglutination is fine-grained in character and is equally intensive with the live and heated cultures (at dilutions of up to 3/4 of the serum O-antigen titre) the culture belongs to the corresponding O group. This phenomenon is observed when the culture contains little or

no B antigen, but such cultures are rarely encountered.

When agglutination does not take place with the heated culture the bacterium belongs to another O group. Agglutination with low titres of the heated culture is possible when there is an antigenic similarity between the culture isolated and the O group of another species of coliform bacteria. The results must be evaluated as negative in such cases. Determination of the nature of the culture requires additional study, employing cross-adsorption of agglutinins with cultures of various species of the coliform group.

When cultures taken from the dishes containing differential media prove to be agglutinable, they are simultaneously subjected to test-tube agglutination and to culturing on nutritive media containing carbohydrates (a composite series) and in columns of semiliquid agar, in order to determine their mobility and to preserve them.

For more detailed study and identification of the E. coli cultures isolated, Ewing has proposed a complete examination, which consists in:

1) agglutination of live bacteria on slides with OB and O antisera of certain enteropathogenic types of E. coli;

2) agglutination of killed emulsions on slides with the same OB and O antisera;

3) test-tube agglutination of heated cultures with O sera in order to confirm their O-antigen group;

4) agglutination on slides with adsorbed O sera in order to differentiate the O-antigen groups and to determine the components of the O-antigen fractions within these groups;

5) titration of K (B)-antigenic suspensions with K (OB)-antisera (in test-tube agglutination);

6) determination of H antigens.

Complete examination of cultures in accordance with this scheme is

not necessary under practical laboratory conditions and is possible only in specialized laboratories. It necessitates having available OB sera, which are obtained by immunizing rabbits with live or killed cultures of enteropathogenic E. coli, O sera, which are obtained from heated cultures containing O antigen, and H sera.

In order to determine the O-antigen content the culture under investigation is heated at 100° for 1-2 hr and an O-agglutination reaction is set up. The results are evaluated after incubating the test-tubes at 37° for 20 hr (after incubation in a water bath at 50° for 20 hr according to Kaufman).

It is helpful to use a mixture of O sera, i.e., several polyvalent sera each containing monovalent O sera. The combination of O groups in the mixture can be selected in such fashion as to avoid group reactions.

Determination of the H antigen makes it possible to detect strains with different H antigens within a serological type. Thus, two different antigens, H_2 and H_{12}, have been found in O group 111, while antigen H_6 has been detected in strains of O group 55 isolated from enteritis patients. Determination of the H antigens of E. coli cultures enables us to use this characteristic in epidemiological analysis (detection of epidemiological chains).

In order to determine the H antigen of a culture which is not sufficiently labile it must be passed through semiliquid agar, which increases its lability. This is done by alternate culturing in U-tubes containing 0.1% agar (18-20 hr) and broth (4-5 hr). When necessary this transplantation is repeated 2 or 3 times, until the culture acquires the requisite lability. H antigens are determined in the usual manner, using a serum mixture consisting of several monovalent H sera, as suggested for determination oof O antigens.

A rough result can be obtained by agglutination on slides with the

mixture and then with each of its constituent monoreceptor sera. Test-tube agglutination is then carried out, with incubation in a water bath at 50° for 2 hr, and the results are evaluated. A.K. Baltrashevich has developed a simplified method of determining H antigens. This technique utilizes semiliquid 0.3% agar in Hottinger's broth or meat-infusion broth to which sufficient monoreceptor serum has been added that the final serum dilution is 1:500 and the serum titre is greater than 1:6400. The lability of the bacteria is preliminarily determined in cultures in semiliquid agar. When growth is diffuse, i.e., when the culture is labile, stab transplants are made directly from the semiliquid agar to a series of test-tubes containing semiliquid agar to which various monoreceptor H sera have been added. The results are evaluated after incubation at room temperature for 1 day. Where the culture corresponds to the H antigen in the test-tube it grows after the fashion of nonlabile cultures, i.e., only along the stab. Growth in the other test-tubes is diffuse, clouding the entire medium.

Complete serological typing of cultures isolated in juvenile colienteritis permits precise determination of their antigenic structure. Following the international classification, these types can be designated as $O_{111}:B_4:H_2$, $O111:B_4:H_{12}$, $O55:B_5:H_6$, $O26:B_6:H_{11}$, etc.

Preparation of sera. OB sera. OB sera are produced by immunizing rabbits with cultures containing a well-developed B antigen. Individual colonies of the same strain may differ substantially in their content of B antigen and it is consequently recommended that the strain be preliminarily cultured on dishes containing meat-infusion agar and 0.1% glucose, then selecting colonies after 24 hr.

Rabbits are immunized with cultures which have been raised on meat-infusion agar slants containing 0.1% glucose and washed off with physiological solution.

The cultures are injected intravenously in doses of 250, 500, 1000, 1500, and 2000 million cells at intervals of one week. During immunization the rabbits receive alternate injections of heated and live cultures, as well as injections of formalinized cultures (exposed to 0.5% formalin at room temperature for 24 hr). Immunization with the alcohol-acetone vaccine proposed by Rashka and used by Zatsepin is also widely employed in practice.

In this technique an equal volume of 96° ethyl alcohol is added to the washings from an agar culture. The mixture is incubated in a heater at 37° for 4 hr and centrifuged and the residue is washed twice with acetone and placed in a heater to dry. The dry bacterial mass is crushed to a powder and, before immunization, suspended in physiological solution by comparison with an optical standard. The immunization scheme is similar to that described above.

O sera. In order to obtain O sera cultures are grown for 24 hr on meat-infusion agar (pH = 7.4-7.6), washed off with physiological solution, and boiled for 2½ hr.

The immunization scheme is the same as for OB sera. The antibody titre should not be less than 1:3200. Since O sera may contain antibodies to the B antigen, titration is carried out to determine the working dilution at which the serum produces clear agglutination with a heated culture but not with a live culture.

H sera. In order to prepare H serum the culture is preliminarily passed through U-tubes in order to increase its lability. It is then transplanted to Hottinger's broth and incubated in a heater at 37° for 4 hr. The lability of the bacteria is checked microscopically. A total of 0.5% of formalin is added to the broth culture. The vaccine thus obtained is administered intravenously to rabbits in increasing doses (from 0.25 to 2.5 ml) at 4-day intervals. In order to remove the O and

B antibodies from the serum it is adsorbed with homologous cultures heated at 100° for one hr.

Bacteriological examination in colibacillary sepsis

When there is a suspicion of colibacillary sepsis 10-20 ml of venous blood is cultured in a flask containing 100-200 ml of 20% bile broth and incubated in a heater at 37° for 48 hr. Material is transplanted from the broth to differential media after the culture has been incubated for 24 and 48 hr.

Bacteriological examination in diseases of the biliary and urinary tracts and peritonitis. The urine, duodenal contents, and peritoneal exudate are cultured directly on Levin's medium, Endo's medium, etc., as well as on bile broth. Transplants are made from the bile broth to solid differential media after 24 and 48 hr of incubation in a heater at 37°. The remainder of the analysis is intended to identify E. coli and its variaties.

Bacteriological examination in food poisonings of a colibacillary nature. Cultures are made on Endo's medium or other differential-diagnostic media and then identified by the usual method (see page 405).

The biochemical and, especially, the serological properties of the E. coli cultures isolated are subjected to careful study, which permits more complete characterization of their antigenic structure and comparison of strains isolated from different sources.

Serological investigation

The blood of some children suffering from colienteritis exhibits specific serological changes (formation of agglutinins to enteropathogenic E. coli of the corresponding type). Although agglutinin formation is noted in only a small percentage (up to 30%) of juvenile colienteritis patients, the agglutinin titre remaining low in the majority of cases, attempts have been made to utilize the agglutination reaction for

diagnostic purposes in both juvenile and adult patients and carriers.

The agglutinatin reaction is set up with live and killed cultures of enteropathogenic E. coli, or a strain isolated from the patient.

In view of the low agglutinin titres observed, especially in children, The reaction is begun at low dilutions (1:10, 1:20). The B-agglutinin reaction is evaluated after the serum and live culture have been incubated for 2 hr at 37° and then kept at room temperature for 18-20 hr. Determination of the O-antibody content, which is carried out after the serum and killed culture have been incubated for 18-20 hr at 37° (at 50° according to Kaufman), is of special importance.

The H antibodies are determined after the serum has been in contact with a formalinized (0.5%) culture at 50° for two hr.

It is recommended that repeated investigations be conducted throughout the illness to evaluate the dynamics of the increase in agglutinins to E. coli.

Hemagglutination reaction. In order to prepare the antigen the strains in question are grown on meat-infusion agar at 37° for 18-20 hr. The growth obtained is washed off with physiological solution and the resultant suspension is brought to a concentration of 2 billion cells per ml and boiled for 2 hr.

Erythrocytes washed 3 times in physiological solution are mixed with a quantity of heated bacterial suspension (1-2 hr at 100°) such that the erythrocyte concentration equals 2.5%. After thorough shaking the mixture is placed in a water bath at 37° for 30 min. The suspension-treated (modified) erythrocytes are then washed 3 times in physiological solution to obtain a clear liquid; this is done to remove the excess antigen, which may hamper hemagglutination or hemolysis. The suspension of treated erythrocytes (in portions of 0.2 ml) is added to 0.2 ml portions of different dilutions of serum obtained from immunized

rabbits or from patients.*

The mixtures are placed in a water bath at 37° for 30 min and then centrifuged for 1-2 min at approximately 1000 rpm. The agglutination is evaluated immediately.

The reaction is accompanied by the following controls: 1) antigen specificity; 2) erythrocytes; 3) serum.

In the hemolytic modification of this method guinea pig complement diluted to 1:20 (in 0.1 ml portions) is added to the treated erythrocytes and serum. The hemolysis is evaluated after incubation at 37° for 30 min. It is also possible to prolong the incubation to 2 hr and then centrifuge the mixture, the result being evaluated from the state of the upper layer.

Centrifugation method. This method was proposed in an attempt to increase the sensitivity and specificity of the agglutination reaction. Inactivated sera are used. In order to determine O antibcdies a heat-killed 20-hr broth culture is added to serial dilutions of the serum; a formalinized culture is added for determination of B agglutinins. The test-tubes are incubated at 37° for 2 hr and centrifuged at 3000 rpm for 10 min; the results are then evaluated.

Accelerated methods for laboratory diagnosis of colienteritis.

Since ordinary bacteriological examination is quite laborious in colienteritis, positive results not being obtained until 3 days after the material is taken, investigations have been undertaken for the purpose of developing accelerated methods for the laboratory diagnosis of colienteritis.

G.G. Yezhova has worked out an accelerated method involving reduced culturing time, centrifuging, and determination of agglutinative properties.

The material under investigation is cultured on Endo's medium in

dishes. After growing for 18 hr at 37° colonies are selected by rough agglutination on slides with a mixture of OB sera. Those colonies which agglutinate are cultured on agar slants (pH = 7.2) and in test-tubes containing 2 ml of Hottinger's broth (pH = 7.2). As much material as possible is used in the cultures and the media are preliminarily heated. After incubation in a heater at 37° for 3 hr the cultures from the agar slants are types on slides and those whose serotypes can be determined are subjected to expanded agglutination with a live bacterial suspension in physiological solution and a broth culture heated at 100° for 1 hr. The reactions are accompanied by culture and serum controls. The test-tubes are kept in a heater at 37° for 45 min and then centrifuged at 2500 rpm for 10 min. The results are evaluated after centrifuging.

Results can be obtained within 24 hr with this technique, as compared to 72 hr for the usual method.

The luminescent-antibody method is based on detection of an antigen treated with specific fluorescent serum by luminescence microscopy. Clinical material is examined in the following manner. Material taken from the patient is cultured on slants of Endo's medium in test-tubes and smears are prepared directly from feces. The smears are subsequently processed by the usual method.

The analysis is regarded as positive when no fewer than 1-2 brightly luminescing (principally at the periphery) greenish cells are detected in each field of view (Fig. 14).

This technique requires 24 hr; its sensitivity varies from 500 to 500,000 bacteria in the volume under investigation, depending on the antigenic characteristics of the strains and the number of extraneous bacteria. Such an analysis must be conducted in parallel with the ordinary bacteriological procedure for laboratory diagnosis of colienteritis.

The triphenyltetrazolium chloride (TTC) method (Kraus) is based on the reduction of triphenyltetrazolium chloride, which is an oxidation-reduction indicator, under the action of the dehydrogenase formed during bacterial growth. The latter is converted from a colorless, soluble, toxic substance to triphenyltetrazolium formasan, which is nontoxic, insoluble, and a deep cherry red in color.

Pea-sized pieces of fecal matter are placed in a test-tube containing 2-3 ml of distilled water. The fecal suspension is shaken and left to settle for 10-15 min. The material to be cultured is taken from the supernatant, avoiding pieces of mucous and fecal matter, and 1 or 2 drops are introduced into test-tubes containing TTC broth to which 4 drops of different OB sera have been added (one serum in each tube). The sera are preliminarily diluted to 1:5 with physiological solution. A control culture is made in a tube containing TTC broth but no serum. All the tubes are incubated in a heater at 37° for 8-12 hr and the results are then evaluated.

When the result is positive a coarse- or fine-grained cherry-red agglutinate forms, settling to the bottom of the tube to leave a clear supernatant.

When the result is negative the medium acquires a cherry red color and is clouded by the colored precipitate, so that the supernatant does not become clear.

It is possible to obtain results within 8 hr when this technique is used for diagnosing colienteritis; however, the fact that a positive result is obtained by agglutination of the live culture makes it impossible to determine its serological type from its O antigen. Kraus' method can consequently be recommended only as a rough or biliary technique for diagnosing colienteritis.

Nutritive Media

2, 3, 5-triphenyltetrazolium chloride broth. A total of 1 liter of distilled water is poured over 500 g of beef cleaned of fat, tendons, and connective tissue and 1% of peptone and 0.5 of sodium chloride are added. This mixture is boiled for 2½-3 hr and its pH is then adjusted to 6.2-6.4. The broth is filtered and sterilized in running steam or under a pressure of 1.5 atm for 30 min. A sterile 2% aqueous solution of 2, 3, 5-triphenyltetrazolium chloride is added to the sterile meat broth in a quantity of 11 ml per 100 ml of broth. The TTC solution is prepared under sterile conditions in distilled water with a pH close to the neutral point. The medium is then poured into sterile test-tubes in 2 ml portions and is readied for use. It is not sterilized after the TTC has been added.

TTC broth is sensitive to the action of light and heat and should be stored in a cold, dark place.

CHOLERA

T.D. Fadeyeva, Candidate of Medical Sciences

The causative agent of Asiatic cholera is V. cholerae asiaticae (Vibrio comma in the modern nomenclature).

Morphology of Vibrio comma

Vibrio comma is a slightly curved, comma-shaped rod ranging from 1 to 4 μ in length and from 0.2 to 0.4 μ in width. These bacteria occur singly, in pairs, and in parallel rows resembling two arcs facing in the same direction, circles, the letter S, or a school of fish.

Vibrio comma has a typical shape and a characteristic arrangement in stained spheres prepared from fecal matter, vomitus, the intestinal mucosa of cadavers, or nutritive media inoculated with these materials; this substantially facilitates the diagnosis of cholera. Rod-like, coccal, spherical, and filamentous forms are encountered together with

the typical form in stained smears prepared from nutritive media inoculated with laboratory strains of V. comma. This bacterium has a single terminal flagellum, which gives it great mobility. V. comma is aerobic, gram-negative, and easily stained with all analine dyes. It has neither spores nor a capsule.

Biology of Vibrio comma; cultural characteristics

A growth of V. comma can be detected on solid nutritive media within 10-12 hr after culturing. Very small, colorless, transparent colonies appear on alkaline agar at this time. After 20-24 hr such colonies are small (1-1.5 mm in diameter), transparent, smooth or slightly granular, and uniform in contour. Older colonies are larger and flatter, have a slightly depressed center, and are granular and nonuniform in contour.

A lustrous, smooth, semitransparent growth with a grayish-blue or slightly creamy color appears on alkaline-agar slants within 24 hr. This growth becomes creamy or yellowish-brown as it ages. After 24 hr in alkaline broth V. comma produces a uniform cloudiness, a light powdery precipitate, and a delicate flocculant film with a whitish-gray color. The film later becomes coarser and denser and settles to the bottom in large flakes and compact granules. After 6-8 hr in peptone water V. comma produces a scarcely noticeable, delicate bluish film and a slight cloudiness. When the test-tube is carefully tilted the film is clearly visible on the surface of the glass.

The biochemical activity of V. comma is very diverse and some of its biochemical properties are of diagnostic value. It liquefies gelatin to form a funnel with an air bubble at the top, clabbers milk (after 24-28 hr), and yields a positive nitrosoindol reaction (cholera red test) as a result of its ability to form indol and to reduce nitrates to nitrites. It forms acid but no gas in Hiss' media containing glucose,

mannose, maltose, sucrose, and mannitol and does not affect media containing arabinose or dulcitol. True V. comma decomposes mannose, sucrose, and starch over a 24-hr period, but leaves arabinose unchanged (Heiberg). It gives a negative Foges-Proskauer reaction and does not hemolyze sheep erythrocytes.

Resistance to physical and chemical agents

V. comma is very sensitive to the action of light, high temperatures, dessication, and chlorine. Direct sunlight kills it within a few hours. In a moist environment it dies within half an hour at 56°, within 5 min at 80°, and instantaneously at 100°. It is killed within 15 min by water containing 1 mg/liter of active chlorine (when the content of organic substances is low). V. comma is comparatively more resistant to low temperatures. It can survive for several days at −5° and some strains are capable of withstanding still lower temperatures (−10 and −31°), surviving for prolonged periods in snow and ice.

Disinfectants and highly dilute acids quickly kill V. comma. Mercuric chloride in a solution of 1:1000 causes it to die instantaneously in media not containing proteins, 1% phenol produces death within 5 min, and sulfuric acid in a dilution of 1:10,000 kills it within a few seconds.

The ability of V. comma to survive in the external world, especially in water, is of great epidemiological significance. An important role in this respect is played by biological factors (antibiosis and bacteriophagia), which can increase or reduce the resistance of V. comma. This bacterium can survive for several weeks or even months in open bodies of water, for 3-5 days in flowing water, for 19 days in sewage, and for 106 days in cesspools. It lives for up to 3 days in fresh feces and for several months in fecal matter kept at low temperatures. V. comma survives for from 8 hr to 13 days in material contam-

inated with the feces of cholera patients (for several weeks in moist material). The survival time of this bacterium in foodstuffs (fruits and vegetables) varies especially widely, ranging from 1 to 30 days.

Variability

Variability is observed in V. comma grown on nutritive media, in the human body, and in the external environment, being manifested in changes in its morphological, cultural, biochemical, serological, and other characteristics. The variability of cultural characteristics takes the form of changes in the structure of the colonies and the manner in which they grow on liquid nutritive media. The normal type of colony for V. comma is the smooth S form, which dissociates to form a smooth S form and a rough R form. S-form colonies are round and transparent, having a uniform contour and a smooth surface. The rough R form consists of cloudy colonies with an irregular surface and a nonuniform contour. Variability of the cultural, biochemical, and serological properties of V. comma is observed in patients who have recovered from cholera and in carriers at the beginning and end of epidemics. In such cases it leads to isolation of rough R variants with an altered sensitivity to bacteriophage, inagglutinable strains, and strains which hemolyzed sheep erythrocytes.

Vibro paracholerae. V. paracholerae (V. pseudocholerae, V. gindha) is similar to V. comma in its morphological and cultural characteristics and certain of its biochemical properties and has the same H antigen. It occurs in various bodies of water and in humans, animals, birds, fish, and crustaceans. Aquatic vibrio, which live in water, can be divided into three groups: alkali-forming, inactive, and acid-forming. Only the acid-forming group is of diagnostic significance, including both V. paracholerae and V. comma.

El Tor vibrio. The El Tor vibrio, which was isolated by Gotsch-

lich in 1905 at the El Tor quarantine station, differs from V. comma in the fact that it hemolyzes goat erythrocytes. This characteristic is assicoated with secrecion of the same hemotoxin produced by V. paracholerae and certain researchers consequently assign this bacterium to the latter species. The majority of investigators now recognize it as identical to V. comma.

Antigenic structure

V. comma is not serologically uniform, consisting of types with common and individual antigens. Japanese authors (1913) sudivided V. irto three serological types: an original type (the Inaba strain, a varient the Ogawa strain), and an intermediate type (the Hikosima strain). All these types have two antigens, a somatic O antigen and a flaggeler H antigen. V. comma and V. paracholerae differ from the other members of the vibrio group in their H antigen and from one another in their O antigens, forming six subgroups according to Gardner and Venkatermen. The true V. comma belongs to subgroup 1-O.

Burroughs subdivided V. comma into four immunological types in accordance with the components making up the O antigen: A, AB (corresponding to the Ogawa strain, AC (corresponding to the Inaba strain), and ABC (corresponding to the Hikosima strain). Of the components of the O antigen (A, B, C, D, and E), only component A is specific to V. comma, the others being encountered in V. paracholerae as well.

Pathogenicity and virulence

V. comma is pathogenic for man and causes cholera in some animals (pigs, susliks, rabbits, puppies, and kittens) only under artificial conditions. This bacterium produces a complex toxin consisting of an exotoxin and an endotoxin. The former separates from the latter under special conditions, regardless of whether or not the cell is broken down. In addition to this toxin, V. comma produces hyalurodinicase and

fibrinolysin. Its virulence varies substantially and can be established roughly in guinea pigs.

Laboratory Diagnosis of Cholera

Bacteriological examination is one of the basic anticholera techniques, permitting precise diagnosis of the disease by isolation and identification of the causative agent and thus facilitating the prompt employment of prophylactic measures. Such examinations are carried out for diagnostic purposes, to detect carriers, and to establish the vectors of the infection. Cholera has now been eliminated in the Soviet Union, but we must always take into account the possibility of its being brought in from abroad.

Material for examination; taking of samples

Feces, vomitus, the intestinal and gall-bladder contents of cadavers, water, foodstuffs, material washed from environmental objects, flies, etc., are examined. Samples of feces and vomitus are taken before administration of cholera bacteriophage. They are collected in bottles or other vessels and 10-20 ml portions are transferred to glass jars with a metal spatula or spoon. The jars are stoppered, sealed with paraffin or wax, and covered with two layers of parchment paper, tying each layer separately. Vessels employed to collect feces are washed with boiling water and used jars are sterilized in an autoclave or by boiling. A tag bearing the requisite data is tied to each jar containing material. When necessary material can be taken from the rectal mucosa with a sterile gauze pad.

When no feces are available pieces of contaminated bedding or underwear can be examined.

In examining healthy persons to detect carriers a laxative (25-30 g of sodium sulfate or magnesium sulfate) is administered and the feces are collected in the usual manner. In mass examinations the fecal

samples are taken with well-flamed sterile thick-walled glass tubes, which are inserted to a depth of 3-4 cm. After it has been withdrawn the tube is immersed in a test-tube or flask containing a small quantity of peptone water. The entire emulsion thus obtained is cultured in peptone water and on dishes containing solid selective media; when the carrier in question has undergone prolonged bacteriophage treatment cultures are also made on solid media containing antiphage serum. In view of the fact that V. comma is localized predominantly in the bile in chronic carriers (G.S. Kulesha and A.G. Nikonov), it is recommended that the bile be subjected to bacteriological examination. A sample is taken with a duodenal probe inserted through the nose or mouth. Greenish-yellow bile enters the bulb of the probe, which is introduced into the duodenum. Bile from the bladder is distinguished by its lighter color and can be obtained 15-20 min after 30-40 ml of 25% magnesium sulfate is introduced through the probe.

Three 10-15 cm segments of the small intestine (from the upper, middle, and lower regions) and the gall bladder are taken from cadavers. The organs removed are placed in two wide-mouthed glass jars.

When there is a suspicion of poisoning some of the material is rendered bacteriologically harmless and sent to a toxicological laboratory for parallel examination.

Fecal matter containing vibrio is preserved in 2% sodium chloride or Venkatermen and Ramakrishnon's preservative (see page 438), especially in warm weather.

When the material must be shipped over long distances the jars are placed in a metal container, padded with wood shavings, paper, or cotton, and placed in a wooden box, which is then sealed. The samples are quickly dispatched to the laboratory by messenger and examined immediately on receipt.

It is recommended that feces be cultured at the patient's bedside and that intestinal and gall-bladder contents be cultured at the autopsy site.

Microscopic examination

Investigation of feces, vomitus, and material from cadavers begins with microscopic examination of stained preparations. In order to make up a smear a piece of feces is spread in a thin layer over a slide, dried in air, fixed, and Gram-stained. Detection of a substantial number of typical, characteristically arranged gram-negative vibrio in the smear indicates that V. comma is probably present.

Bacteriological examination

The material is subjected to bacteriological examination in parallel with the microscopic examination and regardless of its results, being cultured on liquid and solid nutritive media. The biological characteristics of V. comma (its flexibility with respect to nutritive substances, its need for free oxygen, and its ability to grow in alkaline media and in the presence of an excess of salts) are taken into account in preparing nutritive media for its isolation. Liquid media are used for concentrating V. comma. According to the handbooks, these media include peptone water, which yields good results in investigating feces containing large quantities of vibrio. It does not always give satisfactory results in investigating material containing few vibrio (the feces of convalescents or carriers or of patients at the end of an epidemic). Many researchers consequently use bismuth sulfite medium, potassium tellurite medium, or Ottolengi's medium for concentrating V. comma.

Cultures are simultaneously made in the concentration medium and on solid nutritive media. Alkaline and bile-salt agar are selective nutritive media, since they retard the growth of the intestinal and pu-

trefactive microflora and promote that of V. comma. This bacterium, however, grows profusely on alkaline agar only when it is present in large quantities in the material under investigation. Media which promote the growth of V. comma when it is present in small quantities are more useful for diagnostic purposes; such media include Dieudonne's medium, Aronson's medium, Wilson and Reilly's medium, etc. A basic condition for the use of nutritive media is preliminary checking for the intensity and rapidity of growth; this is done by culturing V. comma on them.

Method of bacteriological examination. Culturing in first concentration medium (first peptone water). For culturing, 3-5 ml of liquid feces or a piece of fecal matter weighing 0.3-0.5 g is mixed with 50-100 ml of medium and poured into bottles or Ehrlenmeyer flasks. At the same time, a small quantity of material is successively cultured with the same platinum loop on 2 or 3 dishes containing alkaline agar and Dieudonne's medium. All the inoculated media are placed in a heater at 37°.

The intestinal and gall-bladder contents of cadavers are investigated in the same manner as feces. Before samples are taken the surface of each intestinal segment is cauterized with a red-hot spatula or scalpel and cut with sterile scissors. The surface of the gall bladder is also cauterized and the bile is extracted with a syringe or Pasteur pipette.

Culturing in second concentration medium (transfer from first peptone water). After 5-8 hr the cultures in the first peptone water are examined macroscopically and microscopically (in stained smears) and transferred to the second peptone water. The medium is now slightly cloudy and a delicate, scarcely detectable film appears on its surface, although the cloudiness and film are sometimes lacking. In either case

1 or 2 loopfuls are carefully taken from the surface and transferred to the second peptone water; 1 or 2 dishes containing alkaline agar and Dieudonne's medium are then inoculated. When substantial numbers of vibrio are detected in the smears a hanging drop is prepared to determine their mobility and a microscopic agglutination reaction is set up on a cover slip with a drop of cholera O serum and examined in a hanging drop. The V. comma agglomerate and lose their mobility. An initial preliminary report is made after 6-8 hr, stating whether or not V. comma has been detected.

Transfer from second concentration medium (second peptone water) and isolation of V. comma. The second peptone water is examined 5-8 hr after inoculation (10-16 hr after the investigation begins) and transferred to 1 or 2 Petri dishes containing alkaline agar and Dieudonne's medium; smears and hanging drops are also prepared.

The solid nutritive media inoculated with the initial material are examined after 10-16 hr. The dishes containing the agar inoculated with the first and second batches of peptone water are examined after the same interval (15-24 hr and 20-32 hr after the investigation begins). Suspected colonies are examined microscopically, stained smears and hanging drops are prepared, and a rough agglutination reaction is set up on slides with cholera O serum diluted to 1:400. The film is emulsified in a drop of physiological solution as a control. The results of the reaction are evaluated with the unaided eye after 2-3 min. More rapid results are yielded by an agglutination reaction in broth. Clear colonies with typical properties are transferred to a test-tube containing an alkaline-agar slant in order to produce a pure culture. A second preliminary report is made after 12-16 hr, indicating the titre at which the vibrio detected agglutinated in the broth.

Investigation of pure culture. The pure culture isolated is inves-

tigated 10-12 hr after culturing on the agar slant. Smears are made up and Gram-stained, a hanging drop is prepared, and an agglutination reaction is set up on slides. In order to study the other basic properties of the culture it is transplanted to alkaline agar and peptone water, stab-cultured in gelatin and broth, and used to conduct an agglutination reaction. The agglutination reaction is set up in broth when the quantity of culture is insufficient or when accelerated results are desired. After incubation for 3 hr at 37° the broth culture is used to inoculate two test-tubes containing broth (with bacteriophage added to one), one containing starch broth, one containing broth and sheep erythrocytes, and one containing Hiss' medium. The results are evaluated 20-24 hr after inoculation of the diagnostic media, first adding several drops of Lugol's reagent to the starch broth and 1 ml of sulfuric acid to the peptone water. A final report is made after 30-36 hr, indicating the dilution at which the vibrio detected agglutinated. This report is often limited to the results of the agglutination reaction, the nitrosoindol reaction, and culturing on Ressel's medium. Conclusive identification of V. comma requires no less than 48 hr and is not always successful.

Bacteriophage test. Portions of 0.2 ml of a 3-20 hr broth culture are added to 2 test-tubes containing 10 ml of broth. A total of 0.2 ml of polyvalent cholera bacteriophage is added to one of the tubes, while the other serves as a control. Both tubes are incubated in a heater at 37° and the results are evaluated after 3-20 hr. When the result is positive the tube containing the phage becomes clear, as a result of lysis of the culture, while the control (without phage) remains cloudy. The two tubes are equally cloudy when the result is negative. For tests on a solid nutritive medium the culture under investigation is spread over a dish containing alkaline agar and a drop of cholera bacteriophage

is streaked onto it. When the result is positive no growth can be seen on the streak after 18-20 hr.

The increasing phage-titre reaction can be used for rapid detection of V. comma (see page 437).

Diastatic-activity test. Several drops of an iodine solution (tincture of iodine or Lugol's reagent) are added to 10 ml of a 6-24 hr culture in broth containing dissolved starch (c 5%). The color of the tube containing the inoculated starch medium remains unchanged (sugar is formed from the starch), while the control tube, which contains the uninoculated starch medium, turns blue.

Investigation of hemolytic properties. The culture under investigation is transferred to broth to which 2-3% (with respect to the initial volume) of washed sheep erythrocytes or defibrinated blood has been added. The mixture is incubated in a heater at 37° and the results are evaluated after 24 hr. V. comma does not hemolyze sheep erythrocytes over a 48-hr period. In another method broth is inoculated with the culture and 2-3% of sheep erythrocytes is added after 24 hr. The results are noted after 2 hr and a final evaluation is made on the following day.

Nitrosoindol reaction. A total of 10-20 drops of concentrated sulfuric acid is added to 10 ml of a 24-hr culture in peptone water. When the reaction is positive the medium turns reddish and then bright red. Its color remains unchanged when the result is negative.

Agglutination reaction. Serum of subgroup O-1 is used in moderate titre (1:3200) for the agglutination reaction. A series of two-fold serum dilutions in physiological solution, ranging from 1:50 to 1:600, is made up. Portions of 0.5 ml of each dilution are mixed with 0.5 ml of a 1-billion-cell emulsion of the culture. This doubles each of the serum dilutions and produces dilutions ranging from 1:100 to 1:3200. A

test-tube containing 0.5 ml of physiological solution and 0.5 ml of the emulsion serves as the antigen control, while a tube containing 1 ml of serum diluted to 1:50 serves as the serum control.

The tubes are kept in a heater at 37° for 2 hr and at room temperature or in a refrigerator for 18-20 hr. The results of the reaction are evaluated after 2 hr, when specific O agglutination is clearly visible; a final evaluation is made after 18-20 hr. An agglutination reaction at dilutions of 1:800-1:1600 is regarded as positive.

Agglutination reaction in broth. The agglutination reaction of vibrio grown in broth containing O agglutinative serum is 2-3 times as sensitive as and requires less time than the ordinary agglutination reaction, thus accelerating the diagnosis of cholera (D.V. Chibrikova). The serum is mixed with the broth in dilutions ranging from 1:100 to 1:12,800. An emulsion is prepared from a single colony or from a minimal quantity of the culture (less than one loopful) in 1-1.5 ml of broth and 1 or 2 drops are added to each serum dilution and to the test-tube containing the antigen control (1 ml of broth). The serum control is 1 ml of the 1:100 dilution. The test-tubes are incubated at 37° and the results are evaluated after 3-4 hr; the prolific multiplication of vibrio makes later evaluation difficult. The results are regarded as positive when agglutination occurs at a dilution of 1:6400-1:12,800.

Serological investigation

The agglutination reaction with the patient's serum (Widal's reaction) is of limited value in cholera, since agglutinins do not appear until the 4th-7th day. It can be used only for retrospective diagnosis in convalescence. The blood from which the serum is obtained is taken from a finger with a Francke needle (or from the ulnar vein with a syringe), drawing no less than 1 ml. The serum titre in convalescents should not exceed a dilution of 1:100 and the agglutination reaction is

consequently set up at low dilutions (1:20, 1:40, 1:80). The remainder of the procedure is similar to the ordinary agglutination reaction. The reaction is regarded as positive at a titre of 1:40-1:80.

Accelerated methods for bacteriological examination

In the accelerated method of diagnosing cholera portions of 0.2-0.3 ml of feces are cultured in 3 test-tubes, the first containing peptone water, the second containing peptone water and cholera O agglutinative serum diluted to half-titre, and the third containing peptone water and 0.5% of dissolved starch (Kodam's medium). In a second procedure the peptone water in the third tube is replaced by serum diluted to titre. The feces are simultaneously cultured on dishes containing a solid nutritive medium. When the result is positive a flaky precipitate can be detected on the bottom and a film on the surface of the tube containing peptone water and agglutinative serum after 6-7 hr; the precipitate and film are examined microscopically (in stained preparations). A blue tint does not appear in the tube containing Kodam's medium when several drops of Lugol's reagent are added. When Kodam's medium is not available the result is regarded as positive only at serum dilutions of no less than 1:2000. A report can be made after 6-7 hr. A positive result can be obtained only when a large quantity of vibrio is present in the material cultured.

Annular precipitation reaction with hapten. Precipitation of cholera O serum with a hapten prepared from native material or from a 6-hr agar culture is recommended for rapid diagnosis of cholera (L.A. Papkova). The reaction with the first hapten is positive when the material under investigation contains a substantial number of V. comma, while the reaction with the second hapten is positive when the bacterium is present in small quantities in the material but has been concentrated by culturing on agar. A report can be made after 1-2 hr in the first

case and after 6-7 hr in the second case.

Evaluating the results of the investigation. Both positive and negative results can be obtained in bacteriological examination. When the results are positive and vibrio with typical characteristics have been isolated evaluation of the results is simple. The processing of positive results becomes difficult only when vibrio with altered characteristics are detected. The agglutinative properties of the vibrio isolated are especially important in establishing its nature. Isolation of a nonagglutinative vibrio does not, however, exclude the possibility that it is V. comma, provided that it has the other characteristics of this species. Such vibrio may, under certain conditions (passage through media containing bile or through animals), become agglutinative.

Abnormalities in biochemical and other properties are occasionally encountered. For example, El Tor vibrio which hemolyze sheep erythrocytes and vibrio which are not lyzed by cholera bacteriophage have been isolated. When such characteristics are altered the nature of the vibrio isolated is determined from its agglutinative properties. All abnormal characteristics (nonagglutinability or any other atypical traits) must be noted in the report. When negative results are obtained it is necessary to make a careful evaluation in the light of clinical or epidemiological data. This is due to the fact that V. comma sometimes cannot be isolated in the typical clinical form for obscure forms of cholera. In such cases one must resort to repeated examination, serological diagnosis, and investigation of cadavers (the small intestine and gall bladder). When bacteriological examination yields negative results but the typical clinical pattern of an obscure form of the disease is present, especially among persons who have been in contact with cholera patients, the diagnosis is consequently rendered as "clinical cholera." Only the aggregate of negative laboratory tests and clinical and epidem-

iological data gives grounds for excluding cholera.

Detection of V. comma in the external environment

In order to concentrate V. comma large volumes of water are filtered through No. 3 or 4 membrane filters and the material washed from them is cultured in peptone water; the filters can first be cut into small pieces. It is also possible to place the filters on solid selective media to permit growth of V. comma.

In order to investigate water without filtration a sample of no less than 1 liter is taken, its pH is determined, and saturated sodium bicarbonate solution is added to bring the pH to 7.6-7.8. A total of 50-100 ml of basic peptone solution is then added to 450-900 ml of the water. The inoculated 1% peptone water (the first concentration medium) is poured into flasks or bottles (in 250 ml portions) and kept in a heater for 5-8 hr. The remainder of the investigation proceeds in the usual manner.

For the use of luminescent sera for the rapid identification of V. cholera see page 74.

Beverages (kvass, cranberry juice, lemonade, beer, etc.) are examined in the same manner as water. Milk is cultured in 2 ml portions in 100 ml of peptone water.

Samples of 200 g are taken in investigating solid foodstuffs (vegetables, meat, fish, etc.). Pieces are taken from different areas of the product, combined to make a representative sample (5-10 g), crushed to a pasty consistency in a mortar with a small quantity of physiological solution, and examined in the same manner as feces. In examining fish cultures are made of the intestinal contents. In order to investigate flies, particularly their legs, one needs glass flytraps containing 1% peptone water and 1% sugar. The flytraps and flies are incubated in a heater at 37° for 5-8 hr; the remainder of the investigation

proceeds in the usual manner.

Increasing Phage-Titre Reaction

Prof. D.M Gol'dfarb

The indicator phage is cholera bacteriophage series Nos. 5 and 6 produced by the "Mikrob" Institute. The standard culture is V. comma strain No. 117, Inaba type. The following media can be used in setting up the reaction: 1) Hottinger's broth; 2) 0.7% agar in Hottinger's broth; 3) 2% agar in Hottinger's broth. When titrated by Grazia's method with strain No. 117 the bacteriophage forms distinct clear negative zones within 3-4 hr.

A substantial increase in titre is observed in inoculated Hottinger's broth (18^8-$1.5 \cdot 10^7$ bacteria per ml) to which the indicator phage has been added in a dilution of 1:50 after one hour of incubation and subsequent titration by Grazia's method, i.e., V. comma is detected within 5-6 hr. This phage is highly specific and reacts only with true V. comma.

The increasing phage-titre reaction is very effective for identifying V. comma in water. Before the phage is added to the water under investigation the latter is mixed with the following basic peptone solution (1/10 of the water volume):

Peptone - 100 g

Sodium chloride - 50 g

Potassium nitrate - 1 g

Sodium carbonate - 20 g

Distilled water - 1000 ml

The remainder of the analysis is conducted in the usual manner. Under these conditions V. comma can be detected in the water in concentrations of $5 \cdot 10^6$-10^7 bacteria per ml after 5-6 hr.

Preliminary culturing can be employed to further increase the sen

sitivity of the increasing phage-titre reaction in cholera.

Nutritive Media

Venkatermen and Ramakrishnon's preservative medium. A total of 12.4 g of boric acid and 14.9 g of potassium chloride are dissolved in 800 ml of hot distilled water and the volume of the solution is brought to 1 liter when it has cooled. Now 250 ml of this solution is mixed with 133.5 ml of 5 M sodium hydroxide and 20 g of dry marine salt (or unpurified table salt), filtered through filter paper, poured into wide-mouthed glass jars in 10 ml portions, and sterilized in an autoclave. The sterile boron-buffered solution will stay at pH 9.2 for several months. A spoonful of feces (no more than 3 g) is mixed with 10 ml of the buffer solution or 2% sodium chloride and sent for examination. A total of 10 ml of the mixture is cultured on a concentration medium at the laboratory. In culturing the mixture of feces and 2% sodium chloride several drops of sidum hydroxide solution are added to neutralize the acid reaction of the feces.

Peptone water. A total of 1% of dry peptone, 0.5% of sodium chloride, and 0.01% of potassium nitrate are mixed and sodium carbonate is added to bring the pH of the mixture to 7.6-7.8. Many researchers prefer to use peptone water which has a more alkaline reaction (pH = 8.6-9.2) and contains 1% sodium chloride, which creates better conditions for isolation of V. comma. Peptone water is prepared from the basic peptone solution by diluting it by a factor of 10 (one part of the peptone solution and 9 parts of distilled water).

Basic (10%) peptone solution. A concentrated (10%) peptone solution is used in investigating water. In culturing the water it is diluted by a factor of 10 to produce 1% peptone water (the first concentration medium). The composition of the basic peptone solution is as follows:

Peptone - 100 g

Sodium chloride - 50-100 g

Fotassium nitrate - 1 g

Sodium carbonate - 20 g

Distilled water - 1000 ml

The basic peptone solution can be stored for prolonged periods. The quality of the basic peptone solution and the peptone water depends on the type of peptone and only standard peptone should consequently be used for preparing media.

Ottolengi's medium. A total of 30 ml of 10% crystalline sodium carbonate is added to 1 liter of filtered bovine bile. Portions of 5 ml of the mixture are poured into test-tubes and sterilized for 20 min at 120°.

Bismuth sulfite medium was proposed by Ried (1939) and modified by Wilson and Reilly (1940). Preparation of the medium requires dry bismuth ammonium citrate or a standard solution of this compound, which consists of 60 g of bismuth citrate, 20 ml of a 12.5% solution of ammonium hydroxide, and 500 ml of water. The bismuth citrate is dissolved in 50 ml of the water and the ammonium hydroxide solution and the remaining water are then added. In order to prepare the basic bismuth sulfite solution 20 g of anhydrous sodium sulfite is dissolved in 100 ml of boiling water and 0.16 ml of the standard solution or 0.1 g of dry bismuth ammonium citrate dissolved in 10 ml of water is added to it. At this point 20 g of glucose is dissolved in 100 ml of boiling water and, after it has cooled, the two solutions are mixed. A total of 10 ml of the basic bismuth sulfite solution is mixed with 100 ml of peptone water (at pH 9.1) and 1 ml of absolute alcohol. The concentration of the medium is doubled in culturing the feces of convalescents or carriers.

Potassium tellurite medium. A total of 0.5% of sodium taurocholate is added to peptone water (1% peptone, 0.5% sodium chloride, pH = 9.0). The medium is poured into 25-ml Ehrlenmeyer flasks, filling them almost to the rim, or into test-tubes and tellurite is added in a concentration of 1:200,000. If three test-tubes are to be inoculated the potassium tellurite is added in concentrations of 1:100,000, 1:200,000, and 1:400,000.

Alkaline agar. A total of 30 ml of 10% sodium carbonate is added to 1 liter of 3% meat-infusion agar and the mixture is boiled for 45 min; the pH is then adjusted to 7.8-8.5.

Bile-salt agar. This medium consists of 3% meat-infusion agar, 0.5% sodium chloride, and 0.5% sodium taurocholate. In order to suppress the growth of Proteus 1% sodium taurocholate is added. Small (0.1 mm in diameter), convex, smooth, clear colonies of V. comma grow on bile-salt agar after incubation at 37° for 15-18 hr.

Aronson's medium. A total of 60 ml of 10% sodium carbonate is added to 1 liter of 3% meat-infusion agar and sterilized in running steam for 15 min; 50 ml of 20% sucrose, 4 ml of saturated alcohol fuchsin, and 20 ml of 10% sodium sulfate are then added under sterile conditions. The precipitate is permitted to settle to the bottom and the medium is poured into dishes. V. comma multiplies profusely on this medium within 10-20 hr, initially forming small colorless colonies and then producing large lustrous colonies with red centers and light-red peripheries. Hottinger's agar or Marter's agar at pH 8.0 can be substituted for the meat-infusion agar.

Dieudonne's medium. Various quantities of defibrinated bovine or ovine blood are mixed with one-normal potassium hydroxide and sterilized at 100° for 45 min. Then three parts of this alkaline blood albuminate are mixed with 7 parts of neutral agar cooled to 45° and the

mixture is poured into Petri dishes. Before use the dishes are held at 60° for no less than 2 hr in order to free them of liberated ammonia, which retards the growth of V. comma.

BRUCELLOSIS

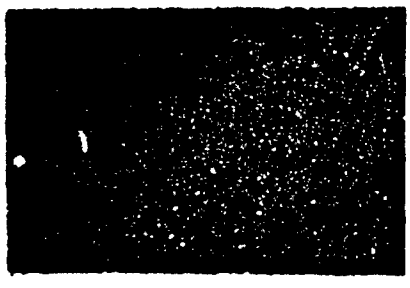
V. I. Kaytmazova, Candidate of Medical Sciences

Brucellosis is an infectious disease of humans and domesticated animals caused by bacteria of the genus Brucella.

It is most convenient to subdivide the genus Brucella into three species, in accordance with their origin (small livestock, cattle, or swine) and certain biological characteristics (see below). This classification makes it possible to call attention to the source of the infection and permits proper planning of prophylactic measures. It is incorporated in the international classification (Bergey). We distinguish the following species: Br. melitensis (Bruce, 1886), which is the causative agent of Brucellosis in small livestock (sheep and goats) and is the most pathogenic for man, playing a basic role in the epidemiology of Brucellosis, Brucella abortus (Bang, 1897), which causes Brucellosis in cattle and both nonsymptomatic and clinically manifest forms of this disease in man, and Brucella suis (Traum, 1914), which causes Brucellosis in hogs and sporadic clinically manifest cases in man.

Morphology of Brucella

Brucella are very small bacteria (0.3-0.6-2.5 μ in diameter) and are spherical, ovoid, or rather elongate in shape.



[illegible] ×20

These bacteria are immobile, do not form spores, and are capable of producing capsules under certain conditions; they are most frequently arrayed randomly in preparations.

Fig. 29. Coarsely granular colonies of Brucella (altered under the influence of bacteriophage). ×20.

Brucella are easily stained by ordinary dyes; they are gram-negative and, in contrast to other bacteria, retain safranine when restained with malachite or brilliant green by Kozlovskiy's method. Kozlovskiy's method for selective staining (as modified by the Institute of Experimental Medicine imeni Gamaleya, Academy of Medical Sciences USSR; Kokorin, 1957) consists in the following: flame-fixed specimens are stained with 0.5% aqueous safranine heated to the boiling point, washed, and restained with a 0.5% aqueous solution of the green dye for 1/2-1 min. Brucella retain the red color of the safranine, while other bacteria take on a green color.

Biology of Brucella; cultural characteristics

Brucella are easily raised on ordinary nutritive media (at pH = = 6.8-7.0-7.2) at temperatures of 35-37°.

Meat and liver agar and broth, potato agar, agar and broth D, and the semisynthetic "Al'bimi" medium can be used as nutritive media. Egg yolks and chick embryos are also good biological media for culturing Brucella.

The first generations of Brucella cultured from an infected organism multiply slowly, the majority of their growth occurring only after 1-2-3 weeks, while laboratory cultures begin to grow within 24-48 hr.

Colonies of Brucella on agar are colorless with a mother-of-pearl lustre, convex, round, regular in contour, and homogeneous or slightly granular (Fig. 28). They gradually become cloudy as they grow and range in size from very small (0.5-0.1-0.01 mm in diameter) to large (3-4 mm in diameter). Various environmental conditions may lead to the

Fig. 30. Striated colonies of Brucella of varying size (altered under the influence of bacteriophage). x20.

ppearance of granular, glassy, eroded, dry, mucoid, or radially striated colonies and colonies which grow into the agar (Figs. 29, 30, 31, and 32).

Fig. 31. Large mucoid colony of Brucella with spindle (altered under the influence of bacteriophage). x20.

When streak-cultured on agar slants Brucella forms a delicate, lustrous clear growth.

Cultures in broth exhibit a uniform cloudiness and do not form films; only an annular growth along the periphery of the glass, slightly above the liquid level, is occasionally observed in older cultures.

Brucella do not liquefy gelatin, do not clabber milk, and act differently on carbohydrates: D. melitensis ferments only glucose, D. abortus ferments glucose, inositol, mannose, and rhamnose, and D. suis ferments glucose, maltose, mannose, and trehalose (Picket and Nelson). It must, however, be kept in mind that this characteristic is apparently unstable.

Dissociation, a phenomenon common in bacteria, also occurs in the Brucella. It may result from frequent reculturing, prolonged culturing in a liquid nutritive medium, or addition of disinfectants, immune se-

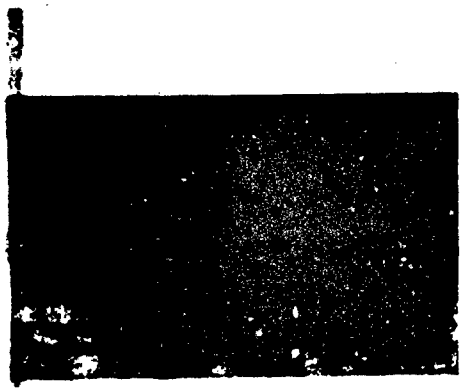


Fig. 32. Granular colony of Brucella (altered under the influence of bacteriophage). ×20.

rum, or other agents to the medium. It has been established that there are both S (smooth) and R (rough) forms of Brucella colonies. Cultures of Br. melitensis dissociate substantially more frequently and more rapidly than those of Br. abortus or Br. suis. This phenomenon must be taken into account in working with Brucella, especially in making up diagnostic preparations and determining the species to which they belong.

Brucella are capable of secreting catalase and of producing hydrogen sulfide.

Resistance to physical and chemical agents

Brucella die rather rapidly under the action of high temperatures; in a moist medium they die within 30 min at 60° and almost instantaneously at the boiling point. A dry heat of 90-95° kills Brucella within 1 hr, while direct sunlight kills them within a period ranging from several minutes to 1 hr. They are more resistant to low temperatures; for example, they may remain viable for 35 days at 5-8°.

Brucella die rather rapidly under the action of various disinfectants. Thus, 2% carbolic acid, 1% creolin, 0.2-1% calcium hypochlorite, 0.5% lysol, 0.2% formalin, or 0.01% chloramine kills Brucella immediately or within a few minutes under experimental conditions. It must, however, be kept in mind that the action of chemicals may be retarded in natural substrates. Thus, in order to kill Brucella soil must be exposed to 5% calcium hypochlorite for 50 min, to 5% freshly slaked lime for 6 hr, to 2% lysol for 12 hr, to 2% creolin for 3 hr, etc.

Antigenic structure; serotypes

It is difficult to distinguish the various species of Brucella from their serological properties. Some serological differentiation is possible only by employing Castelliani's agglutinin-adsorption method or by using monoreceptor sera.

Two antigens, A and M, have been found in Brucella. These antigens are present in different proportions in each species of the genus. The M antigen is found in large quantities in Br. melitensis, while the A antigen occurs in high concentrations in Br. abortus.

The presence of a superficial antigen in Brucella has been postulated, but its role is as yet unknown.

There is as yet no unanimity of opinion on the antigenic structure of Brucella.

Pathogenicity, virulence, and toxin formation

Brucella are pathogenic microorganisms. The different species of the genus, however, are characterized by varying pathogenicity, i.e., a varying ability to induce given pathomorphological changes in infected humans and animals.

Virulence varies both from species to species and within the same species.

It is believed that Br. melitensis is always virulent and Br. abortus facultatively virulent for man; Br. suis varies in virulence. Thus, the Danish strain of Br. suis is of low pathogenicity, while strains isolated in America play a basic role in human brucellosis.

It has not been established that Brucella cultures produce exotoxins and their pathogenic action is consequently associated with an endotoxin.

Laboratory Diagnosis of Brucellosis

Bacteriological examination

Cultures of Br. melitensis and Br. suis can be isolated under ordinary aerobic conditions. Special conditions, culturing in an atmosphere with an increased carbon dioxide content, are necessary to obtain primary cultures of Br. abortus.

The material must be cultured on high-quality, preliminarily checked nutritive media. Agar is suitable only if 20-25 colonies have grown after no more than 5 days of incubation at 37° when 10 Brucella cells (according to the GNKI cloudiness standard) have been cultured in Petri dishes.*

The presence of bacteriophage retards the reproduction of Brucella and thus reduces the [illegible] isolating them, particularly from blood. It is recommended that Brucella antiphage serum be added to nutritive media (solid and liquid) to increase the effectiveness of bacteriological diagnosis (M.S. Dr'shevkina).

In view of the fact that the first generations of bacteria of the Brucella group exhibit poor growth, cultures of the material under investigation in nutritive media are kept in a heater at 37° for no less than 30-35 days. The inoculated media are first inspected after 4-6 days and are then reexamined every 3-4 days. Cultures are made on agar from those test-tubes in which cloudiness appears.

Investigation of blood. Production of hemocultures is a widely-employed basic method for the bacteriological diagnosis of human brucellosis. At the height of the acute stage hemocultures can be obtained in 65-90% of all cases of brucellosis caused by Br. melitensis and in 5-15% of all cases caused by Br. abortus. Hemocultures can sometimes be obtained during the first [illegible] fever; cases in which Brucella have been isolated [illegible] days after the onset of the illness

have been described. We cannot, however, exclude the possibility of reinfection in these cases.

It is recommended that blood be cultured while the patient is in a febrile state. However, it is possible to obtain hemocultures when the patient's temperature is normal or when the agglutination reaction and intracutaneous test are negative.

Blood should not be taken for culturing during antibiotic therapy.

The blood is cultured in the following manner: two flasks containing 50-100 ml of broth and 0.2-0.5% of sodium citrate are inoculated with 5-10 ml of blood taken from the ulnar vein under sterile conditions. One of the flasks is incubated in an atmosphere with an elevated carbon dioxide content, while the other is incubated under normal conditions. The cultures must be kept in the heater for no less than 1 month.

Blood cultures can also be made by Kastaned's method, which consists in the following: portions of 15 ml of agar are poured into square 100 ml flasks, which are then sealed and sterilized. After sterilization the flasks are arranged in such fashion that a uniform layer of agar solidifies on one wall. At this point 10 ml of sterile broth is added to each flask under sterile conditions; 5-10 ml portions of blood are then cultured in each flask. Between the 4th and 7th days the flasks are rocked in order to wet the surface of the agar with the broth. When the results are positive colonies of Brucella appear on the surface of the agar. If no Brucella are detected within one month material is transferred from the broth to a nutritive medium before the end of the experiment.

Uvarov and Khaykina suggest the following method for isolating Brucella cultures from blood. A total of 5-6 ml of blood is mixed with 2 ml of 2% sodium citrate. Half the blood is transferred to 80 ml of

meat-infusion broth containing 1% glucose, while the remainder is cultured in a test-tube containing agar. Both cultures are incubated in a heater for 30 days. Material from the broth is transferred to agar every 3-5 days. At the same time the surface of the agar is wetted with the blood in the test-tube.

Concentration by preliminary culturing in ordinary 3-5-day hen's eggs is recommended for isolation of Brucella from blood, bile, bone marrow, etc. (Odessa Institute of Epidemiology and Microbiology imeni Mechnikov). From 3 to 5 eggs are used in each investigation. A total of 0.2 ml of blood is injected into the yolk with a syringe and the defect in the shell is immediately sealed with sterile paraffin. The infected eggs are incubated at 37° for 5 days and then opened and 0.3-0.5 ml of the yolk is cultured in liquid and solid nutritive media.

Investigation of bone marrow. Isolation of myelocultures is of great value in diagnosing Brucellosis (I.D. Itskovich, 1950; Sh.Kh. Khodzhayev, 1951). Myelocultures are isolated 1½-2 times as often as hemocultures and can be obtained during the acute, subacute, and even chronic stages of the disease. For the techniques of taking and culturing bone marrow see page [illegible].

Investigation of urine. Patient cultures are obtained in 6-14% of all cases of human brucellosis. The urine is collected aseptically with a catheter, mixed with [illegible] of brucellosis agglutinative serum in a titre of no less than 1:[illegible], and centrifuged. The residue is cultured on Petri dishes containing agar to which gentian violet has been added in a dilution of 1:200,000 to suppress the growth of gram-positive flora. The cultures are incubated at 37° for 10-12 days. Egg yolks are also recommended for culturing the urine residue.

Brucella can sometimes be isolated from the urine within 2 weeks after infection, but this usually occurs somewhat later, at the end of

the febrile period or the beginning of convalescence.

Investigation of other materials. Brucella cultures can also be isolated, albeit in a very small percentage of cases, from the feces, bile, milk, cerebrospinal fluid, sputum, bursitic exudate, and other secreta. A suspension is prepared from feces, precipitated with brucellosis agglutinative serum (1:50, 1:100), incubated for 2 hr in a heater at 37°, and centrifuged; the residue is cultured on Petri dishes containing agar to which gentian violet has been added. Other materials can be cultured directly on solid and liquid nutritive media, but it is best to use the biological method (see below) or to culture them on the yolks of hen's eggs.

Identification and differentiation of species of Brucella. Differentiation of species in the Brucella cultures isolated is of great importance for the prophylaxis of brucellosis, for eliminating nidi of this disease, and for prescribing rational therapy.

Study of the cultures isolated is also necessitated by the fact that both typical strains corresponding to their basic host and atypical cultures, i.e., cultures with altered properties, have recently been isolated in the USSR and abroad.

Methods of identifying cultures isolated. Microscopic examination of Gram- and Kozlovskiy-stained smears and study of colony morphology, the agglutination reaction on slides with specific serum, and the action of brucellosis bacteriophage are employed to determine whether or not the cultures isolated belong to the genus Brucella.

For rapid preliminary identification of a culture a drop of specific agglutinative serum is applied to a slide, diluted to 1:25 with carbolinized physiological solution (0.5% carbolic acid), and used to emulsify one loopful of the culture under investigation. When the results are positive agglutination sets in rapidly and distinct flakes are formed,

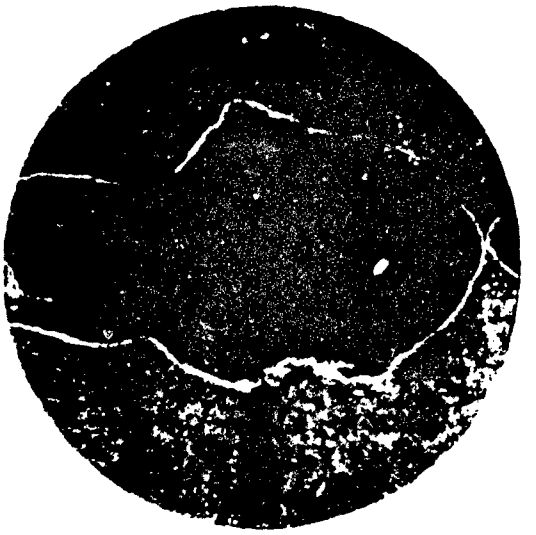


Fig. 33. Zone of brucellar lysis at site of application of bacteriophage.

while when they are negative the suspension remains homogeneous.

In the specific bacteriophage test 0.1 ml of a bacterial suspension consisting of 500 million or 1 billion cells from the culture under investigation in 1 ml of physiological solution is applied to two Petri dishes (experimental and control) containing Hottinger's nutritive (glucose-glycerine) agar, liver agar, or 1.2-1.4% Martin's agar (pH = 7.2). A spatula is used to spread the suspension uniformly over the surface of the medium. When the inoculated agar in the experimental dish has dried a drop of bacteriophage is applied to it with a slender Pasteur pipette. Slightly tilting the dish causes the drop of bacteriophage to run and form a "streak." After the culture has dried the dish is inverted and placed in a heater at 37° together with the control (which contains no bacteriophage). The results are evaluated twice, after 24 and 48 hr. When the results are positive the culture is lyzed along the "streak" (Fig. 33).

Dissociation of Brucella. The simplest and most widely used methods

for detecting the dissociation of Brucella cultures are the agglutination reaction on slides with trypaflavin and the thermoprecipitation reaction.

In order to set up the trypaflavin reaction a drop of a 1:500 salt (0.85%) solution of trypaflavin is applied to a slide and thoroughly mixed with a loopful of the culture under investigation (raised on agar). When the results are positive, i.e., with dissociated strains, agglutination sets in rapidly and distinct flakes are formed. Undissociated strains do not produce agglutination.

In thermoprecipitation 2-3 ml of a 1-billion-cell suspension of a 2-day Brucella culture in physiological solution is heated in a test-tube at 90° in a water bath for 30 min. The results are noted after 30 min, 1 hr, and 24 hr. Marked agglutination occurs over these intervals when R forms are present, while suspensions of undissociated strains remain homogeneous.

Differentiation of species of Brucella. The various species of Brucella can be differentiated by Heddelson's complex method, which involves determination of: 1) the behavior of the first generations of the cultures isolated with respect to carbon dioxide; 2) hydrogen sulfide formation; 3) sensitivity to analine dyes (bacteriostatic method). It is recommended that these procedures be supplemented with: 4) determination of urease activity; 5) study of agglutinability in monospecific serum.

The behavior of the first generations with respect to carbon dioxide clearly distinguishes Br. abortus from Br. melitensis and Br. suis. This characteristic cannot be used to differentiate the latter two species. However, cultures of Br. abortus initially raised in an atmosphere with an elevated carbon dioxide content are capable of growing under ordinary atmospheric conditions when subsequently recultured on

nutritive media.

The following procedure is employed to obtain an elevated carbon dioxide content: 1) a Petri-dish lid containing 0.35 g of sodium bicarbonate per liter of vessel volume is placed on the bottom of the vessel holding the culture; a short test-tube containing an equal quantity of hydrochloric acid is placed in an inclined position on the lid, a short distance from the soda. The vessel is tightly covered and carefully shaken; the reaction between the acid and the soda releases the requisite quantity of carbon dioxide; 2) carbon dioxide is supplied from a cylinder through a gasometer to a hermetically sealed vessel (dessicator) containing the culture until a volume equal to 5-10% of the vessel volume has been added; 3) Novac's method: cultures of Br. abortus are placed in a hermetically sealed vessel with cultures of bacteria which vigorously absorb oxygen (Bac. subtilis, Bac. prodigiosum, etc.).

Differentiation from hydrogen sulfide formation. It has been established that cultures of Br. suis and Br. abortus form hydrogen sulfide during assimilation of proteins and amino acids, while Br. melitensis either fails to produce this gas or forms negligible quantities over short periods.

Br. suis produces hydrogen sulfide most intensively and for the longest period (for from 4 to 10 days). This characteristic is somewhat less pronounced in Br. abortus.

This method can thus be used to differentiate Br. melitensis from Br. suis and Br. abortus. The latter two species cannot be distinguished by this technique. It must, however, be kept in mind that certain strains of Br. abortus and, occasionally, of Br. suis do not form hydrogen sulfide. There are also strains of Br. melitensis which produce hydrogen sulfide. These atypical properties are apparently associated

with incipient dissociation of the culture.

Method of determining hydrogen sulfide. One standard loopful (a 2 mm loop) of a suspension of the culture under investigation in physiological solution is uniformly spread over the surface of a liver-agar slant (pH = 6.8-7.2). The suspension is prepared from a 2-day agar culture and its concentration is brought to 1 billion cells per ml by comparison with a turbidity standard. Paper impregnated with lead acetate is used as the hydrogen-sulfide indicator.

The darkened area of the paper, measured in millimeters, is the index of the intensity of hydrogen sulfide formation. The results are evaluated over a period of 6 days, noting them every 2 days; the darkened paper is replaced with new paper at each reading. The three indices are added to give a final evaluation of the ability of the strain to form hydrogen sulfide. The total hydrogen-sulfide-formation index for Br. suis is 12-18-20 mm, while that for Br. abortus is 5-7 mm.

Differentiation from sensitivity to analine dyes (bacteriostatic method). The most reliable method for differentiating Brucella involves determination of the bacteriostatic action of analine dyes and was suggested by Heddelson. Two stains, basic fuchsin and thionine, are usually used in this differentiation technique. Br. melitensis grows in media containing both dyes, Br. abortus grows only in media containing fuchsin, and Br. suis grows well in the presence of thionine but does not grow in media containing fuchsin.

The most widely known procedure is Mayer-Tsobel's method (as modified by the Institute of Experimental Medicine imeni Gamaleya of the Academy of Medical Sciences USSR), which employs a semiliquid medium and two dyes, fuchsin and thionine. The basic stain solutions are prepared in the following manner: 0.1 g of dye is ground with 20 ml of alcohol and sufficient distilled water is added to make 100 ml, this

yielding a 0.1% solution with a 20% alcohol content. In preparing the stain-bearing medium agar is melted and cooled to 45° and the basic dye solution is added to it under sterile conditions. That dye concentration at which clear differentiation is obtained with standard strains of Brucella is regarded as the working dose;for example, the thionine concentration should be 1:25,000 (4 ml of the basic solution must consequently be added to 100 ml of medium). If the final fuchsin concentration in the medium should be 1:50,000, 2 ml of the basic dye solution must be added to 100 ml of medium.

In doubtful cases, when atypical strains are to be differented, the bacterium under investigation must be cultured in media containing various dye concentrations (e.g., from 1:15,000 to 1:40,000 for thianine). After the dye has been added 4 ml portions of the medium are poured into test-tubes. These are then inoculated with one loopful of a one-billion-cell suspension of a 48-hr agar culture and the resultant cultures are incubated in a heater at 37°. The results are evaluated over a 6-day period, noting them every 2 days.

Brucella can also be differentiated by the bacteriostatic method on solid nutritive media. In this procedure quantities of the dyes sufficient to produce the requisite concentration are added to molten agar and the resultant media are poured into Petri dishes and dried. A suspension of a 48-hr agar culture is prepared by emulsifying one loopful in 0.1 ml of salt solution. One loopful of this suspension is streaked over the surface of the dye-containing agar. As many as 4-6 cultures can simultaneously be raised on a single dish. The dishes are incubated in a heater and the results are noted after 24, 48, and 72 hr.

Differentiation on the basis of determination of urease activity. It has been established that all species of Brucella possess urease activity, but that it varies in extent from species to species. It is re-

commended that Christensen's medium be used for determination of urease activity.

This medium has the following composition: peptone – 1 g, sodium chloride – 5 g, monosubstituted potassium phosphate – 2 g, phenol red – 0.012 g, glucose – 1 g, agar-agar – 20 g, and distilled water – 1000 ml.

After all the ingredients have been dissolved in the water the pH is adjusted to 6.8 and 5 ml portions of the medium are poured into test-tubes and sterilized under a pressure of 1 atm (at 121°) for 20 min. The medium-containing tubes are then cooled to 50° and 20% urea (preliminarily sterilized and passed through a Zeitz filter) is added in a quantity sufficient that its final concentration is 2%. The medium is cooled with the tubes in an inclined position.

This agar medium is inoculated with one loopful of the Brucella culture. The cultures are incubated at room temperature and observed over a 5-hr period, between the 19th and 24th hours.

When Br. suis is present the medium takes on a reddish tint almost immediately and turns red within 15-20 min. When Br. melitensis is present the medium becomes reddish within 20 min and red within 2 hrs. When Br. abortus is present the medium becomes reddish after 18-20 hr and requires at least one day to turn red. Br. suis produces urease more rapidly and more intensively than the other species of Brucella, Br. abortus exhibiting this property to a far lesser extent. Br. melitensis occupies an intermediate position and has a variable urease activity (Renu and Katrefazh).

Determination of urease activity cannot be employed as an independent technique for differentiating the species of Brucella, since it cannot distinguish Br. melitensis from Br. suis and Br. abortus.

In addition to these methods, the species of Brucella can be differentiated serologically, utilizing a direct agglutination reaction

with monospecific sera or agglutinin adsorption by Castelliani's method.

The agglutination reaction with monospecific sera makes it possible to differentiate Brucella strains with typical antigenic structures.

Serological investigation

Serological methods include the volumetric agglutination reaction in test-tubes (Wright's reaction), the rapid agglutination reaction on plates (Heddleson's reaction), the centrifugation-accelerated agglutination reaction, the complement-fixation reaction, and a number of others which are not widely used.

Wright's agglutination reaction is the most common technique for the laboratory diagnosis of brucellosis. It is positive at the very outset of the illness, occasionally even on the first day of fever. High agglutination titres usually persist throughout the febrile period and then decrease sharply. Wright's reaction sometimes remains positive for up to 2-4 years. It must, however, be kept in mind that there are cases of typical brucellosis in which a positive hemoculture is obtained but the agglutination reaction stays negative. It should also be noted that Wright's reaction may be weakly positive in certain other infectious diseases (tularemia, typhus, typhoid, etc.).

So-called proagglutination zones, i.e., a negative result in tubes containing low serum dilutions and a markedly positive result at higher dilutions, may be observed in Wright's reaction; it is also possible for agglutination to fail to occur in the middle of a series of dilutions. The appearance of the zone phenomenon is apparently associated with the presence of incomplete (blocking) antibodies in the blood serum.

The blood serum to be investigated is obtained in the usual manner.

The physiological solution used to dilute the serum and antigen should contain 0.5% carbolic acid.

TABLE 32

Preparation of Turbidity Standard for Evaluating Wright's Reaction

1 № пробирки	2 Основное разведение антигена		5 Стандарт мутности			6 Степень агглютинации	
	3 антиген концентрации 1 млрд. микробных клеток в мл	4 0,5% раствор карболизированного физиологического раствора в мл	6 из каждой пробирки антигена в мл	4 0,5% раствор карболизированного физиологического раствора в мл	7 общий объем в мл	%	плюсы 9
1	1	3	0,5	0,5	1,0	75	+++
2	2	2	0,5	0,5	1,0	50	++
3	3	1	0,5	0,5	1,0	25	+
4	4	0	0,5	0,5	1,0	0	—

1) Test-tube No.; 2) basic antigen dilution; 3) antigen concentration, billions of cells per ml; 4) 0.5% carbolinized physiological solution, ml; 5) turbidity standard; 6) ml of antigen from each test-tube; 7) total volume, ml; 8) degree of agglutination; 9) plusses.

The reaction is set up with 1 ml of each of no less than 5 dilutions (from 1:50 to 1:800). Brucellosis monodiagnostic serum containing 10 billion cells per ml is employed as the antigen. It is preliminarily diluted by a factor of 10 with physiological solution.

The procedure for stepping up Wright's reaction is similar to that for Widal's reaction.

The reaction is accompanied by two controls - serum and antigen. The test-tubes are incubated in a heater for 20-24 hr and then at room temperature for 18-20 hr. The reaction is evaluated with the unaided eye, using a four-plus scale.

That serum dilution which yields agglutination in no less than 50% of the tubes is assumed to be the maximum titre.

The following scheme is recommended for diagnostic evaluation of the results of the reaction: agglutination at a titre of 1:50 - doubtful result, at a titre of 1:100 - weakly positive result, at a titre of 1:200-1:400 - positive result, and at a titre of 1:800 or more - sharply positive result.

When a negative or doubtful reaction (a titre of 1:50) is obtained it is recommended that the patient's serum be reexamined after 7-10 days.

In accordance with a decision of the Expert Committee on Biological Standardization of the Ministry of Public Health, the degree of clarification and hence of agglutination is determined by comparison with turbidity standards prepared in the following manner: portions of 1, 2, 3, and 4 ml of antigen diluted to 1 billion cells per ml are successively added to 4 test-tubes and portions of 3, 2, and 1 ml of carbolinized physiological solution are then successively added to the first three tubes. After the contents have been shaken portions of 0.5 ml of each antigen dilution are transferred to new test-tubes and 0.5 ml of carbolinized physiological solution is added. These turbidity standards correspond to 75, 50, and 25% clarification and no clarification (see Table 34).

The turbidity standards are made up fresh each time a reaction is conducted and are prepared under the conditions which obtain in the basic experiment. It is recommended that the serum titre be determined from the dilution which yields 50% agglutination. When the reaction is evaluated by comparison with turbidity standards there is no need to set up an antigen control. When employing this evaluation method it is very important to use test-tubes of the same diameter in the basic experiment and for the turbidity standards.

Heddleson's reaction is a method for the rapid diagnosis of brucellosis and is specific. Its sensitivity is due to the fact that the reaction takes place with undiluted serum and concentrated antigen. The serum to be tested should be absolutely clear. Undiluted brucellosis monodiagnostic serum of the type used for the volumetric agglutination reaction is employed as the antigen. The reaction is accompanied by two

controls: 1) a serum control for each serum tested; 2) an antigen control for each series of antigen.

Agglutination by the plate method is carried out in the following manner: the sheet of glass on which the reaction is set up is divided into 4 x 4 cm squares. There should be 6 squares in each horizontal row. The left-hand square is labeled with the number of the serum to be tested and quantities of 0.04-0.02-0.01-0.02 ml of the serum are applied to successive squares (with a 1 ml micropipette or graduated pipette). A pipette is used to add 0.03 ml of antigen to each of the first three serum doses. A 0.03 ml portion of physiological solution is added to the last dose (0.02 ml), this being the serum control. The serum is carefully mixed with the antigen, using a glass rod and beginning at the lowest serum dose.

For the antigen control 0.03 ml of physiological solution is added to 0.03 ml of antigen.

The glass is then uniformly heated over an alcohol flame in such fashion that its entire surface reaches a temperature of approximately 37° (which requires about 2 min). When the reaction is positive distinct flakes appear within the first few minutes. The maximum observation time is 5 min.

The results are evaluated with the unaided eye, using the following scale: complete clarification with formation of coarse- or fine-grained flakes - 100% agglutination (++++); almost complete clarification and formation of distinct flakes - 75% agglutination (+++); slight clarification and formation of noticeable flakes - 50% agglutination (++); cloudy liquid with scarcely noticeable granularity (+); uniformly cloudy liquid (-).

The reaction is regarded as positive when the agglutination registers at least two plusses (++) on this scale.

The serum dilutions obtained with the plate method are not comparable to those in the agglutination reaction in test-tubes (Wright's reaction) and cannot be expressed as titres. The following scheme is consequently recommended for diagnostic evaluation of the results: no agglutination at any serum dose – negative reaction; agglutination at first dose (0.04 ml of serum)– doubtful reaction; agglutination at second or third dose (0.02-0.01 ml) – positive result; four-plus agglutination at all doses – sharply positive reaction.

Only a positive result is of value in diagnosing brucellosis.

When a doubtful result is obtained it is recommended that the reaction be repeated after 7-10 days and that an allergy test be conducted. Repetition of the reaction is also recommended when a negative result is obtained in the presence of epidemiological or epizootological indications.

Since the agglutination reaction on glass is a qualitative reaction and cannot be used to determine titre, it is essentially recommended only for mass examinations. The classical agglutination reaction in test-tubes must be employed under hospital conditions and in examining blood donors, when it is necessary to determine the agglutinin titre and its dynamics.

Other reactions have been proposed for the rapid diagnosis of brucellosis, including the centrifugation-accelerated agglutination reaction, the blood-drop method, Noble's reaction, the precipitation reaction, Meinicke's flocculation reaction, etc.

The majority of these reactions have not been studied with sufficient thoroughness and are not widely used. The centrifugation-accelerated agglutination reaction (M.G. Lokhov) is rather interesting. The advantage of this method lies in the fact that it does not require a heater, since centrifugation replaces the action of heat. Moreover, the

final result of the reaction can be obtained 40-60 min after it begins. In this procedure the agglutination reaction is set up in the usual manner, beginning at a serum dilution of 1:50. Standard diagnostic sera are employed as the antigen. The reaction is accompanied by serum and antigen controls. Centrifugation is carried out in ordinary manual or milk centrifuges for 20-30 min. The results are evaluated on the scale used for Wright's reaction.

It should be noted that the agglutination reaction is conducted with cerebrospinal fluid for diagnosis of brucellar damage to the central nervous system.

The complement-fixation reaction is a fully specific test for brucellosis and is of high sensitivity (M.L. Feder).

This reaction appears somewhat later than Wright's reaction, but persists substantially longer. The reaction is set up in the usual manner, using Buaven's antigen, a heat-killed suspension of brucella in physiological solution, or an antigen specially prepared for the complement-fixation reaction.

It is recommended that the prolonged complement-fixation reaction under refrigeration be used to detect small quantities of antibodies. This qualification of the reaction (PCFR) can also be employed to detect brucellosis antigen in the serum of brucellosis patients.

Detection of Brucella by biological testing

If the material under examination is contaminated or has a low Brucella concentration, the only reliable investigative technique is the biological method.

Guinea pigs and white mice are the laboratory animals most susceptible to brucellosis. These animals can be inoculated by any ordinary method, but it is best to inject the material to be studied subcutaneously into the inguinal region in doses of no more than 0.5 ml for

white mice and 1 ml for guinea pigs. The advantage of subcutaneous inguinal injection lies in the fact that it is possible to detect a regional infection in the lymph nodes (inguinal and paraaortic).

Infected white mice must be dissected within 20 days after inoculation and guinea pigs within 30 days.

Bacteriological investigation of the infected animals is most effective when organ fragments and lymph nodes are subjected to mass culturing on nutritive media. The material must be cultured on high-quality media which have preliminarily been checked.

Before dissecting guinea pigs an allergy test is run and blood is taken from the heart for Wright's reaction (the normal agglutination-reaction titre for guinea pigs is less than 1:10).

All the lymph nodes and organ fragments are removed by puncture with a sterile wooden rod (which should be 25-30 cm long and 4-5 mm in diameter) and introduced into a test-tube containing agar; there they are crushed against the wall of the tube with the rod and carefully rubbed into the surface of the medium. The remainder of the culture material is transferred to a test-tube containing broth. The cultures are incubated at 37° for no less than 25-30 days and inspected every 5-6 days. If the broth in any of the tubes becomes cloudy portions are transferred to agar.

Allergic reactions

Burne's intracutaneous allergic test. Burne's reaction is specific and is distinguished by its high sensitivity. It usually becomes positive toward the end of the first month of illness, but may do so even earlier. It remains positive long after all clinical symptoms have disappeared. This test can consequently be used to diagnose both acute and latent forms of brucellosis and to establish retrospective diagnoses.

It must be kept in mind that Burne's reaction becomes positive af-

ter vaccination with live brucellosis vaccine; this reaction develops 1-1½ months after vaccination, is quite pronounced between the 2nd and 13th month, and then begins to fade.

The reaction is set up with brucellin, which is a filtrate of a 3-week broth culture of Brucella.

A dose of 0.1 ml of brucellin is injected intracutaneously into the forearm with a syringe and a fine needle.

A painful swelling, usually accompanied by hyperemia, develops at the site of the injection when the reaction is positive. This edemic swelling is the most characteristic sign of a positive reaction. It varies in size, averaging approximately 4 x 6 cm. The edemic area is usually elongate or oval in shape and is most often distinct in outline and clearly raised above the normal skin; it may, however, be internalized and less pronounced, perceptible only on palpation. The reaction may develop within 7-10 hr, requiring 24 hr in rare cases, and persists for 40-50 hr (occasionally as long as 60-72 hr). A reaction which appears and disappears within 6 hr after the brucellin is injected is regarded as nonspecific. The reaction is evaluated on the day after the injection, by infection (for edema and discoloration) and palpation of the skin (for edema). The size of the edemic area in centimeters (length and width), the degree of painfulness, and the extent of the discoloration are noted. If the reaction is negative after 24 hr it is repeated 48 hr later.

In evaluating the reaction pronounced dermal edema at the site of the brucellin injection is regarded as indicating a positive allergic reaction. Absence of the pain and hyperemia which usually accompany the edema does not exclude the possibility of a positive reaction.

In rare cases involving especially sensitive individuals the local reaction is accompanied by lymphangioitis, a slight swelling of the re-

gional lymph nodes, and, occasionally, a rise in body temperature.

Opsonic-phagocytic test

The opsonic capacity of the neutrophils begins to appear at approximately the same time as the allergic reaction and lasts for an indefinitely long period, especially in persons who remain in contact

TABLE 33

Evaluation of Opsonic-Phagocytic Test

Количество фрунцелл, фагоцитированных одним нейтрофилом 1	Оценка фагоцитоза 2	Количество лейкоцитов (нейтрофилов) 3	Вычисление цифрового показателя 4
0	0	3	3×0=0
1—20	+ (1)	4	4×1=4
21—40 5	++ (2)	6	6×2=12
41 и больше	+++ (3)	12	12×3=36
		25	Показатель 52 6

1) Number of Brucella phagocytized by one neutrophil; 2) evaluation of phagocytosis; 3) number of leucocytes (neutrophils); 4) calculation of numerical index; 5) or more; 6) index.

with brucellosis. High phagocytic indices are also observed in individuals who have received live vaccine.

The opsonic-phagocytic test is conducted and evaluated in the following manner: 1 ml of the blood to be studied is added to a test-tube containing 0.5 ml of a sterile 2% sodium citrate solution. A total of 0.5 ml of special antigen prepared for this test is immediately (or within 6 hr) added to the tube. The contents are thoroughly mixed and the tube is placed in a heater or water bath at 37° for 30 min. After heating the tube is gently shaken and smears are prepared by the usual hematological method. The smears are fixed with Nikiforov's solution and stained by Romanowskiy or Kozlovskiy's method.

The reaction is evaluated by counting the phagocytized Brucella in 25 segmented neutrophils from several areas of the preparation. Each

neutrophil is assigned to a group in accordance with the number of ingested Brucella.

The results of the reaction are expressed by a numerical index, roughly following the scheme shown in Table 33.

The minimum phagocytic-activity index is zero (25 x 0 = 0). The maximum index is 75 (3-plus phagocytosis in all 25 leucocytes).

As a guideline it can be assumed that indices of 10-24 characterize a weakly positive reaction, indices of 25-49 a markedly positive reaction, and indices of 50-75 a sharply positive reaction.

In the majority of healthy persons the phagocytic-activity index is 0 or 1, very rarely reaching 3-5.

Detection of Brucella in environmental objects

Investigation of milk and foodstuffs. Milk and dairy products are one of the basic sources of human brucellosis. Their importance in the epidemiology of this disease is attributable to the fact that Brucella retain their viability for prolonged periods in such substances. The time for which Brucella remain viable in raw milk, dairy products, and meat varies within rather wide limits, from several days to months, years, or longer, depending on the temperature, humidity, method of preservation, and other factors. According to some data, these bacteria remain viable in milk and dairy products for as long as they are fit for consumption.

Methods based on serological principles are now widely used to examine milk for brucellosis. These include the agglutination reaction on plates and the ring test with colored antigen.

The agglutination reaction on plates is specific and sensitive and can be conducted with either whole milk or whey. The latter is obtained by adding rennin or a mixture of 1 g of pepsin, 1 ml of dilute (1:2) hydrochloric acid, and 8 ml of distilled water to the milk and heating it

in a water bath. The whey thus obtained is applied to a slide and Heddleson's antigen is added to it. The mixture is then slightly heated over a Bunsen-burner flame and the results of the reaction are evaluated.

The annular precipitation reaction is used only for investigating whole, fatty milk.

In setting up this test 2 drops of a specially prepared colored brucellar antigen (blue or red) is added to 2 ml of milk. The test-tube is shaken and placed in a heater at 37-38° for 40-60 min. When the results are positive annular precipitation occurs in the surface layer of the milk, a colored ring forming, while when the results are negative no such ring is produced, the milk retaining the color of the antigen.

Bacteriological and biological methods are also used to detect contamination in milk and dairy products, particularly brynza [a Russian cheese], sour cream, etc.

Bacteriological cultures are made in dye-containing nutritive media (in order to retard the growth of extraneous microorganisms). The milk is first centrifuged and cultures are made from the cream and the sediment. In order to speed up the results it is recommended that the material to be examined be preliminarily cultured in egg yolks and then transferred to solid nutritive media.

Biological testing is conducted by inoculating guinea pigs or white mice and subsequently performing autopsies.

Solid foodstuffs (meat, brynza, etc.) are first crushed in a sterile porcelain mortar and then suspended in physiological solution. The suspension is centrifuged to concentrate the Brucella, adding brucellar agglutinative serum beforehand. The centrifugate thus obtained is cultured in nutritive media containing dyes and brucellar antiphage serum and in egg yolks and used to inoculate laboratory animals.

Soil, manure, feeds, water, and other environmental objects become sources of human infection after contamination with various excreta from brucellosis-infected animals (urine, feces, sexual secreta, etc.). Examination of such environmental objects for brucellar contamination is greatly hampered by the presence of extraneous microorganisms, particularly E. coli. In addition to bacteriological investigation by culturing on dye-containing nutritive media and in egg yolks and biological testing, the bacteria must be concentrated on membrane filters.

It is known that the causative agent of brucellosis is retained on No. 1 membrane filters. Better deposition is provided by two sterile No. 1 filters placed one on top of the other and mounted in a Zeitz apparatus. After the material to be investigated has been filtered the membrane filters are transferred to a Petri dish (individually) with a sterile forceps and pressed gently against the surface of the medium. The side of the filter bearing the deposited bacteria should face upward. When colonies suspected of being Brucella appear on the filter they are identified by the usual techniques.

Culturing of pulverized filters in egg yolks is also recommended; in this procedure the pulverized membrane filter is placed in a test-tube, 2 ml of physiological solution containing gentian violet is added, the contents are shaken for 5-10 min, and the tube is left to stand for 30 min. The supernatant is then cultured in egg yolks (this method was developed by the Odessa Institute of Experimental Medicine).

The increasing phage-titre reaction can also be used to investigate environmental objects. This method is, however, of very limited value, since it can be employed only to indicate Brucella in uncontaminated material (e.g., tap water). It cannot be used for objects containing extraneous microorganisms, particularly E. coli, since the latter suppresses the growth of Brucella and retards the increase in phage

titre.

Of particular interest is the technique for detecting Brucella based on the ability of sensitized guinea pigs to react to intracutaneous injection of a Brucella suspension within 24-28 hr.

This method makes it possible to detect Brucella in quantities of 100 million within 24 hr and 10 million within 48 hr when they are mixed with large numbers of other bacteria (A.A. Golubeva).

Increasing Phage-Titre Reaction

Prof. D.M. Gol'dfarb

The increasing phage-titre reaction can be employed for detection of Brucella in certain environmental objects (N.N. Ostrovskaya and D.M. Gol'dfarb). The indicator phage is the typospecific phage for Br. abortus, which was isolated by M.Z. Papkhadze and T.T. Abashidze. This phage is specific within the genus Brucella, since it reacts only with Br. abortus. The phage titre, determined by Grazia's method, is 10^9-10^{10} particles per ml. The phage is characterized by a very long latent period; depending on the experimental conditions, it reproduces in cultures of Br. abortus after 12-24 hr of incubation at 37°. Investigations involving artificially infected broth have shown that it is possible to detect 100,000 cells per ml after 24 hr, 1000 cells per ml after 48 hr, and 10 cells per ml after 72 hr. Subsequent experiments established that it is possible to use the increasing phage-titre reaction to detect Brucella in artificially infected tap water. The procedure for setting up the reaction with water is somewhat different from that for dysentery or typhoid bacteria. In order to suppress the extraneous microflora gentian violet is added to the water under investigation in a dose of 0.1 ml (of a 0.04% solution) per 100 ml. The remainder of the reaction is conducted in the usual manner. The sensitivity of the increasing phage-titre reaction depends on the volume of water to be

studied and is highest at low volumes, of the order of 1 ml. The minimum quantity of Brucella detectable in water after 48 hr of exposure to the indicator phage is 1000 cells per ml. The increasing phage-titre reaction does not give positive results in investigating infected soil or milk, since the phage titre does not rise even as a result of prolonged incubation; this is due to the fact that the material contains large quantities of extraneous microorganisms, which suppress the growth of Brucella and make it impossible for the phage to reproduce. The increase phage-titre reaction can thus be utilized to detect Brucella in environmental objects comparatively uncontaminated with extraneous microorganisms. In such cases the presence of Brucella can be established more quickly than in bacteriological investigation.

Nutritive Media

Liver infusion. Fresh beef liver is cleaned of fat and membranes and passed through a meat-grinder. The ground meat is flooded with tap water (1 liter of water per kg of meat) and left to stand at 25-30° for 3 hr or at 4-10° for 6-10 hr. The mixture is stirred and cooked in blowing steam in an autoclave for 20 min. It can also be cooked in a pot, provided that it is constantly stirred. The scum which forms is removed during the cooking process and the mixture is stirred once more and boiled for approximately 2 hr. After cooking the infusion is filtered through a thin cotton gauze filter.

The liver infusion thus obtained is poured into bottles and sterilized at 115-120° for 20 min.

Liver broth. A total of 500 ml of tap water, 10 g of dry peptone, and 5 g of chemically pure sodium chloride are added to 500 ml of liver infusion.

The mixture is filtered, its pH is adjusted to 7.4, and it is then filtered through filter paper and sterilized at 115-120° for 20 min;

the broth has a pH of 7.1-7.2 after sterilization.

Liver agar. A total of 500 ml of tap water, 10 g of dry peptone, 5 g of chemically pure sodium chloride, and 25 g of agar washed with tap water and thoroughly dried are added to 500 ml of liver infusion. The pH is adjusted to 7.4 and the medium is cooked until the agar dissolves and poured into appropriate vessels (in 1-2 liter portions). It is then autoclaved at 115° for 20 min, permitted to settle, and filtered through a cotton gauze filter (which is preliminarily wetted with warm water). The pH is then readjusted to 7.1-7.2. The medium is poured into vessels of the requisite capacity and sterilized at 115° for 20 min.

"Al'bimi" agar medium (as modified by the Institute of Microbiology and Epidemiology imeni Gamaleya of the Academy of Medical Sciences USSR). A total of 20 g of dry peptone, 10 ml of yeast water, 5 g of chemically pure sodium chloride, and 20 g of agar-agar are added to 1 liter of distilled water. The pH is adjusted to 7.3 and all the ingredients are dissolved by autoclaving; the solution is filtered through a cotton filter (preliminarily wetted with warm water and thoroughly wrung out). At this point 1 g of glucose and 0.1 g of bisulfite are added. The pH is again adjusted to 7.2-7.3 and the medium is poured into appropriate vessels (test-tubes, flasks, etc.) and sterilized in running steam for 40 min and then at 110° for 20 min.

Yeast water consists of 1 kg of baker's yeast and 1 liter of distilled water. The mixture is boiled until the yeast dissolves and then filtered through a linen filter. The solution can be stored under chloroform in a dark place for no more than 2 weeks.

Medium D (method devised by the Institute of Epidemiology and Microbiology imeni Gamaleya of the Academy of Medical Sciences USSR). a) Broth D: 2.5 g of powdered standard broth D is added to 100 ml of cold

distilled water, heated, and thoroughly mixed until it completely dissolves. The solution is then filtered, poured into appropriate vessels, and sterilized at 120° for 20 min; its pH should be 7.1-7.2.

b) Agar D: 5 g of powdered standard agar D is added to 100 ml of cold distilled water; the mixture is heated and stirred until the agar completely dissolves, not permitting it to burn. The medium is then filtered, poured into appropriate vessels, and sterilized at 120° for 20 min; its pH should be 7.2.

Potato medium (formula devised by the Control Institute for Veterinary Preparations). Selected large potatoes are washed and peeled; 1 liter of distilled water is added to 1 kg of the potatoes and they are cooked until done. The liquid is then poured off and measured, sufficient distilled water is added to bring it to its initial volume, and it is filtered through a double gauze filter. The following ingredients are then added:

Peptone - 10 g

Chemically pure sodium chloride - 5 g

Glycerine - 30 ml

Washed agar-agar - 30 g

The mixture is placed in a pot and boiled until the agar has completely dissolved; its pH is adjusted to 7.2 with 10% sodium hydroxide. The medium is then sterilized in an autoclave at 116° for 30 min, filtered, measured, mixed with 10 g of glucose, and resterilized at 116° for 30 min.

Mayer-Tsobell's medium (as modified by the Institute of Epidemiology and Microbiology imeni Gamaleya of the Academy of Medical Sciences USSR). A total of 1 ml of distilled water is added to 900 g of ground meat and the mixture is boiled for half an hour. The resultant decoction is filtered through cotton, brought to its initial volume (1 liter)

with distilled water, and then mixed with 5 g of chemically pure sodium chloride, 2 g of peptone, and 2 g of agar; when the latter ingredients have been dissolved (by heating) the medium is refiltered and its pH is adjusted to 7.2. Portions of 100 ml are poured into flasks and sterilized in an autoclave at 115° for 20 min. The medium should have a pH of 6.8-7.2 after sterilization.

Basic dye solutions are added to the semiliquid medium before use (see above).

LEPTOSPIROSIS

Prof. V.V. Anan'in

The leptospiroses are a group of infectious diseases of man and domestic animals and are etiologically, pathogenetically, pathologoanatomically, and epidemiologically similar. They occur in many of the nations of Europe, Asia, the Americas, and Africa, as well as in Australia, and have been recorded as sporadic cases and epidemics in humans and domestic animals.

The causative agents of these diseases are microorganisms of the genus Leptospira Noguchi, which belong to the family Treponemataceae.

In addition to pathogenic Leptospira, there is a large number of saprophytic species, which live free in artificial and natural bodies of water, in moist soil, etc.

All pathogenic and saprophytic Leptospira are virtually indistinguishable morphologically. At the same time, their morphological characteristics are typical only of Leptospira and are diagnostic for this genus.

Morphology of Leptospira

The basic structural characteristics of Leptospira can be seen in the dark-field microscopy of live Leptospira (in a compressed or hanging drop), in examination of smears stained by the Romanowsky-Giem-

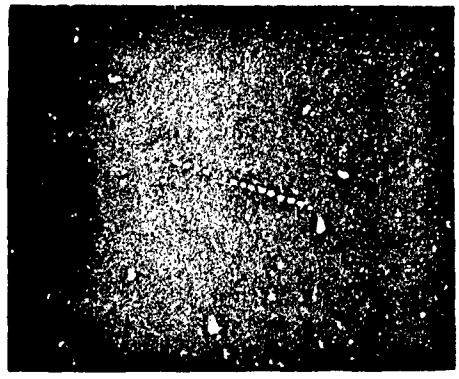


Fig. 34. Leptospira in dark field (photograph with brightness booster). 90 × objective, 5 × eyepiece (photograph by M.Ya. Korn).

Fig. 35. Structural diagram of Leptospira: axial thread and plasma spiral (after Alston and Broom).

sa or other methods, and particularly in electron microscopy.

Unstained live Leptospira are semitransparent, since they refeact light only slightly. Materials containing live Leptospira (urine, blood cerebrospinal fluid, tissue and organ suspensions, cultures, etc.) are consequently examined by dark-field microscopy; this technique shows Leptospira to be silvery white helical threads with hook-like bends at their ends. The fine turns of the helix are closely packed (18 turns in 9 μ), which gives it the appearance of a string of pearls or a "garland of roses" (Fig. 34).

Live Leptospira are very mobile: in liquid media they are charac-

terized by rapid linear movement, circular motions, or stationary rotation. In the latter case the terminal apparatae rotate actively, while the central portion (body) of the microorganism remains rigid. Leptospira in semiliquid media (e.g., semiliquid agar) are characterized by zigzag movement.

The dimensions and structural details of Leptospira are determined in stained preparations and by electron microscopy. They average from 7 to 14 μ in length, but both longer (up to 30 μ) and shorter (3-4 μ) specimens are encountered; their mean diameter is 0.07-0.14 μ and the average pitch of the primary helix is 0.25 μ. Leptospira have a slender rigid axial filament (200-300 A in diameter) and a cytoplasmic helix which surrounds the filament with uniform turns. The cytoplasm is enclosed in a thin unstructured membrane; it is thought that the axial filament is a skeletal structure and motor organ. No flagella, undulating membrane, granules, or other inclusions have been detected (Fig. 35).

The usual laboratory techniques are dark-field microscopic examination of preparations containing live Leptospira and, very rarely, examination of stained preparations.

Biology of Leptospira; cultural characteristics

Pathogenic Leptospira grow in liquid and semiliquid media, all of which must contain native rabbit serum (5-10% by volume). Sera from other animals are less suitable. Water-serum medium, S.I. Tarasov's modification of Fervort-Wolf's medium, Korthoff's medium, etc. (see page 491), are the most common media. Leptospira have also been successfully cultured in semiliquid agar media (see page 492).

Leptospira grow rather luxuriantly in semiliquid agar media, but such cultures are unsuitable for the agglutination reaction.

The optimum temperature for raising Leptospira is 28-30°, but

growth is possible between 22 and 37°. Pasteur pipettes are used to transfer cultures and primary growths. The volume of culture transplanted should amount to approximately 0.5 ml for each 5 ml of medium. Leptospira grow rather slowly; the time required for one generation to pass through the logarithmic phase is 58-68 hr. Maximum growth consequently cannot be obtained in less than 5-10 days. The more frequently reculturing is carried out, the more rapidly a luxuriant growth is obtained.

It is recommended that reculturing be carried out no less than once a month in order to maintain a collection of live Leptospira cultures. Cultures to be stored for extended periods are covered with a layer of sterile vaseline oil or paraffin or are sealed in ampules, which prevents evaporation of the medium and resultant alteration of its pH.

Any possibility of contamination with extraneous microorganisms should be excluded in culturing Leptospira. Cultures of these spirochetes in liquid media are colorless and odorless and, if luxuriant, exhibit an opalescence which can be seen in transmitted light when the test-tube is gently shaken; in semiliquid media a slight annular turbidity is observed 1.5-2 cm below the surface in the region of heaviest growth.

The growth of Leptospira in cultures is checked by dark-field microscopy of compressed drops.

Up to 100 or more actively mobile Leptospira can be detected in a single field of view (10 × eyepiece and 40 × objective) in normally developing cultures.

Antigenic structure; serotypes

Pathogenic and saprophytic Leptospira are differentiated into serological types, which are designated by binary specific names, e.g.,

Leptospira bataviae, L. pomona, etc. The division into species (types) is made with the aid of the agglutination and lysis cross-reaction (ALR). The specialized literature describes more than 50 serological types and subtypes of Leptospira. The antigenic structure of the serological types is very stable and can consequently be used as the basis for differential diagnosis of the individual leptospiroses.

Identification of Leptospira cultures. Newly isolated strains of Leptospira are identified with the aid of the agglutination reaction. For this purpose the laboratory must have available specific antisera for standard Leptospira strains and antiserum for the newly isolated strain.

In order to produce antisera mature rabbits (weighing 2-2.5 kg) are immunized by injecting a culture of the appropriate strain into the marginal vein of the ear in accordance with the following scheme: a 1st injection of 2 ml, a 2nd injection of 3 ml, and a 3rd injection of 5 ml, with intervals of 5-6 days between the injections. Blood is taken from the rabbits to determine the immune-serum titre no less than 10-15 days after the last injection; total exanguination is employed only if the serum titre with respect to the homologous strain reaches 1:3000-1:5000 or more.

An agglutination cross-reaction is conducted with all known serotypes of Leptospira, first with strains of those serotypes known to occur in the nation or region in question.

If the newly isolated strain is agglutinated to titre with serum from one of the standard strains and the latter strain is agglutinated to titre with serum from the new strain, the two strains belong to the same serotype (species). If the new strain does not exhibit any serological affinity with the standard strains in the agglutination reaction it is regarded as an original serotype.

Any difference in the titres of the new and standard strains in the cross-reaction forces us to assume that there are peculiarities in the antigenic structure of the new strain. A more accurate notion of the antigenic structure of the strain can be obtained by cross-absorption of antibodies from antisera, employing killed (Ruis, Shuffner, Bolander, et al.) for live (V.S. Kiktenko, V.V. Anan'in, et al.) Leptospira cultures. In the first case 100 ml of a fully mature 7-8 day culture is killed by adding 0.5 ml of formalin and centrifuged at 10,000 rpm for 30 min. The supernatant is carefully decanted and nine volumes of the residue are added to 1 volume of antiserum (0.9 ml and 0.1 ml). The homologous titre of the depleted serum should not exceed 1:3000; sera with higher titres are preliminarily diluted to the requisite titre with physiological solution.

The antiserum-residue mixture is incubated in a heater at 37° for 18 hr and then centrifuged for 30 min at 10,000 rpm. The serum is regarded as depleted if its titre with respect to the absorbed strain is less than 1% of its initial titre.

The following modification of this method can be employed: approximately 200 ml of a 7-8-day live culture (100 or more specimens per field of view) is centrifuged at 16,000 rpm for one hour (this compacts the culture by a factor of approximately 60-70).

The supernatant is decanted and the residue is collected in a separate test-tube. One volume of the serum under investigation (with a titre of up to 1:5000-1:10,000) is mixed with 49 volumes of the compacted culture (residue) in fractions of 15, 15, and 19 volumes, the additions being separated by intervals of 2 hr. After each portion of the culture is added the mixture is shaken and placed in a heater at 29°. After incubation for 18 hr in the heater it is centrifuged at 16,000 rpm for one hour. The supernatant, which is the depleted serum diluted to 1:50,

is carefully drawn off with a pipette and subjected to an agglutination reaction (together with the corresponding initial serum) to detect antibodies to the absorbed strain.

TABLE 34

First Possible Result

1 Сыворотка / 2 Штаммы	A	B в
а) абсорбированная штаммом В	—	—
б) абсорбированная штаммом А	—	—

1) Serum; 2) strain; a) Absorbed strain B; b) absorbed strain A.

TABLE 35

Second Possible Result

1 Сыворотка / 2 Штаммы	C в	D г
в) абсорбированная штаммом Г	+	—
г) абсорбированная штаммом В	—	+

) Serum; 2) strain; c) Absorbed strain D; d) absorbed strain C.

TABLE 36

Third Possible Result

1 Сыворотка / 2 Штаммы	E Д	F в
д) абсорбированная штаммом Е	+	—
е) абсорбированная штаммом Д	—	—

1) Serum; 2) strain; e) Absorbed strain F; f) absorbed strain D.

Antibody extraction is regarded as complete if the depleted serum does not agglutinate or lyze the strain in question at a dilution of 1:100 (the first possible dilution).

The cross-reaction with depleted sera is set up in the usual manner, employing a series of increasing dilutions (1:100, 1:500, etc., to titre). The following results are possible in antibody cross-absorption.

1. The standard strain (A) completely extracts the antibodies of the antiserum to the strain under study (b). The test strain (B) in turn completely extracts the antibodies from the antiserum to the standard strain (a), as illustrated by the scheme shown in Table 34.

These results indicate that the two strains are identical.

2. The standard strain (C) and the strain under study (D) do not extract the antibodies to heterologous strains from the sera (c and d; Table 35). In this case the strains belong to different serotypes.

3. One of the strains, e.g., the standard strain (E), completely extracts the antibodies from the antiserum to the test strain (f). The antibodies to the homologous strain are retained (to no less than 10% of titre) in the antiserum to the standard strain (e) absorbed by the test strain (F; Table 36).

In this case the strains are of the same serotype, the test strain belonging to the incomplete subtype and the standard strain to the complete subtype.*

Pathogenicity, virulence, and toxin formation

A number of laboratory and wild animals can be used as subjects for diagnosing and studying leptospirosis. Guinea pigs (weighing 100-200 g), suckling rabbits, young white mice (10-14 g), golden hamsters, and susliks are most commonly employed, but 10-30-day-old brook trout (Lagurus lagurus) can also be used (V.V. Anan'in).

TABLE 37

List of Serotypes of Pathogenic Leptospira Found in the USSR

№ п/п	a Серологический тип	b Наименование наиболее известных в СССР штаммов
1	L. icterohaemorrhagiae	c «Судьин», «Белоусов», «Крысиный № 41», «Мич», d «Крыса № 56», «Кондратьев», «Одесса» и др.
2	L. canicola	«Каширский», «Крыса», «Раменка», «Нимфа»
3	L. grippotyphosa	e «Дмитровский», «Стрелок», «Барботин», «РА», «Корнилов», «Витулина», «Тип I» и др.
4	L. tarassovi	f «ДВ-А», «Перепелицын», «Т-384», «Тип III» и др.
5	L. pomona	g «ДВ-В», «Монаков», «Поросенок-2», «Гиацинт», «Жуков», «Тип II» и др.
6	L. batavise	h «Мышь-малютка 167», «Мышь-малютка 867» и др.
7	L. hebdomadis	i «Терехов»
8	L. nero	j «Полевка-экономка 276», «Гамзулин», «Титова», «П-183» и др.
9	L. sejroe	k «БА-1, «Егошин», Mus musculus 529, 751, 753,
10	L. ussuri	l «ДВ-П»
11	L. javanica m	«Сорекс»
12	L. erinacei auriti n	«Еж № 670»
13	L. erinacei europaei o	«Еж № 1».

a) Serological type; b) designations of best-known Soviet strains; c) Sud'in, Belousov, Krysiny No. 41, Mich, Krysa No. 56, Kondrat'yev, Odessa, etc.; d) Kashirskiy, Krysa, Ramenka, Nimfa; e) Dmitrovskiy, Strelok, Barbotin, RA, Kornilov, Vitulina, Type I, etc.; f) DV-A, Perepelitsyn, T-384, Type III, etc.; g) DV-V, Monyakov, Porosenok-2, Giatsint, Zhukov, Type II, etc.; h) Mysh'-malyutka 167, Mysh'-malyutka 867, etc.; i) Terekhov; j) Polevka-ekonomka 276, Gamzulin, Titova, P-183, etc.; k) BA-1, Yegoshin, Mus musculus 529, 751, 753; l) DV-P; m) Soreks; n)Yezh No.

670; o) Yezh No. 1.

The pathogenicity of the individual species (serotypes) of Leptospira varies in different laboratory animals. Thus, for example, young guinea pigs and white mice are very sensitive to infection with virulent cultures of L. icterohaemorrhagiae and less sensitive to infection with L. pomona and L. nero; infection with L. grippotyphosa does not produce a clinically manifest disease.

On the other hand, young rabbits, guinea pigs, susliks, and trout are equally susceptible to the causative agents of both icteric and nonicteric leptospiroses.

Laboratory Diagnosis of Leptospirosis

Material for examination

Microbiological investigation is necessary to pinpoint diagnoses based on clinical and epidemiological data. The blood, cerebrospinal fluid, and urine can be examined. The kidneys, liver, and other organs are also investigated in fatal cases.

Microscopic and bacteriological examination

Investigation of blood and cerebrospinal fluid. Leptospira can be detected in the patient's blood only during the first week of illness. Positive results are most frequently obtained when the blood is examined during the first 1-3 days. It is rather rare that Leptospira can be detected in the patient's blood by direct microscopy, since they are present in small quantities even during the first few days of illness.

V.I. Terskikh recommends that 1 ml of blood taken from a vein or the lobe of the patient's ear be quickly mixed with 2 ml of 1.5% sodium citrate in a test-tube and left to stand for 1 hour. The clear upper layer is examined microscopically (no less than 10 compressed drops). Microscopic examination of the residue obtained by centrifuging this

clear layer for 1 hour at 10,000-12,000 rpm is more reliable. Leptospira are very rarely detected in the cerebrospinal fluid, even by microscopy.

Blood cultures yield the best result. In order to obtain hemocultures blood is taken from the ulnar vein under aseptic conditions and cultured at the patient's bedside in 8-10 test-tubes, each containing approximately 0.5 ml of one of the media described above. The cultures are incubated in a heater for 1 month and checked by dark-field microscopy every 5 days. When Leptospira are detected cultures must be made on a fresh medium to preserve the strain for identification. Cerebrospinal fluid is cultured on nutritive media in the same manner.

In parallel with the culturing, part of the blood or spinal fluid is administered subcutaneously or intraperitoneally to susceptible animals, no more than 1.5-2 ml for large animals (guinea pigs or suckling rabbits) and 0.2-0.3 ml for small animals (trout, etc.).

Investigation of urine. It is best to investigate urine between the 7th-8th day and the 3rd month after the onset of the illness Just-excreted urine or (with great success) the residue obtained by centrifuging 30-50 ml of urine at 10,000-12,000 rpm is examined by dark-field microscopy. A total of 10-20 compressed drops are studied. Portions of 0.5-0.6 ml of urine collected under aseptic conditions are cultured in 5-6 test-tubes containing a sterile medium. The cultures are observed in the usual manner (see above).

At the same time, the urine is administered subcutaneously to sensitive animals, in the same doses as for blood. The following method of inoculating guinea pigs yields the best results for detection of L. icterohaemorrhagiae and L. pomona: the animal is immobilized on an operating table, the hair on its abdomen is removed, and the skin on the shaved area is scarified with a scalpel; the scarified surface is rubbed

with urine and the procedure is repeated 3 or 4 times, permitting the urine to dry in each instance. The animals are observed in the manner described below (page 485).

Investigation of cadaver organs. Microscopy and culturing of tissue suspensions and subcutaneous administration of material to experimental animals are employed to detect Leptospira in cadavers. Positive results can be obtained only when fresh material is examined, since Leptospira rapidly die in corpses; these microorganisms are most frequently detected in the liver and kidneys.

Leptospira can be detected in organ sections by silver impregnation by Levaditi's method.

Serological investigation

As in other infectious diseases, serological methods do not permit an early diagnosis of leptospirosis, since antibodies (agglutinins and lysins) do not appear in the blood before the 7th-8th day of illness. Detection of antibodies at this time does not provide a reliable diagnosis, since they may also be found in the blood of patients who have recovered from leptospirosis at some time in the past. The patient's blood must consequently be reexamined after 3-5 days and at longer intervals: only when there is some certainty of an increase in titre can a diagnosis of leptospirosis be made. The diagnosis should be supported by clinical observations and anamnestic epidemiological data. Antibody formation is retarded in persons treated with antibiotics.

The agglutination and lysis reactions are used almost exclusively for serological diagnosis. Other methods (the complement-fixation reaction, administration of thrombocyte, etc.) have not come into wide use, being insufficiently specific or technically complicated.

For serological diagnosis with the agglutination and lysis reactions the laboratory should have available a collection of Leptospira

cultures of all known serotypes or at least of those which cause leptospirosis in the nation and region where the diagnosis is to be made. Table 37 is a list of the serotypes found in the USSR which must be at hand for serological diagnosis.

The strains constituting the culture collection are stored in special containers or boxes in a dark place at room temperature and are transplanted monthly to a nutritive medium (Fervort-Wolf's medium, etc.). Each strain is simultaneously cultured in 4-6 test-tubes. The agglutination reaction utilizes 7-10-day live cultures raised on a liquid nutritive medium (cultures in semiliquid media are unsuitable for this purpose!) and having a sufficiently luxuriant growth (50-100 or more Leptospira per field of view) without spontaneous agglutination (clumps of Leptospira) or impurities (phosphate residues, etc.). Blood is taken from the patient or convalescent's ulnar vein, finger, or ear lobe.

Technique of the agglutination reaction. The serum to be studied is diluted with physiological solution to 1:50, 1:250, 1:500, 1:2500, 1:5000, 1:12,500, 1:25,000, and 1:50,000 in a series of test-tubes.

Portions of 0.1 ml (or 2 drops) of each of these basic dilutions are transferred to a series of agglutination test-tubes and mixed with 0.1 ml (or 2 drops) of the live Leptospira culture. The final serum dilutions are thus 1:100, 1:500, 1:1000, etc.

The number of agglutination series corresponds to the number of strains (serotypes) included in the reaction. Agglutination test-tubes containing 0.1 ml (or 2 drops) of the corresponding culture and 0.1 ml (or 2 drops) of physiological solution serve as the control.

The tubes are shaken and incubated in a heater at 37° for 2 hr and the results are then evaluated. For this purpose one drop of the mixture is taken from each test-tube with a Pasteur pipette, compressed drops are prepared on slides, and the resultant specimens are subjected

to dark-field microscopy. A separate pipette is used for each series of dilutions, i.e., each strain. Preparation of the specimens begins with the control and runs from the highest serum dilutions to the lowest.

Agglutination takes the form of adhesion of larger or smaller numbers of Leptospira and is manifested in formation of "packets," "bars," or large spherical agglomerates, the latter often covering several fields of view. The microorganisms may retain their mobility (Fig. 36).

Lysis is initially manifested in formation of granularity in both isolated and agglutinated Leptospira. The microorganism then gradually break up into fragments, losing their mobility and becoming amorphous granular structures.

Agglutination and lysis occur simultaneously in the first serum dilutions, but the higher the serum dilution, the longer agglutination predominates. Lysis does not occur at all in the highest dilutions.

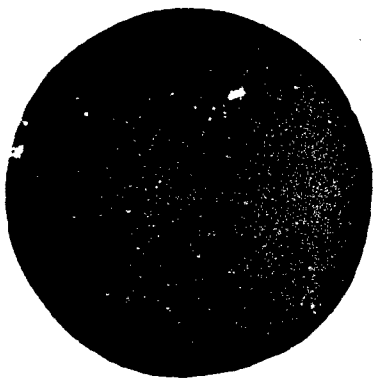


Fig. 36. Agglutination of Leptospira; "packets" of adhering Leptospira.

It is recommended that positive results be indicated by plusses in evaluating the agglutination reaction.

1. The majority of the Leptospira are unlyzed; there are isolated clumps of agglutinated Leptospira (+).

2. From 1/3 to 2/3 of the Leptospira are agglutinated or lyzed (++).

3. The majority of the Leptospira are agglutinated or lyzed (+++).

The serum titre is determined from the highest serum dilution at which the Leptospira undergo single-plus agglutination.

When it is necessary to investigate large quantities of serum at the same time a rough agglutination reaction is set up in dilutions of

1:100 and 1:500. When a positive result is obtained the reaction is conducted with successive dilutions to the serum titre.

Antibodies can be detected both in the patient's serum and in his urine, although the titre in the agglutination reaction is substantially lower in the latter case, amounting to no more than 10% of the corresponding serum titre.

Positive agglutination in a dilution of 1:500 is assumed to be the diagnostic titre. The serum titre usually reaches 1:10,000-1:20,000 or more on the 15-30th day after the onset of the illness, subsequently decreasing. In some cases the agglutination reaction will reveal antibodies in the blood of recovered patients for many months and years, which makes it possible to establish a retrospective diagnosis.

Biological testing

Blood, urine, cerebrospinal fluid, human and animal tissues, and water, foodstuffs, and other environmental objects suspected of being contaminated with Leptospira can be investigated in biological experiments.

Animals can be inoculated intraperitoneally, subcutaneously, intravenously, intracardially, or by scarification of the skin or mucosa (conjunctiva, digestive tract, etc.).

The symptomatology and pathogenesis of leptospirosis and the pathologoanatomic changes which it produces are very similar in different animals, particularly in severe cases.

L. icterohaemorrhagiae and other Leptospira of the icteric group produce leptospirosis with a very typical clinical pattern in guinea pigs.

After an incubation period lasting 2-7 days or sometimes more, depending on the virulence of the strain, the animal's temperature rises to 39-41° and it rapidly loses weight and refuses to eat; the blood ves-

sels of the sclera develop a characteristic congestion. After 2-5 days the animal's temperature drops to a subnormal level against a background of severe fatigue, the sclera and visible mucosa and then the skin become icteric, and shedding of the coat is noted; the animal dies 4-12 days after infection. An autopsy reveals icterus, which is particularly pronounced in the subcutaneous cellular tissue, and hemorrhages in all the internal organs, the skin, and the subcutaneous cellular tissue; the latter are most profuse in the inguinal and axillary regions. Typical petechia are observed in the pulmonary tissue: when viewed against the general ischemic background they resemble the wings of a butterfly.

The liver exhibits necrotic foci, which take the form of reddish-yellow spots. The kidneys are abnormally enlarged, acute nephritis causes stresses in the capsule, and the adrenals are hyperemic.

Leptospira circulate in the blood during the incubation period and throughout the course of the disease, until the animal dies; once icterus has appeared Leptospira can be detected (microscopically) in the internal organs, being present in especially large numbers in the liver.

A clinical pattern similar to that of icterohemorrhagic leptospirosis in guinea pigs is observed in suckling rabbits, young trout, golden hamsters, and susliks after infection with virulent Leptospira which cause nonicteric leptospirosis (L. grippotyphosa, L. pomona, L. nero, etc.). It is consequently preferable that these animals be used in studying and diagnosing leptospiroses of the nonicteric group.

A definite percentage of experimental animals recover after infection, a phenomenon due to a number of factors: an insufficient inoculation dose, low strain virulence, individual resistance, etc.

When the Leptospira are not sufficiently virulent it is necessary to pass them from animal to animal. Blood, peritoneal exudate, tissue

suspensions, etc., can be used for this purpose.

Recovered animals often become carriers of the infection, beginning with the 2nd week of illness: Leptospira persist in the renal tubules and are excreted with the urine. This ability to carry Leptospira may last as long as several months and is accompanied by development of interstitial nephritis.

Observation of the experimental animals is carried out in the following manner: 1) the animals are weighed before the experiment and then daily throughout the entire postinoculation observation period; 2) the temperature of large animals (guinea pigs and rabbits) is taken twice daily, morning and evening; 3) when there is a rise in temperature the peritoneal exudate is examined microscopically for Leptospira. For this purpose an incision is made in the skin of the abdomen (just above the navel, in order to avoid damaging the urinary bladder). The abdominal wall is punctured with a fine capillary (formed by drawing out a Pasteur pipette in a Bunsen-burner flame) through the incision and a drop of peritoneal exudate is collected; 4) at the same time cardiopuncture is carried out with a 1-2-millimeter syringe and portions of 0.2-0.3 ml of blood are cultured in test-tubes containing a nutritive medium. The exudate and blood cultures must be examined repeatedly.

Animals which do not die of the infection and are not killed during the experiment are observed over a one-month period and then sacrificed. Tissue from the renal cortex is cultured and examined microscopically for Leptospira. Blood is taken from the heart for determination of specific antibodies to Leptospira by the agglutination and lysis reactions.

Detection of Leptospira in the external environment

Investigation of animals for leptospiral infection. The microbiological diagnostic methods which have been described are essentially

also suitable for diagnosing leptospirosis in agricultural and commercial animals, as well as in dogs and cats.

The following techniques are employed: 1) microscopic examination of tissue suspensions (kidneys, liver) in physiological solution and of urine and blood in compressed-drop preparations, using a dark-field condenser; 2) microscopic examination of histological sections of the kidneys and liver (silver impregnation); 3) inoculation of laboratory animals with tissue suspensions prepared from the organs of wild animals; 4) culturing of the material to be investigated on special nutritive media and subsequent observation; 5) investigation of the blood (serum) for antibodies to pathogenic Leptospira by the agglutination and lysis reactions.

The corpses of animals which have died or been killed are immobilized on a table for dissection. The pelt is wetted with alcohol and seared.

The instruments used in the autopsy (scissors, forceps, etc.) are kept in alcohol until needed and are flamed before use with an alcohol burner. The pelt is removed from the thorax and abdomen. The abdomen and then the thoracic cavity are opened, and the kidneys and liver are removed and placed in a sterile Petri dish. Several drops of blood are taken from the heart with a Pasteur pipette; 2-4 drops are applied (individually) to a piece of filter paper and preserved for subsequent serological investigation by drying at room temperature.

Tissue fragments cut away with scissors under sterile conditions are then cultured; pieces are cut from the surface of the kidneys (the cortical layer!).

Pasteur pipettes can be used for removing and culturing tissues. The amount of tissue cultured in a single test-tube should be no larger than a grain of millet, since any greater quantity not only fails to

promote the growth of Leptospira but actually prevents it, as a esult of maceration of the tissue and changes in the physicochemical properties of the medium.

For dark-field microscopic examination compressed drops are prepared on slides from a suspension of the tissue in physiological solution. The material to be suspended is removed from the organ in question (the surface layer of the kidneys) with a Pasteur pipette. When the urinary bladder is filled with urine the latter is cultured and examined microscopically.

At the same time that the cultures are made it is recommended that the material (suspensions of renal and hepatic tissue in physiological solution) be administered to guinea pigs, golden hamsters, trout, and other susceptible animals. These are then observed in the manner described above to obtain a pure strain and determine its pathogenicity and virulence.

The biological method is very helpful for obtaining pure cultures from carrier animals which reach the laboratory dead and contaminated with putrefactive bacteria. Laboratory animals are inoculated subcutaneously with the material to be studied (renal tissue, urine, etc.). Pure Leptospira cultures can be obtained from cultures of material (renal and hepatic tissue, blood) from those laboratory animals which die or are killed 10-15 days after inoculation.

The cultures are incubated in a heater for 30 days and checked for Leptospira every 5 days. The strains obtained are reinforced by reculturing and identified in the manner described above. An agglutination reaction is carried out with dried drops of blood no more than one month after the samples are prepared. The initial blood dilution is 1:10 (2 drops of blood and 18 drops of physiological solution), i.e., a serum dilution of 1:20, and the subsequent dilutions are 1:50, 1:100,

1:500, 1:1000, etc. A rough reaction is conducted with the first two dilutions and an expanded reaction (to titre) is carried out when the results are positive. The procedure for setting up the agglutination reaction is the same as if the serological diagnosis of human leptospirosis.

For prompt examination of wild animals it is recommended that a laboratory be set up in the immediate vicinity of the area where they are to be caught. The cadavers must be examined during the first few hours (no more than 12 hr) after death, since contamination with putrefactive bacteria makes it difficult to detect and isolate Leptospira.

Detection of pathogenic Leptospira in water. Leptospira can be detected in water and silt (from reservoirs, rivers, marshes, etc.) by Appelman and Van Tyl's method. This technique was proposed for isolating L. icterohaemorrhagiae, but has also proved suitable for isolating L. pomona (V.V. Anan'in and A.A. Varfolomeyeva).

Sterile glass vessels are used to collect portions of 2-3 liters of water and mud for examination from areas where humans and animals are suspected of being infected. The guinea pigs to be used in the experiment, which should weigh 150-250 g, are then treated in the following manner: the coat is shaved from the abdomen and 10-20 cm^2 of skin in the shaved area is scarified with a scalpel, making closely-spaced longitudinal and transverse incisions. The water is heated to 30° and poured into an enameled vessel (a basin or tray) and the guinea pigs are immersed in it. The scarified skin should be wetted. The animal is immersed for 1 hr, transferred to a warm place, and taken to the vivarium when it has dried off.

Observation of the animals is conducted in accordance with the usual rules (see above); it must be kept in mind that the illness produced in guinea pigs by L. pomona and other nonicteric Leptospira is

manifested only in a brief rise in temperature or a loss of weight or is entirely symptomless. Regardless of the animal's condition, it is consequently necessary to culture blood from the heart to produce hemocultures and to conduct microscopic examinations of the peritoneal exudate for Leptospira from the 4th-5th day to the 20th day after immersion.

This method makes it possible to detect L. icterohaemorrhagiae in water containing 1 cell in 2 ml or L. pomona in water containing 10 cells per ml.

The concentration media proposed by Sultzer, Walker, Heindle, et al., are suitable only for direct culturing of saprophytic Leptospira from water and are totally unsuitable for isolating pathogenic Leptospira.

Nutritive Media

1. The water-serum medium is the simplest to prepare. Distilled tap, well, or river water is poured into test-tubes (in 5 ml portions) and sterilized. When it has cooled 0.5 ml of fresh rabbit serum inactivated in a water bath at 56-58° for 30 min is added to each tube.

The water used to prepare the medium should have a pH of 7.0-7.3.

2. Fervort-Wolf's medium, as modified by S.I. Tarasov.

Distilled water - 900 ml

Sodium chloride - 0.5 g

Peptone - 1 g

Sorensen's phosphate buffer mixture at pH 7.2 - 100 ml

The medium is autoclaved in a flask at 120° for 30 min. A few days after autoclaving it is twice filtered through a two-layer paper filter and 5 ml portions are poured into test-tubes. These are then autoclaved for 30 min at 120°. After they have cooled all the tubes which appear cloudy or contain a residue are discarded. Approximately 0.5 ml of rab-

bit serum is added to each tube containing clear medium and the mixture is heated in a water bath at 56-58° for 30 min.

Sorensen's buffer mixture at pH = 7.2 is prepared from 1/15 M $Na_2HPO_4 \cdot 2H_2O$ (bimetallic sodium phosphate) and 1/15 M KH_2PO_4 (monometallic potassium phosphate). In order to obtain 1 liter of buffer solution at pH = 7.2 720 ml of the first solution is mixed with 280 ml of the second solution. The mixture must be stored in a refrigerator to avoid bacterial contamination.

Korthoff's medium:

Redistilled water – 500 ml

Witte's peptone – 400 mg

Sodium chloride – 700 mg

Sodium bicarbonate – 10 mg

Potassium chloride – 20 mg

Calcium chloride – 20 mg

Monobasic potassium phosphate (KH_2PO_4) – 90 mg

Dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) – 480 mg

The solution is heated at 100° for 20 min. When it has cooled it is filtered and then reheated for 30 min at 100°. After it cools 8% of fresh rabbit serum is added and the mixture is poured into test-tubes in 8 ml portions. The tubes are heated in a water bath at 56° for 1 hr.

Semiliquid agar medium: 1 g of agar-agar thoroughly washed in distilled water (left to stand for 24 hr) is added to 500 ml of distilled (or tap) water and boiled for 30 min. Portions of 5 ml are poured into test-tubes and sterilized in an autoclave; when they have cooled 0.5 ml of sterile rabbit serum is added to each tube and heated in a water bath at 56° for 30 min.

In adding the serum to any of the media described above it must be carefully mixed with the water or water-salt base by thoroughly shaking

the test-tube.

The optimum medium pH is 7.2-7.6. It is best to use test-tubes and other vessels of neutral glass for preparing nutritive media. Before using ordinary glass test-tubes they are filled with 1-2% hydrochloric acid, left to stand for 24 hr, rinsed with 3-5% sodium bicarbonate, carefully washed with tap water, filled with distilled water, and left to stand for 2-3 days.

Tubes in which no leptospiral growth is detected are discarded.

Test-tubes and other vessels containing Leptospira cultures, as well as used slides and cover slips, are disinfected for 24 hr in 1% hydrochloric acid. After disinfection the vessels are thoroughly scrubbed with brushes and soap, repeatedly rinsed with tap water, filled with water, left to stand for 24 hr, dried, stoppered with cotton plugs, and sterilized.

LISTERIOSIS

Prof. N.G. Olsuf'yev, Corresponding Member of the Academy of Medical Sciences USSR

The causative agent of Listeriosis* is the bacterium Listeria monocytogenes Murray, Webb, et Swann. Modern taxonomy assigns this microorganism to the family Corynebacteriaceae of the order Eubacteriales. The Listeria are easily distinguishable from other pathogenic bacteria, but are very similar to the causative agent of erysipeloid, Erysipelothrix rhusiopathiae.

Morphology of Listeria

The Listeria are straight, short (0.3-0.5 × 1-2 μ), ovoid rods and are sometimes almost coccoid in shape (Fig. 37); they are found singly or in clumps in smears prepared from agar cultures. Longer rods and filamentous forms may be encountered in older cultures, especially of dissociated strains. These bacteria are gram-positive, form neither

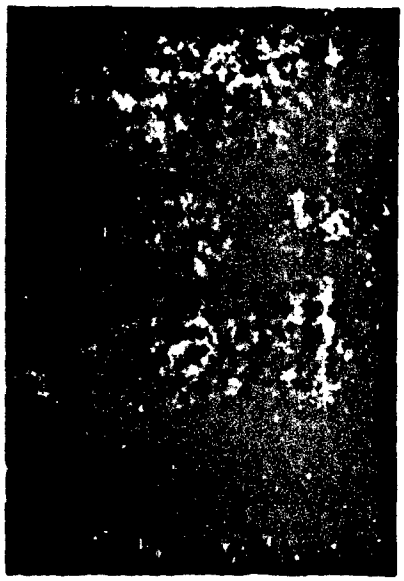


Fig. 37. Listeria in a smear prepared from an agar culture. x1000.

spores nor capcules, and are slightly mobile. They have 1-4 flagella attached to the lateral surface of the cell or, more rarely, to its terminal region. The mobility of Listeria is most manifest when they are cultured at room temperature.

Biology of Listeria; cultural characteristics

Listeria are easily cultured on ordinary slightly alkaline nutritive media, but do not grow profusely. Day-old colonies on nutritive agar are small, round, slightly convex, smooth in outline, semitransparent, and slightly chromogenic in transmitted light (Fig. 38). Listeria grown in broth make the medium slightly cloudy; they do not form a film or annular ring. A narrow zone of beta-hemolysis is found around colonies on blood agar. Listeria ferment glucose, maltose, rhamnose, levulose, esculin, and salicin to acids (without gas formation), ferment sucrose, glycerine, and lactose slowly (this is not true of all strains), and have no effect on mannitol, dulcitol, arabinose, inulin, raffinose, and sorbitol. They do not form indol or hydrogen sulfide, clabber milk, or liquefy gelatin. They are attenuated when cultured on artificial nutritive media, being converted from the virulent S form to the low-virulence R form. The rods become somewhat longer and rougher under these conditions and filamentous forms are often found among them; the colonies become larger and less transparent, with a striated or nodular surface and a nonuniform serrate or ramified margin. Some strains may lose their mobility.

Antigenic structure; serotypes

A total of 5 serotypes (1, 2, 3, 4a, 4b) of Listeria, differing in their complement of flageller and somatic antigens, are known. Serotypes 1 and 4b are most commonly encountered. The same serotype may be detected in the most diverse animals and in man, exhibiting no special preference with respect to host species.

Fig. 38. S colonies of Listeria on meat-infusion agar; day-old culture. ×35.

Bacteriophagia is known in Listeria. Bacteriophages lyze strains belonging to the different serotypes in different fashions.

Resistance to physical and chemical agents

Listeria are rather stable in the external environment. They are capable of surviving for 3-6 months in soil and for a year or more in water (at low temperatures). They not only survive but reproduce in meat-milk and are able to reproduce even when such products are stored at low temperatures (4-6°). An outstanding biological characteristic of Listeria is their great epidemiological significance. They can withstand heating at 58°, but die within 20-30 min at 70° and 3-5 min at 100°. During the winter they are capable of surviving for protracted

periods in the frozen corpses of rodents, in straw, and in other materials. Ordinary concentrations of disinfectants have a lethal action on Listeria.

Pathogenicity

Listeria are pathogenic for many species of mammals, including rodents, for insects, for carnivores and ungulates, and for birds. The majority of species exhibit a moderate sensitivity to listeriosis, although the disease may be fatal for individuals. In nature listeriosis has been detected in voles, mice, water rats, gray rat, hares, gerbils, shrews, wild boar, dear, foxes, raccoons, capercaillie, partridge, and other animals. Among domestic animals it has been recorded in sheep, goats, pigs, cattle, horses, dogs, cats, chickens, geese, ducks, turkeys, pigeons, canaries, and parrots and, on breeding farms, among silver-gray foxes, guinea pigs, rabbits, and white mice.

Laboratory Diagnosis of Listeriosis

Bacteriological investigative techniques form the basis for the laboratory diagnosis of human listeriosis, serological methods being used only in an auxiliary capacity. Listeria can be isolated and identified in ordinary bacteriological laboratories. The danger of infection, however, makes it necessary to exercise care in shipping pathological material to the laboratory and in handling cultures and, especially, listeriosis-infected experimental animals.

Material for examination

In examining patients cultures are made from the pathologically altered areas of the mouth (when angina is present), the blood, puncture specimens from enlarged lymph nodes, the suppurative discharge from the eyes, and the cerebrospinal fluid (when meningoencephalitis is present). In performing autopsies cultures are made from the brain, liver, and spleen.

The most reliable period for isolating Listeria from listeriosis patients is the first 7-10 days of illness. In listeriosis of newborns the fetus, the umbilical blood, the amniotic fluid, and, especially, the meconium are investigated, as is the placenta.

Microscopic examination

This method can be used for investigating the organs of newborns which have died of listerial sepsis. Bacterioscopic examination of the cerebrospinal fluid is possible in the meningoencephalitic form of listeriosis. This technique involves smears stained by the Gram method. Since Listeria have no characteristic morphological peculiarities by which they can be reliably identified in tissue smears, bacterioscopy can be employed as a diagnostic method only in conjunction with culturing and biological testing.

Bacteriological investigation

Ordinary nutritive media, such as meat-infusion agar or broth at pH = 7.0-7.2, are completely suitable for culturing; several bacteria are the minimum culture dose for such media. When a large quantity of material is cultured on agar the listerial growth is continuous and easily detectable with the unaided eye after 18 hr of incubation in a heater at 37°; when only a small amount of material is cultured isolated colonies sometimes become visible after 2-3 or more days. Growth in meat-infusion broth, which makes the medium moderately cloudy, is usually noted after 18-24 hr of incubation, rarely requiring longer to become visible. Material in which Listeria may be mixed with extraneous bacteria (e.g., oral mucous) must be cultured on a nutritive medium containing 0.05% potassium tellurite, which retards the growth of gram-negative microorganisms and for the most part has no effect on the growth of Listeria (or of streptococci and certain other gram-positive bacteria).

Since Listeria are capable of reproducing in dead tissue, small pieces of the latter may be placed in sterile test-tubes and kept in a refrigerator (at 4-6°) for 5-10 days or more, which increases the culturability of the Listeria (Gray's method).

Serological investigation

Agglutination reaction. This reaction has repeatedly been used by various authors for serological diagnosis of listeriosis in man (and domestic animals), but it is of little value in view of its low specificity. In completely healthy persons (or animals) the reaction with Listeria antigen may be positive at serum dilutions of 1:50-1:200 and, in some cases, at a dilution of 1:1000. This nonspecific agglutination is attributable to heteroantibodies, which are formed in the body under the influence of bacteria with an antigenic similarity to Listeria, such as certain forms of Enterococcus, Staphylococcus, etc.

Diagnostic serum, which is a suspension of formalin-killed Listeria, is used for the agglutination reaction.

Agglutinins to Listeria can be detected in human listeriosis patients from the second week of illness onward, their titre reaching 1:500-1:1000-1:5000 at the height of antibody production; however, in certain cases they may be low in titre or completely lacking throughout the entire course of the illness. Only an agglutination titre of 1:320 or more or an increase in titre by at least 2 dilutions on repeated examination should be regarded as positive in diagnosing human listeriosis.

Indirect hemagglutination reaction. This reaction is more specific for listeriosis than the agglutination reaction. The antigen is extracted from the Listeria culture by heating it for 1 hr in a boiling-water bath and sensitizing sheep erythrocytes with the extract obtained (whole or diluted to 1:5); 2.5% of the erythrocytes is added to the ex-

tract. The treated-erythrocyte suspension is then subjected to hemagglutination with the serum under investigation, employing the usual technique.

Paul-Bunnel's reaction. This reaction consists in agglutinating the serum under investigation with washed sheep erythrocytes. It may be positive at serum dilutions of 1:10-1:20 or more, as high as 1:160 in certain cases. According to indications in the literature, this reaction is positive in approximately 1/3 of all cases of listeriosis, but, being nonspecific, may be positive in diseases such as infectious mononucleosis of viral etiology.

Complement-fixation reaction. This reaction is more specific for listeriosis than the agglutination reaction. The diagnostic serum titre for humans is 1:10 or more; this titre may also be of anamnestic significance. On recovery the complement-fixation reaction often quickly becomes negative or drops to a low positive level. The success of the reaction is affected by the quality of the antigen, which is prepared by treating a suspended Listeria culture with ultrasound, followed by preservation with phenol to a final concentration of 0.5%. Alcoholic antigen can also be used, but it reacts with the antibodies in the complement-fixation reaction only at low immune-serum dilutions; heated and, particularly, formalinized antigen has poor fixation properties and a pronounced anticomplimentary action.

Biological testing

Pathological material from patients or cadavers can be investigated biologically. White mice, which are given the material (preliminarily ground and mixed with physiological solution) subcutaneously, are usually used for such tests. Biological investigations are carried out in parallel with culturing. It must be kept in mind that the sensitivity of white mice to listeriosis is limited (the DCLM for virulent strains

injected subcutaneously is usually no less than 100,000-1 million cells), so that it is not always possible to detect Listeria in the material being studied. Their susceptibility to this disease can be sharply increased by a single intramuscular injection of cortisone (4-5 mg 4 hr before the test is conducted). The trout Lagurus lagurus is highly susceptible to listeriosis (with a DCLM of 10-100 cells) and is consequently to be recommended for biological tests for Listeria; these trout reproduce well in captivity.

The time required for the animals to die after subcutaneous injection of the material under investigation depends on its infectivity and varies from 3 to 51 days in white mice (not treated with cortisone) and from 3 to 8 days in trout. Listeria can be detected bacterioscopically in the liver, spleen, etc., of animals which die of listeriosis within a short time (10-20 days) after infection and cultures are easily made from these organs. Bacterioscopy is usually negative in protracted cases, but cultures, especially those from the brain, may be positive.

Considering the ability of Listeria to reproduce in dead tissue, part of the pathological material under investigation (pieces of parenchymatous organs, the brain, etc.) should be placed in sterile test-tubes, kept in a refrigerator (at 4-6°) for 5-10 days, and then subjected to biological investigation once more.

The experimental animals which survive must be killed (white mice no earlier than the 20th day and trout no earlier than the 10th day after inoculation). Pieces of the liver, spleen, and brain are cultured on meat-infusion agar and broth. If the organs of the killed animals exhibit no marked pathologoanatomic changes (necrotic foci, etc.) no further cultures are made and the investigation is regarded as finished.

Conjunctival test in guinea pigs. A drop of a day-old broth cul-

ture is applied to the extended lower lid of one eye; the reaction is regarded as positive when suppurative conjunctivitis develops after 2-3 days.

Detection of Listeria in the external environment

Methods for detecting Listeria in the external environment have not been sufficiently well studied and few data are available. Biological tests with white mice form the basis for such techniques and can be used to investigate water, material washed from straw and grain, etc.

Laboratory diagnosis of listeriosis in animals

The same investigative techniques are used as in diagnosing this disease in man. The organs of dead domestic or wild animals (brain, liver, spleen, and enlarged lymph nodes) are investigated by bacterioscopy, culturing on nutritive media, and biological testing. When the brains and parenchymatous organs of domestic animals are to be examined separately it is recommended that the altered portions of the tissue (necrotic foci, inflamed, hyperemic, or edemic areas, etc.) be cut away and carefully ground in a mortar with a small quantity of sterile sand and meat-infusion broth or physiological solution. Successful diagnosis is ensured by culturing large quantities of material (e.g., 2-5 ml of carefully ground suspension) in flasks containing 15-50 ml of meat-infusion, liver, or serum broth. Sick domestic animals can be investigated by serological methods. There are indications that it is possible to utilize an allergic reaction for diagnosing listeriosis in domestic animals (e.g., pigs). The antigen is injected intracutaneously in a dose of 0.2 ml and an infiltration 2 x 3 cm in size forms within 72 hr in animals suffering from listeriosis and in carriers.

Biological testing in white mice is employed to detect Listeria in the organs of live rodents and other small mammals.

TOXOPLASMOSIS

D.N. Zasukhin, Doctor of Biological Sciences, and A.K. Yygiste, Candidate of Medical Sciences

Toxoplasmosis is a parasitic disease of man and is also widespread among mammals and birds.

The causative agent of toxoplasmosis is the parasitic Protozoa Toxoplasma gondii Nicolle et Manceaux, 1909.

We distinguish two forms of this disease in man, congenital and acquired. In congenital toxoplasmosis the causative agent enters the developing fetus through the placenta and multiplies, damaging various organs and tissues. Intrauterine death of the fetus, developmental anomalies and damage to the central nervous system (hydrocephalia, microcephalia, foci of calcification in the brain, etc.), damage to the eyes (corioretinitis, etc.), abortion, and improper development of individual organs are characteristic of congenital toxoplasmosis. In some cases the infant exhibits a pattern of encephalitis.

Mothers who bear such infants may be clinically healthy carriers or have toxoplasmosis in its acute or chronic form.

Acquired toxoplasmosis also has diverse clinical manifestations. We can distinguish lymphadenopathic, exanthemic, cerebrospinal, and ocular forms. In addition to acute, clearly manifest toxoplasmosis, there are mild forms and the symptomless form exhibited by carriers, the latter occurring rather widely, especially among the various groups of the populace which come into contact with infected material (veterinary workers, hunters, rabbit breeders, slaughterhouse workers, and certain others).

Morphology of toxoplasma

Toxoplasma is crescentic or oval in shape, with one pole of the body pointed and the other rounded. In dry smears it is 4-7 μ long and

2-4 μ wide. In preparations stained by Romanowskiy-Giemsa's method the protoplasm takes on a blue color, while the centrally located nucleus is stained red. The latter (which is 1-2 μ in diameter) gives a positive Feulgen's reaction. No flagella or other motor organelles have been detected. Toxoplasma is an obligatory intracellular parasite and is found in the liver, spleen, lungs, brain, spinal cord, muscles, intestinal wall, and other organs and tissues. It cannot always be detected in the blood, even in acute cases. Toxoplasma is capable of active movement and active penetration of various cells of the host.

This microorganism reproduces by longitudinal binary fission. It multiplies only in the protoplasm of the host's cells.

In addition to free intracellular forms, so-called pseudocysts and cysts may be found in man and animals. These usually occur in the brain (near the ventricles), eyes, and myocardium. Pseudocysts and cysts are aggregations of parasites, usually within a single cell. Cysts are enclosed by a clearly visible membrane.

Cysts and pseudocysts may survive for prolonged periods (years) in man and animals; they are oval or round in shape and there is not always a clearly distinguishable boundary between the individual parasites. A single cyst, 30-150 μ in diameter, may contain several hundred Toxoplasma. Deposits of calcium salts surround the cyst, such foci being called calcifications; they range from 1 mm to 1 cm in diameter and can occasionally be detected during x-ray examination.

Laboratory Diagnosis of Toxoplasmosis

The laboratory diagnosis of toxoplasmosis involves a set of parasitological investigations involving attempts to detect the causative agent in smears, organ prints, and histological sections, biological tests in laboratory animals (white mice, hamsters, susliks, etc.), immunological reactions (with Sabin-Feldman's stain), the complement-fix-

ation reaction, the hemagglutination reaction, the diffusion reaction in a gel, cutaneous tests, the fluorescent-antibody reaction, etc.

Parasitological (microscopic) investigation

Biopsy specimens, puncture specimens, the cerebrospinal fluid, and various exudates can be examined. In performing autopsies it is best to take material from the brain, liver, spleen, lungs and lymph nodes.

In microscopic examination of organ fragments smears or prints are prepared on slides. Fluids are preliminarily centrifuged (at 2000-3000 rpm for 10-20 min). The smears and prints are fixed in methyl alcohol or Nikiforov's solution (alcohol and ether) and stained by Romanowsky-Giemsa's method. The preparations are examined with an oil-immersion objective. In studying smears and prints it must be kept in mind that in the majority of cases there will be few parasites in the preparation, even when the process is acute.

Parasitological differential diagnosis. Toxoplasma are structurally (morphologically) similar to certain other Protozoa, from which they must be distinguished. In man these include Leischmania, Trypanosoma cruzi, Sacrosporidia, yeasts, and similar organisms. In addition to the aforementioned, in animals Toxoplasma must be distinguished by Encephalitozoon, Coccidia, Hepatozoon, and M organisms.

There are usually few parasites in the smear and careful examination of many fields of view is consequently necessary. It must be noted that Toxoplasma is not always found in the blood and that it occurs in small quantities when it is present. Detection of this microorganism when examining blood preparations is consequently purely accidental.

Histological investigation. Fragments of organs and tissues (the brain, liver, spleen, lymph nodes, eyes, etc.) are fixed in the usual manner (with formalin, Carnot's solution, etc.). Subsequent processing is carried out by histological methods. Sections are stained with hema-

toxylin-eosin and by Romanowsky-Giemsa's and other methods. The resultant preparations are examined with an oil-immersion system. This processing causes Toxoplasma in such preparations to be substantially smaller than in smears and prints, as well as more circular in shape. The nuclei of Toxoplasma in specimens stained with hematoxylin-eosin are dark violet, while their cytoplasm is red.

Toxoplasma cysts in histological sections are stained with azocarmine by Heidenhain's method (S.G. Vasina). The cysts can be easily distinguished from the surrounding tissue in such stained sections. The cyst membranes are stained dark blue, the nuclei of the Toxoplasma red, and their cytoplasm a blue of varying intensity.

Culturing of Toxoplasma

Toxoplasma are cultured on 8-12-day-old chick embryos.

The chorioallantois is used for inoculation. Smears are prepared for examination from this membrane and from the liver and spleen of the embryo.

Toxoplasma can also be raised in tissue cultures, but this method has not yet come into wide use for diagnosing toxoplasmosis.

Serological investigation

Reaction with Sabin-Feldman's stain. This reaction is based on the fact that the cytoplasm of live extracellular Toxoplasma suspended in serum which does not contain specific antibodies is stained blue by an alkaline solution of methylene blue. The toxoplasma do not pick up the dye when specific antibodies are present in the serum. The following ingredients are necessary for the reaction.

1. Live Toxoplasma. Peritoneal exudate from white mice infected 3-4 days previously is used as the source of live Toxoplasma. The exudate is passed through a syringe with a fine needle several times to free the microorganisms from the host cells.

2. Test serum (which need not be inactivated).

TABLE 38

Scheme of Reaction with Sabin-Feldman's Stain

1 Компоненты / № пробирки 2	1	2	3	4	5	3 Контроль
Исследуемая сыворотка 4	2	$2_{1:2}$	$2_{1:4}$	$2_{1:8}$	$2_{1:16}$	
Физиологический раствор 5	—	—	—	—	—	2
Активатор 6	4	4	4	4	4	4
Взвесь токсоплазм 7	2	2	2	2	2	2
8 Пробирки помещают в водяную баню при 37° на 1 час						
Щелочной раствор метиленового синего 9	6	6	6	6	6	6
Окончательное разведение сыворотки 10	1:4	1:8	1:16	1:32	1:64	

Note: The amounts of the ingredients are measured in drops.

1) Ingredients; 2) test-tube No.; 3) control; 4) test serum; 5) physiological solution; 6) activator; 7) Toxoplasma suspension; 8) test-tubes incubated in water bath at 37° for 1 hr; 9) alkaline methylene blue solution; 10) final serum dilution.

3. Fresh human serum containing no specific antibodies (the so-called auxiliary factor or activator), without which the reaction cannot be conducted. When frozen (at -30°) the activator retains its properties for 2-3 months. Repeated freezing and thawing reduce its quality.

4. Alkaline (pH = 11.0) methylene blue solution. This ingredient is made up as needed from saturated alcoholic methylene blue and a buffer solution of sodium bicarbonate and borax.

Azure A or B, toluidine blue, brilliant cresol blue, or thionine can be used as the stain.

It is, however, necessary to adhere to the commonly accepted method, so that the data of different laboratories are comparable.

Table 38 shows the manner in which the reaction is set up (after Beverly and Bitty).

Evaluation of results. A drop from the bottom of the test-tube is placed on a slide and covered with a cover slip; the number of unstained extracellular Toxoplasma is then counted under a microscope. Any serum dilution (or titre) at which more than 50% of the Toxoplasma remain unstained is regarded as positive. In making this evaluation it is necessary to take into account the number of unstained parasites in the control; for example, if 10% of the Toxoplasma in the control are unstained the serum is regarded as positive when 60% of the parasites on the experimental slide are unstained.

Complement-fixation reaction. This reaction is set up in the same manner as the classical Bordet-Gengou reaction.

1. The antigen is prepared from peritoneal exudate from Toxoplasma-infected white mice. The working dose is 1 antigen unit (the greatest antigen dilution which yields complete arrest of hemolysis with positive serum).

2. The serum to be investigated is inactivated at 56° for 30 min.

3. Fresh normal guinea pig serum is used as the complement. The complement dose is determined by titrating it in the presence of the antigen.

4. The hemolytic system consists of equal parts of a 3% suspension of washed sheep erythrocytes and hemolytic serum from a rabbit immunized with sheep erythrocytes, which is diluted to one-third of titre. In order to sensitize the erythrocytes the suspension is placed in a heater at 37° for 30 min. Table 39 shows the scheme of the complement-fixation reaction.

Evaluation of results. The extent to which hemolysis is retarded is indicated on a 4-plus scale. The serum (or its titre) is regarded as positive if the extent of this retardation is evaluated as no less than two plusses.

TABLE 39

Scheme of Complement-fixation Reaction

1 Ингредиенты (в мл) / 2 № пробирки	1	2	3	4	5	6	Контроль 3
Разведения исследуемой сыворотки . . 4.	0,25 (1 : 5)	0,25 (1 : 10)	0,25 (1 : 20)	0,25 (1 : 40)	0,25 (1 : 80)	0,25 (1 : 160)	0,25 (1 : 5)
Антиген . . . 5.	0,2	0,2	0,2	0,2	0,2	0,2	—
Комплемент . 6	0,15	0,15	0,15	0,15	0,15	0,15	0,15
Физиологический раствор . . . 7	8 До 0,75	До 0,75	До 0,75	До 0,75	До 0,75	До 0,75	До 0,75
9 В холодильник при 4° на 16—18 часов							
Гемолитическая система . 10.	0,5	0,5	0,5	0,5	0,5	0,5	0,5
11 В термостат при 37° на 30—40 минут							

1) Ingredients (in ml); 2) test-tube No.; 3) control; 4) diluted test-serum; 5) antigen; 6) complement; 7) physiological solution; 8) up to; 9) in a refrigerator at 4° for 16-18 hr; 10) hemolytic system; 11) in a heater at 37° for 30-40 min.

Intracutaneous allergic test with toxoplasmin. This test is set up in the same manner as Mantoux's reaction. The toxoplasmin is usually prepared from diluted peritoneal exudate from Toxoplasma-infected white mice. It is processed by a special method and injected intracutaneously in a dose of 0.1 ml. Subcutaneous injection of an equal quantity of sterile physiological solution serves as the control.

In evaluating the results the test is regarded as positive if an infiltration no less than 10 x 10 mm in size develops at the site of the toxoplasmin injection within 48 hr.

Evaluation of data obtained. There is now no doubt of the specificity of the serological reactions and allergic skin test used in toxoplasmosis.

The complement-fixation reaction becomes positive 3-4 weeks after the onset of the illness and remains so for from 2 to 4 years.

The stain reaction becomes positive during the second week of ill-

ness, but remains so for a more protracted period. This reaction stays positive at low titres throughout the entire lifetime of the animal in the opinion of some researchers and for 4-5 years after infection in the view of others. The reaction with toxoplasmin becomes positive at the end of the 4th week after infection and probably remains so until the animal dies. The intracutaneous test does not yield reliable results in children less than 2 years old and adults more than 60 years old, since the body is areactive to toxoplasmosis antigen.

The titre of complement-fixing antibodies in the serum is always substantially lower than the antibody titre detected in the stain reaction (provided that both reactions are positive).

Isolation of Toxoplasma by biological testing

The same materials are used for biological testing as for microscopic examination (the brain, liver, spleen, lymph nodes, cerebrospinal fluid, etc.); they should be sterile. Pieces of the organs in question are ground in a mortar and suspended in a small quantity of physiological solution, to which antibiotics (penicillin and streptomycin) should be added to suppress the concomitant microflora.

White mice, hamsters, susliks, guinea pigs, and other laboratory animals susceptible to toxoplasmosis are used for inoculation.

Rodents are usually inoculated intraperitoneally with 0.5-1 ml of the organ suspension, but they may also be inoculated subcutaneously, intracerebrally, etc. It must be kept in mind that there are strains of Toxoplasma which are of low virulence for one species of animal and more virulent for other species. Hence it follows that the chances of isolating Toxoplasma from the material under investigation are enhanced if it is used to inoculate different laboratory animals. Infected animals are subject to generalization of the pathological process. Toxoplasma are easily detected in various organs, especially the liver,

by examining stained smears.

A large quantity of exudate (up to 2.5 ml) containing many Toxoplasma is formed in the abdominal cavities of mice. The microorganisms can easily be detected by examining a compressed drop of the exudate at low magnification or by studying smears fixed and stained in the usual manner.

In some cases it is necessary to make 3-5 passes (over a period of 7-10 days) to increase the virulence of the toxoplasma.

A diagnosis of toxoplasmosis can be made only in accordance with clinical symptoms, positive serological and allergic reactions, and anamnestic data. Serological investigations should be conducted several times over the course of the infection. An increase in antibody titre is an important characteristic of toxoplasmosis. A single serological investigation is of clinical value only when the stain reaction is positive at a titre of 1:64 or more and the complement-fixation reaction is simultaneously positive at a titre of 1:5. If these reactions are accompanied by a negative skin test it is very probable that the infection is recently incurred. Detection of low antibody titres in the first investigation followed by a rise in titre after 3-4 weeks is an important indication of recently incurred toxoplasmosis.

If the antibody titre does not rise on subsequent examination of the patient's serum, the disease in question is obviously not toxoplasmosis.

A proper diagnosis of toxoplasmosis can be made when the appropriate clinical symptoms are present, the causative agent has been isolated or detected in preparations, and the serological data satisfy the aforementioned requirements.

DISEASES CAUSED BY PATHOGENIC INTESTINAL PROTOZOA

Prof. Sh.D. Moshkovskiy, Corresponding Member of the Academy of Medical Sciences USSR, and Ye.A. Pavlova, Candidate of Medical Sciences

Amebiasis

Six species of ameba are parasitic in the human intestine: Entamoeba histolytica, Entamoeba hartmanni, Entamoeba coli, Endolimax nana, Jodamoeba butschlii, and Dientamoeba fragilis.

Of these amebae only Entamoeba histolytica (the dysentery ameba) is pathogenic, being the causative agent of amebiasis, a disease produced by the multiplication of this protozoan in the tissues of the large intestine, or, less frequently, other organs.

The acute form (stage) of intestinal amebiasis is characterized by a frequent stool and by appearance of mucous and blood in the feces. Periods of acute symptoms often alternate with periods of remission and the disease has a prolonged relapsing course.

Entamoeba histolytica may leave the large intestine and reproduce in the skin around the anus or a fistula or in the tissues of other organs (the liver, lungs, brain, etc.); this is referred to as extraenteric amebiasis.

Amebae are found in the human body in the vegetative stage (feeding and reproducing) and in the cyst stage. Vegetative amebic forms die soon (within 15-20 min) after being excreted from the body, while cysts may retain their viability for a longer time.

Dysentery amebae may reproduce for a protracted period in the large intestine without penetrating its tissues or producing clinical symptoms (making the animal a carrier). In carriers the ameba reproduces in a small vegetative form, the forma minuta (lumen form), which produces cysts and has 4 nuclei when mature.

Only cysts are detected in formed feces in the normally function-

ing intestine. Vegetative lumen forms can also be detected when the intestinal function is disrupted, as manifested in the appearance of a liquid or semiformed stool.

In ulcerative conditions of the large intestine caused by dysentery amebae the vegetative stages are larger (tissue form, or forma magna) than the lumen form and engulf erythrocytes. Large tissue forms of Entamoeba histolytica are detected in masses in the mucoid, bloody feces observed during the acute phase of the illness, which is accompanied by formation of extensive ulcers in the large intestine; they also occur in lesser numbers in small pieces of mucous embedded in the apparently normal feces of persons with only slight ulcerations of the large intestine.

Laboratory Diagnosis of Amebiasis

Material for examination; taking of samples

Feces or mucous taken from the rectum or sygmoid colon during rectoscopy or romanoscopy are examined.

The feces should be collected in a clean dry vessel, in portions of 10-15 g.

In extraenteric amebiasis pus obtained by puncturing or opening the abscesses in the liver, lungs, or other organs is examined.

The samples must be examined for vegetative amebic forms immediately after they are taken, since these forms die and decompose within 15-30 min after they are isolated from the body.

Feces containing cysts can be kept in a refrigerator for several days. Nevertheless, it is not desirable to postpone the examination, since isolated cysts undergo changes which hamper diagnosis.

Cysts in feces can be preserved in Barbagallo's solution (4% formalin in physiological solution), using 5-8 parts of solution to one part of feces. The cysts of E. coli survive for several years, but the

majority of the cysts of other amebae, including E. histolytica, become granular after several days in this solution, which makes it hard to distinguish their nuclei.

Parasitological (microscopic) investigation

The clinical manifestations of amebiasis are very diverse and are often quite similar to the symptoms of intestinal disorders of other etiologies. A diagnosis of amebiasis should consequently be confirmed parasitologically. The following rules must be observed in parasitological investigation.

1. The material to be examined must be fresh, since amebae are not resistant to the action of environmental agents.

2. The individual conducting the investigation should be thoroughly familiar with the investigative techniques and the morphological characteristics of the pathogenic ameba E. histolytica and of the other species of ameba encountered in the human intestine.

3. The investigation must be repeated several times when a negative result is obtained. Amebae are isolated from the intestine in a nonuniform fashion during the various stages of the disease and a single negative analysis does not permit the investigator to exclude the possibility of amebiasis. Failure to observe any of the aforementioned rules may lead to diagnostic errors.

Before preparing specimens for microscopic examination the material should be subjected to careful macroscopic inspection, noting its consistency and the presence of mucous or blood. Native smears and fixed, stained preparations are examined microscopically.

Preparation of native smears. These preparations are made up on clean slides. A small piece of fecal matter is taken with a wooden rod from the most suspicious area (that containing mucous and blood), carefully mixed with a drop of physiological solution, applied to a slide,

and covered with a cover slip. Care must be taken that the suspension contains no large particles, which prevent uniform distribution of the material between the two pieces of glass. The final preparation should be of moderate thickness. If it is too thin it contains only a small quantity of material, while if it is too thick the large number of different elements hampers detailed examination of the protozoa. A native preparation must be examined with the illumination slightly reduced (the diaphragm closed).

When the illumination has been properly adjusted the amebae and cysts in the native preparation appear as somewhat greenish refractive bodies. A preliminary inspection is conducted at low magnification; all small refractive structures detected at this magnification should then be examined at high magnification (with a 40-power objective). In order to observe the movement of vegetative amebae it is necessary to avoid cooling the specimen. For this purpose the preparation must be made up on a heated slide and observed on a heated stage or with the microscope in a heated box.

Microscopic pattern of native preparations. Large vegetative tissue forms of E. histolytica are observed in specimens prepared from bloody mucous taken from amebiasis patients with ulcerations of the large intestine. These amebae are round or oval in shape when at rest and range from 15 to 30-40 μ in diameter. Elongated forms may be of greater length. The cytoplasm is divided into two distinct layers. The outer layer, which is highly refractive, transparent, and homogeneous, is called the ectoplasm. This ectoplasm forms pseudopodia, which are protuberances usually pointing in the direction in which the ameba is moving.

In fresh fecal matter the tissue form of E. histolytica is characterized by active progressive movement. This type of active movement is

not observed in the nonpathogenic amebae of the human intestine. The inner layer, the endoplasm, is less transparent and granular. This layer contains the nucleus, whose outlines are usually indetectable in fresh preparations. The endoplasm of fresh tissue forms of E. histolytica do not contain bacteria, which distinguishes it from the other intestinal amebae, whose cytoplasm always contains bacteria. E. histolytica is capable of phagocytizing erythrocytes. Amebae containing engulfed erythrocytes can always be detected when mucous and blood are present in the feces in acute amebiasis. Tissue amebae which phagocytize erythrocytes are referred to as hematophages or erythrophages. Detection of hematophages is very important in diagnosing the acute phase of amebiasis, since the other intestinal amebae do not engulf erythrocytes under normal conditions. Vegetative lumen forms of E. histolytica can be detected in semiformed nonsanguinous feces obtained from convalescents, after exacerbation from persons suffering from protracted relapsing amebiasis, and from carriers with undamaged large intestines.

The lumen form of E. histolytica is 12-20 μ in diameter. Its cytoplasm is divided into ectoplasm and endoplasm. Its movement is less active than that of the tissue forms, but it exhibits sudden intermittent formation of transparent pseudopodia. A small number of engulfed bacteria are sometimes visible in the endoplasm. The lumen form of E. histolytica does not phagocytize erythrocytes.

Cysts of E. histolytica can be detected in the formed feces of persons who have recovered from amebiasis and of carriers. The cyst membrane is clearly visible in native preparations. The cysts are round or sometimes slightly oval in shape, very rarely being irregular. They range from 6 to 20 μ in diameter, averaging 10-12 μ. The cytoplasm is fine-grained, with no differentiation into endoplasm and ectoplasm; there are never any engulfed particles. The nuclei cannot be seen in

native preparations. Inclusions, taking the form of colorless, highly refractive rods or masses with rounded ends, can be clearly distinguished in some cysts. These inclusions are a reserve of nutritive material and are called chromatid bodies.

Differentiation of the various species of amebae and, especially, their cysts is facilitated by examination of specimens prepared with Lugol's reagent (1:2:100). This solution is stored in a brown-flask bottle. It should be made up fresh every 12-14 days.

The technique for preparing specimens with Lugol's solution is the same as for native specimens, except that a drop of Lugol's reagent is used instead of a drop of physiological solution. Two preparations are usually made up on the same slide, one native and the other with Lugol's reagent. The native smear is investigated first, the vegetative form being identified their movement and the character of the engulfed particles; when cysts are detected the preparation is studied with Lugol's solution to determine the species to which they belong. In the latter preparation the cytoplasm of the cysts is stained a greenish-yellow color. The structure and number of the nuclei can be determined in iodine-stained cysts. The nuclei of E. histolytica cysts take the form of rings of small refractive granules, with a small circular karyosome in the center. The number of nuclei in these cysts ranges from 1 to 4 (there are eight in mature E. coli cysts). In iodine-treated preparations the glycogen vacuole of the cyst can be seen as a brownish spot with diffuse margins. Iodine does not stain the chromatoid bodies.

In order to determine the viability of the amebae and cysts a particle of fecal matter is mixed with a drop of 2% eosin. Live amebae and cysts do not pick up the dye, while dead amebae are stained a reddish color. The nucleus is stained first as the cell dies, followed by the cytoplasm.

Preparation of permanent stained specimens. It is sometimes difficult to identify amebae and their cysts in fresh preparations. In some cases a conclusive diagnosis can be made only from stained specimens. Permanent stained preparations of protozoa clearly show the structural details of the nuclei and other elements. Such preparations can be stored for long periods and can be subjected to repeated examination. In making up permanent stained preparations the material used must be fresh and the smears should be properly fixed. The smears are made on cover slips or, less frequently, on slides, the former being more convenient. The material used for the specimens is carefully inspected and areas containing mucous and blood are selected. The smears are made with a platinum loop or wooden rod. When no mucous is present a small quantity of fecal matter is smeared on the edge of a cover slip and the latter is then drawn (under light pressure) along another cover slip held by one of its corners. The smear should be as uniform in thickness as possible. Care must be taken that the specimen does not dry out during its preparation or its subsequent transfer from liquid to liquid. The cover slip is carefully dropped with its smeared side down so that it floats on the surface of a portion of Shaudin's fixative* in a large watch-glass or a salt-shaker with a concave bottom.

After a few seconds the cover slip (smear down) drops to the bottom of the watch-glass and is then transferred, smeared side up, to a Petri dish containing fresh Shaudin's solution. The cover slips are arranged smeared side up in a row on the bottom of the dish, in order to avoid damaging the smears. After 20-25 min the preparations are transferred (in the same position) to a dish containing 70° alcohol, where they are left for 5 min; in order to remove any residue of the mercuric chloride they are then placed in 70° alcohol containing iodine (tincture of iodine is added to the 70° alcohol until it reaches the

color of port wine or thoroughly steeped tea). The specimens are then twice left to stand for 5 min in 70° alcohol. If staining cannot be carried out immediately, the preparations can be stored in 70° alcohol for a prolonged period (several months).

Staining method. Staining by Haydenhayn's method. The 70° alcohol is rinsed from the fixed specimens with distilled water and they are placed in a 4% solution of ferrous ammonium sulfate [$NH_4Fe(SO_4)\cdot 12H_2O$], which is the mordant, for 6-12 hr.

The alum solution is prepared as needed from amethyst-colored crystals. Crystals covered with a yellowish deposit are carefully washed with distilled water to remove it. After they have taken on a pure amethyst color they are dried with filter paper and the requisite quantity is weighed out. A 20% alum solution can be prepared beforehand and will not deteriorate for several months. This 20% solution is diluted by a factor of 5 before use. After the mordant has been applied the specimen is quicly washed with distilled water and transferred to the hematoxylin solution for 12-24 hr.

The hematoxylin solution is prepared in the following manner: 1 g of hematoxylin is dissolved in 10 ml of 96° alcohol, sufficient distilled water is added to make 100 ml, and the solution is kept in a heater at 37° for 3-4 days to age. It can then be stored in well-stoppered bottles.

After application of the hematoxylin the specimen is rinsed with water and differentiated in 1-2% ferrous ammonium sulfate. This is done in the following manner: the specimen is placed smear down on a bridge formed by gluing two strips of glass to a slide with Canadian balsam.

The alum solution is poured beneath the specimen. The specimen must be checked microscopically (at low and then high magnification) until the structure of the nucleus and cytoplasm can be clearly seen.

After differentiation the specimen is thoroughly washed with distilled water and then rinsed with tap water under a faucet or in a Petri dish, changing the water frequently over a period of 30-45 min. The specimen is dehydrated by being successively placed in alcohol of increasing strength (70, 80, and 96°) for 3-5 min. From the 96° alcohol it is transferred to a carbol-xylol mixture (one part of chemically pure phenol and two parts of xylol) for clarification, rinsed in pure xylol, and sealed in Canadian balsam. There are a number of modifications of this method which require less time, but it is not always sensible to save time at the expense of the quality of the results obtained.

Faust's modification. The specimen is:

1. Fixed for 2 min in Shaudin's solution heated to 60°;
2. Placed in 70° alcohol, 70° alcohol containing iodine, 70° alcohol, and 50° alcohol for 2 min each;
3. Rinsed with water for 2 min;
4. Placed in 2% aqueous alum heated to 40° for 2 min;
5. Rinsed with water for 3 min;
6. Placed in 0.5% aqueous hematoxylin for 2 min;
7. Rinsed with water for 2 min;
8. Differentiated in cold 1-2% aqueous alum;
9. Rinsed with water for 10-15 min;
10. Placed in 70°, 80°, 90°, and 100° alcohol for 2 min each;
11. Clarified in xylol and sealed in balsam.

Hematoxylin staining by Hansen's method. The specimen is fixed in Shaudin's solution in the same manner as for staining by Haydenhayn's method.

The 70° alcohol is rinsed off with distilled water and the specimen is transferred to Hansen's hematoxylin (preliminarily filtered) for from 30 sec to 2 min.

Hansen's hematoxylin is prepared in the following manner: 10 g of chrome alum is dissolved in 250 ml of distilled water and boiled until a greenish color appears. A total of 1 g of crystalline hematoxylin is dissolved in 10-15 ml of hot water and then added to the alum solution or dissolved directly in the hot alum solution, stirring it constantly. The mixture takes on a dark, almost black color. When the mixture has cooled 5 ml of 10% aqueous sulfuric acid is added to it. It is then mixed with 0.55 g of potassium dichromate preliminarily dissolved in 20 ml of warm water. The potassium dichromate solution must be added drop by drop, stirring the mixture constantly. The solution is then boiled for 2-4 min and is ready for use when it has cooled. It is stored in tightly-stoppered bottles.

The specimen is then rinsed with distilled water and tap water (see above) in a dish for 10-30 min. Dehydration, clarification, and sealing in balsam are carried out in the manner described above.

Hematoxylin staining by Delafield's method. The specimen is fixed in Shaudin's solution. After being removed from the 70° alcohol the specimen is rinsed with distilled water and transferred to Delafield's hematoxylin for 6-10 hr. Before use 2 or 3 parts of distilled water are added to one part of the dye.

Delafield's hematoxylin is prepared in the following manner. Two solutions are made up: 1) 4 g of hematoxylin is dissolved in 25 ml of absolute alcohol; 2) a saturated solution of potash alum is made up in 400 ml of distilled water. The two solutions are mixed in a wide-mouthed jar and left to stand in a light place for 3-4 days. The mixture is then filtered and 100 ml of glycerine and 100 ml of methyl or 96° ethyl alcohol are added to it.

The solution is left to stand in a heater in a loosely covered bottle for from several days to 6 weeks, periodically being shaken during

this period. It is then filtered and stored in bottles with ground stoppers.

After application of the hematoxylin the specimen is rinsed for 5-10 min and differentiated in 70° alcohol acidified with acetic acid (2-3%). It is then immersed for several minutes in 70° alcohol to which ammonia has been added (one drop per 100 ml of alcohol) to remove the acid. Staining can also be carried out with Delafield's solution diluted by a factor of 200. In this case the specimen is periodically inspected until a stain of sufficient intensity is achieved; differentiation is omitted.

Dehydration, clarification, and sealing in balsam are conducted in the same manner as in the other methods.

Delafield's hematoxylin stains cysts well.

Permanent stained specimens prepared in this manner can be stored for several years.

Microscopic pattern of stained specimens. In stained specimens the vegetative tissue form of E. histolytica is round or oval in shape. The differentiation into ectoplasm and endoplasm is easily seen: the ectoplasm appears as a light ring, while the endoplasm is stained a grayish-blue, is fine-grained, and sometimes contains small bacteria-free vacuoles. The erythrocytes in the endoplasm are round, black inclusions of varying diameter, depending on the stage of digestion. In contrast to native preparations, where the basic characteristics are movement and the character of pseudopod formation, the differential-diagnostic characteristic which serves to distinguish E. histolytica from nonpathogenic amebae in permanent specimens is the structural details of the nucleus. The nucleus of E. histolytica is a vesicle approximately 5 γ in diameter (4-9 μ), is enclosed by a thin membrane, and stains black. The inside of the membrane is lined with fine chromatin granules arrayed in a single

row, which stain black (the peripheral nuclear chromatin of E. coli consists of larger, coarser granules). The small, dark, round karyosome always lies in the center of the nucleus in E. histolytica, the larger karyosome of E. coli is usually located eccentrically.

During the degeneration of vegetative forms of E. histolytica, which occurs shortly after they are isolated from the body, the nucleus undergoes changes reflected in its structure. The peripheral chromatin granules are arrayed randomly under the nuclear membrane and the karyosome becomes irregular in shape and appears larger. Degenerated nuclei of E. histolytica divide in a manner similar to that exhibited by the nuclei of E. coli, which makes it impossible to distinguish the two species. The specimens must consequently be prepared from fresh material and properly fixed.

The vegetative lumen form of E. histolytica is smaller (12-20 μ in diameter) than the tissue form. The differentiation into ectoplasm and endoplasm is less distinct than in the tissue form and the endoplasm contains no erythrocytes. The structure of the nucleus is similar to that observed in the tissue form.

Cysts of E. histolytica. The majority of the cysts of this species retain their spherical shape and their diameter is the same as in fresh preparations (6-20 μ, averaging 10-12 μ). The cyst membrane does not stain, but the cytoplasm stains a bluish-gray, while the nuclear chromatin, karyosome, and chromatoid bodies stain black. The number of nuclei ranges from 1 to 4, mature cysts containing 4. The structure of the nucleus is similar to that observed in the vegetative forms. In cysts containing 4 nuclei the peripheral chromatin forms a crescentic mass in the nucleus. A substantial percentage of the cysts of E. histolytica (especially in fresh material) exhibit rod-like chromatoid bodies with rounded ends, which stain an intense black. The presence of chromatoid

bodies facilitates a diagnosis, since they are considerably less common in the cyst of E. coli and have pointed ends when they do occur.

For a precise diagnosis of the cyst of E. histolytica it is necessary to find mature cysts containing 4 nuclei (mature cysts of E. coli have 8 nuclei). In making microscopic examinations it must be kept in mind that the nuclei often do not lie in the same plane and consequently can be examined and counted only when the micrometer screw is turned.

The vegetative forms and cysts of E. histolytica and other intestinal protozoa are well preserved in the following mixture:

Glycerine – 1.5 ml

Glacial acetic acid – 5 ml

Shaudin's fixative (without acetic acid) – 93.5 ml

Powdered polyvinyl alcohol – 5 g

The mixture is heated to 75° in a water bath and shaken until the powder has completely dissolved. The resultant solution can be stored for several months.

In order to preserve material containing intestinal protozoa one part of fecal matter is mixed with 3 parts of this solution. Permanent stained specimens can be prepared from the mixture after weeks or months. For this purpose it is thoroughly shaken and smears are made on slides. The latter are dried at 37° for 3 hr or more, transferred to alcohol containing iodine, and then treated by Haydenhayn's method.

Methods of concentrating cysts. Methods of concentrating the cyst in the material under investigation have been proposed in order to reduce the time involved in the investigation and the number of specimens which must be examined and to increase the probability of detecting cysts.

Concentration methods are employed only for formed feces assumed to contain cysts, since any vegetative amebae are always destroyed when

the material is processed. The proposed techniques are based either on deposition of the cysts in water or flotation on a liquid with a higher specific gravity than the cysts.

Deposition method. 1. Settling method. Pieces of feces weighing approximately 5 g are carefully mixed with a small quantity of tap water, transferred to a 500 ml graduate filled to overflowing with water, shaken, and permitted to stand for 10-15 min. The large particles of fecal matter settle to the bottom during this period. The cloudy liquid at the top is poured off into another graduate and permitted to stand for 24 hr. The cysts settle to the bottom during this period and the supernatant is discarded. The residue is examined. 2. Centrifugation method: the deposition of the cysts can be accelerated by centrifuging the liquid after it has stood for 10-15 min. Centrifuging is carried out for from 45 sec to 1 min at 1000 rpm and the residue is examined.

Cyst-flotation methods. The residue left in the cylinder after the suspension has stood for 24 hr is freed of the fecal particles deposited with the cyst by repeated centrifuging with water. When the supernatant becomes clear it is discarded and a 30% sucrose solution or 33% zinc sulfate solution is added to the residue. The mixture is thoroughly shaken and centrifuged for 2 min at 100 rpm. The film containing the cyst is examined.

The preparations produced by all these concentration methods are examined with Lugol's reagent.

Examination of carriers. Persons employed in the foodstuffs industry, medical workers, and employees of pediatric institutions should be examined to determine whether they are cyst carriers. The material to be examined for protozoa should be taken when specimens are collected at bacteriological stations where persons in the aforementioned categories are examined for bacterial dysentery.

Feces, which are collected in covered boxes, serve as the material to be investigated and can be stored in a refrigerator for 24 hr. The investigation is conducted with native smears and smears stained with Lugol's solution. In doubtful cases no less than 3 permanent stained specimens are prepared from each sample.

Investigation of cadavers. Autopsies should be performed as soon after death as possible. Affected portions of the large intestine, lungs, liver, and other organs are subjected to histological treatment. Amebae occur in the tissues and smears are prepared from the affected areas to permit their detection. The smears are processed in the manner described above.

Culturing of amebae

Culturing of material on nutritive media to obtain an amebic growth is a supplemental diagnostic procedure and is employed after all the aforementioned methods have been exhausted. Negative culturing results do not permit the investigator to exclude the possibility of amebiasis.

The media used for culturing amebae can be divided into two groups: media with a solid substrate and liquid media. The most satisfactory media are Beck and Drbolav's solid medium and Ye.A. Pavlova's liquid medium.

Pieces of feces weighing approximately 0.1 g (or pieces of mucous if it is present) are forced to the bottom of a test-tube containing medium heated to 37°. The culture is incubated at 37° and the amebae multiply on the bottom of the tube. The first transplants are made after 24 hr and subsequent transplants at 48-hr intervals. A Pasteur pipette is used for transferring material to fresh medium, collecting a small quantity of the ameba-containing sediment from the bottom.

Detection of amebae by biological testing

This method is rarely employed for diagnostic purposes. Kittens,

puppies, rabbits, rats, guinea pigs, etc., are used as laboratory animals for infection with E. histolytica. If the inoculation material contains vegetative forms (or is assumed to contain them) it is administered per rectum or injected directly into the cecum with a syringe (by laparotomy). When the results are positive the animals develop diarrhea containing mucous and blood within 3-8 days. Microscopic examination reveals E. histolytica.

Material containing cysts obtained by concentration methods is administered per os. The fecal matter is washed from the cysts and they are mixed with the animal's food. When the results are positive diarrhea containing mucous and blood develops after an incubation period lasting for from several days to several weeks. Microscopic examination reveals vegetative E. histolytica.

Ulcerations containing tissue forms of E. histolytica are detected in the large intestine and, when the cecum is inoculated, primarily in this organ.

Infected animals are kept separate, observing safety measures.

Detection of E. histolytica in the external environment

Water is examined for amebic cysts. The settling and centrifugation methods can be employed if the water is contaminated. If the water appears to be pure it is filtered through membrane filters and material scraped from them is then examined.

Balantidiasis

Balantidium coli causes severe anatomic damage to the large intestine; asymptomatic carriers are also observed. B. coli is usually found in the vegetative stage in the human intestine, cysts rarely being encountered.

The techniques for collecting material, examining it, and preparing permanent stained specimens are the same as for amebiasis.

Microscopic pattern. Vegetative forms of B. coli are oval in shape, 30-150 μ long and 30-80 μ wide. The entire soma of B. coli is covered with rows of cilia. Live specimens exhibit vigorous movement. The slit-like mouth (cytostome) is located in the anterior, narrower end of the body and is surrounded by long cilia, while the anus is at the posterior end. There are two nuclei: the macronucleus is a thick, curved whip-like structure, while the micronucleus is small and round and lie very close to the macronucleus. It is often impossible to detect the micronucleus. The cysts are oval in shape and 45 × 65 μ in size and are covered with a distinct double membrane.

B. coli grow well on the media used for culturing intestinal amebae.

Intestinal Flagellates

Trichomonas hominis is oval (sometimes circular) in shape and 3-20 μ in diameter. The enlarged anterior portion of its soma bears 3-4 free flagella. Along the margin of the body is a sinuous membrane, whose edge bears a filament terminating in a free flagellum. There is a spine-like projection at the posterior end of the body. The small vesicular nucleus is in the anterior half of the soma. This microorganism does not form cysts.

Chilomastix mesnili is piriform in shape, 7-20 μ long, and 3-10 μ wide. The blunt anterior end of the soma bears 4 free flagella. This microorganism forms piriform cysts somewhat smaller than the vegetative form. It reproduces in the large intestine and is of no pathogenic significance.

Lamblia intestinalis is piriform with truncated poles, 10-20 μ long, and 5-15 μ wide. The flat side of the blunt anterior end bears an adhesive disk, with which the parasite attaches itself to the epithelium of the small intestine. There are 2 oval, vesicular nuclei with karyosomes in the center and 4 pairs of flagella. The double axial filament

of the axostyle passes along the body.

The cysts of this microorganism contain 2 or 4 nuclei and the coiled flagella and axostype can be seen within them. They are enclosed in a double membrane and are 8-12 μ long and 7-10 μ wide.

Intestinal flagellates are diagnosed in the same manner as amebae.

Vegetative forms of Lamblia are also found in the duodenal contents, which are sampled by probing.

Lamblia are of no great pathogenic significance in the overwhelming majority of cases. Detection of these microorganisms in persons suffering from intestinal disorders is not an absolute proof that they play an etiological role.

A diagnosis of lambliosis cannot be made without first excluding diseases of bacterial (Sh. dysenteriae, Salmonella, pathogenic Escherechia coli, etc.), amebic, and viral etiology or of a noninfectious nature.

In rare cases the etiological role of Lamblia can be established from the effectiveness of atebrin therapy, but this is not of itself always a precise indication (Table 40).

Nutritive Media

Beck and Drbolav's medium. A total of 4 eggs are broken into a vessel containing glass beads and mixed with 50 ml of Locke's solution:

Sodium chloride - 9 g

Calcium chloride - 0.24 g

Potassium chloride - 0.42 g

Sodium carbonate - 0.2 g

Glucose - 1 g

Distilled water - 1 liter

The mixture is poured into test-tubes and permitted to coagulate in an inclined position at 70°. It is then sterilized in an autoclave

for 20 min and 5-6 ml of a mixture of Locke's solution and inactivated human or horse serum in a ratio of 1:8 (one part of serum to 8 parts of Locke's solution) is added to each test-tube under sterile conditions.

Ye.A. Pavlova's liquid medium:

Sodium chloride – 4.25 g

Disubstituted sodium phosphate – 0.3 g

Monosubstituted potassium phosphate – 0.23 g

Distilled water – 500 ml

Portions of 8-9 ml of the solution are poured into test-tubes and sterilized in an autoclave; 0.5 ml of serum and a loopful of rice starch preliminarily ground in a mortar and sterilized by dry heat are then added to each tube.

TABLE 40

Comparative Characteristics of Human Intestinal Amebae

1 Признаки	2 Вид амеб			
	Entamoeba histolytica		Entamoeba hartmanni	Entamoeba coli
3 Вегетативные:	7 Тканевая	8 Просветная		
4 Размеры (в микронах)	15—40	12—20	6—15	15—60
5 Движение	9 Быстрое поступательное с образованием крупных псевдоподий из эктоплазмы, которое бывает видно у амебы, находящейся в спокойном состоянии	Более вялое, чем у тканевых форм. Разделение на экто- и эндоплазму имеется 10	Может быть поступательным, псевдоподии образуются толчками 11	Медленное, псевдоподии в виде наплывов из эктоплазмы, которая в спокойном состоянии не заметна 12
6 Включения в цитоплазме	15 Эритроциты	16 Эритроцитов не бывает. Единичные бактерии 17	Бактерии 18	Бактерии, кусочки клетчатки, крахмал, лейкоциты и др. 19

Признаки	Вид амеб					
	Entamoeba histolytica	Entamoeba hartmanni	Entamoeba coli	Endolimax nana	Jodamoeba butschlii	Dientamoeba fragilis
Ядро (по препаратам, окрашенным гематоксилином) 20	Под оболочкой ядра тонкий ободок периферического хроматина. Кариосома маленькая, расположена центрально 26	Ядро сходно по строению с ядром E. histolytica, но имеет меньшие размеры 27	Под оболочкой ядра периферический хроматин в виде грубых зерен. Кариосома большая, неправильной формы, лежащая эксцентрично 28	На оболочке ядра хроматин не видно. Кариосома большая, состоит из нескольких глыбок в цисте, собранных в плотный комок 29	На оболочке ядра хроматин в виде очень нежного ободка. Кариосома большая круглая. Иногда лежит эксцентрично. Занимает значительную часть ядра, окружена крупноячеистой сетью, заполняющей весь остаток ядра 8—15 30	Оболочка ядра в виде одно тонкого ободка. Периферического хроматина нет. Кариосома из отдельных зерен, собранных в центре ядра розеткой 31
21 Цисты: Размеры (в микронах) 22	8—20 (в среднем 12)	До 10 32	10—35 (в среднем 15—20) 34	5—14 (в среднем 7—8) 34	(в среднем 9) 34 35	Цисты не известны 33
Число ядер 23	1—4	1—4	38 1—8	39	Одноядерные	
Хроматоидное тело 25	Имеет расплывчатые контуры, занимает малую часть цисты 36 В виде 2—3 брусков, реже палочек с тупыми концами, имеется в значительном большинстве цист 41	Имеет расплывчатые контуры 37 Многочисленные, в форме крупных кокков и палочек 42	Имеется в молодых (одно- и двухъядерных). Занимает почти всю массу 43 В виде палочек с острыми, как будто расщепленными, концами (встречаются редко)	Имеется не всегда. Контуры расплывчатые В виде тонких изогнутых полосок (встречаются очень редко) 44	Имеется во всех цистах; имеет резко очерченные контуры Не бывает 45 40	

1) Characteristics; 2) species of ameba; 3) vegetative; 4) size (in microns); 5) movement; 6) cytoplasmic inclusions; 7) tissue; 8) lumen; 9) rapid progressive movement accompanied by formation of large ectoplasmic pseudopodia, which can be seen in resting ameba; 10) movement more sluggish than in tissue forms. Differentiation into ectoplasm and endoplasm visible; 11) movement may be progressive, pseudopodia formed intermittently; 12) slow movement, pseudopodia in form of ectoplasmic protuberances, not visible in resting state; 13) slow; 14) rapid movement, pseudopodia maple-leaf-shaped; 15) erythrocytes; 16) erythrocytes not usually present; 17) isolated bacteria; 18) bacteria; 19) bacteria, cellular detritis, starch, leucocytes, etc.; 20) nucleus (in hamatoxylin-stained preparations; 21) cysts; 22) size (in microns); 23) number of nuclei; 24) glycogen vacuole; 25) chromatoid bodies; 26) peripheral chromatin in delicate ring beneath nuclear membrane. Karyosome small, centrally located; 27) nucleus similar in structure to that of E. histolytica, but smaller in size; 28) peripheral chromatin in coarse granules beneath nuclear membrane. Karyosome large, irregular in shape, eccentrically located; 29) chromatin not visible on nuclear membrane. Karyosome large, consisting of several clumps collected into dense mass; 30) chromatin in form of very delicate ring on nuclear membrane. Karyosome large, circular, occasionally eccentrically located, occupying substantial portion of nucleus, surrounded by large-celled network occupying remainder of nucleus; 31) nuclear membrane in form of scarcely detectable ring. No peripheral chromatin. Karyosome consisting of individual granules collected into rosette-shaped structure in center of nucleus; 32) up to 10; 33) cysts unknown; 34) averaging; 35) mononuclear; 36) diffuse in outline, occupying small portion of cyst; 37) diffuse in outline; 38) present in young (mononuclear and dinuclear) cysts, occupying almost entire cyst; 39) not always present, diffuse in outline; 40) present in all cysts, distinct in outline; 41) forming 2-3 block-like structures or, less frequently, rods with blunt ends, present in substantial majority of cysts; 42) numerous, forming large spheres and rods; 43) forming rods with sharp, pointed ends (rarely encountered); 44) forming narrow curved bands (very rarely encountered); 45) not present.

MELIODOSIS

Prof. Ya.Ye. Kolyakov, Distinguished Scientist

Meliodosis is a glanderous pyemic disease of certain species of animals and of man and is encountered in a number of the nations of Southeast Asia (Malaya, Viet-Nam, Indonesia, Thailand, etc.), Ceylon, and Australia. Isolated cases have been detected in South America and Panama. Meliodosis is also known in the literature as Rangoon glanders, glanderous disease, Stanton's disease, Fletcher's disease, and pneumoenteritis.

The international classification system includes the causative

agent of meliodosis, Pasudomonas pseudomallei, in the family Pseudomonadaceae of the order Pseudomonodales. Its synonyms include Bacillus whitmori, Malleomyces pseudomallei, Loefflerella whitmori, and Bacillus pseudomallei. It is often called Whitmore's bacterium, after the scientist who discovered it.

Morphology of Whitmore's bacterium

Whitmore's bacterium is a well-structured rod 1-6 μ long and 0.5 μ wide, with rounded ends, and is mobile. Longer filamentous forms and coccoid forms are also encountered. It is gram-negative and easily stained with aniline dyes; staining is nonuniform and often bipolar. Staining by Romanowsky-Giemsa's method is recommended.

Biology of Whitmore's bacterium; cultural characteristics

This microbe can be raised on any nutritive medium and is aerobic, but will also grow under anaerobic conditions. The optimum culturing temperature is 37.5°.

In meat-infusion broth it produces a uniform turbidity and forms a film on the surface of the medium. It grows well in slightly acid and slightly alkaline media, the optimum pH being 7.6. Glycerine and blood cerum promote its growth.

On meat-infusion agar Whitmore's bacterium forms smooth, circular, cream-colored colonies, which later become rough and corrugated, acquiring a yellowish-brown hue after 4-7 days. The best growth is obtained on 5% glycerine agar.

In meat-infusion gelatin it produces moderate infundibuliform liquefaction (at 20°) after 4-5 days.

On potato medium Whitmore's bacterium forms a profuse creamy or yellowish-cream growth. It is best to use glycerinized (5%) medium.

This microorganism peptonizes milk slowly, without clabbering it.

Whitmore's bacterium ferments glucose, lactose, sucrose, maltose,

maltose, xylose, manitol, dulcitol, and inulin to form an acid but no gas. It produces hydrogen sulfide but not indol, has a denitrifying action, and reduces methylene blue.

Special care must be employed in differentiating the causative agents of glanders and meliodosis, since they are so closely related.

Pseudomonas mallei	Pseudomonas pseudomallei
Cells granular	Often bipolar when stained
Nonlabile	Labile
Translucent grayish-white growth with pearly luster on agar	Rough creamy growth on agar
Does not liquefy gelatin	Moderate crateriform liquefaction of gelatin when stab-cultured
Honey-colored growth on potato medium	Profuse creamy growth on potato medium
Rats insusceptible	Rats very susceptible
Rabbits relatively insusceptible	Rabbits susceptible

In differentiating these two species of bacteria it must be kept in mind that their antigenic properties differ little in the complement-fixation reaction and the agglutination reaction when they are cross-tested with glanders and meliodosis sera (since they have the same O antigen). Pseudomonas pseudomallei has a flagellar H antigen and its somatic O antigen may be the same as or different from (type 2) that of Pseudomonas mallei.

Resistance to physical and chemical agents

Whitmore's bacterium is thermolabile and dies after 15 min at 58° or 2-3 weeks under refrigeration (at -4°). It is resistant to desiccation. It survives for from 8 to 27 days in putrescent material and for up to 44 days in water; 1% phenol or 0.1% formalin kill it within 24 hr.

Laboratory Diagnosis of Meliodosis

Bacteriological examination

Bacteriological diagnosis (isolation of a pure culture of the caus-

tive agent) is of basic importance in diagnosing meliodosis. For this purpose cultures are made of pus from the abscesses, the nasal discharge, exudates, the blood, the urine, and, in exceptional cases, the cerebrospinal fluid. The material is cultured in the usual nutritive meat-infusion agar, meat-infusion broth, meat-infusion gelatin, and potato medium.

The accelerated method of bacteriological diagnosis consists in staining bacterial smears with fluorescent antibodies (Moody, Goldman, and Thomson, 1956). For details see page 74.

Serological investigation

In chronic cases the complement-fixation reaction and the agglutination reaction with meliodosis antigen can be used for diagnostic purposes. The positive agglutination titre ranges from 1:80 to 1:640. It is necessary to take into account the antigenic similarity of the causative agents of glanders and meliodosis and the similarity of the allergens prepared from cultures of these two bacteria. In view of the essentially identical antigenic structure of these pathogenic bacteria, serological diagnosis of meliodosis is of limited practical value. Bacteriological diagnosis is of decisive importance.

Isolation of Whitmore's bacterium by biological testing

At the same time that the pathological material (pus, blood, urine) is cultured on nutritive media, it is used to inoculate male guinea pigs intraperitoneally and by rubbing ground material into scarified areas of the skin. When an isolated culture is to be studied it is injected subcutaneously. An ulceration develops at the site of the injection, while intraperitoneal inoculation causes suppurative orchitis and periorchitis (the "scrotal sign"). The animals die within 10-20 days. Rabbits are also very sensitive, dying after 8-10 days. When autopsies are performed on guinea pigs and rabbits numerous caseous nodules, from

which a pure culture of the causative agent can be isolated, are found in various organs (the liver, spleen, and lungs). In connection with the susceptibility of rats to meliodosis, it is expedient to inoculate them in order to differentiate pseudomonas pseudomallei from Pseudomonas mallei.

FOOD POISONINGS CAUSED BY Cl. PERFRINGENS

Prof. K.I. Matveyev and B.D. Bychenko, Candidate of Medical Sciences

Outbreakd of food poisonings caused by Cl. perfringens have a sporadic or epidemic character. Strains of type A (see page 909) cause a brief, acute illness involving an incubation period of 6-24 hr, abdominal pain, and diarrhea. The patient may evacuate as often as 20 times a day; all the symptoms disappear after 24-48 hr and fatalities are extremely rare. Cases of necrotic enteritis caused by strains of type F take a more severe course. In its acute forms this disease may be fatal within 12 hr. The most characteristic symptoms are abdominal pain and a liquid stool passed as often as 20 times a day, sometimes with an admixture of blood. Such patients often end up on the operating table with a diagnosis of "intestinal obstruction." In its chronic form the disease lasts several days. Fatalities are common (33%). Cases of infectious enterotoxemia caused by strains of type D have been described in man.

Out breaks of enterotoxemia occur among animals in certain pasturelands, where the soil contains highly toxigenic Cl. perfringens of type B, C, D or E. These bacteria may be spread throughout the area in the feces of sick animals or healthy carriers. Specific antitoxic immunity is the principal factor in protecting against enterotoxemia.

The role of thermolabile strains of Cl. perfringens in food poisonings should not be underestimated, since experiments on volunteers have shown that these strains can produce symptoms of infection.

Cl. perfringens is found everywhere under natural conditions: in the soil, dust, human and animal feces, the water of rivers and lakes, mud, various objects, etc.

These microorganisms are apparently capable of vegetating in different soils, especially those rich in humus substances. The number of Cl. perfringens per gram of soil averages several tens of thousands (10,000-70,000). They multiply very rapidly in the intestines of man and animals and, in some cases, one gram of feces may contain more than 1 million cells. The natural distribution of the individual types of Cl. perfringens has not been sufficiently well studied. The type most commonly encountered in the soil and in human and animal feces is type A. The other types (B, C, D and E) are found in the soil of areas in which the livestock industry is highly developed and in the feces of sick and healthy animals. Nothing has yet been found in which type F occurs consistently. This bacterium has been isolated from tinned meats, fish pies, and the organs of persons who died of necrotic enteritis in Germany (1946-1948).

The morphology, biology, cultural characteristics, and toxicity of Cl. perfringens are discussed in detail on page 909.

Laboratory Diagnosis of Diseases Caused by Cl. Perfringens

An investigation must be made for Cl. perfringens in all sporadic and epidemic cases of food poisoning, in order to confirm or exclude these bacteria as an etiological factor. This investigation has a twofold purpose: first, direct detection of the toxins of Cl. perfringens in the patient's feces or the contents of the small intestine and the peritoneal fluid in the case of cadavers and, second, detection of the causative agent.

Material for examination; taking of samples

Vomitus, the fluid obtained by gastric lavage, 10 ml of blood from

the ulnar vein, or 200-300 g of feces can be examined.

The foodstuffs which can cause poisoning include meats, fowl, fish, canned fruits and vegetables, meat and fish soups and other dishes, sauces, sausages, grease, milk, etc.

The following samples are taken from cadavers: 200-300 g of the contents of the small intestine, 100 ml of peritoneal fluid (to be investigated for toxins), the intestinal and mesenteric lymph nodes, pieces of the liver, and 10 ml of blood from the heart.

In convalescence blood is taken from the ulnar vein (10-14 days after the onset of the illness), to be investigated for specific antitoxins in the serum (this investigation can be conducted only at scientific-research institutes of microbiology, in accordance with the special instructions for cases of mass poisoning).

All the samples are collected in sterile, hermetically sealed vessels and sent to the laboratory as soon as possible. In individual cases the material can be stored in a refrigerator for no more than 24 hr.

Bacteriological examination

Direct examination for toxins. A large portion of the patient's feces or the intestinal contents of the cadaver is carefully ground in a mortar with one or two parts of physiological solution, quickly filtered through coarse filter paper, and centrifuged at 5000 rpm for 30 min to obtain a clear or slightly opalescent liquid. Peritoneal fluid is freed of particles by centrifuging. If no centrifuge is available all these materials can, after preliminary coarse filtration, be filtered through a Zeitz filter or a fine simple paper filter. This procedure is best carried out at low temperature (4°), in order to retard inactivation of the toxins. Filtration usually reduces toxin strength to a greater extent than centrifuging.

During the analysis the clear liquids thus obtained are stored at 4° in test-tubes or flasks sealed with rubber stoppers.

In order to determine the toxins portions of 0.5 and 0.1 ml of the centrifugate are administered intravenously (or intraperitoneally) to two pairs of white mice weighing 22-24 g and 0.1 ml is given intracutaneously to a white guinea pig or rabbit. The animals' behavior is observed for the first 3-4 hr. If they become ill and die, exhibiting convulsions, during this period or if signs of necrotic changes develop at the site of the intracutaneous injection after 40-50 min, a neutralization reaction must be set up with Cl. perfringens antitoxic sera: portions of 2.4 ml of the liquid under investigation are poured into 6 test-tubes and portions of 0.6 ml of antitoxic sera to Cl. perfringens types A, B, C, D and E are added to 5 of them. The sixth tube serves as the control (Table 41). Each ml of serum should contain no less than 100 units of antitoxin.

The mixtures are incubated at 20° for 40 min and portions of 0.5 ml or 1 ml from each tube are administered intravenously to 2 white mice weighing 22-24 g. When mice are not available the reaction can be set up with guinea pigs or rabbits; in this case 0.2 ml of the mixture is injected intracutaneously into the skin of the side.

The neutralization reaction is preliminarily evaluated during the first 5 hr. A final evaluation is made after 24 hr.

It is customarily assumed that:

1) the principal lethal toxin of Cl. perfringens type A (lecithinase C) has been detected if the mice which received the mixture of test fluid and type A serum survive, while the control animals die (the results obtained by administering the other mixtures are of no significance).

There is no necrosis at the site of the intracutaneous injection

TABLE 41

Scheme of Neutralization Reaction for Principal Lethal Toxins of Cl. Perfringens

1	2	3 Антитоксические сыворотки					5
		A	B	C	D	E	
№ пробирки	Количество исследуемой жидкости, мл	4 α-антитоксин, мл	α + β + ε-антитоксин, мл	α + β-антитоксин, мл	α + ε-антитоксин, мл	α + ι-антитоксин, мл	Физиологический раствор, мл
1	2,4	0,6	—	—	—	—	—
2	2,4	—	0,6	—	—	—	—
3	2,4	—	—	0,6	—	—	—
4	2,4	—	—	—	0,6	—	—
5	2,4	—	—	—	—	0,6	—
6	2,4	—	—	—	—	—	0,6

1) Test-tube No.; 2) quantity of test fluid, ml; 3) antitoxic sera; 4) antitoxin, ml; 5) physiological solution, ml.

of the mixture of test fluid and type A serum, but necrosis does develop at the site of the injection of liquid from the control tube (No. 6).

An additional proof of the presence of lecithinase is the development of hematuria in the control animals, a positive reaction with lecithovitellin, or hemolysis of human, mouse, sheep, or rabbit erythrocytes (see the section headed "Special Investigative Techniques");

2) the principal lethal toxins of Cl. perfringens type B (β and ε toxins) have been detected if the mice are protected by type B serum and the other mice used in the experiment die. Intracutaneous injection of the mixture of test fluid and type B serum does not produce necrosis, the skin becoming necrotic in all other cases;

3) the principal lethal toxin of Cl. perfringens types B, C, and F (β toxin) has been detected if the mice which received the mixture of

test fluid and types B and C sera survive and the other animals die.

Intracutaneous injection of the mixture of test fluid and types B and C sera does not produce necrosis, the skin becoming necrotic in all other cases;

4) the principal lethal toxin of Cl. perfringens type D (ε toxin) has been detected if the mice which received the mixture of test fluid and type D (and possibly B) serum survive and the other animals die.

TABLE 42

Scheme of Neutralization of Test Fluid with Cl. Perfringens Antitoxic Sera

Действие в реакции нейтрализации антитоксических сывороток типа 1										Контроль, смесь испытуемой жидкости с физиологическим раствором 3		4 Обнаруженный токсин
A		B		C		D		E				
α-антитоксин 2		α + β + ε-антитоксины		α + β-антитоксины		α + ε-антитоксины		α + ι-антитоксины				
0	0	ô	ô	ô	ô	ô	ô	ô	ô	+	+	Лецитиназа (альфа-токсин) 5
+	+	0	0	+	+	+	+	+	+	+	+	Смесь бета- и эпсилон-токсинов 6
+	+	0	0	0	0	+	+	+	+	+	+	бета-токсин 7
+	+	0	0	+	+	0	0	+	+	+	+	эпсилон-токсин 8
+	+	+	+	+	+	+	+	0	0	+	+	йота-токсин 9

Symbols: 0) Mouse survives, no necrosis after intracutaneous administration to guinea pig; +) mouse dies, necrosis develops after intracutaneous administration to guinea pig; ô) mouse dies or survives, necrosis not always noted after intracutaneous administration to guinea pig.

1) Neutralizing action of antitoxic sera of type; 2) antitoxin; 3) control – mixture of test fluid and physiological solution; 4) toxin detected; 5) lecithinase (α toxin); 6) mixture of β and ε toxins; 7) beta toxin; 8) epsilon toxin; 9) iota toxin.

Intracutaneous administration of the mixture of test fluid and type D (and possibly B) serum does not cause necrosis, the skin becoming necrotic in all other cases;

5) the principal lethal toxin of Cl. perfringens type E (toxin) has been detected if the mice which received the mixture of test fluid and type E serum survive and the other animals die.

Intracutaneous injection of the mixture of test fluid and type E serum does not produce necrosis, the skin becoming necrotic in all other cases (Table 42).

Isolation of a pure culture of Cl. perfringens. In order to detect Cl. perfringens the material under investigation is cultured on one of Weinberg's liquid nutritive media (page 967), casein-mushroom medium (page 964), meat broth containing cotton and 0.5% glucose (page 968), sugar blood agar (page 970), or bile agar (page 970).

Toxic foodstuffs, feces, vomitus, the contents of the small intestine, lymph nodes, pieces of the liver, and other materials are ground in separate mortars with one or two parts of physiological solution to obtain a homogeneous suspension. Each suspension is divided into two parts, one of which is heated at 80° for 15 min (blood from a patient or cadaver is cultured without heating). The heated and unheated material is then cultured in 1 ml portions on a liquid nutritive medium in test-tubes or in 5 ml portions in flasks.

The heated suspensions are also streak-cultured with a platinum loop in Petri dishes containing blood agar and in long columns of Wilson-Blair's medium and of agar in slender test-tubes.

The cultures are incubated for 24 hr at 37° in anaerostatic devices, under a vacuum (400 mm Hg). When such devices are not available the inoculated dishes are flooded with a layer of no less than 2 mm of agar, which is melted and cooled to 45-50°, and then placed in an ordinary heater.

The character of the growth in the media is observed. The individual strains of Cl. perfringens may develop rapidly (within 4-8 hr) in

liquid and solid media, exhibiting intensive gas formation, darken and split Wilson-Blair's medium, and produce disk-like discontinuities in long agar columns in narrow test-tubes. Colonies which turn green when exposed to the air are formed on dishes containing blood agar within 12-16 hr; in the majority of toxigenic strains these are surrounded by one or two zones of hemolysis on blood agar and by a lustrous precipitation zone on bile agar.

In order to determine the thermostability of strains of Cl. perfringens types A and F, portions of 1 ml of suspensions of foodstuffs, feces, or intestinal contents are poured into 4 test-tubes containing a liquid nutritive medium (Weinberg's medium, casein-mushroom hydrolyzate, or meat broth containing a piece of cotton) and 0.5% glucose. The first tube is heated in a water bath at 100° for 1 hr, the second for 2 hr, the third for 3 hr, and the fourth for 4 hr. The tubes are then cooled to 37° and incubated in a heater. The growth in them is observed over a period of 10-15 days. The thermostability of the spores is expressed in hours. For example, if growth is observed only in the first tube, the thermostability of the strain is 1 hr, if growth is observed only in the second tube it is 2 hr, etc.

All the cultures which exhibit intensive growth accompanied by gas formation must be investigated. Smears are prepared from mature cultures, Gram-stained, and examined microscopically. Those cultures in which large numbers of gram-positive rods similar to Cl. perfringens are found are checked for toxicity. If the initial anaerobic cultures are heavily contaminated with common microorganisms the strain must be purified and obtained in isolated colonies; it is then transferred to a liquid medium and its toxigenicity is checked.

The strain is purified by transplanting the contaminated culture to a solid medium (glucose-blood agar, bile agar, Wilson-Blair's medium,

or a long agar column prepared by Veyon-Vinial's method).

In order to determine whether the strain belongs to the species Cl. perfringens, its activity with respect to litmus milk, carbohydrates, and gelatin is checked (see Table 72).

The toxicity of the culture fluids obtained from the strain is tested before and after activation with trypsin. For this purpose 2 ml of 5% trypsin is added to 2 ml of a centrifugate of the culture and the mixture is incubated in a heater at 37° for 30 min.

For details of the neutralization reaction with inactivated and trypsin-activated culture fluids see page 540.

An increase in the toxicity of the culture fluid after treatment with trypsin (more rapid death of mice or enlargement of the zone of dermal necrosis in guinea pigs) indicates that , or ε protoxins are present; these can be identified with only two antitoxic sera, types D and E. The type to which the strain belongs is determined by neutralizing its toxins with antitoxic sera. Types A, D, and E are easily identified in this manner. Differentiation of strains which produce β toxins (types B, C, and F) requires special supplemental methods for determining spore thermostability and the agglutination of types B and F (see below).

Many strains of type B are easily differentiated from types C and F by their ability to produce an ε toxin. In contrast to type F, strains of types B and C do not have thermostable spores. Finally, it must be noted that agglutinative sera of types B and F agglutinate and precipitate the majority of strains of the type in question.

Diseases caused by Cl. perfringens can be diagnosed bacteriologically by the neutralization reaction more rapidly (within 3-5 hr) if toxin of sufficient strength (no less than 10 MLD/ml for white mice) is detected in the feces, intestinal contents, or peritoneal fluid. When

no toxin can be found in these materials complete bacteriological analysis for Cl. perfringens requires from 8 to 24 hr.

The following accelerated scheme can be used to reduce the time and work involved in the analysis:

1) only the material heated at 80° for 15 min is cultured on litmus milk containing 0.5% glucose, which permits a rough determination of Cl. perfringens within a few hours from the change in the milk;

2) the thermostability of the Cl. perfringens spores in the material under investigation is checked by boiling 1 ml of suspended material in 10 ml of litmus milk containing 0.5% glucose and then incubating the mixture in a heater at 37°;

3) thewhey of milk clabbered by a growing culture of Cl. perfringens is investigated for toxicity before and after activation with trypsin;

4) the neutralization reaction is set up only with type A antitoxic serum, since the majority of the naturally encountered strains of Cl. perfringens belong to this type;

5) a complete study is made only of those strains which have thermostable spores and those whose toxins are not neutralized by type A serum.

Cl. perfringens is detected in the external environment by complete or accelerated bacteriological analysis involving isolation of pure cultures, determination of their toxicity, and identification of the type of bacterium.

In order to determine the extent to which water and various liquids are contaminated with spores and vegetative cells of Cl. perfringens successive ten-fold dilutions are made up from the liquid under investigation and 1 ml of each dilution is cultured in 9 ml of molten Wilson-Blair's agar cooled to 50°.

Solids (soil, foodstuffs, etc.) are ground in a mortar with one or two parts of physiological solution, ten-fold dilutions in physiological solution are made up from the resultant suspension, and 1 ml of each dilution is cultured on the same media and in the same order as for liquids.

The cultures are incubated in a heater at 37° for 24 hr.

The approximate titre of Cl. perfringens is determined from the last tube containing from 10 to 50 black colonies on the Wilson-Blair medium. The number of colonies in this tube is multiplied by the dilution, thus obtaining the total number of Cl. perfringens per ml or g of material.

Since many species of bacteria may alter Wilson-Blair's medium, it is necessary to isolate several colonies, check the morphology of the cells, and determine the behavior of isolated cultures with respect to litmus milk, glucose, galactose, levulose, lactose, sucrose, mannitol, and dulcitol.

Special investigative techniques for determination of certain types of Cl. perfringens. In food poisonings caused by Cl. perfringens type A it is necessary to determine the spore thermostability of strains isolated from foodstuffs and feces, as well as their ability to produce lecithinase and θ hemolysin.

In order to check spore thermostability 1 ml of fresh culture is transferred to 10 ml of a liquid medium containing pieces of marrow or meat (pH 8-8.2), but no glucose. A day-old culture of Cl. perfringens raised at 37° is stored for 5 days at 20° in a dark place. Smears are prepared from the cellular detritus and examined microscopically for free spores. Cultures containing large numbers of free spores are shaken and 1 ml portions are transferred to 4 test-tubes containing fresh medium and 0.5% glucose. All the tubes are heated in a water bath and

incubated in the manner described for determination of spore thermostability in samples taken from patients.

The ability of the strain to produce lecithinase is checked by reacting it with lecithovitellin (a 5% solution of egg yolk in physiological solution) and in a hemolysis reaction with mouse or human erythrocytes.

The simplest method for qualitative determination of lecithinase is to culture Cl. perfringens on yolk agar (agar containing a 5% emulsion of egg yolk and 1% glucose) and glucose-blood agar. The Cl. perfringens colonies, which produce lecithinase in yolk agar, are surrounded by a lustrous precipitation zone, while on blood agar they are surrounded by a zone of incomplete hemolysis.

For quantitative determination of lecithinase successively decreasing two-fold dilutions of Cl. perfringens culture fluid are made up in one-milliliter portions of physiological solution containing 0.005 M of calcium chloride. A total of 0.9 ml of the culture fluid and 0.1 ml of Cl. perfringens type A specific diagnostic serum are mixed in a separate control tube. The tubes are incubated at 20° for 40 min and 0.2 ml of lecithovitellin (a 5% emulsion of egg yolk in physiological solution and 0.005 M calcium chloride heated at 60° for 20 min and filtered through a Zeitz or talc filter to obtain a clear opalescent liquid) is then added to each tube. The mixture is incubated in a heater at 37° for 2 hr. If the liquid becomes cloudy and a white turbidity forms near the surface, the control reaction being negative, lecithinase is present. The last test-fluid dilution which decomposes the lecithovitellin is the lecithinase titre.

It is convenient to use the following method for lecithinase determination when a large number of cultures are to be investigated.

Yolk agar containing 0.005 M calcium chloride is made up and poured

into Petri dishes in 2 mm layers. After the agar has cooled a sterile metal tube with a pointed end and an outside diameter of 0.5 cm is used to punch symmetrically arrayed holes in it.

For this purpose the dish is placed on a drawing and the holes are made to correspond to circles in the diagram. The wells are filled with centrifugates of the Cl. perfringens cultures to be studied and are each labeled with an appropriate numeral or letter. The dish is covered, placed in a heater for 2 hr, and then left to stand at room temperature for 24 hr.

The lecithinase activity is expressed in millimeters, being determined from the extent of the lustrous precipitation zone. The specificity of the reaction can be checked with type A antitoxic serum, which is preliminarily added to the agar or to the appropriate wells.

The ability of Cl. perfringens to produce θ hemolysin is determined by studying cultures raised for 6-8 hr in a liquid medium not containing pieces of meat.

The successive two-fold dilutions of the culture fluid under investigation are made up with a phosphate-chloride buffer (0.038 M disubstituted sodium phosphate, 0.032 M $NaH_2PO_4 \cdot H_2O$, and 0.078 M sodium chloride at pH 6.8) containing 0.016 M l-cysteine hydrochloride. The phosphates are added to suppress the action of the lecithinase, while the cysteine is necessary to reduce the oxidized θ hemolysin.

The test-tubes containing the various dilutions are kept at 37° for 25 min to reduce the θ hemolysin and 0.2 ml of a 5% suspension of horse erythrocytes in physiological solution is then added to each tube. The θ-hemolysin titre is established from the dilution of the test fluid caused by lysis of approximately half the horse erythrocytes (as determined from the erythrocyte sediment on the bottom of the tube).

In order to differentiate Cl. perfringens types B, C, and F, which

produce the lethal, necrotic β toxin, it is necessary to determine the thermostability of their spores (type F forms thermostable spores) and to set up an accelerated agglutination reaction on slides and a precipitation reaction (in test-tubes) with types B and F specific agglutinative (precipitative) sera prepared in the diagnostic laboratory (see page 135).

The antigen for the precipitation reaction is prepared by boiling a 20-billion-cell suspension in physiological solution or by decomposing it with ultrasound for 10 min.

Before it is overlayered on the whole serum the antigen is centrifuged to obtain a clear liquid.

Detection of any lethal Cl. perfringens toxin in feces, intestinal contents, or peritoneal fluid in the presence of clinical symptoms of food poisoning must be regarded as proof of the etiology of the illness.

When direct identification of the toxin is impossible, detection of any highly toxigenic Cl. perfringens of types A, B, C, D, E or F in foodstuffs, vomitus, or other materials from the patient or cadaver is of great diagnostic value.

Isolation of thermostable, slightly toxigenic strains of type A from foodstuffs or specimens from patients must be regarded as proof that these bacteria are the etiological factor in the infection when analyses for other pathogenic microorganisms (Salmonella, Staphylococci, Cl. botulinum, etc.) are negative.

BOTULISM

Prof. K.I. Matveyev, T.I. Bulatova, Candidate of Medical Sciences, T.I. Sergeyeva, Candidate of Medical Sciences, N.N. Sitnikova, and V.A. Sidorova

Botulism (from the Latin botulus, or sausage), or sausage poisoning, is a special form of food poisoning etiologically associated with sporogenic anaerobic bacteria.

Cases of botulism in the USSR now develop almost exclusively as a result of consumption of home-canned foodstuffs (fish, ham, etc.). In the Baltic republics, the Ukrainian SSR, and the Byelorussian SSR botulism is most frequently caused by pork sausage and ham, while in the Caspian and Azov regions it most often results from home-salted and home-cured red-meat fish (sturgeon, white sturgeon, and starred sturgeon). Outbreaks of botulism caused by consumption of salted Baykal whitefish are observed in the Baykal region and other areas of Siberia. Outbreaks have occurred in the northern and far-eastern regions as a result of consumption of canned fish, particularly flounder.

Commercially canned meats, fish, and vegetables rarely cause botulism, but their production and distribution should be carried out under strict sanitary inspection.

Botulism is a food poisoning caused by botulism bacteria and their toxins. A total of 6 types of botulism bacteria are now known: Cl. botulinum types A, B, C, D, E, and F. Types A, B, and E are of the greatest significance in human pathology in the Soviet Union. Type C has been detected in the soil and in botulism of mink in the USSR.

Morphology of Cl. botulinum

The causative agents of botulism are small rods from 4 to 9 μ long and from 0.6 to 0.8 μ wide, with rounded ends, which form subterminal or terminal spores; spore-bearing bacteria resemble tennis rackets (Figs. 39, 40, and 41). The bacteria in young cultures are gram-positive, while gram-negative specimens predominate in smears prepared from older cultures. The rods are mobile and have from 4 to 35 flagella arrayed over the entire surface of the cell (are peritrichal; Figs. 42 and 43). Their mobility is rapidly attenuated when they are exposed to air. Cl. botulinum does not form capsules.

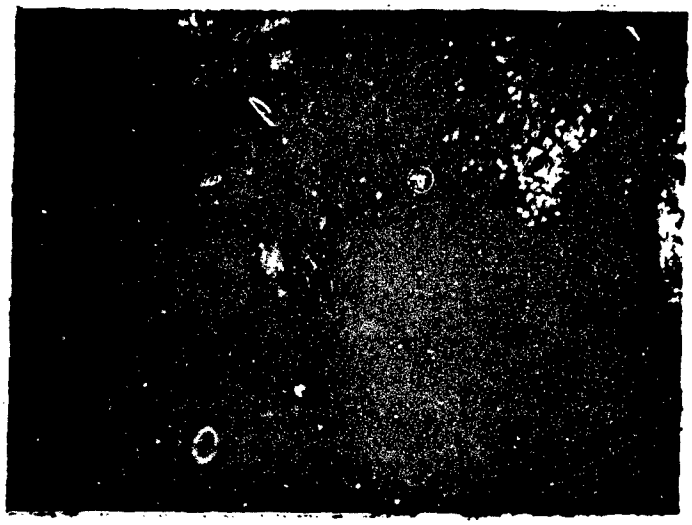


Fig. 39. Cl. botulinum A in a 48-hr culture on agar containing rabbit blood. × 1900.

Biology of Cl. botulinum; culturing properties

The causative agents of botulism are strict anaerobes. In special liquid nutritive media their growth is sometimes, although not always, accompanied by development of turbidity and formation of gas; the culture gives off an odor of rancid butter. The optimum growth temperature for types A, B, C, and D is 35°, while that for types E and F is 28-30°.

When cultured in long agar columns Cl. botulinum forms lentiform colonies or colonies resembling balls of cotton with thickened centers (Fig. 44). When cultured on blood agar it produces clear colonies with a diameter of several millimeters, smooth or serrate edges, and a lustrous surface, which are surrounded by zones of hemolysis (Figs. 45, 46, and 47). Cultures can be raised on Petri dishes in anaerostatic equipment, after the oxygen has been removed by physical or chemical means.

When transferred to sugar-containing media fresh cultures of Cl. botulinum may ferment glucose, levulose, fructose, maltose, dextrin,

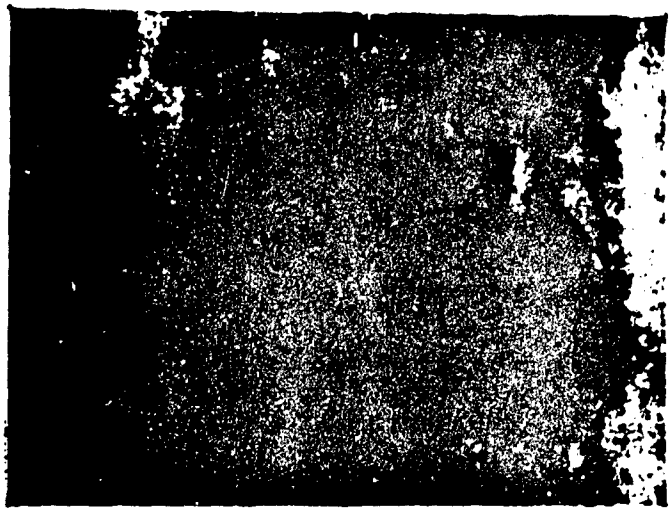


Fig. 40. Cl. botulinum E in 48-hr broth culture. x 1800.

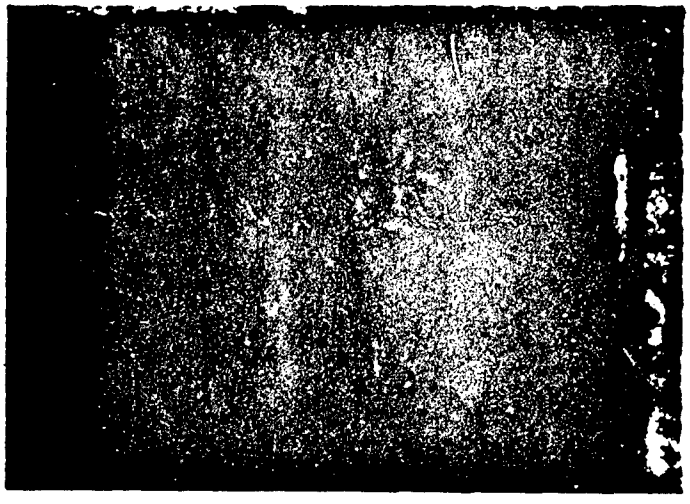


Fig. 41. Cl. botulinum B in 6-day culture on liver agar. x 1800.

salicin, adonitol, and inositol to form gas and acid, but do not decompose galactose, sucrose, dulcitol, mannitol, arabinose, or rhamnose; these characteristics are, however, very unstable and cannot be used as a basis for identifying Cl. botulinum or for differentiating its individual types.

Cl. botulinum types A and B have strong proteolytic properties and

Fig. 42. Cl. botulinum A in two-day broth culture. Flagella visible. Electron-microscopic photograph. x 11,500.

Fig. 43. Cl. botulinum B. Flagella visible. Stained by Levinson's method. x 1800 (after Weinberg, Nativel, and Prevo, 1937).

liquefy pieces of liver or ground meat in media of the Tarocci type. Type F has less pronounced proteolytic abilities, while types C, D, and E are capable of only weak proteolysis (Table 43).

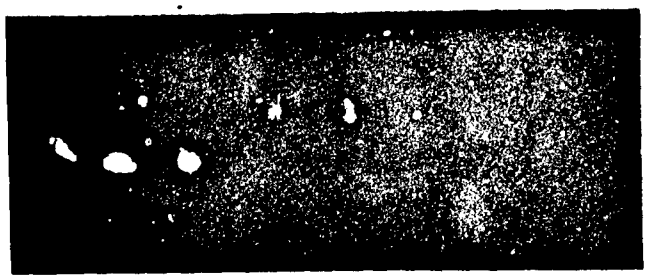


Fig. 44. Flaky and lentiform colonies of Cl. botulinum B in agar column. Day-old culture. × 2.

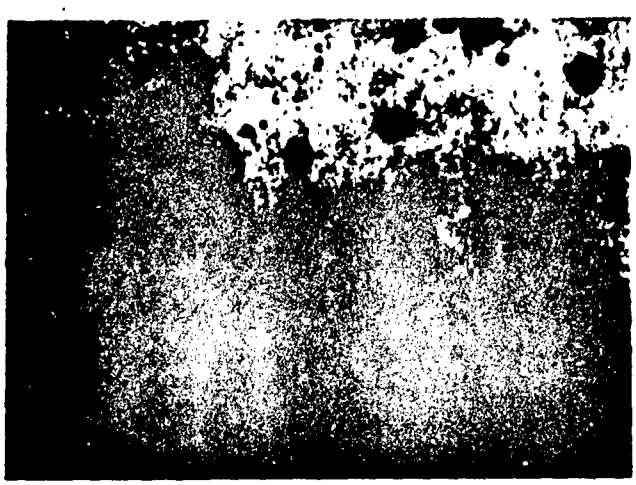


Fig. 45. Smooth form of Cl. botulinum A colonies on agar containing rabbit blood and glucose. Three-day culture. × 8.

Toxin formation

Under anaerobic conditions Cl. botulinum produces powerful toxins in various canned foodstuffs, forage, the human body, animals, and artificial nutritive media.

The toxins of Cl. botulinum are quite resistant to external influences (temperature, light, pH, agitation, concentrations of various salts, etc.). They break down when boiled for 10-15 min. Toxin-containing material must be stored in darkness at 4-5°.

The proteolytic enzymes of the gastrointestinal tract (pepsin and

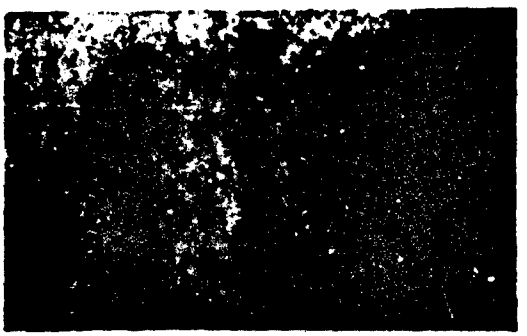


Fig. 46. Rough form of Cl. botulinum B colonies on liver agar. Two-day culture. × 16.

trypsin) do not decompose types A, B, C, D and F toxins and greatly intensify the activity of type E toxin. This is due to the fact that Cl. botulinum produces prototoxin in large quantities and toxin in smaller quantities. The prototoxin is activated by pancreatine and trypsin, as well as byCl. sporogenes protease, so that high-strength toxins neutralizable with specific serum are obtained from weakly toxic filtrates.

Resistance to physical and chemical agents

Cl. botulinum occurs widely in nature. This bacterium consistently inhabits the soil, whence it enters water, fresh fruits and vegetables,

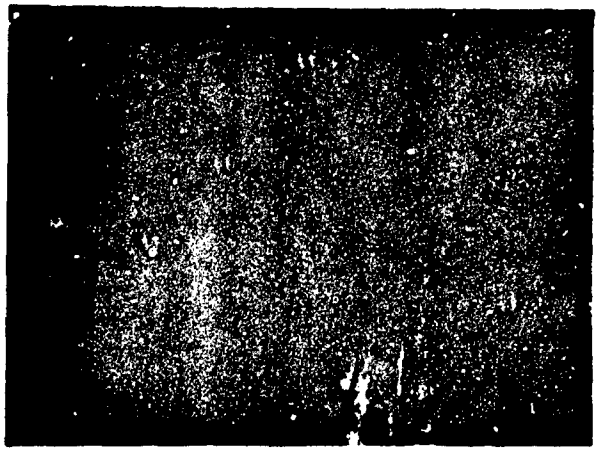


Fig. 47. Colonies of Cl. botulinum E on liver agar, Two-day culture. × 16.

TABLE 43

Saccharolytic and Proteolytic Characteristics of Cl. botulinum Types A, B, C, D, E, and F

1 Наименование субстрата	2 Тип Cl. botulinum					
	A	B	C	D	E	F
3 Желатина	+	+	(+)	(+)	(+)	+
4 Белок	+	+	—	—	—	+
5 Лакмусовое молоко	+	+	—	—	—	+?
6 Глюкоза	кг	кг	(к)	к	кг	кг
7 Галактоза	—	—	(кг)	(к)	—	—
8 Лактоза	—	—	—	к	—	
9 Левулеза	кг	кг	—	к	кг	кг
10 Мальтоза	кг	кг	(кг)	(к)	кг	
11 Маннит	—	—	—	—	—	
12 Дульцит	—	—	—	к	—	кг
13 Фруктоза	кг	кг	(кг)	к	кг	(кг)
14 Глицерин	(кг)	(кг)	(кг)	к	(кг)	(кг)
15 Салицин	(кг)	(кг)	—	—	(кг)	(кг)
16 Декстрин	(кг)	(кг)	(кг)	(к)	—	+
17 Сероводород	+	+	—	+	(+)	

18

Symbols: kg) Forms acid and gas; (kg)) not all strains form acid and gas; +) liquefaction or peptonization, positive reaction; –) no liquefaction or peptonization; (+) slow or irregular reaction.

1) Substrate; 2) type of Cl. botulinum; 3) gelatin; 4) protein; 5) litmus milk; 6) glucose; 7) galactose; 8) lactose; 9) levulose; 10) maltose; 11) mannitol; 12) dulcitol; 13) fructose; 14) glycerine; 15) salicin; 16) dextrin; 17) hydrogen sulfide; 18) kg.

foodstuffs, and forage and thus the intestines of humans and animals, birds and fish, where it multiplies.

In soil, artificial media, foodstuffs, and fodder the causative agent of botulism forms spores resistant to chemical and physical agents. These may remain viable for decades when dried. When conditions are favorable the spores germinate and the bacteria multiply and produce toxin.

The spores of Cl. botulinum can withstand very low temperatures, surviving even at -190°. They easily endure a temperature of -16° for a year, although some of the spores break down and release their toxin; foodstuffs containing large numbers of spores may consequently be poisonous after freezing.

The resistance of spores to high temperature is very great and depends on the composition of the substrate; they are far more resistant in substrates containing considerable quantities of fat than in fat-poor substrates.

The spores of Cl. botulinum are also very resistant to various chemical bactericides; 10% hydrochloric acid requires an hour to kill them at room temperature, while 40% formalin diluted to 1:2 requires 24 hr and they may remain viable in ethyl alcohol for 2 months.

Laboratory Diagnosis of Botulism

Investigation of the patient's excreta and the foods which he has eaten is intended to establish the type of Cl. botulinum causing the illness, to confirm the clinical diagnosis, and to permit prescription of proper therapy.

Material for examination; taking of samples

The fluid obtained by gastric lavage and the patient's vomitus, blood, and feces are examined in all illnesses with symptoms characteristic of botulism.

Blood must be taken before the patient is given therapeutic serum, 6-8 ml being drawn off from a vein into a sterile test-tube. The blood can be mixed with 4% sodium citrate in a ratio of 3:1.

A total of 50-100 ml of lavage water and 50-60 g of feces are collected in glass jars sealed with rubber or cork stoppers; preservatives should not be added. The material to be examined must be stored in a refrigerator, since botulin toxin decomposes at room temperature.

Pieces of the liver weighing 50-60 g, segments of the small intestine, and the stomach and its contents are taken from cadavers for examination. When there is a suspicion that botulin toxin has entered through the respiratory passages 50-60 g of the lungs, the liver, and 8-10 ml of blood must be taken from the cadaver for investigation. Organ samples are taken under sterile conditions; the surface of the organ is cauterized with a red-hot spatula and pieces are then cut from its interior with sterile scissors. The specimens from each organ are placed in separate jars, which are sealed with stoppers or lids.

Various canned foods are investigated to detect Cl. botulinum and its toxins: 1) raw materials are checked to be certain that they are safe for preparation of canned products. If they are found to be infected with Cl. botulinum their use for preparation of canned goods is prohibited; 2) the output of enterprises producing canned meats, fish, and vegetables is checked in accordance with the instructions for bacteriological checking of industrial processes in the preparation of canned meats, meat-vegetable products, vegetables, and fish prepared by the Scientific Research Institute of the Canning Industry and adopted by the State Sanitary Inspection Commission USSR in 1951; 3) products in stores and restaurants and leftovers are investigated for Cl. botulinum, as well as for Salmonella, Staphylococci, and the Cl. perfringens group, in all cases of food poisoning with symptoms resembling those of botu-

lism, even mild forms, and in all cases of severe food poisoning.

It must be kept in mind that poisonous foodstuffs contaminated with Cl. botulinum often remain unchanged in appearance, odor, and taste. The specific odor of rancid butter is sometimes noted and the cans containing the goods may be burst.

The material to be examined is collected under sterile conditions in a glass jar, which is sealed with a tightly-fitted glass, rubber, or cork stopper or a screw-on lid. Solids can be wrapped in several layers of waxed or parchment paper for shipment.

When sterile vessels are not available the samples can be collected in any jars which have been preliminarily boiled for 15 min. No less than 100 g of material should be taken for examination and, when possible, several samples should be taken from different areas.

When it is impossible to collect the recommended amount of material smaller samples can be taken. Preservatives should not be added to the specimens.

Labels are pasted onto the samples and they are numbered, sealed, and carefully packed. The material is shipped as soon as possible and kept under refrigeration at the laboratory until it is examined, since if it contains botulin toxin the latter may decompose at room temperature.

Bacteriological examination

Samples received at the laboratory are simultaneously investigated for botulin toxins and Cl. botulinum, as well as for bacteria of the Cl. perfringens group (see page 536).

Detection of botulin toxins. Pieces of food in fluids obtained by gastric lavage are ground in a sterile mortar. Two-thirds of this ground sample is used for detection of botulin toxins and one-third for isolation of Cl. botulinum.

The first portion of the ground sample is left to stand at room temperature for 1 hr to permit extraction. The extract is then filtered through a cotton-gauze filter or centrifuged at 3000 rpm for 15-20 min. The filtrate or centrifugate thus obtained is used to set up a neutralization reaction.

Citrated blood or undiluted blood serum is investigated for botulin toxins with the neutralization reaction; citrated blood can only be administered intraperitoneally to mice.

In order to detect toxins in the patient's feces a sample of 10-15 g is taken, ground in a sterile mortar with two parts of physiological solution, and left to stand at room temperature for 1-1½ hr to permit extraction of the toxin. The liquid is then filtered through a cotton-gauze filter or centrifuged and used to set up a neutralization reaction. Extracts of cadaver organs are prepared in the same manner.

Neutralization reaction. Detection of toxins is carried out with 4 mice weighing 16-18 g. Since the material under investigation may contain any one of the four known types of botulin toxin, a preliminary reaction must be conducted with a mixture of types A, B, C, and E antibotulin diagnostic sera.

Doses of 0.5-0.8 ml of the filtrate or centrifugate to be studied are administered intraperitoneally or intravenously (in the caudal vein; centrifugates can be administered only intravenously) to two mice. Another pair of mice receives a mixture consisting of 0.5-0.8 ml of the material under investigation and 0.2 ml of a mixture of types A, B, C, and E monovalent antibotulin diagnostic sera (0.05 ml of each type of serum). Dry typospecific diagnostic sera, whose titre is no less than 1000 units per ml for each type, are produced for this purpose.

Before administration the mixture of material and sera is left to stand at room temperature for 30 min. The quantities of material admin-

istered with and without serum should be identical. The animals are observed for 4 days, but if they become ill or die before this period has elapsed a neutralization reaction is set up with monovalent sera. When botulin toxin is present the two mice which received the filtrate but no sera die, while the other two mice survive.

The pattern of illness and death is usually very characteristic: accelerated respiration, complete muscular relaxation, retraction of the abdominal muscles ("wasp waist"), paralysis, and agonal convulsions.

When all four mice, i.e., those which received the filtrate with and without serum, die it is necessary to repeat the neutralization reaction with extracts diluted 5, 10, 20, and even 100 times.

Guinea pigs can be used instead of mice in this experiment. One guinea pig is given 0.5 ml of the mixture of A, B, C, and E sera and 3 ml of the filtrate (or centrifugate) under investigation subcutaneously or intraperitoneally, while the control guinea pig is given 3 ml of the material to be tested.

When botulin toxin is detected in this experiment an expanded neutralization reaction is immediately set up with typospecific diagnostic sera to determine the type of toxin. Portions of 2.4 ml of the filtrate to be studied are poured into 5 test-tubes and 0.6 ml of serum is added to each tube, type A to tube No. 1, type B to tube No. 2, type C to tube No. 3, and type E to tube No. 4; 0.6 ml of physiological solution is added to tube No. 5. After the mixture has been left to stand for 30 min at room temperature portions of 1 ml from each test-tube are administered intravenously or intraperitoneally to 2 mice.

A separate syringe is used for each serum. The results are evaluated after 4-6 hr, 24 hr, and 4 days. When botulin toxin is present the mice which received the mixture of toxin and homologous serum survive, while the other mice die. The type of serum which neutralizes the

toxin indicates the type of toxin.

For example, when all the mice except those given the contents of test-tube No. 2 die, the material under investigation contains B toxin.

Detection of Cl. botulinum. In order to detect Cl. botulinum cultures are made in liquid nutritive media. It is best to use casein-mushroom medium, Hottinger's broth, or pepsin-peptone broth containing 0.5% glucose (see media for anaerobes) for the primary cultures. The pH should be between 7.2 and 7.4. It is necessary that meat media contain ground meat or liver and that casein-mushroom medium contain boiled millet and cotton.

Pepsin-peptone medium is most suitable for isolating Cl. botulinum types A and B, while Hottinger's broth is best for isolating types C and E. All the types of Cl. botulinum grow well and produce quite strong toxins on casein-mushroom medium.

Cultures are made in 100-200 ml flasks, into which a layer of vaseline oil 1 cm thick is poured. When only meat media are available in the laboratory the materials to be studied are cultured in 2 flasks containing pepsin-peptone medium and 2 flasks containing Hottinger's broth. The media are heated in a boiling-water bath for 20 min, quickly cooled, and inoculated.

After inoculation one flask containing each medium is heated in a water bath at 80° for 20 min to free it of extraneous asporogenic microorganisms.

If casein-mushroom medium is available the material is cultured in 2 flasks, one of which is heated after inoculation.

All the flasks are incubated in a heater at 35-37°. If Cl. botulinum type E is detected during the investigation further culturing must be carried out at 28°, the optimum temperature for this type.

If the Cl. botulinum in the material under investigation is pre-

dominantly in the vegetative form the majority of the growth will be in the unheated flasks, while if the material contains spores growth will be observed in the heated flasks, permitting immediate isolation of a pure culture in some cases. After culturing all the initial specimens must be stored in a refrigerator until the investigation is terminated. The cultures are examined for Cl. botulinum after 48 hr.

The growth of Cl. botulinum is sometimes characterized by intensive gas formation and proteolysis of pieces of liver or ground meat; these characteristics are, however, not consistent and gas formation and proteolysis may be absent in certain media. Samples (10-15 ml) taken from such flasks under sterile conditions are investigated by preparing smears, staining them by Gram's method or with anaerobic stains, and examining them microscopically.

A neutralization reaction is set up with the culture fluid and polyvalent antibotulin serum of types A, B, C and E. When positive results are obtained a neutralization reaction is set up with each individual serum. If no growth is noted in the flasks after 2 days incubation must be prolonged and the investigation is repeated after 4, 6, and 10 days. When rods with the morphology typical of Cl. botulinum or botulinum toxin is detected in the culture under study it is concluded that the material is contaminated with this microorganism. A pure culture need not be isolated in such cases.

If bacteria with a morphology similar to that of Cl. botulinum are detected in the cultures but they contain no toxin, the culture fluid must be activated with pancreatine or trypsin by the method described on page 566; this permits detection of type E botulin toxin and isolation and study of a pure culture.

Food samples received at the laboratory should be examined for botulin toxin and Cl. botulinum.

The samples should also be investigated for bacteria of the Cl. perfringens group and other anaerobes.

A portion of 25-30 g of the product to be examined is carefully ground in a sterile mortar with physiological solution or with distilled water if it is salted (the material is easier to grind if sterile quartz sand is added). One or two parts by weight of physiological solution or distilled water are used, depending on the character (density) of the product.

A total of 2/3 of the ground sample is used for detection of botulin toxins, while 1/3 is used for isolation of Cl. botulinum.

The portion to be investigated for toxins is left to stand at room temperature for 1-2 hr to permit extraction of the toxins and then filtered through a cotton-gauze filter or centrifuged at 2500-3000 rpm for 30 min. Filtration through a talc filter is impermissible, since talc adsorbs botulin toxins.

A toxin-neutralization reaction is set up with the filtrates or centrifugates thus obtained and antitoxic serum, using the method described above.

Cl. botulinum is detected in foodstuffs by the method described in the section on investigation of specimens from patients. Portions of 5-10 ml of the material under investigation, which is ground with physiological solution or distilled water, are cultured in flasks containing a nutritive medium.

Culturing in long agar columns. Clear 1% agar containing glucose, which is prepared in Martin's broth, Hottinger's broth, or pepsin-peptone broth and poured into test-tubes 0.8 cm in diameter and 15-18 cm long, is used for culturing. Before inoculation the agar is melted and cooled to 45-50°. The tip of a Pasteur pipette is immersed in the material, taking care not to chip the pipette, and successively trans-

ferred from tube to tube; the agar is then thoroughly mixed by rolling the tubes between the palms. The cooled, inoculated tubes are incubated in a heater at 35-37°. A total of 5-8 agar columns is used for each culture. If the growth in the initial culture is not very profuse the Pasteur pipette must be broken and a little of the culture taken up in the capillary; inoculation is then carried out in the manner described above.

If the initial culture contains a massive growth of extraneous microorganisms and few typical spore-bearing botulin rods it is necessary to transfer 5-10 ml of the culture to a test-tube and heat it in a water bath at 80° for 20 min. It is then recultured in the agar columns.

Colonies resembling pieces of cotton, flakes with thickened centers, or regular lentiform disks appear in the test-tubes after 1-2 days. Suspicious colonies are transferred to a liquid or semiliquid nutritive medium containing 0.5% glucose under a layer of vaseline oil in test-tubes. The remainder of the colonies are examined microscopically.

There are two methods for transferring the colonies from the test-tubes.

1. The agar column is punctured from the top with a Pasteur pipette with a broken tip and the desired colony is removed.

2. The bottom of the tube containing the agar column is slightly heated in a Bunsen-burner flame; the vapor produced by the boiling liquid forces the agar out into a sterile Petri dish.

The suspicious colony is removed with a Pasteur pipette with a broken tip.

The culture raised from the colonies in the liquid medium is examined microscopically and checked for toxin by setting up a neutralization reaction in mice.

Culturing on dishes. A drop of the liquid under investigation is

applied to a thin layer of sugar-blood or liver agar in a Petri dish, gently rubbed into the agar with a spatula, and successively transferred to 2-3 other dishes. The dishes are placed right side up in a microanaerostat (of any available brand) and incubated at 35-37°.

In order to maintain a complete vacuum an open Petri dish containing an alkaline solution of pyrogallol is placed on the bottom of the microanaerostat. After 24 hr the Cl. botulinum colonies appear as clear smoky-gray drops 0.1-0.2 cm in diameter, surrounded by zones of hemolysis.

When anaerostatic equipment is not available anaerobes can be raised by the dish method, in which air is simply excluded from the nutritive agar; the inoculated, slightly cooled agar is poured into the lid of a sterile Petri dish and the other half of the dish is placed on it (without permitting it to solidify) in such fashion that the bottom of the dish presses tightly against the surface of the agar. The edge of the dish can be flooded with paraffin. In this technique the surface of the glass is in close contact with the entire surface of the agar, thus creating conditions favorable for growth of the strictest anaerobes in the agar layer between the two pieces of glass.

Colonies raised in this manner are examined with a magnifying glass or a MBS-1 stereoscopic microscope. Some of the colonies are used to prepare smears, which are examined microscopically.

The colonies are removed from the surface of the medium with a loop or Pasteur pipette and transferred to test-tubes containing broth and 0.5% glucose. The cultures thus produced are checked for purity by microscopic examination and for toxins by titration and by setting up a neutralization reaction in mice.

Their proteolytic and biochemical characteristics are also studied (see Table 43).

Activation of Cl. botulinum type E prototoxin. Cultures of Cl. botulinum type E produce a prototoxin, which is of itself not toxic to animals when administered intravenously or subcutaneously.

Activation with proteolytic enzymes, trypsin or pancreatine, is consequently employed to detect this prototoxin.

Preparation of proteolytic enzymes. Pure dry trypsin is dissolved in physiological solution in a ratio of 1:100 (initial solution) before use. For activation this solution is taken in a concentration of 0.5% with respect to the total volume of culture fluid to be activated.

The trypsin can be replaced by dry high-activity (no less than 50 units) medical pancreatine, 4 g of which is dissolved in 100 ml of physiological solution and left to stand in a refrigerator at 4° overnight.

Before use the liquid thus obtained is filtered through a dense paper filter and then through a sterilized Zeitz filter to produce a clear opalescent liquid.

A dose of 0.5 ml of the final solution should not kill a white mouse weighing 17-18 g when administered intravenously. When 0.2 ml of this solution is administered intracutaneously to a guinea pig it usually causes dermal necrosis (over an area of 0.5 × 0.5 cm).

The activity of the preparation must be preliminarily checked with a standard strain of Cl. botulinum type E.

The final pancreatine solution can be stored at 4° for 2 weeks.

Activation of Cl. botulinum type E prototoxin. A 4-5-day culture of Cl. botulinum in a liquid meat or casein medium is centrifuged or filtered. The final trypsin solution is added in a concentration of 0.5% of the total volume of the culture fluid. If pancreatine is used instead of trypsin the culture fluid is mixed half and half with the final pancreatine solution.

The mixtures thus obtained are incubated in a heater at 37° for 1 hr.

After incubation the activated liquid is titrated in white mice by intravenous injection and neutralization with type E serum.

The toxin titre of inactivated culture fluid from the same culture is checked in the same manner.

The results are preliminarily evaluated after 5 hr and a final evaluation is made after 24 hr. The activation index, i.e., the ratio of the number of MLD (minimum lethal doses) in the activated culture fluid to that in the inactivated culture fluid, is then determined. If the activation index is positive and larger than 2 the culture fluid undoubtedly contains prototoxin. A negative activation index indicates that the strain in question does not produce prototoxin and does not belong to type E.

Accelerated methods of bacteriological examination

Determination of botulin toxins by biological testing and the neutralization reaction is the most reliable method, but when the mater-

TABLE 44

Scheme of Experiment for Detection of Botulin Toxin in Blood (Expressed in Volumes)

1 № пробирки	2 Ингредиенты		5 Антиботулинические сыворотки				Взвесь стафилококка 6
	цитратная кровь белого 3	физиологический раствор 4	A	B	C	E	
1	3	1	—	—	—	—	1
2	3	—	1	—	—	—	1
3	3	—	—	1	—	—	1
4	3	—	—	—	1	—	1
5	3	—	—	—	—	1	1

1) Test-tube No.; 2) ingredients; 3) citrated blood; 4) physiological solution; 5) antibotulin sera; 6) staphylococcal suspension.

ial under investigation contains only small doses of toxin the animals do not die for 48-96 hr; moreover, toxin doses sublethal for the exper-

imental animal cannot be detected. Accelerated methods for toxin detection were consequently developed long ago and have now been introduced into practice.

Method of determining phagocytic index (S.M. Minervin et al., 1959). This technique makes it possible to detect the presence of botulin toxin and simultaneously establish its type within a comparatively short period (approximately 3 hr). It is based on the fact that types A and B botulin toxins, when added to blood under test-tube conditions or administered to animals, sharply reduce the phagocytic activity of leucocytes as a result of their own leucocytic properties. The phagocytic capacity of the leucocytes of animals suffering from botulism decreases by a factor of 3, 5, 10, or even 20, depending on the extent of the poisoning. In very severe cases of intoxication the phagocytic index may drop to zero. When homologous antitoxic serum is added to blood exposed to botulin toxin, either under test-tube conditions or in an animal, the phagocytic capacity of the leucocytes is in large measure restored, a phenomenon not observed when heterologous sera are added.

The following equipment and reagents are required for the phagocytosis reaction:

1. Small-diameter agglutination tubes;

2. The smallest-diameter pipettes from a Panchenkov apparatus;

3. 3% sodium citrate;

4. Types A, B, C and E typospecific botulin antitoxic diagnostic sera;

5. A culture of Staphylococcus aureus (strain No. 209);

6. Blood as a source of leucocytes. Either human or guinea pig blood can be used, but rabbit blood gives very unclear results.

7. A fixative for the smears – Nikiforov's solution (equal volumes of ethyl alcohol and ether), methyl alcohol, or absolute ethyl alcohol.

Methyl alcohol is the best of these.

8. Stains for the smears: the best results are obtained with Romanowsky-Giemsa's stain and Romansky's azure-eosin.

Method: the patient's blood is mixed with 3% sodium citrate in a ratio of 2:1 in a test-tube. The experiment is carried out in 5 test-tubes, 5 volumes of the ingredients being reacted in each tube. Table 44 shows the experimental set-up, with the various quantities expressed in volumes. The ratio of the reagents should be the same in all the tubes. Panchenkov pipettes with capillaries of equal length must be used for this purpose. One volume is usually assumed to be 1/4 of the length of the Panchenkov pipettes (from the tip to division 75). The manner in which the ingredients are mixed is of great importance in obtaining clear results. All the ingredients must be introduced at the bottom of the tube, since they may otherwise remain on the wall and dry out without reaching the bottom. During the actual reaction the rack containing the tube is shaken every 8-10 min.

The first step is to introduce 3 volumes of citrated blood into each tube; one volume of physiological solution is then added to tube No. 1 and types A, B, C and E diagnostic sera to tubes Nos. 2, 3, 4 and 5 respectively. After the reagents have been mixed the tubes are placed in a heater for 30 min to permit neutralization of the toxin in the tube to which the homologous serum has been added.

After incubation for 30 min one part of the staphylococcal suspension is added to each of the tubes and they are returned to the heater for 20 min to permit phagocytosis to go to completion. Smears are then prepared from the contents of each tube and used to determine the phagocytic index.

The smears should not be too thin, since a great deal of time is required to calculate the phagocytic index if they are. Fixation with

methyl alcohol should not be too prolonged; it is sufficient to immerse the smears in the alcohol for several seconds and then immediately stand them on edge to dry. The specimens must be stained very carefully, since overstained or understained smears are unsuitable for determination of the phagocytic index. It is necessary to try to make the cocci within the leucocytes clearly visible.

A mechanical stage, which ensures successive inspection of the entire smear, should be employed in determining the phagocytic index. This completely excludes any possibility of counting the same leucocyte more than once.

The phagocytic index is the average number of cocci engulfed by one leucocyte. In calculating the index the number of cocci in 50 leucocytes is counted and the result is divided by 50.

If the patient's blood contains botulism toxin the phagocytic indices of all the blood specimens (test-tubes) are low, except for that portion in which the toxin type corresponds to the serum type added to it.

Determination of the phagocytic index cannot be employed to detect botulin toxins in foodstuff or organs from cadavers, since uncontaminated products and organs give a positive reaction in a large percentage of cases.

Indirect hemagglutination reaction (T. Rytsay, 1956; V.A. Sinitsyn, 1960; R.Ye. Konnikova, 1961; R.Kh. Yafayev, 1961). This reaction is based on the method devised by Boyden (1951), who established that erythrocytes preliminarily treated with a tannin solution adsorb proteins, including antibodies and antigens. It is a new technique and can be used for guideline purposes. A biological test must be simultaneously conducted in mice, as in the phagocytosis-suppression reaction, to check the results obtained.

Essentially, all the authors who have employed this reaction have set it up in the same manner, except for slight differences. The simplest method is that proposed by R.Kh. Yafayev: fresh washed sheep erythrocytes are treated with a 1:20,000 tannin solution in physiological solution. The tannin can be stored under refrigeration for several weeks at a concentration of 1:1000 (10 mg in 10 ml of physiological solution) and then diluted to 1:20,000 before use.

One part of the 1:20,000 tannin solution is added to one part of a 2.5% suspension of the washed sheep erythrocytes. The mixture is placed in a heater at 37° for 10 min and then centrifuged, the supernatant is poured off, and the residue is washed once with ordinary physiological solution. At this point 3 ml of typospecific antibotulin antitoxic serum diluted to 1:10 with buffered physiological solution at pH 6.4 is added to 0.1 ml of the tanninized erythrocytes. Antitoxic sera purified by the Diaferm-3 method or by combined dialysis are suitable for the reaction, regardless of their titre. Types A and B sera with titres of from 2500 to 16,000 units per ml have been tested.

Since not all series of sera give a positive reaction with toxins it is necessary to collect suitable series in preliminary experimets with known toxins.

After 3 ml of the 1:10 serum has been added to the tannin-treated erythrocytes the mixture is placed in a heater at 37° for 1 hr and then centrifuged; the erythrocyte residue is washed twice, first with buffered physiological solution at pH 6.4. The supernatant is discarded after centrifuging. The residue is resuspended (second washing) in buffered physiological solution at pH 6.4 containing inactivated normal rabbit serum diluted to 1:100. The mixture is centrifuged, the supernatant is discarded, and the residue is resuspended in 5 ml of the same buffered solution containing rabbit serum.

The suspension of tannin-treated erythrocytes sensitized with immune serum is used to set up the indirect hemagglutination reaction, adding 0.1 ml to each of a series of tubes containing 0.5 ml of the material to be investigated.

The hemagglutination reaction is set up by pouring portions of 0.5 ml of successive two-fold dilutions of the material into ordinary bacteriological test-tubes. Extracts of foodstuffs and other material objects are prepared in the manner described on page 572. The material to be studied is diluted in buffered phosphate solution* at pH 7.0 containing 0.4% normal rabbit serum.

The reaction is evaluated 2 or 3 hr after incubation at 37° for 45-60 min.

In working with extracts of foodstuffs a positive reaction is also observed in samples contaminated with Cl. sporogenes. The sensitivity of this reaction cannot be precisely established, since it is governed not by the quantity of botulin toxin in the sample, but by the amount of specific antigen, which incorporates the active toxin and soluble, nontoxic bacterial antigens. Types A and B botulin toxins give cross-reactions with the corresponding sera. Certain series of botulin antitoxin are absolutely harmless to animals but give a positive hemagglutination reaction. This method is consequently of only guideline value.

Some authors recommend the complement-fixation reaction as a guideline for determining botulin toxins. This reaction is not very sensitive to toxins and also indicates specific proteins. It can be used to detect only weak botulin toxins.

FOOD POISONINGS OF STAPHYLOCOCCAL ETIOLOGY

N.P. Nefed'yeva, Candidate of Biological Sciences

Food poisonings of staphylococcal etiology are acute gastrointestinal illnesses which develop after consumption of food containing

staphylococcal enterotoxin.

The clinical symptoms of staphylococcal poisoning include a very short incubation period, occasionally lasting only 30 min but usually 2½-3 hr long, rarely reaching 6 hr. The duration o. the incubation period is undoubtedly influenced by the amount of enterotoxin involved and the individual sensitivity of the victims.

The most frequently observed symptoms are repeated vomiting, sometimes with an admixture of blood and mucus, pain of varying severity in the epigastric region, and, in many cases, signs of cardiac weakness and cold sweats. Diarrhea is not always present, occurring only once or twice in the majority of patients. Body temperature is usually normal, but sometimes subnormal; a rise in temperature is exceptional, lasting only a few hours and never going above 38°. Headaches, chills, and convulsions are often noted. Fatalities are extremely rare, occurring predominately in children, persons weakened by other diseases, and the elderly. Recovery is rapid, the patient feeling well within 2-3 days.

Morphology of Staphylococci

Staphylococci are round or oval cells with a diameter of from 0.5 to 1 μ. They are found in clusters resembling bunches of grapes, but isolated and paired cells and sometimes short chains are encountered in cultures on solid and liquid media. Staphylococci do not form spores or capsules and have no flagella. They are easily stained with aniline dyes and are gram-positive.

Biology of Staphylococci; cultural characteristics

Staphylococci are very easily cultured in vitro, are unselective with respect to nutritive media, and are facultative anaerobes but grow better in the presence of oxygen. The optimum medium pH is 7.2-7.8, the optimum temperature is 37°, and the temperature limits of reproduction lie at 12 and 43°. Addition of blood or glucose to the nutritive

medium accelerates staphylococcal growth. Staphylococci actively form a golden, enamel-white, or lemon-hellow pigment (lipochrome), especially at room temperature (20°) and in the presence of air. Colonies of these bacteria on solid nutritive media have the form of regular disks from 2 to 4 mm in diameter. The margins of the colonies are smooth and their surface is slightly convex and lustrous; they are opaque and have the color of the pigment produced by the strain. Staphylococcal colonies may form clarified zones of hemolysis on blood agar. In liquid media these microorganisms produce strong diffuse turbidity, gradually forming a sediment.

Staphylococci most frequently clabber and then peptonize milk.

These bacteria ferment many carbohydrates (lactose, glucose, maltose, etc.) to form acids but not gas. They release hydrogen sulfide but do not produce indol.

Resistance to physical and chemical agents

Staphylococci are rather stable with respect to physical and chemical agents. They withstand desiccation and exposure to direct sunlight quite well.

In liquid media Staphylococci can endure heating at 70° for 60 min or at 80° for 10 min. Phenol (5%) or mercuric chloride (0.1%) requires at least 15-25 min to kill them. These bacteria are very resistant to sodium chloride in the medium on which they are growing, multiplying even at salt concentrations of 7-10%, and also withstand high sugar concentrations (up to 47%) well.

Staphylococci very quickly produce strains resistant to antibiotics and the number of such strains is continually increasing as various antibiotics come into wide use.

Pathogenicity, virulence, and toxin formation

Staphylococci may be pathogenic or nonpathogenic for man and ani-

mals. A general pathogenetic characteristic of pathogenic Staphylococci is their ability to induce diverse suppurative and inflammatory processes. Rabbits are the laboratory animals most sensitive to Staphylococci. They develop abscesses when the bacteria are injected subcutaneously and soon die when they are injected into the bloodstream.

The difficulty of establishing the etiological role of Staphylococci in food poisonings is attributable primarily to the fact that these bacteria occur very widely in nature; they are consistent inhabitants of humans and animals. Staphylococci can almost always be detected on the skin of a healthy person and often in his saliva, mouth, nose and feces; they can always be isolated from pus produced by inflammations. These bacteria occur in air, water, and sewage and are often found in substantial numbers in various foodstuffs, which can be eaten without unfavorable consequences.

Pathogenic Staphylococci produce a variety of toxins (Table 45).

All the toxins shown in Table 45, with the exception of the last, are true soluble exotoxins. The enterotoxin occupies a different position; its properties have been rather widely studied, but its pharmacological and chemical nature and antigenic characteristics remain a matter of dispute.

TABLE 45

Staphylococcal Toxins and Their Action

Токсин a	b Патогенное действие
1. Гемолизин	Растворение эритроцитов c
2. Дермотоксин	Местное воспаление кожи и нагноение d
3. Летальный токсин	Гибель животного e
4. Фибринолизин	Растворение фибрина f
5. Лейкоцидин	Растворение лейкоцитов g
6. Энтеротоксин	Пищевое отравление; поражение пищеварительного тракта h

1) Hemolysis; 2) dermotoxin; 3) lethal toxin; 4) fibrinolysin; 5) leucocidin; 6) enterotoxin. a) Toxin; b) pathogenic effect; c) dissolution of erythrocytes; d) local inflammation and suppuration of skin; e) death; f) dissolution of fibrin; g) dissolution of leucocytes; h) food poisoning, damage to alimentary tract.

Only certain pathogenic Staphylococci are capable of producing enterotoxin in many types of food without altering its appearance, color, or taste.

The antigenic and immunogenic properties of the enterotoxin are not like those of a true bacterial exotoxin. Experimental immunization of animals produces a slight, irregular acclimatization to the enterotoxin. Active immunization of humans against the enterotoxin is also ineffective.

Man displays the greatest sensitivity to staphylococcal enterotoxin; 0.5 ml of toxic filtrate from a Staphylococcus culture, administered per os, may be sufficient to cause a positive reaction. Individual sensitivity varies, however. Animals are considerably less sensitive to the enterotoxin. Monkeys become ill when given 50 ml of filtrate, while 2-3-month kittens require a dose of 20-25 ml (Jordan and MacBrown).

The question of how often strains capable of producing enterotoxin are encountered among the pathogenic Staphylococci has not yet been conclusively resolved. According to the data of certain authors (Dolman), few Staphylococci form enterotoxin; however, experiments on monkeys have shown that 75% of the strains of coagulase-positive Staphylococci are capable of producing enterotoxin (Ivens et al.).

The principal characteristic of the enterotoxin is its thermostability, which must be taken into account in investigating food poisonings and in taking prophylactic measures. The data of a number of authors have shown that staphylococcal enterotoxin is very thermostable, capable of withstanding not only prolonged boiling but even autoclaving in some cases.

The enterotoxin, when dried, can be stored for up to 8 months with no loss of toxicity. When it is kept at low temperatures (e.g., 4°) it can be stored for more than 2 months without losing its strength.

Staphylococci do not die in frozen foods, but do not form enterotoxin. If enterotoxin is produced in the product before it is frozen it is not inactivated and the product may cause food poisoning.

Alcohol, formalin and chlorine do not decompose the enterotoxin, which is also resistant to acids and alkalies. It remains active over the pH range 4.5-8.2.

Laboratory Diagnosis of Food Poisonings Caused by the Enterotoxins of Pathogenic Staphylococci

Material for examination; taking of samples

In studying staphylococcal toxicosis the following materials are subjected to bacteriological investigation: a) the remains of food eaten by the patients and, if possible, the original products; b) vomitus, lavage fluids, and feces from the victims; c) washings and scrapings from inventory stock, equipment, and containers and washings from the hands and samples of oral and nasal mucus from persons occupied in the production of food.

A wide range of foodstuffs can cause poisoning. Staphylococci multiply equally well in media rich in carbohydrates and in media rich in proteins.

Almost any type of foodstuff can produce food poisoning of staphylococcal etiology.

Not all foods in which Staphylococci multiply well are, however, equally favorable media for enterotoxin formation; these bacteria reproduce well in certain foods, but still not produce enterotoxin.

Milk and milk products play a special role in food poisoning caused by Staphylococci. There are many ways in which milk can be contaminated with pathogenic Staphylococci. It is sufficient to recall that these bacteria are the usual cause of mastitis in cows, a condition in which their milk is heavily infected with Staphylococci.

Cream products play no small part in the etiology of streptococcal food poisoning, both in the Soviet Union and abroad.

Meat and meat products often also figure as sources of staphylococcal food poisoning, such substances being good nutritive media for microorganisms.

Epidemiological observations usually make it possible to establish that the personnel engaged in preparing the food that served as the source of infection included persons carrying pathogenic Staphylococci in the nacopharynx or persons with impetiginous diseases.

All the samples to be investigated are collected in sterile vessels, observing the usual rules of asepsis.

In culturing solid foodstuffs (meat, fish, cheese, etc.) a piece of the product, taken under sterile conditions and weighing 5-10 g, is ground in a sterile mortar with sterile physiological solution or sterile distilled water in a ratio of 1:10. Sterile glass beads or sand is used for more uniform grinding. The resultant mass serves as the initial material for culturing.

Cream, butter, ice cream, melange, etc., are melted before culturing, being placed in a heater or water bath at 45-50° for a short time.

Liquids, including vomitus and lavage fluids, are cultures without preliminary processing. Foods with an acid pH are neutralized with 10% sterile sodium bicarbonate until they give a slightly alkaline reaction.

Bacteriological examination

The bacteriological investigation can be divided into separate stages.

Stage I: The material to be studied is cultured (immediately or after appropriate processing) on two successive solid nutritive media - milk-salt agar (page 904) and blood agar. The cultures are made by thoroughly rubbing 2 drops of the material into the surface of the milk-

salt agar with a spatula, which is then used to transfer the material to the surface of the blood agar. Cultures are simultaneously made in concentration media: salt broth containing 6.5% sodium chloride, salt broth containing 10% sodium chloride, and sugar broth containing 1% glucose. No more than 1/10 of the volume of the nutritive medium is inoculated. All the cultures are incubated at 37° for 18-24 hr.

Cultures are not made in salt media when investigating products containing large quantities of salt (herring, sprat, etc.).

Cultures are not made in sugar broth when investigating products with a high sugar content (cream, ice cream, etc.).

Stage II: a) After 18-24 hr the cultures are inspected and specimens are made up from individual typical colonies, Gram-stained, and examined microscopically. When gram-positive cocci are detected the colonies are transferred to one sector of dishes containing blood agar, in order to obtain a pure culture and check its hemolytic properties. The dishes are incubated at 37° for 18-24 hr.

Note: After incubation at 37° cultures on milk-salt agar must be kept at room temperature for an additional 48 hr, since the elevated salt content of the medium may have a retarding influence on the growth of both the concomitant microflora and the Staphylococci.

b) Regardless of whether or not colonies suspected of being Staphylococci develop on the solid media, material is transferred from the concentration media to one sector of dishes containing blood agar.

The cultures are incubated for 18-24 hr at 37°.

When no growth occurs within 24 hr secondary cultures are made on blood agar prepared with salt broth.

Stage III: The cultures isolated from the solid media and transferred to the blood agar (see Stage II) are studied in the following manner: 1) specimens are prepared, Gram-stained, and examined micro-

scopically. During the examination the morphology of the culture is determined and its purity is checked; 2) the presence or absence of hemolysis is noted; 3) a plasma-coagulation reaction is set up; 4) the pure culture is used to inoculate agar slants in order to determine its phagotype.

The plasma-coagulation test is carried out by thoroughly mixing sterile 5% sodium citrate with fresh rabbit or human blood in a ratio of 1:5. After centrifugation or settling under refrigeration the plasma is drawn off and diluted with physiological solution, adding 4 parts of the latter to 1 part of plasma. Portions of 0.5 ml of the diluted plasma are poured into sterile stoppered test-tubes and mixed with one loopful of a day-old agar culture. One tube of plasma is left uninoculated as a control. The tubes are incubated in a heater at 37° and the results are evaluated after 1, 2, 3, and 24 hr.

When coagulase-positive Staphylococci are not found on the solid media colonies isolated from the concentration media are studied in the manner described above (Stage II).

Pathogenic Staphylococci coagulate blood plasma and usually have hemolytic properties.

TABLE 46

Type Phages

Литическая группа 1	2 Фаги
Первая 3	29, 52, 52A, 79,80
Вторая 4	3A, 3B, 3C, 55, 71
Третья 5	6, 7, 42E, 47, 53, 54, 75, 77, 83A
Четвертая 6	42D
Смешанная 7	187, 81

1) Lytic group; 2) phages; 3) first; 4) second; 5) third; 6) fourth; 7) mixed.

Hemolysis is more reliably detected when Staphylococci are raised on blood agar in a carbon-dioxide atmosphere.

The extent to which the foodstuffs being studied are contaminated may be of importance in evaluating the results of investigations for Staphylococci; it is determined by culturing the material directly on solid media and is customarily designated as "massive colonial growth," "diffuse growth," or "growth of isolated colonies." When there is no growth on the solid media Staphylococci detected on the concentration media are regarded as isolated colonies.

Phage typing of Staphylococci

Use of phage typing in studying Staphylococci has shown that this technique is of undoubted value in establishing their etiological role in diseases and food poisonings. Epidemiological study of staphylococcal infections is no longer possible unless this method is employed.

Phage typing of bacteria isolated from suspected foods and food-preparation personnel in outbreaks of food poisoning has made it possible to identify Staphylococci in a number of cases.

For phage-typing of coagulase-positive Staphylococci it is necessary to have available a set of type phages and bacterial strains sensitive to them. The phages and cultures must be stored in a lyophilized state. Any nutritive medium can be used for titrating the Staphylococci with the phages. The best results are obtained with Hottinger's broth at pH 7.2-7.4 to which 0.4% glucose and 0.004% calcium chloride have been added, meat agar produced by adding 1% agar to this base, and dry standard nutritive media.

There are 22 phages in the type set presently employed (see Table 46).

Before titrating the strains it is necessary to determine the test dose for each phage. The test dose is assumed to be that phage dilution at which complete, continuous lysis of the sensitive strain does not occur even though the preceding dilution causes continuous lysis. In or-

der to determine this dose the phage filtrates are diluted ten-fold (to 1:100,000) and a measuring loop (2 mm in diameter) is used to apply one drop of each dilution to a dish which has been preliminarily dried and inoculated with a 4-6-hr broth culture of the sensitive strain; the dish is then incubated at 30° for 20 hr. If the phage is in dry form 1 ml of Hottinger's broth is added to an ampule opened under aseptic conditions (yielding a dilution of 10^{-1}) and further dilutions are then made up. The strains to be typed are first reacted with test doses of the phages.

Petri dishes with grids of squares drawn on their bottoms are filled with agar, dried at 37° for 30-40 min, and then inoculated with a uniform layer of a types 4-6-hr broth culture, removing the excess with a pipette.

TABLE 47

№ штамма 1	2 № фага						3 Результат типирования
	6	7	42Д	47	53	54	
1	++	±	−	++	++	++	6/47/53/54 +
2	++	++	−	++	++	++	6/7/47/53/54 } одни и тот же штамм 4
3	++	−	−	++	±	±	6/47+
4	−	++	±	++	−	−	7/47+ } 4 идентичен 5 5
5	−	++	±	+	+	−	7+
6	−	−	++	−	−	−	42Д — особый штамм 6

1) Strain No.; 2) phage No.; 3) typing result; 4) same strain; 5) strains 4 and 5 identical; 6) separate strain.

The broth culture is obtained by transferring a 24-hr culture from a nutritive-agar slant. The dishes are again dried for 20-30 min and the phages are applied with a measuring loop (which must be flamed after each phage), one to each square of the grid. For convenience in evaluating the results the phages should always be applied in the same order. After the drops of phage have dried the dishes are incubated at 30° for 18-20 hr or at 37° for 5-6 hr and then held at room temperature for 18-20 hr. If the strain to be typed does not give a lytic reaction with test doses of any of the phages it is checked with the phage fil-

trates at 100 times the test doses.

Instead of drawing the grid on the bottom of the dish it is possible to use a grid drawn on paper. The prepared dish is placed bottom down on the paper, which has an arrow to indicate the direction in which the phages are to be applied.

The results are evaluated on the following scale:

++ more than 50 negative phage colonies, semicontinuous or continuous lysis with or without secondary growth;

+ 20-50 negative phage colonies;

± less than 20 negative phage colonies;

– negative result.

In order to detect nonspecific lysis a drop of physiological solution is applied to one square. The results of the typing are presented as a list of all the phages which lyzed the strain to no less than 2 plusses on the aforementioned scale. If weak lytic reactions took place with other phages a plus sign (+) is appended to the phage formula. For example, if the strain was lyzed by phages 6++, 7++, 47++, 53+, and 70± its phage formula, or phagotype, is 6/7/47+. Different subcultures of the same strain may exhibit variations in their lytic reactions. In order to avoid erroneous evaluation of such reactions it is necessary to type simultaneously all cultures isolated in a case of staphylococcal illness and to make a rather detailed study of the typing results. Attention must be devoted to both strong and weak lytic reactions. An example of the typing of Staphylococci isolated during an outbreak of food poisoning is presented in Table 47.

Analysis of the data in this table shows that strains Nos. 1, 2, and 3 are identical, even though there are qualitative differences in the strength of their lytic reactions; strains Nos. 4 and 5 are identical, while strain No. 6 differs from all the others.

Serological reactions (agglutination) are not set up with the patient's blood in staphylococcal infections.

Detection of Staphylococcal enterotoxin by biological testing

Researchers have suggested the use of a number of animals (monkeys, kittens, mature cats, puppies, frogs, piglets, mice, etc.) and various administration techniques for studying the characteristics of staphylococcal enterotoxin. However, the only laboratory animals which can be reliably recommended for determination of enterotoxicity are monkeys, cats, and kittens.

Biological tests are conducted: 1) to study the ability of Staphylococcus cultures to form enterotoxin when raised on nutritive media; 2) to detect staphylococcal enterotoxin in foodstuffs. Kittens (1½-2 months) and mature cats can be used as experimental animals.

Biological testing in kittens. Kittens are fed the foodstuff to be studied or a 5-day milk culture of previously isolated Staphylococci (20-25 ml) on an empty stomach. If a kitten will not eat the suspected product the latter must be suspended in distilled water (1:1), thoroughly ground, and administered in a dose of 20-25 ml with a pipette or spoon. At least 2 or 3 kittens should be used.

The animals' reaction is regarded as positive when vomiting develops within 30-60 min. The vomiting is sometimes accompanied by diarrhea and general prostration. Vomiting occurring within 5-10 min is nonspecific. The kittens are observed over a period of 4-5 hr. If no reaction appears within this time the test is assumed to be negative.

Biological testing in cats. Enterotoxicity is determined in cats by intravenous injection of the suspected material, a convenient and sufficiently sensitive technique. One drawback of this method, however, is the fact that it can be used only for direct detection of enterotoxin in foodstuffs.

The enterotoxin is obtained with the aid of 0.75% agar in veal broth on Hottinger's broth containing 0.25% glucose (pH 7.4). Dishes inoculated with Staphylococcus cultures are placed in a desiccator containing 20% carbon dioxide and kept in a heater at 37° for 2 days.

In order to obtain a 20% carbon dioxide content in the desiccator its bottom is filled with 1 g of sodium bicarbonate per liter of vessel volume. The dishes are loaded into the desiccator and, leaving its lid slightly ajar, a Mohr pipette is used to drop 10% hydrochloric or sulfuric acid directly on the soda (8-9 ml of acid per g of soda). After the acid has been added the lid is quickly closed and fitted tightly in place.

After 2 days the dishes are removed and 10 ml of physiological solution is poured over the agar, which is mashed to a uniform pasty consistency with a spatula and left to stand at room temperature for 2 hr. The extract is placed in test-tubes and centrifuged to obtain a clear supernatant, which is drawn off. The staphylococcal centrifugate is heated in a boiling-water bath for 30 min and administered to unanesthetized cats through the marginal vein of the ear or the femoral vein in a dose of 0.5 ml per kg of body weight.

Development of vomiting and diarrhea, or of vomiting alone, within 30 min to 3 hr indicates that the material contains enterotoxin. Each cat can be used 3 or 4 times. When negative results are obtained in a cat which has previously been used for similar determinations it is necessary to run a check with a fresh cat.

At present, intravenous administration of suspected material to cats can be employed to detect enterotoxin only in certain foods (canned sprat, cod in oil, cream, semolina, etc.). The product under investigation is diluted to 1:1 with water, the resultant suspension is centrifuged at 2000-3000 rpm for 1-1½ hr, and the centrifugate is drawn off,

heated in a boiling-water bath for 30 min, and administered intravenously to cats in a dose of 0.5-1 ml per kg of body weight.

A cat intravenously injected before the enterotoxin experiment with identical uncontaminated material treated in the same manner serves as the control.

DISEASES CAUSED BY ENTEROCOCCI

A.P. Kalina and Prof. G.P. Kalina

Enterococcus is the common name of Streptococcus faecalis, a member of the genus Streptococcus of the family Lactobacillaceae, and belongs to a special group of bacteria with only a morphological similarity to the other species of Streptococcus (a tendency to form chains). Enterococcus differs widely from the other species of Streptococcus in a number of its biological and cultural characteristics.

The name "enterococcus" was suggested by Thyrselin in 1889. The binomial scientific name under which the bacterium is now known, Streptococcus faecalis, was assigned to it by Andrews and Horder in 1906.

Recently, however, it has been established that the term enterococcus has become a subject of dispute, including a group of similar but not identical microorganisms; in addition to Str. faecalis, at one time or another Str. liquefaciens, Str. zymogenes, Str. durans, Str. bovis, and Str. faecium have been described as enterococci. It is now thought that there are three basic species of enterococci in nature: 1) Str. faecalis, with its varieties var zymogenes and var. liquefaciens; 2) Str. faecium;* 3) Str. durans. As for Str. bovis, a number of its characteristics differ sharply from those of the other species of enterococci and are intermediate between the latter and other Streptococci. However, the existence of a large number of enterococcal strains with diverse biochemical traits, which did not fit into the aforementioned classification, led to the establishment of a broad unclassified group

(Ray et al.) and even of an independent species (biotype in Kolober's classification), Str. innominatus, to which Kolober assigned those varieties whose characteristics make it impossible to include them in one of the species mentioned above.

Enterococci inhabit the intestines of humans and warm-blooded animals where, like E. coli, they are arbitrarily pathogenic. These bacteria may cause acute intestinal affections of the enterocolitis type in children, as well as suppurative processes, wound infections (often in association with other suppurative and purulent microbes), cholecystitis, pancreatitis, osteomyelitis, dental caries, inflammations of the middle ear, and meningitis; a substantial percentage of all cases of endocarditis are attributable to enterococci. A great deal of doubt has recently been cast on the etiological role of enterococci in food poisonings (by the detailed studies made by Silliker and Deible in 1960 and 1963, by Temper in 1962, etc.). In a number of countries enterococci are used as indicator bacteria in evaluating the sanitary-epidemiological condition of environmental objects, particularly water.

Morphology of enterococci

These cocci are usually arranged in pairs or small clusters in cultures on solid media (Fig. 48) and in diplococcoid forms or short chains in smears prepared from liquid media or liquid pathological material. Each coccus in a pair is often slightly elongate and sometimes has tapered ends (a trait previously ascribed exclusively to Pneumococci; see Fig. 54). Enterococci are gram-positive and are characterized by a high degree of polymorphism, especially in specimens prepared from pathological material or from cultures in media to which bacteriostatic substances have been added (even when these substances have no direct bacteriostatic action on enterococci): the cocci become distended and the indiv dual cells of pairs and chains are not uniform in size or

shape. The cells may become so elongated that they resemble coccobacteria or even bacilli (Figs. 49, 50 and 51). Chains may increase in length; in 40% bile, for example, very long chains, taking the form of tangled knots or filaments, may traverse the entire field of view (Fig. 52).

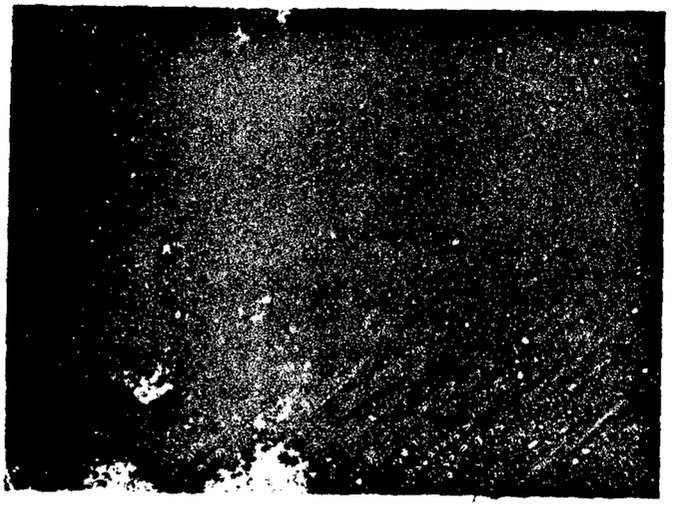


Fig. 48. Day-old agar culture of enterococci. × 1350.

Enterococci do not form spores or capsules. They are usually immobile, although mobile varieties have been described; when mobility does occur it results from the presence of from 1 to 4 flagella (Fig. 53).

Fig. 49. Enterococci; formation of rod-like forms. × 1000.

Fig. 50. Enterococci; rod-like forms; polymorphism. × 1100.

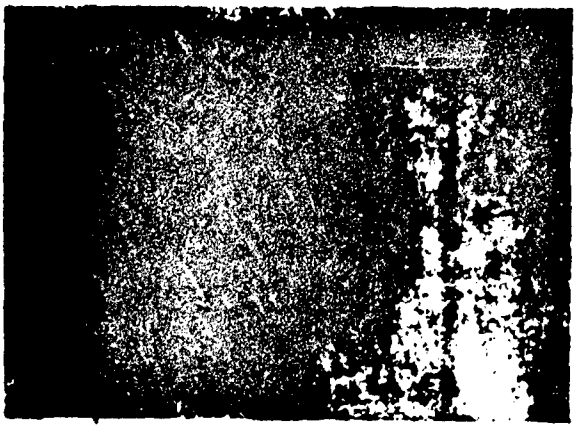


Fig. 51. Enterococci; polymorphism on medium containing potassium tellurite. × 1350.

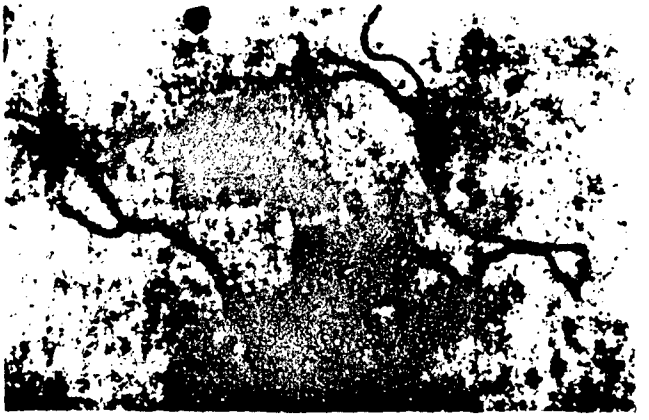


Fig. 52. Enterococci; chains in broth containing 40% bile, × 900.

Biology of Enterococci; cultural characteristics

Enterococci are the least selective of all the Streptococci, growing on any ordinary nutritive medium. Their growth is improved and accelerated by adding glucose, mannitol, animal or human proteins, or certain growth substances (e.g., yeast extract or yeast autolyzate), although enterococci grow rather slowly even under the most favorable conditions and reach their maximum growth only after 36-48 hr of culturing at 37°. In contrast to other species of Streptococci grow at any

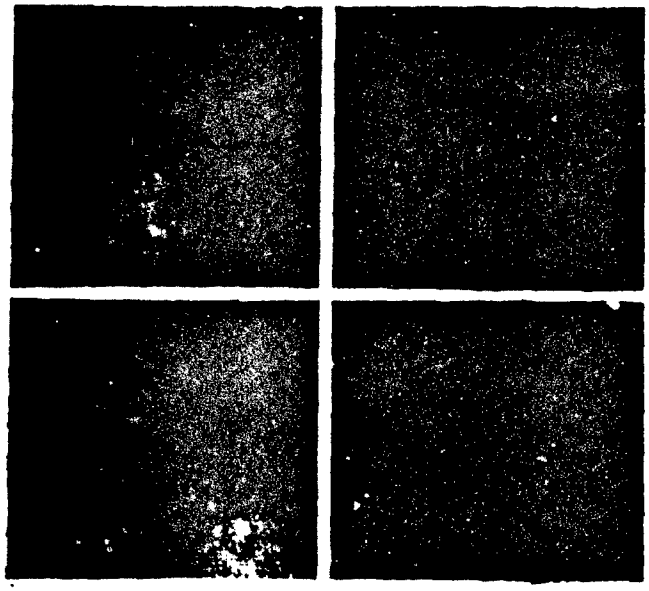


Fig. 53. Enterococci (Str. faecium); flagella. 1) Diplococcus with two lateral flagella; 2) single coccus with three terminal and one lateral flagella; 3) diplococcus with one lateral flagellum; 4) diplococcus with three or more lateral flagella (after Langston, Guthiere, and Bouma, 1960). x 1800.

Fig. 54. Enterococci; electron-microscopic photograph. x 10,000 (after Duka, Alexandrescu, Birtz, and Hut, 1962).

temperature between 10 and 45°. They are facultative aerobes, but all metabolic processes proceed intensively under anaerobic or relatively anaerobic conditions.

Enterococcal colonies on nutritive agar are small, but fuse to

Fig. 55. Enterococcal colonies on sugar agar; fused colonies visible. × 20.

form a continuous growth when present in large numbers, which distinguishes them from colonies of hemolytic and other Streptococci (Fig. 55). They are convex, circular, and homogeneous and refract light well, although they are somewhat cloudy. Kolober (1962) has described the yellow pigmentation of colonies of certain strains of enterococci. In liquid media these bacteria form a diffuse turbidity whose intensity depends on whether or not growth-stimulating substances are present in the medium; thus, the turbidity is more intensive in media containing yeast autolyzate than in ordinary or sugar-containing meat-infusion broth. Older cultures form a mucoid sediment, which does not break up when shaken but rises and coils into a corkscrew form.

Enterococci are rather active biochemically, decomposing glucose and mannitol without forming gaseous products; other carbohydrates are broken down by various species of enterococci (see page 596). Str. liquefaciens liquefies gelatine; Str. zymogenes causes hemolysis on blood-containing media, while the other species of enterococci leave such media unchanged or (more rarely) turn them green within 48-72 hr or some-

times later. Individual species may or may not clabber milk. Litmus or methylene blue in milk are usually reduced before clabbering occurs.

Resistance to physical and chemical agents

Enterococci are the most resistant of all the Streptococci: they can withstand heating to 55-60° for 1 hr and resist the bactericidal or bacteriostatic effects of many substances with a selective action on gram-positive bacteria, such as basic aniline dyes and many antibiotics. They remain viable in the external environment (water, soil, etc.) for substantially longer than other species of Streptococci.

Antigenic structure; serotypes

The enterococci are a rather homogeneous group and are designated by the letter "D" in Lensfield's serological classification.

Lensfield distinguished 3 subtypes within this group, while Sylvester and Benedict differentiated 4, as did Foley and Willer. Barber et al. (1962) recently described group-specific nucleoproteins, typo-specific polysaccharides, and species-specific proteins in group D.

This work has still not proved to be of practical value.

Laboratory Diagnosis of Diseases Caused by Enterococci

Material to be examined; taking of samples

Fecal matter, pus, blood, discharges from ulcerations, urine, secreta from the female genitalia, cerebrospinal fluid, lavage fluids, and vomitus are examined, in accordance with the localization of the enterococcal process. Samples of pathological material are taken in the same manner as in other streptococcal diseases.

Microscopic examination

Microscopic examination of smears prepared from pathological material permits rough determination of whether or not the process is of enterococcal etiology. The presence of sharply polymorphic diplococci and short chains, especially in ulcerative discharges, pus, or cerebro-

spinal fluid, sometimes makes it possible to establish a preliminary diagnosis. The final diagnosis is based on isolation of a pure culture of the causative agent and determination of its species. The smears must be Gram-stained.

Bacteriological investigation

Pathological material is usually examined for enterococci in the same manner as for Streptococci in general (page 751). Material from ulcerations, pus, urine sediment, etc., are cultured on dishes containing blood agar. Parallel cultures can also be made on Belen'kiy's medium (page 600) to permit differentiation of enterococci from other Streptococci.

Blood is cultured in sugar broth, observing the usual rules for this procedure (the amount of medium should exceed the amount of culture material by a factor of no less than 10).

When the material is heavily contaminated with extraneous microorganisms and contains relatively few enterococci selective media, both solid and liquid, are used. Hain and Perry's medium (page 601), Henny and Norton's medium (page 602), Shattock and Hirsch's medium (page 602), Rote's medium (page 603), Weng Lang-Liu and Stuart's medium (page 603), Litsky, Malman, and Fifield's medium (page 603), and G.P. Kalina's "AEM" medium (page 604).

Chapman's medium (page 601), Slanetz and Bertly's medium (page 603), and Kolober and Morelli's medium are recommended solid media(p.604)they are poured into dishes both for direct culturing and for culturing from liquid nutritive media or for raising bacteria on filters. All these media retard the growth of gram-negative microbes to some extent, while some of them retard that of many gram-positive bacteria (Sarcina, sporogenous anaerobes, occasionally Staphylococci, and, usually, Streptococci of other species). The ability of a medium to inhibit the develop-

ment of gram-negative bacteria results from the presence of sodium azide or potassium tellurite.

Certain dyes (ethyl or crystal violet) are added to the media to suppress the growth of other gram-positive bacteria, but they may also hamper and retard the development of enterococci to some degree.

Considering the shortage of sodium azide, a liquid selective medium (the "AEM" medium; see page 604) has been developed whose selectivity is based on elevated alkalinity (pH 10.2) and inclusion of polymixin M (Soviet preparation), which retards the growth of gram-negative microorganisms, particularly E. coli. Since sporogenous aerobes and Sarcina are capable of growing, albeit slowly, on this medium, it is necessary to make confirmatory transplants from it to sugar-yeast agar containing crystal violet in a concentration of 1:800,000.

Transplants are made from liquid selective media to solid selective media or to sugar or blood agar, depending on the type of material and the extent to which it is contaminated with extraneous bacteria, after 24, 48 and 72 hr, taking into account the slow growth of enterococci in general and especially of those isolated from pathological material and environmental objects. The slight inhibiting action of selective media on enterococci must also be taken into consideration.

The following criteria are employed in investigating the culture thus isolated (Sherman).

Sherman's criteria are characteristics which clearly differentiate the enterococcal group (except Str. bovis) from Streptococci of other serological groups (Str. haemolyticus, Str. viridans, Str. mitis, Str. salivarius, etc.) and are shown in Table 48.

Differentiation within the enterococcal group. Table 49 shows the basic characteristics suggested for differentiating the individual species and varieties of enterococci.

TABLE 48

Sherman's Criteria

Признак 1	2 Энтерококки	3 Стрептококки других серогрупп
Температурные пределы роста 4	10—45	25—38
Рост на средах pH=9,6 (в присутствии 0,05 м Na_2CO_3) 5	+	—
Рост в питательной среде с 40% желчи 6	+	—
Рост в средах с 6,5% поваренной соли 7	+	—
Рост в молоке с 0,1% метиленового синего 8	+	—
Термоустойчивость (при 60° в течение 30 минут) 9	+	—

1) Characteristics; 2) enterococci; 3) Streptococci of other serological groups; 4) growth-temperature range; 5) growth on media at pH 9.6 (containing 0.05 M Na_2Co_3); 6) growth in nutritive medium containing 40% bile; 7) growth in media containing 6.5% sodium chloride; 8) growth in milk containing 0.1% methylene blue; 9) thermoresistance (at 60° for 30 min).

TABLE 49

Differentiation of Species of Enterococci

Вид 1	2 Теллурит калия (1:2500)	3 Редукция 2, 3, 5-трифенилтетразолия хлорида	4 Маннит	5 Сахароза	6 Сорбит	7 Арабиноза	8 Рафиноза
Str. faecalis	+	+	+	+	+	—	—
Str. faecium	—	—	+	+	—	+	—
Str. durans	—	—	—	—	—	—	—
Str. bovis	—	—	—	—	—	+	+

1) Species; 2) potassium tellurite (1:2500); 3) reduction of 2, 3, 5-triphenyltetrazoline chloride; 4) mannitol; 5) sucrose; 6) sorbitol; 7) arabinose; 8) raffinose.

The varieties of Stre. faecalis, var. zymogenes and var. liquefaciens, have characteristics identical to those of the parent strain, except that the first is capable of lyzing erythrocytes and (sometimes) liquefying gelatin, while the second is capable of liquefying gelatin.

Str. liquefaciens, in addition to its ability to liquefy gelatin, is characterized by peptonization (proteolysis) of milk, which can be clearly seen in a solid medium containing molk and yeast autolyzate.

Indices of carbohydrate metabolism are the most indecisive criteria. In addition to the basic species, Morelli and Kolober have described a large number of varieties occupying a position between Str. faecalis and Str. faecium. Certain strains of Str. faecium may also be able to liquefy gelatine or lyze erythrocytes.

The most reliable criteria, usually accurate, are behavior with respect to potassium tellurite (Str. faecalis grows in the presence of potassium tellurite in a concentration of 1:2500, reducing it and forming black colonies; Str. faecium does not grow at this potassium tellurite concentration, but forms small, light-colored colonies at a concentration of 1:5000) and reduction of 2,3,5-triphenyltetrazolium chloride (TTC). The entire differential series shows negative characteristics for Str. durans. Str. bovis is easily distinguished by the fact that it exhibits none of Sherman's criteria except an ability to grow at 45° and in broth containing 40% bile.

Detection of enterotoxin in the external environment

Detection of enterococci in foodstuffs is significant in suspected food poisonings, as is occurrence of these bacteria in water, since their presence is assumed to be a supplemental proof of contamination in a number of nations.

Foodstuffs are examined for enterococci by direct culturing on dishes containing solid media (nutritive sugar agar, Belen'kiy's medium, or blood agar). Solid selective media containing penicillin or crystal violet (page 599) are used when the product to be investigated is heavily contaminated with gram-positive flora, while media containing sodium azide or polymyxin M are employed when it is contaminated with gram-negative flora.

Cultures can simultaneously be made in liquid concentration media, taking into account, however, the fact that detection of enterococci,

as with other microorganisms responsible for food poisonings (Salmonella, Staphylococci, etc.), is significant only when the product is heavily contaminated; the presence of small quantities of enterococci, detectible only with liquid concentration media, need hardly be given consideration.

The investigative technique proposed by Ray et al., (1961) can be employed when foodstuffs are to be examined in order to determine whether they are sanitary (the quantity of enterococci is to be established).

Ray's procedure for examination of foodstuffs for enterorocci.

1. Ten-fold dilutions are made up in a concentration medium (Rote's azide-dextrose broth; page 603) and incubated for 48 hr at 37°.

2. Transplants of 3 loopfuls of material are made from those test-tubes in which growth is detected to a confirmatory medium (Litsky, Malman, and Fifield's medium) and incubated at 37° for 48 hr.

3. Streak cultures are made from those tubes in which growth is detected on dishes containing Barnes' medium (TTC-glucose agar) and incubated at 37° for 48 hr.

A) Colonies with red centers are Str. faecalis and its varieties; B) colorless colonies are Str. faecium, Str. durans, Str. bovis, and unclassified strains.

A successful technique for investigating water and foodstuffs is to culture ten-fold dilutions of the material on AEM medium (page 604) and then to make transplants of all the dilutions (regardless of whether or not growth has occurred) to sectors of a dish of nutritive agar containing crystal violet and triphenyltetrazolium chloride (page 605). For further identification of the species of enterococci, colonies with red centers can (but need not) be transferred to sectors of blood (page 601) and milk (page 601) agar, while colorless colonies are transferred to a composite series (mannitol, sorbitol, arabinose, and raffin-

ose).

Reliable differentiation of enterococci from Str. lactis is of decisive importance in investigating milk products. Since Str. lactis does not grow at 45° and has little resistance to a severely alkaline medium pH, an effective method of isolating enterococci mixed with this bacterium involves culturing in Chesborough and Ivens' medium, modifications of this medium, or AEM medium (page 604) and incubation at 45°.

When culturing water, especially for the purpose of making a quantitative determination of enterococci, cultures in liquid selective media are of decisive importance. Titration is carried out in the manner usually employed for E. coli. The membrane method, which involves filtration of different quantities of material (different dilutions in the case of contaminated water) through membrane filters, which are then placed on solid selective media (slanetz and Bertly's medium, Chapman's medium, or Kolober and Morelli's medium), is a convenient technique for determining the enterococcal index of water.

Accelerated methods for bacteriological examination

Enterococci grow slowly, even on media quite favorable to their development. The results are usually evaluated no less than 36-48 hr after culturing, during which time the material is incubated at 37° or 45° (the selective temperature). All procedures which promote the forced development of enterococci are consequently to be welcomed as techniques for accelerating the diagnosis of enterococcal diseases and the detection of enterococci in environmental objects. Addition of 2-3% yeast autolyzate or extract substantially accelerates enterococcal growth. Morelli and Kolober suggested that relatively anaerobic culturing conditions be created on a solid medium to permit accelerated development of enterococci, the results being evaluable within 8-14 hr. This is done by placing a membrane filter through which the enterococcus-con-

taining material has been passed on the bottom of a dish and flooding it with esculin medium (page 604). The layer of medium should be no more than 2-3 mm thick.

Black enterococcal colonies can be detected on the surface of the filter within 8 hr and are especially distinct when the dish is illuminated with a powerful light source. Since only enterococci form black colonies on the medium, a colony count and rough microscopic examination of selected colonies make it possible to obtain results within 10-14 hr.

One of the most reliable criteria of whether a given bacterium belongs to the enterococci (provided that its morphology is appropriate) is whether it reduces methylene blue in milk. In order to accelerate this reaction, which usually requires two days, it is recommended that a day-old agar culture of the microbe to be investigated be washed with milk containing methylene blue and the washings poured over butter. Complete or almost complete (in the lower 2/3 of the test-tubes) reduction of the dye and decclorization of the milk take place within 2-4 hr.

Nutritive Media

Differential diagnostic media

Chesborough and Ivens' medium (1959). The basic medium contains 4 g of tryptone, 2 ml of yeast extract, 4 g of glucose, 1 g of sodium chloride, and 100 ml of distilled water. A 0.02 M solution of Na_2CO_3 in 100 ml of distilled water and 1% K_2HPO_4 in 100 ml of water are made up separately. The basic medium and the two solutions are sterilized under a pressure of 1 atm for 12 min. All the ingredients are combined under aseptic conditions, the mixture is brought to pH 9.6-10.0 with 2 N NaOH or H_3PO_4, sufficient distilled water is added to make 400 ml, and portions of 5 ml are poured into test-tubes under sterile conditions. Enterococci grow on this medium, producing a diffuse turbidity, but no other Streptococci will develop.

Modified Chesborough and Ivens' medium (G.P. Kalina). The tryptone and distilled water in the basic medium are replaced by 100 ml of meat-infusion broth, while 2 ml of yeast autolyzate is substituted for the yeast extract. It is expedient to add 0.8 ml of 1.6% alcoholic bromthymol blue to the medium after its ingredients have been mixed in order to permit evaluation of culture growth (turbidity) and of acid formation from glucose decomposition.

Bile broth. A total of 40 ml of bovine bile is added to 60 ml of nutritive broth and the mixture is poured into test-tubes and sterilized under a pressure of 1 atm for 10 min.

D.E. Belen'kiy and N.N. Popova's medium (1929). A total of 40 ml of bile is added to 60 ml of 2.5% nutritive agar and the mixture is sterilized under a pressure of 1 atm for 10 min; the agar is cooled to 50°, mixed with 5 ml of defibrinated or citrated blood, and poured into dishes.

Medium containing 6.5% sodium chloride. A weighed batch of 6 g of sodium chloride is dissolved in a small amount of distilled water, sterilized under a pressure of 1.5 atm for 20 min, and mixed with 100 ml of nutritive broth or glucose broth; 5 ml portions are poured into test-tubes under sterile conditions.

Milk containing methylene blue for determination of reductive characteristics. Portions of 5 ml of sterile milk (whole if possible, since a layer of cream on the surface of the medium aids in creating relatively anaerobic conditions favorable to enterococci) are poured into test-tubes and 0.1 ml of 1% (according to Scadhog) or 5% (according to Sherman) methylene blue is added to each tube under aseptic conditions.

Medium for detection of proteolysis in milk. This medium contains 80 ml of 2% meat-infusion agar, 2 ml of yeast autolyzate, and 20 ml of milk.

The molten nutritive agar, containing the yeast autolyzate, is mixed with the milk, and, after thorough stirring, poured into dishes in a thin layer.

Barnes' medium containing 2,3,5-triphenyltetrazolium chloride (TTC) (1956). This medium contains 1 g of Ivens peptone, 1 g of Lemko LAB, 0.5 g of sodium chloride, 1.2 g of agar-agar, and 100 ml of distilled water; the pH of the mixture is adjusted to 6.0-6.1 and it is sterilized at 1.5 atm for 20 min. A total of 1 g of glucose and 0.01 g of TTC are dissolved separately in a small quantity of water and sterilized in running steam for 30 min. Meat-infusion broth containing 2% yeast autolyzate can be successfully substituted for the Ivens peptone and Lemko LAB. Colonies of Str. faecalis have cherry-red centers, while those of other enterococcal species are colorless.

Medium containing potassium tellurite. Immediately before portioning into dishes 100 ml of 1.2% meat-infusion agar at pH 7.2 containing 2% yeast autolyzate is mixed with 0.04 g of potassium tellurite preliminarily dissolved in a small amount of distilled water. The temperature of the agar should not be above 50°, since potassium tellurite decomposes at higher temperatures.

Selective media

Hain and Perry's medium (1943). This medium contains 2 g of bactotryptone, 0.5 g of sodium chloride, 0.5 g of glucose, 0.4 g of K_2HPO_4, 0.15 g of KH_2PO_4, 0.05 g of sodium azide (NaN_3), 0.2 ml of 1.6% alcoholic bromcresol purple, and 100 ml of distilled water. It is sterilized at 1 atm for 15 min.

Chapman's medium. This medium contains 1 g of bactotryptose, 0.5 g of No. 3 peptone bactoprotease, 5 g of sucrose (e.g., table sugar), 1.5 g of bacterial agar, 0.1 g of dextrose, 0.4 g of anhydrous K_2HPO_4, 0.75 ml of 1% trypan, 0.08 ml of 0.1% crystal violet, and 100 ml of dis-

tilled water. The mixture is sterilized at 1 atm for 15 min. After it has cooled to 50-55° potassium tellurite dissolved in a small amount of water is added to produce a final concentration of 1:2500 and the medium is poured into dishes.

Henny and Norton's medium (1947); the American standard for investigation of drinking water). This medium contains 1 g of peptone, 0.5 g of sodium chloride, 0.2 g of K_2HPO_4, 0.5 g of glucose, 0.3 g of yeast extract, 0.025 g of sodium azide, 0.2 ml of 1.6% alcoholic bromcresol purple, and 100 ml of distilled water; the pH of the mixture is adjusted to 6.6-6.8 and portions of 5 ml are poured into test-tubes and sterilized at 1 atm for 15 min. For culturing large quantities of material (such as water) the medium is made up double strength and poured out in portions of 10 and 50 ml, doubling the quantity of test liquid used to dilute the medium.

Shattock and Hirsch's medium (1947). Solution I contains 1 g of Lemko dextrose broth, 1 g of Lemko LAB, 1 g of Ivens peptone, 0.5 g of sodium chloride, and 100 ml of tap water; its pH is adjusted to 7.0 and it is sterilized at 1 atm for 15 min. Solution II contains Clark's buffer solution, 0.7505 g of glycine, 0.585 g of sodium chloride, and 100 ml of freshly boiled distilled water. Six parts of this solution are mixed with four parts of 10 N sodium hydroxide. A total of 10 ml of solution II is added to 90 ml of solution I and the pH of the mixture is adjusted to 9.6 with one-normal sodium hydroxide. It is then left to stand overnight in a stoppered flask under refrigeration to permit complete settling, filtered through a Zeitz filter, and quickly poured into flasks under aseptic conditions, leaving as small an air space as possible. The medium is ready for use after 48 hr. An uninoculated control flask must be set aside and its pH determined before and after incubation. The alkalinity of the medium should not drop by more than 0.04.

Str. durans and Str. bovis will not grow on this medium.

Rote's medium (1948). This medium contains 1.5 g of Difco tryptose, 0.45 g of Difco meat extract, 1.5 g of dextrose, 1.5 g of sodium chloride, 0.02 g of sodium azide, and 100 ml of distilled water; the pH of the mixture is adjusted to 7.2.

Weng Lang-Liu and Stuart's medium (1951). This medium contains 0.7 g of Difco tryptone, 0.3 g of tryptose, 0.4 g of K_2HPO_4, 0.5 g of glucose, 0.5 g of yeast extract, 0.04 g of sodium azide, 0.1 ml of 1.6% brothymol blue, and 100 ml of distilled water; the mixture is sterilized at 1 atm for 15 min.

Litsky, Malman, Fifield's concentration medium (1953). This medium contains 2 g of Difco tryptose, 1.5 g of dextrose, 0.5 g of sodium chloride, 0.27 g of K_2HPO_4, 0.27 g of KH_2PO_4, 0.02 g of sodium azide, and 100 ml of distilled water.

Litsky, Malman, and Fifield's confirmatory medium (1953). This medium has the same composition as the preceding one, but contains 0.04 g of sodium azide and sufficient 1% ethyl violet to give a final concentration of 1:1,200,000 (or sufficient crystal violet to give a final concentration of 1:800,000). The material to be investigated is cultured in the concentration medium for 48 hr and 3 loopfuls are then transferred to the confirmatory medium for 48 hr.

Slanetz and Bertly's medium (1957). This medium contains 2 g of Difco tryptose, 0.5 g of yeast extract, 0.2 g of glucose, 0.4 g of potassium phosphate, 0.04 g of sodium azide, 0.01 g of 2,3,5-triphenyltetrazolium chloride (TTC), 1 g of agar-agar, and 100 ml of distilled water. The first 5 ingredients are dissolved in the water, the pH is adjusted to 7.2, the agar is added, the mixture is boiled until the agar melts and then slightly cooled, the TTC is added (as a 1% solution), and the medium is poured into sterile dishes. The authors do not men-

tion sterilization. This is a good medium for raising enterococci on membrane filters.

Modification of foregoing medium. The Difco tryptose can be replaced by meat-infusion broth with no detriment to the results: 2 g of yeast autolyzate can be used instead of the 0.5 g of yeast extract.

Kolober and Morelli's selective medium (1958). A weighed portion of 250 g of beef heart, cleaned of fat and connective tissue, is cut into small pieces and ground in a mortar with 200 ml of distilled water to produce a liquid paste; 200 g of demembranized beef brain and an additional 200 ml of water are then added and the mixture is ground to a homogeneous mass. It is then transferred to a pot, the residue is washed from the mortar with 400 ml of distilled water, which is also poured into the pot, and the contents of the latter are boiled for 10 min, stirring from time to time. The mass is filtered through hygroscopic cotton, sufficient distilled water is added to the filtrate to bring it to 800 ml, and it is placed over a burner; 10 g of Vaillant's peptone B, 5 g of sodium chloride, 2.5 g of $Na_2HPO_4 \cdot 12H_2O$, 2.5 g of glycine, and 15 g of agar-agar are added to it and its pH is adjusted to 9.2 with 50% sodium hydroxide, carefully boiling it until the agar dissolves. A total of 2.25 g of sodium azide, 1 g of esculin, and 2 g of ferrous citrate are dissolved separately in 200 ml of water. The cooled solution is mixed with the basic medium, sufficient distilled water is added to bring the total volume to 1000 ml, the pH is checked (9.2 is correct), and the medium is poured into flasks and sterilized at 115° for 20 min. The pH should be 9.0 after sterilization. The finished medium is poured into dishes and the residue is carefully decanted.

Alkaline enterococcal medium (AEM) for concentration of enterococci from highly contaminated environmental objects and feces (G.P. Kalina). This medium is a modified Chesborough and Ivens' medium (page 599)

altered in the following manner: its pH is adjusted to 10.2. The pH can be determined with 0.1% phenolphthalein in a buffered solution containing 0.72 g of $Na_2CO_3 \cdot 10H_2O$ and 0.21 g of $NaHCO_3$, using doses of 100 ml (3 drops in 5 ml). The antibiotic polymyxin M is added (after all other ingredients have been mixed0 to produce a final concentration of 200 units/ml.

Material is transferred from this medium to meat-infusion agar containing 1% glucose, 2% yeast autolyzate, crystal violet in a concentration of 1:800,000, and 0.01% 2,3,5-triphenyltetrazolium chloride (TTC).

MYCOTIC FOOD POISONING

Yu.I. Rubinshteyn, Candidate of Biological Sciences

Mycotic food poisonings are intoxications resulting from the consumption of food containing toxins of fungal origin. Mycoses are associated principally with damage to grain crops by microscopic fungi, which produce toxic substances resistant to high temperatures under favorable temperature and humidity conditions.

The causative agents of mycotoxicoses in man are primarily members of the subdivision Sporotrichiella of the genus Fusarium.

Morphology and biology of the causative agents of mycotoxicosis; cultural characteristics

The principal traits used for identification of the subgenera, species, and varieties of fungi included in the genus Fusarium are cultural characteristics (character of growth and pigmentation), conidial morphology, and type of sporulation. The species of the genus Fusarium are among the conidial fungi; the structure of the conidial sporulation organs is one of the most important characteristics for differentiating these species. It is necessary to use various nutritive media to detect sporulation (see page 622) and one is sometimes forced to make cultures

on a series of media; there are cultures which will not exhibit sporulation and consequently must remain unidentified.

In studying Fusaria it is necessary to isolate as many cultures as possible from the substrate under investigation, in order to obtain strains which sporulate.

When examining grain for Fusarium damage one cannot be content with determining merely the genus. It is necessary to establish the subgenus, species, and variety of Fusarium causing the injury and in order to do so one must first obtain a culture displaying typical sporulation.

The species of the genus Fusarium vary in their formation of aerial mycelia; the latter may be highly or poorly developed or entirely absent, in which case only a pyonnota (a continuous mucoid layer on the surface of the medium) is formed.

The pigment produced on different nutritive media is one of the characteristics used for distinguishing the subgenera. The media used for determining pigmentation usually include Chapek's medium, potato agar containing 2% glucose (PAG), slices of potato, and rice grains. The simplest and least variable index is the pigment formed on rice, which is determined in 15-day cultures.

Laboratory Diagnosis of Mycotoxicosis

The toxicity of grain crops overwintered in the field and of products manufactured from them is determined in accordance with Instruction No. 289-59, adopted by the Ministry of Public Health USSR in 1959.

Material to be investigated; taking of samples

Grain overwintered in the field (millet, wheat, polygonum, oats, barley, and other crops), as well as flour, bran, bread, and other products suspected of having been prepared from overwintered grain, are investigated.

Samples are taken from each incoming batch of overwintered grain, in accordance with State Standard 3040-55.

Method of taking and rating grain samples.

Grain left in the field in windrows or as a standing crop is sampled in the following manner.

a) If the grain has been left standing under a snow cover areas of 1 m^2 of grain are cut at 5-10 points in the field (or more, depending on the size of the field of unharvested grain); the grain is air-dried in sheaves and hand-threshed.

b) If the grain is left in windrows until the fall-winter or spring period (under a snow cover) areas of 1 m^2 of grain are cut at 5-10 points in the field (or more, depending on the size of the unharvested field), sheaved, dried, and threshed (see Paragraph a).

c) If the grain is intended for late harvest or has been overwintered under a snow cover it is harvested with a combine and stored after threshing, samples being taken in accordance with State Standard No. 3040-55.

All the samples should be labeled with the following information: a) sampling site (oblast, rayon, sovkhoz, kolkhoz); b) name of grain crop (millet, wheat, etc.); c) origin of sample (standing grain, sheaves, storehouse); d)sampling date; e) size of batch, if sample has been taken from stored grain.

The samples are sent to the nearest public-health food laboratory for examination, indicating the rayon in which the grain was overwintered, the quantity of grain from which the sample was taken, the sampling site and date, and the weight of the sample.

A weighed specimen of approximately 100 g is taken from the sample to be investigated, dried at 30-40° for 24 hr, and ground.

Biological testing (determination of toxicity in grain)

An extract is obtained by soaking 50 g of the ground material in pure ethyl ether in a Soxhlet apparatus for 6 hr. After the ether has been refluxed the tincture is transferred to a jar or wide-mouthed test-tube and the ether is evaporated at 40-50°.

When no Soxhlet apparatus is available 50 g of the ground material is placed in a bottle and flooded with ethyl ether in such fashion that it is covered to a depth of 1-2 cm. Extraction is continued for 1-3 days at room temperature, periodically shaking the mixture, and the extract is then filtered and the ether evaporated at a temperature of no more than 40-50°.

The extract is tested by setting up a dermal reaction in rabbits.

Preparation of the animal. Rabbits weighing no less than 1.5 kg, with unpigmented skin, are suitable for the test. An area of skin 3 × 3 cm in size is carefully shaved for each experiment. No more than 4 or 5 reactions can be set up on each side of the animal's body and the shaved areas should be at least 2 cm apart. The shaving is done on the day before the test; traumatized areas are unsuitable for the reaction.

Procedure for dermal reaction. Extract obtained in the manner described above is applied to the animal's skin twice, at an interval of 24 hr; reactions are simultaneously conducted in 2 rabbits. A glass spatula is used to apply the extract, which is gently spread in a uniform layer over the shaved area. Two controls are set up to determine each rabbit's reactivity, employing extracts prepared from good grain (negative control) and from highly toxic grain (positive control).

In order to keep the animal from licking off the extract a heavy cardboard collar is placed about its neck after the first application of extract and removed 24 hr after the second application.

Determination of results of dermal reaction. The character of the

dermal reaction varies.

a) When the reaction is negative there are no inflammatory changes and the skin remains normal or occasionally exhibits slight exfoliation, which disappears after 1 or 2 days.

b) When the reaction is positive edema, erubescence, and yellowing of the skin develop within 24 hr after the extract is applied, although sometimes requiring as long as 3-5 days to appear, and a superficial necrotic process sets in, subsequently forming a necrotic crust.

c) A doubtful reaction is accompanied by development of slight edema and erubescence, followed by yellowing but not by formation of a necrotic crust. In such cases it is necessary to repeat the investigation with a second extract prepared from the same sample. A second doubtful reaction is regarded as positive, while a negative reaction following a doubtful reaction is considered to be negative. The rabbit is kept under observation for 7 days when a doubtful reaction is obtained.

The method adopted by the Ministry of Public Health USSR in 1959 must be used for checking the toxicity of late-threshed grain left in the field during heavy rains or early snows. Such grain, just like that overwintered in the field, is investigated by skin testing in rabbits. Since the dermal reaction in rabbits may not be as clear in such cases as when testing overwintered grain, supplemental experiments involving feeding of the grain to cats must be carried out if the dermal reaction is inconclusive.

Experiments on cats are specific for detection of grain damaged by Fus. sporotrichiella, which may cause a toxic alimentary aleukia. Each specimen is tested in 2 cats weighing 1.5-2 kg. Before the experiment the animals are weighed, their temperatures are taken, and a clinical analysis (hemoglobin, number of erythrocytes and leucocytes per mm^3,

leucocytic formula, and sedimentation rate) is made of their blood; blood samples are collected by making a small incision in the ear. The experiment lasts for one month, during which period the animals are fed the grain to be tested in a daily dose of 10 g per kg of body weight. The ground grain is fed as a cereal or mixed with meat and soup (the cats are kept on a normal diet).

The animals' condition, temperature, and weight are observed throughout the experimental period (they are weighed no less than once a week). Consideration must be given to the condition of the gastrointestinal tract (vomiting, diarrhea), the occurrence of bloody discharges from the nose and mouth, the state of the gums (bleeding), and the appearance of cutaneous hemorrhages, convulsions, paralysis, or disruptions of motor coordination. Systematic hematological investigations are conducted at intervals, depending on the animals' condition and hematological indices.

A pathologoanatomic autopsy must be carried out when an animal dies; pathohistological investigation of the internal organs, brain, and bone marrow is desirable if facilities are available.

Development of leucopenia and anemia, general exhaustion, disorders of the gastrointestinal tract, and other symptoms, or of leucopenia alone, with no accompanying clinical symptoms, indicates that the grain under investigation is toxic.

Experiments on pigeons. Pigeons are very sensitive to the toxins produced by Fus. sporotrichiella. Birds weighing 250-350 g are used to determine the toxicity of grain, feeding them daily portions of 2-3 g for 14 days. Each sample is tested on 2 pigeons (which are kept in separate cages). The bottom of each cage is lined with a piece of clean white paper, on which the water dish is placed. The birds react with vomiting from 15-30 min to 4-12 hr after being fed the grain, depending

on its toxicity. Vomitus shows up clearly on the white paper, which should be changed daily. A liquid stool is noted in some cases; highly toxic grain causes the pigeons to die. When no reaction develops within 14 days the observation period is continued for 5 days after the last feeding of the grain under study and its toxicity is then evaluated.

Mycological investigation

Cultures for detection of superficial and internal damage to grain. Chapek's medium, potato agar containing glucose, and moisture chambers containing filter paper are used for isolating fungi of the genus Fusarium from grain.

In order to detect surface contamination portions of 10 grains are placed on Petri dishes containing the aforementioned media. The grains are arranged so as to be equidistant and a total of no fewer than 200 grains is investigated. The dishes are placed (right side up) in a heater at 18-22°; the fungal colonies growing around each grain are examined after 3-5 days. The total yield is determined on the 5th day of growth by counting the number of grains affected by a given fungus, summing the numbers for each species of fungus, and calculating the percentage contamination from the initial number of grains. Grains are also spread out in Petri dishes containing filter paper previously cut to the size of the dishes and sterilized. The paper is wetted with sterile water and 10-20 grains are placed on it; a total of 200 grains is laid out and the dishes are incubated at 18-22°. They are inspected daily and fresh sterile water is added, taking care that the paper is always kept moist. Fusaria grow on the filter paper, forming white, reddish, or red mycelia; good growth and pigmentation are usually observed after 5-10 days.

In order to obtain pure cultures Fusaria are transplanted from any nutritive medium to Chapek agar slants or glucose-containing potato

agar; white, reddish, and red colonies are used for transplantation, taking as large a quantity of material as possible. After incubation at 18-22° for 5-7 days colonies from the test-tube are examined microscopically; pure cultures are subjected to further investigation.

In order to detect internal (deep) damage the grains are treated by one of the methods described below to kill the superficial microflora before being spread out on medium-containing dishes. In treatment with mercuric chloride (1:1000) 200 grains are placed in a jar, sufficient mercuric chloride is added to cover them, and they are soaked for 2 min (gently shaking them all the while); they are then rinsed with sterile water or cooled freshly-boiled water and dried between sheets of filter paper. In treatment with 0.5% potassium permanganate the grains are soaked for 20 min, thrice rinsed with water, and dried in the same manner as after treatment with mercuric chloride. In treatment with alcohol (70°) the grains are soaked for 1-3 min, thrice rinsed with water, and dried in the manner described above. Grains treated by one of these methods are cut in half with a sterile scalpel, which is flamed before cutting each grain; the halves are placed cut side down on a nutritive medium (10-20 per dish) and the dishes are incubated at 18-22°. The colonies are counted after 7-8 days, depending on the growth rate. All growing Fusaria cultures are transferred to test-tubes containing Chapek agar slants and potato agar with glucose.

Both superficial and internal damage must be taken into account in evaluating the quality of the grain; it is generally easier to obtain pure Fusaria cultures from treated grain.

In order to isolate Fusaria from flour a small quantity of the latter is taken on the tip of a sterile scalpel and placed in heaps (8-10) on Chapek's medium. The colonies are raised and transplanted in the same manner as when investigating grain.

Determination of subgenera, species, and varieties of Fusaria.

The Fusaria cultures raised on the Chapek agar and the glucose-containing potato agar are examined microscopically and compressed-drop specimens are prepared in tap water or physiological solution from those tubes in which no extraneous growth is noted. Freshly prepared specimens are examined with a dry system; when weak objectives are used it is necessary to lower the condenser, which gives a clearer image of the hyphae and spores. For better observation it is recommended that blue or matte filters be inserted into the condenser-diaphragm adapter. f the culture is found to be pure it is transplanted to various media for determination of the subgenera and species of the genus Fusarium. If extraneous microorganisms are detected the culture must be immediately purified, since other fungi, growing rapidly, will otherwise suppress the Fusaria and make it impossible to obtain the strain in question in pure form.

Cultures are purified by transplantation to dishes containing Chapek's medium. A small piece of a mycelium is placed in a test-tube containing 3-5 ml of sterile physiological solution or tap water, the tube is shaken or rolled between the palms of the hands to separate the tangled hyphae, and a Pasteur pipette is used to apply 4-5 drops of the liquid to 4-5 sectors of a dish containing Chapek's medium; purification should be carried out in 2-3 dishes. The dishes are incubated at 18-22° and pure Fusaria colonies are transferred to Chapek agar slants (after preliminary microscopic examination).

Young (3-4-day) Fusaria colonies should be selected in order to avoid growth on the dishes of any extraneous fungi present in the initial test-tube. The tubes containing the cultures are incubated at 18-22° and the growth and pigmentation are noted; when no contamination is observed microscopically or macroscopically the strain is subjected

to identification procedures. All strains of the subgenus Sporotrichiella, which are identified by their piriform or limoniform conidia (see identification key), are transplanted and investigated on various media in order to determine the species and varieties to which they belong and to establish their toxicity. The cultures are stored on Chapek's medium or in test-tubes containing sterile grain at 18-20° and 5°; they are transplanted every 3-6 months.

Identification of the species and varieties of Sprotrichiella is based on a complex of characteristics pertaining to pigmentation during growth on various media, type of sporogenesis, and the morphological features of the macroconidia and microconidia.

Cultures should be simultaneously made on the following media: Chapek's agar, potato agar (PA), acid potato agar (APA), potato slices, grains of rice (all in test-tubes), and Bilai's synthetic medium. Identification must not be postponed, since storage under laboratory conditions may to some extent alter the properties of the culture. The culture material must be taken from a spore case or pyonnota or, when these are lacking, from an aerial mycelium. A piece of the mycelium or spore case is placed in a test-tube containing 5 ml of sterile physiological solution, the tube is shaken, and a Pasteur pipette is used to apply 5 drops of the liquid to each of the aforementioned media. The liquid should be uniformly spread over the surface of the slants and the potato slices and rice grains should be saturated; the cultures are incubated at 18-22°.

A final evaluation is made of the growth obtained 15 days after culturing. For media containing agar the color of the aerial mycelia, the appearance of the mycelia (puffy, delicate, powdery), the color of the substrate, and the type of sporogenesis (presence of spore cases and pyonnota) are noted. For the rice the color of the mycelia and the

grains, the border around the grains, and the presence of sclerotia are noted. For the potato slices the color of the mycelia and the slices and the presence of sclerotia are noted. Material for determination of the size and shape of the conidia is taken from the Chapek's agar; a microscope is used to note the presence and shape of macroconidia and microconidia and the presence of chlamydospores. Macroconidia with different numbers of septa and with many septa are noted. The percentages of conidia with 1, 2, 3, 4, 5, etc., septa are computed, counting 50-100 macroconidia in different fields of view. The size (length and width) of the macroconidia and microconidia is determined with an ocular micrometer; the dimensions of macroconidia with the same number of septa are established. The minimum, maximum, and most common dimensions are noted. Material from different areas of the culture (aerial mycelia, spore cases, etc.) should be examined microscopically. The data obtained furnish a basis for identifying the species and variety to which the strain in question belongs, using Bilai's key.

Varieties are identified by testing their pathogenecity on appropriate plants.

Determination of toxicity. Culturing on fat-containing medium. It has been established that there is a direct relationship between the toxicity of Sporotrichiella cultures and their ability to decompose fat while growing on a synthetic medium of definite composition (Yu.I. Rubinshteyn, 1950).

Determination method: 3 ml of sterile sunflower oil is added to 100 ml of hot melted synthetic medium, the mixture is vigorously shaken for 8-10 min, 0.2-0.3 ml of 1% aqueous methylene blue is added, and the mixture is again shaken and poured into Petri dishes. Pieces of mycelium are cultured on 4 sectors of a dish, placing them on the surface of the medium; a separate dish is used for each strain. The dishes are in-

cubated at 18-22° and the observation period lasts 6-8 days. Toxic cultures form rather large colonies with distinct mycelia within 3-4 days after culturing. The colonies continue to grow and merge after 6-8 days, the entire dish being covered with whitish or dirty-red mycelia; subsequent growth is so intensive that the mycelia reach the rim of the dish and creep onto its lid. The agar takes on a dirty-red color; the cultures retain characteristically shaped macroconidia and microconidia on this medium. Nontoxic cultures grow poorly, forming only isolated colonies at the inoculation sites, which do not merge. The mycelia are usually white and the agar either retains its original color or turns blue. Toxic and nontoxic strains of Fus. sporotrichiella can thus be differentiated by the character of their growth.

Luminescence method. It has been established that there is a direct relationship between the ability of Fus. sporotrichiella to produce an orange luminescence and their toxicity (Yu.I. Rubinshteyn, 1956).

Primary luminescence is determined in the following manner. After 14-21 days of growth at 18-22° cultures on agarized Chapek's medium in test-tubes (it should first be established that neither the glass nor the cotton plugs luminesce) are examined under a PRK-4 mercury-quartz lamp with a Wood filter. Luminescence develops slowly and becomes more intense after 3-5 min; the mycelia and sometimes the medium luminesce. Highly toxic strains emit a bright orange light, weakly toxic strains produce a light orange or lilac light or do not luminesce, and nontoxic strains produce a light lilac light or do not luminesce.

In addition to the method based on the primary luminescence of Fus. sporotrichiella, it has been shown that luminescence microscopy and the use of fluorochromes, permit more detailed and highly differentiated study of the structural characteristics of mycelia and conidia

than ordinary microscopy (Yu.I. Rubinshteyn, 1960).

Determination of culture toxicity

Skin test in rabbits. Grain contaminated with the culture to be tested is prepared for the dermal reaction by introducing the culture into 1-liter flasks containing 100 g of sterile grain (wheat or millet). For this purpose culture material from Chapek's medium (pieces of mycelium, with some of the nutritive medium if possible) is added to flasks containing 50-100 ml of sterile physiological solution, the mixture is thoroughly shaken, and 10 ml of the resultant suspension is added to each of the grain-containing flasks. The latter are vigorously shaken and the cultures are incubated at 18-22° for 21 days. After this 21-day period has elapsed flasks containing growing cultures are autoclaved in running steam (100°) for 1 hr and cooled to room temperature and material is transplanted to Chapek agar slants in order to make sure that the cultures have been killed. The contents of the flasks are carefully removed (with a wooden spatula), spread in a thin layer on sheets of filter paper, and dried at 37-40° (in a heater). The dried grain is stored in jars with ground stoppers, to be used for skin tests and for feeding to experimental animals.

The ether extract for the skin test is made up in the manner described for testing native grain, the only difference being that 20-30 g rather than 50 g of grain is used; the procedures for setting up the reaction and evaluating the results are the same as in investigating native grain.

Experiments involving feeding of grain to animals. This technique utilizes the same material (contaminated grain) as skin tests. The experimental animals may be white mice (for establishing a general toxic action), growing white rats (as a model for reproducing harvest disease), or cats (for reproducing toxic alimentary aleucia). Pigeons are sensi-

tive to the toxins of Fus. sporotrichiella and can also be used to establish the general toxicity of cultures.

Monkeys furnish a good model for reproducing toxic alimentary aleucia (Yu.I. Rubinshteyn and L.S. Lyass, 1948), while puppies are suitable for reproducing harvest disease (Yu.I. Rubinshteyn, 1953).

A special probe (a needle and bulb) is used to feed white mice a suspension of contaminated grain in physiological solution; this suspension is prepared by grinding grain contaminated with the strain to be tested with a small quantity of physiological solution and then adding sufficient of the latter to bring the total volume to 100 ml per 10 g of grain (a 1:10 suspension). The entire mass is transferred to a funnel containing a piece of gauze and the latter is thoroughly wrung out; the suspension obtained after wringing out the gauze is fed to the experimental animals (it should be prepared no more than 2 days before feeding) and should be stored at 4-5°. The suspension is taken up with a syringe and probe and daily doses of 0.5-1 ml are administered orally to the mice (which should weigh 18-20 g). Each strain is tested in no fewer than 5 mice; the experiment is conducted over a 30-day period. A suspension is simultaneously prepared in the same manner from uncontaminated autoclaved grain and administered in the same way to 5 mice over a 30-day period, thus serving as a control. Before the experiment the mice are weighed and their blood is investigated (taking samples from the tail), determining the hemoglobin, leucocyte, and erythrocyte counts and the leucocytic formula. During the experiment the animals' condition, their weight, their appetite for the usual diet, the state of the gastrointestinal tract, and the hematological pattern are noted. Mice react to administration of various specific toxins with disruptions in the central nervous system (convulsions, tremors, paresis, extremital paralysis) and gastrointestinal tract (diarrhea) and changes

in the hematological pattern, developing anemia, leucopenia, and neutropenia.

Autopsies are performed on all the mice which die or are killed before the end of the experiment and their organs are investigated; it is desirable to prepare and examine microscopic specimens.

Affection by Fusaria toxins produces hyperemia and hemorrhaging in the gastric and intestinal mucosa, necrosis and hemorrhaging in the liver, atrophy in the spleen, focal changes in the lungs, and hemorrhaging and hyperemia in the cerebral blood vessels.

White rate provide a good model for studying affections of the skeletomotor apparatus by the toxins of certain forms of Fus. var. poae. The experiments are performed on young animals (weighing 40-50 g), which are given daily doses of 1-2 ml of material prepared in the same manner as for experiments on white mice. The material is administered over a period of 6 months. The knees of the hind legs are x-rayed before and during the experiment and any changes in the epiphyseal zone are noted. Experiments on rats are conducted when grain and cultures are to be investigated in connection with harvest (Kaschin-Beck's) disease.

White rats also exhibit changes in the peripheral blood, but these animals are less sensitive to the toxins of Fus. sporotrichiella than mice or cats.

Cats provide a good model for reproducing the basic symptoms of toxic alimentary aleukia; they are generally highly sensitive to the toxins of Fus. sporotrichiella and react with general exhaustion, disruption of hemopoiesis, disorders of the gastrointestinal tract, and changes in the liver, kidneys, and spleen. Mature (2-2.5 kg) or young (800-900 g) animals are used; they are weighed, the morphological pattern of their blood is studied, and the sedimentation rate is deter-

mined. In addition to their other food (cereal, soup, meat) they are given daily doses of 0.1-1 g of grain contaminated with the strain to be tested per kg of body weight. The experiment lasts 30 days; the animals' general condition, their weight, the state of the gastrointestinal tract (vomiting, diarrhea),,the occurrence of bloody discharges from the nose or bleeding from the gums, and the pattern of the peripheral blood are noted. Autopsies are performed on all the cats which die or are killed after the experiment, a macroscopic examination is made of all the internal organs, and the condition of the bone marrow is determined (specimens are taken from the lower third of the femur or the upper portion of the tibia); microscopic examination of the organs and bone marrow is also desirable.

Experiments on pigeons are conducted in the same manner as in testing native grain, feeding them grain contaminated with the strain to be tested.

Experiments involving parenteral administration of grain to mice. A single intraperitoneal injection of filtrates of toxic cultures of Fus. sporotrichiella kills mice within 18-72 hr; intraperitoneal and oral administration of the same material produces the same clinical symptoms and similar changes in the internal organs.

Chapek-Doxa's medium containing autolyzate, millet broth containing autolyzate, or grain can be used for raising Fus. sporotrichiella and producing toxins. Portions of 200 ml of liquid media (Chapek-Doxa's medium, millet broth) are poured into 1-liter flasks, which are inoculated with the culture to be tested and incubated at 18-22° for 20 days. The film which forms is removed from the culture fluid and the latter is filtered through a Zeitz asbestos filter or a F-3 filter; the resultant filtrate is administered intraperitoneally to 3-5 mice in single doses of 1 ml. In order to determine the thermostability of the toxin

the filtrate is heated in a water bath at 100° for 20 min, cooled, and administered in the same dose (1 ml) to a second group of 3-5 mice; the toxins of Fus. sporotrichiella are thermostable.

If cereal grains are used as the substrate they are inoculated by the same method used to produce material for the skin test. The dried contaminated grain is ground in a mill and the flour is mixed with physiological solution (10-15 g of flour and 100 ml of physiological solution); the resultant mixture is agitated in a shaking machine for 40 min and filtered first through a paper filter and then through a Seitz or F-3 filter. Doses of 1 ml of unheated and heated (at 100° for 20 min) filtrates are administered intraperitoneally (using 3-5 mice for each filtrate). Toxic cultures cause the mice to die within 18-72 hr.

In all the experiments the surviving animals are observed for 7 days and then killed.

Autopsies should be performed on all the animals which die or are killed and their organs are investigated macroscopically (microscopic examination is also desirable).

The toxicity of the cultures under investigation is evaluated from all the data obtained by the above-described methods. The experiments involving feeding of the grain to animals are decisive.

Key to the subgenus Sporotrichiella*

Subgenus Sporotrichiella Wr. emend. Bilai.

Macroconidia fusiform-falciform, sometimes fusiform or linearly lanceolate, with gradually narrowing upper cavity and more or less distinct stem, usually having 3-7 septa; formed in aerial mycelia, less frequently in spore cases and pyonnotae, reddish-yellow, yellow ochre, or golden orange in color. Microconidia piriform or limoniform, spherical-ovoid, sometimes fusiform-elliptical, for the most part with a

single cavity, less frequently with 1-3 septa; produced in aerial mycelia, occasionally taking the form of chains, but often heaped up like a powder. Aerial mycelia usually well developed, long, cobwebby, often powdery, white, reddish, or yellowish in color. Chlamydospores usually many in number, sometimes lacking in mycelia and conidia. Stroma carmine purple or yellowish-ochre in the majority of cases.

Key to species and variaties

1. Macroconidia usually with 1-3-5 septa, fusiform-falciform with gradually narrowing conical upper cavity: F. sporotrichiella.

– Macroconidia usually with 5-7 septa; with 5 septa, 28-60 = 3-6; with 7 septa, 45-70 = 4-6: F. sarcochroum.

2. Microconidia dominant. Macroconidia with 1-3 septa formed sporadically in small numbers in aerial mycelia; with 1 septum, 9-5-20 = = 3-4; with 3 septa, 17-32 = 3.8-5: F. sporotrichiella var. poae.

– More or less profuse formation of macroconidia in aerial mycelia, pyonnotae, and spore cases – 3.

3. No chlamydospores. Macroconidia usually with 3 septa, 24-70 = = 2.5-5: F. sporotrichiella var. anthophilum.

– More or less profuse formation of chlamydospores – 4.

4. Macroconidia usually with 3 septa, 25-35 = 3.8-4.8: F. sporotrichiella var. tricinetum.

Macroconidia typically with 3-5 septa; with 3 septa, 30-45 = 4.5-5; with 5 septa, 32-45 = 3.8-5.5: F. sporotrichiella var. sporotrichiodes.

Nutritive Media

Chapek's medium:

Glucose – 20 g

Sodium nitrate ($NaNO_3$) – 2 g

Monobasic potassium phosphate (KH_2PO_4) – 1 g

Magnesium sulfate ($MgSO_4$) – 0.5 g

Potassium chloride (KCl) – 0.5 g

Ferric sulfate ($FeSO_4$) – 0.001 g

Distilled water – 1000 ml

Agar – 20 g

The mixture is sterilized at 112° for 20 min.

Potato agar: 200 g of cleaned, sliced potato is flooded with 1000 ml of tap water and placed in a Koch apparatus or an autoclave (running steam) for 40 min. The liquid is then poured off, boiled for 30-40 min, filtered through cotton gauze, brought up to its initial volume, and mixed with 20 g of agar. The agar mixture is sterilized at 120° for 20 min.

Potato agar containing glucose. A total of 2% of glucose is mixed with prepared potato water and 2% of agar is added after the glucose has dissolved. The mixture is sterilized at 112° for 20 min.

Acid potato agar (acidified immediately before use). A Pasteur pipette is used to add 1-2 drops of 50% sterile citric acid to 10 ml portions of sterile hot potato agar prepared in the manner described above and poured into test-tubes; the mixture is thoroughly stirred.

Rice. Portions of 5 g of rice grains are poured into test-tubes, mixed with 8-10 ml of tap water, and sterilized twice, first in running steam for 1 hr and then at 120° for 20 min on the following day.

Potato slices. Small, cylindrically cut slices of peeled potato are placed on the bottoms of test-tubes (one piece per tube) and sterilized twice, first in running steam for 1 hr and then at 120° for 20 min on the following day.

Medium containing fat:

Sodium nitrate ($NaNO_3$) – 2 g

Potassium chloride (KCl) – 0.5 g

Dibasic potassium phosphate (K_2HPO_4) – 1 g

Magnesium sulfate ($MgSO_4$) – 0.5 g

Ferric sulfate ($FeSO_4$) – 0.001 g

Distilled water – 1000 ml

Agar – 20 g

The medium is divided into 100 ml portions and sterilized at 120° for 20 min. Sunflower oil is added to the tubes in doses of 5-6 ml and the mixture is sterilized at 112° for 20 min. Before inoculation the medium is melted and, while it is still hot, 3 ml of sunflower oil and 0.2-0.3 ml of previously boiled 1% aqueous Nile blue are added per 100 ml of medium; the resultant mixture is agitated for 5-10 min and poured into dishes.

Bilai's medium:

Monosubstituted potassium phosphate (KH_2PO_4) – 1 g

Potassium nitrate (KNO_3) – 2 g

Magnesium sulfate ($MgSO_4$) – 0.5 g

Potassium chloride (KCl) – 0.5 g

Ferric sulfate ($FeSO_4$) – 0.001 g

Soluble starch – 0.1 g

Sucrose – 0.1 g

Glucose – 0.1 g

Water – 1000 ml

Portions of 5 ml are poured into test-tubes and a strip of filter paper is inserted into each tube in such fashion that it is immersed in the medium for less than half its length. The medium is sterilized at 112° for 20 min.

Chapek-Doxa's medium containing autolyzate:

Sucrose – 20 g

Sodium nitrate ($NaNO_3$) – 8 g

Monobasic potassium phosphate (KH_2PO_4) – 1 g

Magnesium sulfate ($MgSO_4$) – 0.5 g

Potassium chloride (KCl) – 0.5 g

Ferric sulfate ($FeSO_4$) – 0.001 g

Yeast autolyzate – 20 g

Distilled water – 1000 ml

The mixture is sterilized at 112° for 20 min.

Millet broth containing autolyzate. A total of 90 g of ground millet (flour) is mixed with 900 ml of tap water and left to stand for 24 hr. The liquid is decanted through cotton gauze (in a funnel) and the latter is thoroughly wrung out. A total of 0.5% of sodium chloride and 2% of yeast autolyzate are added and the mixture is heated until they dissolve. The medium is sterilized at 120° for 30 min.

Grain to be inoculated (for skin tests in rabbits and feeding to experimental animals). Portions of 100 g of millet, wheat, or some other grain are poured into 1-liter flasks, flooded with 100 ml of tap water, and sterilized at 120° for 30 min. Each new batch of grain should be preliminarily checked by skin tests in rabbits to make sure that it has not been damaged by Fusaria and to determine its toxicity. Only grain which gives a negative dermal reaction in rabbits and which has not been affected by Fusaria can be used. The grain is wetted with water and sterilized at 120°, for 30 min.

INFECTIONS OF THE RESPIRATORY TRACT

TUBERCULOSIS

Prof. A.I. Togunova

The current classification (Bergey, 1957) divides the pathogenic acid-fast Mycobacteria into 7 species: 1) Mycobacterium tuberculosis L.N., or M. tuberculosis typus humanus; 2) M. bovis Bergey, or M. tub. typ. bovinus; 3) M. microti Reed (Vole bacillus); 4) M. avium Chesteri, or M. tuberculosis avium; 5) M. paratuberculosis (Johnes bacillus) – the causative agent of hypertrophic enteritis in cattle; 6) M. leprae hominis; 7) M. leprae murium. The new classification assigns Mycobacteria parasitic in poikilotherms (M. poikilotermum) to the potentially pathogenic Mycobacteria (see below). This large group comprises Mycobacteria isolated by a number of authors in skin diseases of man (from abscesses in so-called tank disease) and cattle, from poikilotherms, and from the external environment, soil, etc. The members of this group most frequently mentioned in the foreign literature are M. fortuitum Cruz or Minetti, M. marinum Aronson, M. themnopheus Aronson, M. platypoecillus Becker and Hagen, M. Balnei Linel and Norden, etc.

The so-called "atypical" or "abnormal" acid-fast Mycobacteria (Odurua) constitute a special group and have recently attracted a great deal of attention. Their nature and their significance in human pathology have still not been conclusively determined; they are often isolated from pathological material taken from tuberculosis patients. Investigative techniques which make it possible to distinguish true tuberculosis Mycobacteria (even those altered by factors such as antibacterial therapy) from the so-called "atypical" Mycobacteria and the latter

from the saprophytes with which they are often mixed are consequently of great value.

The so-called "atypical" acid-fast Mycobacteria are now customarily divided into 4 basic groups (Renon et al.), depending on whether they carry out chromogenesis under illumination or independently of light: I) photochromogenic Mycobacteria (Kansas type) are usually isolated from sick humans; during growth under illumination cultures produce a lemon yellow pigment, which later changes to orange and then to brick-red; they do not produce pigment when cultured in darkness. A brief exposure to light is sufficient to cause subsequent pigmentation (during incubation at 37°); the colonies are smooth. II) scotochromogenic Mycobacteria acquire a bright orange color when grown in darkness. They are usually isolated from the patient in company with other microorganisms, sometimes in a mixed culture. III) Nonphotochromogenic Mycobacteria (Betti Sea type) do not generally produce pigment. IV) Fast-growing Mycobacteria (3-5 days at room temperature).

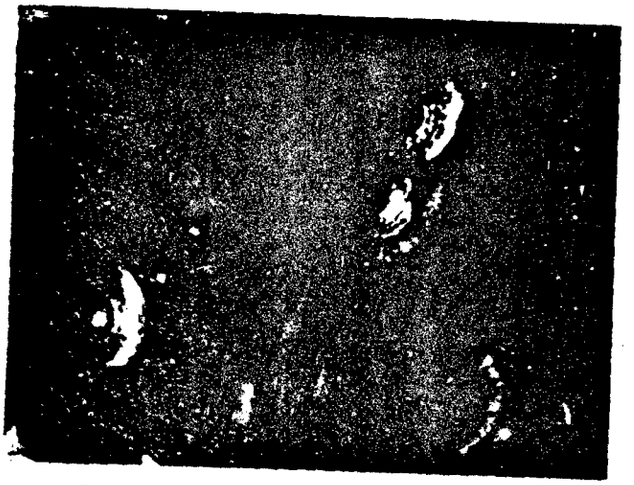
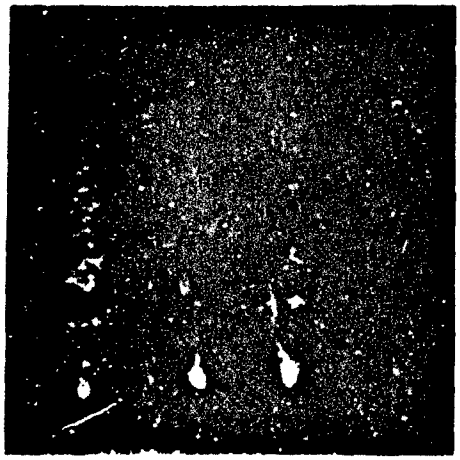


Fig. 56. Colonies of saprophytic Mycobacteria isolated from air on Petragnani's medium (dark orange pigment).

General characteristics: growth on solid media is smooth, continuous, finely plicated, and often oily. These bacteria grow more rapidly than tuberculosis Mycobacteria on the same media (see below), but may also grow on less favorable media (not containing glycerine). In liquid media (synthetic and others) growth at the bottom takes the form of flakes, while that at the surface usually takes the form of a thin oily film. The bacterial cells are polymorphous and vary in size. No flagella are formed in microcultures (see below) or liquid media. The majority of strains are nonpathogenic and are not virulent for guinea pigs and rabbits, although some are pathogenic for white mice when administered intravenously or intraperitoneally and for hamsters when administered intraperitoneally. Tuberculin reactions are inconsistent (+ or −) in humans and experimental animals infected with these Mycobacteria. The histomorphological changes are atypical, but generally retain the elements of tuberculoid tissue. Atypical Mycobacteria are more resistant to many antituberculosis drugs than tuberculosis Mycobacteria.

The last category of acid-fast Mycobacteria comprises the true acid-fast saprophytes and is a broad group of microorganisms morphologically and tinctorially similar to tuberculosis Mycobacteria. Saprophytes may be found in the soil, plants, manure, water, foodstuffs, milk, butter, the skin and excreta of sick and healthy persons and animals, etc. They are polymorphous rods of varying diameter and are for the most part relatively acid-fast, although less alkali- and alcohol-fast than tuberculosis Mycobacteria. They grow rapidly (2-3 days) on the majority of special media for tuberculosis Mycobacteria, but can also be raised on ordinary nutritive media at temperatures of 20-28° (Figs. 56 and 57). Their growth-temperature range is from 52 to 10°, although this does not hold for all strains. The following are the principal saprophytes.



1a *2a* *1б* *2б*

Fig. 57. Cultures of a nonpathogenic strain (Raven 852_2). 1) On glycerine-containing agar at 18° (a) and 37° (b); 2) on agar without glycerine at 18° (a) and 37° (b).

Fig. 58. Culture of the acid-fast saprophyte Mycobacterium phlei. Moist, coarsely plicated film on surface, sediment at bottom.

1. M. phlei L.N.: synonym [illegible]bacillus (timothy bacillus) or Grassbacillus. This bacterium [illegible]ly in nature and takes the form of polymorphous rods; it [illegible]ly poorly acid-fast. Cultures take the form of a smooth [illegible] with a gray or yellow color and become plicated as they [illegible]. Its growth-temperature range

is 28-52° and it will not grow at 10°. Growth requires 2 or 3 days. This bacterium is nonpathogenic for all species of experimental animals.

2. M. smegmatis L.N. Growth of this bacterium requires 3-4 days on all media at 28-45°. It is isolated from smegma contaminated with urine. Cultures take the form of a luxuriant, oily, cream-colored continuous growth. The bacterium appears as relatively short, thin polymorphous rods and is highly acid-fast, but poorly alkali- and alcohol-fast. It is nonpathogenic. In investigating material from patients (urine, lavage fluids, sputum, bronchoectasis, etc.) it is necessary to take special account of this and other saprophytes.

Morphology of tuberculosis Mycobacteria

The type variety is M. tuberculosis hominis (human type); this bacterium takes the form of straight or curved rods 1.5-4 microns long and 0.3-0.5 microns in diameter, which stain red by Ziehl-Neelsen's method, violet by Gram's method, and violet by Much's and Much-Weiss's methods (it is iodophilic). Brighter red (Zihl-Neelsen's method) or violet (Much's and Much's-Weiss's methods) granules are noted in the rods when these staining techniques are employed. Electron microscopic (× 30,000) examination of preparations of shadowed rods reveals round structures and a rather compact cell wall (Fig. 59). Electron microscopic investigation of ultrathin sections of tuberculosis Mycobacteria has made it possible to establish (Basserman) that the cell wall consists of 3 layers and that the cytoplasm contains nuclei and granules, which are probably mitochondria or chondriosomes. In preparations made up from cultures, especially those raised on liquid media, virulent tuberculosis Mycobacteria are arrayed in plaits. Both red cells and lighter violet cells (which are less acid-fast) are detected in smears prepared from very young and old cultures and stained by Ziehl-

Neelsen's method. The round structures (granules) almost always vary in the extent to which they take up the dye (acid-fastness). They must not be confused with Much granules, which stain by Gram's method and not by Ziehl-Neelsen's method, or with filterable forms of Mycobacterium tuberculosis.

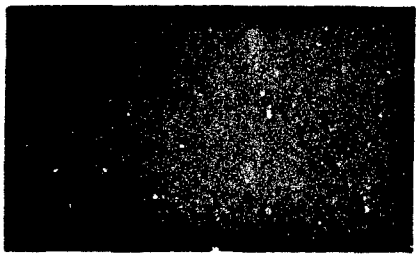


Fig. 59. Mycobacterium tuberculosis. Electron-microscopic photograph (bacterium shadowed). × 30,000.

Staining of tuberculosis Mycobacteria. Acid-fast Mycobacteria are stained slowly by aniline dyes. They must consequently be stained with the aid of a mordant (carbolic acid) while being heated. The most common staining techniques are Ziehl-Neelsen's method, Ziehl-Hughes's method, Much's method, Much-Weiss's method, and Spengler's method.

Ziehl-Neelsen's method (for ordinary bacterioscopic investigation): a) a smear is prepared on a slide and fixed by flaming it three times; b) Ziehl's carbol fuchsin is applied to the preparation through a strip of filter paper and the slide is carefully heated over a Bunsen-burner flame until vapor is given off (usually 3 times); c) the paper is removed and the preparation is rinsed with distilled water; d) the stained smear is decolorized by application of 10% sulfuric or 3-20% nitric acid or by dipping the slide into a beaker containing one of these acids (for several seconds); e) the slide is rinsed with water; f) the preparation is restained with aqueous methylene blue or Loeffler's blue.

Ziehl-Neelsen's method as modified by Hughes. The specimen is: a) stained with carbol fuchsin as in the preceding method; b) decolorized in 3% alcoholic hydrochloride; c) rinsed with water; d) restained with

aqueous methylene blue. The rods appear red against a blue background. These two methods are the most commonly employed in practice.

Much's method (modified Gram's method). The specimen is: a) flame-fixed and stained with carbol methyl violet through filter paper while being carefully heated; b) flooded (without rinsing) with a solution of iodine in potassium iodide (Lugol's solution) for 5 min; c) rinsed with water; d) transferred to 5% nitric acid for 1 min; e) immersed in 3% hydrochloric acid for 10 min; f) placed in an acetone-alcohol mixture until completely decolorized; g) rinsed with water; h) restained with aqueous fuchsin. Tuberculosis Mycobacteria appear as violet granular rods ("atypical" Mycobacteria appear solid).

Much-Weiss's method: a) 3 parts of carbol fuchsin are mixed with one part of carbol methyl violet in a test-tube; b) the preparation is stained with this mixture heated to the boiling point (carefully!), changing the dye 4 times; c) the stained specimen is immersed in Lugol's solution for 5-10 min; d) the preparation is transferred to 5% nitric acid for 1 min and then e) to 3% hydrochloric acid for 10 sec and f) to acetone-alcohol until decolorized; g) the specimen is rinsed with water. Mycobacteria appear red, containing dark violet granules of varying size.

Biology of tuberculosis Mycobacteria; cultural characteristics

Pure cultures of tuberculosis Mycobacterium can be grown outside the organism on solid and liquid media with a good air supply; they can also be raised under relatively anaerobic conditions, but grow very slowly, sparsely, and atypically. On special liquid nutritive media the majority of true tuberculosis Mycobacteria form a plicated film on the surface of the medium (R form), creeping up the walls of the flask (Fig. 60). Only in media containing detergents (twin-80®) do they yield a uniform growth throughout the medium (especially when frequently or con-

Fig. 60. Culture of Mycobacterium tuberculosis var. hominis on synthetic medium. Dry plicated film on surface of medium and walls of flask. Medium clear.

а б в г д

Fig. 61. Mycobacterium cultures on glycerine-potato medium. a) Mycobacterium tuberculosis var. hominis (5709); b) Mycobacterium tuberculosis var. bovis (Valee); c) Mycobacterium microti; d) facultatively pathogenic strain (pigmented); e) saprophyte from butter (L. Rabinovich).

stantly agitated). Under such conditions these Mycobacteria, which are hydrophobic (as a result of the presence of superficial cerclipid-), become hydrophilic, or wettable, so that they produce a diffuse growth (Dubos's medium). Tuberculosis Mycobacteria grow on nutritive media containing mineral salts, amino acids, carbohydrates, and egg whites or yolks and especially well on media containing glycerine. They will also grow (although more sparsely) on media composed of mineral salts, es-

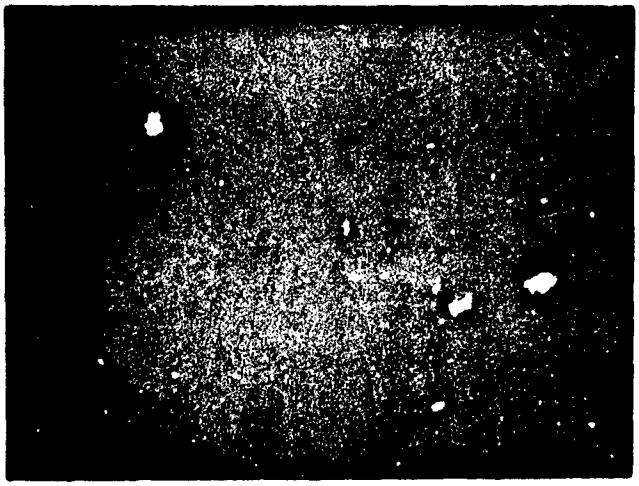


Fig. 62. Colonies of Mycobacterium tuberculosis var. hominis on Petragnani's medium. R form.

pecially ammonia (L.M. Model'). The optimum growth temperature is 37-38°. Growth is slow (20-30 days) in the initial cultures isolated from the organism.

Mycobacterium tuberculosis var. hominis produces a dry, plicated or scaly, light cream growth on solid nutritive media (Fig. 61); the cultures have a very characteristic aromatic odor. They are difficult to pick up with a loop and are consequently transferred with a special narrow spatula; as a result of their high lipid content they crack when placed over a flame (a characteristic trait). In transplanting a culture the material should be spread out with a spatula and gently rubbed over the surface of the medium. Colonies of this Mycobacterium are plicated and dry and usually have slightly raised centers and nonuniform margins (Fig. 62). They produce a fine flaky suspension (R type) in physiological solution. The character of the cultures changes on media containing substances such as bile (a grayish oily growth is produced and the rods are longer and branched). Mycobacterium tuberculosis var.

hominis is characterized by glycerinophilia, i.e., it grows more profusely and rapidly (eigonically) on media containing glycerine. This bacterium may on rare occasiona form smooth colonies (see the section on variability). Mycobacterium tuberculosis var. bovis does not require glycerine. Cultures grow slowly (disgonically) and are finely plicated, dry, and grayish. The colonies are small and hemispherical or planoconvex, becoming finely plicated as they age (Fig. 63). This bacterium grows slowly on the surface of liquid media, forming isolated islands which gradually merge to produ a film (Fig. 64). Its odor is usually aromatic. The rods are acid-fast and shorter and thicker than those of the preceding type. The first few generations after isolation from the organism exhibit the sort of growth described above. Acclimatized cultures grow more rapidly, especially on glycerine media. Mycobacterium avium has a very characteristic type of growth. This bacterium is in rare cases found to be the causative agent of human tuberculosis. The rods are long, slender, sometimes branched, and acid-fast. The optimum growth temperature is 40-42°, which is to some extent a differential-diagnostic characteristic. Cultures of Mycobacterium avium grow relatively rapidly (10-12 days) and profusely on nutritive media containing glycerine, taking the form of a smooth, oily continuous growth on solid nutritive media. The colonies are hemispherical, smooth, and lustrous. The cultures can be smeared on slides and readily give a homogeneous suspension in physiological solution; they are white or cream in color, with an easily distinguished orange variant. In liquid nutritive media this bacterium produces a diffuse growth with a coarse sediment on the bottom and sometimes a moist fatty film at the surface with filaments reaching into the medium. The cultures are white or yellowish and have an optimum pH of 6.8-7.3.

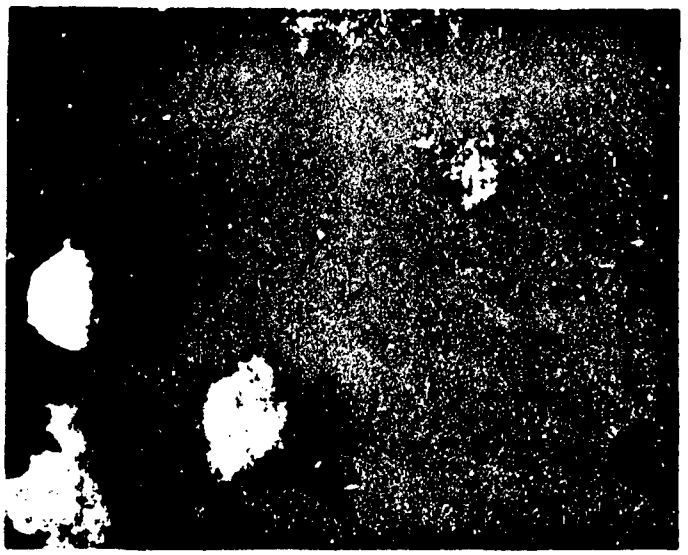


Fig. 63. Colonies of Mycobacterium tuberculosis var. bovis on Petragnani's medium. R and S forms.

Fig. 64. Culture of Mycobacterium tuberculosis var. bovis. Slowly growing segments of film. Medium clear.

Mycobacterium tuberculosis microti is of no significance in human pathology; these rods are ovoid or round, semicircular or circular. In smears they resemble fish roe. They are acid- and alcohol-fast and cause a tubercular disease in voles, from which they were isolated in

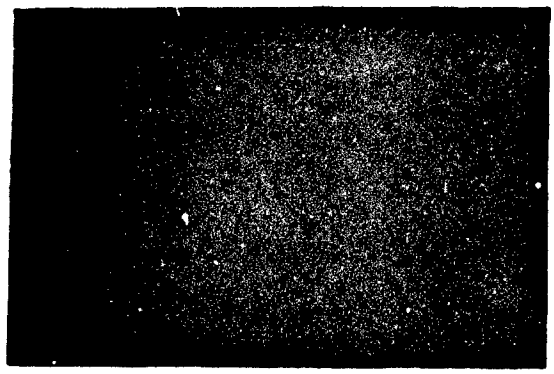


Fig. 65. Colonies of Mycobacterium microti on Petragnani's medium.

1937 by Wells during an epizootic in Scotland (Fig. 65).

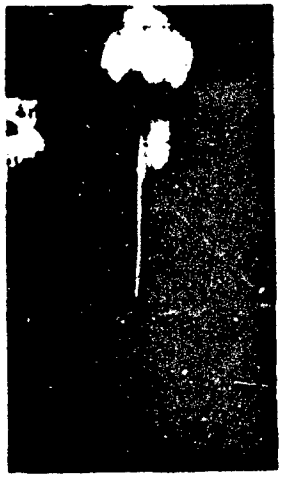


Fig. 66. Culture of chromogenic variant (137/p). Moist, oily film. Sediment on bottom.

Typical and atypical cultures of tuberculosis Mycobacteria. The types of tubercular cultures described above are usually isolated from material obtained from humans and animals suffering from tuberculosis. These cultures are not, however, always typical (especially when the patient receives antibiotic therapy); they may be somewhat moister or contain individual smooth or pigmented colonies. Such cultures may be true tuberculosis Mycobacteria altered in the patient's body or so-called abnormal or atypical acid-fast Mycobacteria. The tubercular nature of atypical cultures is best established by the "mouse test" (injection of 1 and 0.1 mg of culture into the caudal veins of 2 white mice; death occurs within 2-4 months in the presence of symptoms of generalized tuberculosis) and by determining

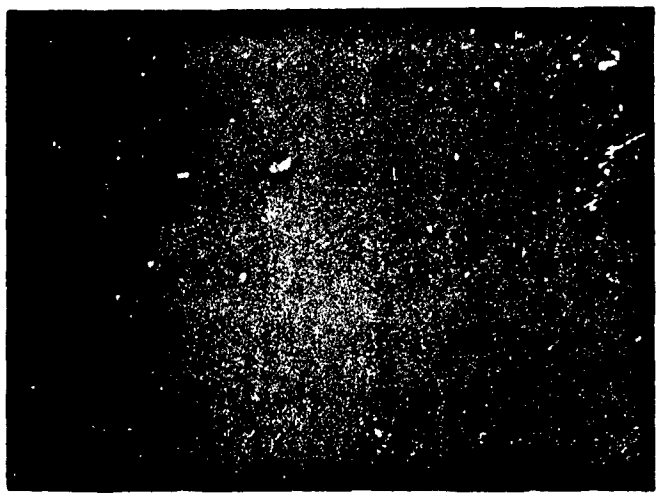


Fig. 67. Dissociation of Mycobacterium tuberculosis var. hominis. Flat, spiral, plicated cream and orange colonies.

their pathogenicity in guinea pigs.

TABLE 50

Properties of soft (S) and hard (R) Variants of Tuberculosis Mycobacteria (principally M.t. humanus)(after A.I. Togunova)

Properties of Variants	Soft (S) variant	Hard (R) variant
Character of growth on nutritive media	Moist, soft, sometimes chromogenic	Dry, hard
Appearance of colonies	Hemispherical, smooth, moist, occasionally flat, slightly plicated or in pigmented spirals	Rough, planoconvex in center, nonuniform at margins, hard or compact, lustrous, cream-colored
Formation of secondary colonies	Forms such colonies	Rarely forms such colonies, which alter the morphological characteristics of the basic colony (lysis)
Character of growth on liquid media	Fine moist film on surface giving rise to filaments, coarse sediment on bottom	Slowly developing plicated "film" culture on surface, creeping up walls of flask
Suspension in 0.85% sodium chloride	Homogeneous, smears well on slides	Nonhomogeneous, in small clumps, smears poorly on slides

Growth rate on nutritive media	Relatively rapid, 8-10 days	Slow, 16-20-30 days
Shape of bacterial cells	Elongated rods, sometimes branched, with reduced acid-fastness and few or no "granules"	Short, slender, straight or slightly curved rods with granules ("granular") and pronounced acid-fastness;
Virulence for laboratory animals (guinea pigs)	Low virulence	Marked virulence
Presence of "poured factor," growth in microcultures	Poured factor moderate or absent, forms coarse "plates" or "flakes" in microcultures	Signs of "poured factor" quite distinct, dense tangles in microcultures
Catalase activity	Pronounced in most cases	Relatively low, variable
Neutral red test (Dubos and Middlebrook's reaction)	Light red or reddish	Pronounced (carmine red)
Antigenic properties	Moderate	Pronounced
Pigmentation	Entire culture, coloration often associated with exposure to light	Center of colony, unstable
Relationship to phagocytosis	Accelerated reaction, does not affect myability of phagocyte	Ordinary reaction, engulfed rods cause phagocyte to die in the majority of cases
Resistance to antibacterial drugs	Primary resistance usually pronounced	Produces resistant forms on prolonged exposure
Cytomorphological reaction of organism (guinea pig)	Development of viable, specific, usually productive granulation tissue, often limited to local foci, tendency toward involution	Typical specific productive-exudative granuloma accompanied by necrosis and caseation, generalization of process.

Variability of tuberculosis Mycobacteria. Dissociation. Under certain conditions (infrequent transplantation, prolonged storage at room temperature, exposure to antibiotics, spontaneous lysis, etc.) all

types of tuberculosis Mycobacteria may form variants differing from the initial culture in a number of characteristics. Mycobacterium tuberculosis var. hominis is characterized by R. form cultures (the so-called hard variant), which are dry and plicated, produce a fine flaky suspension in physiological solution, and are virulent for guinea pigs. On dissociation this bacterium produces a S form (soft variant) and forms a moist film on liquid media (Fig. 66). The colonies are hemispherical or spiral (Fig. 67), smooth, and moist and yield a homogeneous suspension in physiological solution; they are of low virulence for guinea pigs. Table 50 shows the principal characteristics of the hard and soft variants.

Pathogenicity, virulence, and toxin formation

Under experimental conditions M. tubercul. typ. human. (Mycobacterium tuberculosis var. hominis) is pathogenic for guinea pigs and monkeys (producing a progressive tubercular process) and less pathogenic for rabbits, cattle, birds, and mice. M. tubercul. typ. bov. (Mycobacterium tuberculosis var. bovis) may also cause tuberculosis in humans, especially children; under experimental conditions it is more pathogenic for rabbits than for guinea pigs. Under natural conditions it also causes a tubercular disease of hogs and birds (in addition to cattle).

The differential characteristics of the various types of tuberculosis Mycobacteria are sometimes not sufficiently distinct. In order to identify the type of Mycobacterium isolated it is then necessary to determine its pathogenicity and virulence in laboratory animals.

Principal methods of identifying types of tuberculosis Mycobacteria. The various types of tuberculosis Mycobacteria are identified from a complex of their morphological and cultural characteristics and their pathogenicity for laboratory animals. It should be noted that such

identification is most conclusive when carried out with the primary cultures isolated from the organism. The identification procedure involves intravenous injection of 2 rabbits with 0.1 and 0.01 mg of fresh culture suspended in physiological solution. Mycobacterium tuberculosis var. bovis causes the rabbits to die within 2-3 months, exhibiting symptoms of pronounced tubercular damage to the lungs; Mycobacterium tuberculosis var. hominis is not fatal when administered in these doses. When administered intravenously to rabbits in a dose of 0.1 mg Mycobacterium avium causes acute septicemia ("Yersin type") resulting in death within 10-15 days. M. tub. avium is nonpathogenic and nonvirulant for guinea pigs. The foregoing can be used as substitutes for the principal international biological methods of identifying tuberculosis Mycobacteria in rabbits (Kossel's method, Jensen's method, and Griffiths's method).

Method of determining culture virulence in animals. In typing cultures by pathogenicity it is necessary to take into account their virulence for the corresponding animals. The virulence of tuberculosis Mycobacteria is determined from the minimum lethal dose (MLD) of a 20-21-day culture that causes guinea pigs to die (on subcutaneous injection) of generalized tuberculosis within a definite period. The inoculation dose is measured in fractions of a milligram of semidry culture, while the number of bacteria is evaluated by comparison with a bacterial standard (e.g., the BTsZh); the latter is also calculated (subsequently) from the number of colonies growing on egg-milk medium. On the average, 1 mg of semimoist (gently pressed between sheets of filter paper) culture contains approximately 40,000,000 bacteria. It is recommended that from 0.001 to 0.00001 mg be administered subcutaneously to guinea pigs for a rough determination of culture virulence. Highly virulent cultures cause death within 3-4 months at small doses (0.00001

mg) and within 2 months at 0.001 mg; low-virulence cultures cause death within 5-6 months at 0.001 mg and within 7-8 months at 0.00001 mg. Larger doses (1 mg or more) must be used to determine the virulence of altered and weakened strains. Vaccine strains are tested for so-called residual virulence, i.e., their ability to cause development of a specific tissue reaction and to survive by vegetating (0.001, 0.01, and 0.1 mg).

Preparation of culture suspension: A specimen of the culture is taken with a spatula at a definite stage of its growth (after incubation for 20-21 days on glycerine-potato medium) and pressed between sheets of sterile filter paper. A quantity of this semimoist culture is weighed to the nearest milligram on a chemical scales, using a sterile box or paper packet. The weight of the box or empty packet is subtracted from the result and the culture is transferred to a previously prepared sterile jar containing glass or stainless steel beads, which should fill no less than 1/3 of the jar (the number of milligrams of culture is noted). The culture is ground by rotating the jar manually or by agitating it in a shaking machine, adding no liquid at first (the culture should not be smeared on the walls of the jar above the beads!). After 10-15 min sterile physiological solution is added to the jar (1 ml per mg of culture), pouring in first a small quantity and then the entire calculated amount. Administration of a given dose requires that the suspension be further diluted, so that the requisite amount of culture is contained in 1 ml of physiological solution. It is convenient to make up successive 10-fold dilutions in jars containing beads. Guinea pigs of standard weight (250-300 g) are inoculated subcutaneously in the right groin and then kept under constant observation (change in weight, general condition, development of regional lymphadenitis or periadenitis, time of death, pathogoanatomic pattern

TABLE 51

Acid-Fast Mycobacteria

Особенности 1	2 Патогенные микобактерии					3 Потенциально-патогенные микобактерии	4 «Атипичные» микобактерии	5 Сапрофиты
	Mycobacterium tuberculosis							
	M. tub. typ humanus	M. tub. typ. bovinus	M. tub. avium	M. microti (vole bacillus)	M. lepras hominis			
6 Кислотоустойчивость	Выражена 14	Выражена	Выражена	Выражена	Выражена 17	15 Большей частью выражена	Большей частью выражена	Большей частью выражена
7 Щелочеустойчивость	То же 16	То же	То же	То же	Нет	То же	18 Умеренно	19 Слабо или не выражена
8 Спиртоустойчивость	» »	» »	Умеренно выражена 18	» »	Нет	» »	То же	То же
9 Устойчивость к обесцвечиванию активным хлором	» »	» »	То же	?	Нет 25	» »	То же	20 Отсутствует
10 Морфологии	Тонкие, зернистые, слегка изогнутые палочки 21	Короткие толстоватые палочки 22	Тонкие, более длинные зернистые, иногда ветвятся 23	Короткие, изогнутые, овоидные, полукружия 24	Прямые или изогнутые, полиморфные, «осколки сигар» (в тканях)	Прямые или изогнутые полиморфные палочки 26	Полиморфные микобактерии 27	Полиморфные, толстые или тонкие, больший частью прямые палочки 28
11 Характер роста 12 на плотных средах	Морщинистый суховатый, эйгонический, глицеринофильность 29	Слегка морщинистый, суховатый, дисгонический, глицерин не нужен 30	Гладкий, мягкий, блестящий, белый или желтоватый 31	Белый или кремоватый, складчатый, слегка влажный 32		Гладкий или складчатый влажный, белый, серовато, хромогенный 33	Фото-ското- и нехромогенный, влажный, мелкосладчатый, маслянистый 34	Слегка влажный морщинистый или гладкий, белый или хромогенный 35
13 на жидких средах	Суховатая морщинистая пленка на поверхности 36	Медленно развивающаяся пленка на поверхности 37	Влажная пленка на поверхности, частью в глубине среды 38	Слегка влажная пленка на поверхности 39		Влажная пленка на поверхности или хлопьевидный осадок на дне 40	Влажная пленка, рост в среде, осадок на дне 41	Пленка складчатая, влажная, или в толще среды слизистый рост 42

Форма колоний в ткане	Сухие шероховатые плосковыпуклые с неровным краем	Плоские и округлые, гладкие и зернистые, суховатые	Полушаровидные гладкие, мягкие	В виде «бараночек» слегка влажные, частью плосковыпуклые		Полушаровидные, большей частью гладкие	Полушаровидные гладкие, частью пигментированные	Гладкие полушаровидные, позже складчатые
Форма роста вирулентного варианта	R	R (и S)	S	R (не вирулентны)		S (не вирул.)	S (не вирул.)	S (редко R), не патогенны
Скорость роста	20—30 дней	20—25—30 дней	10—12 дней	20—25 дней		12—15 дней	6—10—25 дней	2—3—5 дней
Оптимальная температура роста	37—38°	37—38°	41—42°	37°		22—37°	22—37°	10—20°—28—45—52°
Корд-фактор	+	+	±	±		—	—	—
Патогенность и вирулентность в эксперименте: Морские свинки	Сильная вирулентность	Выраженная вирулентность	Слабая или отсутствует	Слабая	Не вирулентны	Отсутствует	Редко преходящие местные инфильтраты	Нет
Кролики	Слабая вирулентность	Сильная вирулентность	Сильная (тип Иерсена)	Не вирулентны	То же	То же	Нет	То же
Куры	То же	Слабая	Сильная	То же	» »	» »	То же	» »
Мыши белые	Вирулентность выражена	Умеренно выражена	Слабая	Слабая	» »	Потенциально патогенны, абсцессы, «болезнь хвоста»	Часто патогенны (в/в, в/бр)	» »
Рогатый скот	Вирулентность слабая	Сильно выражена	Слабая		?	Местные абсцессы	?	Нет
Хомячки золотистые	Сильно выражена	То же	Выражена умеренно	Не вирулентны	?	Потенциально патогенны	Часто патогенны	
Животные холоднокровные	—	—	—	—	—	Условно патогенны	Не патогенны	То же
Иммуногенность	+	+	+—	Выражена	Слабо выражена в тканях	Нет	Нет	» »
Значение в патологии человека	+	+	+—	—	+	+—	+ фотохром +—	—

1) Characteristics
2) pathogenic Mycobacteria
3) potentially pathogenic Mycobacteria
4) "atypical" mycobacteria
5) saprophytes
6) acid-fastness
7) alkali-fastness
8) alcohol-fastness
9) resistance to active chlorine
10) morphology
11) character of growth
12) on solid media
13) on liquid media
14) pronounced
15) pronounced in most cases
16) the same
17) none
18) moderate
19) weak or moderate
20) absent
21) slender, granular, slightly curved rods
22) short thick rods
23) slender, elongate, granular, sometimes branched
24) short, curved, ovoid, semicircular
25) straight or curved, polymorphous, "cigar bands" (in tissues)
26) straight or curved polymorphous rods
27) polymorphous Mycobacteria
28) polymorphous, thick or slender, straight rods for the most part
29) plicated, dry, eigonic, glycerinophilic
30) slightly plicated, dry, disgonic, glycerine not required
31) smooth, soft, lustrous, white or yellowish
32) white or cream, plicated, slightly moist
33) smooth or plicated, moist, white or gray, chromogenic
34) photochromogenic, scotochromogenic, or nonchromogenic, moist, finely plicated, oily
35) slightly moist, plicated or smooth, white or chromogenic
36) dry plicated film on surface
37) slowly developing film on surface
38) moist film on surface, often within medium
39) slightly moist film on surface
40) moist film on surface or flaky sediment on bottom
41) moist film, growth into medium, sediment on bottom
42) plicated moist film or mucoid growth within medium
43) form of type colonies
44) type of growth of virulent variant
45) growth rate
46) optimum growth temperature
47) chord factor
48) pathogenicity and virulence under experimental conditions
49) guinea pigs
50) rabbits
51) chickens
52) white mice
53) cattle
54) golden hamsters
55) poikilotherms

56) immunogenicity
57) significance in human pathology
58) dry, rough, planoconvex with nonuniform margins
59) flat and round, smooth and granular, rough
60) hem'spherical, smooth, soft
61) in form of "steering wheel," slightly moist, often planoconvex
62) hemispherical, smooth in most cases
63) hemispherical, smooth, often pigmented
64) smooth, hemispherical, plicated on aging
65) or
66) nonvirulent
67) S (rarely R), nonpathogenic
68) days
69) highly virulent
70) marked virulence
71) weak or absent
72) weak
3) not virulent
4) absent
75) transient local infiltrations in rare instances
76) none
77) strong (Yersin type)
78) weak
79) strong
80) virulence pronounced
81) moderate
82) potentially pathogenic, abscesses, "tail disease"
83) pathogenic in some cases (v/v, v/br)
84) weak virulence
85) highly virulent
86) local abscesses
87) potentially pathogenic
88) some pathogenic
89) facultatively pathogenic
90) nonpathogenic
91) weak in tissues
92) photochrome

on dissection, and whether dead of specific process or killed after a given interval). When dissecting the animals it is convenient to record the pathologoanatomic changes on individual cards, providing each animal with corresponding documentation.

Toxicity. Tuberculosis Mycobacteria do not produce exotoxin. However, filtrates of cultures of these bacteria on liquid nutritive media are toxic for animals suffering from tuberculosis; this results from release into the medium of exotoxin liberated from lyzed bacterial cells (specific proteins and lipids soluble in petroleum ether and

chloroform). Tuberculosis Mycobacteria also produce volatile toxins. One peculiarity of these toxins is the fact that they have a specific action only on humans and animals infected with tuberculosis (Table 51).

Determination of drug resistance of tuberculosis Mycobacteria

So-called drug-fast or resistant Mycobacteria are found in patients undergoing antibacterial chemotherapy and sometimes develop spontaneously (primary resistance). In some cases resistant tuberculosis Mycobacteria are quite variable (in their growth capacity and form on nutritive media, metabolism, virulence, and morphology).

Determination of resistance of tuberculosis Mycobacteria on solid medium containing various quantities of antibacterial drugs. Appropriate preliminarily prepared dilutions of streptomycin or phthvicide are added (to the coagulation point) to Helberg's or Petragnani's egg-milk medium (Table 52).

Each flask is labeled with the antibiotic concentration which it contains. The medium is poured into test-tubes (which are labeled)and the latter are then placed in a Koch coagulation apparatus in a semi-inclined position and coagulated at 85° for 45 min. The finished medium can be stored under refrigeration for no more than 1-1 1/2 weeks. In the direct method the pathological material is treated with 4% NaOH or 2% H_2SO_4. After the centrifugate has been washed with sterile physiological solution or neutralized with hydrochloric acid (when treated with NaOH) it is mixed with 2 ml of physiological solution; 5 test-tubes containing the aforementioned medium are inoculated with 0.2 ml of the resultant suspension. An equal number of test-tubes containing medium and corresponding antibiotic concentrations are left uninoculated as controls. If an isolated culture is to be tested (indirect method) it is suspended in sterile physiological solution by

comparison with a BTsZh bacterial standard (5 mg per ml) and 0.1 ml of the suspension is added to each test-tube. The tubes are placed in a heater in an almost horizontal position and removed after 1-2 days; they are then sealed with paraffin or rubber stoppers and incubated for 3-4 weeks, after which an initial evaluation of the cultures is made. If the experimental tubes contain growth a positive report is made. If no growth is present observation is continued for 2 or more months. The resistance of the Mycobacteria to the drug in question is determined from the number of colonies in the experimental tubes containing different antibiotic concentrations and in the corresponding controls. Determination of the drug resistance of tuberculosis Mycobacteria on Gerrold's yolk-agar medium (see below) containing various quantities of antibacterial drugs is carried out in the same manner.

Determination of drug resistance of Mycobacteria by the depth method.

a) Determination of streptomycin resistance. A total of 10 ml of distilled water is added to a flask containing 100,000 or 200,000 units of streptomycin. This is the "basic dilution"(10,000 or 20,000 units per ml of solution), which can be stored for 1 month at +5 or 10°, and is used to make up dilutions containing 1000, 100, 50, 10, 5, 2, and 0.5 γ of streptomycin per ml. Penicillin solution is added to the nutritive medium (see below) used to prepare the dilutions; this is done by adding 10 ml of nutritive medium to a flask containing 200,000 units of penicillin. After the penicillin has dissolved 0.1 ml of the solution is added to 200 ml of nutritive medium. The subsequent streptomycin dilutions are made up as follows:

b) Determination of phthvicide resistance. Phthvicide dilutions.

1. In order to obtain the "basic" dilution 12 mg of phthvicide is weighed out and mixed with 6 ml of alcohol and then with 6 ml of dis-

TABLE 52

Streptomycin Dilutions and Addition to Nutritive Medium (γ = 0.001 mg)

1 № п/п	2 Разведение стрептомицина			6 Приготовление среды с разными концентрациями в ней стрептомицина		
	3 количество стрептомицина	количество дистиллированной воды 4	получ. концентрация 5	количество питательной среды в колбе 7	3 количество стрептомицина	8 количество стрептомицина в 1 мл среды
	9 Основной флакон					
1	200 000 ед	4 мл 11	50 000 γ/мл		12	
2	2 мл № 1	8 »	10 000 »	50 мл	0,5 мл разведения № 2	100 γ/мл
3	2 » № 2 10	2 »	5 000 »	50 »	То же № 3	50 »
4	1 » № 2	9 »	1 000 »	50 »	» » № 4	10 »
5	2 » № 4	2 »	500 »	50 »	» » № 5	5 »

13

14 Схема разведения фтивазида и добавления его к питательной среде

№ п/п 1	15 Разведение фтивазида			18 Приготовление среды с различными концентрациями фтивазида		
	16 количество фтивазида	17 количество растворителя	5 получ. концентрация	19 количество среды в колбе	16 количество фтивазида	20 концентрация фтивазида в мл среды
1	70 мг 21	22 7 мл спирта	10 000 γ/мл	50 мл	13 0,5 мл разведения № 1	100 γ/мл
2	12 3 мл № 1	23 3 мл дист. воды	5 000 »	50 »	14 То же № 2	50 »
3	1 » № 1	9 »	1 000 »	50 »	» » № 3	10 »
4	1 » № 3	9 »	100 »	50 »	» » № 4	1 »

1) Test-tube No.; 2) streptomycin dilution; 3) amount of streptomycin; 4) amount of distilled water; 5) resultant concentration; 6) preparation of medium containing different streptomycin concentrations; 7) amount of nutritive medium in flask; 8) amount of streptomycin in 1 ml of medium; 9) basic flask; 10) units; 11) ml; 12) 0.5 ml of dilution No. 2; 13) the same; 14) phthvicide dilutions and addition to nutritive medium; 15) phthvicide dilution; 16) amount of phthvicide; 17) amount of solvent; 18) preparation of medium containing different phthvicide concentrations; 19) amount of medium in flask; 20) phthvicide concentration in 1 ml of medium; 21) mg; 22) ml of alcohol; 23) ml of distilled water.

tilled water. The preparation is dissolved over a period of 3-5 min (producing a clear yellowish liquid). The resultant dilution amounts to 1000 γ/ml and is used to prepare dilutions of 100, 50, 10, 1, 0.1, and 0.05 γ/ml. Portions of 2 ml of nutritive medium containing the corresponding phthvicide dilutions and penicillin are added to all the test-tubes except the controls, to which 2 ml of medium without phthvicide

Порядковые разведения 1		2 Исходные разведения стрептомицина			3 Питательная среда	4 Содержание стрептомицина в 1 мл
А	А	5 Основное (10 000 γ/мл)	6 —0,5 мл		9,5 мл	500 γ/мл
В	Б	Разведение А (500 »)	—2	»	8 »	100 »
С	В	7 » Б (100 »)	—3	»	9 »	25 »
Д	Г	» » (100 »)	—1	»	9 »	10 »
Е	Д	» Г (10 »)	—4	»	4 »	5 »
F	Е	» Г (10 »)	—1	»	9 »	1 »
G	Ж	» Е (1 »)	—2	»	8 »	0,25»

1) Sequential dilutions; 2) initial streptomycin dilutions; 3) nutritive medium; 4) streptomycin content per ml; 5) basic; 6) ml; 7) dilution.

is added. In direct determination 0.2 ml of a suspension of pathological material treated in the manner described above is added to each of the tubes, both experimental and control, after preliminarily mixing the washed residue (centrifugate) with 2 ml of the nutritive medium. After incubation in a heater for 20 days the contents of one of the control tubes are centrifuged and smears are made on slides, dried, heat-fixed, stained by Ziehl-Hughes's method, and examined microscopically. When acid-fast Mycobacteria are present in the smears, in the form of "plates," the residues (centrifugates) in the other tubes are examined and the microscopic pattern observed is used to evaluate the resistance of tuberculosis Mycobacteria to the preparation in question. When there is no growth in the control tube after 20 days the entire series of experimental tubes is left in the heater for an additional 10 days, after which the growth in the control is reexamined. If there is no growth the experiment is repeated.

Determination of resistance to paraaminosalicylic acid. A total of 10-15 mg of paraaminosalicylic acid is dissolved in 10-15 ml of Soton's medium, VKL medium, or semisynthetic medium based on casein hydrolyzate, yielding a solution containing 1000 γ/ml; this "basic" solution is used to make up paraaminosalicylic acid dilutions of 250,

100, 50, 10, 1, and 0.1 γ/ml. Resistance to this drug is determined in the manner described above.

The microculturing method is also recommended for determining the drug resistance of tuberculosis Mycobacteria (see page 663 for a description of the technique employed).

Sensitivity and resistance of tuberculosis Mycobacteria. Those tuberculosis Mycobacteria which grow only at streptomycin concentrations of up to 5 γ/ml (0.25, 1, 2 and 5 γ/ml) are considered sensitive to this drug. Resistant bacteria grow at streptomycin concentrations of more than 5 γ/ml, the extent of their resistance being expressed by the corresponding concentrations (10, 25, 100, and 1000 γ/ml). Phthyvazide-sensitive microbacteria grow in concentrations of preparation up to and including 1 γ/ml (0.05, 0.1 and 1 γ/ml), while resistant forms live at concentrations above 1 γ/ml (2 and over 5 γ/ml; 10 and 20 γ/ml).

In testing drug resistance Helberg's, Petragnani's, and Gerrold's media should be used as solid media and Soton's synthetic medium (with plasma), blood medium (see below), VKL synthetic medium containing serum (or plasma) and semisynthetic media based on casein hydrolyzate as liquid media.

Laboratory Diagnosis of Tuberculosis in Humans

Material for examination; taking of samples

In order to establish the specific etiology of a disease suspected of being tuberculosis material taken from the patient (sputum, cerebrospinal fluid, pleural fluid, pus, urine, feces, puncture specimens from the lymph nodes, etc.) is subjected to clinical and microbiological investigation. The material is examined bacterioscopically, bacteriologically, and biologically for tuberculosis Mycobacteria.

Preparation of vessels to hold material. In collecting material

it is necessary to take measures to prevent contamination with extraneous acid-fast Mycobacteria. The vessel should be thoroughly washed and sterilized in a dry-air chamber (at 160° for 1 hr) or boiled for 1-2 hr. Jars, test-tubes, etc. are sealed with cotton stoppers, paper covers, etc., and autoclaved at 120° for 20 min. Glass vessels previously used for infected material are filled with 5% carbolic acid or chloramine for 24 hr and then with a chromate mixture (50 g of potassium dichromate in 1 liter of concentrated sulfuric acid) for 2-4 hr; they are then thoroughly rinsed in tap water and finally in distilled water. Rubber tubes and probes are boiled and thoroughly rinsed.

Examination of sputum for tuberculosis Mycobacteria. The sputum to be examined should be as fresh as possible. The most highly suppurative lumps (poured off into a Petri dish) are transferred to clean degreased slides with a tweezers or spatula and thin smears are made by crushing them between two slides; the smears are air-dried and immediately flame-fixed. The resultant preparation is stained with carbol fuchsin (through filter paper), rinsed with water, and decolorized with 15-25% sulfuric acid; after rinsing with water (to remove the acid) it is treated with 3% alcoholic sulfate, again rinsed with water, and restained (for 5 min) with aqueous methylene blue. After the specimen has dried it is examined microscopically with an oil-immersion objective. Approximately 100 fields of view, covering virtually the entire preparation, are inspected. Tuberculosis Mycobacteria appear bright red, slender, and delicate and are found singly or in groups, principally extracellularly. When differentiation from saprophytes is doubtful the smears are also treated by Teleman's or Weichselbaum's method or with 1% Javel water (see below). If no acid-fast rods are detected in the smears it is helpful to make thicker smears and stain them by Spengler's method or to employ one of the concentration tech-

niques. The most widely used of the latter is the flotation method, including culturing of flotation rings.

Technique of the flotation method. An equal volume of 1% sodium hydroxide is added to the bottle containing the sputum, which is then tightly stoppered and shaken manually or in a shaking machine for 15-25 min, until the sputum has completely dissolved. The liquid is poured into a sterile 400 ml flask or a bottle, which is sealed with a rubber stopper and heated in a water bath at 55° for 30 min. At this point 1-2 ml of xylol and 200 ml of distilled water are added and the bottle is shaken for 10 min and left to stand at room temperature for 10-20 min. A creamy froth forms on the surface and is carefully collected with a sterile Pasteur pipette equipped with a rubber band or bulb and transferred to a clean slide placed on a glass plate in a warm-water bath (60°). As the slide dries new portions of froth are overlayered on it for concentration (until all the flotation material has been used). The dry preparation is flooded several times with ether from a pipette in order to degrease it. After the ether has evaporated the smear is fixed, usually by heating. Benzine, ligroin, benzene, gasoline, and petroleum ether, but not kerosene can be substituted for the xylol. The flotation rings can be cultured (see below).

Investigation of lavage fluids. Suppurative lumps are examined bacterioscopically; further investigation is carried out in the manner described below.

Investigation of smears from the throat or mouth. If no sputum is expectorated an attempt can be made to obtain it with a laryngial probe or from the mouth with a sterile pad (in children). Suppurative lumps are smeared on a slide and the smear is stained by Ziehl-Neelsen's method and examined bacterioscopically.

Investigation of urine. The urine residue produced by centrifuga-

tion serves as the material for examination. Special attention is paid to differentiation from Mycobacterium smegmatis.

Cerebrospinal fluid is taken under aseptic conditions, immediately discharged from the syringe into a sterile test-tube, and left to stand overnight under refrigeration. A thin fibrous film ("web") is formed and usually contains any tuberculosis Mycobacteria present. The film is carefully "drawn out" onto a slide, air-dried, fixed, and stained by Ziehl-Neelsen's or Ziehl-Hughes's method. If no film is formed the investigation is repeated with the residue obtained by centrifugation (for 30 min at 2000-3000 rpm).

Use of the luminescence method for detecting tuberculosis Mycobacteria

Use of luminescence microscopy for detecting tuberculosis Mycobacteria has a number of advantages over direct bacterioscopy for smears stained by Ziehl-Neelsen's method. When the investigator has acquired a certain skill the luminous rods are quickly detected against the dark field; examination of the smear at low magnification (approximately 280 x) permits inspection of a larger field of view and reduces eyestrain, while the color of the luminescence sometimes aids in making a rough differentiation of tuberculosis Mycobacteria from acid-fast saprophytes. Luminescence microscopy is thus a rapid method, albeit rough, for detecting tuberculosis Mycobacteria.

The following fluorochrome dyes are the principal ones used in examination for tuberculosis Mycobacteria: 1) auramine (1 g); 2) rhodamine B (0.1 g in 1 liter of distilled water); 3) acridine orange (0.1 g in 1 liter of distilled water).

The fluorochromes are diluted in distilled water which has been boiled and cooled 3 times.

Smears are prepared (from sputum and other materials) on thin, clean, degreased slides and air-dried for 1-2 hr (well-dried smears are

not washed during the subsequent operations). The drying time can be reduced by carefully heating the preparation or by playing a stream of air over it (with a fan). One smear is stained by Ziehl-Neelsen's method and another with auramine-rhodamine for 10 min. The specimens are then thoroughly rinsed with tap water, differentiated with alcoholic sulfate for 15 sec, and again rinsed with water; the background is darkened with an acid fuchsin solution (1 g of fuchsin, 1 ml of acetic acid, and 500 ml of distilled water) for 1 min and the smear is rinsed with water.

Tuberculosis Mycobacteria usually luminesce golden orange against a black background (the "starry sky" pattern); acid-fast saprophytes appear greenish with an orange tinge (this trait is not consistent). According to Strugger's data, liver tuberculosis Mycobacteria luminesce red and dead ones green when stained with acridine, but this has not been confirmed by subsequent investigations (N.N. Bobrov, M.I. Meysel', et al.).

In investigating cerebrospinal fluid for tuberculosis Mycobacteria positive results can be obtained 12.5 times as often by luminescence microscopy as by examination of specimens stained by Ziehl-Neelsen's method (A.A. Batyreva).

Bacteriological examination

Isolation of cultures of tuberculosis Mycobacteria from pathological material. Bacterioscopic examination does not always permit detection of tuberculosis Mycobacteria. Isolation and study of cultures make it possible to conduct a more complete investigation. Culturing of material, generally sputum, must be conjoined with a method intended to kill or retard the growth of extraneous bacteria. Methods based on treatment of the material with an acid or alkali which does not affect tuberculosis Mycobacteria at the concentrations used are employed for

this purpose. The techniques devised by Levenstein-Sumiyoshi, Cohn, Petrov, Mazur, Helberg-Mazur, etc., are most frequently used.

Levenstein-Sumiyoshi's method (as modified by M.V. Trius and A.A. Klebanova). Several milliliters of sputum are mixed with 5-6 ml of 15% sterile sulfuric acid in a sterile jar (containing glass beads). The mixture is shaken for several minutes, until it is completely homogenized, poured into centrifuge tubes, and centrifuged. The sulfuric acid should not be permitted to act for more than 15-20 min. Observing strict asepsis, the residue is thrice rinsed with sterile physiological solution during centrifugation and then transferred with a spatula to test-tubes containing glycine-potato or egg-milk medium (Petragnani, Helberg). Inoculation is carried out by rubbing a large quantity of the residue into the surface of the medium.

Cohn's method. An equal amount of 6-12% sulfuric acid is added to the material in a sterile jar containing beads (as above), which is shaken with circular motions (if the material is not heavily contaminated with extraneous microorganisms it is best to use 3% sulfuric acid). The material is then transferred to centrifuge tubes and centrifuged in such fashion that the total action time of the sulfuric acid does not exceed 20 min. The liquid is poured off and the residue (without washing off the acid) is carefully spread with a spatula on an egg-milk medium (Cohn's, Petragnani's, or Helberg's medium).

Mazur's method. Sputum or other pathological material is transferred to a short, broad sterile test-tube with a tightly fitted rubber stopper. A total of 3-4 ml of 2-3% sterile sulfuric acid is added and the stoppered tube is vigorously shaken "up and down" for 3 min. A froth, which traps the tuberculosis Mycobacteria, is formed and is transferred to egg medium with a platinum spatula.

Culturing of flotation rings. Sputum or other material is treated

in a narrow-mouth bottle (see above). The material is flooded with an equal quantity of 0.5% potassium or sodium hydroxide, shaken until completely homogenized (10 min), mixed with 100 ml of sterile distilled water and 0.5 ml of xylol or benzine (filtered through a cotton filter), and shaken vigorously for 5 min; the bottle is then filled to the base of the neck with distilled water. The flotation ring thus formed is collected with a sterile Pasteur pipette and transferred to a sterile slender test-tube. An equal quantity of sterile 3-6% sulfuric acid is added and the tube is sealed with a rubber stopper, vigorously shaken for 5 min, and left to stand for 10 min, until a compact ring has formed. The ring is transferred to egg medium with a platinum spatula and cultured in the usual manner.

Mycobacteria appear within 3-4 weeks with all these culturing methods; in order to accelerate bacteriological diagnosis it is helpful to employ micromethods for isolating tuberculosis Mycobacteria from pathological material.

Price's method (as modified by A.S. Khol'tsman and R.A. Shkol'nikova). Thin smears of the material to be examined are made at one end of clean (flamed) narrow slides. After the preparations have been dried several drops of sterile 2-6% sulfuric acid are applied, permitted to act for 5 min, and then washed off with sterile physiological solution (carefully!). The slides are transferred (smear down) with a sterile forceps to previously prepared and labeled test-tubes containing nutritive medium, using 4 tubes (4 slides) for each batch of material. The tubes and slides (smears) are incubated in a heater for 2-4 weeks. After 7-10 days one slide is stained by Ziehl-Hughes's method and examined microscopically. One smear from each batch of material (the control) is examined immediately after preparation (without incubation) for acid-fast Mycobacteria. The nutritive medium can be: 1) blood med-

ium; 2) a semisynthetic medium based on casein hydrolyzate and containing 5% human serum. A macroscopically visible colonial growth can be seen on the slides when clear media are used.

Depth culturing in blood medium (Shkol'nikova's method). The material is preliminarily treated with 3-5% sterile sulfuric acid by centrifugation (for no more than 20 min) and the residue is then washed with sterile distilled water (for 5 min), transferred to blood medium in test-tubes (4 ml) with a sterile Pasteur pipette, and incubated at 37-38° for 10-20-30 days. The residue is stained by Ziehl-Hughes's method for the time indicated above and examined microscopically. Cultures can be made on a solid medium (egg medium). This technique is sufficiently effective (for culturing sputum, pus, pleural fluid, etc.).

Biopsy material is shipped in a sterile jar or test-tube without preservative. It is cut into small pieces with a curved scissors in a sterile mortar (aseptically) and then ground with sand, adding a small quantity of 3% sterile sulfuric acid. The homogenate is centrifuged and washed twice with sterile physiological solution to remove the acid. The time for which the material is in contact with the sulfuric acid, including the washing period, should not exceed 20 min. It is cultured on egg media.

Investigation of milk: 30-50 ml of aseptically collected milk is centrifuged for 20 min and the residue is treated with 3-5% sulfuric acid (as described above) and cultured on Cohn's, Petragnani's, or Helberg's medium. If there is very little residue the acid is not washed off.

Investigation of feces. Examination of feces for tuberculosis Mycobacteria is of no special diagnostic value for bacillary patients, who swallow their sputum. If it is necessary to establish tubercular damage to the intestine (presence of ulcers) special attention must be

paid to mucous and membranous material in the feces, which are subjected to bacterioscopic and bacteriological examination.

Investigative technique. 1. Approximately 5 g of fecal matter is ground with sand in a mortar, adding small portions of 25% aqueous sodium chloride to obtain a semiliquid mass, which is filtered through sterile gauze. The filtrate is poured into centrifuge tubes, filling them 2/3 full, 2 ml of a mixture of sulfuric ether and ligroin is added to each tube, and the tubes are sealed with sterile rubber stoppers, shaken, and centrifuged for 10-20 min. The surface layer of liquid is poured off and the residue is shaken with 4% sodium hydroxide, left to stand for 3 hr in a heater, and centrifuged; the supernatant is discarded. The centrifugate is neutralized with several drops of 8% hydrochloric acid and cultured on egg media.

2. The feces are processed by the flotation method. Suppurative lumps are ground in a mortar with a small quantity of distilled water (20 ml) and filtered through a cotton-gauze filter; the liquid floats (as in the case of sputum) and the flotation ring can be cultured.

Examination of blood for tuberculosis Mycobacteria. 1. A blood specimen of 3-5 ml, taken aseptically from a vein, is added to a test-tube containing twice as much sterile distilled water. The hemolyzed blood is incubated in a heater for 4-6 weeks to permit growth of tuberculosis Mycobacteria.

2. A total of 5 ml of venous blood is mixed with 3 ml of 10% sodium citrate in a sterile test-tube and centrifuged. The citrated plasma is drawn off with a sterile Pasteur pipette and the entire residue is transferred to a test-tube containing 4 ml of sterile distilled water, shaken, and centrifuged for approximately 10 min. The supernatant is poured off and 1 ml of 3% sterile sulfuric acid is added to the residue, which is shaken for 3 min, mixed with 5 ml of sterile distilled

water, and again centrifuged. The supernatant is discarded, 1 ml of sterile physiological solution is added to the centrifugate, and the entire suspension is transferred with a pipette to 2-3 test-tubes containing egg-milk medium. The tubes are incubated in a heater for 3-4 weeks.

Culturing technique for solid and liquid media. Pure cultures are transferred to solid media with a platinum (or stainless steel) spatula, which is used to rub them gently into the surface of the medium. Liquid media are inoculated with a pierced spatula: 1) by transferring a fragment of a young film from a liquid medium to the medium to be inoculated (the film should not be immersed in the liquid); 2) by transferring a thin film grown on a liquid phase (glycerine water) Pavlovskiy's potato medium in a Roux test-tube; 3) by inoculating a liquid medium with a suspension of the culture. In the latter case a given quantity of a young culture is transferred under rigidly aseptic conditions to a sterile jar containing beads, which is shaken for 1 min with no liquid; 5-10 ml of sterile medium or physiological solution is then added. Inoculation is carried out with a Pasteur pipette or siphon (for large cultures). After shaking, the cultures are placed in a heater for 3-5 days and the flasks are then carefully tilted to bring the cultures to the surface from the bottom. The flasks are left to stand, growth appearing on the surface after 8-10 days, as first a film and then a thicker film.

Methods of studying the characteristics of acid-fast Mycobacteria isolated from pathological or other material. The cultures may be: 1) true tuberculosis Mycobacteria of a given type; 2) altered tuberculosis Mycobacteria; 3) so-called "atypical" acid-fast Mycobacteria; 4) acid-fast saprophytes. The methods of distinguishing them are morphological, tinctorial (resistance of stained smears to decolorization by acids,

alkalies, alcohol, and Javel water), cultural, biochemical, and biological.

The morphology of Mycobacteria is not a sufficiently reliable trait. However, the presence of delicate, slender, rather uniform rods which stain red by Ziehl-Hughes's method always points toward a tubular nature in Mycobacteria isolated from material from patients by staining.

Among the basic methods for direct differentiation of tuberculosis Mycobacteria from saprophytes and other species of the group (genus) we should point out those devised by Ziehl and Hughes (see above), Teleman, and Weichselbaum, which are based on the lower alkali- and alcohol-fastness of a number of Mycobacteria.

Teleman's method. The specimen is: a) stained with Ziehl's fuchsin heated to the vaporization point (see above); b) decolorized with alcoholic potassium hydroxide (one part of 30% potassium hydroxide in 3 parts of 60° alcohol); c) restained (after rinsing with water) with methylene blue.

Weichselbaum's method. The specimen is: a) stained with heated Ziehl's fuchsin; b) rinsed with water and air-dried; c) stained in saturated alcoholic methylene blue for 5 min; d) rinsed with water. Tuberculosis Mycobacteria are stained red, while Mycobacterium smegmatis and other alcohol- and alkali-sensitive Mycobacteria are stained blue. In particularly doubtful cases smears stained by Ziehl-Hughes's method are tested for resistance to the (decolorizing) action of Javel water, acids, and alkalies.

Method: Stained smears containing acid-fast rods are immersed in beakers filled with the following solutions: 1)sulfuric acid (5, 10, and 25%); 2) potassium or sodium hydroxide (5, 10, and 25%); 3) Javel water (0.01, 0.1, and 1%). After predetermined intervals (5, 30, and

60 min and 3, 6, 12, and 24 hr or longer if decolorization has not taken place) the slides are removed from the solutions, thoroughly rinsed with water, restained with aqueous methylene blue, and examined microscopically with an immersion objective for acid-fast Mycobacteria.

Saprophytes usually lose their color rapidly, especially when exposed to concentrated solutions of sodium hydroxide or Javel water. True tuberculosis Mycobacteria retain the dye for several days under the action of acids and for 2-24 hr under that of alkalies (depending on the concentration), while the majority of "atypical" Mycobacteria lose their color after a few minutes or hours.

The test for resistance to acids and alkalies permits a rough differentiation of true tuberculosis Mycobacteria from saprophytes in stained smears, but is not always sufficient for a complete differential-diagnostic characterization of Mycobacteria. It is consequently necessary to resort to isolation of cultures. The following characteristics are significant in this case: growth rate, type of colonies and cultures, most favorable growth temperature (10, 20, 37, or 41°), character of growth on selected favorable and unfavorable media, and ability to produce a homogeneous or lumpy suspension in physiological solution. These traits permit a rough assignment of the cultures isolated to one of the basic types of tuberculosis Mycobacteria or to one of the other species of the genus. All the aforementioned indices can be studied by tests for virulence in vitro and in vivo, which are carried out by testing for the chord factor or for Dubos and Middlebrook's virulence factor. It is convenient to observe the character of the mycobacterial growth in microcultures, checking them microscopically.

Microculturing technique: a suspension of 1 mg per ml (determined by comparison with a standard or by weighing) of Mycobacteria from colonies or cultures (6-13-15 days) is made up in sterile physiological

solution; the suspension is freed of large clumps of bacteria by standing for 1 hr under refrigeration and mixed with an equal quantity of sterile 5% bovine or human serum in physiological solution. Portions of 0.03 ml of the suspension are applied to one end of narrow slides (cut longitudinally) preliminarily sterilized in covered Petri dishes. The drops are carefully smeared with the fused tip of a sterile Pasteur pipette over an area of the slide the size of a Kopeck. The dishes (covered) are placed in a heater for 40-60 min to dry (this operation must be carried out under aseptic conditions!). The dry smears are left in the Petri dishes and treated (fixed) with 2-5% sterile sulfuric acid (for 3 min), which is then thoroughly washed off by repeated application of sterile distilled water.* One smear is immediately stained (by Ziehl's method) and examined microscopically for Mycobacteria, which should occur singly or in very small groups (control smear). The slides, smear down, are immersed in nutritive medium in test-tubes (5-6 ml), which are placed in racks in a heater (2 rows). The nutritive medium can be semisynthetic medium based on casein hydrolyzate and containing 5% human or bovine serum, VKL medium containing 5% serum, or citrated bovine or ovine blood in a dilution of 4:1.5. At various intervals (8-16-20 days) one slide is removed from the test-tubes, the nutritive medium is thoroughly washed off, and the smear is fixed in Nikiforov's solution, stained by Ziehl-Neelsen's or Ziehl-Hughes's method, and examined microscopically. The following are the principal types of growth (Fig. 68): 1) plates of close-packed, linearly arrayed acid-fast rods, which are slow-growing, thickened, and intertwined (virulent tuberculosis Mycobacteria); 2) colonies which grow rapidly (usually 6-8 days) on slides and are visible macroscopically. Rather loose, long plates intertwined in places (resembling curls) can be detected microscopically in stained preparations (tuberculosis Mycobac-

teria of reduced virulence; chromogenic strains are variants); 3) slender, long or short curved "plates," which are slow-growing and acid-fast (nonpathogenic tuberculosis Mycobacteria with residual virulence; BTsZh etc.); 4) after 3-6-12 days clumps of nongranular polymorphous rods appear against a background of an amorphous nonacid-fast substance; the rods are often acid-fast, free-lying "granules" (so-called "atypical Mycobacteria" and nonpathogenic and nonvirulent variants; 5) after 4-6 days weakly acid-fast polymorphous rods accumulate randomly against a background of an amorphous light blue (when stained by Ziehl-Hughes's method) substance (acid-fast saprophytes).

Among the indirect tests for virulence in tuberculosis Mycobacteria is Dubos and Middlebrook's cytochemical test with neutral red: virulent tuberculosis Mycobacteria give a strongly positive reaction, staining dark red, while those with attenuated virulence stain reddish and saprophytes stain yellow (as a result of the presence of different dehydrogenases). The neutral-red reaction serves as a guideline, the value and accuracy of its virulence "readings" being relative.

Method: individual colonies or a small quantity of culture material is carefully ground with a glass rod in a sterile test-tube and twice washed with 50% methanol at 37° (during centrifugation). Approximately 3 ml of 1% barbiturate buffer in 5% sodium chloride (pH 9.0-9.4) and a drop of aqueous 1% neutral red are added to the residue. The supernatant slowly changes from red to yellow and the residue takes on a red, reddish, or yellow color over the next 15-30 min. In experiments with chromogenic cultures clearer results are obtained by washing young cultures with 50% methanol in ether and then adding the barbiturate buffer.

A second test, also based on the varying enzymatic activity of Mycobacteria, is determination of their catalase activity, which is most

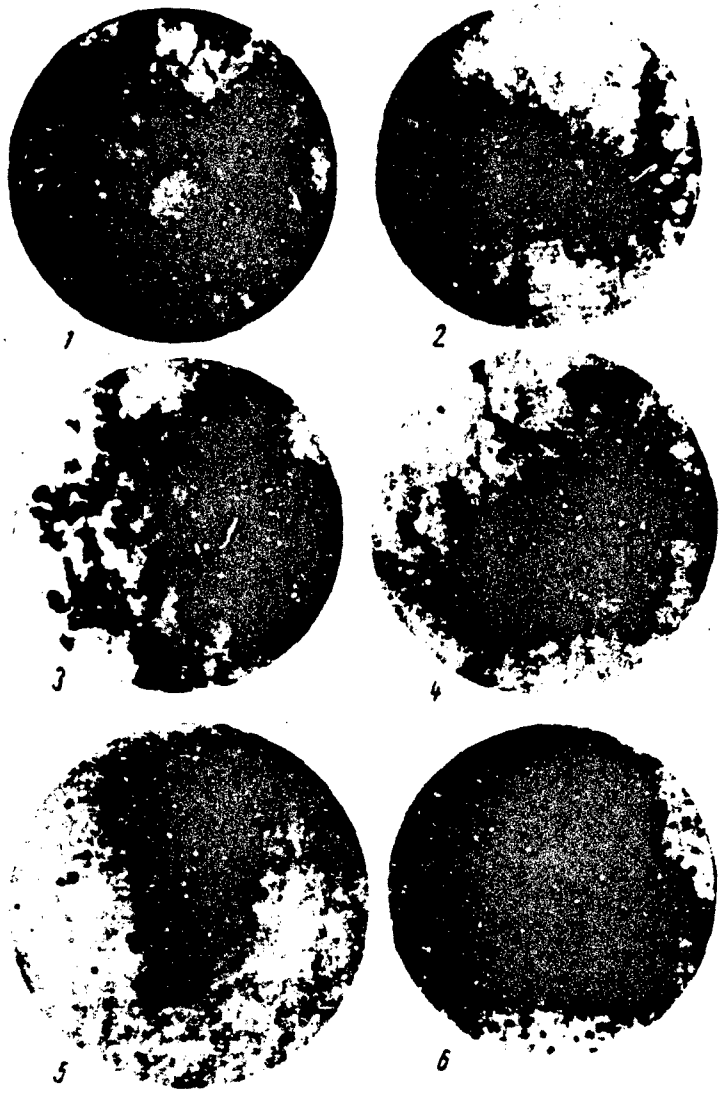


Fig. 68. Microcultures on slides. 1) Virulent tubercular strain; compact plates; 2) attenuated immunogenic variant (137/2); loose, curly plates; 3) chromogenic nonpathogenic variant (137 p); loose plates; 4) BTsZh-Mcro vaccine strain; growth in form of loose flakes and short filaments; 5) nonpathogenic, nonvirulent "atypical" Mycobacterium strain No. 8 (isolated from tuberculosis patient); loose flakes; 6) chromogenic strain (isolated from tuberculosis patients); coarse flakes of cyanophilic substance with acid-sensitive and acid-fast rods (saprophyte).

pronounced in saprophytes, "atypical" Mycobacteria, attenuated tuberculosis Mycobacteria, and strains resistant to preparations of iso-

nicotinic hydrazide.

Method of determining catalase activity. Middlebrook's qualitative method (adopted by the International Committee on Laboratory Methods): a mixture of equal parts of 30% hydrogen peroxide (pro analysi) and 10% Twin's solution is made up. Approximately 1 ml of this mixture is added to a 4-5-week culture (on M.U.I.T. medium or any egg medium) with a Pasteur pipette, keeping the test-tube in an almost horizontal position. The relative activity is determined from the rate of gas-bubble evolution over a 3-min period. Catalase-negative colonies do not decompose the peroxide, even when given 5-10 min. Only the 30% hydrogen peroxide (without the Twin solution) should be used for young cultures. The most common method for quantitative determination of catalase activity is iodometric titration: a weighed portion of washed bacteria is transferred to a cooled mortar and ground with glass beads for 5 min. Cold buffer solution (15 M phosphate solution at pH 6.98) is added to produce a concentration of 50 mg of bacteria per ml and the mixture is left to stand at 0° for 1 hr; the resultant suspension is filtered and 1 ml of the filtrate is added to 25 ml of IV/100 H_2O_2. One test-tube is held at 0° and another at 10°. Portions of 5 ml of the liquid are transferred to flasks containing 5 ml of 10% sulfuric acid after 3, 6, and 9 min. A total of 10 ml of 10% potassium iodide and a drop of 1% ammonium molybdate are added to each flask. The remaining undecomposed hydrogen peroxide is titrated with N/200 Na_2O_3 containing starch. The quantity of undecomposed H_2O_2 characterizes the catalase activity. Peitser and Widelock's relatively simple colorimetric method (1955) for determining catalase activity (in isoniacide-fast tuberculosis Mycobacteria) is based on the change produced in the intensity of the yellow color of an ammonium molybdate solution by hydrogen peroxide, which depends on the extent to which the latter is decomposed

by the mycobacterial catalase.

The most precise method for determining the pathogenicity and virulence of the cultures isolated is administration of definite doses to guinea pigs, rabbits, and white mice (see page 642 for a description of this technique).

Serological investigation

The following serological reactions are employed: 1) the complement-fixation reaction; 2) the indirect-hemagglutination reaction; 3) the double-diffusion reaction in a gel. These reactions are of only relative diagnostic value. They are used principally as indirect indices of immunological dynamics in tuberculosis patients (the complement-fixation and hemagglutination reactions) and to establish an antigenic similarity among acid-fast Mycobacteria, usually atypical strains, isolated from the patient.

The complement-fixation reaction is set up in the usual manner, titrating the complement doses (page 154); the most specific and sensitive antigens are phosphatide antigens prepared by Negra and Bocke's method and those prepared from the polysaccharides of tuberculosis Mycobacteria.

The less cumbersome indirect hemagglutination reaction proposed by Dubos and Middlebrook is more frequently employed. Extracts of tuberculosis Mycobacteria prepared by Dubos and Middlebrook's method or purified dry tuberculin is used as the antigen for sensitization of the sheep erythrocytes. The serum of humans and animals suffering from tuberculosis reacts in an average titre of 1:60-1:150, while that of healthy individuals or individuals suffering from other diseases reacts in an average titre of 1:5-1:15. The serum of hyperimmune animals reacts in a titre of 1:360 or more.

Method. 1. Blood is taken aseptically from a sheep and mixed with

Olsver's solution in a ratio of 1:1.2. When stored at a temperature of no more than 14° the mixture is usable for 2 1/2-3 months. 2. Before setting up the reaction the erythrocytes are washed 3 times (by centrifuging) with physiological solution to remove the preservative. 3) The erythrocyte residue is stored at 4° (it remains usable for 3 days). 4) The washed erythrocytes are treated with tannin solution (1:20,000) for 10 min, centrifuged, and washed with buffered physiological solution. 5) The tanninized erythrocytes are sensitized: a) with an extract of tuberculosis Mycobacteria; 10 ml of neutral isotonic extract of tuberculosis Mycobacteria is added to 0.5 ml of the residue of washed sheep erythrocytes and the mixture is thoroughly shaken and incubated at 37° for 2 hr. The supernatant is discarded and the residue is washed three times with saline solution. The washed, sensitized erythrocytes are suspended in 100 ml of sterile buffered saline solution to produce a 0.5% suspension (which is usable for the next 3 days); b) with purified dry tuberculin (containing polysaccharides) diluted to 1:1000 for 2 hr at 37°; the erythrocytes are then washed with buffered saline solution in the manner described above and a 1% suspension is made up. 5) Preparation of serum: blood serum is mixed with sodium merthiolate to obtain a concentration of 1:10,000 and held in a water bath at 56° for 30 min (it can be inactivated without addition of merthiolate). 6) the heteroagglutinins are removed (adsorbed) from the sera to be tested. For this purpose fresh sheep erythrocytes are suspended in each serum in a ratio of 1:2 and the mixture is held at room temperature for 20 min and then centrifuged (in practice a drop of sheep-erythrocyte residue is added to serum diluted to 1:2, which is then centrifuged after 30 min). 7) portions of 0.4 ml of the serum dilutions (1:2 to 1:320 or more) are poured into test-tubes (10 mm in diameter) and 0.4 ml of a 0.5% suspension of the sensitized erythro-

cytes is added to each tube. Four controls are set up: 1) normal serum and erythrocytes; 2) normal serum and antigen-sensitized erythrocytes: 3) buffered physiological solution and erythrocytes; 4) buffered physiological solution and sensitized erythrocytes.

The test-tubes are incubated at 37° for 2 hr, shaken, and held at room temperature until evening or overnight; the results are evaluated from the residue of agglutinated erythrocytes (provided the controls are negative). Positive result (++++): the liquid is clear and a compact "dome" is formed on the bottom; large clumps of erythrocytes are produced when the tube is gently shaken. Positive result (+++): the liquid is clear and the "dome" on the bottom is less compact; the clumps of agglutinated erythrocytes are smaller. Weakly positive result (++): the "dome" is diffuse and the clumps of agglutinated erythrocytes are small. Doubtful result (+): no "dome" is formed and there are only a few clumps of erythrocytes in the residue. Negative result (−): the erythrocytes do not precipitate and are not agglutinated.

Preservative for erythrocytes (Olsver):

Dextrose or glucose − 2.5 g

Sodium citrate − 0.8 g

Chemically pure sodium chloride − 0.42 g

Distilled water − 100 ml

This mixture is sterilized in running steam for 10 min on 3 consecutive days or made up ex tempore in a flask and boiled for 15-20 min.

Buffered NaCl solution at pH 7.2.

The following phosphate solutions are prepared:

a) M/15 KH_2PO_4 − 9.1 g in 1000 ml of distilled water;

b) M/15 Na_2HPO_4 − 12 g in 1000 ml of distilled water. A total of 700 ml of the first solution and 300 ml of the second are combined and 8.5 g of chemically pure NaCl is added to 1 liter of the mixture, which

is sterilized at 120° for 20 min.

The precipitation reaction in test-tubes, which involves overlayering of the antigen on the serum (annular precipitation), is carried out with undiluted serum and Kodam's antigen, which contains polysaccharides from tuberculosis Mycobacteria: 0.5 ml of antigen is overlayered on 0.2 ml of active serum with a Pasteur pipette. This reaction is of limited diagnostic value in tuberculosis.

The precipitation reaction in a gel makes it possible to study the complex antigenic relationships between normal tuberculosis Mycobacteria and altered "atypical" Mycobacteria, saprophytes, etc.There are two basic modifications: a) Uden's method in an agar column; b) Ouchterloni's method on a sheet of agar in a Petri dish and on slides. The latter modification, "counter-diffusion in a gel" (agar), is more convenient and indicative. The serum (antibody) and antigen are placed in different reservoirs and diffuse through the gel (agar) toward one another at different rates. The meeting of equivalent quantities in a certain area (the equivalence zone) causes precipitation, in the form of bands or "precipitation lines." Each line corresponds to an antigen-antibody complex. The work of Parlett and Yumans, Seibert and Figuroa, A.I. Togunova and V.M. Dolzhanskiy, and others has shown that antigens extracted from tuberculosis Mycobacteria consist of a whole series of antigenic complexes, which precipitate with the corresponding antibodies of serum from experimental animals and tuberculosis patients or persons suffering from other mycobacterial diseases. In practice, this reaction permits differentiation of true tuberculosis Mycobacteria from altered Mycobacteria, many "atypical" or "abnormal" forms, and the serologically isolated true acid-fast saprophytes by their antigenic composition.

Preparation of 1% agar. A total of 20 g of dry agar (Japanese) is

soaked overnight in 1 liter of distilled water in a flask. On the following day the agar is melted in a water bath and 50 ml of 10% $CaCl_2$ is added. The hot agar is filtered through a cotton-gauze filter and poured into Petri dishes in a thin layer, where it is permitted to solidify. Small cubes are cut from the agar, placed in a gauze sack with a forceps, and washed in running water for 72 hr. The washed agar is placed in a flask, melted in a water bath, and mixed with an equal volume of 1.6% NaCl (16.5 g of NaCl in 1 liter of distilled water). The prepared 1% agar is sterilized in an autoclave at 110° for 30 min and merthiolate is added to it to produce a final concentration of 1:10,000.

Setting up the diffusion-precipitation reaction in Petri dishes. A small quantity of molten agar (containing merthiolate) is poured into sterile Petri dishes with smooth bottoms and spread in a thin layer (approximately 2 mm thick) by tilting the dishes. This creates a so-called "underlayer," which keeps the reagents from flowing beneath the gel layer in which precipitation takes place. After the agar has solidified a stencil indicating the desired arrangement of the wells is placed beneath the dish. Sterile metal cylinders 5-8 mm in diameter are placed on the "underlayer" (following the stencil) and a new layer of agar approximately 3 mm thick is poured into the dish. After the agar has solidified a forceps is used to remove the cylinders, grasping them by their upper ends; this leaves wells with smooth edges and a thin underlayer at the bottom. The distance between the wells (which will contain different reagents) is usually 15 mm. If different sera are to be tested with the same antigen the latter is poured into a central well and the sera are contained in peripheral wells; if a serum is to be tested it is placed in the central well and different antigens are poured into the peripheral wells. Other well arrangements are also possible. The ingredients are dropped into the wells with Pasteur pipettes.

Active sera (from hyperimmune rabbits) and good antigens form distinct precipitation lines within 1-2 days. The dishes are placed in a desiccator and incubated in a heater at 31°; the reaction proceeds somewhat more rapidly at 37°. Reagents are added to the wells as they dry out, usually at intervals of 24-48 hr (a total of 5 times) and the observation period lasts 8-10 days, noting the precipitation lines as they appear (1-3-5 or more bands). The dishes containing the agar for precipitation are best prepared 3-4 days before the reaction is carried out.

Preparation of antigens. The antigens are prepared from tuberculosis Mycobacteria by the following method (A.I. Togunova and V.M. Dolzhanskiy): a weighed portion of a compressed culture (20 days for tuberculosis Mycobacteria and 16 days for BTsZh) is transferred to a sterile 250 ml jar containing stainless-steel balls (150 g). The culture is shaken (ground) for 30 min with circular motions, 0.5 ml of distilled water is added, and shaking is continued. Depending on the moistness and total quantity of the culture, 2-4 ml of distilled water is added over the 2-hr shaking period. The culture is then diluted with sterile physiological solution to a content of 20 mg/ml of liquid (using the initial weight as a basis). The completeness with which the antigen decomposes acid-fast Mycobacteria is checked by microscopic examination of smears stained by Ziehl-Neelsen's method: the smaller the number of undecomposed rods, the more active the antigen. The suspension is left to stand in test-tubes under refrigeration for several days, the tubes are then placed in a heater at 37° for 1 hr, and the liquid is poured off from the residue or the suspension is preliminarily centrifuged. The opalescent liquid, which contains polysaccharides, is the antigen.TThis procedure (an accelerated method) can be used to prepare antigen from the culture under investigation.

Antigens from ultrasound-treated Mycobacteria: a weighed portion

of the culture (approximately 1 g) is vigorously homogenized in a jar containing beads for 15 min. The suspension (20 mg/ml of distilled water) is transferred with a sterile pipette to an alcohol-disinfected cell for irradiation, which is carried out in a UL-1 apparatus at 1200 kc/sec for 2 hr. The entire suspension, including any undestroyed Mycobacteria, is utilized.

"Moist extracts" of Mycobacteria (A.I. Togunova and V.M. Dolzhanskiy). In order to prepare these antigens a film culture is raised on a synthetic medium (VKL) or on a semisynthetic medium based on fungal casein hydrolyzate (15-25 days). The bacterial mass is filtered aseptically through a Buchner funnel under a vacuum, washed from the medium with sterile distilled water, and vacuum-dried on the funnel. A weighed portion of 5-10 g of fresh bacteria is transferred to a sterile 3-liter bottle containing 2-3 kg of stainless steel balls, maintaining aseptic conditions; the bottle is stoppered and placed on a rotating device to create a ball mill (60 rpm), thoroughly grinding the mass for 18 hr (in a refrigerator at 5-8°). Portions of 10-15 ml of sterile physiological solution are added to the bottle and it is shaken manually; the total quantity of physiological solution should yield an antigen concentration of 100 mg/ml (based on the initial weight of the culture). The liquid is permitted to stand or centrifuged (maintaining asepsis), poured off from the sediment, and preserved with merthiolate. For the reaction the antigen is diluted to a concentration of 20 mg/ml (based on the initial weight). The "moist extracts" are opalescent and contain up to 40% polysaccharides. They are most active in precipitation reactions with specific sera (yielding up to 10-12 precipitation bands). The antigens are prepared from live cultures of nonpathogenic Mycobacteria. Cultures of true virulent tuberculosis Mycobacteria are preliminarily killed by autoclaving or by adding 3% phenol (on 3 suc-

cessive days, to cultures on liquid media). All the antigens are preserved by adding merthiolate (to a concentration of 1:10,000).

Filtrates of the culture fluid concentrated to 1/10 do not differentiate the antigenic complexes as well.

The precipitative sera are prepared by immunizing rabbits with live (nonpathogenic Mycobacteria, BTsZh) or killed (virulent tuberculosis Mycobacteria) cultures. A 2 mg/ml suspension of the culture in physiological solution, obtained by shaking in a jar containing beads (for 15-20 min), is administered intravenously to a rabbit in a dose of 2 ml; on 4 occasions thereafter, at 6-day intervals, 1 ml of the suspension is injected subcutaneously and 1 ml is injected intravenously an hour later. The blood used to produce the serum is taken 14-16 days after the last injection. The serum is tested with the antigens in dishes or on slides (microreaction).

Detection of tuberculosis Mycobacteria by biological testing

Although culturing is now justifiably the most rational diagnostic procedure, simultaneous administration of the initial material to guinea pigs is a reliable and relatively rapid method in a number of especially important cases. Material not contaminated with extraneous microorganisms can be injected subcutaneously or directly into the abdominal cavity. Material containing extraneous bacteria should first be processed in the manner described above (treatment with sterile 3-5-9% sulfuric acid and examination for bacterial contamination). After the acid has been washed off with physiological solution during centrifugation the resultant suspension (in a dose of 2-3 ml) is administered subcutaneously in the inguinal region to 2 guinea pigs weighing 250-300 g. When the material contains virulent tuberculosis Mycobacteria a consolidation usually forms beneath the skin at the injection site after 10-12 days, subsequently becoming a chronic ulceration. The re-

gional lymph nodes become thickened and then enlarged, collecting in a clump. The animals die of generalized tuberculosis after 2-4 months. In order to accelerate the diagnosis a guinea pig can be killed after 2-4 weeks and the extent of the regional lymphadenitis determined; an intracutaneous tuberculin test must also be carried out on the opposite paw (using 0.1 ml of 1:10 tuberculin). The process is generalized more rapidly when the material is administered intraperitoneally, the primary process being observed in the omentum and adjacent lymph nodes and organs. The pathological process develops slowly if the diagnostic material contains few tuberculosis Mycobacteria or Mycobacteria of reduced virulence. In such cases one of the experimental guinea pigs is killed (after 3-4 weeks), the enlarged regional lymph nodes are removed under aseptic conditions and ground in a sterile porcelain mortar, a small quantity of physiological solutions is added to the resultant paste, and the suspension is filtered through a double layer of sterile gauze (on a funnel) and injected subcutaneously into the inguinal region in one or two fresh guinea pigs (concentration method). Some of the past is cultured on an egg-milk medium after treatment with dilute sulfuric acid. A more pronounced tubercular process should develop in the animals given the concentrated material. Only a rather atypical regressive regional process involving local abscesses and infiltrations may develop when the material contains altered or atypical Mycobacteria (e.g., phthvicide-fast bacteria). A through study of the culture is then necessary to obtain a complete characterization.

Accelerated biological test with a regional lymph node. An inguinal or cervical lymph node of a young guinea pig (150-200 g is palpated through the skin and gently kneaded between the fingers; several drops of the material under investigation are then injected into it with a syringe. After 8 10 days the infected lymph node is easily felt and

must be excised and prints examined bacterioscopically for tuberculosis Mycobacteria.

As was noted above, intravenous administration of 1 mg of material to white mice is a sensitive test for detection of virulence in altered tuberculosis Mycobacteria.

Allergic reactions

Tuberculin. The tuberculin preparation devised at the end of nineteenth century by Koch is widely used for diagnostic purposes. It is referred to as "old Koch tuberculin" or ATK (Alt tuberculin Koch) and consists of a filtrate prepared nutritive medium from beneath 2-2 1/2-month film cultures of tuberculosis Mycobacteria and condensed to 1/10 of its initial volume.

The following preparations are also used: a) purified dry tuberculin (M.A. Linnikov, USSR); b) a purified protein derivative of tuberculin (the PPD of foreign authors). Purified dry tuberculin contains principally specific proteins (tuberculoproteins) and a few polysaccharides (approximately 30%), while ATK contains extraneous additives and glycerine (from the medium). According to Brehmy, 1 ml of liquid ATK contains 100,000 tuberculin units, while the international PPD unit equals 0.00002 mg.

Specific local (allergic) reactions develop when tuberculin is administered cutaneously or intracutaneously to humans and animals infected with tuberculosis. When 1 ml of tuberculin is administered subcutaneously or intraperitoneally to guinea pigs a shock reaction develops, terminating in death toward the end of the first day.

Priquet's dermal reaction is carried out principally in children and involves application of a tuberculin solution to scarified skin on the forearm; the tuberculin dilutions (50, 25, and 10%) are made up in sterile carbolized (0.25%) physiological solution. The specific tuber-

culin reaction, which takes the form of hyperemia, swelling, edema, and central ischemia, develops within 12-24 hr; an evaluation is made after 48 hr. The test is regarded as positive if the dermal reaction appears over an area with a diameter of no less than 5 mm.

Mantoux's intracutaneous reaction is generally carried out when the skin test is negative (at dilutions IV and II); 0.1 ml of the corresponding tuberculin dilutions are injected intracutaneously. When the result is positive hyperemia, swelling, and a blister are observed within 8-12 hr; an evaluation is made after 48-72 hr.

Plaster test: a tuberculin plaster is applied to the skin; specific papules (which are quite transient) appearing on the skin within 24 hr indicate a positive result.

All tuberculin diagnoses should be made by qualified personnel, following special instructions and observing the strictest asepsis.

The intracutaneous tuberculin test (Roemer's reaction) is employed in experimental work. A total of 0.1 ml of a 10-fold dilution of a given tuberculin preparation is injected into the skin of a guinea pig's hind paw (shaved on the night before the test); a graduated test (using solutions of different concentrations) is set up in the skin of the side in guinea pigs. The ophthalmic reaction is employed in rabbits (applying 0.1 ml of tuberculin to the conjunctiva). Hyperemia and swelling of the conjunctiva, sometimes accompanied by a serous or even suppurative discharge, is noted after 12-24 hr. The ophthalmic reaction and an intracutaneous reaction in the scapular region are employed in monkeys. Dry purified tuberculin should be diluted in accordance with the instructions packed with the preparation.

Nutritive Media

A.D. Pavlovskiy's medium. A white potato is carefully washed with a brush and soap and wiped dry. It is then peeled and cut in half with

a flamed knife. Cylindrical pieces equal in width to the diameter of the test-tubes to be used and 5-6 cm long are cut from the potato with a special sterile punch or a knife. The cylinders are then cut diagonally into 2 wedges, which are immediately immersed in sterile 5% glycerine water for 1-2 hr (if the potato is young it must first be soaked for 1 hr in sterile 2% sodium bicarbonate). The wedges are dried between sheets of filter paper and a forceps is used to insert them into sterile Roux test-tubes (which have a constriction) half-filled with sterile 5% glycerine water (at pH 7.0-7.2) in such fashion that the lower portion of the wedges is in contact with the liquid. The tubes are sterilized in an autoclave at 110° for 10 min. After sterilization the hot tubes are placed in an inclined position, with the surface of the wedges down, so that they remain moist. In order to check its sterility the medium is placed in a heater (in a vertical position) for 2 days; it is stored under refrigeration (4-6°).

Petragnani's medium. A total of 0.25 liter of fresh whole milk (at the neutrality point) is poured into a sterile 1-liter flask containing glass beads and 6 g of potato flour, 1 g of peptone, and one finely cut potato the size of a hen's egg are added, constantly stirring the mixture. The flask is immersed in a boiling-water bath and heated for approximately 10 min, until its contents have uniformly thickened, agitating it with circular motions all the while. After the temperature of the mixture has dropped to 60° 4 fresh hen's eggs and 1 yolk are stirred into it. A total of 12 ml of sterile glycerine and 6 ml of 2% malachite green (in distilled water) are then added and the mixture is thoroughly stirred, filtered through a double layer of sterile gauze (in a funnel) and poured into sterile test-tubes in uniform portions; it is permitted to coagulate for 30 min in an inclined position at 85° in a Koch apparatus. The tubes are heated at 75° for 15 min on

each of the next 2 days. The final medium is light green in color;;its surface is smooth and has sufficient condensation water. It is stored under refrigeration and is used as fresh as possible.

Hon's egg medium. Fresh hens eggs are carefully washed with alcohol, dried, and broken under aseptic conditions into a sterile flask containing glass beads. 3 parts of egg are mixed with 1 part of 10% nutritive glycerine broth. The mixture is thoroughly stirred, filtered through sterile gauze, poured into sterile test-tubes in uniform portions, and heated in a Koch coagulation apparatus at 75° for 30 min on 3 successive days. After coagulation 0.5 ml of broth without glycerine is added to each tube (under aseptic conditions).

Helberg's egg medium. The constituents of the medium are prepared as follows: 1) salt solution: dibasic potassium phosphate – 1 g, potassium citrate – 1 g, magnesium sulfate – 1 g, peptone – 5 g, chemically pure glycerine – 30 ml, and sufficient distilled water to make 1000 ml. The salts are dissolved in a small quantity of water and the glycerine and remaining water (to make 1000 ml) are then added. The mixture is slightly heated, filtered through a folded paper filter, and poured into flasks. It is then sterilized in an autoclave at a pressure of 1 atm for 20 min.

2. Cow's milk is poured into flasks (in portions of 110 ml) and sterilized fractionally over 2 days, for 10 min at 105° (1 atm) on the first day and for 15 min in running steam on the second day.

3. Potato extract: a potato is peeled, cut into slices, flooded with a quantity of tap water equal to twice its own weight, brought to the boiling point, and boiled for 15 min. After settling the extract is filtered through a cotton-gauze filter, poured into flasks (in 110 ml portions), and sterilized at 120° for 20 min.

4. A 2% aqueous solution of malachite green is sterilized at 120°

for 20 min.

Method of preparing Helberg's medium: 5 fresh eggs are carefully washed with warm water, soap, and a brush, wetted with alcohol, and quickly flamed. The yolks of two eggs are separated under aseptic conditions, dropped into a sterile jar containing beads, and broken by shaking. The contents of the remaining 3 eggs (whites and yolks) are then added and the entire mixture is thoroughly shaken with the beads. To the resultant egg mixture are added 50 ml of the salt solution, 50 ml of the milk, 50 ml of the potato extract, and 2.5 ml of sterile 10% citric acid. These ingredients are stirred, 3.5 ml of the 2% malachite green is added, and the suspension is again thoroughly mixed. The mixture is filtered through a sterile gauze filter on a funnel, poured into test-tubes in 3-6 ml portions (depending on the tube diameter), and sterilized once for 1 hr at 85° in a Koch coagulation apparatus (the sterilization time should be prolonged to 1 hr 15 min when broad test-tubes are used). The final medium is kept in a heater for 2 days to check its sterility. It is stored under refrigeration and can be used for 1 week after preparation.

Nutritive medium devised by the International League for the Prevention of Tuberculosis (Milieu Union International Tuberculeau - M.U.I.T.). Salt solution:

Monobasic potassium phosphate - 2.4 g

Magnesium sulfate - 0.24 g

Magnesium citrate - 0.6 g

Asparagine - 3.6 g

Redistilled glycerine - 12 g

Distilled water - 600 ml

This mixture is sterilized for 2 hr in running steam at 100°. A total of 20-22 fresh eggs are carefully washed, soaked for half an

hour in 5% soda (Na_2CO_3) containing soap, and again washed in tap water until the latter becomes clear. The contents of the eggs are dropped into a large vessel, shaken vigorously, and filtered through gauze. Then 1 liter of the eggs and 600 ml of the salt solution are mixed and 20 ml of the malachite green is added. The mixture is left to stand for 1 hr, until the bubbles disappear. Portions of 7 ml are poured into sterile test-tubes (160 x 16 mm). The medium is coagulated in an inclined position at 85° for 40 min. The cotton stoppers are then hermetically sealed with paraffin, to which 0.1% sorbic acid is added to prevent mold. The medium is stored in a refrigerator at 4° and is usable for 6 months.

Levenstein-Jensen's medium:

1) salt solution:

monobasic potassium phosphate - 0.4 g

magnesium sulfate - 0.04 g

magnesium citrate - 0.1 g

asparagine - 0.6 g

chemically pure glycerine - 2 g

distilled water - 100 ml

This mixture is sterilized in running steam for 2 hr.

2) The contents of 22 fresh hen's eggs (carefully prepared by the method described above) are dropped into a sterile 1-liter flask containing beads, mixed, and filtered through sterile gauze. At this point 600 ml of solution No. 1 and 1 liter of mixture No. 2 are combined, 20 ml of sterile 2% malachite green is added, and the mixture is thoroughly stirred and poured into test-tubes under aseptic conditions; the tubes are placed in a Koch apparatus in a semihorizontal position and coagulated at 85° for 40 min. The medium is then placed in a heater to check its sterility.

Finlayson's medium. The white and yolk of an egg are separated under aseptic conditions and poured into individual sterile flasks. A sterile graduate is used to combine 60 ml of yolk, 15 ml of white, 2 ml of sterile physiological solution, 1 ml of glycerine, 1 ml of Congo red, and 1 ml of 1% malachite green, which are thoroughly mixed. The medium is poured into test-tubes and coagulated in the manner described above.

Glycerine meat-infusion broth. A total of 5-10% of chemically pure glycerine is added to ordinary meat-infusion broth (1% peptone, 0.5% chemically pure sodium chloride); the medium should have a pH of 7.2.

Soton's synthetic medium:

Disubstituted potassium phosphate (K_2HPO_4) - 0.5 g

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) - 0.5 g

Ferric ammonium citrate ($FeNH_4C_6H_5O_7$) - 0.05 g

Citric acid - 2 g

Asparagine - 4 g

Chemically pure glycerine - 60 ml

Distilled water - 940 ml

The mixture is heated in a water bath at 100° until the salts have completely dissolved; when it has cooled pure ammonia is used to adjust the pH to 7.2-7.3 and the medium is filtered through cotton, poured into flaks or test-tubes, and sterilized at 120° for 30 min. Its sterility is checked in an incubator.

VKL glycol medium containing zinc:

Citric acid - 0.65g

Glycol- 5 g.

Disubstituted potassium phosphate - 5 g

chemically pure sodium chloride - 5 g

Sodium citrate - 2 g

Glucose - 2 g

Magnesium sulfate - 1 g

Ferric sulfate and ferric oxide - 0.05 g

Chemically pure glycerine - 40 g

0.1% zinc sulfate - 1 ml

distilled water - sufficient to make 1000 ml

The pH is adjusted to 7.0-6.8 with 5% citric acid and the medium is sterilized at 110° for 10 min. It should be light yellow in color after sterilization. Darkening of the medium indicates caramelization of the glucose as a result of overheating.

L.M. Model's medium:

Dibasic potassium phosphate - 5 g

Ammonium oxalate - 5 g

Magnesium sulfate - 0.5 g

Ferric sulfate - 0.05 g

Glycerine - 50 ml

Distilled water - 950 ml

The medium is alkalized to pH 7.2 with ammonia and sterilized at 120° for 30 min.

Semisynthetic medium based on fungal casein hydrolyzate (A.I. Togunova and M.I. Loginova):

Liquid casein hydrolyzate - 90-100 ml (amine nitrogen content - 75-80 mg-%)

Ferric ammonium citrate (or ferric sulfate) - 0.05 g

Magnesium sulfate - 0.05 g

Sodium citrate - 0.15 g

Chemically pure sodium chloride - 0.25 g

Chemically pure glycerine - 50 ml

Distilled water - sufficient to make 1 liter

The pH is adjusted to 7.0-7.2 with 10% citric acid and the following phosphates are added:

Na_2HPO_4 – 5 g; KH_2PO_4 – 1.0-1.25 g (both first dissolved in small quantities of water).

The medium should have a final pH of 7.0-7.1.

The mixture is sterilized at 120° for 20 min. It is straw yellow in color and absolutely clear.

The basic hydrolyzate is obtained by digesting casein with the proteases of the fungus Aspergillus terricola (I.N. Vinogradov; see page 961).

Semisynthetic casein medium containing plasma and glycerine (R. A. Shkol'nikova):

Monobasic potassium phosphate (KH_2PO_4) – 1.5 g

Dibasic sodium phosphate (Na_2HPO_4) – 2.5 g

Magnesium sulfate – 0.5 g

Sodium citrate – 1.5 g

Ferric ammonium citrate – 0.05 g

Enzymatic casein hydrolyzate – 80 ml

Glycerine – 30 ml

Distilled water – 890 ml

The salts are dissolved in distilled water in the indicated order, gently heating the solution in a water bath. The medium is filtered through filter paper, its pH is adjusted to 7.0-7.2, and it is poured into flasks or test-tubes in 2 ml portions and sterilized at 120° for 30 min. Before inoculation 0.2 ml of plasma (taken from citrated human blood) and 0.3 ml of penicillin solution (10-20 units per ml) are added to each tube. Preparation of the hydrolyzate is described on page 960.

Blood medium: Citrated donor blood (without antiseptics) can be used. Immediately before inoculation the sterile citrated blood is di-

luted to 1:2 or 1:3 with distilled water and 3 ml portions are poured into short sterile test-tubes; penicillin solution is added in a concentration of 20 units per ml of medium (dissolving 200,000 units of penicillin in 10 ml of sterile distilled water and, before inoculation, further diluting it by adding 0.1 ml of the initial solution to 9.9 ml of distilled water; 0.3 ml of this dilution is added to the 3 ml of blood medium in each tube).

Jerrold's egg-agar medium. A total of 3% of agar is added to glycerine meat-infusion broth and 135 ml portions of the mixture are poured into flasks and sterilized in an autoclave for 20 min at a pressure of 1.5 atm. The yolk of one egg and 2 ml of sterile 2% malachite green are added under aseptic conditions to each flask of agar, which is melted and cooled to 48-52°. In order to test the drug resistance of Mycobacteria an antibacterial preparation is added to all but one of the flasks in the dose indicated above (see the section on determination of drug resistance); the contents of the flasks are thoroughly stirred and poured into test-tubes, in which the medium is slanted and cooled.

The last 4 media are used primarily for determining drug resistance.

Preparation of Javel water: 25 g of calcium hypochlorite (approximately 25% active chlorine) is flooded with 150 ml of distilled water and thoroughly shaken; 14 g of potassium carbonate (potash) is flooded with 300 ml of distilled water and thoroughly shaken; after 6-8 hr the two solutions are filtered separately through cotton or gauze and the resultant filtrates are combined.

The interaction of the calcium hypochlorite and the potassium carbonate produces potassium hypochlorite in a 1% solution. A 0.01% solution is used for decolorization.

DIPHTHERIA

N.I. Apanashchenko, Candidate of Medical Sciences

The causative agent of diphtheria, Corinebacterium diphtheriae, was discovered by Klebs in 1883 and a pure culture was isolated by Loeffler in 1884.

Morphology of C. diphtheriae

These bacteria vary in size, from 1 to 6 microns in length and 0.3 to 0.8 microns in diameter.

A distinctive characteristic of C. diphtheriae is the diversity of individual forms within the same strain: in addition to long, curved, delicate "typical" rods, cultures may contain short, thick rods with knobby protuberances at the ends and, occasionally, coccal cells (Fig. 69). Nonuniform staining of each rod is characteristic, occasionally giving the cells a chain-like or zebra-striped appearance. C. diphtheriae may produce long-lived L-forms, which sometimes revert to the bacterial form (Fig. 70).

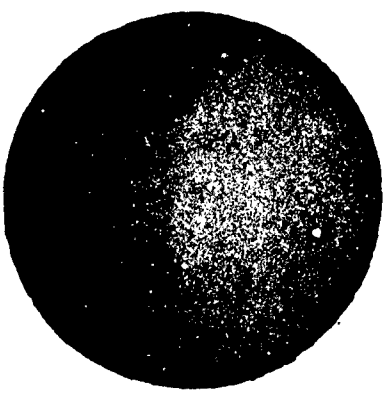


Fig. 69. Corinebacterium diphtheriae. Volutin granules are visible at the poles. Stained with crystal violet.

The ends of the rods, which often bear knob-like protuberances, give them a resemblance to clubs (the Greek coryne means club). The

protuberances often contain volutin granules (Babes-Ernst bodies). Chemically, volutin is a nucleic-acid derivative. The volutin granules are quite distinct in young cultures raised on coagulated serum or Klauberg II medium. They cannot always be detected in broth cultures.

The appearance of preparations is characteristic, especially if the smears contain many bacteria. The microbes occur in large groups, the entire field occasionally appearing as a mass of tangled rods and resembling a matted coat of hair. The general arrangement of the bacteria in the field is also reminiscent of a bundle of clubs. The arrangement of the bacteria in stained smears is very characteristic: they may occur in pairs, at an acute or right angle to one another (Fig. 71). C. diphtheriae are immobile, do not form spores, have no flagella, and are gram-positive, although they are more easily decolorized than many other gram-positive microorganisms.

Staining. C. diphtheriae stains well with basic analine dyes, the volutin granules staining especially intensely. Loeffler's alkaline methylene blue, acetic toluidine blue, crystal violet, and Neisser's method (see page 41) are usually employed for staining smears of diphtheria cultures. Nonuniform staining of the bacteria, which have a striated appearance, is evident when alkaline methylene blue or especially toluidine blue is used; the volutin granules take on a more intense blue color. A characteristic feature of staining by Neisser's method is the fact that the volutin granules are stained blue-black, contrasting with the light brown color of the rest of the cell. Granules which stain by Neisser's method are sometimes indetectable in preparations stained with methylene blue. Intensely staining granules are not characteristic only of C. diphtheriae. Thus, certain cocci, which often give a metachromatic reaction with methylene blue, sometimes stain equally intensely; other bacilli may exhibit a similar

granularity. Moreover, cultures of C. pseudodiphthericum yield a negative result when stained by Neisser's method: isolated granules are visible in the preparation, but the rods do not have the characteristic beaded appearance. It is best to employ methylene or toluidine blue and Neisser's method in parallel. This technique is particularly valuable when dealing with small forms of diphtheria bacilli, whose structural details are not shown by methylene blue. When a preparation stained with methylene blue contains only a small number of C. diphtheriae the latter can easily be inspected, but they are easier to detect in a smear stained by Neisser's method.

Fig. 70. L forms of C. diphtheriae. Phase-contrast microscopy. 1350 x (prepared by G.Ya. Kagan).

Biology of C. diphteriae; cultural characteristics

Diphtheria bacillae develop well when oxygen is freely available an grow at temperatures of from 15 to 40°, the optimum growth temperature being 33-35° and the optimum pH 7.6-8.0. They will grow on ordinary nutritive media, but develop better and exhibit characteristic morphological traits on media containing blood or serum from any species of animal. C. diphtheriae appear on such media within 8-10-12 hr, growing more rapidly than other bacteria. The most common

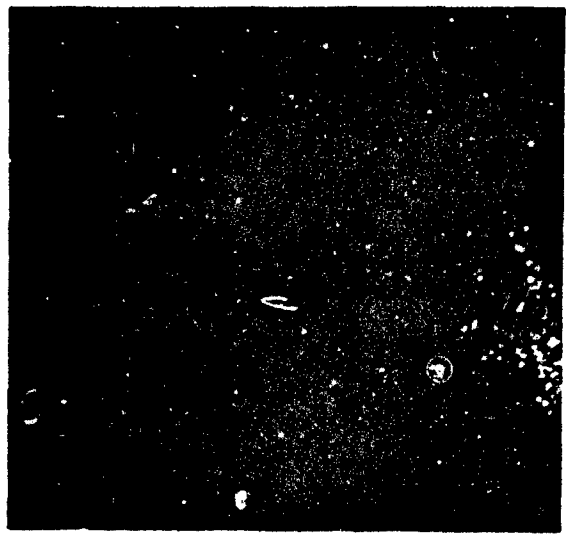


Fig. 71. Corinebacterium diphtheriae. Typical angular arrangement of bacteria. Anoptral microscopy. 2000 x.

selective nutritive media are: a) coagulated horse serum (Roux's medium); b) a mixture of 3 parts of serum and one part of meat broth containing 1% glucose and 1% peptone and subjected to slow coagulation (Loeffler's medium); c) tellurium media (Klauberg II medium, blood-tellurium agar, and serum-tellurium agar). The general appearance of growths of C. diphtheriae on slants of coagulated serum media in test-tubes is very characteristic: the colonies do not merge and the entire culture takes the form of scattered granules, resembling shagreen leather. The colonies are round, smooth or slightly granular, and opaque, with a translucent periphery and smooth margins, which subsequently become sinuous or even serrated. The size and shape of the colonies depend to a substantial extent on the quality of the nutritive medium and the size of the culture. The wide use of potassium tellurite for bacteriological diagnosis of diphtheria has caused C. diphtheriae to become highly resistant to this compound; they produce a luxuriant growth at concentrations sufficient to retard the development of the

concomitant microflora. They form dark gray or black colonies on tellurite media, reducing the tellurite to metallic tellurium. This process takes place within the bacterial cell and crystals of metallic tellurium can be detected intracellularly by electron microscopy.

Types of C. diphtheriae. Three types of diphtheria bacillus are distinguished on the basis of their cultural characteristics: gravis (severe), mitis (moderate), and intermedius (intermediate). The gravis type produces a granular sediment and a film in broth and forms flat mat colonies of irregular outline, resembling daisies, on solid media. The mitis type uniformly clouds broth and forms convex translucent colonies. The intermedius type has certain of the characteristics of the first two types and is referred to as intermediate for the reason. Atypical cultures are also often encountered.

The view advanced by Anderson et al., which holds that the severity of the clinical course of the disease is determined by the type of C. diphtheriae, has not been confirmed by extensive research conducted in the Soviet Union. It has been established that the gravis type most frequently causes group outbreaks within individual families and epidemics, while the mitis type is usually associated with episodic cases of diphtheria.

Just as other microorganisms, diphtheria bacilli form smooth (S), rough (R), and intermediate (RS) colonies. The R form generally predominates in highly toxic strains (see Figs. 72 and 73).

Diphtheria bacilli produce acid but no gas on media containing glucose, maltose, and galactose, but not on those containing lactose, sucrose, and mannitol. Fermentation of starch and glycogen is a characteristic of the gravis type. Many strains cause hemolysis on blood agar and lyze erythrocytes added to a culture. The "Park-Williams No. 8" strain, which was isolated by Park and Williams in the United

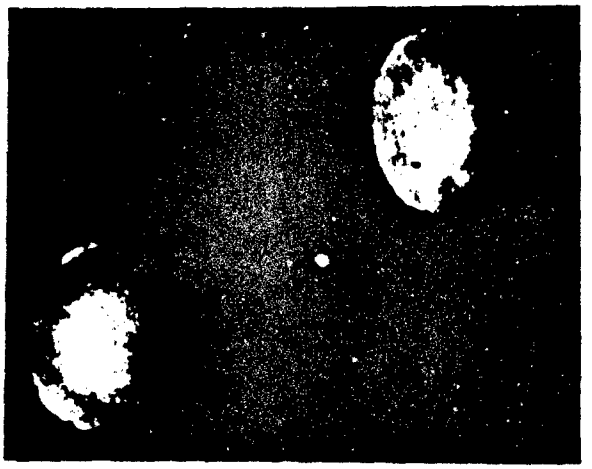


Fig. 72. Corinebacterium diphtheriae. Colonies on dishes containing blood agar. S-form colonies. 14 x.

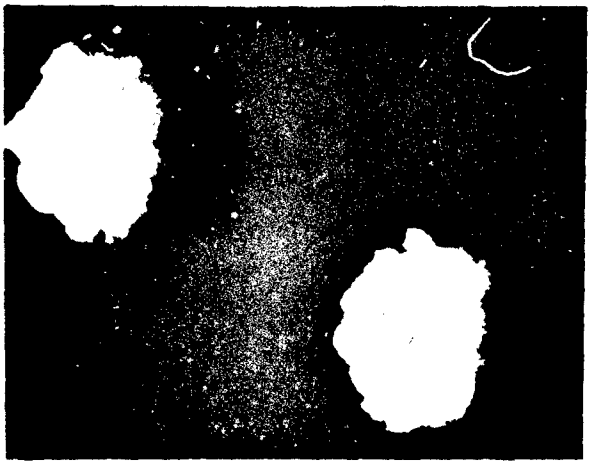


Fig. 73. Corinebacterium diphtheriae. Colonies on dishes containing blood agar. R form. 14 x.

States in 1894, belongs to the intermediate type and is enzymatically less active than its variants, "Toronto," "Massachusetts," and "Weissenzee."

Resistance to physical and chemical agents

C. diphtheriae is sensitive to disinfectants: 10% hydrogen peroxide kills it within 3 min, while 1% mercuric chloride, 5% carbolic

1 Обозначение серологических типов по новой номенклатуре	2 Типы			6 Примечание
	3 Робинсона и Пенни	4 МНИИВС им. Мечникова	5 Московский НЭМГ	
1	I	I	—	Основные типы 7
2	II	IV	A	
3	III	III	—	
4	IV	—	—	
5	V	—	—	
6	—	VI	Д	
7	—	II	—	
8	—	—	B	Дополнительные типы 8
9	—	—	c	
10	—	—	E	
11	—	—		

1) Serological types, following new nomenclature; 2) types; 3) Robinson and Penny; 4) MNIIVS imeni Mechnikov; 5) Moscow IEMG; 6) notes; 7) basic types; 8) supplemental types.

acid, or 50-60% alcohol kills it within 1 min. The majority of antibiotics, particularly penicillin, are lethal for this bacterium in the human body and on nutritive media. Low temperatures (down to −190°) require extended periods to kill diphtheria bacilli: they also remain viable after repeated freezing and thawing. Light, moisture, or high temperatures cause them to die more rapidly. They are killed within a few days under the action of direct sunlight. In a liquid medium C. diphtheriae is killed within 10 min at a temperature of 60°, but an absolutely dry film will survive heating to 98°. Diphtheria bacilli entering the external environment in drops of moisture expelled by a patient or carrier exhibit considerable resistance when a certain combination of conditions (principally absence of light, moisture, and high temperatures) exists; they consequently survive for months in the dry state on articles kept in darkness (the patient's dishes, pencils, pens, toys, etc.). Bacteria adhering with saliva to the rim of a glass retain their viability for up to 15 days (Esmark). The usual rinsing of glasses with cold water under a tap and even scrubbing with a towel

will not ensure removal of diphtheria bacilli. C. diphtheriae can be killed only by treatment with hot water for more than 5 min. These bacteria die after 4-8 hr in diffused light. They retain their viability for up to 5 weeks in dust, for up to 6-20 days in water and milk, and for up to 2 weeks in cadavers.

Antigenic structure; serotypes

Serological types. Diphtheria bacilli are heterogeneous with respect to antigenic structure, but they all produce the same toxin. Serological heterogeneity may be observed within a single type and there is no serological relationship among the three types (gravis, mitis, and intermedius). In 1961 the Ministry of Public Health USSR approved a new nomenclature, utilizing Arabic numerals, for the various serological types.

An agglutination reaction is carried out on slides with pure cultures. The agglutinative serum is diluted to 1:25 with saline solution at pH 7.6 (3 g of sodium chloride is added to 100 ml of distilled water and the pH is adjusted by adding 5% sodium phosphate). The culture to be studied is mixed in a drop of the saline solution and collected with a sterile Pasteur pipette with cotton in its tip to serve as a filter.

For a culture control, a preparation is set up with saline solution but no serum. A positive reaction is characterized by rapid appearance of an agglutinate (2-3 min), which appears the form of flakes, these being especially distinct when the preparation is viewed from above the meniscus (see page 118).

Toxin formation

All three types of C. diphtheriae produce the same toxin, which is easily neutralized by specific diphtheria antitoxic sera. The optimum toxin-formation conditions differ from the optimum growth con-

ditions. Meat extracts containing peptone were formerly the principal media for toxin formation. Study of bacterial nutrition has, however, shown that neither peptone nor proteases are necessary for toxin formation. Meat-extract media can successfully be replaced by certain amino acids and growth agents. Addition of maltose or glucose to the medium promotes an intensification of toxin production.

Determination of the toxigenicity of diphtheria bacilli. Determination of the toxocity of diphtheria cultures is intended to determine the danger presented to others by diphtheria carriers.

Toxicity is determined in vivo in guinea pigs (by the subcutaneous or intracutaneous method) or in vitro on solid nutritive media in Petri dishes.

a) Subcutaneous method: in this technique the culture must be incubated on coagulated serum for 18-24 hr. The bacterial growth is best washed off with nutritive broth, since even physiological solution breaks down the cells. Both mixed and pure cultures can be used for the subcutaneous test. The experimental and control guinea pigs should weigh 250-300 g. The control animal is given 100 units of antidiphtheria serum on the day before the experiment. The bacterial suspension is administered to both animals in a dose of 0.2 ml. If the strain under investigation is toxigenic the unimmunized guinea pig dies within 2-5 days, exhibiting characteristic pathologoanatomic changes (a serous-hemorrhagic infiltration at the injection site, severe hyperemia of the adrenals, and an exudate in the pleural and abdominal cavities and pericardium). The control animal, which received the same culture dose, survives. The subcutaneous method is distinguished by the fact that it is more accurate than the intracutaneous method.

b) Intracutaneous method: this method makes it possible to test several strains on one animal. It is preferable to use a pure culture,

which is washed from coagulated serum with 1-4 ml of broth or physiological solution, depending on the extent of the growth. The suspension is diluted in such fashion that 1 ml contains approximately 500 million bacteria, as determined by comparison with an optical standard. The test is performed on 2 guinea pigs weighing 400 g (preferably white), shaving the coat from the sides and back over an area which permits simultaneous testing of 8-10 cultures. On the day before the experiment one of the guinea pigs (the control) is given 1000 units of antitoxic antidiphtheria serum intraperitoneally. The suspended test cultures are injected intracutaneously in doses of 0.2 ml. Known toxigenic and nontoxigenic strains are administered at the same time, as a control. In order to keep the experimental guinea pig from dying during development of the characteristic dermal reaction 100 units of antidiphtheria serum is injected intraperitoneally after 4 hr. Toxigenic strains cause formation of an inflammatory infiltration at the injection site within 2-5 days and subsequent necrosis. The results are evaluated after 24-48-72 hr.

c) Method of determining toxigenicity in vitro: this method is accurate, simple, inexpensive, and rather rapid (the results can be evaluated within 24 hr in the majority of cases). It permits detection of moderately toxigenic cultures, which may give a negative result when the intracutaneous method is used. This technique has come into wide practical use in recent years.

A total of 12 ml of nutritive medium which has been melted and cooled to 50° is poured into sterile Petri dishes 10 cm in diameter. Strips of filter paper 1.5 x 8 cm in size are wrapped in paper packages, 4 strips to a package, and sterilized in an autoclave under pressure at 120°. The paper strips are placed in sterile Petri dishes as needed and moistened with Diaferm 3 diphtheria antiserum diluted

Fig. 74. Completed precipitation reac ion in agar. A nontoxic strain is in the center.

with physiological solution to a content of 500 units per ml. The diluted serum can be stored under refrigeration for up to 10 days. Each strip is moistened with 0.25 ml of antiserum. The serum-impregnated paper is transferred to the center of a dish containing solidified nutritive medium with a sterile tweezers. The dishes are dried in a heater for 15-20 min.

The cultures to be tested are applied to the surface of the solid nutritive medium in spots 1 cm in diameter and 0.3-0.5 cm from the paper, 2 spots on each side of the strip. One dish is simulteneously inoculated with 3 strains, one of which is known to be toxigenic and serves as the control. Use of strain PW8 for this purpose is not recommended. The inoculated dishes are incubated in a heater at 37° (Fig. 74). The results are evaluated daily for 48 hr. After 18-24 hr the dishes are examined with a magnifying glass in transmitted light against a dark background. On subsequent days the lines become more compact and are visible to the unaided eye. The control strain is stored on coagulated serum at 4-6° and is recultured at least every 10- 14 days. When the morphological properties of the culture are al-

tered (coccoid forms appear) it is passed through blood agar.

The secondary precipitation lines, which appear 3-4 days after culturing, are regarded as nonspecific. They merge with the secondary line of the toxigenic control strain or cross its primary line.

Laboratory Diagnosis of Diphtheria

The morphological characteristics of C. diphtheriae are very unstable, depending both on the conditions in the host organism and the culturing conditions. Morphological properties may vary as a result of passage through an immune organism or of sanation of the mucosae with antibiotics. Hence it follows that the bacteriological diagnosis of diphtheria cannot be limited to identification procedures based solely on study of traits as unstable as morphological characteristics. The purpose of bacteriological investigation is to isolate a pure culture and to identify it by studying a complex of biological characteristics.

Material for examination; taking of samples

Dry sterile cotton swabs (sterilized by dry heat at 160° for 40 min) are used for taking samples; when the material is to be shipped over long distances or in a hot climate the swabs should be moistened with 5% glycerine in physiological solution (the pH of the solution is adjusted to 8.0 with 20% Na_2HPO_4). It is best to take the samples on an empty stomach for no less than 2 hr after the last meal (care should be taken that the mouth has not been rinsed with disinfectants shortly before the material is taken). When carriers have been subjected to sanation procedures or patients have received antibiotic therapy the material to be cultured should be taken no less than 3 days after termination of treatment. Before taking material the stopper must be loosened in the test-tube so that it can be removed quickly; the root of the tongue is held down with a tongue depressor and samples are taken from the most severely affected area under visual

monitoring, without touching the tongue, the mucosa of the cheek, or the teeth. When incrustations are present material should be taken from the boundary between the affected and healthy tissue, pressing the swab gently against it. If a diphtheritic plaque is present it is best to remove a small fragment with a tweezers and to culture this material.

Analyses for diphtheria requires simultaneous investigation of films and mucous from the mouth and nose; in diphtheria of rare localizations (eyes, ears, vagina, skin, or wounds) the oral and nasal mucous must be examined in addition to material from the affected area. In taking samples from the nose it is first carefully cleaned with a dry cotton swab, which is inserted deep into the nasal passages. Two swabs must be used for taking samples from the mouth and nose; material can be taken from both nostrils with a single swab.

The samples should be taken by the physician or his assistant (dresser, nurse, epidemiolcgical assistant, or laboratory technician). The material should be sent to the laboratory as quickly as possible (no more than 3-4 hr after it is taken) and cultured soon after it arrives. When distance makes it impossible for the laboratory to receive the samples within 3-4 hr the material must be taken with a glycerine-impregnated swab or cultured at the patient's bedside. In such cases the laboratory should supply tubes containing coagulated serum in addition to the swabs for taking the material. During the winter the medium should be protected against freezing.

Bacteriological investigation

The material from each swab is cultured on coagulated serum and on a dish containing blood-tellurium agar or Klauberg II medium (the medium should be heated in an incubator before inoculation). Use of both coagulated serum and blood-tellurium agar permits diagnosis of

diphtheria in a large percentage of cases. The swab is inserted into a test-tube containing coagulated serum and moistened with condensation water; the material is then rubbed into the surface of the medium with circular motions. The culture is incubated at 37° and examined after 18-24 hr. An inspection can be made earlier, e.g., after 6-12 hr, in emergency cases. Although culturability is 5-10% higher on tellurium medium than on Loeffler's serum, parallel use of Loeffler's medium or coagulated serum is extremely desirable, since these media make it possible to determine whether the swab is suitable for examination: insufficient bacterial growth indicates that the patient's throat was treated with antibiotics or that the material was incorrectly taken or was overlong in transit. Diphtheria bacilli develop more rapidly on Loeffler's medium than on tellurium medium and a preliminary diagnosis can be made sooner, often within 8 hr. One drawback of Loeffler's medium is the pausity of the growth of C. diphtheriae in certain cases and the consequent difficulty of isolating it from among the other bacteria present. The principal drawback of tellurium medium is the slow growth of certain strains of the mitis type.

Colonies raised on dishes for 24-48 hr are studied macroscopically with a magnifying glass and suspected colonies are examined microscopically and cultured on Petri dishes containing a special medium for determination of toxigenicity. For this purpose 5 or 6 colonies are cultured in separate streaks or spots. Several (3-4) of these colonies are then cultured in test-tubes containing coagulated serum to obtain pure cultures, as well as in test-tubes containing Hiss's carbohydrate media (sucrose and glucose). Cultures on Hiss's medium containing starch are made on the following day if necessary. When only a few (1-3) suspected colonies are present they are cultured only in tubes containing coagulated serum, in order to isolate a pure cul-

ture, all of whose characteristics are then studied.

On the following day (i.e., 48 hr after the initial culture is made) the results of culturing on the media for determination of toxigenicity and biochemical properties are evaluated and the pure culture from the coagulated serum is examined microscopically. If a nontoxigenic culture which decomposes carbohydrates is isolated it is necessary to conduct additional tests for cystinase (Pisou's test) and urease (culturing in broth containing urea or Saks's test) and an agglutination reaction with polytypic diphtheria serum.

The urease test permits rapid (within 20 min) identification of pseudodiphtheria bacilli, 90% of which possess this enzyme. Diphtheroid bacilli contain urease in 25% of all cases, while Loeffler's bacilli do not produce this enzyme at all.

The serological type is determined only when requested by an epidemiologist. An agglutination reaction is set up on slides with monotypic diphtheria sera.

When direct culturing on tellurium agar does not produce colonies of diphtheria bacilli or when these bacilli are not sufficiently typical morphologically in smears it is necessary to make a microscopic examination of smears of a mixed culture raised on coagulated serum. When rods with typical or suspicious morphological characteristics are detected the mixed culture is transplanted to Petri dishes containing blood-tellurium agar or Klauberg II medium. In clinically suspicious cases the investigator cannot be satisfied with a single negative result, but must make a second culture.

In prophylactic examinations cultures on coagulated serum are examined after 24 hr. When the growth is scanty or indetectable smears are made from the condensation water. If diphtheria bacilli are detected the cultures are again incubated and investigated after

48 hr.

Table 53 can be used for identifying the bacterium isolated.

Times for formulating and making reports in investigations for diphtheria. 1. When morphologically typical diphtheria bacilli are found in cultures of material from the mouth and nose in dishes or in a mixed culture on coagulated serum a report of "diphtheria bacilli detected, investigation continuing" is made after 24-48 hr.

2. If rods morphologically similar tc diphtheria bacilli are detected on coagulated serum or in colonies a report of "rods suspected of being diphtheria bacilli detected, investigation continuing" is made after 24-48 hr.

TABLE 53

Properties of Diphtheria Bacilli and Similar Corinebacteria

1 Название вида	2 Сахароза	3 Глюкоза	4 Крахмал	5 Токсигенность	6 Дополнительные признаки: 7 проба Пизу	8 проба на уреазу	9 агглютинация
Дифтерийные палочки 10	13 — (изредка +)	+	+ (gravis) или — (mitis) 14	+ или —	++ ++	—	+ (изредка —)
Дифтероиды 11	+	+	—	—	— или +	— или +	—
Ложнодифтерийные палочки 12	—	—	—	—	—	+	—

1) Species; 2) sucrose; 3) glucose; 4) starch; 5) toxigenicity; 6) additional traits; 7) Pisou's test; 8) urease test; 9) agglutination; 10) diphtheria bacilli; 11) diphtheroid bacilli; 12) pseudodiphtheria bacilli; 13) rarely; 14) or.

3. If no diphtheria bacilli are detected on tellurite media in dishes or coagulated serum a negative report is made.

4. When material taker from a rarely affected area is investigated a report is made only when the culture isolated has been identified from the aggregate of its properties (after 48-72 hr).

5. In examining patients and carriers in accordance with epidemiological indications a final report can be made after 48 hr (culture toxigenic) or 72 hr (culture nontoxigenic) when diphtheria colonies are detected in the Petri dishes after 24 hr. When colonies appear in the dishes after more than 24 hr the report is made correspondingly later.

6. If diphtheria bacilli are first detected only on the coagulated serum (principally in prophylactic examinations) a final report is made after 48-72 hr (if toxigenicity is detected in a mixed culture or 72-96 hr (if toxigenicity is not detected in a mixed culture).

7. When the preliminary report has indicated detection of diphtheria bacilli and subsequent study shows them to be diphtheroid the final report is formulated as "the bacillus detected proved to be diphtheroid on further investigation."

8. When the preliminary report has indicated detection of diphtheria bacilli and subsequent study shows them to be other bacteria (except diphtheroid) the final report is formulated as "presence of diphtheria bacilli not confirmed by further investigation."

9. When the preliminary report has indicated detection of morphologically suspicious rods the final report depends on the result obtained in isolating a pure culture and is formulated as "diphtheria bacilli not detected" when negative.

10. When the analysis is positive the final report is formulated as "toxigenic (or nontoxigenic) diphtheria bacilli isolated;" in studying cultural-biochemical characteristics the report indicates whether the bacterium belongs to the gravis or mitis type, while in serotyping the serological type is noted.

11. In all cases where diphtheria bacilli are detected the laboratory must notify the institution which sent the material for an-

alysis.

12. In all cases where diphtheria bacilli are first detected in material not sent to the laboratory from medical institutions the former must notify the regional epidemiologist so that the necessary antiepidemic measures can be taken.

13. In making a report by telephone it is necessary to note the date and the surnames of the persons placing and receiving the call.

Determination of sensitivity or insensitivity to diphtheria (Schick test)

Specially prepared aged, purified, and stabilized diphtheria toxin is used for the Schick test, being injected intracutaneously in a dose of 0.2 ml, which contains 1/40 MLD. A positive Schick test is usually regarded as a sign of sensitivity to diphtheria, indicating that antitoxin is either completely lacking or present only in small quantities in the circulating blood. A negative reaction (immunity present) is consistently registered at an antitoxin content of 1/30 unit per ml or more; a positive reaction (immunity absent) takes place at 1/1000 unit per ml or less. The Schick test can be either positive or negative between these two antitoxin contents. This test makes it possible to evaluate the immunological condition of a group of children after a course of immunization and, with proper organization, the effectiveness of the preparation used for active immunization. There is no need to conduct a Schick test before immunization. The reaction usually becomes negative 4-12 weeks after immunization.

The actual quantity of diphtheria antitoxin circulating in the blood can be determined by titration in rabbits or guinea pigs.

Determination of quantity of diphtheria antitoxin by Jensen's method. Intracutaneous titration of diphtheria antitoxin is based on

the law of strict proportionality in the neutralization of diphtheria toxin by specific antitoxin. This law was first demonstrated by Roemer (1909) and was studied in detail by Jensen (1933). The latter suggested that titration be carried out at different levels, depending on the antitoxin content of the sera to be tested. The precision with which the serum titre can be determined is considerably reduced when the toxin is highly diluted.

Titration of antidiphtheria immune sera, a procedure employed in various investigations connected with the study of antitoxic immunity in diphtheria, is usually carried out at 1/300 unit or 1/3000 unit, depending on the assumed titre of the serum under investigation. Sera containing from 0.5 to 100 units per ml are generally titrated at 1/300 unit, while sera containing less than 0.5 unit per ml are titrated at 1/3000 unit. The minimum serum titre which can be determined at 1/300 unit is 0.05 unit, provided that whole (undiluted) serum is used, but since the serum is diluted to 1:9 with buffer solution in order to conserve the material, the minimum antitoxin titre determinable at 1/300 unit is actually 0.5 unit per ml. The minimum titre determinable at 1/3000 unit is less by a factor of 10, i.e., 0.005 unit* when undiluted serum is used and 0.05 unit per ml when the serum is diluted to 1:9.

The experimental reacting dose of diphtheria toxin (LR) is the least quantity of toxin which, when mixed with 1/300 unit (for titration at 1/300 unit) or 1/3000 unit (for titration at 1/3000 unit) of standard antidiphtheria serum (containing 10 units/ml), causes a dermal erubescence 10 mm in diameter at the site of an injection of 0.1 ml of the mixture after 48 hr.

Diphtheria toxins prepared in peptic- or tryptic-extract broth containing approximately 200-250 MLD per ml (carbolized with 0.25%

phenol) and aged for no less than 2 years in a dark place at a temperature of +4° are used for this purpose. The experimental toxin doses for titration at 1/300 and 1/3000 unit are determined simultaneously in 2 rabbits. The experiment is repeated 2 or 3 times and an average is taken.

In order to exclude individual variations associated with the sensitivity of the rabbits a series of control tubes (a total of 11) containing mixtures of a given quantity of toxin with different quantities of standard antitoxin is prepared. An amount of antitoxin corresponding to the titration level, i.e., 1/300 or 1/3000 unit and 0.1 ml of mixture, is always added to tube No. 5.

The sera are titrated in volumes of 3 ml or 0.3 ml, depending on the amount of serum available. It is preferable to titrate them in volumes of 3 ml.

Jensen's buffer (pH 7.38) is used to dilute the sera and toxin.

Titration in volumes of 3 ml is carried out in ordinary test-tubes, while titration in volumes of 0.3 ml is conducted in agglutination tubes. The pipettes and syringes should be accurately graduated (their calibration should be checked).

Preparation of rabbits. Rabbits of the chinchilla or white strain with a well-developed subcutaneous cellular tissue and weighing no less than 3 kg are used for titration. The animals should be kept in individual cages for 2-4 weeks before titration. In order to promote better development of the subcutaneous cellular tissue they must be fed concentrated feeds (fish meal, dry milk, oatmeal, vitamins, and fresh vegetables) throughout this period. There should be no scars on the skin and the undercoat should be dark. The coat is carefully clipped with scissors on the day before titration, taking care not to traumatize the skin; if the rabbits are depilated or

shaved this is done 4-5 days before the experiment. The skin should be smooth and have no scratches or blotches.

Before the experiment a pen is used to mark out a grid on the rabbit's skin, which is duplicated in a notebook. Each square of the grid is 2 x 2 cm in size.

The results of titration of the test sera are evaluated after 36-48 hr. The control series is examined first, measuring the diameter of the erubescence with paper graduated in millimeters. In this series the mixture from tube No. 5, which contains 1/300 units, should give a reaction with a diameter of 10 mm; the titre of the test serum corresponds to its dilution in this case, yielding a reaction 10 mm in diameter. If the mixture from tube No. 5 of the control series gives a reaction more or less than 10 mm in diameter that control dilution which gives a reaction 10 mm in diameter is selected and a correction factor by which the titration results in units are to be multiplied is introduced. The correction factor is (numerically) the quantity of standard serum diluted 100-fold contained in the mixture which gives a reaction 10 mm in diameter.

If all the reactions in the control series have diameters more or less than 10 mm, none precisely equaling 10 mm, the average of the two closest values is taken. In this case the correction factor is the arithmetic mean of the quantities of diluted standard serum contained in these tubes.

It can be seen from Table 55 that an erubescence 10 x 10 mm in size lay between tubes Nos. 3 and 4; the values for these tubes were consequently averaged and a correction factor introduced. The amount of antitoxin per ml of test serum is calculated in the following manner: if an erythema 10 x 10 mm in size is detected within 48 hr after intracutaneous injection of 0.1 ml of a mixture con-

TABLE 54

Set-up of Titration Experiment at 1/300 Unit in Volumes of 3 ml

1	2	3	4	5	6	7	8	9
№ пробирки	Сыворотка	АЕ/мл	Разведение сыворотки	Количество разведенной сыворотки, мл	Количество буфера Иенсена, мл	Количество разведенного токсина, мл	Диаметр реакции через 48 часов, мм	Результат
10 Контрольный ряд 1	11		0,25 : 24,75	1,6	0,4	1,0		
2				1,4	0,6	1,0		
3	Стандартная ГКИ. Серия №	10,0		1,25	0,75	1,0		
4				1,1	0,9	1,0		
5				1,0	1,0	1,0		
6				0,9	1,1	1,0		
7				0,8	1,2	1,0		
8				0,71	1,29	1,0		
9				0,63	1,37	1,0		
10				0,56	1,44	1,0		
11				0,5	1,5	1,0		
12	Испытуемая №	13	14					
		0,05	Цельная	2,0	—	1,0		
13		0,1	»	1,0	1,0	1,0		
14		0,13	»	0,75	1,25	1,0		
15	12	0,2	»	0,5	1,5	1,0		
16		0,4	»	0,25	1,75	1,0		
17		0,5	1:9	2,0	—	1,0		
18		1,0	1:9	1,0	1,0	1,0		
19		1,5	1:9	0,75	1,25	1,0		
20		2,0	1:9	0,5	1,5	1,0		
21		4,0	1:9	0,25	1,75	1,0		
22		5,0	1:99	2,0	—	1,0		
23		10,0	1:99	1,0	1,0	1,0		
24		20,0	1:99	0,5	1,5	1,0		

Предполагаемый титр

1) Test-tube No.; 2) serum; 3) units/ml; 4) serum dilution; 5) quantity of diluted serum, ml; 6)quantity of Jensen's buffer solution, ml; 7) quantity of diluted toxin, ml; 8) diameter of reaction after 48 hr, mm; 9) result; 10) control series; 11) GKI standard; series No.; 12) test serum No.; 13) assumed titre; 14) whole.

taining 0.5 ml of diluted (1:9) test serum, 1.5 ml of buffer solution, and 1 ml of diluted diphtheria toxin, 0.1 ml of this mixture contains 1/300 unit and 3 ml contains 30 times as much, i.e., 1/10 unit. If the 0.5 ml of 10-fold diluted test serum in the mixture corresponds to 1/10 unit, 1 ml of the serum will contain 20 times as much antitoxin, i.e., 2 units.

The titration is carried out and the results evaluated in precisely the same manner at 1/3000 unit, the only difference being the fact that the standard serum is diluted by a factor of 1000.

The experimental toxin dose for titration at 1/3000 unit can-

not be calculated arithmetically from the dose for 1/300 unit, but is determined in rabbits, since the experimental dose is somewhat greater than one-tenth that for titration at 1/300 unit.

TABLE 55

Example of Titration at 1/300 Unit in Volumes of 3 ml

1	2	3	4	5	6	7	8	9
№ пробирки	Сыворотка	АЕ/мл	Разведение сыворотки	Количество разведенной сыворотки, мл	Количество буфера Иенсена, мл	Количество разведенного токсина, мл	Диаметр реакции, мм	Вычисление контрольного коэффициента
10 Контрольный ряд 1	11 Стандартная	10,0	1:99	1,6	0,4	1,0	0	
2				1,4	0,6	1,0	0	
3				1,25	0,75	1,0	8×8	1,25+1,1=2,35
4				1,1	0,9	1,0	13×13	2,35 : 2=1,17
5				1,0	1,0	1,0	14×14	
6				0,9	1,1	1,0	21×21	
7				0,8	1,2	1,0	25×25	
8				0,71	1,28	1,0	30×30	
9				0,63	1,37	1,0	31×31	
10				0,56	1,44	1,0	32×32	
11				0,5	1,5	1,0	32×32	
12	Испытуемая	0,5	1:9	2,0	—	1,0	—	14
13	№ 1	1,0	1:9	1,0	1,0	1,0	8×8	2АЕ×1,17= =2,34 АЕ
14	12	2,0	1:9	0,5	1,5	1,0	10×10	
15		4,0	1:9	0,25	1,75	1,0	20×20	
16		0,5	1:9	2,0	—	1,0	—	
17	№ 1, № 2	1,0	1:9	1,0	1,0	1,0	8×8	1+2=3
18		2,0	1:9	0,5	1,5	1,0	12×12	3:2=1,5
19		4,0	1:9	0,25	0,75	1,0	20×20	1,5×1,17= =1,75 АЕ
		13						

1) Test-tube No.; 2) serum; 3) units/ml; 4) serum dilution; 5) quantity of diluted serum, ml; 6) quantity of Jensen's buffer, ml; 7) quantity of diluted serum, ml; 8) reaction diameter, mm; 9) calculation of correction factor; 10) control series; 11) standard; 12) test serum; 13) assumed titre; 14) units.

Accelerated methods for bacteriological diagnosis of diphtheria

Preliminary bacterioscopy. Preliminary bacterioscopy of smears of material taken with swabs is carried out only when requested by the physician who sent the material for examination. Two swabs are employed for this purpose, one being used for culturing and the other for preparation of several smears for bacterioscopy.

Method of raising diphtheria bacilli on a serum-impregnated swab

(Folger, 1902; Sole; S.Ya. Arkavin and S.Ya. Dynina, 1937; et al.). This accelerated method makes it possible to establish a bacteriological diagnosis within 4 hr. Anordinary cotton swab is immersed in heated undiluted sterile serum (containing no antiseptics). The excess serum is pressed out against the wall of a test-tube and the remaining serum is then coagulated by gentle heating over a burner or in a heater at 80° for 15 min. The mouth, including the affected area, is wiped with the serum-impregnated swab, which is then placed in a heater at 37°; smears are prepared from it after 3-4 hr and stained in the usual manner. There have been reports that positive results can be obtained in 80% of all cases after 2 hr of incubation and in 95% of all cases after 4 hr.

Preparation of serum-impregnated swabs (D.D. Lebedev and A.I. Titova's method):

a) an ordinary sterile cotton swab on a wooden stick or stainless-steel wire is immersed in equine or bovine serum coagulated to a jelly-like consistency (by preliminary heating in a water bath at 60-62° for 40-60 min);

b) the swab is then placed in a sterile test-tube, on the bottom of which are pieces of cotton wetted in sterile physiological solution or distilled water to keep the serum-impregnated swab from drying out. The prepared swabs are again placed in a water bath at 85-90° or in a Koch apparatus for 30 min to permit final coagulation of the serum. This procedure (wetting the swab in semicoagulated serum and then permitting it to coagulate) is repeated 3 times. Swabs prepared by this method are smooth and moist and remain usable for more than 2 weeks. In taking samples with such swabs care must be exercised that the entire surface of the cotton is covered with mucous. The principal drawback of this method is the fact that the morphology of C. diphtheriae

is substantially altered: the rods become shorter and often lack granules or inclusions;

c) accelerated diagnosis with potassium tellurite (Mannulo) is based on the ability of C. diphtheriae to reduce potassium tellurite: a 2% aqueous solution of potassium tellurite is smeared over the affected area of the body in a film. If diphtheria bacilli are present at the surface they cause the film to turn black within 5-10 min. When smearing a potassium tellurite film in the mouth care must be taken that it does not come into contact with the tongue.

Proper preparation and storage of both the potassium tellurite and the 2% solution are extremely important in obtaining accurate results with the tellurite test.

Nutritive Media

Klauberg's medium. A total of 3 ml of 2% potassium tellurite, 10 ml of glycerine mixture, and 50 ml of laky blood are added to 100 ml of melted 3% nutritive agar (or 7.5 g of dry nutritive agar in 100 ml of distilled water).

Preparation of laky blood: 16 ml of defibrinated bovine or human blood is added to 34 ml of sterile distilled water.

Preparation of glycerine mixture: 20 ml of chemically pure sterile glycerine is added to 40 ml of defibrinated bovine or human blood or diluted dried blood. The mixture is stored in a refrigerator for 3-6 weeks.

Blood-tellurite agar. A total of 5-10% of defibrinated human (donor or placental) or animal (cattle, sheep, rabbit, or guinea pig) blood and 1 ml of 2% potassium tellurite (K_2TeO_4) are added to 100 ml of 2-3% meat-infusion agar (at pH 7.6) which has been heated and cooled to 50°. The mixture is thoroughly stirred and poured into dishes. Sterile blood clots obtained by Wasserman's or Widal's reaction can be used for preparing blood agar. Two or 3 such clots are shaken with

3 ml of meat-infusion broth in a broad test-tube. The resultant suspension is drawn off with a pipette and added to 50 ml of meat-infusion agar (at pH 7.6) which has been melted and cooled. Dried blood can be employed instead of fresh blood.

Dry nutritive agar (5-7.5 g of dry nutritive agar in 100 ml of water) can be substituted for the meat-infusion agar.

Serum-tellurite agar. A total of 20 ml of equine or 30 ml of bovine serum and 1 ml of 2% potassium tellurite are added to 80 ml of 2% meat-infusion agar (at pH 7.6) which has been melted and cooled to 50°. The medium is poured into sterile Petri dishes.

Preparation of 2% potassium tellurite: Prepared 2% potassium tellurite in 40% glycerine in ampules can be used for making up blood or serum agar; this solution is used for the clinical tellurite test.

When commercial potassium tellurite solution is not available it is prepared in the following manner: 2 g of potassium tellurite is dissolved in 100 ml of sterile distilled water. Heating the tellurite solution to above 50° causes the compound to decompose. Powdered potassium tellurite must be stored in a brown-glass jar with a ground stopper.

Before using the media the dishes are dried in a heater for 15-20 min and then inoculated. Colonies of diphtheria bacilli grow on tellurite media within 24-48 hr and are black or dark gray in color, as a result of the reduction of tellurium salts to metallic tellurium. After 24 hr colonies of C. diphtheriae on blood-tellurite media are 0.5-2 mm in diameter, convex, and slate black. Colonies of diphtheroid bacilli are identical morphologically to those of C. diphtheriae. Colonies of pseudodiphtheria bacilli are gray, lustrous, convex, and conical. Staphylococci grow in moist black lustrous colonies.

After 24 hr colonies of C. diphtheriae on serum-tellurite agar

are light gray, with small, black, slightly raised centers. Colonies of pseudodiphtheria bacilli are light-colored, with brownish centers. Colonies of diphtheroid bacilli are identical to those of C. diphtheriae. Colonies of Staphylococci are black and lustrous, with a white margin.

Medium for determination of Corinebacterium cystinase (Pisou's test). A total of 2 ml of cystine solution (1% cystine is carefully mixed into 0.1% sodium hydroxide and an equal volume of 0.1% sulfuric acid is added) is added to 90 ml of melted 2% meat-infusion agar at pH 7.6. The medium is sterilized at 112° for 30 min. It is then melted and cooled to 50°, 1 ml of 10% lead acetate (twice sterilized in live steam) is added, the two are stirred, and 9 ml of normal equine serum is added. Portions of 2 ml of the medium are poured into small test-tubes under sterile conditions. Cultures are made by the stab method.

Determination of urease for identification of pseudodiphtheria bacilli and certain diphtheroid bacilli.

Preparation of urea-containing broth. A total of 1 g of urea and 0.2 g of cresol red (1.6% alcoholic solution) are added to 100 ml of meat-infusion or Hottinger's broth (the pH must be precisely 7.0). Portions of 2-3 ml of the mixture are poured into sterile test-tubes and sterilized in live steam for 10 min. The results are evaluated 20-24 hr after inoculation. If the medium becomes red the strain under investigation contains urease.

Preparation of reagents for determination of urease by Saks's method.

Reagent A:

Urea – 2 g

96° ethyl alcohol – 2 ml

Distilled water – 4 ml

Reagent B:

0.2% phenol red – 1 ml

Monosubstituted potassium phosphate (KH_2PO_4) – 0.1 g

Disubstituted potassium phosphate (K_2HPO_4) – 0.1 g

Distilled water – 100 ml

Sodium chloride – 0.5 g

Reagent A is stored unsterilized at 4-10°. Reagent B is sterilized in an autoclave in live steam. The two reagents are mixed ex tempore, taking one part of solution A to 19 parts of solution B; 0.1 ml portions of the mixture are poured into slender test-tubes, which are then placed in a heater for 30 min. When the reaction is positive, i.e., when the bacteria contain urease, the mixture turns red. There is no change in color when the reaction is negative.

Medium for determination of toxigenicity. Preparation of nutritive agar: aqueous agar is melted, mixed half and half with Martin's broth, poured into sterile test-tubes in 10 ml portions, and sterilized in live steam for 30 min.

Preparation of cresol-red paper: 0.1 g of cresol red is dissolved in 100 ml of 5% alcohol and left to stand at 37° for 24 hr, shaking the solution frequently. On the following day strips of filter paper are wetted with the solution and quickly dried. The paper becomes bright yellow in color, turning various shades of red in alkaline media.

Before being poured into Petri dishes the nutritive agar in each tube is melted and cooled to 50° and 2 ml of normal equine serum or 3 ml of native bovine serum is added.

WHOOPING COUGH

Prof. M.S. Zakharova

The causative agent of whooping cough, Bordetella pertussis (Hemophilus pertussis), was isolated from the sputum of a child suffering

from this disease by Bordet and Gengou in 1906. In 1937 Eldering and Kendrick isolated a bacterium from whooping-cough patients which was similar but not identical to B. pertussis; they named this organism B. parapertussis.

Morphology of B. pertussis

These bacteria are short, ovoid gram-negative rods 0.2 x 0.4-1.2 microns in size (Fig. 75). Bipolarly located metachromatic granules are detected on staining with toluidine blue. B. pertussis also exhibits a capsule.

Biology of B. pertussis; cultural characteristics

Freshly isolated virulent cultures of B. pertussis, which are S-form or phase I in Leslie and Gardner's classification, grow only on a nutritive medium consisting of potato-glycerine agar to which blood has been added (Bordet-Gengou's medium) or a semisynthetic medium based on an amino-acid complex in the form of casein or bean hydrolyzate. Colonies of B. pertussis on Bordet-Gengou's medium are smooth, lustrous, clear, dome-shaped, pearly or mercurous in color, and approximately 1 mm in diameter. Colonies of B. parapertussis are very similar in appearance to those of B. pertussis, but are larger and appear sooner.

Atypical variants of B. pertussis grow on ordinary nutritive media, as do B. parapertussis and B. bronchiseptica.

Colonies of B. pertussis on a semisynthetic medium, such as casein-carbon agar (KUA medium), are small (0.5-1 mm in diameter), convex, distinct in outline, lustrous, smooth, grayish-cream in color, and viscous in consistency (Fig. 76).

Colonies of B. pertussis and B. parapertussis on Bordet-Gengou's medium are surrounded by a characteristic zone of hemolysis (this zone is not clearly delimited and extends diffusely into the surround-

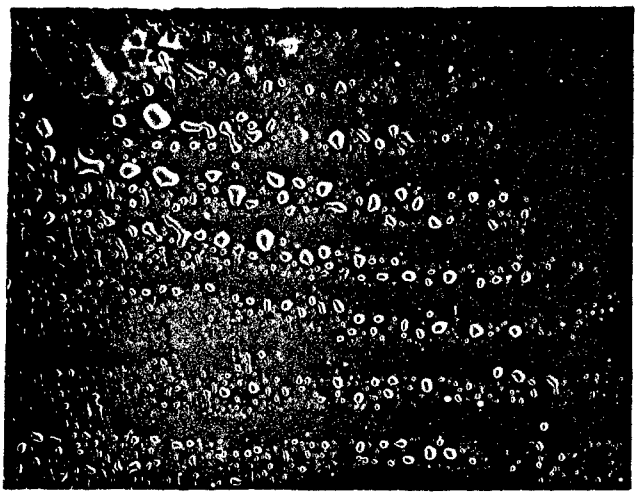


Fig. 75. B. pertussis. Day-old culture on KUA medium. 1500 x.

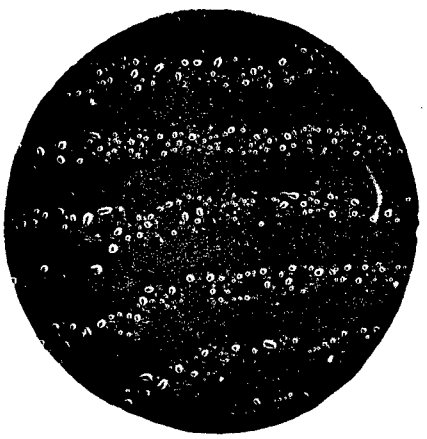


Fig. 76. B. pertussis colonies on KUA medium.

ing medium; Fig. 77).

B. pertussis does not ferment sugars, produce indol, reduce nitrates, or utilize citrates. Up to 70% of the strains of this bacterium are catalase-positive, while all B. parapertussis are catalase-positive. In litmus milk B. parapertussis causes alkalization only after 10-14 days, while B. parapertussis requires 1-4 days.

B. pertussis is a facultative anaerobe and has an optimum culturing temperature of 35-36°.

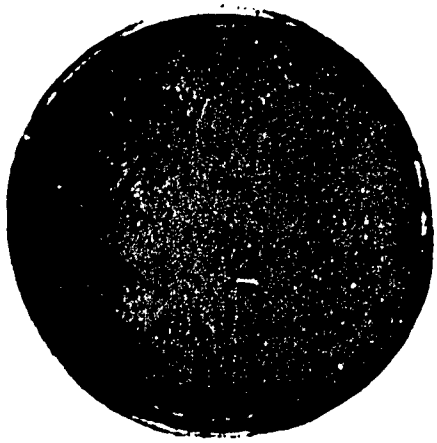


Fig. 77. B. pertussis colonies on Bordet-Gengou's medium.

TABLE 56

Differential Characteristics of Species of the Genera Bordetella and Haemophilus

Признак 1	2 Вид микроба					
	B. pertussis	B. parapertussis	B. bronchisepticus	Brucella	Alcaligenes	H. influenzae
3 Рост на простом агаре	–	+	+	+	+	–
Образование коричневого пигмента (КУА)	–	±	17–	±	–	–
4 Образование уреазы	–	±	Медленное	±	–	–
5 Использование цитратов	–	±	±			+
6 Гемолиз	+	±	±	–	±	–
7 Подвижность	–	–	+	–	+	–
8 Восстановление нитратов	–	–	+	+	±	+
9 Потребность в X- и V-факторах крови	–	–	–	–	–	–
10 Потребность в тиамине	11–	–	–	+	–	–
12 Лакмусовое молоко (коагуляция)	–	±				
13 Капсула	+	–	–			18
14 Проба Дольда	Некроз	16 Некроз				Инфильтрация, помутнение
Агглютинация с сыворотками: 15						
B. pertussis	++	±	–			
B. parapertussis	±	++	–			

1) Characteristic; 2) species of bacterium; 3) growth on simple agar; 4) formation of brownish pigment (KUA medium); 5) urease formation; 6) citrate utilization; 7) hemolysis; 8) mobility; 9) reduction of citrates; 10) requires x and v factors of blood; 11) requires thiamine; 12) litmus milk (coagulation); 13) capsule; 14) Dold's test; 15) ag-

glutination with sera; 16) necrosis; 17) slow; 18) infiltration, erubescence.

Differential characteristics of B. pertussis, B. parapertussis, and B. bronchiseptica. The principal differential characteristics for these bacteria are serological (agglutination reaction) and biochemical.

Since an agglutinative cross-reaction may take place at a relatively high titre, especially when it is set up on slides, it is desirable to use species-specific adsorbed antisera or ordinary serum diluted beyond the limit for the cross-reaction.

Table 56 shows the basic characteristics which permit differentiation of the 3 species of Bordetella, as well as of the members of the genus Haemophilus, which previously included the Bordetella.

The principal traits which permit differentiation of B. pertussis and B. parapertussis cultures are as follows: B. parapertussis will grow when first cultured on simple agar (without blood), casein-carbon agar, meat-infusion agar (especially with tyrosine added), and pieces of potato, forming a brownish pigment; it decomposes urea (as a result of the presence of urease; see below) and utilizes citrates. B. pertussis does not usually grow on simple agar (growth is noted only when the culture dissociates), forms no pigment, and does not decompose urea.

B. parapertussis cultures agglutinate to approximately 1/10 of titre with B. pertussis antiserum.

Urease test. The urease test is employed to differentiate B. pertussis from B. parapertussis.

Reagents: 2% aqueous urea (stored for no more than 2 weeks) and 0.1% alcoholic phenolphthalein.

Procedure for reaction: 0.3 ml of the urea solution and 0.3 ml of

a suspension of the culture to be tested are poured into a test-tube. The suspension should have a density of no less than 10 billion cells per ml; the denser the suspension, the more rapidly the reaction proceeds. The culture can also be added with a loop.

To this mixture is added 2-3 drops of phenolphthalein and the test-tube is shaken and placed in a heater. The reaction (a color change) may take place within 15-20 min. The final result is evaluated after 2 hr.

A positive reaction (obtained when the liquid turns a rasberry red) is characteristic of cultures of B. parapertussis.

Resistance to physical and chemical agents

B. pertussis is an obligative parasite and can survive for only a very short period outside the human body. It remains viable in dry sputum for several hours, but dies within 30 min at a temperature of 50-55°. It is very sensitive to ultraviolet light and chemical antiseptics. Both B. pertussis and (to a lesser extent) B. parapertussis are sensitive to antibiotics, the most effective of which are polymixin, terramycin, levomycetin, biomycin, and streptomycin.

Antigenic structure

The various B. pertussis are identical in antigenic properties, but differ in the complexity of their antigenic structure. As many as 12 antigenic components can be isolated from phase I strains of B. pertussis.

B. pertussis has both specific antigens and antigens in common with B. parapertussis and B. bronchiseptica, the latter being shared with either both species or only one of them.

When the investigator has available a predetermined set of strains it is possible to obtain species-specific sera by sequential adsorption, which is very important in the differential diagnosis of

these bacteria.

Individual fractions with immunogenic properties (agglutinogens, hemagglutinins, histamine-sensitizing factors, and protective antigens) have been isolated from B. pertussis.

Pathogenicity, virulence, and toxin formation

Only humans are susceptible to whooping cough under natural conditions. A disease symptomatically quite similar to whooping cough has been induced in monkeys under experimental conditions by inoculating them nasally or intratracheally with a culture of B. pertussis. It has proved possible to induce prolonged coughing in puppies by inoculation with B. pertussis. Mice develop pneumonia after nasal administration of a culture of this bacterium and encephalitis after intracerebral administration. Chick embryos, rats, and guinea pigs die of intoxication after intraperitoneal injection of a culture.

Intracutaneous administration of a culture of B. pertussis to rabbits or guinea pigs causes development of necrosis as a result of the liberation of the toxic compounds of the bacteria as they are broken down. This reaction is characteristic of B. pertussis and B. parapertussis and distinguishes them from members of the genus Haemophilus (H. influenzae), which cause only erubescence and an infiltration, even when injected in large doses.

Laboratory Diagnosis of Whooping Cough

Material for examination

B. pertussis reproduces in the mucosae of the respiratory tract. It can be detected (by culturing) in material excreted from the respiratory tract during all phases of the disease, but is most frequently isolated during the early period. During the catarrhal stage and the first few days of the spastic stage it can be isolated in 40-100% of all cases with a typical or latent course.

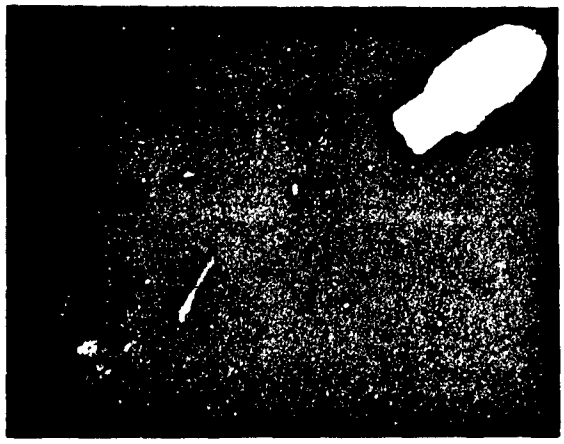


Fig. 78. Nasopharyngeal swab.

Culture material can be obtained in two ways: by the "cough-plate" method and by collecting it with a nasopharyngeal swab. The latter procedure is employed for nursing infants.

Cough plates: As soon as a coughing fit develops an open dish containing nutritive medium is placed 8-10 cm from the child's mouth and held there for several seconds (6-8 coughs). It is desirable to inoculate two dishes. When the coughing spasm is brief the procedure must be repeated with the same dish. Artificial initiation of coughing is not recommended. In using this method the investigator should not breathe on the open dish and the lid is held with its inner surface down (to avoid contamination with air-borne bacteria), taking care not to touch this surface with the fingers.

The dish is covered as quickly as possible after the material has been collected, in order to avoid contamination, and immediately placed in an incubator. If the material is not taken at the laboratory and the dishes must be shipped this should be done as rapidly as possible, wrapping them in cotton when the weather is cold; a hot-water bottle can be packed in the box with the dishes. If it is impossible to ship the dishes immediately they must be kept in a cold place (at a tempera-

ture of no more than 36°).

Cultures from nasopharyngeal swabs. The swab is a small piece of gauze or cotton fastened tightly to the end of a fine flexible wire (nichrome wire is quite suitable; Fig. 78).

Before sterilization the swab is carefully checked (it should be smooth and present no danger to the patient) and placed in a stoppered test-tube. The sterilized swab is removed from the tube and carefully inserted into the child's nostril (immobilizing its head in the hands of an assistant) until the posterior wall of the pharynx is reached, whence a bit of mucous is taken. When this procedure causes frequent coughing leaving the swab in place for a short time during a coughing fit increases the chances of obtaining a positive result. Children withstand this operation quite well. Taking material through the mouth with a swab, using a tongue depressor, is more complex and detrimental to the child, having no advantages over use of a nasopharyngeal swab.

The swab is removed from the nostril and immediately used to inoculate a dish containing nutritive medium. The culture material, applied to the surface of the medium with the swab, is thoroughly rubbed over the entire dish with a glass spatula or platinum loop. The same spatula is employed to inoculate a second dish.

The cultures should be made within 1-2 hr after the material is taken. Shipment of the swabs over long distances is not recommended, since they rapidly dry out.

Bacteriological investigation

The inoculated dishes, which are wrapped in paper and placed in a box or case together with a small vessel containing water to keep them from drying out, are incubated in a heater at 35°; the dishes are incubated for 48 hr and inspected several times during this period to permit detection of molds and other bacteria, especially those which

produce a creeping growth. Those portions of the agar overgrown with such microorganisms are cut away with a sterile scalpel or needle.

After incubation for 48 hr the dishes are checked for B. pertussis daily for 5 days, using a magnifying glass or a binocular microscope.

Colonies of B. pertussis usually appear on casein-carbon agar (see page 733) or Bordet-Gengou's medium (page 734) within 70-80 hr. Colonies on casein-carbon agar are smooth, 0.5-1 mm in diameter, convex, distinct in outline, lustrous, smooth, grayish-cream in color, and viscous in consistency, like thick sour cream; they are easily removed from the surface of the nutritive medium with a loop and can be rather easily suspended in physiological solution.

Colonies of B. parapertussis are very similar in appearance to those of B. pertussis, but are larger and appear within 24 hr.

Colonies of B. pertussis on Bordet-Gengou's medium are smooth, raised, lustrous, have a mother-of-pearl tint, and are almost transparent and no more than 1 mm in diameter. Colonies of gram-positive cocci on this medium are usually dull, dark in color, and more opaque. Colonies of B. pertussis are surrounded by a characteristic zone of hemolysis.

When the colonies are removed with a loop and suspended in physiological solution they form flakes, which rapidly break up to form a homogeneous suspension when the tube is shaken.

Smears are prepared from suspicious colonies for Gram-staining and the remainder of the material (or separate colonies when there is a considerable number on the dish) is used for an agglutination reaction on slides with antipertussis serum.

B. pertussis is easily decolorized when Gram-stained. Microscopic examination of the smears reveals small, weakly stained coccoid bacilli uniformly scattered over the entire field of view, for the most part as

isolated cells, rarely as pairs, and sometimes as short chains. They often stain bipolarly. B. parapertussis are gram-negative bacteria which are ovoid in shape or take the form of short rods; they are sometimes somewhat larger than B. pertussis and are frequently polymorphous. The two species are usually morphologically indistinguishable when first isolated.

When strains are stored on an artificial nutritive medium for an extended period the colonies change from the smooth to the rough form and pronounced polymorphism (formation of filaments and larger cells) is observed.

Immune antibacterial antipertussis serum (phase I) diluted to approximately 1/100 of titre is used for agglutination. For example, if the serum has a titre of 1/16000, it must be diluted to 1:160.

A total of 2 drops, one of physiological solution and one of agglutinative serum, are applied to a slide. A colony is removed from the medium with a loop and suspended in physiological solution; several drops of this suspension are then mixed with serum. B. pertussis agglutinates rather rapidly, agglutination taking place within 5-10 min in very rare cases. When the drops on the slide have dried they can be stained and examined microscopically in place of specially prepared smears.

If the suspected colonies on the dishes are very small or isolated and an agglutination reaction cannot be set up they must be transplanted to casein-carbon agar or Bordet-Gengou's medium in a test-tube or on a sector of a dish and the cultures then examined after 24-48 hr.

Results of bacteriological investigation. A positive report is made if colonies of bacteria which correspond morphologically and culturally to B. pertussis and are agglutinated by specific (antipertussis) serum appear on the dishes.

A negative report is made if no colonies corresponding to the genus Bordetella are found on the dishes after 4-5 days. It is recommended that the analysis be repeated in such cases (especially when clinical and epidemiological indications are present).

If the dishes contain only a very sparse growth or are solidly overgrown with extraneous bacteria the report is formulated as "B. pertussis not detected." The reason for the report is indicated and it is recommended that the investigation be repeated. It should be kept in mind that when the material has been correctly taken and cultured on a nutritive medium of suitable quality B. pertussis can be isolated during the catarrhal period in 80-100% of all cases.

Identification of pure cultures. The procedures for identifying B. pertussis are based on the morphological, biochemical, serological, toxic, and pathogenic properties of the cultures isolated.

In order to preserve cultures they must be regularly recultured on casein-carbon agar or Bordet-Gengou's medium. This should be done no less than every 7-10 days. Dried cultures retain their principal biological properties for more than 2 years. Drying is carried out by freezing in the presence of stabilizers (serum, skimmed milk, 10% sucrose in 1% gelatin, etc.).

Freshly isolated strains and strains properly stored in the laboratory are serologically identical. Changes in basic biological properties (morphological, cultural, serological, antigenic, and immunogenic) are observed within a very short time on prolonged culturing in artificial nutritive media or in complete media, i.e., those which do not satisfy growth requirements. This phenomenon is to some extent analogous to the transition from the S to the R form in other species of bacteria, although the morphological changes in colony structure are less pronounced than in other species.

Fig. 79. Accelerated agglutination on a plate.

Bordet and Schleswick described the serological types of B. pertussis as I (freshly isolated cultures, smooth form) and II (rough form).

Leslie and Gardner distinguish 4 phases characterized by definite morphological and serological properties. Phase I is the virulent S form and is the most complete antigenically and immunogenically, phase IV is nonvirulent and corresponds to the R form, and phases II and III are intermediate phases, or serological variants. Strains of B. pertussis isolated from the body are most frequently in the smooth S form (Leslie and Gardner's phase I), thus differing sharply from H. influenzae. In addition to phase I, however, phase I and an intermediate phase, an intermediate phase alone, or phase III and an intermediate phase may be isolated in examining pertussis patients during different periods of the disease (Kasuga, 1956). An intermediate phase (Leslie and Gardner's phases II and III) is most frequently encountered during the later stage of the illness. According to the data of Leslie and Gardner, of 20 freshly isolated strains 18 were phases I and II and strains intermediate between phases I and II. Many authors (Kendrick, Leslie and

Gardner, et al.) have written that it is principally strains of Leslie and Gardner's phase I which are isolated from patients, dissociated variants being formed only as a result of culturing under laboratory conditions. The aforementioned data should be used as a basis for diagnostic work, concluding that B. pertussis has been isolated only when bacteria with the basic characteristics of phase I have been isolated. According to Lawson's data, the smooth form (phase I) appears as small, gram-negative, monomorphic coccoid bacilli 0.2-0.3 microns wide and 0.4-1.2 microns long; they are occasionally arrayed in pairs, are immobile, and have a capsule. Colonies on Bordet-Gengou's medium are lustrous, pearly gray in color, convex, and 0.25-1 mm in diameter. Phase I B. pertussis do not grow on chocolate, blood, or ascitic agar or Loeffler's medium, do not form indol in peptone water, and do not ferment carbohydrates. The cataphoretic rate varies from 1.2 to 2.2 microns per sec at 1 volt/cm. Phase I B. pertussis are agglutinated by phase I serum to titre; intracutaneous administration to rabbits causes necrosis, while intraperitoneal administration to animals (rabbits, guinea pigs, or mice) causes death. Intratracheal, intranasal, or intralaryngeal administration to mice produces lethal pneumonia accompanied by bacteriemia.

Skin test on rabbits. A bacterial suspension of 5-10 billion cells per ml in physiological solution (prepared from a 2-day culture raised on casein-carbon agar or Bordet-Gengou's medium) is used for the skin test. It is administered intracutaneously in a dose of 0.2 ml to a rabbit (albino or gray with a white undercoat or a thoroughly clipped or shaved guinea pig).

A necrotic area 1-2 cm in diameter should be formed at the center of the injection site within 48-72 hr (Fig. 80).

Serological investigation

Serological reactions (the agglutination, complement-fixation, and opsonophagocytic reactions) to some extent aid in confirming the diagnosis in pertussis with an atypical course (antibodies appear in low titres during the 2nd-3rd week of illness) and in establishing a retrospective diagnosis. These serological indices are of very limited value when mass immunization against whooping cough has been carried out.

Blood is taken for examination (with a Pasteur pipette) from a finger, drawing a total of 0.8-1 ml and observing the usual rules of asepsis.

For the complement-fixation reaction the serum is heated for 30 min at 56° (an agglutination reaction can also be set up with the heated serum).

Agglutination reaction. The agglutination reaction is set up with a 2-day culture raised on casein-carbon agar or on Bordet-Gengou's medium. The density of the suspension should be adjusted to 1 billion cells per ml by comparison with a glass pertussis standard. The serum is diluted to titre; the appropriate serum dilution and the bacterial suspension are mixed in equal volumes and the mixture is thoroughly shaken and incubated at 35-37° for 2 hr; it is then left to stand overnight in a refrigerator or at room temperature.

The results are evaluated after 24 hr, designating the intensity of the reaction by plusses (++++, +++, ++, +). The serum titre is assumed to be twice the last dilution yielding ++ agglutination.

Accelerated agglutination is the most convenient method, especially for determining agglutinins in the serum of inoculated children.

Special slides with wells (15 mm in diameter and 1.5 mm deep) or polysterol plates (produced by the "Estonplast" plant, Tyori Street, Tallin) are used for this purpose. The antigen and serum are poured

out in one-drop portions and a bacterial suspension with an approximate density of 20-30 billion cells per ml is prepared. A control consisting of the culture and physiological solution is simultaneously set up (to confirm the absence of spontaneous agglutination). In setting up the reaction the wells should be covered with cover slips. The wells in a polysterol plate are far deeper and cannot be covered (Fig. 79).

In order to accelerate the reaction the plate or slide is shaken or rotated on a special device (rotators or shuttle apparatuses of various types) at a varying amplitude and frequency (from 40 to 200-280 cpm, with an amplitude of approximately 1.8 cm). The agitation time varies from 4-5 to 15 min, depending on conditions. When no agitation device is available this operation is carried out manually.

Fig. 80. Skin reaction in a rabbit. necrosis.

After agitation the plate is transferred to a heater for 1 hr or left to stand for the same period at room temperature and is then evaluated in the usual manner. The results obtained by the accelerated method should be identical to those obtained by the test-tube method.

In addition to live cultures, diagnostic serum (a suspension of bacteria killed by exposure to merthiolate in a concentration of 1:10,000 for 8–10 days, with a density of 20–30 billion cells per ml) can be used as the antigen.

Methylene blue is added to the bacterial suspension to furnish contrast for the reaction. The diagnostic serum is usable for 2 years after preparation.

In setting up the agglutination reaction with the test serum (especially with diagnostic serum) a control (serum known to be positive) must be included in each experiment.

Production of agglutinative serum. Rabbits are immunized in accordance with the following scheme to obtain agglutinative serum.

Cycle I: 5 billion B. pertussis killed with merthiolate (merthiolate concentration in suspension – 1:10,000, exposure time – 7–10 days) or formalin (concentration – 0.1%, exposure time – 22–24 hr) subcutaneously.

2nd and 3rd injections – 5 billion cells of live culture intravenously.

4th and 5th injections – 10 billion cells of live culture intravenously.

Cycle II – 10 days after cycle I: 1st and 2nd injections – 10 billion cells of live culture intravenously.

The intervals between injections are 4–7 days. The animal is bled 6–7 days after the last injection. The titre is usually about 1:50,000 with this immunization scheme. Antiserum to B. pertussis phase I gen-

erally agglutinates B. parapertussis to 1/10 of titre.

A killed bacterial suspension can also be employed for all the injections.

Nutritive Media

Casein-carbon agar, a semisynthetic nutritive medium which does not contain blood, is recommended for cultures. This medium and Bordet-Gengou's medium yield equally good results in isolating B. pertussis from patients, but the former is advantageous for its simpler method of preparation.

Bordet-Gengou's medium milk-blood agar, casein-hydrolyzate blood agar, Bordet-Gengou's medium containing diamidine compounds, etc., require addition of blood and are consequently inconvenient for wide use and have no advantages over casein-carbon agar.

A. Casein-carbon agar. Preparation of casein hydrolyzate. The following ingredients are recommended for preparation of casein hydrolyzate: a) high-grade or first-grade technical ascitic casein (State Standard No. 1211-41), ascitic nutritive casein (TU-153-54; fat content no greater than 1.5-2%), and casein acetate of the Hammerstein type produced by the "Molochnoye" Plant of the Velogda Milk Institute; b) type A) purified activated wood charcoal (State Standard No. 4453-48); c) chemically pure hodrochloric acid with a specific gravity of 1.19, Before hydrolysis tbe casein (of the first two types) is washed in the following manner: one part of casein is mixed with eight parts of 0.2% acetic acid. The solution is changed 3 times during the first day and twice daily for the next 2-3 days. The casein is then washed with distilled water in a metal sieve covered with gauze and adjusted to pH 6.0 (until a yellow tinge is obtained with gamma-dinitrophenol or a blue tinge with bromcresol green). The washed casein is left on the gauze for 2-3 hr (until no more water runs off). It can

then be used to prepare hydrolyzate or is dried at 60-70°, in which form it can be stored for a prolonged period.

The casein is hydrolyzed with concentrated hydrochloric acid under pressure in a glass bottle with a cotton stopper covered with a paper cap (one part of hydrochloric acid is used to 1.25 part of casein).

The mixture is autoclaved for 3 hr at a temperature of 127°. After autoclaving the hydrolyzate is diluted to 1:2 with distilled water and filtered through a linen filter.

The filtrate is then diluted with distilled water taken in a volume equivalent to 10 times the initial weight of the casein (10 liters per kg), poured into glass bottles, and clarified with activated charcoal. For this purpose charcoal is added to the diluted hydrolyzate (20 g per liter) and the mixture is autoclaved at 110° for 10 min and filtered through linen.

The hydrolyzate is a clear liquid with a slightly yellowish tinge. The degree of protein decomposition in the hydrolyzate should be no less than 90%. The final hydrolyzate contains 735-1000 mg-% amine nitrogen, 820-1080 mg-% total nitrogen, and 4.5-6.3% chlorides. It can be stored for several months by adding 0.5% chloroform.

Preparation of yeast dialyzate. Preparation of dialyzate requires fresh pressed baker's yeast, 1 kg of which is mixed with 1 ml of distilled water to produce a homogeneous mass. This suspension is poured into 4 cellophane bags (40-45 cm long and 9 cm wide), 500 ml in each, and the ends of the bags are tied in knots. The bags are thoroughly washed with distilled water before filling and rinsed with running tap water and then distilled water after filling. All the bags are immersed in a covered vessel of a chemically inert material filled with 2 liters of sterile distilled water. They should be completely immersed in the liquid and should not touch one another.

Dialysis is carried out at a temperature of 78–80° for 7 hr. On completion of the process the contents of the pot are poured into a sterile bottle and cooled, chloroform is added, and the dialyzate is stored in this form at a temperature of 5–7° for up to 3 months.

Preparation of casein-carbon agar. Casein-carbon agar is prepared from the following formula, which provides for a total quantity of 1 liter.

Casein hydrolyzate – 150–170 ml (so that the final medium contains 150–170 mg-% of amine nitrogen).

KH_2PO_4 – 0.5 g

$MgCl_2$ – 0.4 g

Soluble starch – 1.5 g

$FeSO_4 \cdot 7H_2O$ – 0.01 g (1 ml of a 1% solution)

$CuSO_4 \cdot 5H_2O$ – 0.005 g (0.5 ml of a 1% solution)

Cystine – 0.03 g (2 ml of a 1.5% solution)

Yeast dialyzate – 50 ml

Agar-agar – 25–30 g

Activated charcoal – 2 g

The hydrolyzate is poured into an enameled or aluminum pot or glass bottle of appropriate capacity, diluted by a factor of 4–5 with sterile distilled water, and neutralized to pH 7.0 with concentrated sodium hydroxide. All the remaining ingredients except the agar-agar and activated charcoal are then added. The starch is preliminarily dissolved in a small quantity of hot distilled water. The pH is again adjusted to 6.8–7.0 (as indicated by bromthymol blue) and sterile distilled water is added to make up the total medium volume. The amine nitrogen and chloride contents are then determined. If the chloride content is less than 0.6% sodium chloride is added to obtain a concentration of 0.9%. The mixture is heated in live steam in an autoclave

for 20 min, until the agar dissolves, and left to stand for 30 min (no more than an hour); the medium is then carefully poured into another vessel (the residue is discarded), the pH is adjusted to 7.3* (as indicated by metanitrophenol) and the activated charcoal is added in the form of a 20% aqueous suspension (which is first autoclaved for 1 hr at a pressure of 1.5 atm, thoroughly stirring it all the while. The medium is then filtered through a 2-layer gauze filter, poured into test-tubes or flasks (in order to prepare Petri dishes), and sterilized for 30 min at 110°. After sterilization it is thoroughly mixed (by rotating the tubes or flasks) until the charcoal is uniformly distributed and poured into dishes. The temperature at which the medium is remelted should not exceed 70°.

The final medium is black in color and the condensation water should contain no charcoal particles. It can be stored for an extended period (a month or longer) if it is kept from drying out.

B. Bordet-Gengou's potato-glycerine agar.

A total of 1 liter of distilled water and 40 ml of chemically pure glycerine are added to 500 g of finely sliced peeled potato (white or sweet). The potato is boiled in water until soft and sufficient distilled water is added to bring the volume up to its initial level; the extract is then filtered (strained) through gauze and left to stand until clarified. To 500 ml of this clear potato extract is added 1.5 liter of a salt solution with the following composition: K_2HPO_4 – 2.25 g, KH_2PO_4 – 0.75 g, KCl – 1.5 g, $MgSO_4$ – 0.075 g, and NaCl – 7.5 g. At this point 60 g of agar-agar (i.e., 3%) is added and the mixture is boiled on an asbestos pad, stirring it until the agar dissolves. The pH is adjusted to 7.1-7.2 and the medium is filtered, poured into appropriate vessels (flasks or test-tubes), and sterilized for 30 min at 110° (0.5 atm) or for 25 min at 120°.

This basic medium can be stored for a prolonged period if it is kept from drying out.

Before use the basic medium is melted and cooled to 45° and sterile defibrinated blood (sheep, rabbit, horse, or human) or the broken-up clots left after the serum has been drawn off are added to produce a concentration of 15-20%.

The medium is thoroughly mixed with the blood and 15 or 20 ml portions are poured into dishes. Several dishes from each batch are placed in a heater for 24 hr as a sterility control. Blood can be used for preparing the medium for no more than 5-6 days after it is taken (it is preferable to use it within the first 72 hr). Well-prepared dishes should be light cherry-red in color and should contain no air bubbles or chunks of agar. The dishes can be used as long as they retain their red color and remain moist (usually 2 to 3 weeks when stored under refrigeration). Addition of 0.25-0.5 units of penicillin per ml of casein-carbon agar or Bordet-Gengou's medium considerably reduces the growth of gram-positive microorganisms on the dishes. Growth of B. pertussis is inhibited by addition of one unit or more of penicillin per ml of medium.

Penicillin in the aforementioned concentration can be applied to the surface of the nutritive medium (in Petri dishes) and rubbed in with a spatula half an hour before inoculation. Penicillin in solution has a reduced activity and it is consequently recommended that a freshly diluted solution be used.

STREPTOCOCCAL INFECTIONS

I.M. Lyampert, Doctor of Medical Sciences

Streptococcus was discovered by Bilroth (1874) in erysipelas and wound infections and by Pasteur (1879) and Ogston (1881) in sepsis and suppurative infections. Fehleisen (1882) obtained a pure strepto-

coccal culture and induced erysipelas in humans.

Streptococcus causes a large number of infections in man and animals. There are nonpathogenic varieties saprophytic in the human mouth and intestines. Attempts to classify Streptococci on the basis of the area from which they were isolated, the length of the chains, and their ability to ferment carbohydrates have proved unsuccessful.

Anaerobic strains are only slightly pathogenic and are usually found in the human mouth and digestive tract. In some cases they produce chronic inflammatory processes (abscesses of the appendix) and wound infections. Of substantially greater importance in the pathogenesis of human streptococcal infections are the facultative anaerobes, which can be divided into 4 types in accordance with the character of the hemolysis which they produce on blood agar: 1) colonies of type β hemolytic Streptococci are surrounded by a clear zone of hemolysis; 2) colonies of type α hemolytic Streptococci are green in color and produce partial hemolysis; 3) type α_1 hemolytic Streptococci produce a less distinct, cloudier zone of hemolysis than the β-hemolytic strains; 4) type γ nonhemolytic Streptococci do not produce hemolysis on solid blood-containing nutritive media.

The β-hemolytic Streptococci are the most pathogenic, causing the majority of streptococcal infections of man and animals. The α-hemolytic Streptococci (green) are less pathogenic. They are found in the oral mucosa of healthy persons and in some cases are detected in chronic sepsis, subacute septic endocarditis, and focal infections of the mouth.

The nonhemolytic Streptococci are saprophytic in the upper respiratory passages and intestinal tract of man. In some cases they cause subacute septic endocarditis, infections of the urinary tract, and wound infections.

Morphology of Streptococci

Streptococci are immobile spherical or oval cocci 0.8-1 micron in diameter, which form chains of varying length and stain positively by Gram's method. Some strains produce capsules.

The length of the chains is related to the culturing conditions. They are longer in liquid nutritive media and are often quite short or fragmentary on solid media. The cocci may be ovoid before division. a process which takes place perpendicular to the chain. Each coccus divides in 2, so that the chain may take on the appearance of a row of diplococci. Counting the number of colonies obtained by culturing on solid nutritive media determines not the number of individual live cells, but the number of chains, since the entire chain participates in colony formation.

Biology of Streptococci; cultural characteristics

On blood-containing agar Streptococci form small (1-2 mm in diameter), translucent grayish or colorless colonies, which do not grow into the agar and are easily removed with a platinum loop. The extent of the zone of hemolysis varies from strain to strain and from group to group. Strains of group A usually form a zone of beta-hemolysis, whose width is somewhat greater than the diameter of the colony. Certain members of group C produce a substantially larger zone of hemolysis. Cultures in which the zone of hemolysis takes the form of a narrow ring around the colony are encountered among the various groups. Type α Streptococci form a greenish, sometimes greenish-brown zone of hemolysis, which is cloudy or clear and varies in size and coloration. In some cases the colony itself takes on a greenish hue.

Strains of group A Streptococci may produce 3 types of colonies:

1. Mucoid colonies. These are large, lustrous, and viscous, resembling drops of water. They are characteristic of freshly isolated

capsule-forming virulent strains.

2. "Matte" (rough) colonies. These are flat and larger than mucoid colonies and have a nonuniform or broken granular surface. They are characteristic of freshly isolated virulent strains containing substance M.

3. "Glossy" (smooth) colonies. These are smaller, lustrous, and round, with a smooth surface and margins. They are characteristic of nonvirulent laboratory cultures.

Streptococci characteristically grow at the bottom in liquid nutritive media, often creeping up the walls of the vessel. The culture breaks up into granules or flakes when agitated. Some strains produce a homogeneous suspension when shaken.

The optimum growth temperature is 37°. Cultures, especially those which have just been isolated, do not grow at all or produce a very sparse growth on media not containing blood or serum. The customary culture media are meat-infusion agar containing 5-7% defibrinated rabbit or sheep blood, semiliquid agar containing serum, and Martin's broth containing 0.2% glucose (pH 7.4-7.6).

Good growth and toxin formation can be ensured by "combined broth" (one part of Poup's peptone and 9 parts of Martin's peptone) or media containing casein hydrolyzate and yeast extract. It is recommended that strains of group A be stored on blood broth (meat-infusion broth containing 5% rabbit erythrocytes). Such strains can be kept on this medium without reculturing for up to 6 months at 5-8° and in sealed medium-containing ampules for more than a year. They can easily be preserved when subjected to lyophilic drying with a sugar-gelatin stabilizer. Strains which produce α-hemolysis grow poorly in liquid nutritive media and cannot be stored in blood broth. Semiliquid agar is recommended for raising and preserving such cultures.

Autoclaving the medium reduces its quality. Fractional sterilization in live steam or sterilization filtration is recommended. Dialyzed media can be used (Stock, 1939).

Hemolytic Streptococci metabolize glucose to form lactic and other acids, this being a factor which limits their growth in nutritive media. Profuse growth can be obtained in a highly buffered glucose-containing medium, in which the lactic acid is neutralized.

Resistance to physical and chemical agents

The maximum and minimum temperatures at which group A streptococci will grow are 42 and 20°.

Hemolytic Streptococci of the various groups usually die when heated at 56° for 30 min. Group D Streptococci can survive heating at 60° for 30 min. Strains of groups B and D will grow on media containing 40% bile, which strains of the other groups will not do. Potassium tellurite in a concentration of 1:2500 suppresses the growth of Streptococci, except those of group D. See page 592for a discussion of the resistance of enterococci (group D streptococci) to physical and chemical agents.

Group A Streptococci may survive for prolonged periods in objects touched by the patient and in dried form in dust. These cultures, however, usually lose their virulence while retainirg their viability (Wannemaker, 1958).

Group A Streptococci are highly sensitive to penicillin, which has a bactericidal action on them, and acquire no resistance to this drug under practical conditions. The Streptococci of other groups, except group D, are also sensitive to penicillin. Sulfanilamides have a bacteriostatic action on group A Streptococci.

Antigenic structure; classification

Serological groups. The current classification of Streptococci is

based on their serological differences (Lensfield). A total of 17 serological groups (A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, R, and S) are known. The division into groups is based on the presence of a specific polysaccharide (substance C) in the members of the different groups.

Group A Streptococci are pathogenic for man, the characteristic representative of this group being Streptococcus pyogenes. Infections of the upper respiratory passages and urogenital tract may also be caused by members of groups C, G, F and B in certain cases. Enterococci (group D) normally inhabit the human intestine. Groups C, G, F and H Streptococci are widely encountered in the mouths of healthy persons. Groups N (lactic-acid), H, D, K and Q Streptococci cause septic endocarditis. Many cultures, especially of strains exhibiting type α hemolysis, cannot be assigned to any of these groups, since they do not contain a group polysaccharide (Str. salivarius, Str. mitis, et al.) and their role in human pathology has been the subject of little research. Certain of them are detected in the blood of persons suffering from septic endocarditis. The various groups of Streptococci not only differ in their ability to cause morbidity in humans and animals and their natural habitats, but also in their biochemical and cultural characteristics. The latter are, however, neither clear nor consistent. Biochemical and cultural characterization is of secondary importance when a strain contains a group polysaccharide. In addition to serological differences, the following indices are usually taken into account in differentiating strains: point of isolation, character of hemolysis, ability to form soluble hemolysins, resistance to various temperatures, ability to grow in milk containing methylene blue, growth at various temperatures and medium pH's and at elevated medium sodium chloride and bile contents, change in litmus milk, decomposition of sodium hippurate

and arganine, NH_4 formation, decrease in medium pH, fermentation of sugars, and liquefaction of gelatin.

Table 57 shows the characteristics of the various groups of Streptococci, as well as of green Streptococci, which have no group polysaccharide.

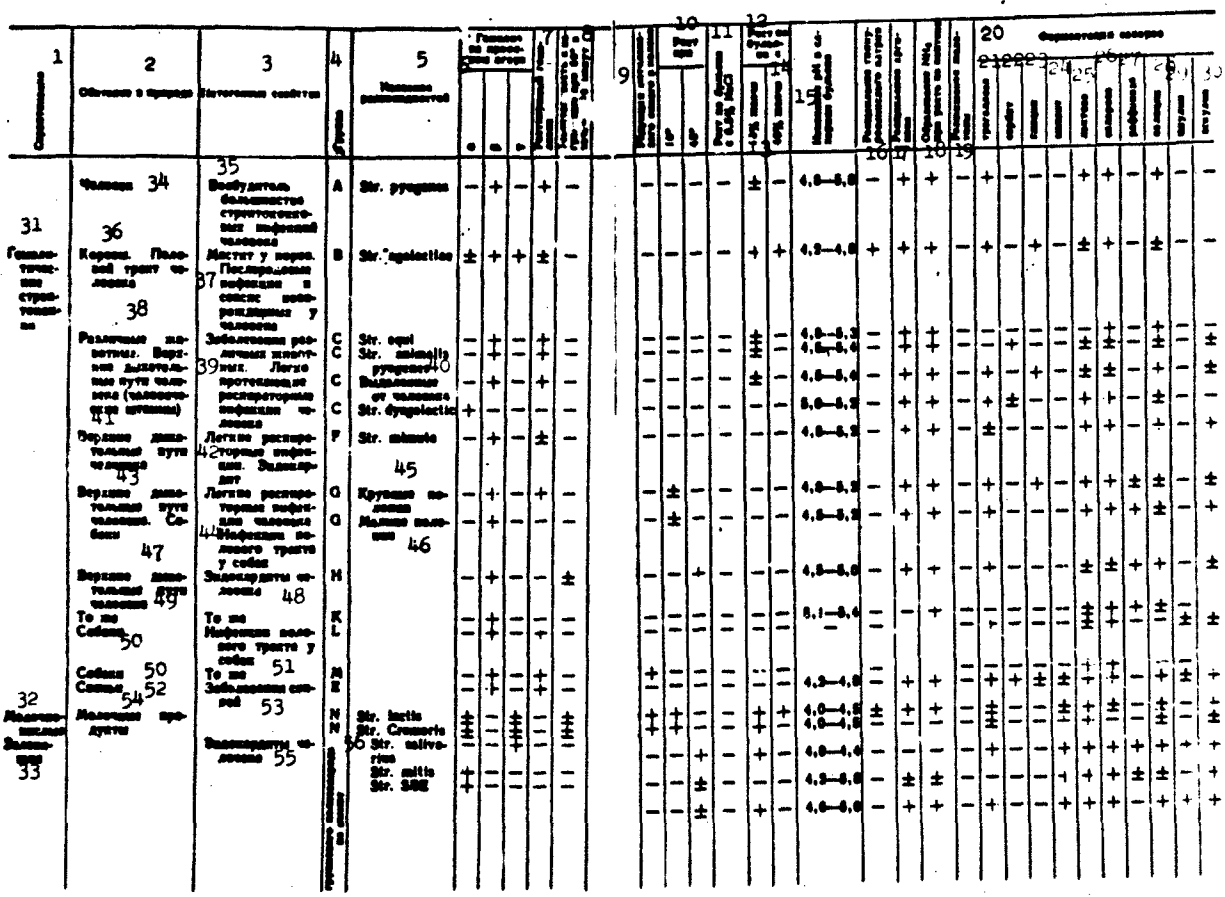
Serological types. Agglutination reactions on slides have made it possible to divide strains of beta-hemolytic Streptococci isolated from patients with scarlet fever and other Streptococcal infections and from healthy carriers into 50 serological types (Griffiths, 1926; Williams, 1925; Wasselhoff and Weinstein, 1945; et al.). Cultures of 46 types belonged to group A, type 7, types 7, 20, and 21 belonged to group C and type 16 to group G. Type differences have also been found among the strains of other groups. Streptococci can be typed only by precipitation reactions with hydrochloric-acid extracts obtained from group A streptococci, using sera from which the group antibodies have been removed by adsorption (Lensfield). Agglutination on slides takes place principally with type antigen T and only partially with the other typospecific substance, substance M, and the corresponding antibodies. The division of Streptococci into types by precipitation reactions with hydrochloric-acid extracts is based solely on differences in substance M (Lensfield and Todd, 1928).

Typing of Streptococci by the precipitation reaction is not possible in all cases, since not all strains contain substance M. Virulent strains freshly isolated from patients with streptococcal infections contain this substance more often and in larger quantities than laboratory strains.

Typing by the agglutination reaction and the precipitation reaction usually gives identical results. Streptococci of the same type are found in different streptococcal infections and in healthy carriers.

TABLE 57

Natural Habitats and Pathogenic, Biochemical and Cultural Characteristics of Streptococci of Different Groups (compiled from the data of various authors)



Note: Certain groups (e.g., group C) contain several varieties with different biochemical properties, which have been described under different names. In addition to the three varieties shown in the table, the green Streptococci, which have no group polysaccharide, contain other varieties (a total of 9). Streptococci of groups O, P, and Q are

not included in the table, since little research has been done on them.
See page for group D streptococci.

1) Streptococci
2) Natural habitat
3) Pathogenic properties
4) Group
5) Variety
6) Hemolysis on blood agar
7) Soluble hemolysin
8) Resistance to heating at 60° for 30 min
9) Reduction of methylene blue in milk
10) Growth at
11) Growth on broth containing 6.5% NaCl
12) Growth on broth containing
13) 10% bile
14) 40% bile
15) Change in pH in sugar broth
16) Decomposition of sodium hippurate
17) Decomposition of arganine
18) NH_4 formation when grown on peptone
19) Liquefaction of gelatin
20) Fermentation of sugars
21) Trehalose
22) Sorbitol
23) Glycine
24) Mannitol
25) Lactose
26) Sucrose
27) Raffinose
28) Salicin
29) Inulin
30) Exulin
31) Hemolytic Streptococci
32) Lactic-acid Streptococci
33) Green Streptococci
34) Man
35) Causative agent of the majority of human streptococcal infections
36) Cattle; genital tract of man
37) Mastitis in cows; postnatal infections and sepsis of human newborns
38) Various animals; upper respiratory passages of man (human strains)
39) Various animal diseases; mild respiratory infections of man
40) Isolated from humans
41) Upper respiratory passages of man
42) Mild respiratory infections; endocarditis
43) Upper respiratory passages of man; dogs;
44) Mild respiratory infections of man; infections of the genital tract in dogs
45) Large colonies
46) Small colonies
47) Upper respiratory passages of man
48) Human endocarditis
49) The same
50) Dogs
51) Infections of the genital tract in dogs
52) Hogs

53) Diseases of hogs
54) Milk products
55) Human endocarditis
56) No group polysaccharide

One, 2, or 3 types (the so-called leading types) usually predominate in scarlet fever. Different leading types may be encountered in different nations and even in different cities of the same nation. These types change with time.

Streptococci of types 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 24, 25, 27, and 29 are most frequently found in scarlet fever and Streptococcal angina in the Soviet Union.

Antigenic structure of Streptococci of group A and other groups. Group A Streptococci have a distinct cell wall, whose principal constituent is group polysaccharide C, or heptene (Lensfield, 1928; Barculis and Jones, 1956; et al.). Antibodies to this substance can be obtained only by immunization with intact cells. The typospecific substances, M and T, are also superficial antigens. Substance M can be obtained together with the group polysaccharide by extraction of intact cells or of cell walls in an acid medium (Lensfield, 1943; Barculis and Jones, 1957; et al.). This substance is a protein and resists heating at 120° for 20 min in acid and neutral media, but is decomposed by trypsin and pepsin. It is the principal virulent factor of group A Streptococci and is present in cultures freshly isolated from patients with streptococcal infections. Substance M is usually entirely lacking or present in only small quantities in laboratory cultures (Lensfield and Perlman, 1952; et al.). It has a leucotoxic action, preventing phagocytosis and reducing leucocyte mobility. Strains containing substance M become resistant to phagocytosis, which explains their ability to grow and multiply in human blood (Macsted, 1956; Foley and Wood, 1959).

Substance M is the principal immunizing component of Streptococcal cells. Antibodies to this substance have a protective effect, while antibodies to other streptococcal antigens do not act in this manner (Rothbart, 1945; Willy and Wilson, 1956; et al.).

Substance M can be detected in the overwhelming majority of streptococeal strains isolated in scarlet fever, especially those of the leading type, which indicates the considerable virulence of such cultures. In rare cases it is observed in strains of rheumatic origin and, exceptionally, in cultures obtained from healthy carriers (I.M. Lyampert and O.I. Vvedenskaya, 1961).

The other type antigen (substance T) is a protein resistant to the action of proteolytic enzymes. It decomposes when heated in an acid medium, so that hydrochloric-acid extracts obtained at high temperatures and containing substances M and S do not contain substance T. This antigen is nonvirulent and antibodies to it do not have protective properties.

Streptococci of group A may have sapsules consisting of hyaluronic acid; these capsules prevent phagocytosis and are consequently one of the factors in the virulence od the with you. Capsules are usually detected in strains freshly isolated from the body, most frequently in young 4-6-hr cultures. Antibodies are not formed to the samples

The cytoplasm of Streptococci contains proteins and a large quantity of nucleotides and nucleic acids, which are the nuclear material of the cell (MacCarthy, 1958). The nucleoproteins of hemolytic Streptococci react with sera obtained by immunization with nonhemolytic Streptococci, as well as with antipneumococcal and, to a lesser extent, antistaphylococcal sera. Little research has been done on these components and their biological action is unknown.

The antigenic structure of the Streptococci of the other groups

has been the subject of little study. Strains of groups B, D, F, and G contain typospecific substances, some which are polysaccharides. Streptococci of group C form capsules, which, like those of group A Streptococci, consist of hyaluronic acid. Strains of group B form capsules consisting of polysaccharides.

Antigenically identical substances have been detected in strains belonging to groups A, C, and G; this is quite interesting, since Streptococci of these groups are detected in human infections more frequently than any other Streptococci not belonging to group A (N.M. Kornilova-Ivanova).

Formation of toxins and other extracellular products

Streptococcal toxin and allergen (thermostable fraction). Streptococcal, scarletinous, or erythrogenic toxin was discovered by Savchenko (1905). Immunization of children with vaccine containing this toxin produce symptoms characteristic of the early period of scarlet fever (Gabricheskiy, 1906).

Dick and Dick (1923,1938) cited a number of data regarding the role of this toxin in the pathogenesis of scarletina: 1) scarletina is produced by administration of toxin for immunogenic purposes; 2) it was established that intracutaneous injection of small doses of toxin causes local erubescence in persons susceptible to scarletina (Dick's reaction); 3) the Dick test is negative in persons immune to scarletina, as a result of neutralization of the toxin by antitoxin.

Native toxin contains two fractions, a dermolabile fraction (the scarletinous toxin, and a relatively thermostable fraction with the properties of an allergin.

The true erythrogenic toxin is a protein (Stock, 1939). This is the streptococcal exotoxin, which causes a scarletinoid eruption and a positive Dick test in subjects susceptible to scarletina.

The erythrogenic toxin is a sympathicotropic poison. Rabbits, which react to it when it is administered subcutaneously (in skin tests) or intravenously, develop a scarletina-like syndrome. Large doses cause the animals to die (I.M. Lyampert, 1956).

Purified erythrogenic toxin is used for skin tests intended to determine the level of antitoxic immunity. The toxin unit is the skin dose (SD), i.e., the least quantity of toxin which, when injected intracutaneously in a dose of 0.1 ml, will cause a positive Dick reaction in no less than 60% of scarletina patients during the first 5 days of illness and in no more than 2-5% of scarletina convalescence beginning with the 20th day of illness (Verzhikovskiy, 1936). The toxin is titrated by skin testing in rabbits, using the standard prepared by the State Control Institute for purposes of comparison. A technique has been proposed for titrating erythrogenic toxin by complement-fixation under refrigeration (M.A. Zelkina, 1953). The majority of strains of group A isolated from scarletina patients, patients with other infections, and carriers produce erythrogenic toxin.

The level of antitoxic immunity can be determined by the Dick reaction (with 1 sd) or by V.I. Ioffe's titration method (skin reactions with different toxin doses).

The level of antitoxic immunity determines susceptibility to scarletina (V.I. Ioffe, 1948; et al.).

The thermostable fraction (allergin) is a nucleoprotein (Verzhikovskiy, 1936). It has been established that sensitivity to this fraction increases throughout the course of scarletina (Ando and Osaki, 1930; Verzhikovskiy et al., 1936; M.G. Danielovich, 1948; V.A. Parfenova, 1947; et al.). Sensitivity (as shown by skin tests) increases in rheumatic fever and other streptococcal diseases. One skin dose of streptococcal allergen is arbitrarily assumed to be the least quantity

which, when injected intracutaneously, will produce a positive reaction 10-15 mm in diameter in 100% of scarletina convalescence. A number of investigators have employed dermal titration with various of allergen (0.1, 0.3, 1 and four sd) to determine sensitivity to this fraction. It has been shown that thermostable-fraction preparations and thermostable fraction in decantates of streptococcal cultures can be titrated by complement fixation under refrigeration with serum from animals immunized with this fraction (I.M. Lyampert, M.N. Smirnova, and O.N. Gryzlova, 1960).

Streptococcal hemolysins and enzymes. In addition to erythrogenic toxin, hemolytic Streptococci produce hemolysins (streptolysin O and S) and enzymes (hyaluronidase, streptokinase, diphosphopyridinenucleotidase, deoxyribonuclease, ribonuclease, proteinase, amylase, and lipase). Some of these substances participate in cellular metabolism, while others are aggressive factors and facilitate invasion, having a toxic action on the macroorganism or suppressing its natural protective mechanisms. A number of them have a leucotoxic action (diphosphopyridinenucleotidase, streptolysin S), which apparently plays a large role in streptococcal infections, since the body's principal protection against Streptococci is phagocytosis.

Streptolysin O is inactivated by atmospheric oxygen. It produces hemolysis within rather than at the surface of blood agar and deep within erythrocyte-containing broth. Reducing agents containing sulfhydryl groups reduce streptolysin O. This hemolysin has antigenic properties and antibodies to it are formed in all infections associated with group A Streptococci. Detection of these antibodies indicates that the patient has had a streptococcal infection (Todd, 1932; V.I. Ioffe, 1948; P.V. Smirnov et al., 1950).

Streptolysin S is not decomposed by atmospheric oxygen. It pro-

duces hemolysis at the surface of blood-containing agar. This hemolysis can be intensified by adding ribonucleic acid to the medium. Streptolysin S has no antigenic properties. The normal streptolysin-S inhibitor, which is detected in the serum of healthy persons, is reduced in titre in the presence of streptococcal infections.

Hyaluronidase. The principal initial propagation factor (Durand-Reynals) of Streptococci is the enzyme hyaluronidase, which hydrolyzes hyaluronic acid, the basic substance of human and animal connective tissue, and increases the permeability of such tissue (Chain and Duty, 1939). Streptococcal hyaluronidase differs antigenically from the hyaluronidase of other bacteria and testicular hyaluronidase.

Methods for detecting hyaluronidase are based either on determination of the decrease in substrate viscosity under its influence (viscosimetric method) or on determination of the loss of the ability of hyaluronic acid to produce insoluble complexes with protein after acidification with acetic acid. The latter reaction can be evaluated from the absence of turbidity (turbidometric method), or clots in a medium containing hyaluronic acid, proteins, and hyaluronidase after acidification with acetic acid. This technique (MacCline's method) is the simplest and most widely used procedure for titrating hyaluronidase and determining antibodies in the blood.

The role of hyaluronidase in the pathogenesis of streptococcal infections is unclear. Its presence in a culture is not an indication of pathogenicity or virulence, since it is found in strains not belonging to group A and in cultures isolated from carriers. According to the data of some authors, hyaluronidase facilitates invasion and exacerbates the course of experimental streptococcal infections. According to those of other authors, hyaluronidase protects against Streptococci, apparently because it breaks down the hyaluronic acid in the

bacterial capsule, so that the Streptococci are more subject to phagocytosis.

Streptokinase. Filtrates of streptococcal cultures lyze fibrin clots (Tillet and Garner, 1933). Plasmin, which is present in human blood in the form of plasminogen, has a fibrinolytic action. Plasminogen is converted to plasmin under the action of a preactivator, which is itself activated by an enzyme, streptokinase (Kaplan, 1946; A.P. Konnikov, 1953).

Streptokinase activates the preactivator of human blood, but does not have a similar effect on that of animal blood. The majority of the strains of group A and certain of those of groups C and G produce streptokinase. This enzyme is a protein with antigenic properties.

Deoxyribonuclease. This enzyme depolymerizes deoxyribonucleic acid (MacCarthy, 1949; Wannemaker, 1958). Group A Streptococci produce 3 different enzymes, which differ serologically and in their optimum pH. All three are antigens. A large percentage of rheumatic fever patients have antibodies to type B deoxyribonuclease. Antibodies to enzymes A and C are rarely encountered. These enzymes have scarcely any toxic effect in streptococcal infections, since they do not affect living cells. They are obviously significant in bacterial nutrition.

Role of group A Streptococci in the pathogenesis of human infections

Group A Streptococci cause a large number of infections of man, which differ both in their clinical course and their epidemiological characteristics.

The diversity of clinical forms results from the fact that there are various points of entry (for example, wound infections and infections of the upper respiratory passages), as well as from the fact that immunity to the erythrogenic toxin may or may not be present. Absence of antitoxic immunity leads to development of scarletina. Persons

immune to the toxin but not to the bacteria develop angina when infected with virulent cultures. Autoantibodies apparently play some role in the number of streptococcal infections with a chronic character (rheumatic fever, nephritis). Development of allergies to the various streptococcal antigens is of great significance in the pathogenesis of streptococcal infections. A previous infection sensitizes the patient to subsequent diseases. The characteristics of the various strains of Streptococci (virulence, toxigenicity, enzymatic properties, typological differences) are apparently of considerably less importance than the state of the body in the development of a given disease.

Laboratory Diagnosis of Streptococcal Infections

Bacteriological investigation

Material collected from the oral or nasal mucosa with a swab is cultured on a Petri dish containing blood agar. The swab is pressed against the agar and the material is then spread over the entire surface with light strokes, using a platinum loop or a Pasteur pipette bent into a spatulate shape. The material should not be rubbed into the agar. The following technique ensures the highest percentage culturability for Streptococci: the swab bearing the material is immersed in a test-tube (to the bottom) containing semiliquid agar immediately after the sample is taken. A total of 2 or 3 drops of defibrinated sheep or rabbit blood are preliminarily introduced at the bottom of the tube. After incubation at 37° for 3-4 hr cultures are made on a dish containing blood agar, using the procedure described above. When Streptococci are present in the mouth characteristic colonies appear on the agar within 24 hr. For microscopic examination an isolated culture is transferred to a liquid nutritive medium (meat-infusion broth containing serum, Martin's broth, etc.) and examined after incubation at 37° for 24 hr. Staining by Gram's method or with Loeffler's methyl-

ene blue is recommended for smears.

The first generations of Streptococci containing α-hemolysin usually grow extremely sparsely in liquid nutritive media. In this connection it is recommended that semiliquid agar containing normal rabbit serum be used for the transplants from the blood agar.

The procedure described above can also be used for investigating pus and discharges from wounds.

In investigating blood a clot or 5-7 ml of whole blood is cultured in 100-150 ml of meat-infusion broth containing serum, Martin's broth, or combined broth* Tarocchi's medium containing semiliquid agar is also recommended, since this creates the most favorable conditions (semianaerobic) for culturing. Blood cultures must be incubated for a protracted period (up to 3-4 weeks) and material should be periodically transferred to dishes containing blood agar during this time.

Strains isolated from the mouth, blood, or wound discharges must be grouped, which permits evaluation of their pathogenicity for man. When no group antigen is present the biochemical characteristics of the culture are studied. Determination of the sensitivity of the strains to antibiotics is important in dealing with Streptococci not belonging to group A. Culturing of blood and determination of the antibiotic sensitivity of the strain isolated are of great practical value in septic endocarditis.

Study of the biochemical characteristic of cultures. When cultured in sterile skim milk containing 2 ml of 1% methylene blue per 100 ml cultures which reduce this dye decolorize the medium within 24 hr. The behavior of Streptococci with respect to bile is studied by culturing them in broth containing 40% or 10% bile. In order to determine the terminal pH cultures are made in broth containing 1% glucose.

Ability to ferment carbohydrates is studied on Hiss's medium, to

which 1% of various carbohydrates is added.

In order to determine hydrolysis of sodium hippurate a culture is raised on broth containing 1% of this compound. After incubation for 4 days 1 ml of decantate is mixed with 0.5 ml of 10% ferric chloride acidified with hydrochloric acid. A precipitate forms when the reaction is positive.

The biochemical properties of the strains of the various groups were described above (see Table 57).

Grouping of strains is the principal method of determining their pathogenicity for man. Grouping is employed in studying cultures isolated from patients and carriers, as well as from milk and milk products. Differentiation of group A Streptococci, which are pathogenic for man, from the other groups is important in practice. Detection of group D Streptococci in studying cultures isolated from the blood of patients with septic endocarditis is significant, since such cultures are not sensitive to penicillin.

In order to obtain group sera rabbits or jackasses are immunized with heat-killed vaccine (heated at 56° for 2 hr).

The bacterial suspension is carefully washed from the nutritive medium.

The complete immunization cycle involves intravenous injection of the vaccine on 4 consecutive days followed by a 3-day interval and 4 more injections, the entire procedure being repeated for 1 month. Serum of sufficiently high titre is usually obtained after 2 cycles. Blood is taken 7-8 days after the last injection. The vaccine doses for rabbits progressively increase from 1 billion to 6 billion cells. Sera in liquid form can be stored for no more than 1½ years.

Various modifications of Lensfield's method are usually employed to obtain extracts containing the group substance. The modification de-

vised by the Institute of Epidemiology and Microbiology imeni Gameleya runs as follows: a 6-hour culture of hemolytic Streptococcus raised on blood broth is transplanted to 25 ml of combined broth containing 0.2% glucose. A 24-hr culture on this medium is centrifuged and the residue is washed with 0.85% sodium chloride and suspended in 0.5 ml of 20 N hydrochloric acid made up in 0.85% sodium chloride solution. The suspension is placed in a boiling-water bath for 15 min and periodically shaken; it is then cooled and the bacteria are removed by centrifuging. A drop of bromthymol blue is added to the decantate and it is neutralized with 1/20 N sodium hydroxide until its color changes from yellow to greenish. The precipitate produced during neutralization is removed by centrifuging. The extracts are tested by precipitation with group sera in test-tubes 2-3 mm in diameter or in capillary tubes. The undiluted extract is overlayered on undiluted sera of different groups. The reaction is evaluated after no more than 15 min. Later evaluation may permit cross-reactions with sera of other groups. The reaction is regarded as positive when a grayish-white ring appears at the boundary between the two layers. The hydrochloric-acid extracts are simultaneously tested with normal rabbit serum as a control. The group sera must be preliminarily tested with extracts prepared from Streptococci of different groups. Both direct tests (with the homologous strain) and cross tests (with strains of other groups) should be conducted, in order to establish whether or not the sera give cross-reactions.

Determination of type of Streptococcus. Determination of the type of Streptococcus at hand is significant in epidemiological analyses conducted to determine the source of an infection.

This determination is made: 1) in an agglutination reaction on slides by Griffiths's method (reaction between antigen T and T antibodies and partially between substance M and M antibodies); 2) in a

precipitation reaction by Lensfield's method (reaction between substance M and M antibodies).

The latter method also permits determination of the virulence of the strain, since presence of substance M in a culture indicates that it is virulent.

a) Agglutination reaction on slides. The following modification of Griffiths's method can be employed for determining the type of a Streptococcus.

The vaccine is prepared and animals immunized to obtain type sera by the same method used to produce group sera. The immunization should however, be more prolonged (3-4 cycles at 2-week intervals). The sera can be stored for more than 3 years in liquid form.

The sera, diluted with 0.85% sodium chloride solution (from 1:50 to 1:1200 or more), are titrated with cultures of the homologous types in agglutination reactions on slides.

The sera are diluted in test-tubes and each dilution is applied to a slide. Sera diluted to one-fourth of the maximum titre are used in typing strains, since they usually give cross-reactions in lower dilutions.

In order to obtain a homogeneous suspension a culture raised on blood broth at 37° for 24 hr is transferred to salt-free meat-infusion broth, incubated at 22° for 48 hr, and centrifuged; the supernatant is discarded and, after thorough shaking, the suspension is used for the agglutination reaction.

Single drops of the various types of sera are mixed with drops of the bacterial suspension on slides. The reaction is evaluated after the slides have been rocked for 15-20 min.

The suspension is tested with nonimmune rabbit serum as a control.

b) Precipitation reaction with type sera (modification of Lens-

field's method employed at the Institute of Epidemiology and Microbiology imeni N.F. Gamaleya, Academy of Medical Sciences USSR).

The sera are prepared by immunizing rabbits with type strains containing substance M. The animals are immunized with heat-killed vaccine for 2 weeks and then with a live culture for 2 weeks. No fewer than 4 immunization cycles should be conducted. The sera are adsorbed on a suspension of type I Streptococci and autoclaved for 1½ hr at 130° (substance C survives autoclaving, while substances M and T are decomposed). A total of 360 billion bacteria is added to 1 ml of serum and the mixture is placed in a heater at 37° for 2 hr, left to stand at 4° for 18-20 hr, and centrifuged to remove the bacterial mass. The adsorbed sera should not contain group antibodies (should not give an agglutination reaction with an autoclaved culture or a precipitation reaction with hydrochloric-acid extracts of cultures of heterologous types).

The sera retain T and M antibodies and consequently agglutinate with live cultures of the homologous type and precipitate with hydrochloric-acid extracts of homologous cultures containing substance M. Precipitation reactions are set up between a hydrochloric-acid extract of the culture to be tested and adsorbed sera of various types. A positive reaction with one of the type sera indicates that substance M is present, since hydrochloric-acid extracts do not contain substance T.

The preparation of the hydrochloric-acid extracts and the set-up and evaluation of the reactions are described on page 753.

Serological investigation

Determination of streptococcal antigens in the patient's body can be employed to detect streptococcal infections of various nosological types.

Determination of streptococcal antigens in blood by the comple-

ment-fixation reaction (CFR) under refrigeration, using serum from a rabbit immunized with a streptococcal culture (V.I. Ioffe), has been employed by a number of investigators in various modifications for studying streptococcal infections such as scarlet fever, angina, rheumatic fever, nephritis, etc. The animals are immunized by the same method used for producing group sera and vaccine from group A Streptococci. The sera should contain a high titre of group antibodies (undiluted serum should give a positive precipitation reaction with a hydrochloric-acid extract of group A Streptococcus diluted to 1:600 or more). It is necessary to dry the serum by the lyophilic method, since it will otherwise become anticomplementary during storage. Its titre is determined by a complement-fixation reaction with a hydrochloric-acid extract prepared from group A Streptococcus in the manner described above. Different serum dilutions are tested with different hydrochloric-acid extract dilutions. The antigens in a patient's blood are determined with immune serum in that dilution at which it reacts with the highest hydrochloric-acid extract dilutions and is still anticomplementary.

In determining antigens the patient's serum, in different dilutions or a single dilution, is tested with rabbit serum in a complement-fixation reaction under refrigeration. The patient's serum (antigen) should be freshly prepared or dried by the lyophilic method. As a control this serum is tested with nonimmune rabbit serum taken in the same dilution as the immune rabbit serum. The complement-fixation reaction is evaluated as soon as hemolysis develops in the control tubes. The reaction is set up under refrigeration with 1, 1.5, and 2 doses of complement or only with 1.5 doses.

Streptococcal antigens in the urine (I.M. Lyampert) are determined by precipitating the patient's urine with a set of antistreptococcal sera (the 16 types most frequently encountered in scarlet fever and

angina in the Soviet Union).

The animals are immunized over a period of no less than 3-4 months, following the scheme described above for production of typospecific sera.

A morning portion of urine clarified by centrifuging is used for the test. When antigens are present a positive precipitation reaction is obtained with one of the type sera when undiluted urine is overlayered on undiluted sera. The reaction is evaluated after incubation at room temperature for 1 hr.

Antigens are detected in the urine of virtually all scarletina patients during the first few days of illness. This method can be used to differentiate scarletina from scarletinous rubella and allergic phenomena (drug rashes). Its principal drawback is the fact that positive reactions occur in streptococcal angina and in a substantial percentage of cases of diphtheria, which is often conjoined with a streptococcal infection.

Determination of antihyaluronidase and anti-O-streptolysin in a patient's blood. Determination of the anti-O-streptolysin and antihyaluronidase content of the blood is employed for diagnosing and evaluating the activity of rheumatic fever. This disease can be more clearly differentiated from other streptococcal infections by the antihyaluronidase level than the anti-O-streptolysin level.

Determination of anti-O-streptolysin and antihyaluronidase can be employed to differentiate rheumatic fever from infectious polyarthritis, septic endocarditis, and cardiac damage caused by various infections, as well as to evaluate the activity of the process. These procedures can be employed for practical purposes by using dry concentrated preparations of O-streptolysin and hyaluronidase (Institute of Epidemiology and Microbiology imeni Gameleya, Academy of Medical Sciences USSR).

1) Method for determination of antihyaluronidase (see instructions for preparation and use of hyaluronidase). Streptococcal hyaluronidase is an enzyme concentrated by precipitation from culture filtrates and freeze-dried; it is titrated by comparison with the antihyaluronidase standard prepared by the State Control Institute. The activity of the preparation is expressed in experimental doses, one experimental dose being the greatest quantity of hyaluronidase which is completely neutralized when mixed with 1 AEHyS of the antihyaluronidase standard prepared by the State Control Institute and which does not prevent coagulation of a working dose of hyaluronic acid acidified with acetic acid. The AEHyS is the unit of the antihyaluronidase standard prepared by the State Control Institute.

Various dilutions of the patient's serum are mixed with one experimental dose of hyaluronidase and one working dose of hyaluronic acid. The latter is prepared from the umbilical cords of newborn infants. The working dose is selected by determining the relative viscosity of the hyaluronic acid in comparison with distilled water (Mogilevskiy).

When the serum contains antihyaluronidase clots form in the test-tubes on acidification with acetic acid. The tube with the least quantity of serum in which the hyaluronic acid coagulates to no less than ++ contains 1 AEHyS.

Method of determining anti-O-streptolysin (see instructions for preparation and use of streptolysin O).

Streptolysin O is a preparation concentrated by precipitation with ammonium sulfate from culture filtrates; it is freeze-dried with a reducing agent.

The preparation is titrated by comparison with a anti-O-streptolysin standard. Its activity is expressed in working doses per ml of preparation, the latter being diluted in accordance with the instruc-

tions on the packing slip.

The working dose is assumed to be the greatest quantity of streptolysin O which is completely neutralized when mixed with ½ AEStO of antistreptolysin standard and which does not hemolyze 0.2 ml of a 5% rabbit-erythrocyte suspension.

The AEStO is the unit of anti-O-streptolysin standard, equaling the international anti-O-streptolysin unit. Various dilutions of the patient's serum in a phosphate buffer solution at pH 6.5-6.7 are mixed with one working dose of streptolysin O and 0.2 ml of a 5% rabbit-erythrocyte suspension. Hemolysis does not occur when the blood contains antistreptolysin. The tube with the least quantity of serum in which marked retardation of hemolysis takes place (+ hemolysis) contains ½ AEStO.

Evaluation of results obtained. An antihyaluronidase titre of up to 300 AEHyS may be detected in essentially healthy persons. A slight (up to 500 AEHyS) or moderate (to 600-700 AEHyS) increase may occur in a number of streptococcal diseases, principally toward the end of the illness and in convalescence.

Active rheumatic fever is characterized by high antihyaluronidase titres (800-1000 or more AEHyS) during the first few days of illness. Persons convalescing from acute streptococcal infections, especially chronic tonsillitis accompanied by high antihyaluronidase levels for several months, must be suspected of having rheumatic fever when other laboratory and clinical indications are present.

An anti-O-streptolysin titre of up to 250 AEStO per ml is observed in essentially healthy persons (the titres are given in international units; one international unit equals two of the units previously used in certain clinics and laboratories).

Elevated anti-O-streptolysin titres indicate that the patient has

had a streptococcal infection. These antibodies reach especially high titres in rheumatic fever and nephritis, two diseases which are characterized by detection of high anti-O-streptolysin titres (500 AESt0 or more) during the first few days of illness, a phenomenon which can be used as a diagnostic aid.

High antihyaluronidase and anti-O-streptolysin levels make it possible to differentiate rheumatic fever from rheumatoid polyarthritis and septic endocarditis, in which these levels are relatively low, and from cardiac damage not associated with streptococcal infections.

Determination of antibodies can be employed for evaluating the activity of the rheumatic process during the period between attacks, in selecting patients for surgery to treat rheumatic heart defects, and for detecting latent rheumatic fever in prophylactic examinations of school children, pregnant women, and other groups.

Determination of C-reactive protein (CRP). CRP appears in the blood in rheumatic fever, other infections, and certain noninfectious diseases (myocardial infarct, malignant tumors, cirrhosis of the liver, etc.). CRP was discovered by Tillet and Francis in the blood of patients with croupous pneumonia, using a precipitation reaction with the somatic pneumococcal C-polysaccharide in the presence of a Ca^{++} salt, whence the name C-reactive protein.

Appearance of CRP in the blood, cerebrospinal fluid, and exudates is an indication that a pathological process associated with inflammation and necrosis of the tissues is taking place in the body. It disappears on recovery. Appearance of CRP in the blood is a nonspecific acute-phase reaction and can be utilized for evaluating the activity of the rheumatic process. CRP is detected during the first few days of illness (the acute period of rheumatic fever) and is not observed during the period between attacks, when no active process is present. At

the end of an attack the CRP normally disappears before the sedimentation rate is normalized. Determination of the CRP level is of especial value in evaluating antirheumatic therapy and in selecting rheumatic-fever patients for surgery to correct cardiac defects.

CRP can be detected by a precipitation reaction with somatic pneumococcal C-polysaccharide or by a capsule-distension reaction with Pneumococci. Determination of CRP with serum from animals (rabbits or sheep) immunized with CRP is the more sensitive reaction.

Method of determining CRP.* CRP antiserum is obtained by immunization of rabbits (the CRP is isolated from serous exudates taken from lymphogranulomatosis patients, treated with alcohol, and precipitated with C_x pneumococcal polysaccharide).

The precipitation reaction with specific serum is set up in capillary tubes (0.4 x 90 mm), which are filled 1/3 full of antiserum and 1/3 full of the patient's serum. The ends of the tubes are gripped in the fingers and they are shaken and placed in a vertical position in a rack (as in determination of the sedimentation rate). A preliminary evaluation of the reaction is made after incubation at 37° for 2 hr and a final evaluation is made after the tubes have stood overnight in a refrigerator.

When CRP is present in the patient's serum a precipitate forms, the amount depending on the quantity of CRP in the serum. A sediment 1 mm deep is evaluated as +, 2 mm deep as ++, 3 mm deep as +++, and 4 mm or more deep as ++++.

The micromethod can be employed for determining CRP by the precipitation reaction. The blood is taken from a finger, the lobe of the ear, or the heel with a capillary tube. The lower end of the tube is sealed by inserting it vertically into wax, paraffin, or plasteline. After the blood has coagulated it is centrifuged and the supernatant

drawn off with a capillary tube. The serum is used for the reaction.

The precipitation reaction with antiserum makes it possible to detect as little as 0.01 mg/ml of CRP. A still more sensitive method is the complement-fixation reaction with specific serum.

CRP can be determined in an agglutination reaction with diagnostic serum, which consists of antiserum adsorbed on latex. When the test serum contains CRP the latex particles agglutinate (Singer, Plotz, et al.).

Evaluation of results obtained. Detection of CRP in the blood in rheumatic fever indicates that the process is active. Rheumatism cannot be differentiated from rheumatoid polyarthritis in this manner, since CRP is also present in the latter case. Disappearance of the CRP from the blood during a rheumatic attack indicates that the process has improved and that antirheumatic therapy has been effective. Prolonged detection of CRP at the end of an attack is a poor pronostic sign, since relapses are possible in such cases. It should be taken into account that the reaction may sometimes be positive in cardiac deficiency accompanied by symptoms of circulatory disturbances and in normal pregnancy.

Determination of rheumatoid factor (RF) (Waaler-Rose's reaction). The Waaler-Rose reaction is of practical value for differential diagnosis of rheumatic fever and infectious polyarthritis. When the reaction is positive the patient's serum agglutinates sheep erythrocytes sensitized with antiserum obtained by immunization with sheep erythrocytes. A positive reaction is observed in 60-95% of all cases of infectious polyarthritis and in 4-5% of all cases of rheumatic fever and other diseases and of healthy persons (Heller, 1952, 1954; Jacobson, 1953; A.I. Nesterov et al.; V.I. [illegible]). Positive reactions are obtained somewhat more frequently in syphilis, hepatic diseases, and

helminthary invasions. Such reactions may also be obtained in the parents of children suffering from rheumatoid arthritis, even when they themselves exhibit no clinical symptoms of the disease.

A positive reaction results from the fact that the blood of patients with infectious polyarthritis contains macroglobulins, which are apparently autoantibodies to normal γ-globulin. The autoantibodies to γ-globulin react with serum γ-globulin adsorbed on erythrocytes when the latter are treated with antiserum containing antibodies to sheep erythrocytes. The substances found in the blood of rheumatoid arthritis patients are referred to as the rheumatoid factor.

Method of setting up the Waaler-Rose reaction. The sheep erythrocytes are stored in Olsver's solution (2.05 g of glucose, 0.8 g of sodium citrate, 0.42 g of sodium chloride, and 0.55 g of citric acid are added to 100 ml of distilled water and the solution is adjusted to pH 6.1 by alkalization with sodium hydroxide and sterilized at 110° for 10 min).

Before use the erythrocytes are washed three times in 0.85% sodium chloride and a 0.5% suspension is made up. The serum for sensitizing the erythrocytes is prepared by immunizing rabbits with sheep erythrocytes. The ability of the serum to agglutinate the erythrocyte suspension is preliminarily determined by testing different dilutions. The maximum serum dilution which clearly causes the erythrocytes to agglutinate is the agglutination titre. The erythrocytes are sensitized at 1/20 of the agglutination titre, i.e., at a serum dilution which will not agglutinate the 0.5% erythrocyte suspension in the absence of serum from rheumatoid arthritis patients.

In sensitizing the erythrocytes equal volumes of serum diluted to 1/20 of titre and of the 0.5% erythrocyte suspension are mixed and the mixture is left to stand at room temperature for 15 min. The test sera

are inactivated at 56° for 30 min and adsorbed on unsensitized sheep erythrocytes. One part of washed erythrocytes is added to 4 parts of serum diluted to 1:4 and the mixture is left to stand at room temperature for 3 hr and centrifuged; the serum is drawn off from the erythrocyte residue. The completeness of adsorption is checked by testing the resultant sera with normal unsensitized sheep erythrocytes.

In order to determine the rheumatoid factor the patient's serum is diluted to 1:10, 1:20, 1:40, etc., after being adsorbed on the erythrocytes. An equal volume of the erythrocyte suspension is added to each serum dilution. When the reaction is positive the erythrocytes agglutinate after being incubated at 37° for 1 hr and standing in a refrigerator for 16–18 hr.

The reaction is usually negative in rheumatic fever. A positive reaction of varying intensity (at dilutions of from 1:10 to 1:1000 or more) is obtained in infectious polyarthritis.

There are modifications of this reaction more convenient for practical use; these employ diagnostic sera consisting of homogeneous suspensions of latex or bentonite particles on which human or rabbit γ-globulin has been adsorbed.

Methods for determination of antitoxic immunity. Antitoxic immunity is determined to detect persons susceptible to scarlet fever and to establish the antitoxic immunity acquired as a result of previous illness or active immunization.

The Dick reaction (with 1 sd of toxin) or V.I. Ioffe's titration method (skin tests with 1/10, 1/3, 1, 4, and 10 sd of toxin) is used to determine antitoxic immunity. The level of this immunity can also be established by titrating the antitoxin in the blood.

Procedure for setting up skin tests. The toxin for skin tests should be highly purified. Toxin heated at 100° for 15 min should give

positive skin tests in no more than 2% of scarletina convalescents, since it contains only a small amount of the thermostable fraction (Verzhukovskiy).

Various doses of purified toxin in 0.1 ml of solution are injected intracutaneously into the underside of the forearm. A separate tuberculin syringe should be used for each toxin dilution (the syringes and needles are boiled for no less than 30 min and the needles are also boiled for no less than 30 min after each injection; before use both syringes and needles are rinsed with the appropriate toxin dilution). The reaction is evaluated 24 hr after the toxin is administered.

TABLE 58

Evaluation of Antitoxic Immunity with the Titration Skin Test (after V.I. Ioffe)

Иммунологическая категория 1	Количество токсина, сд 2	Реакция (+ или −) 3	Оценка результатов 4
I	1/10	+	Высокая чувствительность к токсину 5
II	1/8	+	Повышенная чувствительность к токсину 6
III	1	+	Малая чувствительность к токсину 7
IV	1	−	Антитоксический иммунитет минимальной напряженности 8
V	4	−	Выраженный антитоксический иммунитет (обнаруживается у подавляющего большинства реконвалесцентов после скарлатины) 9
VI	10	−	Иммунитет высокой напряженности (обнаруживается в половине случаев у реконвалесцентов) 10

1) Immunological category; 2) quantity of toxin, sd; 3) reaction (+ or −); 4) evaluation of results; 5) high sensitivity to toxin; 6) elevated sensitivity to toxin; 7) low sensitivity to toxin; 8) minimal antitoxic immunity; 9) pronounced antitoxic immunity (detected in the overwhelming majority of scarletina convalescents); 10) high immunity (detected in half of scarletina convalescents).

Evaluation of results obtained. The reaction is regarded as positive when an erubescence more than 10 mm in diameter develops. Hyperemia less than 2 cm in diameter is evaluated as +, from 2 to 3 cm as ++, and

from 3 to 4 cm as +++. V.I. Ioffe (1938, 1948) and Ittsigson (1940, 1953) suggested the evaluation scheme shown in Table 58.

The incidence of scarlet fever is 0.9% when the reaction at 1 sd is negative, 5.6% when the reaction at 1 sd is positive, and 17 and 23% when the reactions at 1/5 and 1/10 sd respectively are positive.

Method of determining antitoxin in blood. The antitoxin in the blood is titrated on the skin of rabbits of the chinchilla strain weighing more than 3.5 kg and having white skin which develop a pronounced erythema 15-25 mm in diameter when given one skin dose of standard toxin. When the reaction at 1 sd is weak or when a reaction is obtained at 0.1 sd the animal is unsuitable for titration. Each serum is titrated in 2 or 3 rabbits at the same time. The injections are given in the back, 1-1.5 cm from the spinal column, beginning at the upper margin of the scapula and ending at the upper margin of the pelvis. Titration is carried out with respect to 1 sd of standard scarletina-streptococcal toxin prepared by the State Control Institute by comparison with the standard serum produced by this institute. One unit of standard serum neutralizes 100 sd of standard scarletina toxin.

The test sera are diluted to 1:5, 1:10, 1:20, etc., with 0.85% sodium chloride. The standard serum is diluted to a content of 1/100 unit in 0.05 ml. The diluted standard toxin should contain 1 sd in 0.05 ml. The diluted test sera and standard serum are mixed with equal volumes of the diluted standard toxin. The following mixtures are prepared as controls: 1) diluted toxin and an equal volume of 0.85% sodium chloride; 2) the lowest test-serum dilution and an equal volume of 0.85% sodium chloride; 3) diluted toxin and an equal volume of diluted standard serum. The mixtures are incubated at 37° for 1 hr and administered intracutaneously to the rabbits. The reaction is evaluated after 24 hr. Since 1/100 unit 1 sd, the greatest serum dilution which

neutralizes the standard toxin should contain no less than 1/100 unit. The serum dilution is determined by establishing the number of units per ml of undiluted serum. The reaction cannot be evaluated when there is no reaction with the standard toxin (control No. 1), when erubescence develops as a result of administration of the test serum without toxin (control No. 2), and when 1 sd of standard toxin is not neutralized by 0.01 unit of standard serum (control No. 3).

Evaluation of results obtained. Stable antitoxic immunity is characterized by a content of 1-2 units per cm^3 of serum, a content of 5 units or more indicating a high immunity (I.I. Levin and V.I. Ioffe).

Use of skin reactions with toxin and allergen for diagnosing scarletina. Certain authors determine the increase in antitoxic immunity and sensitivity to the thermostable allergen fraction in diagnosing latent and ambiguous cases of scarletina (V.I. Ioffe, 1948). The patient is given 1/3 and 1 skin dose of toxin intracutaneously during the first few days of illness and 1, 4, and 10 sd at later times (larger doses should be used at the beginning for older children). The thermostable fraction is injected intracutaneously at the same time.

Scarletina is characterized by negative skin tests to increasing toxin doses and by an increase in allergen sensitivity.

DISEASES CAUSED BY CAPSULAR BACTERIA

Prof. B.Ya. El'bert, Honored Scientist of the BSSR

The genus Klebsiella (family Enterobacteriaceae) includes a broad group of immobile gram-negative bacilli, which usually have capsules, do not form spores, and produce a mucoid layer on nutritive media. The principal species of this genus are Frisch and Volkovich's Bacillus scleromatis (rhinoscleromatis), or Klebsiella scleromatis (rhinoscleromatis), Abel and Lewenberg's Bacillus ozaenae, or Klebsiella ozaenae, and Friedlander's bacillus, or Klebsiella pneumoniae; Es-

cherich's gas-forming capsular bacillus Aërobacter aërogenes, or Klebsiella aërogenes, El'bert's nonfermenting capsular bacillus, or Klebsiella anfermentans (possibly identical to Plen's Bacterium ciprinicida), and the capsular bacilli described by Dimoch and Edwards (Klebsiella genitalium [synonymous with Encapsulatus genitalium]) and by Ballas, Thomas, and Cane (Klebsiella paralytica) have been described but not included in the international classification.

Capsular bacteria are found in the nasal and oral mucous of patients with scleroma and ozena, in the respiratory excreta and pulmonary tissues of patients with Friedlander's pneumonia, in the urine in infections of the urinary tract, in human excrement, and in environmental objects (on the leaves of plants, in soil and water samples, etc.).

Morphology of capsular bacteria

Bacteria of the capsular group are short rods with rounded ends 2-3 μ long and 0.5-1 μ wide, occurring singly and often in pairs. They have no flagella and do not form spores. The mucoid form is enclosed in a broad oval or circular capsule, which is easily visible in ink preparations (Fig. 81). Various techniques have been proposed for staining this capsule (see page 52).

Fig. 81. Mucoid forms of K. scleromatis. Ink preparation with supplemental staining.

1. Smears are air-dried but not fixed, stained with 1% aqueous crystal violet under refrigeration, rinsed with 20% aqueous copper sulfate, and dried with filter paper.

2. A drop of india ink is applied to a slide with a loop and mixed with an equal quantity of water; a small amount of an agar or broth culture is introduced into the resultant liquid with a plati-

num loop and the two are thoroughly mixed. A smear is prepared with a polished slide, following the procedure used for blood smears. After air-drying the smear is fixed in methyl alcohol for 3 min or in concentrated mercuric chloride for 1 min and stained with carbol thionine or carbol fuchsin for 5-10 min. The capsule is clearly defined in such an ink preparation, the bacterial soma being stained.

Since the capsular substance has a weak affinity for solutions of analine dyes, which are easily washed off,the capsule is stained by treating the preparation with ethyl or methyl alcohol mixed with acetic acid and salts of certain heavy metals.

Biology of capsular bacteria; cultural characteristics

Capsular bacteria grow and multiply well on simple nutritive media. They utilize ammonium salts, glucose, or lactic acid as sources of the nitrogen and carbon necessary for protein synthesis. Strains of K. scleromatis assimilate glucose, levulose, galactose, maltose, mannitol, sucrose, raffinose, arabinose, mannose, starch, dextrin, inositol, and salycin, as well as certain organic salts (sodium citrate, levo and dextro sodium succinate, and sodium mucate) in synthetic media under aerobic conditions (El'bert).

In assimilating the aforementioned substances K. scleromatis and K. ozenae form acids but no gaseous metabolic products, in contrast to the majority of strains of Friedlander's bacillus, which form both acids and gases. Of all the capsular bacteria only K. scleromatis forms a large quantity of alkali, raising the medium pH to 7.7, or leaves the reaction of the medium unchanged when raised in peptone water containing 1% lactose. All the other species of Klebsiella, except K. anfermentans, acidify peptone water containing lactose to pH 6.35 or less. In determining the enzymatic activity of Klebsiella, the investigator can limit himself to three carbohydrates - lactose, glucose, and su-

crose (Table 59).

The biochemical criteria for assigning a strain of capsular bac-

TABLE 59

Enzymatic Properties of Bacteria of the Genus Klebsiella

Вид 1	Лактоза 2	Глюкоза 3	Сахароза 4	5 Примечание
Палочка склеромы 6	К— Г— 10 11	К+ Г—	К+ Г—	Кислота образуется лишь на 3—4-е сутки или позднее 12
Палочка озены 7	К+ Г—	К+ Г—	К+ Г—	
Диплобацилла Фридлендера 8	К+ Г+[1]	К+ К+[1]	К+ К+[1]	
Aerobacter. aërogenes	К+ Г+	К+ Г+	К+ Г+	
Неферментирующая палочка 9	К— Г—	К— Г—	К— Г—	

*Also K+G-.

1)Species; 2) lactose; 3) glucose; 4) sucrose; 5) notes; 6) K. scleromatis; 7) K. ozaenae; 8) Friedlander's bacillus; 9) K. anfermentans; 10) K; 11) G; 12) acid is formed only after 3-4 or more days.

terium to the species K. scleromatis are: 1) does not decompose lactose; 2) usually decomposes all carbohydrates and alcohols except erythritol, dulcitol, inulin, and melezitose to form a acid but no gas; 3) does not decompose amygdaline, salts of tartaric acid, or sodium citrate; 4) decomposes sucrose to form an acid after 3-4 or more days. Fermentation of sugars must be observed over a period of seven days, noting the results daily.

K. scleromatis and other capsular bacteria do not decompose tryptophan and do not form indole.

On solid nutritive media with a neutral or slightly alkaline reaction capsular bacteria product typical viscous convex colonies, which often merge to form a continuous mucoid layer with a mother-of-pearl lustre. Clear, round nonmucoid colonies with a bluish tint,

i.e., noncapsular (S) and rough (R) colonies, are sometimes found among the mucoid (M) colonies. Formation of nonmucoid S colonies can be observed on microscopic examination of young colonies 3-4 hr after culturing on Petri dishes containing meat-infusion agar, as well as macroscopically after exposure of a mucoid culture to specific bacteriophage, immune serum, antibiotics (gramicidin), or bovine bile. Figure 82 shows the character of young colonies of K. scleromatis. Microscopic examination of young colonies on meat-infusion agar makes it possible to establish whether a capsular bacterium belongs to one of the species of the genus Klebsiella. This method is based on the characteristics of the division of the bacteria during reproduction. K. scleromatis is characterized by young colonies with a concentric form, K. ozaenae by a diffuse-concentric form, Friedlander's bacillus by a loop-like form, Aerobacter aerogenes by a terrace-like form, K. anfermentans by a stellate form, Mucosus vulgaris, a variant of K. scleromatis, by a stellate-concentric form.

Young colonies can be isolated with a special needle for production of pure cultures.

As a result of their domed shape and considerable mucus production mature colonies of capsular bacteria cannot be subjected to thorough microscopic examination. At the same time, the architectonic characteristics of flat young aggregates of capsular bacteria are rather easily distinguished. For this purpose a small drop of a 24-hr broth culture is spread over the surface of meat-infusion agar in a Petri dish with a Pasteur pipette bent at an obtuse angle. The dish is then incubated in a heater at 37° for 2-3 hr and left to stand overnight at room temperature. A rectangular strip of agar is cut from that in the dish with a flamed lancet and placed on a slide. A 50-100-candlepower electric bulb is used as the light source for examination, stopping

down the condenser diaphragm. The preparation is inspected first with a low-power objective and then with a 40-power.

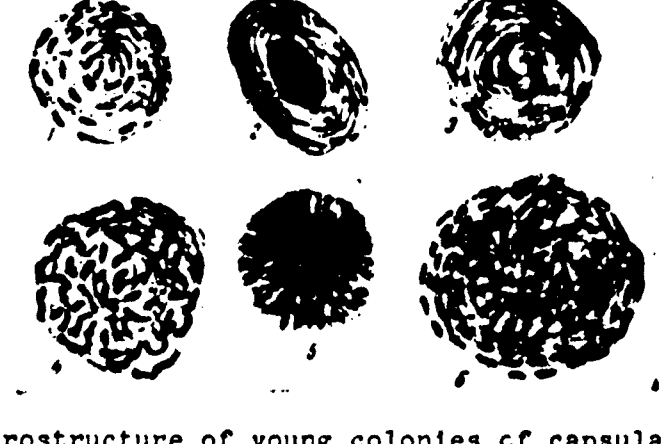


Fig. 82. Microstructure of young colonies of capsular bacteria on meat-infusion agar. 1) Concentric (K. scleromatis); 2) terrace-like (Aerobacter aerogenes); 3) diffuse-concentric (K. ozaenae); 4) loop-like (K. pneumoniae); 5) stellate (K. anfermentans); 6) radial-concentric (K. scleromatis, variant Mucosus vulgaris)(after V.Ya. El'-bert).

Under natural conditions the only source of infectious scleroma is apparently scleroma patients in prolonged and intimate contact with healthy persons. At the same time, carriers of K. scleromatis are not observed among healthy individuals, even those in close contact with patients, although this bacterium is detected in cultures of the nasal and pharyngeal mucus in 95% of patients. Thus, even though it is the causative agent of the pathological process in question, the virulence of this bacterium is very low. It is possible that development of the process requires the individual's reactivity to meet certain as yet undetermined conditions associated with a deficit of certain factors (trace elements, vitamins, etc.).

It has been suggested that K. scleromatis only breaks the ground for the true causative agent, which has not as yet been discovered. The fact that scleroma is not clearly contagious has given rise to the

view that the bacterium detected in the mucus and tissues of the upper respiratory passages is unable to produce morbidity in man, being a nonpathogenic variant of K. scleromatis.

Resistance to physical and chemical agents

K. scleromatis is the least resistant of the capsular bacteria. An important differential-diagnostic characteristic of the microbe

TABLE 60

Antigenic Structure of Bacteria of the Genus Klebsiella (after Kaufman)

O-группы 1	2 Антигены O	K		3 Штаммы
1	1	1		A=4208, A=5054, 380
	1	2		B=5055, 243, 412
	1	3		C=5056, C=10273, 915
	1	7		AER=4140
	1	8		N=1015 и др. 4
	1	10		N=919 и др.
	1	12		N=313
2A	2a	2		B=4209
	2a, 2b	2		B=7380, 4631, 1360
	2a	3		Rhin. 5046 и др.
	2a	8		N=1126
2B	2a, 2c, 2d	4	Ozaena	D=5050
	2a, 2c	5?	Ozaena	E=5051
	2a, 2c	6	Ozaena	F=5052
3	3	11		N=390, 674
		9		N=55 и др.
		13		N=1470, 1995
				N=1193

1) O-groups; 2) antigens; 3) strains; 4) etc.

is its inability to withstand the action of bovine bile, which is in contrast to the other species of the genus Klebsiella. K. scleromatis is differentiated from other capsular bacteria by inoculating agar mixed half-and-half with bile with a drop of a broth culture; K. scleromatis does not grow on this medium, while other capsular bacteria produce a luxuriant growth (El'bert).

Citral has a bactericidal action on K. scleromatis in a dilution of 1:5000, on Friedlander's bacillus in a dilution of 1:3000, and on K. ozaenae and Aerobacter aerogenes in a dilution of 1:1000.

K. scleromatis does not grow when 0.1 ml of broth containing 1 million cells is cultured on an agar slant with a citral concentration of 1:10,000. The other bacteria of the capsular group grow well under these conditions. The growth of capsular bacteria in broth is arrested after 18 hr at the following citral concentrations: K. scleromatis 1:40,000, Friedlander's bacillus 1:10,000, K. ozaenae 1:20,000, and Aerobacter aerogenes 1:3000. Citral thus has a bacteriostatic and bactericidal action on capsular bacteria, its most pronounced effect being on K. scleromatis (D.Ye. Zebitsker).

In test-tube experiments, streptomycin has a bacteriostatic action at concentrations of 2-4 units per ml of medium. The effect of this drug is most marked during the first hour after culturing, during the logarithmic phase of reproduction (3 hr after culturing), and during the period of maximum stationary growth (after 48 hr).

Intramuscular injection of 300-1400 units of streptomycin 16 hr after intranasal inoculation of a mouse with K. scleromatis prevents death. Later injection of 500 units of streptomycin has a weak therapeutic effect (Ye.A. Izraitel').

In addition to streptomycin, biomycin, aureomycin, and mycostatin have an antibiotic effect.

Even at very high concentrations, penicillin does not retard the growth of K. scleromatis and has no therapeutic effect. Gramicidin in a concentration of 12.5-25 micrograms per ml and sanasine in a concentration of 25-50 micrograms per ml have a bactericidal action on K. scleromatis. Prodigiosine has a similar effect.

Mercuric chloride diluted 1:400,000 or chloramine diluted to

1:5000 kills K. scleromatis within 3 hr, while phenol diluted to 1:500 requires 24 hr.

Heating capsular bacteria to 67-70° in aqueous suspension causes them to die within 1 hr.

Antigenic structure; serotypes

Capsular bacteria are distinguished by a considerable diversity of antigenic structure, which is associated with the characteristics of the somatic (S and R) and capsular (K) antigens. More than 60 capsular types have now been found (Kaufman, Edwards, et al.) and this is apparently not the final count. One of the O-antigens (2) has partial antigens (a, b, c) and each of the 5 O-antigens often is associated with many capsular antigens. K. scleromatis is serologically homogeneous and all its strains belong to the same antigenic group [O(2a) : : K(3) according to Kaufman's formula]. There are several serotypes of K. ozaenae (K-4, 5 and 6) and many serotypes of Friedlander's bacillus, which has numerous capsular antigens (Table 60).

All the foregoing forces us to conclude that only utilization of a complex investigative technique, incorporating morphological, cultural, biochemical, and antigenic data on the bacterium under investigation, permits us to assign a given strain to a definite species or group within the genus Klebsiella.

Laboratory Diagnosis of Scleroma

Laboratory diagnosis of scleroma is based on pathohistological, cytological, bacteriological, and serological methods.

Microscopic investigation

Pathohistological investigation makes it possible to detect the Mikulicz's hydropic cells, hyaline globules, and plasma cells typical of scleromatous granulomas in sections and prints of infiltrations.

Bacteriological investigation

Diagnosis of Scleroma is based on detection of K. scleromatis in the nasal, oral, tracheal, laryngeal, and bronchial mucous and in biopsy specimens of infiltrations and on isolation and study of a pure culture in accordance with the investigative scheme which has been developed (B.Ya. El'bert).

The principal stages in the analysis are:

1) taking of samples for examination;

2) culturing on 2 or 3 Petri dishes containing weakly alkaline meat-infusion agar (pH 7.2) or glycerine agar;

3) isolation of colonies on agar slants and production of a pure culture;

4) investigation of young colonies by agar microscopy to determine the type of structure present;

5) culturing in a color series (lactose, glucose, and sucrose) to determine the type of fermentation;

6) study of the sensitivity of the bacteria when cultured on agar mixed half and half with bovine bile;

7) investigation for indol formation;

8) determination of serological properties with antiserum 1 by the complement-fixation reaction.

The following are supplemental methods:

9) determination of culture virulence in white mice;

10) isolation of a nonmucoid variant by culturing in Petri dishes and setting-up of an agglutination reaction between the noncapsular culture and antiserum 1;

11) study of the sensitivity of the bacteria to the lyzing action of specific bacteriophage;

12) investigation of the patient's serum by a complement-fixation

reaction with the culture isolated and with laboratory antigen and by an agglutination reaction with the nonmucoid form;

13) determination of the pH of peptone water in which a culture of the bacterium under investigation was grown;

14) reaction with acetylmethylcarbonal (Foges,Proskauer) and with methyl red.

In bacteriological investigation of infiltrate fragments the material is pulverized with a forceps and immersed in a test-tube containing meat-infusion broth. The culture is incubated at 37° for 24 hr, material is transplanted to a Petri dish containing neutral agar, and a pure culture is isolated and identified by the method described above.

Bacteriophage. K. scleromatis bacteriophage is especially easy to detect during the summer, immediately after filtration of a suspension of K. scleromatis from agar or broth cultures.

Scleroma bacteriophage was isolated from old broth cultures by V.M. Gerkes and from agar cultures by N.A. Izraitel'. This bacteriophage is able to lyze cultures of the bacteria most sensitive to it at titres of 10^{-7}-10^{-8}. Complete lysis of young 5-hr broth cultures is observed after 7-8 hr.

Since scleroma bacteriophage is strictly specific, lysis is employed in the bacteriological diagnosis of scleroma. Agar in Petri dishes is inoculated with mucoid cultures isolated from the nasal mucous; after incubation at 37° for 2 hr scleroma phage is applied to the growth and the dishes are left to stand overnight in the incubator. Presence of sterile areas indicates that the cultures consist of K. scleromatis. Other capsular bacteria are not lyzed by scleroma phage.

Serological investigation

Serological diagnosis of scleroma is based on investigation of the patient's serum by a complement-fixation reaction with antigen prepared from a mucoid culture of K. scleromatis and by an agglutination

TABLE 61

1 Гомологичные сыворотки	2 Культуры Klebsiella											
	3 палочка склеромы						4	5 палочка Фридлендера		Aerobacter aerogenes		
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
I	3200	3200	3200	3200	3200	3200	3200	—	—	—	—	—
II	.	3200	3200	3200	3200	.	3200	—	—	—	—	—
III	3200	3200	3200	3200	.	3200	3200	—	—	—	—	—
IV	3200	3200	3200	3200	3200	3200	3200	—	—	—	—	—
V	3200	3200	3200	3200	.	3200	3200	—	—	—	—	—
VI	3200	.	3200	3200	3200	3200	3200	—	—	—	—	—
VII	3200	3200	3200	3200	3200	3200	3200	—	—	—	—	—
VIII	—	—	200	—	—	—	—	3200	3200	—	—	—
IX	200	—	200	—	200	—	—	3200	3200	—	—	—
X	—	—	—	—	200	—	—	—	—	3200	3200	3200
XI	—	—	200	—	200	200	—	—	—	3200	3200	3200
XII	—	—	—	—	—	—	200	—	—	3200	3200	3200

Note: The figures represent the maximum serum dilution at which a positive agglutination reaction is noted.

1) Homologous sera; 2) Klebsiella cultures; 3) K. scleromatis; 4) in noncapsular form; 5) Friedlander's bacillus.

reaction with antigen prepared from a nonmucoid culture of this same bacterium.

1. The complement-fixation reaction is sensitive and strictly specific in scleroma. It agrees with the clinical data in 95% of all cases.

The antigen is a day-old suspension of K. scleromatis heated at 60° for 1 hr and brought to a concentration of 500 million cells per ml by comparison with a standard; previously prepared scleroma diagnostic serum for complete K. scleromatis antigen can also be used.

When freshly prepared polyvalent bacterial antigen is used it is

necessary to inoculate agar slants with 3 or 4 strains of K. scleromatis selected for this purpose on the day before the experiment; the day-old growth is washed off, brought to a concentration of 500 million cells per ml with physiological solution, by comparison with a bacterial standard, and heated at 60° for 1 hr. The procedure for setting up the complement-fixation reaction is described on page 154 .

Agglutination reaction. The serum of scleroma patients contains antibodies to the somatic O antigen, which makes it possible to employ an agglutination reaction, using a suspension of noncapsular bacteria as the antigen. The specificity of the O antigens of K. scleromatis is quite evident in Table 61.

The antigen consists of nonmucoid K. scleromatis. It must be kept in mind that nonmucoid capsular bacteria are divided into: 1) smooth forms capable of agglutination in immune sera; 2) rough forms corresponding to the R forms of other bacteria. The smooth forms are divided into two variaties: alpha forms, which are characterized by growth of translucent colonies in dishes, and beta forms, which produce clear colonies. Highly agglutinable smooth, nonmucoid strains of the clear form of K. scleromatis are the most suitable for the agglutination reaction. Either a suspension of live noncapsular bacteria or a formalinized diagnostic serum can be used.

A titre of 1:1600 is assumed to be diagnostic. A reaction at this titre is evaluated as weakly positive, while those at higher titres, beginning with 1:3200, are evaluated as positive (S.I. Gladyshevskaya).

In some cases the serum of healthy persons or of persons suffering from other infections gives an agglutination reaction with scleroma antigen at titres of 1:200-1:800. A positive agglutination reaction is obtained in approximately 85% of scleroma patients and a weakly positive reaction in 10%.

Opsonophagocytic reaction. Serum from scleroma patients substantially intensifies the phagocytosis of K. scleromatis. The opsonophagocytic test is a valuable method of diagnosing scleroma, supplementing the complement-fixation and agglutination reactions. It can also be employed to determine the dynamics of the process, since it is impossible to evaluate the course of the infection from the complement-fixation reaction, whose intensity remains almost unchanged over periods of many years.

The opsonophagocytic reaction is regarded as sharply positive in scleroma if the minimum phagocytosis index is 70 or more and the opsonophagocytic index is at least 12. It is considered positive when the minimum phagocytosis index is 25-70 and the opsonophagocytic index at least 9.5. Minimum phagocytosis indices of from 10 to 15 and opsonophagocytic indices of 9 indicate a weakly positive result.

A precipitation reaction can be employed in investigating infiltrates and oral mucous from scleroma patients (the material is treated by Buaven's method to obtain a hapten and the latter is then overlayered on rabbit antiscleroma serum in a test-tube; B.L. Botbinik).

It is also recommended that the culture isolated be tested for virulence. Intramuscular administration of 0.5 ml of a suspended day-old agar culture (with a density of 1 billion cells per ml) to a white moust almost without exception causes the animal to die and the bacteria are found to have spread to all its organs and lymph nodes.

Laboratory diagnosis of diseases caused by Friedlander's bacillus, detection of K. ozaenae in ozena, and investigations for capsular bacteria in urine (especially when neoplasms of the bladder or various inflammatory processes are present), the intestinal contents, the environment, etc., are based solely on data obtained by bacteriological analysis (culturing on meat-infusion agar with subsequent differentia-

tion and description of the morphological, cultural, biochemical, and antigenic properties of the microorganisms isolated).

EPIDEMIC MENINGITIS

Prof. P.V. Pavlov

The generally accepted classification assigns the causative agent of epidemic human meningitis, Meningococcus (Neisseria meningitis), to the genus Neiseria of the family Neiseriaceae.

Fig. 83. Meningococci. Two-day culture on serum agar.

Meningococci may be detected in the nasopharynx and sometimes cause rhinopharyngitis. They merit special attention in human pathology as the causative agent of meningeal inflammations (cerebrospinal meningitis) and occasionally of sepsis.

Morphology of Meningococcus

Meningococci are spherical gram-negative cells 0.6-0.8 microns in diameter and occur in pairs. This paired arrangement can be seen quite clearly in examining smears prepared from the cerebrospinal fluid. They often occur intracellularly in this fluid and resemble coffee grounds. Substantial numbers may be seen extracellularly in some cases. Meningococci in smears prepared from cultures are arrayed

in pairs and occasionally in tetrads. A characteristic feature of these bacteria is their polymorphism. They appear as small spherical cells in smears from young cultures (Fig. 83); in addition to these small spherical cells, a substantial number of giant cells may be observed in old (3-5 day) cultures.

Biology of Meningococci; cultural characteristics

Meningococci are aerobic; their biochemical activity is relatively weak and, of the many carbohydrates,they decompose only glucose and naltose to form acids. The optimum temperature for good growth is 35-37°, while the optimum pH is 7.4-7.6. Meningococci require nutritive media containing native human or animal proteins, especially during the first few generations. As a rule, these bacteria are cultured on solid (agar-agar) nutritive media containing ascitic fluid, human or rabbit blood, or hydrocele fluid. Meningococci grow well on nutritive media such as Loeffler's medium and various egg media. Dorset's egg medium (see page 791) is quite often used for isolating and culturing Meningococci.

Fig. 84. Meningococcus colonies on serum agar. Day-old culture.

Fig. 85. Meningococcus colonies on serum agar. Seven-day culture.

Meningococci grown on ascitic agar or agar containing formalinized

serum (see page 792) form small, slightly convex, bluish semitransparent colonies with uniform margins (Fig. 84). When the cultures are incubated for 2-5 days the colonies increase in size, become less transparent, and acquire uneven margins (Fig. 85).

Resistance to physical and chemical agents

Meningococci have a very low resistance to various physical and chemical agents. Just as Gonococci, Meningococci die within 5 min or less at a temperature of 55°. They are more resistant to low temperatures (0-2°), although also dying within a short time. A 1% phenol solution or 0.1% mercuric chloride solution kills these bacteria within 1-2 min. Just as Gonococci, Meningococci are sensitive to sulfanilamides and, to a somewhat lesser extent, to penicillin and streptomycin.

Antigenic structure; serotypes

According to the French classification proposed by Nicol, Meningococci are divided into types A, B, C, and D. This classification also includes parameningoccccal strains. The English classification (Gorion and Murray)divides the Meningococci into 4 serological types. It has been established that type A of the French classification corresponds to types I and III of the English classification and that type B of the former corresponds to types II and IV of the latter.

Types C and D are parameningococcal strains.

Two types of Meningococci, A and B, are usually encountered in the Soviet Union. Type C is isolated in extremely rare cases.

Laboratory Diagnosis of Meningococcal Infections

Material for examination; taking of samples

Cerebrospinal fluid, fluid from the lateral ventricles (in infants), blood, nasopharyngeal mucous, tissue fluid, and roseolae from patients with epidemic cerebrospinal meningitis can be examined for Meningococci.

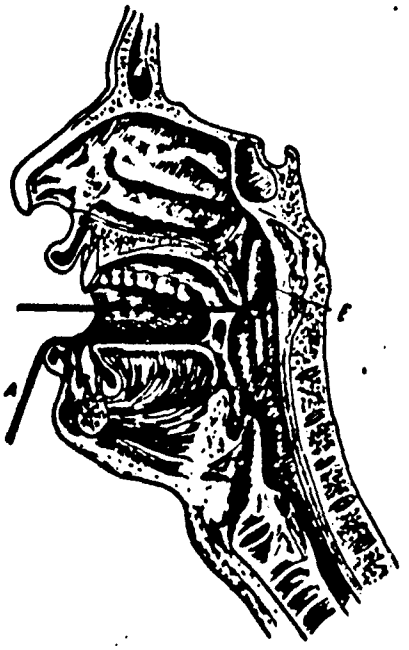


Fig. 86. Taking mucous from the nasopharynx for examination for Meningococci. A) Tongue depressor; E) swab for collecting material.

In examining persons who have died of Meningococcal infections pus from the meninges and material from the inflammatory foci in the organs are investigated.

In conducting a bacteriological examination for Meningococci it must be remembered that, as a result of the wide use of sulfanilamide drugs for treating cerebrospinal meningitis, the culturability of these bacteria may be greatly reduced.

For more successful bacteriological isolation of Meningococci it is recommended that a solution of paraaminobenzoic acid, which is antagonistic to sulfanilamides and blocks their action, be added to the nutritive medium used for culturing the material.

Mucous is collected from the nasopharynx with a sterile cotton swab fastened to a metal wire and bent through a 45° angle with a sterile forceps before insertion. In taking samples the root of the tongue is held down with a tongue depressor held in the left hand and the

swab is introduced past the soft palate with the right hand and rotated (Fig. 86).

In withdrawing the swab from the nasopharynx care must be taken that it does not touch the oral mucosa, which would contaminate it.

Investigation of the cerebrospinal fluid is of primary importance for detecting Meningococci in cerebrospinal meningitis. For this purpose approximately 5-10 ml of spinal fluid is collected in a sterile syringe by lumbar puncture, observing strict asepsis, and transferred to a test-tube under sterile conditions. In cerebrospinal meningitis the spinal fluid is usually cloudy as a result of the large number of leucocytes which it contains. In rare cases the spinal fluid remains clear despite the presence of Meningococci.

Spinal fluid taken from patients should be protected from cold and examined bacterioscopically and bacteriologically as soon as possible. For this purpose part of the fluid is taken from the test-tube under sterile conditions and centrifuged and smears are prepared from the centrifugate for bacterioscopic examination; part of the sterile residue is transferred to 2 or 3 Petri dishes containing a nutritive medium (agar and formalinized serum) and spread with a spatula by Drigal'skiy's method. Some of the residue can be used to inoculate test-tubes containing serum-agar slants or Dorset's medium, observing the rules of asepsis.

The tube containing the remaining spinal fluid is placed in a heater at 37° and incubated for 24 hr. The first cultures are sometimes negative when working with freshly drawn fluid; the number of positive results increases after incubation. However, it is not recommended that the spinal fluid be left at 37° for more than 24 hr, since more prolonged incubation (48 hr) greatly reduces the viability of Meningococci.

Microscopic examination

The smears are stained by Gram's method and with methylene blue or dilute fuchsin (by Pfeyffer's method) and examined microscopically.

When gram-negative diplococci are detected intracellularly or even extracellularly a single bacterioscopic examination is sufficient for the investigator to conclude that Meningococcus is responsible for the disease, since gram-negative diplococci in the spinal fluid of cerebrospinal meningitis patients can only be Meningococci.

Bacteriological investigation

It is preferable to use clear nutritive media for isolating Meningococci, since these permit ready observation of the type of colonies and simpler differentiation of meningococcal colonies from those of other, similar bacteria. The most widely used of these media are ascitic agar (3 parts of 3% agar and one part of ascitic fluid) and serum agar (prepared in the same manner as ascitic agar).

It sometimes proves impossible to obtain the first generations of meningococcal cultures on ascitic agar; moreover, use of this medium is limited by the need for a regular supply of sterile ascitic fluid. An extremely convenient nutritive medium for isolating and culturing Meningococci is agar containing formalized serum prepared by Legroux's method (see page 791), which is a fully suitable substitute for ascitic fluid.

Bacteriological investigation of the spinal fluid is of secondary importance; it is employed if bacterioscopic investigation reveals no gram-negative diplococci. Bacteriological investigation confirms the bacterioscopic analysis and may be of supplemental value in clarifying the biochemical properties of the culture isolated and in determining the type of Meningococcus.

In general, if culturing of the spinal fluid yields small, bluish,

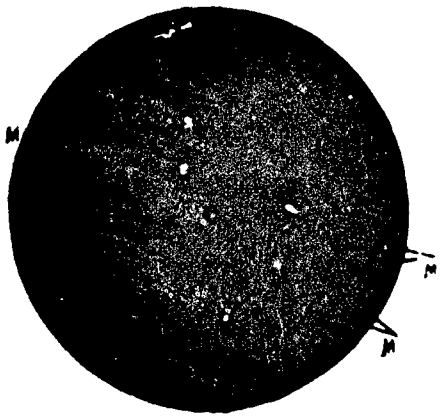


Fig. 87. Bacterial colonies obtained by culturing a nasopharyngeal discharge on serum agar, after incubation for 24 hr. M) Meningococcal colonies.

slightly raised colonies with uniform margins and gram-negative diplococci are detected in smears prepared from these colonies, no further investigation need be made of the fluid for diagnosis of Meningococci, the results obtained being regarded as completely conclusive.

Investigation of blood. In investigating blood for Meningococci it is cultured in Petri dishes containing ascitic agar or agar and formalinized serum. A total of 10 dishes are inoculated, each with 1 ml of blood, which is spread over the surface of the medium with a spatula. At the same time, 10 ml of blood taken from a vein with a syringe under sterile conditions is cultured in a flask containing 150 ml of ascitic or serum broth. A positive result is obtained in 25% of all cases during the first week of illness.

Investigation of nasopharyngeal mucous. The nasopharyngeal mucous is investigated for Meningococci principally in examinations conducted to detect carriers and, in rare cases, for the purpose of diagnosing cerebrospinal meningitis.

It must be kept in mind that investigation of mucous is more complex than investigation of spinal fluid, since, in addition to various

bacilli, the nasopharynx contains a considerable number of gram-negative diplococci, including Gonococci, which are difficult to distinguish morphologically from meningococci.

In this connection morphological diagnosis of meningococci isolated from the nasopharyngeal mucous is unreliable and must be supplemented by bacteriological investigation and subsequent study of the morphological (by Gram-staining), biochemical, and serological (by the agglutination reaction) characteristics of the diplococci isolated.

The mucous-bearing swab is immersed in a test-tube containing a small quantity of sterile broth and the mucous is washed off. Culturing is carried out at the patient's bedside. When it is necessary to ship the material to the laboratory it should be protected from cold. Several (2-3) drops of the broth containing the mucous are placed on a dish containing ascitic agar or agar and formalinized serum and rubbed into the surface of the medium with a spatula; the remaining material is successively transferred to 2-3 dishes with the same spatula. The inoculated dishes are incubated at 37° for 24 hr and the colonies produced are then inspected. From among the numerous colonies growing on the dishes those which are small, clear, and bluish in color are transferred to appropriate nutrutive media for further investigation (Fig. 87).

The colonies taken from the dishes are examined bacterioscopically on the following day and, when gram-negative diplococci are detected, their biochemical properties are checked. Biochemical differentiation of diplococci by their sugar-fermenting capacity on a solid nutritive medium (see page 792) is a very convenient procedure (Lingelsheim). The surface of the medium is inoculated with the cultures to be tested in radial streaks, using a platinum loop.

Fermentation is observed over a 48-hr period, incubating the

TABLE 62

Fermentation of Carbohydrates by Members of the Genus Neisseria and Similar Diplococci

1	2	3	4	5	6
N. meningitidis	+	0	0	+	0
N. catarralis	0	0	0	0	0
N. cenerius	0	0	0	0	0
N. flavus I	+	+	0	+	0
N. flavus II	+	+	0	+	0
N. flavus III	+	0	0	+	0
Diplococcus siccus	+	+	0	+	0
Diplococcus crassus	+	+	+	+	+
N. gonorhoeae	+	0	0	0	0

Symbols: + ferments carbohydrates; 0) does not ferment carbohydrates.

1) Bacterium; 2) glucose; 3) levulose; 4) sucrose; 5) maltose; 6) lactose.

dishes at 37°.

For a more detailed biochemical analysis the enzymatic properties of the cultures can be tested with respect to 5 carbohydrates: glucose, levulose, sucrose, maltose, and lactose. The enzymatic capacity of various diplococci is shown in Table 62.

Dorset's liquid medium (see page 791) can be used to confirm identification of meningococcal strains. In order to preserve such strains, cultures are made in a liquid nutritive media and incubated at 37° for 24 hr; smears are then made and examined for Meningococci. If these bacteria are present the medium bearing the culture is flooded with sterile paraffin oil to a depth of 1-2 cm. The test-tubes containing the cultures are stored in a heater at 37°, reculturing material to fresh medium no less than once a month. Meningococci retain their viability and cultural characteristics for 6 months or more on this medium.

Serological investigation of Men'ngococci (typing). Monotypic

horse or rabbit agglutinative sera can be used for typing Meningococci. There are two methods commonly employed for this purpose.

In order to determine the type of a Meningococcus a day-old culture is washed from Dorset's medium or ascitic agar with a quantity of physiological solution sufficient to produce a thick suspension (containing 5 billion cells per ml, as determined by comparison with an optical standard). It is best to type Meningococci by Noble's agglutination reaction (see page 119), since this reaction yields results within a few minutes; group agglutination does not occur in this case.

Nutritive Media

Dorset's medium: carefully washed hens eggs are wiped with alcohol and broken under aseptic conditions; their contents are poured through a sterile funnel into a sterile flask containing glass beads. A total of 10 ml of sterile Ringer's solution is then added to the flask for each egg. The mixture is thoroughly stirred, until completely homogeneous, and 5-6 ml portions are poured into sterile test-tubes, observing strict asepsis. The tubes are placed in a Koch coagulation apparatus and heated at 58° for 1 hr on 2 consecutive days. In order to avoid contaminating their walls the tubes should not be removed from the apparatus. On the 3rd day the medium is sterilized by gradually raising the temperature to 100°, permitting it to coagulate completely. In order to obtain a liquid medium it is sterilized in a Koch apparatus at 58° for 1 hr on 3 consecutive days.

After sterilization the medium-containing tubes are placed in an incubator at 37° for 3-5 days to check their sterility.

Legroux's medium: 1 ml of commercial formalin is added to 500 ml of horse serum. The contents of the flask are stirred and, after it has stood at room temperature for 5 min, 1 ml of ammonia (22° Baume) is added to the mixture to neutralize the remaining formaldehyde. The

treated serum is then mixed with 2 parts of sterile distilled water, thoroughly stirred, and filtered through filter paper into flasks of the desired volume. The latter are sterilized at 110° for 15 min.

The sterile formalinized serum can be stored under laboratory conditions for an indefinite period. In order to prepare the nutritive medium one part of 3% agar preliminarily melted and cooled to 56° is mixed with 1/3 part of formalinized serum. The mixture is thoroughly stirred (in order to avoid formation of air bubbles) and poured into Petri dishes or into test-tubes to obtain slants. This nutritive medium is quite suitable for isolating the 1st generation of meningococcal cultures, for subsequent maintenance of strains, and for diagnostic purposes.

Lingelsheim's solid medium for determination of biochemical properties: agar containing formalinized serum or serum agar is mixed with 10% of the desired carbohydrate and tincture of litmus is added until the mixture takes on a light blue color. The medium is sterilized at 100° for 5 min on 3 consecutive days and 15 ml portions are poured into Petri dishes and permitted to cool.

COCCAL PNEUMONIA

L.B. Balanyan, Candidate of Medical Sciences

Pneumonia may be caused by various microorganisms, which enter the human respiratory passages from the surrounding atmosphere (infection) or inhabit the oral and nasal mucosae of persons to all intents and purposes healthy (carriers), becoming pathogenic to the host only under unfavorable conditions (cold, trauma, disease, etc.): these include coccal (Pneumococci, Streptococci, Staphylocicci, and, according to some data, catarrhal Micrococci) and bacillary (Friedlander's and Pfeyffer's bacilli; pneumonia of newborns caused by E. coli has been described) forms. This disease can also be produced by

associations of the aforementioned bacteria (so-called mixed infections), as well as by viruses.

One of the most dangerous and common (responsible for more than 90% of all cases) causative agents of pneumonia, especially the lobar form, is Pneumococci. However, in recent years, a substantial change has been noted in the character of the microflora observed in pneumonia, both in children and adults. Pneumococci, which have long occupied first place among the causative agents of pneumonia, are now encountered quite rarely (up to 30% rather than 90-96%). The proportion of Staphylococci and E. coli has increased greatly, especially in young children.

This change is partially attributable to the spread of strains of bacteria resistant to the action of drugs (antibiotics, sulfanilamides).

Such changes may also be only apparent. When hospitalization is delayed the patient receives various drugs on admission, which influence the microflora of the mouth and upper respiratory tract. Bacteria which succumb quickly to drugs cannot be detected on examination, so that a high percentage of more resistant forms is observed.

Laboratory Diagnosis of Pneumonia

Material for examination

Sputum, mucous taken from the nasopharynx with a sterile swab, pus, various exudates, puncture specimens, and blood can be examined. Sputum is the usual subject of investigation, nasopharyngeal mucous being employed when none is available.

Microscopic examination

Before beginning to process material taken for examination smears are made on two slides for preliminary microscopy (bacterioscopic diagnosis); one smear is stained by Gram's method and the other with dilute carbol fuchsin (Pfeyffer's stain).

When lanceolate diplococci surrounded by light unstained capsules are found in the smears a preliminary diagnosis of pneumococcal etiology is made.

A preliminary diagnosis of streptococcal infection is made when gram-positive cocci arranged in a chain are detected.

Gram-positive cocci with a characteristic morphology arranged in groups resembling bunches of grapes furnish a basis for diagnosing staphylococcal infection.

Detection of small gram-negative rods, which often form nest-like aggregares and stain polarly, indicates that Pfeyffer's bacillus is present.

Detection of gram-negative diplobacilli of varying size surrounded by capsules taking the form of light-colored orioles indicates that Friedlander's bacillus is present.

This microscopic diagnosis is rough and preliminary. Study of cultures on blood and other special media and isolation and identification of pure cultures prepared from isolated colonies permit precise determination of the microflora present.

Pneumococcal pneumonia

Bacteriological investigation

For bacteriological investigation the material to be studied is suspended in 1 ml of slightly alkaline (pH 7.6) sterile 1% peptone water or r at-froth. Martin's broth containing no more than 0.02% glucose at pH 7.6 is the most suitable medium for this purpose.

One drop of the suspended material is transferred with a spatula to preliminarily dried Petri dishes containing agar to which 5-7% of defibrinated rabbit blood has been added (see below for the procedure for detecting Staphylococci).

Cultures on blood agar are examined after incubation in a heater

at 37° for 1-2 days. At the same time that the appearance of the cultures is studied material is transferred to liquid media and examined microscopically.

Colonies of Pneumococci on blood agar take the form of small, translucent greenish-gray aggregates, which are especially distinct in a thin layer of agar. When a colony is carefully inspected in incident light a small compact mound at the center and a light periphery can be detected. Colonies are often surrounded by a seemingly luminescent oriole. When a colony is removed from nutritive agar the latter bears traces of so-called false or incomplete hemolysis caused by the vital activity of the bacteria, which results in conversion of the hemoglobin of the blood agar to methemoglobin.

Colonies of type III Pneumococci are distinguished by a mucoid consistency and substantially greater diameter, up to 2 mm; they are slightly cloudy and tend to merge.

In liquid media Pneumococci produce a moderate uniform cloudiness, forming no film or sediment. A small amount of precipitate is detected in some cases, rising from the bottom in the form of a light powdery turbidity when the tube is gently shaken.

Microscopic examination reveals slightly elongated paired gram-positive cocci with pointed outward ends, which resemble a candle flame or lancet, consequently being called lanceolate diplococci. Pneumococci consisting of two regularly ovoid or even round cocci are, however, occasionally encountered, varying in size from 0.5 x 0.75 to 1 x 1.5 microns. The diplococci occur singly, especially just after being isolated from the body, but may also form short chains consisting of 3-4 pairs of bacteria. Pneumococci form capsules, which surround both cocci with a light ring, since they cannot be stained by the usual methods.

Differentiation of Pneumococci from Streptococci often presents great difficulties, as a result of their close relationship and morphological similarity. The simplest and most convenient of the existing differentiation methods is based on the differing behavior of these two types of bacteria with respect to bile. Pneumococci are easily lyzed in the presence of bovine bile and bile salt, while Streptococci are unaffected. The bile-resistant test is conducted in the following manner.

A Pasteur pipette is used to inoculate 100% bile broth with 2-3 ml of a 18-20-day culture of the bacterium to be tested. This causes the broth to become cloudy, taking on the appearance of milky tea. If the broth becomes clear after incubation at 37° for 24 hr the bacteria are lyzed) the culture consists of Pneumococci. If no clarification takes place the culture consists of Streptococci. This technique for differentiating Pneumococci from Streptococci gives a precise identification when the requisite condition (use of pure cultures) is met.

In some cases Pneumococci detected in investigating cultures are subjected to further study (when specific serum therapy is needed, in order to solve certain epidemiological problems, etc.). This usually involves determination of the type of Pneumococcus, of its virulence for white mice, and of its resistance to antibiotics.

The type of Pneumococcus is determined by agglutinating the pure culture with specific sera of the first three fixed types. Cultures which do not agglutinate with these three types of sera belong to group X. The reaction is set up in the following manner: a 18-20-hr broth culture is poured into agglutinative tubes in 0.5 ml portions and equal volumes of sera diluted to 1:5 (with physiological solution) are added. The control consists of 2 tubes, one containing the test

culture mixed with normal rabbit serum and the other containing culture alone. The contents of the tubes are thoroughly shaken and incubated in a heater at 37° for 2 hr; a preliminary evaluation of the reaction is then made. The final results are noted after additional incubation at room temperature for 20 hr.

The agglutination is evaluated as four-plus if the contents of the tube are completely clarified and the agglutinated culture forms a dense film, which breaks up into 3-4 fragments only on vigorous shaking, 3-plus agglutination has occurred if the contents of the tube are completely clarified and the agglutinated culture is easily broken up; two-plus agglutination has occurred if no clarification takes place and particles of the agglutinated culture are clearly visible to the unaided eye in the cloudy contents of the tube; in one-plus agglutination a finely granular suspension of agglutinated Pneumococci is present in the tube. No visible agglutination is observed when the reaction is negative; the contents of the tube are uniformly cloudy after shaking. The first three fixed types are detected principally in croupous pneumonia. Group X Pneumococci are encountered in acinous pneumonia, principally in young children (broncho pneumonia).

The Neifeld distension phenomenon is used for rapid typing without isolation of a pure culture; this procedure is based on the fact that the capsules of Pneumococci are greatly enlarged (distended) in the presence of serum of the same type as the culture under investigation. The reaction is set up in the following manner.

One drop of the test material, one drop of rabbit diagnostic serum types I, II, and III, and one drop of methylene blue are applied to each of three cover slips. The drops are thoroughly mixed with a loop or the fused tip of a Pasteur pipette and the slips are covered with a slide containing three wells, first smearing the edges of the

slips with vaseline. Microscopic examination with an oil-immersion lens reveals substantial enlargement of the light-colored zone around the bacteria when the Pneumococci and the specific serum are of the same type. No enlargement is observed when the bacteria and serum do not correspond.

The virulence of the pneumococcal culture is determined in white mice weighing 18-22 g. For this purpose a 18-20 hr broth culture is diluted down to $1:10^{-8}$ with 1% peptone water or slightly alkaline broth and doses of 0.5 ml of the various dilutions are administered intraperitoneally to groups of mice, using 2 mice for each dilution. The animals are then observed for 4-5 days. Any culture which kills the mice at a dilution of $1:10^{-7}$ or more is regarded as highly virulent, any which kills them at dilutions of from $1:10^{-4}$ to $1:10^{-6}$ is considered moderately virulent, and any which kills them at dilutions of $1:10^{-3}$ or less is regarded as weakly virulent. Nonvirulent strains of Pneumococci do not kill mice. In order to eliminate the possibility of animals dying of intercurrent causes blood from the hearts of the animals which succumbed is cultured to permit detection of Pneumococci.

Nutritive media containing various drugs are employed to determine the drug resistance of the Pneumococci isolated.

Either the minimum quantity of drug which the nutritive medium must contain to suppress culture growth and reproduction for the minimum number of bacteria capable of growing and multiplying in the presence of a given amount of drug is determined.

In the first case a series of test-tubes containing equal volumes of nutritive medium (5 ml) and increasing quantities of drug are inoculated with equal amounts (0.1 ml of a 1:100 dilution) of a 18-20 hr broth culture. That tube in which no growth can be seen with the unaided eye or in which it lags greatly behind the control (which contains

no drug) is noted after 24 hr.

In the second case a series of tubes containing equal volumes of nutritive medium and a given amount of drug are inoculated with 0.1 ml portions of different dilutions ($1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$, etc.) of a 18-20 hr broth culture. That tube in which no growth is visible or in which it is greatly reduced in comparison with the control (which contains no drug) is noted after incubation in a heater at 37° for 24 hr.

Both of these methods are sufficiently simple and permit evaluation of the resistance of the strain isolated to sulfanilamides and antibiotics in vitro, although the resistance determined in vitro in the laboratory and that shown by clinical data (where the principal index is whether or not the drug in question has any therapeutic effect) do not always correspond (doing so in approximately 50% of all cases). It is consequently recommended that data obtained in determining the drug resistance of bacteria in vitro be used principally for comparative evaluations.

Serological investigation

Detection of Pneumococci in the material under investigation does not furnish sufficient grounds for assigning them an etiological role. The bacteria responsible for a disease cause responses in the body, which appear as formation of various types of antibodies, changes in dermal sensitivity to specific antigens, etc. A proper and conclusive evaluation of the role of the Pneumococci isolated in the etiology or pathogenesis of the disease in question can consequently be made only by setting up a number of serological reactions.

A skin test using heat- or formalin-killed Pneumococci suspended in physiological solution as the antigen is of great value. The suspension is administered intracutaneously. No reaction is observed at the injection site in patients with pneumococcal pneumonia (this is

true in approximately 90% of all cases). In the overwhelming majority of healthy persons and persons suffering from pneumonia of nonpneumococcal etiology erubescence and an infiltration appear at the injection site within 20-24 hr. This reaction is not typospecific. The majority of authors regard it as specific and suggest that it be used for diagnostic purposes. Another diagnostic procedure is to agglutinate cultures isolated from the patient with his serum. It has been established that the agglutinin titre of the patient's blood, low at the beginning of the illness, usually increases gradually as it develops.

Precipitation of the patient's urine (antigen), which is concentrated by boiling and filtered, with typed pneumococcal sera is of some diagnostic value.

Little research has been done on these reactions and they have consequently not come into wide practical use.

Detection of Pneumococci by biological testing

At the same time that the material under investigation is cultured on blood agar doses of 0.5 ml are administered intraperitoneally to 2 white mice weighing 18-20 g.

One of the mice is killed 5-6 hr after inoculation and blood taken from its heart under sterile conditions with an elongated Pasteur pipette is cultured in a test-tube containing Martin's broth prepared in accordance with the formula given above, to which 5% of unpreserved normal horse serum is added.

After the blood from the heart has been cultured the abdominal cavity is opened, the spleen is removed, pieces are cut from it with a sterile scissors, and prints are made on a slide by repeatedly pressing the cut surface against it until an impression free of blood is obtained. The resultant prints are stained by Gram's method and inspected for microorganisms. This technique is very useful when the bacteria

are not particularly virulent and thus do not cause septicemia, so that they cannot be detected in blood cultures.

The second mouse is permitted to die a natural death of septicemia. If it has not died after 2 or 3 days it is killed and examined in the same manner as the first mouse.

Streptococcal pneumonia – see page 735.

Staphylococcal pneumonia

In many cases the widespread occurrence of Staphylococci greatly complicates bacteriological diagnosis of the diseases which they cause. Such diagnosis is reliable only when the material investigated is taken from the affected area; the clinician thus usually establishes that pneumonia is staphylococcal in nature from its clinical and x-ray characteristics. Bacteriological investigation is usually employed to confirm or disprove the clinical diagnosis.

Bacteriological investigation

Milk-salt and blood agar (see page 904) are used for bacteriological investigation.

Staphylococci are detected by examination of material (puncture specimens, discharges, pus, exudates) taken directly from the focus of the disease (the pleural and pulmonary cavities) and of blood cultures made under absolutely sterile conditions. Investigation of the oral and nasal mucous is of secondary value and merely supplements the general pattern.

A total of 2 or 3 drops of the material to be investigated are placed on the surface of a dish containing milk-salt agar and uniformly distributed with a sterile spatula; the spatula is then rubbed over the surface of blood agar. When the growth on the milk-salt agar is overly profuse fewer bacteria grow on the blood agar, leaving individual colonies accessible for isolation.

The cultures a e incubated at 37°. The blood cultures are kept in an incubator until growth (c'.oudiness) appears. If no bacterial growth is detected after 4 or 5 days (daily shaking is recommended) the cultures are regarded as negative and destroyed.

After 24 hr the cultures are removed from the incubator and the growing colonies are studied.

Staphylococcal colonies on solid media are regular disks with smooth margins, a moderately convex lustrous surface, and a diameter of 2-4 mm. They are easily removed from the agar, are sometimes rather viscous,and are an opaque golden-orange, lemon-yellow, or white in color. It should be kept in mind that certain strains of Staphylococcus require a longer time and a source of light for pigment formation. When colonies of unpigmented Staphylococci are observed they are consequently assigned to the species Staphylococcus albus only after being left to stand at room temperature for 3-4 days.

Pathogenic strains of Staphylococcus are usually surrounded by a zone of more or less pronounced hemolysis on blood agar.

Staphylococcal colonies having nothing in common with those described above are encountered, albeit rarely. They are very large and flat, have nonuniform serrated edges, a matte surface, and often a yellowish tint, and are nonpathogenic (they do not coagulate plasma, do not produce toxin, do not hemolyze erythrocytes, and cannot be types with phages). Only microscopic examination shows the characteristic clusters of gram-positive, rather large cocci.

In liquid media Staphylococci produce a strong diffuse turbidity; some strains form a film. A sediment is deposited at the bottom of the test-tube when it is permitted to stand, but rises in a viscous mass when it is gently shaken. Staphylococci are gram-positive; staining by Gram's method is obligatory in investigating Staphylococcal cultures.

Under the microscope Staphylococci are rather regular spherical cocci 0.6-0.9 microns in diameter. They occur singly, in pairs, and even in short chains of 2-4 cocci, but the principal form is irregular clusters resembling bunches of grapes. Pathogenic strains usually appear lilac in color, while nonpathogenic strains are an intense blue.

Strains of Staphylococci isolated from single colonies (no less than 3 cultures from each patient) are investigated for pathogenic properties and sensitivity to antibiotics. Material is transferred daily from the blood cultures to blood agar and the resultant cultures are subjected to the same investigative techniques.

There is no single generally recognized pathogenic trait common to all strains of Staphylococcus. In practice, the pathogenicity of a staphylococcal culture is determined from a complex of its properties: pigment formation, hemolysis of erythrocytes, coagulation of blood plasma, production of exotoxin, and lysis by type phages. Pathogenicity is determined in vivo only for special studies. This test is not obligatory in establishing a bacteriological diagnosis.

In laboratory diagnosis an obligatory criterion of the pathogenicity of staphylococcal cultures is their plasma-coagulating ability (see page 902).

All cultures which coagulate plasma over a given observation period are regarded as pathogenic; cultures which do not coagulate plasma are considered nonpathogenic and not investigated further.

The pathogenicity of staphylococcal cultures is also characterized by their ability to produce alpha-hemolysin of varying strength. The ability of cultures to produce exotoxin in liquid media is determined in the following manner. The strains to be investigated are cultured with a loop in test-tubes containing casein broth (1 liter of broth contains 600-660 ml of acid casein hydrolyzate, 200 ml of yeast extract,

140 ml of wheat-bran autolyzate, and 0.5 g each of Na_2HPO_4 and KH_2PO_4; the mixture is sterilized at 110° for 30 min). The tubes are placed in a desiccator in an inclined position and stored in an incubator for 5 days. At the beginning and end of each working day 20% of carbon dioxide by volume is introduced into the desiccator. At the end of the 5-day period the tubes are removed from the incubator and the minimum quantity of toxin which hemolyzes rabbit erythrocytes (Dhm) is determined in the live culture. This reaction is set up in the following manner.

Toxin samples are diluted by factors of 5, 10, 20, 40, 80, 160, 320, 640 and 1280. For this purpose physiological solution is added to 9 test-tubes (for each culture), pouring 1.6 ml into the first tube and 1 ml into the remaining 8. A total of 0.4 ml of the toxin to be tested is added to the first tube (yielding a dilution of 1:5) and thoroughly stirred; 1 ml of the material from this tube is transferred to the second tube (yielding a dilution of 1:10), the mixture is stirred, 1 ml is transferred to the third tube (yielding a dilution of 1:20), etc. When the final tube is reached 1 ml of the dilution is removed, so that the reaction is conducted in volumes of 1 ml. One drop of thrice-washed rabbit erythrocytes diluted by a factor of 3 is added to each tube, the contents of the tubes are mixed by shaking the rack, and the tubes are placed in an incubator for 1 hr and then left to stand at room temperature for an additional hour. The results are then evaluated; the toxin titre is assumed to be that dilution which causes 2-plus hemolysis of the erythrocytes. The toxin is considered strong if it hemolyzes the erythrocytes at a dilution of 1:320 or more, moderately strong if it hemolyzes them at a dilution of 1:80-1:320, and weak if it hemolyzes them at a dilution of 1:40 or less.

An indirect index of the pathogenicity of Staphylococci is their

lyzability by staphylococcal type phages, since it is known that nonpathogenic strains cannot be typed. Moreover, typing the cultures under investigation aids in identifying them and in elucidating the sources and propagation modes of the infection; it is consequently both useful and necessary to set up experiments for determining the phage types of the cultures (see page 581).

It is also necessary for practical reasons (correct selection of antibiotics for therapy, identification of different strains isolated from the same patient, etc.) to determine the sensitivity of the cultures to antibiotics. A rough sensitivity determination by the disk method is sufficient for this purpose. In essence, this method rests on the appearance of zones of retarded culture growth on the surface of agar around disks impregnated with antibiotics. It must be kept in mind that the sensitivity to antibiotics determined experimentally in vitro and that observed clinically do not completely correspond. A culture resistant to a certain antibiotic in vitro (as determined by the disk or serial-dilution method) may easily be suppressed under clinical conditions, since in the latter case it is subject not only to the action of the antibiotic,but to that of the entire complex of the body's protective adaptations.

Serological investigation

Determination of the staphylococcal-antitoxin level of the patient's blood serum under dynamic conditions, i.e., at the beginning and end of the illness, is the most widely employed of the serological reactions.

In this case the titration is based on neutralization of the hemolytic properties of staphylococcal toxin with the antitoxin in the patient's serum. In setting up this reaction it is necessary to have standard toxin with a precisely titrated hemolytic limit (Lh), i.e.,

the minimum quantity of toxin which will cause almost complete hemolysis of erythrocytes when mixed with 1 antitoxic unit (AU).

Before setting up the experiment all the sera to be tested are heated at 56° for 30 min to destroy the hemolysins to rabbit erythrocytes present in some human sera.

Sera containing one or more antitoxin units are titrated with 1/5 Lh of toxin. Portions of 1 ml of the various serum dilutions are mixed with 1 ml of dilute toxin corresponding to 1/5 Lh. After shaking 1 drop (0.05 ml) of thrice-washed erythrocytes diluted by a factor of 3 with physiological solution is added to each tube.

When the antitoxin content of the serum may be less than 1 unit it is diluted only by a factor of 5, 1 ml portions are poured into test-tubes, 1 ml of toxin diluted to a content of 1/10, 1/20, or 1/40 Lh per ml is added, the contents of the tubes are mixed by shaking the rack, and 1 drop of erythrocytes is added to each tube. The total reaction volume is 2 ml. The following controls are set up in titrating sera: 1) test serum – 2 ml of serum diluted to 1:10; 2) erythrocytes – 1 drop of washed rabbit erythrocytes diluted by a factor of 3 in 2 ml of physiological solution; 3) toxin – 1/20 and 1/40 Lh, each in a volume of 2 ml.

The results are evaluated after incubating the mixture at 37° for 1 hr and at room temperature for 1 hr, noting all hemolysis with an intensity of 2 plusses or less. The results are regarded as reliable when the controls react properly, i.e., when hemolysis does not occur in the tubes containing the test serum and erythrocytes. The control toxins should cause hemolysis, 3-plus (almost complete hemolysis) at 1/40 Lh and 4-plus (complete hemolysis) at 1/20 Lh.

The serum titre is considered to be that dilution which prevents intensive hemolysis of the rabbit erythrocytes when mixed with the

toxin (the hemolysis should be of no more than 2-plus intensity). In order to determine the titre of the undiluted serum its dilution factor is multiplied by the toxin dose used. Thus, for example: 1) if 1 ml of serum diluted by a factor of 5 neutralized 1/40 Lh of toxin its titre is 5 x 1/40, i.e., 0.125 AU; 2) if 1 ml of serum diluted by a factor of 40 neutralized 1/5 Lh of toxin its titre is 40 x 1/5, i.e., 8 AU.

The antitoxin content of the patient's blood usually rises throughout the course of a staphylococcal infection, thus confirming the etiological role of Staphylococcus in the disease in question. The antitoxin titre may fail to increase in severely weakened patients and in certain other cases (anergia).

DISEASES CAUSED BY H. INFLUENZAE

Prof. K.I. Matveyev

Haemophilus influenzae (Afanas'yev-Pfeyffer) belongs to the family Brucellaceae, and the order Eubacteriales. It is found rather commonly in the mucosa of the upper respiratory passages in man; when the body's resistance to infection is lowered it may cause meningitis (especially in children), acute inflammation of the respiratory passages, pneumonia, empyema, bronchitis, laryngitis, conjunctivitis, acute and chronic otitis, and other diseases.

Morphology of H. influenzae

All the species of bacteria belonging to the genus Haemophilus are short rods with an almost coccoid shape (Fig. 88) 1-1.5 micron long and 0.3-0.4 micron wide. They are very polymorphous and may form filaments (Fig. 89). They are immobile, do not form spores, and are gram-negative. When isolated from the body the majority of species grow only on media containing certain factors found in blood or plant tissues.

These bacteria stain slowly with analine dyes; 5-15 min is re-

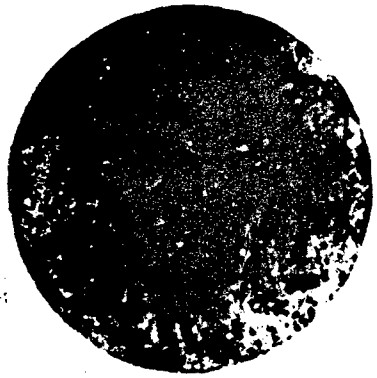


Fig. 88. Day-old culture of H. influenzae on solid medium. 1000 x.

Fig. 89. Day-old culture of H. influenzae on solid medium, filamentous forms. 1000 x.

quired for staining with carbol fuchsin diluted by a factor of 10.

Biology of H. influenzae; cultural characteristics

The growth factors or growth vitamins known as the X and V factors play a large role in the growth of haemophilus on nutritive media. The X factor is found in blood, is thermostable, and is not decomposed when blood or its products are autoclaved. The blood of various animals or an aqueous solution of hematin fluoride is used as a source of the X factor. The V factor is present in the tissues of plants and animals and is produced by many bacteria. It was assumed that this thermolabile factor was a vitamin and it was consequently named the V factor. It is now known that the V factor is coenzyme 1 or coenzyme 2. A solution of pure coenzyme 1 in high dilutions can be used. The V factor is usually obtained from baker's yeast: a suspension of 100 g of yeast is made up in 400 ml of distilled water (pH 4.6), boiled for 10 min, centrifuged or filtered through paper (pH 7.0), sterilized by filtration, and stored in sterile form in a glass vessel with a glass stopper.

When culturing these bacteria on liquid media from 3 to 10% of

TABLE 63

Biological Properties of Species of the Genus Haemophilus

Вид 1	Морфология 2	3 Капсула	4 Потребность в X-факторе	5 Потребность в V-факторе	6 Гемолиз	7 Биохимическая активность: 8 индол	9 декстроза	10 левулоза	11 галактоза	12 мальтоза	13 лактоза	14 сахароза	15 маннит	16 ксилоза	17 глюкоза	18 редуцирует цитраты
H. influenzae (M. N. Afanas'yev, 1891; Pfeiffer, 1892)	Коккобактерии или нити	+	+	+	−	±	+	+	+	+	−	+	−	−	−	+
H. aegyptius (Koch, 1883; Weeks, 1886)	То же	−	+	+	−	−	−	−	+	−	−	−	−	−	+	+
H. suis (Lewis, Shope, 1931)	» »	−	+	+	−	−	±	−	±	±	−	±	−	−	−	+
H. haemolyticus (Pitchette, Silliman, 1919)	Бактерии или нити	−	+	+	+	±	?	?	?	?	?	?	?	?	?	+
H. parainfluenzae (Rivers, 1922)	Бактерии часто с заостренными концами	−	−	+	−	±	+	±	±	+	−	+	−	±	±	+
H. parahaemolyticus (Pittman, 1963)	Большие толстые бактерии или нити	−	−	+	+	−	−	−	−	+	−	+	−	−	+	+
H. haemoglobinophilus (Friedberger, 1903)	Коккобактерии или короткие нити	−	+	−	−	+	−	−	+	−	−	+	+	+	+	+
H. influenzae-murium (McKie van Rooyen, Gilroy, 1933; Kairies, Schwartzer, 1936; Ivanovics and Ivanovics, 1937)	Толстые бактерии могут иметь заостренные концы, или нити	−	+	−	−	−	−	−	−	+	+	+	−	−	+	?
H. ducreyi (Ducrey, 1890)	Длинные тонкие палочки; часто короткие цепочки	−	+	−	(+)	?	±	−	−	±	−	±	−	−	−	−
H. aphronihilus (Khairat, 1940)	Коккобактерии или нити	−	+	−	−	−	−	−	−	+	+	−	−	−	+	+
H. vaginalis (Gardner, Dukes, 1955)	То же	−	+	?	±	?	?	?	?	?	?	?	?	?	?	?

Symbols: +) Positive; −) negative; ±) may or may not be positive; ?) not investigated; [±]) weakly positive.

1) Species; 2) morphology; 3) capsule; 4) requires X factor; 5) requires V factor; 6) hemolysis; 7) biochemical activity; 8) indol; 9) dextrose; 10) levulose; 11) galactose; 12) maltose; 13) lactose; 14) sucrose; 15) mannitol; 16) zylose; 17) glucose; 18) reduces citrates; 19) M.I. Afanas'yev; 20) coccobacteria or filaments; 21) the same; 22) bacteria or filaments; 23) bacteria, often with pointed ends; 24) large thick bacteria or filaments; 25) coccobacteria or short filaments; 26) thick bacteria, sometimes with pointed ends, or filaments; 27) long thin rods, often short chains.

Fields's decomposed blood, which contains the X factor, or yeast extract, which contains the V factor, is added to them.

Four of the species of the genus Haemophilus isolated from man

require both the X and V factors, 2 species require only the V factor, and 4 species require only the X factor (Table 63).

In culturing these bacteria it should be kept in mind that yeast extract often contains both the V factor and small quantities of the X factor sufficient for raising certain X-dependent strains.

Antigenic structure; serotypes

The first generations of S-form H. influenzae have capsules when isolated from the body. Such capsular strains can be preserved in fresh rabbit blood at 4°. If these conditions are not met they may lose their capsules as a result of dissociation to the R form. Freshly isolated noncapsular strains are variants of the rough form. The capsules of the individual strains differ in antigenic properties, since they contain 6 different polysaccharides. H. influenzae is divided into 6 serotypes (a, b, c, d, e, and f) in accordance with its capsular antigens. Serotype b is most frequently isolated from man. Capsular strains are more pathogenic for mice than noncapsular strains.

Precipitation-agglutination reaction. The capsular antigens can be used to obtain specific antisera with high titres, which permit precise determination of the serological types of H. influenzae. A drop of serum is applied to a slide and a small quantity of bacteria from a typical colony is introduced into it. After a few minutes precipitation and agglutination occur with the serum of the type to which the bacterium isolated belongs. The reaction is first set up with type b serum. In some cases slow granular agglutination takes place as a result of the reaction between the common somatic antigen and agglutinins to it. This reaction is nonspecific.

The precipitation-agglutination reaction can be set up in test-tubes. For this purpose a culture is incubated overnight on blood broth and various serum dilutions are added to it; the culture and se-

rum are taken in equal volumes (0.5 or 1 ml). The tubes are placed in a heater for 4 hr and a preliminary evaluation is then made; they are then left to stand overnight in a refrigerator and the final results are evaluated in the morning. At low serum dilutions (1:20) a disk of precipitate forms at the bottom of the tube, breaking up when the tube is shaken.

Laboratory Diagnosis of Diseases Caused by H. Influenzae

Material for examination; taking of samples

In diseases of the respiratory passages and inflammations of the ear mucous and pus are taken with a swab, in pneumonia sputum is collected in a Petri dish, in empyema the intrapleural exudate or pus is investigated, and in meningitis the spinal fluid is examined.

Microscopic and bacteriological investigation

For culturing of mucous and sputum on nutritive media suppurative lumps are rinsed in physiological solution and cultured on Lewinthal's blood (chocolate) agar (see page 814) and on fresh blood agar containing 10% rabbit or horse blood. H. influenzae is very unstable outside the body, rapidly dying when the mucous, sputum, or pus dries out; the cultures must consequently be made shortly after the material is taken.

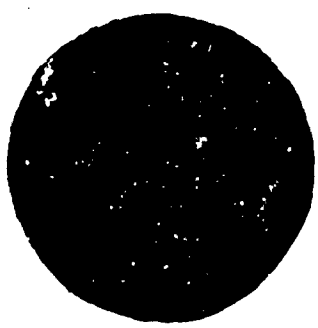


Fig. 90. Colonies of H. influenzae. Two-day culture. 8 x.

Smears are prepared on slides from the suppurative lumps, flame-fixed, stained for 5-10 min in carbol fuchsin diluted by a factor of 10, and examined microscopically.

Colonies of hemoglobinophilic bacteria grow to rather large size on Lewinthal's medium, which facilitates their recognition. After incubation at 37° for 24 hr colonies of capsular strains are 1-3 mm in diameter;

they are substantially larger than the colonies of noncapsular strains and slightly whitish in color, have a smooth glossy surface and uniform margins, and are round in shape (Fig. 90). Colonies of noncapsular strains on this medium reach a diameter of 0.5-1 mm, having nonuniform margins and a convex center. Smears are prepared on slides from colonies grown on blood agar and Lewinthal's medium, stained with Ziehl's fuchsin (1:10), and examined microscopically. Typical bacteria are subjected to serological investigation and transplanted to liquid media for further investigation.

When cultured on liquid blood media (Lewinthal's or Fields's broth) from colonies H. influenzae produces a homogeneous growth with a slight sediment at the bottom. Coccobacterial forms produce a uniform turbidity, while filamentous forms form flakes.

Precipitation reaction. When H. influenzae is raised in liquid media or when a suspension in physiological solution or distilled water is prepared from cultures on solid media the specific capsular antigen dissolves in the liquid. It can be detected in such solutions by a precipitation reaction with typospecific sera. The reaction is set up in slender test-tubes, using absolutely clear liquid and serum. In order to obtain a clear fluid from a culture or the patient's excreta it is centrifuged. The fluid is overlayered on a small quantity of serum with a Pasteur pipette. A white ring appears at the boundary between the serum and the fluid after a few minutes. If the appearance of the ring is delayed the tubes are placed in an incubator for 10-20 min.

Precipitation reactions can be set up with various fluids obtained from the patient. Annular precipitation can be employed to investigate the spinal fluid in meningitis or the serous fluid from the pleural or abdominal cavity. In a prolonged illness the patient's

Blood serum will give a precipitation reaction as a result of the capsular antigen accumulated in it. The precipitation reaction can be used to detect the specific capsular polysaccharides of H. influenzae in the patient's urine.

In setting up a reaction with fluids from the patient it is necessary to keep in mind the fact that there may be a cross-precipitation reaction between type a H. influenzae capsular serum and pneumococcal antigen of subgroup 6B, between type B serum and pneumococci of subgroups 6, 6A, 6B, 15A, 29, 35B, and between type c serum and pneumococci of subgroups 11, 11B, 19, 21, and 33B.

Neifeld's reaction. A hanging drop is prepared with a loopful of a young culture from Lewinthal's broth and a drop of typospecific H. influenzae capsular serum and a drop of Loeffler's methylene blue are introduced into it. On microscopic examination the capsules are clearly seen around the bacteria, which are stained blue. There are no cross-reactions between the sera of different types of H. influenzae. The broth culture must be taken at an age of 5-6 hr, before the capsular substance has dissolved in the liquid and while it still surrounds the bacterial cells. If the laboratory has available capsule-forming type strains a, b, c, d, e, and f they can be used to detect antibodies to the capsular antigen in the patient's serum a week or more after the onset of the illness.

Differentiation of H. influenzae from other hemoglobinophilic bacteria. The fact that H. influenzae rapidly dissociates to the R form makes it quite difficult to differentiate it from other hemoglobinophilic bacteria. Some species can be distinguished by the appearance of their colonies in semiliquid (0.15%) agar. Thus, colonies of H. aegyptius are comet-shaped, fluffy, and broadened at the base, colonies of noncapsular H. influenzae are granular in structure, and colonies

of capsular H. influenzae are always large and fluddy.

H. aegyptius agglutinates 0.5% suspensions of erythrocytes of all human blood groups when equal volumes of culture and erythrocytes are mixed and incubated at room temperature. No other species of Haemophilus agglutinates erythrocytes.

Diffusion in agar (see page 148) can be employed to determine the antigenic properties of the bacteria isolated. This technique permits conclusive differentiation of the various species of Haemophilus.

H. haemolyticus forms large colonies surrounded by zones of hemolysis on blood agar; this clearly distinguishes it from H. influenzae, which does not cause hemolysis.

In differentiating hemoglobinophilic bacteria it is necessary to determine all the other characteristics of the individual species, which are shown in Table 60.

Nutritive Media

Lewinthal's medium: 10% of defibrinated blood is added to agar preliminarily melted and cooled to 60° and the mixture is thoroughly stirred, placed in a water bath, and held at 75-80° until the agar takes on a chocolate color.

CONTAGIOUS INFECTIONS

PLAGUE

N. N. ZHUKOV-VEREZHNIKOV, PROFESSOR, MEMBER OF THE AMN USSR,
AND S. I. KHARLAMPOVICH, CANDIDATE OF BIOLOGICAL SCIENCES

Plague is one of the particularly dangerous infections and is a typical zoonotic disease with natural focus. The disease in humans, as a rule, is preceded by epizootic disease of rodents. Rodents (gophers, marmots, mice, rats, etc.) are the infection reservoir in nature and transmit it from one to another mainly via fleas. Humans can be infected by sick rodents, also via the fleas, which subsequently leads to flareups of plague among the population. The infection mainly assumes the bubonic form of plague which can change to the pulmonary form which has a tendency to rapid spreading in the form of an epidemic.

In our country, immediately after the Great Socialist October Revolution, the Communist Party and the Soviet Government began to carry out widespread sanitary measures. The rapid growth of culture and the wellbeing of the working masses and also the prophylactic measures resulted in the liquidation of plague in the Soviet Union.

However, plague is still encountered in certain countries bordering on the USSR, where epidemics of this disease sometimes assume impressive dimensions. Furthermore, plague foci still occur in the East and Southeast of the USSR among rodents. It is thus clear that Soviet medical workers must have a good knowledge of this disease to be able to recognize the first cases of plague in time and to organize the implementation of antiepidemic measures.

Morphology of P. pestis

The plague microbe (Pasteurella pestis) usually has an ovoid shape, a length of 1 to 2μ and a width of 0.3 to 0.7μ and is gram negative, with a typical bipolar stain (when the central part of the body of the microbe is stained less intensely than the ends). This form predominates in the smears from tissues and excretions of humans and animals and also from young broth cultures (V.M. Tumanskiy, 1958). Only in protracted cases of plague in humans (festering abscesses, ulcers) and also in old cultures are modified plague bacteria sometimes found in the form of poorly stainable individuals, spherical or elongated forms, etc. In the smears from one or two day old agar cultures, small bacilli with either weak bipolarity, sometimes without any, are seen either singly or in small groups (Fig. 91). In the smears from

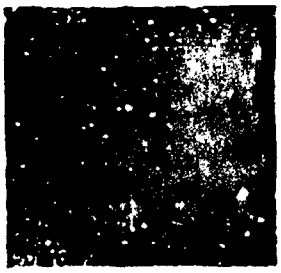


Fig. 91. Past. pestis. Two day old agar culture.

Fig. 92. Past. pestis. Broth culture. Chains; marked bipolarity.

broth cultures, the plague bacteria are grouped in chains of varying length, normally with marked bipolarity (Fig. 92). On agar containing 3% common salt, odd involutionary forms can be seen regularly. When grown on artificial nutrient media at elevated temperature (37°), the plague microbe forms a capsule (Fig. 93). The capsule forms more readily on moist and slightly acid nutrient media. Capsules are very rarely

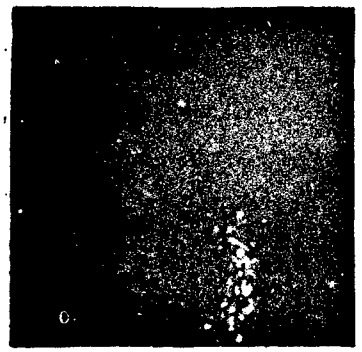


Fig. 93. Past. pestis. Culture at 37°. Clearly visible capsules.

found in the organs of cadavers, in sputum and the fluid from the abscesses (N. N. Zhukov-Verezhnikov, 1944). Plague microbes do not form spores. Cilia are absent.

Biology of P. pestis; culture properties

The usual nutrient media (meat peptone agar, broth) with a pH = 6.9-7.2 are suitable for growing P. pestis. The temperature optimum is between 28-30°. Growth is possible at a temperature of 5-10° and at 37°. The culture is usually kept at 28°. It is advisable for stimulating the growth of plague microbes, to add to the nutrient medium sodium sulfite, hemolyzed blood or Fields' broth, which are substances capable of restoring the redox potential (sodium sulfite) and of stimulating the synthesis of the respiration enzymes (hemin). The growth of plague microbes is visible on agar plates within only 24 hours in the form of a frail grayish bloom. The colonies on agar belong to the R form. The beginning development of colonies is detected by the appearance of very small soft lumps and later of flat lamellar formations with irregular outlines, reminiscent of lace kerchiefs (Fig. 94). They are only slightly hyaline. In transmitted light the color is grayish-white with a bluish shade and in reflected light, white-gray. Under low magnification, noticeable chromogenicity is observed, imparting a brownish or yellowish tinge to the center of the colonies and a ragged periphery and also a highly characteristic tuberosity to the surface (Fig. 95). The colonies are viscid, particularly when grown at 37°. Typical for the colonies is a considerable polymorphism (see Fig. 95). Thus, the ragged fringe is often absent, the surface of the colonies is altered, etc. Old colonies are very polymorphous. The Twort phenome-

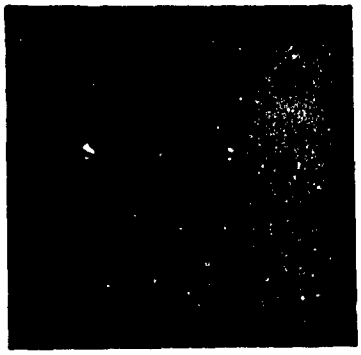


Fig. 94. Young growth of Past. pestis on agar plate.

non is sometimes observed (Fig. 96): hyaline regions of the colonies as seen macroscopically, and striation of these regions under the microscope.

On broth, the culture grows in the form of flakes, suspended, as a rule, in completely transparent fluid, with a loose sediment on the bottom. Upon prolonged storage, the media form the so-called plague stalactites which hang down from the surface of the medium. The plague microbe is capable of fermenting various sugars and alcohols without gas evolution and in the virulent form secretes fibrinolysins. According to the data of I. V. Domoradskiy and G. A. Yaromyuk, fibrinolysins can also be detected in the avirulent forms, albeit in smaller quantity. Three subspecies of the plague microbe are found: one of them (P. pestis, var. marmiottae) ferments glycerol and is isolated in continental foci of wild plague, where pulmonary plague often occurs, and also contains the special PT antigen (T. D. Fadeyeva); the other (P. Pestis, var. ratti) does not ferment glycerol, does not contain the above mentioned antigen and is isolated in oceanic, mainly "rat" foci (for example, in India), where primary pulmonary plague is rare; the third subspecies (P. pestis, var. citelli) differs from the first two by not having any nitrogen-fixating capacity. It was first isolated from the organism of the small gopher.

Characteristic for the plague microbe is an adaptive, dissociative and mutational variability. It is capable of entering into sexual recombination with E. coli Hfr and to acquire under artificial conditions the capacity of hydrolyzing lactose (F. Jacob). The species-forming variability expresses itself mainly in the direction of the formation

Fig. 95. Day-old colonies of Past. pestis. Typical presence of ragged edge zone in some colonies and its absence in others (polymorphism). 20x.

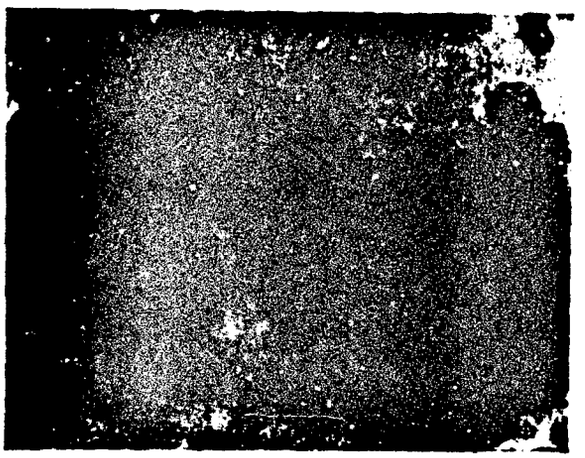


Fig. 96. Parts of Past. pestis colonies, subjected to the action of phage. The striation of the colonies can be seen (Twort phenomenon).

of forms very similar to Bact. pseudotuberculosis rodentium (A. A. Bezsonova, G. N. Lenskaya). The plague microbe and the closely related pseudotuberculosis microbe belong to the group of germs responsible for hemorrhagic septicemia (Pasteurella), but occupy a special position.

Laboratory Diagnosis Of Plague

Peculiarities of the laboratory examination of material, containing P. pestis

Plague is enormously contagious and is one of the diseases which must be immediately reported to the local authorities and the Health Ministry of the USSR as soon as it is detected.

The taking of sample material from patients (particularly with the pulmonar form), as all laboratory tests, must be carried out with the observation of measures which fully ensure the safety of the laboratory personnel. Among these measures are explanations to the personnel concerning the pathways by which the infection can spread and the methods of personal prophylaxis, the correct use of the antiplague suit, reliable continuous disinfection and timely disinfection of material, reliable final disinfection, correct storage of infected material, infected animals and cultures, and, finally, limitation of the number of persons working with infected material.

The antiplague suit consists of an overall, properly protecting the back and neck, a gown, rubber or leather (impermeable) boots, protective goggles (of the driver's or pilot's type), very convenient being clean and smooth cellophane (which does not dim like goggles), a second gown, rubber gloves, a towel, moistened with a disinfectant solution (tucked into the belt on the right side) and, finally, the most important element of the suit - the cotton-gauze antiplague mask. Necessary is also a kerchief which covers completely all exposed parts of

the face and head.

The cotton-gauze mask should be sufficiently large to cover the nose, mouth and chin.

To prepare the cotton-gauze mask, a continuous layer of white hygroscopic cotton wool with a thickness of 1.5 cm, a length of 25 cm and a width of 15 cm is covered with a double layer of gauze cloth, the ends of the latter cut, thus forming four straps, two of which are tied at the back of the head and two on the forehead.

When the mask has been donned, a cottonwool tampon is inserted into the space around the wings of the nose. It is best to wrap every mask together with the tampon in a separate paper bag and to use it after it has been sterilized. When the work is completed, all the parts of the suit are disinfected by complete immersion in 5% lysol. The goggles are soaked in 70° ethyl alcohol. The basic principle of current disinfection consists in the fact, that everything which can be burnt (for example, cadavers of rodents) is disposed of in this manner or autoclaved. The remainder is disinfected by soaking in 5% lysol for 24 hours. The syringes and autopsy instruments are boiled in lysol for 40 minutes. The floor, walls and the laboratory table are wiped from time to time with 5% lysol. The hands are steeped in 3% lysol.

Finally, a complete wet disinfection of the entire room and equipment with 10% lysol is carried out. Cultures and infected material must be kept in safes, which, like the thermostats, must be sealed. All inoculations of media are recorded daily in a journal and the remaining number of all types of cultures is recorded at the end of each working day.

The experimental animals (guinea pigs) are kept in a separate room in high glass jars, each animal separately. The jars are wound

with two-layer gauze soaked in lysol and wrung out. Mice are kept in jars placed into a wire mesh box with a sheet metal lid. The animals and the work carried out with them are recorded daily. All work in the laboratory is carried out only in the complete antiplague suit.

The laboratory work with plague cultures is regulated by special instructions of the Health Ministry of the USSR.

Materials for examination; taking of specimens

The following materials may be brought into the laboratory: 1) the contents of the bubo (bubonic form of plague); 2) the discharge from the ulcer or a specimen obtained by puncture of a carbuncle (cutaneous form); 3) matter from the oral cavity taken with a tampon and sputum (pulmonary form of plague); 4) autopsy material (parts of the organs of cadavers, blood); 5) live rodents; 6) cadavers of rodents; 7) fleas from rodents and humans; 8) water; 9) food products. The air is also tested in some cases. These materials must be taken prior to prescribing therapeutic preparations because etiotropic preparations (for example, antibiotica or sulfonamides) make the subsequent isolation of a culture very difficult. Besides, when the sputum and the bubo contents are microscopically examined after administration of these preparations, the number of bacteria in the smears decreases and they soon become undetectable.

The importance of the microbiological diagnosis is enormous, particularly for detecting the first cases of plague, whose diagnosis on the basis of the clinical pattern alone is almost impossible. The preliminary diagnosis is established on the basis of the microscopic examination of the material, and the final diagnosis on the basis of the isolation and identification of the culture.

Macroscopic Examination

The smears are fixated by complete immersion into Nikiforov's

solution (mixture of equal parts of alcohol and ether) for 20 minutes and the alcohol residues are then burned.

Gram staining is necessary in all instances. At the same time it is useful to stain the smears with Löffler's methylene blue because this method shows the bipolarity more clearly (overstaining must be avoided!).

A few fixed smears should be left unstained as reserves.

Bacteriological investigation

Isolation of cultures is essential in all cases, where the establishment of a reliable diagnosis is necessary.

In typical cases, normal inoculation on agar plates (pH = 7.2-7.3) makes it possible to isolate the culture. It is preferable to carry out the inoculation with a loop by applying the material in streaks. More rarely, the material is distributed with a spatula. The examination of the inoculated plates is carried out within only 10-12 hours. When the cultures are kept at 25-28°, growth in the form of kerchiefs can be observed. Later, within 24-48 hours, individual colonies or growth in streaks are formed. The cultures are kept in the thermostat for 5-7 days.

A complication in the normal course of isolation of a pure culture can occur in cases, when other microorganisms grow on the agar, during examination of putrescent matter and the presence of plague bacteriophage in the starting material.

It is particularly difficult to isolate a culture when a complex of the corresponding antibiotics and sulfonamide preparations has been used for therapy (N. N. Zhukov-Verezhnikov and N. K. Zav'yalova, 1959). Special measures must then be taken for isolating the culture. Intense inoculation of a medium with high sensitivity (see further on) and also an increase in the number of samples taken from the same patient,

are to be considered as the principal measures of this kind.

When colonies of plague bacteria are detected among extraneous bacterial flora, it is necessary to examine carefully all colonies, first with the unaided eye and then under very low magnification under the microscope. The plague colonies normally grow more slowly than the others, particularly during the first 24 hours. They can be discerned among large colonies of extraneous microflora and a certain quantity of culture can be taken with a loop in such a manner as to contain the suspected colonies, and this mixture of microbes can then be inoculated on an agar dish and it can also be used to infect guinea pigs subcutaneously and by rubbing it into the depilated skin. In this case the cultures must be kept at 28° as usual. If possible, it is advantageous to maintain part of the cultures at 15°, which slows down the growth of the extraneous microbes. The isolation of a culture from putrescent material requires special measures. It is possible to obtain a pure culture of P. pestis by inoculating with the object under examination agar which has been mixed with an alcoholic solution of gentian violet or the same dye with sulfite (selective medium, see p. 59). When examining putrescent corpses or cadavers of camels or rodents for plague, it is recommended to inoculate selective and standard media with marrow from the tubular bones. We have succeeded in this manner in isolating a pure culture from putrescent corpses even 4-5 months after burial in summer.

The dye addition markedly retards the development of extraneous flora and particularly of proteus which always threatens specific cultures because of their slow growth. Gentian violet sometimes also inhibits the growth of the plague culture slightly, hence it is necessary to prepare cultures on standard agar as well.

The development of the bacteria during their isolation is often

inhibited by bacteriophage. It can appear even in pure cultures, causing a visible disappearance of growth. To surmount this obstacle, it is recommended to use antiphage serum, applying 0.1 ml of it and distributing it in a uniform layer over the agar surface prior to inoculation with the test material. Subculturing on media with antiphage serum should be carried out as long as the culture has not acquired the capacity to grow on standard media.

When it is believed that the test material contains only small numbers of microbes, high sensitivity media must be used. To this end, 0.1% rabbit or horse blood is added to the nutrient medium. Fields' agar is also used.

Sodium sulfate is added to the medium for the same purpose. V.M. Tumanskiy worked out a selective high-sensitivity medium (blood or antiphage serum with gentian violet).

When the pure culture has been isolated by direct inoculation or as a result of infection of guinea pigs, identification can be carried out on the basis of the following data: a) the external appearance of the colonies on agar; b) typical growth on broth; c) typical morphology of the microbes in smears and gram-negative stain; d) typical pathoanatomical pattern observed on laboratory animals when infected with the pure culture; e) agglutination with specific serum; f) behavior with respect to the specific bacteriophage. Agglutination need not be carried out in this case.

The investigation of the enzymatic properties, the mobility, etc. is carried out only in special cases for differential diagnosis to distinguish it from related species of bacteria (see Tables 64 and 65).

Agglutination with somatic sera must be carried out in a volume of 1 ml in wide test tubes with cotton wool plugs. Dilutions 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280. The emulsion, prepared

from a live culture, grown in the cold, is added in such quantity that a final concentration of 200,000 microbes per 1 ml is obtained. The results are noted after it has been kept at 37° for 2 hours and again after the test tubes have stood in the cold for 12-18 hours.

The agglutination with capsule serum is carried out in the same manner but the culture in this case is grown at 37° and checked for the presence of capsules.

The titers of the somatic sera vary within the limits 1:160 - 1:1280, rarely higher, those of capsule sera within the limits 1:20 - 1:160.

Owing to its greater specificity and greater accuracy of the reaction, agglutination with capsule serum is preferable.

The antiplague sera give a group reaction with the microbe of rodent pseudotuberculosis.

The phage test is carried out on solid media by placing a drop of phage on a fresh culture and on liquid media by adding phage to a three-hour broth culture in a quantity corresponding to 1/10 of the culture volume.

A final conclusion is arrived at on the basis of a study of the complex of characteristics of the test culture. The phenomenon of variability must be taken into account.

The differentiation of the plague microbe and the microbe of rodent pseudotuberculosis can be achieved only by comparison of all the distinguishing characteristics. To these belong mainly the morphology of the colonies, the minimum inoculation dose, the mobility, the behavior with respect to rhamnose, adonitol, to bacteriophage and the pathological pattern of the animals. Each of these characteristics is in itself not constant.

Examination of rodents for plague is usually carried out in a

specially equipped room. Field rodents (gophers, marmots, gerbils, etc.) are caught by means of various methods and the rodent cadavers are also collected on the plains.

Domestic rodents (mice, rats) are caught in traps. The rodents are collected with observation of all the rules for ensuring the safety of the collecting personnel (rubber gloves, lysol, buckets fitted with lids for the collected rodents, etc.). Live rodents are killed with chloroform. For this purpose it is sufficient to throw a piece of cotton wool, soaked in chloroform, into the jar and to close it tightly.

Prior to autopsy, the rodents are immersed in kerosene for several minutes for disinfection and pinned down on a board, half submerged in a tray filled with lysol. The autopsy is performed with sterile instruments kept in a beaker with alcohol. The alcohol on them is ignited when they are to be used. All manipulations are carried out in the complete antiplague suit. The use of syringes and pincers allows complete avoidance of touching the rodents with the hands. After the autopsy, the rodent cadavers are burned or autoclaved; the table, board or floor around the table are drenched with 5% lysol. The instruments are boiled for 40 minutes.

The lymph nodes, spleen, liver, lungs, blood and exudates are examined during the autopsy. Pieces of organs are cut out with scissors and inoculation on agar is carried out by touching its surface with the piece of tissue and subsequent streaking with a loop. The blood taken from the heart with a pipette is used to inoculate agar and broth. Then contact smears - are made. Investigation is carried out in all cases by infecting experimental animals. If a large number of rodents are examined, the socalled biological group test is used. This consists in injecting a guinea pig with an emulsion from the spleen

and lymph nodes of 15-20 of the rodents under examination. The rodents are combined into such groups only when they are of the same species and have been caught in the same place.

If the pathoanatomical pattern, bacterioscopy or other data give rise to even the slightest suspicion of plague, individual infection of test animals is obligatory. In this case, the guinea pig is injected with an emulsion made from pieces of spleen, liver and lymph nodes.

When rodent cadavers which are already decomposed to some degree, are subjected to examination, measures are taken for suppressing the extraneous microflora (see above) and also the highly effective method of inoculating with bone marrow, where the secondary microflora penetrates last during putrefaction is applied.

For this purpose, the femural bone of the rodent is freed of the soft tissues, its surface disinfected with alcohol, which is then burned, and the bone is cut into halves with sterile shears. The surface of the bone marrow taken with a syringe, pipette or thick loop.

Diagnosis of bubonic plague. An inflamed lymph node with its surrounding tissue is termed a bubo. The bubo is the main symptom in this form of plague and appears during the first days of the disease.

The bacteriological diagnosis of bubonic plague in humans is carried out by examination of the contents of bubos and of the blood.

The material is extracted from the bubo with a syringe following preliminary treatment of the skin with alcohol or iodine, from the central and also the peripheral part of the bubo in view of the circumstance that it is more difficult to isolate the germ from the central part when softening of the tissues (necrosis) has taken place. If the bubo is open, the material removed from the ulcer and the firm tissue at the periphery is examined. It is best to effect the inoculations and smears directly with the syringe. 1-2 ml of broth is then taken

with the syringe and given to a guinea pig subcutaneously, intraperitoneally or by inunction. The latter is particularly important, when the smear shows the presence of a mixed infection. When a patient recovers, repeated examination of the bubo contents is necessary, because the patient can be discharged only after two negative findings on the bubos with intervals of 5-6 days a month after clinical recovery.

Inoculation with blood is effected with regard to premature bacteriemia or septicemia appearing at the end of the disease.

It is difficult to determine the optimum ratio of blood and broth volume in each case. Hence, one ought to proceed in the following way: 2 ml of blood, taken with a syringe from the ulnar vein vein of the patient, is placed into a test tube with 2 ml of broth. Having mixed it, 4 ml of the mixture is taken with a graduated pipette, and transferred into the next tube with 16 ml of broth, etc., until the eighth fivefold dilution has been achieved. All test tubes are placed into the thermostat at 28° for 5 days and examined daily. When a growth appears, a control test must be carried out to determine its specificity (smears and inoculations on agar). This method can be used to determine the degree of bacteremia, because the maximum dilution in which growth can still be observed, increases in proportion to the increase in the number of microbes in the blood.

Diagnosis of the septic form of plague. The septic form appears as a result of entry of a highly virulent plague microbe into the organism with a diminished resistance or when infection with plague from human fleas takes place (in some of the cases).

The bacteriological diagnosis of the septic form of plague is established on the basis of the blood test. The inoculations are carried out on broth and on agar plates. The guinea pigs are infected subcut-

aneously or intraperitoneally. Smears are also prepared because with this form of the disease, the microbes can be directly observed in them and sometimes even when present in small numbers (G. A. Wolfertz).

Diagnosis of primary pulmonary plague. When the plague microbe gets into the lungs, primary plague pneumonia develops. The bacteriological diagnosis of this form of plague is established on the basis of tests on the sputum or, more rarely, material from the pharynx.

The material from the pharynx is taken with a sterile cottonwool swab, as far as possible trying to take it from the tonsils and the deep parts of the pharynx; the inoculations and smears are made directly with the swab. The material is then washed off the swab into a test tube with 1 ml of broth by carefully but strongly rubbing it in the latter. When the swab is taken out of the test tube it is pressed against the walls in order to extract the maximum amount of material. The suspension thus obtained is used for infecting guinea pigs by subcutaneous inunction, and intraperitoneally. It should be emphasized that the preliminary microscopic examination of smears sometimes does not give a positive result, because, on the one hand, few plague germs may be present and, on the other, a microflora is present which gives a similar microscopic pattern. It is important to keep this in mind during treatment. Since the time, when pulmonary plague became curable, diagnosis has become more difficult, particularly when the patient has already been given sulfonamide preparations. However, when gram negative ovoids with marked bipolarity are found, the smears must be considered to be suspect.

The sputum is collected in a wide Petri dish; small clots of it (preferably taken from bloody parts at different points) are transferred to the agar surface close to the edge of the dish and there carefully rubbed in on a small area with the pincers. The pincers with the

remaining material are then placed in a sterilizer with 5% lysol for boiling and the material distributed over the whole dish with a loop, not covering more than 1/5 of the inoculated surface with the first streaks. The smears (about 10) are prepared from different parts of the sputum. Pieces of sputum are carefully mixed with pincers into 2-2.5 ml of sterile broth for infecting animals with sputum. The suspension is collected with a syringe and given to guinea pigs subcutaneously (several guinea pigs) and intraperitoneally. When infection is carried out by inunction, a little sputum can be applied to the surface of the shaven and scarified skin and rubbed in as indicated above. The microscopic examination of smears from sputum is of great importance in the diagnosis of primary pulmonary plague, particularly in presence of epidemiological and clinical indications because in combination with these indications it is the basis for establishing a tentative diagnosis and taking the first measures (isolation of the patient and those which have been in contact with him, quarantine of his living quarters, etc.).

The number of plague microbes in the smears during the early fever period is very small, various cocci and other microflora being predominant. In these cases it is essential to sample the sputum repeatedly. Even a few hours after the first sputum samples have been taken from an untreated patient, an increase in the number of bipolar gramnegative organisms and a decrease in the other microflora are observed. This change is evidently rapid and in proportion to the aggravation of the disease, may terminate with a complete disappearance of the secondary microflora from the sputum which then contains a pure culture of plague microbes. The differential diagnosis, distinguishing between primary pulmonary plague and other kinds of pneumonia is often of great importance. A complex scheme of sputum examination has been worked out for

these cases, which enables a differential diagnosis to be established, distinguishing plague, anthrax, glanders, streptococcus, Friedländer and pneumococcus pneumonia and also psittacosis and melioidosis. The complex method is as follows (N. N. Zhukov-Verezhnikov, 1944).

1. 20 smears from sputum are prepared (or, if there is no sputum, from the pharynx by means of a swab). The sampling is carried out repeatedly. Contact smears from lungs, spleen, liver, blood, etc., are prepared after autopsy. They are gram-stained or stained with methylene blue and Romanovskiy-Giemsa dye.

2. Cultures of sputum or pieces from organs are carried out: a) on several dishes with normal agar (pH = 7.2-7.3), the cultures being grown at 28 and 37°; b) on 0.5% blood agar; c) on agar with gentian violet and sulfite; d) on 2% glycerol agar.

3. Infection of the animals is carried out by different methods; a) guinea pigs - subcutaneously, intraperitoneally and by inunction (it is desirable to infect 2 guinea pigs by each method). Infection with a suspension of sputum or a ground-up mixture of pieces of lung, spleen and liver; b) 2 white mice subcutaneously, one intraperitoneally (i.e. excluding coccus and some other types of pneumonia). When the animals die, an autopsy is carried out on them and cultures from the organs and smears are prepared. When necessary, animals are infected with organs from those that have died. When there are several guinea pigs, infected subcutaneously with the same material, one of them is killed 48-55 hours later and smears and cultures prepared from the injection site, the nearest lymph gland and organs.

4. From the grown colonies, new cultures and smears are made and stained by the Gram and Burri-Gin's methods. The mobility is determined by the hanging drop method.

5. The cultures thus obtained are again injected into mice and

guinea pigs subcutaneously and the species identified (staining series, phage test, agglutination, etc.).

6. Even if the isolated germ does not belong to any particularly dangerous type (for example, pneumococcus, streptococcus) its identification is nevertheless completed in the prescribed sequence. The characteristics of germs subject to differentiation are given in Table 65.

7. By means of separate subcultures, the resistance to antibiotics and chemotherapeutic preparations is determined as early as possible. To accelerate the procedure, the qualitative method of growing a microbe gason on agar plates with circles of filter paper, impregnated with a certain preparation is used. During the further course of investigation of the strains, the resistance is definitely determined by allowing different concentrations of the preparations to act on them. Finally, it is useful in some cases to determine the sensitivity of individual colonies.

Diagnosis of the cutaneous form of plague. The bacteriological diagnosis of the cutaneous form of plague is made on the basis of an examination of tissues from the pharynx and the thickened tissue around an ulcer or puncture of a plague carbuncle. Otherwise the procedure is the same as with the bubonic form.

When intestinal infection is suspected, the feces are tested. When blood is present, the bloody part of the feces is taken. Otherwise the procedure is the same as in the examination of putrescent material.

Diagnosis of meningeal affections. If plague meningitis is suspected, spinal fluid taken under sterile conditions is used as initial material. Otherwise, the procedure is the same as with the septic form.

Examination of cadavers. During autopsy on a cadaver, which must be carried out in the antiplague suit and with observation of all the rules, the blood and pieces of the lungs, spleen, liver, and glands

(bubos) are taken. The parts of the organs in which the affection is suspected to be present, are taken, and pieces are cut from every lobe of each lung. Large, wedgeshaped pieces are cut out from the bubos and the spleen.

The autopsy material is then further examined in the same manner as the cadaver material obtained from rodents (see above). It is necessary, however, to make a large number of smears and cultures from each piece of organ and also to use a large number of guinea pigs for infection by means of all three methods (subcutaneous, intraperitoneally, by inunction).

Detection of plague bacteria by means of the biological test

Guinea pigs are most easily infected. White mice can be used for differential diagnosis or when guinea pigs are not available. The infection is carried out by different methods, depending on the nature and type of material. Subcutaneous infection must always be carried out. The intraperitoneal injection of the material speeds up the diagnosis considerably but is relevant only in cases when it is assumed that the material is not contaminated, and only in parallel with the above described methods. Putrescent material is rubbed into the skin of a guinea pig at the same time.

Infection by inunction is carried out in the following manner. A skin area of 4 x 5 cm in the region of the abdomen of a healthy guinea pig is shaven. The area thus freed of hairs is then moistened with sterile physiological solution (not with alcohol) as well as the hair around it. The skin is then scarified with a scalpel fairly energetically but not so strongly that it bleeds. 2-3 drops of a preferably very thick emulsion prepared from the test material, are then applied to the scarified area with a pipette and rubbed in with the flat part of the scalpel until the scarified part becomes dry. The material is

applied and rubbed in under a large glass funnel. The subcutaneous infection is carried out by injection on the internal surface of the thigh or the lower part of the abdomen after the hair has first been removed from this area and it has been disinfected with alcohol. When intraperitoneal infection is carried out, it is preferable to pierce the skin first with a sharp, sterile needle, then, using the opening thus formed, to inject the material with a blunt needle. The careful selection of the parts of the syringe and care in carrying out the injection are essential. The assistent should immobilize the animal properly.

When a sufficient number of microbes is present in the test material, the guinea pigs infected by inunction, usually die on the 5-7th day, those infected subcutaneously, on the 4-6th day and those infected intraperitoneally, on the 3-5th day. These are average times. They can vary within wide limits. The guinea pigs may even survive, hence it is recommended to kill the animals on the 7-9th day after infection. In addition to preparing cultures, fresh guinea pigs can then be infected with the emulsion from the organs of the killed animal. When the presence of phage is suspected, it is recommended to mix the emulsion with an equal volume of antiphage serum and to inject this mixture, after having let it stand for an hour, into a guinea pig subcutaneously.

Earlier diagnosis is possible by examining one of the two guinea pigs, which have been infected subcutaneously, after only 1-2 days: the region of the lymph nodes near the injection site is palpated and when the nodes are enlarged or when several infected animals are available, even without this symptom, the animals are killed with chloroform and an autopsy is performed on them.

Contact smears and cultures are made and, if necessary, repeated

infection of animals is carried out, using material from the point of injection on the lymph nodes and the tissue around the injection site, which is usually edematous. The pathoanatomical pattern must be observed during the autopsy of the animals.

The following elements of the pathological pattern are of diagnostic importance: infiltration of the subcutaneous blood vessels, increase and hemorrhagic infiltration of the regional lymph nodes, presence of necrotic nodules in the spleen (more rarely in the liver), dystrophy of the parenchymatous organs and exudative phenomena (with hemorrhagic appearance). In the case of intraperitoneal infection, the lesions of the mesenteral lymph nodes and the formation of necrotic nodules are usually less marked and may be completely absent. A viscous exudate is found in the abdominal cavity which is relatively clear. The parenchymatous organs show a marked granular dystrophy. In typical cases, an enormous number of gram-negative, bipolar, ovoid, and polymorphous microorganisms are found in the contact smears from the parenchymatous organs.

Rapid methods of bacterialogical examination

I. The method of rapid detection of the plague germ by means of bacteriophage, introduced into the test material (V.M. Tumanskiy and coworkers) is used for examining objects which are of basic practical importance: material from patients, material from fresh or putrescent cadavers of humans and rodents, ectoparasites, water, dust, soil, food products, etc.

Analysis method: the test material is placed on 3 agar plates (Hottinger or Martin agar, with 0.2% hemolysed blood and gentian violet 1:100,000 - Tumanskiy medium) in a quantity of 0.05 ml (1 drop). By means of a loop, plague bacteriophage (diluted 1:10) is applied immediately to the test material on the first agar plate and distributed

uniformly on the agar in the dish by means of a glass spatula. The test material in the second agar dish is uniformly distributed over the entire surface with a glass spatula and then on the gason thus obtained, a drop of plague bacteriophage is applied and allowed to flow in such a manner as to form a track across the agar plate. In the third agar dish, the test material is distributed with a spatula, as in the first two cases, but bacteriophage is not added (control).

The results can be observed only $2\frac{1}{2}$-3 hours after the plates have been placed in the thermostat.

When a large number of plague microbes is present in the test material, small bacteriophage colonies are visible on the initial growth of the plague microbe (first dish) and a sterile streak on the second plate, after only 2 hours.

II. The method of rapid plague diagnosis is based on the property of plague bacteriophage of multiplying quickly (30-40 minutes) in presence of plage microbes (I.V. Domoradskiy and coauthors).

Analysis procedure: Sufficient bacteriophage is added to 25-50 or 100 mℓ of the test and control liquid to obtain a phage dilution of 1:50 - 1:100.

The test and control sample are left in the thermostat for 45-60 minutes at 37°, then 0.5 mℓ of the fluid is taken from each sample and diluted 10 x with broth. 10 consecutive dilutions (up to 10^{-10}-10^{-11}) are made up from the first dilution. Separately, 0.5 mℓ is taken from the test and control dilutions and added to 2.5 mℓ of semiliquid agar (0.7%), heated previously ro 48-50°. 0.1-0.2 mℓ of 24 hour old indicator culture (vaccine strain of the plague microbe) is also added; the concentration of this suspension is 20 billions microbes per 1 mℓ.

The mixture is carefully stirred and poured in a uniformly thick layer on the surface of agar in a plate (2% Hottinger agar). The cul-

tures are incubated at 37°. The results can be gauged after 3-3½ hours. Sterile spots are visible on the massive growth of the indicator culture. The results are evaluated on the basis of the difference in the titer of the phage on the dishes of the control and test series.

III. Method of rapid plague diagnosis, based on the quick growth of cells of P. pestis on an enriched and selective medium for this microbe (Ye. I. Korobkova).

Analysis method:

1. The suspected material is used in a quantity of 0.2-0.3 ml to inoculate 4 test tubes containing enriched medium (semiliquid agar with blood and gentian violet), and 0.2-0.3 ml of bacteriophage is placed on the surface of the medium in one of the inoculated test tubes. All test tubes are incubated at 30-35°. The remaining test material (0.1 ml) is inoculated on agar dishes of the same composition.

2. After 3 hours of growth, the test tubes are examined and where macroscopic growth is apparent, 2-3 drops of the suspension is transferred by means of a loop to 2 object glasses. The smears are carefully dried and fixed in Nikiforov's mixture, stained with Löffler's methylene blue and by the gram method and allowed to dry a little without drying with a filter paper. When the test is positive, chains are visible in the smears, consisting of gram-negative bipolar bacilli. This kind of growth, however, is absent in the test tube containing the phage. At the same time, the bacterial growth (turbid layer of the medium) is drawn off from the surface of the medium in one of the test tubes and this suspension injected intraperitoneally (0.4 ml) into several white mice. 8-10 hours later, the agar dishes are examined, and the initial growth of P. pestis can be seen.

3. 10-12 hours after infection, the mice are killed and semiliquid agar of the same composition is inoculated with the exudate from the

peritoneal cavity and pieces of organs (liver, spleen, lymph nodes). Agar dishes are inoculated at the same time. Examination is begun 4 hours (test tubes) and 8 hours later (dishes). Thus, a preliminary answer can be given 4 hours after the beginning of the test and a final diagnosis, 18-20 hours later.

TABLE 64

Differential-Diagnostic Characteristics

	Microbe	
	Past. pestis (R-form)	Past. pseudotuberculosis rodentium (R-form)
Mobility	Immobile	Mobility appears during growing of the culture in the cold
Shape of colonies	Tuberous, coarse, sometimes with a fringe which is sharply distinguishable from the center, cloudy, chromogenic (typical strains	Tuberous, coarse with a fringe which is not sharply distinguishable from the center, more transparent, more often chromogenic
Inoculation dose (minimum)	Grows on ordinary media only when inoculated with several thousand cells	Grows on ordinary media when inoculated with some tens of cells
Growth with inoculation in the form of a continuous streak of pure culture	Grows on 60-70% of the streaks, the growth is markedly diminished in proportion to the distance from the first streak (Typical strains)	Grows, as a rule, on all streaks, growth uniform
Pathogenicity for laboratory animals	Kills guinea pigs and rats. Typical changes in the guinea pigs	Kills guinea pigs and, more rarely, rats. In guinea pigs infected subcutaneously, nodules in the liver and spleen are found in typical cases; with intraperitoneal infection, a thickening of the omentum and coating on the intestines are observable

Agglutination with anti-plage serum	Agglutinates to the full titer. New strains may not be aglutinated	Is often agglutinated to the full titer by somatic serum, more rarely by capsule serum
Behavior towards plague bacteriophage	Lysed up to the titer (except the modified strains)	Not lysed, as a rule
Fibrinolytic properties	Has fibrinolytic properties	As a rule, does not have fibrinolytic properties
Modification of the pH of the medium	Slow alkalinization at initial pH = 5.7	More rapid alkalinization at initial pH = 5.7
Lactose	-	-
Glucose	+K	+K
Glycerine	Some strains - Some strains +K	+K
Maltose	+K	+K
Adonite	Usuall -	+K
Rhamnose (isodulcite)	- (rarely +K)	+K
Raffinose	-	Sometimes +K, more often -
Dextrose	-	Sometimes +K, more often -
Sucrose	-	Sometimes +K, more often -

Symbols: -) No fermentation of carbohydrate; +K) fermentation of carbohydrate to form acid.

IV. The fluorescence-serological method by means of which it is possible to detect P. pestis in air, water, food products, etc. (see p. 74) deserves great attention.

V. The method of rapid diagnosis, based on the antibody neutralization reaction (M.I. Levi and A.G. Momot) can be used with sucess in the examination of coarse putrescent material. For this reaction, a suspension from spleen, liver or bone marrow of dead rodents is required. 1% (40%) formalin is added to the suspension. The suspension thus obtained is transferred to a sterile test tube and kept at 56° for 30 minutes. The heated suspension is filtered through paper.

28 test tubes are required for this test, arranged thus: 1st

series 10 test tubes, 2nd, 3rd and 4th series, 6 test tubes each.

0.25 ml of the filtrate with the index 2 is added to each of the test tubes of the 2 first series. Series 3 tubes (0.25 ml each) are titrated against previously known positive antigen (a suspension of plague microbes of strain EV76 is incubated for 72 hours at 37° with a concentration of 10 million microbes per 1 ml, killed with formalin).

0.25 ml of antiplague serum (1-4 serum units) is added to each test tube of the 1st (test), 3rd and 4th series.

0.25 ml of solvent (1% solution of normal rabbit serum) is added to each test tube of the 2nd series.

Following incubation at a temperature of 37° for 1-2 hours, a drop of erythrocytes (sensitized by capsule antigen of the plage germ) is added to each of the 28 test tubes, then the test tubes are shaken carefully and allowed to stand at room temperature. The reaction is observed after 14-18 hours. Typical for a positive reaction is the settling of the erythrocytes to the bottom of the test tubes in the form of a button. The negative reaction does not give an agglutination of the erythrocytes and the button phenomenon is absent.

Detection of the germ in the environment

Examination of fleas and other ectoparasites. Paramount importance is being attached at the present time to the investigation of fleas. The fleas are sorted out into species for the purpose of the microbiological investigation. Subsequently, individual fleas and even small groups (30-50 specimens) from each species can be examined. Every flea on which an autopsy is to be performed, is immersed in 96° alcohol for a few minutes and then rinsed two or three times with sterile physiological solution. It is then dissected under a magnifying glass and the stomach and intestine removed and made into an emulsion in physiological solution. The suspension thus obtained is injected into

laboratory animals and used to inoculate nutrient media (V. A. Bychkov and A. K. Borzenkov).

When small groups of fleas are to be examined, they are washed with several portions of sterile physiological solution, ground in a mortar and the suspension thus prepared injected intraperitoneally into laboratory animals, while part of the suspension is used to inoculate media containing antiplague antiphage serum. The same method is used when ticks and their larvae, nymphs, bugs and lice are investigated.

Water is tested by culturing and infection of guinea pigs. The material in the water is concentrated first by two parallel methods, by methods of precipitation and by means of membrane filters.

The testing of air to determine the presence of suspended droplets and particles containing microorganisms (for example, in wards where patients with pulmonary plague are present) is carried out, as indicated on p. 266.

TABLE 65

Differential-diagnostic Characteristics of the Germs Causing Acute Pneumonia

		Culture from the sputum of a patient or the lungs of cadaver									
Gram-stained smear from the patient's sputum	Smear from lungs of a cadaver, gram stained	On normal agar and 2% glycerol agar at 28°	On normal agar and 2% glycerol agar at 37°	On 0.5% blood agar at 37°	Results of infection of guinea pigs (subcutaneously, by inunction, intraperitoneally)	Results of the infection of white mice, subcutaneously	Smears from a pure agar culture, gram-stained	Smears from pure agar culture, stained according to Burri-Gin	Growth of the isolated pure culture on meat-peptone broth	Growth on broth containing bile	Test with specific plague bacteriophage: drop of phage on the surface of agar, freshly inoculated with a suspension of pure culture.
I. Pulmonary plague											
At the climax of the disease numerous gram-negative staining ovoid rods without capsules, partly bipolar	The same as in Col. 1	On normal agar 24-48 hours later growth of small, fairly delicate typical colonies	On normal agar, the same as Col. 3 but the consistency of the colonies is slightly more viscid. Growth slowed down. More copious secondary microflora. In the smears stained by the Burri-Gin's method, small capsules can be discerned	It is difficult to observe the plague colonies. Normally copious growth of secondary microflora	With subcutaneous infection, death on the 4-6th day. When infection is carried out by inunction, on the 5-7th day	Death on the 4-7th day	Fairly uniform rods with rounded ends; gram-negative. Bipolarity not very marked	If the culture was grown at 28°, capsules are absent. In smears from cultures, grown at 37°, small capsules can be seen	The broth remains clear, growth is observed in the form of flakes which gradually accumulate on the bottom, where a loose precipitate forms. In the smears, the rods may be arranged in chains	-	At the point, where the drop has been applied, sterile spot, which gradually increases in size. Around the spot, typical growth of plague culture.
II. Pneumonia, caused by Friedländer pneumobacillus											
Varying number of gram-negative rods with rounded ends. Usually, but not always, marked capsules Sometimes bipolar forms are encountered, normal secondary microflora (cocci), etc.)	Normally numerous gram-negative rods with rounded ends and capsule. Individuals without capsule are also found Bipolar forms can be seen in large numbers. Rods with inclusions of blue color occur in rare cases	After 24 hours, growth of small, not highly typical colonies, which are transformed only 32-48 hours later into very large, white-gray, circular, smooth, transparent colonies, which are moist and shiny in consequence of the presence of abundant slime. The colonies usually fuse	The same as Col. 3 but the typical slimy large colonies grow faster (within 18-32 hours)	The same as Col. 4. Blood interferes to some degree with the identification of the colonies	With subcutaneous infection, death on the 3-7th day	Death within 18-32 hours. Autopsy reveals increase in the size of lymph glands and spleen. In the smears, usually copious numbers of gram-negative rods with capsule. Individual rods with bipolar stain	Uniform, sometimes elongated rods with rounded ends, gram-negative. Slight washed-out contours indicate presence of capsules	At 28 and 37°, a massive capsule is observed, sometimes attaining very large dimensions	The mature culture (24 hours) clouds the broth uniformly: strands of slime which gradually settle on the bottom, can be seen	-	At the point of application of the drop of plague bacteriophage, typical slimy growth as in the remainder of the dish.
III. Croupous or Catarrhal Pneumonia; Caused by Pneumococcus											
At the climax of the disease, gram-positive diplococci, enclosed in capsule. Varying quantity of secondary microflora	Usually numerous gram-positive diplococci, enclosed in capsule	Growth of pneumococci is absent or extremely sparse	The same as Col. 5	After 18-48 hours, growth of small, circular, flat colonies with dark tinge. The blood around the colonies is altered and has a dark-green tinge	With subcutaneous and intraperitoneal infection, death on the 3-8th day. In the smears, capsular, gram-positive diplococci. When infection has been carried out by inunction, death only in exceptional cases	Death after 18-49 hours. In the smears everywhere, including the blood, numerous gram-positive diplococci, surrounded by capsule	Gram-positive diplococci, rarely arranged in chains	Diplococci with marked capsule	On broth with bile, growth, as a rule, absent	-	The plague bacteriophage has no effect
IV. Pneumonia Caused by Streptococci											

are found among the secondary microflora	cocci, arranged in chains, partly in the form of diplococci	very small, round, protuberant, opaque, grainy colonies. In the smears, gram-positive cocci	lies, described in Col. 1	of streptococcus	days. In the smears, gram-positive streptococci	gram-positive streptococci, sometimes with capsule	together in pairs	with slimy streptococcus	suspended growth, flakes which gradually settle on the bottom	flakes.	has no effect

V. Pulmonary Form of Anthrax

Large, gram-positive rods with truncated ends	Large, gram-positive rods, sometimes surrounded by capsula	After 24-48 hours growth of fairly large grayish-blue colonies with coarse center and periphery, consisting of twisted threads	The same as Col. 1 but after 18-32 hours	The same as in Col. 4	With subcutaneous infection, death on the 3-6th day, with intraperitoneal infection, on the 24th day	Death after 24-48 hours. Degeneration of the organs. In the smears, numerous typical gram-positive rods, surrounded by capsule	Fairly large gram-positive rods with truncated ends	Typical rods, more or less marked capsule	The broth usually remains clear, growth in the form of flakes; precipitate on the bottom. In the smears, partially chains.	-	The plague bacteriophage has no effect

VI. Psittacosis-type Pneumonia

Secondary, sparse, more rarely, copious microflora. The germ cannot be detected	The germ is not visible. In the sections of pieces of lung tissue, desquamative catarrh of the alveolar wall with disengagement of the alveolar epithelium in the lumen of the alveole	The germ cannot be cultured on normal media	The same as in Col. 3	The same as in Col. 3	The guinea pigs fall ill, but die only rarely	Death on the 5-14th day; in the smears from the organs, very small, hard-to-distinguish, bodies of the germ (0.2 × 0.35), in the form of cocci or elongated forms. Bipolarity possible in the latter case. Typical small necrotic foci in the spleen. The cultures remain sterile or show contamination	Cannot be grown on ordinary media	The same as in Col. 8	The same as in Col. 8	-	The plague bacteriophage has no effect

VII. Glanders Pneumonia

Small, sometimes granular gram-negative rods may be found in addition to the secondary microflora	Gram-negative rods, sometimes granular, may be found	On glycerol agar, after 24-48 hours, growth in the form of slimy, round, grayish-white colonies, gradually assuming a yellow-brown tinge, may be found. Cultures on potato, on which the glanders microbe grows in the form of honey-drop like colonies, is indispensable. Later on, the culture assumes a dark reddish-brown color	The same as Col. 4 but growth is more rapid		Death on the 8-20th day. In the males infected subcutaneously and particularly those infected intraperitoneally, necrotic orchitis (Strauss phenomenon) develops after 2-4 days: the scrotum reddens, the testes become considerably enlarged and necrotic nodes form in them. Ulcers are formed on the scrotum. The pus taken from incisions in the scrotum, contains glanders microbes, which are easy to grow on glycerol agar or potato	Death at different times	Small, gram-negative rods. Granularity is observed sometimes		Cloudy growth within 5 days, slimy film, slimy deposit on the bottom.	-	The plague bacteriophage has no effect

Phage Titer Crowth Reaction

D.M. Gol'dfarb, Professor

Plague bacteriophage, supplied by the Institute "Mikrob", has been proposed as indicator phage, as standard culture, the avirulent strains: EB, No. 1 and No. 17 (Domoradskiy).

The application of the phage titer growth reaction for the indication of plague microbes is based on experimental studies, carried out by Domoradskiy and coworkers, who showed that by using the phage titer growth reaction, it is possible to detect 1 million plague rods within 3-3½ hours. Water, blood, organ extracts, bubo contents, etc., can be used as test material. Pieces of tissue and mechanical admixtures do not interfere with the indication of plague bacteria. Because the concentration of microbe bodies in different test objects is unknown, the test material must be taken in a volume of 3-5 liters when closed water reservoirs, washings from external objects or soil extracts are to be tested. The phage titer growth reaction is carried out with 50 mℓ without pretreatment of the liquid, 50-100 mℓ is incubated for growing bacteria in it and in the remainder of the test liquid, the plague bacteria in it and in the remainder of the test liquid, the plague bacteria are concentrated by adsorption on aluminum hydroxide or starch. To 3-5 liters of fluid, 10% alum solution is added in a quantity of 10-25 mℓ per 1 liter of fluid. The flakes of aluminum hydroxide appear very quickly. If this precipitate is not formed, 5-10% sodium carbonate solution is added to the test liquid dropwise. Following the settling of the coagulant, the supernatant liquid is drawn off by means of a syphon and the precipitate which contains the bacteria, is transferred to a flask containing 50 mℓ broth. If the phage titer growth reaction is carried out on the residue, the control samples contain aluminum hydroxide obtained by precipitation in a liquid which is known to be

free of plague germs. The sensitivity of the phage titer growth reaction increases considerably if preliminary concentration of the plague microbe or preliminary growing is carried out in the test material. In the latter case, gentian violet (1 ml of 0.1% water-alcohol solution per 100 ml of medium) is added to the entire material in order to suppress the secondary microflora which prevents the growing of the plague germ.

Because the plague bacillus is present in large numbers in the organs of animals which have died of the plague, in human cadavers, the sputum of patients and bubo contents, all these objects can be examined without preliminary treatment and only in the case of a negative result is it essential to carry out another test involving growing from the remaining portions of test material which have been stored in the cold. When solid objects (organs, tissues) are to be analyzed, they are first ground up in a mortar with glass or sand and then used to inoculate broth, using 50 ml per 3-5 g of the material.

The method of analysis does not differ in principle from the scheme of phage titer growth reaction, described for other infections. As the plague phage is thermally less stable than other indicates phages, the test and control samples, following their dilution before the titration according to the method of Gracia, are kept at 50° for 60 minutes or at 58° for 10 minutes.

Nutrient Media

Selective medium of G. N. Lenskaya. To 100 ml of melted agar 1 ml of 2.5% freshly prepared acqueous solution of sodium sulfite and 1 ml of saturated alcoholic gentian violet solution, diluted 1:100 with distilled water, is added.

Selective medium of Ye. I. Korobkova. To broth made up from Hottinger's digest, 0.15% agar is added. To sterile semiliquid agar, 0.3%

hemolyzed blood and 1:200,000 gentian violet is added. 5 ml of the medium is filled into each test tube.

TULAREMIA

N. G. Olsuf'yev, Professor, Member of the AMN USSR

The germ of tularemia, Francisella tularensis (McCoy et Chapin), belongs to the family Brucellaceae of the order Eubacteriales. Differences of opinion existed in the literature for a long time concerning its position in the system of microorganisms; some authors (Berdzhi [?], 1934, 1957) assign it to the genus Pasteurella, others (Topley and Wilson, 1929, 1955; Stutzer, 1936) to the genus Brucella. In 1947, K. A. Dorofeyev published a proposal to assign the tularemia germ to a special genus Francisella, based on the considerable differences between this microbe from Pasteurella and Brucella. This proposal was subsequently supported by Olsuf'yev and coauthors (1959), Philippe and Own (1961) and other researchers.

The American strains of the tularemia microbe differ from the European-Asian by their greater pathogenicity for laboratory animals and humans and also by certain biochemical properties. This indicates the presence of two geographical subspecies of the tularemia microbe - an American subspecies (New World or neoarctic) and European-Asian (Old World or paleoarctic). The European-Asian subspecies has recently been given the name Francisella tularensis palaearctica, while the American subspecies retained its traditional name Fr. tularensis tularensis.

Morphology of Fr. tularensis

The tularemia bacteria have very small dimensions, 0.3-0.5μ, and even very small cells with a diameter of 0.1-0.2μ, which can pass through certain bacterial filters (for example, the Seitz filter) are sometimes encountered. When grown on artificial nutrient media, the

tularemia microbes usually have the shape of a very small coccus, while they are most often found in animal organs in the form of coccobacteria (Fig. 97). In cultures on nutrient media, the tularemia bacteria show a polymorphism, which is particularly marked in the American subspecies. The microbe is immobile, does not form spores and has a small capsule. Typical for the cultures is the formation of slime by the bacteria, which is easily detected when smears on glass slides are prepared.

The tularemia bacteria can be stained with all the dyes normally used in laboratory practice but less vividly than many other bacteria. Tularemia bacteria are stained gram-negative. On contact smears from animal organs, tularemia bacteria are not stained in a bipolar manner which distinguishes them considerably from the microorganisms of the genus Pasteurella.

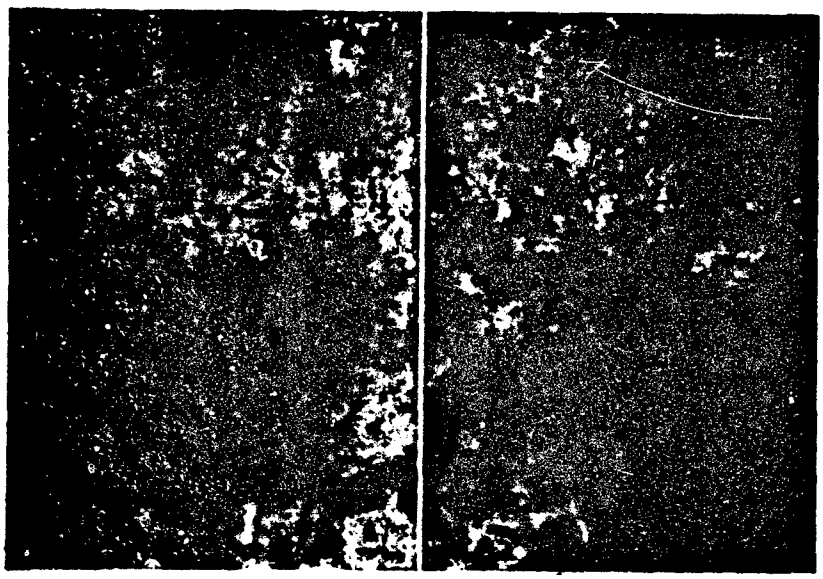


Fig. 97. a) Tularemia bacteria from a culture on coagulated yolk medium. 1000×; b) tularemia bacteria in the blood of a water rat, found dead in its natural habitat. 800×.

Biology of Fr. tularensis; culture properties

The tularemia microbe is capricious with regard to growing on artificial nutrient media. It will not grow on normal meatpeptone agar on in broth. The microbes can be grown on yolk media and also on agar media to which cystine and other nutrients, particularly blood, have been added. The temperature optimum is 36-37°. It is strictly aerobic. Isolated colonies can be readily obtained when dishes with Yemel'yanova (see p. 875) or Francis (see p. 876) medium are inoculated with it. The colonies on these media are of a whitish color with bluish tinge, circular, with a smooth fringe, protuberant, smooth, shiny, attaining (after several days growth) a diameter of 1-2 mm and over (Fig. 98) when inoculation is carried out with dilute material. The colonies of virulent tularemia bacteria have the same morphology and biological properties as the S-form. In liquid nutrient media, the tularemia bacteria proliferate considerably less well than on solid media, growth being observed only on the surface of the medium, which is evidently connected with the aerobic nature of these bacteria. Satisfactory culture results can be obtained either by adding colloids to the liquid medium (yolk from hen's eggs, agar, etc.) or by aeration of the medium.

The capacity of fermenting carbohydrates and alcohols is limited in tularemia microbes and can be reliably determined only when special solid media with limited protein content and accurately determined pH are used. Hiss' media are not suitable for this purpose, because the tularemia bacteria do not proliferate in them. Tularemia bacteria can ferment to an acid glucose, maltose, in some cases also levulose and mannose, while the American strains can also ferment glycerol, Lactose, saccharose, rhamnose, mannitol and a number of other substances are not fermented by tularemia bacteria. The tularemia bacteria form hydrogen sulfide and reduce certain dyes.

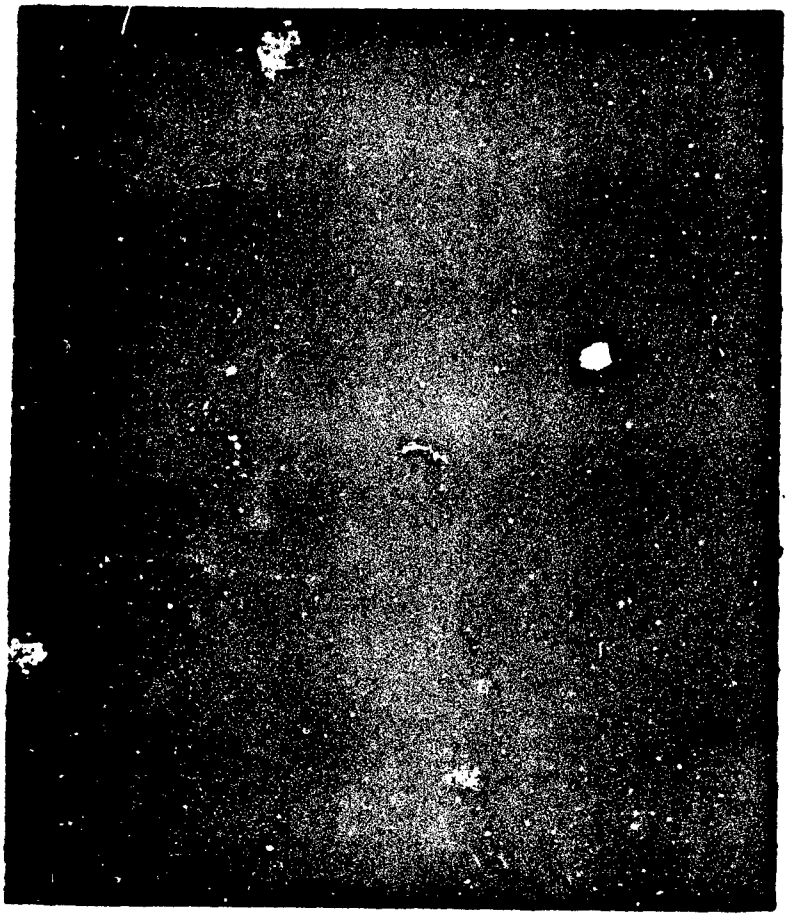


Fig. 98. Colonies of tularemia bacteria (S-type) on blood fish-yeast agar (with cystine and glucose). 7×.

Stability towards physical and chemical factors

In contrast to other gram-negative pathogenic bacteria, the tularemia microbe survives for relatively long periods (without proliferation) in water and also in other objects of the environment under conditions of low temperature. Thus, at 4°, tularemia bacteria can survive in water or soil over 4 months without any decrease in virulence. When the temperature is increased, for example to 20-25°, the microbes die in water within 10-15 days. In corn and straw, tularemia bacteria survive at a temperature below 0° for up to 6 months and at 20-30°, up

to 20 days. Tularemia germs have been often detected under natural conditions in the water of streams and wells and also in straw and other objects, which is of great epidemiological significance.

The pathogenic organism can survive for more than 3 months in frozen cadavers of rodents which have died of tularemia, but when these thaw out and the ambient temperature rises, these survival times are correspondingly shortened.

Tularemia bacteria are sensitive to high temperatures - boiling kills the microbes instantly and heating to 60° causes their death within 20 minutes. Under the action of direct sunlight, tularemia bacteria die within 20-30 minutes, while their viability in disperse light is preserved for up to 3 days.

Tularemia microbes are not resistant to the usual disinfectants lysol, phenol, chlorine, corrosive sublimate, etc. Tularemia bacteria are particularly sensitive towards ethyl alcohol and die within a minute when exposed to it.

Antigen structure

A differentiation of Fr. tularensis into serotypes is unknown to date and serological differences between the American and Euro-Asian subspecies of this microbe have not been found. The tularemia bacteria contain two antigen complexes: a cell wall (Vi) and somatic (O) complex. The virulent and immunogenic properties of the cell are connected only with the cell wall antigens; when they are lost, the tularemia bacteria become avirulent and nonimmunogenic.

During Vi-agglutination, typical for virulent cultures, a stable agglutinate settles out on the bottom of the test tube, which is broken up into coarse flakes by shaking; during O-agglutination, which occurs only in avirulent cultures, an unstable agglutinate is precipitated which is easily broken up into small flakes or an almost homogen-

eous suspension by shaking.

The tularemia bacteria show an antigenic similarity with brucellae: the specific tularemia agglutinating serum with high titer can agglutinate brucellae, when the dilution is not too great, and brucella serum can agglutinate tularemia bacteria. Some saprophytic bacteria also have the capacity to be partly agglutinated by tularemia serum. Bacteriophagy can be observed on museum strains of tularemia bacteria but this phenomenon can be observed only when dishes with media specially selected for this purpose are inoculated.

Pathogenicity

The strains of tularemia bacteria, isolated in natural foci from rodents, ticks and other objects and also from human patients, have very similar characteristics, including the virulence. Differences are observed only between strains which belong to different geographical subspecies of the germ - the American and Euro-Asian. When culturing is carried out on artificial nutrient media, an attenuation of the tularemia bacteria takes place and their transformation from the virulent S-form to the avirulent and nonimmunogenic R-form. The vaccine strains of the tularemia microbe are an intermediate form of variability between the virulent S-form and the avirulent R-form, and are termed SR-variant. They have a residual virulence for animals susceptible to tularemia, for example, white mice.

The pathogenic properties of the tularemia microbe are connected mainly with toxic substances representing endotoxins.

The tularemia microbe is pathogenic for many species of mammals and particularly rodents but the degree of its pathogenicity is not the same for all species. Field mice, water rats, rabbits, hamsters, gerbils, domestic mice, shrews and some other rodents and insectivorous animals have the greatest susceptibility and infection sensitivity

to tularemia (group I). With these animals, even if infection takes place with minimum doses, the disease assumes the form of acute septicemia, they discharge the germ in large numbers with the urine and feces and die with extraordinary intense bacterial insemination of the internal organs and the blood.

Much less sensitive to tularemia are gophers, marmots, gray and black rats, hedgehogs and other animals (group II). When these are infected with tularemia, they normally survive and their excretions contain only small quantities of bacteria.

Among carnivores, such as foxes, cats, polecats, etc., the disease may proceed without visible clinical symptoms (group III) even when infected with massive doses.

For ungulates and also birds and coldblooded animals, the tularemia microbe is only slightly pathogenic in most cases.

The human organism is similar to that of the animals of group II with regard to its susceptibility and infection sensitivity to tularemia. In humans, tularemia is a feverish disease with relatively benign course which does not represent an infection hazard for other people. The germ can enter the human organism by various pathways, including the integument of the skin, the mucosa of the eyes, the oral cavity, the gastrointestinal tract and the respiratory pathways which is also the reason for the great diversity in the clinical forms of this disease. The mortality of tularemia in the USSR is under 0.5%, in contrast to the US and Canada, where it is 5-6% (without treatment), which is connected with the greater virulence of the germ, occurring on the territory of North America.

Laboratory Diagnosis of Tularemia in Humans

The use of two reactions is entirely sufficient for the diagnosis of tularemia in a patient: the cutaneous tularemia test and the agglu-

tination reaction (using the volume method). Other immunological tests are either technically more complex (for example, the complement fixing reaction) or as yet insufficiently standardized (for example, the hemagglutination test). Only specially equipped laboratories can carry out isolation of germ cultures from the patient and this method is used mainly for research purposes.

Serological investigation

Agglutination reaction. The most widely used and at the same time quite accurate method of serological tularemia diagnosis is the agglutination test. It is used in the examination of tularemia patients or of reconvalescents (retrospective diagnosis) but it can also be used for the study of the immunological state of the organism, vaccinated against tularemia.

The agglutination test is negative or only slightly positive in patients during the first 7-10 days after the beginning of the disease, the titer of the serum increases towards the 3rd week and in the 4-6th week of the disease attains the maximum values (1:400 – 1:800, rarely more, 1:1600 – 1:3200). Having attained its maximum, the agglutination titer begins to decrease slowly which is normally observed from the 8th week since the beginning of the disease onwards; within 6-12 months, the serum titer has dropped to 1:50 – 1:400, then to 1:10 – 1:40 and may remain on this level for some years.

Relatively late detection of agglutinins in the blood (during the 2nd week of the disease) makes it difficult to use the agglutination test for an early diagnosis of tularemia, while the long retention of the agglutinins makes it possible to use the test for retrospective diagnosis. The specific agglutinins are absent in the blood of persons who have not contracted tularemia and have not been vaccinated against it.

The blood for the test is taken from patient's ulnar vein or from the head of a finger with observation of the rules of asepsis. The agglutination test is carried out by the generally used volume method. Tularemia diagnosticum (a suspension of tularemia bacteria killed with formalin) is used as antigen, which is diluted to a concentration of 1 billion microbial cells per 1 ml.

0.5 ml serum, diluted with physiological solution 1:12.5; 1:25; 1:50; 1:100; 1:200, and 1:400 is placed into test tubes. 0.5 ml of the diluted diagnosticum is added to each test tube. In this manner, an actual dilution of the serum of 1:25; 1:50; 1:100; 1:200; 1:400, and 1:800 is obtained, containing 500 millions microbe cells in 1 ml in every test tube. In addition, a control test tube, containing 0.5 ml antigen and 0.5 ml of physiological solution (antigen control) is set up. To check the serum, the original dilution without addition of antigen is used. When the additions have been carried out, the stand with the test tubes is shaken and placed into the thermostat (37°) for 2 hours, after which the preliminary recording of the results is carried out. The test tubes are then left to stand at room temperature overnight. The reading of the agglutination test results is carried out with the unaided eye 18-24 hours after the beginning of the test. The evaluation is carried out on the basis of the degree of transparency of the fluid in the test tubes and the quantity of microbes (agglutinate) settled on the bottom:

1) marked positive reaction - complete clarification of the fluid with microbe bodies settled on the bottom in the form of an "umbrella", designated by four plus signs (++++);

2) positive reaction - considerable clarification of the fluid as compared with the control tube and formation of a clearly visible precipitate which rises from the bottom in flakes upon slight shaking of

the test tube, designated by three plus signs (+++);

3) weakly positive test - agglutination visible with the unaided eye with slight precipitate, designated by two plus signs (++);

4) doubtful reaction - agglutination visible with the unaided eye, but without precipitate, designated by one plus sign (+);

5) absence of an agglutination reaction and clarification of the fluid relative to the control test tube, designated by a minus sign (-).

The agglutination titer of the serum is calculated on the basis of the maximum dilution which gives a clear reaction (no less than three plus). If the titer of the serum is determined by means of the last dilution, in which the reaction had been weakly positive (++), or doubtful (+), this must be indicated when giving the titer.

Typical for tularemia is a stable Vi-agglutination: the agglutinate formed on the bottom of the test tube (when the reaction was ++++ or +++), is broken up into coarse flakes by shaking, while the fluid remains clear or almost clear.

If a clear agglutination reaction (++++ or +++ with formation of a stable agglutinate) does not take place in any of the dilutions, the result is doubtful. When recording the results, the rare possibility must be kept in mind of a delay in the agglutination (proagglutination zone) in not very strongly diluted serum.

In the serological diagnosis of tularemia on humans, a titer of not less than 1:100 may be considered as positive, but the increase in the titer must be observed (see further on).

It is essential to take into account the similarity between the tularemia and brucella microbes in consequence of which the serum of a tularemia patient can, if not greatly diluted, agglutinate brucella a and vice versa. However, the test with the homologous culture is normally carried out at much higher serum dilutions than with heterologous

serum, which is also the basis of differential diagnosis.

The laboratory which carries out the examination of the patient's blood by the serological method, should submit the results of the investigation to the interested institute in writing not later than 2 days after the material has been received by the laboratory.

Rapid methods of serological diagnosis of tularemia. The blood drop test on a glass slide is used for this purpose. Normal tularemia diagnosticum can be used as antigen but a drop of distilled water (which is necessary for the lysis of the erythrocytes) must be added to the drop of blood. In positive cases, when the titer of the patient's serum is 1:100 and over, the agglutination on the glass slide begins immediately after mixing of the blood with the antigen. However, if the titer of the patient's serum is lower (1:50), the agglutination on the glass takes place with some delay, during the first 2-3 minutes, and is also not very marked. When the reaction is strongly positive, coarse flakes are formed, with weakly positive reaction, small flakes. The blood drop test can also be carried out with a dried blood drop. The result is read 2-3 minutes after mixing of the blood with the antigen and designated by one, two or three plus signs depending on the time of appearance of the agglutination and the degree of its intensity.

The micro serum reaction can also be used for rapid tularemia diagnosis on humans. For the purpose of this test, instead of a drop of blood, a drop of blood serum from the patient is applied to the glass slide and the same quantity of antigen added (undiluted tularemia diagnosticum with a concentration of 5 billions microbe cells in 1 ml). In positive cases, flakes are also precipitated. This reaction often becomes positive on the 9-10th day of the disease, but sometimes even earlier, when the titer of the serum with volume agglutination is

1:10 - 1:20. The positive microtest on the glass slide may serve as a tentative diagnosis while final conclusions should be based on the results of the agglutination test as carried out by the volume method.

Allergic reactions

The reaction is highly specific. The alle gic skin reaction in human tularemia patients becomes positive usually on the 3-5th day of the disease, more rarely later. It is obtained in all clinical forms of tularemia and, as a rule, occurs earlier than the agglutination reaction. Following recovery, the allergic reaction is retained by the organism for many years, in most cases for life, which makes it possible to employ the tularemia test for the purpose of retrospective diagnosis of a past disease. One has to take into account, however, the possibility of a weakening or in some cases, an extinction of the reaction in the course of years, particularly individuals which have contracted the disease in childhood. The allergic reaction also appears in persons vaccinated with live tularemia vaccine but it develops somewhat more slowly than in persons which have had the disease and is extinguished in some persons within 5-6 years. In persons who are not immune to tularemia, the allergic reaction to tularemia antigen is completely absent.

The allergic test is carried out on humans intradermally and epicutaneously, using the respective antigen.

Intradermal allergy test. The corpuscular preparation tularin is used for the intradermal test on humans. This is a suspension of tularemia bacteria in physiological salt solution (with 3% glycerol added), killed by keeping them at 70° for an hour. 1 ml of the preparation contains 100 million microbe cells (according to the standard of the State Control Institute). Tularin is prepared from virulent or vaccine cul-

tures. The preparation is stable in storage.

The intradermal test is carried out on the palmar side of the forearm (between the upper and middle third), which has first been cleaned with alcohol. 0.1 m/ tularin is injected strictly intradermally with a sterile syringe with a fine needle. A whitish blister with a diameter of 3-4 mm which is subsequently reabsorbed within half an hour, should appear at the injection site immediately. If the tularin is injected subcutaneously, the blister is not formed and the intradermal test must be repeated in this case on the other arm.

The allergic skin reaction in the form of hyperemia and infiltrate normally appears within 24 hours, but sometimes becomes noticeable after only 6-10 hours after injection of the tularin. The reaction is evaluated 24-48 hours after the test has been administered. In order to evaluate the intensity of the reaction, the dimensions of the reddened and infiltrated skin area is measured in centimeters in two mutually perpendicular directions. It is considered to be positive when the infiltrate and hyperemia has a diameter of 0.5 cm and over. The absence of any skin alteration or the presence of hyperemia without infiltrate which disappears within one day, is evaluated as a negative result. In doubtful and suspected cases, the test must be repeated because a negative result may be due to the circumstance that the examination was carried out in an early stage.

In some cases the allergic skin reaction assumes the form of the formation of a pustule or shorlived lymphangitis with some painfulness, slight swelling of the local lymph nodes and even a rise of the body temperature by 1-1.5° lasting for 1-2 days. Cases in which necrosis (with subsequent cicatrization) at the site where the tularin had been injected, are also known.

Epicutaneous allergy test. Tularin, 1 m/ of which contains 2 bil-

lion microbial bodies is used as antigen for the exodermal test. Tularin for exodermal application is prepared from a vaccine strain, the method of preparation being analogous to that for intradermal application. General reactions to the epicutaneous test are considerably more rare than to the intradermal, and local side reactions (necrosis, lymphangitis) are never observed.

The epicutaneous test is carried out on the external surface of the left shoulder (in its middle third). The ampoule containing the exodermal tularin is shaken until a uniform turbidity is visible. A drop of the preparation is applied with an eye dropper on an area of the skin on the shoulder which has been carefully cleaned with alcohol. Two parallel incisions are made across the drop of tularin thus applied with a sterile vaccination quill, each with a length of 8-10 mm, keeping a distance of 4-5 mm between incisions and the drop of tularin rubbed in for a short time with the flat side of the vaccination quill. The incision should not be too superficial, a few drops of blood should appear after they are made, coloring the tularin drop a light red. The reaction is evaluated after 48 hours by visual examination and measurement across the incision the limits of the skin erythema in centimeters. A positive reaction is indicated by edema of the skin around the incision and by erythema. The edema and erythema sometimes appear only 24 hours after the application of the tularin. Within 48-72 hours, the reaction is usually marked and then gradually fades, having disappeared completely towards the 7-12th day. In rare cases, vesicles appear along the incisions, which disappear again within 2-3 days. The reaction is considered to be positive if the reacting skin area has a diameter of 0.5 cm or over or when a clearly apparent reddening and slight edema are visible along the incisions (ridge).

Rapid allergy test. The soluble antigen "tuallergen" which is

extracted by a chemical method from a virulent or vaccine culture of the tularemia microbe can be used for rapid allergic diagnosis of tularemia. 0.1 ml of this antigen solution is injected intradermally into the palmar surface of the forearm, using the same technique as for the tularin injection. The response reaction in the form of hyperemia appears within 15-30 minutes after injection of the preparation, the size of the reacting skin region attaining 1-2 cm and over. The reaction is read within half an hour of administration of the test.

The application of the methods of laboratory examination and their evaluation

The allergic skin test can be used as a method of early tularemia diagnosis because it is positive in most tularemia patients from the 5-6th day of the disease onwards. Cases are encountered, however, in which the allergy reaction can have anamnestic importance, for example individuals who have recovered from tularemia or who have previously been vaccinated. Special attention must be directed to this circumstance because of the present great number of vaccinated persons in the tularemia foci. Hence the tularemia diagnosis should not be based only on the results of a single allergy test. The detection of agglutinins for tularemia microbes in a serum dilution of 1:50 - 1:100 early in the disease in the absence of an increase in the serum titer during subsequent checks also does not provide sufficient grounds for diagnosing a fresh case of tularemia. The presence of agglutinins in the patient's blood has merely anamnestic significance. The two diagnostic methods - the allergic and the serological - should be used in combination and nonobservation of this rule may lead to diagnostic errors.

In order to discover any increase in the agglutination titer of the serum, the patient's blood must be examined at least twice: the first time immediately after the patient has asked for medical assistance or upon hospitalization, and the second time, 7-10 days after

the first check. When inconclusive results are obtained, the examination of the patient's blood must be carried out a third time, 7-10 days after the second check. The increase in the agglutination titer of the serum confirms the diagnostic importance of the skin test and resolves the problem of the nature of the disease while an absence of any increase in the serum titer indicates the anamnestic nature of the reaction.

The use of the allergic and serological methods for examination of the patient in combination with the clinico-epidemiological data helps to establish a correct diagnosis of tularemia; when the results of the laboratory tests turn out negative, the presence of this disease must be doubted. It is necessary only to keep in mind the possibility of a belated appearance of the serological reactions in severe cases of tularemia, particularly those involving pneumonia. Rare cases of a belated appearance of the allergy reaction as compared with the agglutination reaction are known. Hence, when the test results are negative during an early stage of the disease, repeated examination is necessary. The treatment, including the use of antibiotics, as a rule does not interfere with the results of the laboratory examination of the patient because normally the development of the immunological reactions has gone far enough before moment when therapy is begun.

Only a plain increase in the agglutination titer of the blood serum and also the presence of clinical symptoms, typical for tularemia, can serve as a basis of the diagnosis of a fresh tularemia case in persons previously vaccinated against tularemia or having recovered from this disease. The examination of the patient should include the elucidation of the circumstances under which he was infected with tularemia. A diagnosis of a tularemia relapse must be based on a careful clinical, laboratory and epidemiological examination of the patient, it being

particularly important to exclude the possibility of reinfection. In view of the inadequateknowledge concerning relapses of tularemia, an exhaustive diagnosis must be based as far as possible on the use of other laboratory methods of investigation, involving the isolation of a culture of the germ from a bubo or other pathologically altered tissues, using for this the biological method (see further on).

When autopsy material is being examined, the isolation of a culture of the germ by means of biological tests represents a confirmation of the diagnosis. Pieces of the enlarged lymph nodes, altered parts of the lungs and spleen are separately ground up in sterile mortars, physiological salt solution is added and 0.3-0.5 ml of this suspension injected subcutaneously into white mice or guinea pigs. The dead animals are dissected and contact smears made from their organs for microscopy and inoculation of nutrient media and the observed pathoanatomical alterations are also described in detail. Surviving test animals are killed (the mice on the 15th day and the guinea pigs on the 25th day) and cultures on nutrient media made from their organs.

Detection of tularemia bacteria by means of the biological test

This method is the most sensitive for detecting tularemia bacteria in any test material. The white mice and guinea pigs used as biological test animals contract tularemia with fatal issue even when a suspension containing only a few tularemia microbes are injected into them subcutaneously. A disadvantage of the method consists in the fact that in some cases the response may require 5-7 days or longer to appear. Dead animals found in nature are subjected to individual examination. A piece of spleen with a size of 3 × 3 mm and altered (enlarged or hyperemic) lymph nodes (submaxillary, cervical, inguinal, etc.) are taken for the biological test, placed into a sterile mortar, carefully crushed and ground with a pestle (care being taken to prevent excessive dry-

ing!), physiological salt solution added and mixed into a homogeneous suspension. The quantity of physiological salt solution added in the mortar varies from 0.5 to 5 mℓ, depending on the quantity and size of the pieces of organs (calculating approximately 0.5 mℓ of physiological salt solution per 0.1 g of organs). 0.5 mℓ of the suspension is injected into white mice, and 1-2 mℓ into guinea pigs. Part of the unused spleen and lymph nodes are placed into a sterile test tube and kept several days in the cold, as long as the results of the culture and biological examination are not yet clear. If the test animal dies prematurely from some other cause and the culture has not been successful, the organs kept in the refrigerator can be used for another biological test. When the organs of the test animal have begun to decompose, the marrow from a tubular bone is taken for the biological test and when the decomposition is very advanced, the exodermal method of infection of the test animal is used. In the last case, pieces of spleen (or some other less decomposed organ) which have been cut into small pieces with the scissors, are applied to shorn part of the skin in the area of the abdomen (with guinea pigs, the hairs are plucked out) and rubbed in carefully (until reddening appears) with the scalpel blade. A suspension from the organ can also be rubbed in. It must be remembered that the method of infection by inunction is much less sensitive than the subcutaneous method and it should be used only if absolutely indispensable.

When the biological test is carried out, strict precautionary measures must be adopted to prevent the transfer of tularemia bacteria from one analysis to another. Every dead animal (found in its natural habitat or having died in the laboratory) must be dissected with separate instruments, the physiological salt solution for each analysis must be prepared previously in sterile syringes and not poured into

the mortar from a flask or with a pipette after grinding of the test material. The preparation of the suspensions for inoculation and infection of the biological test animal must be completed before the autopsy of the next animal is begun. The board for the autopsy and the edges of the trough must be carefully burnt off with an alcohol swab, the hands (gloves) rubbed with alcohol and only then can the next autopsy be undertaken.

The test animals are kept singly in high glass or sheet metal jars, covered with lids of wire mesh. Taking the animals out of the jars and also the bedding must be carried out with precautions to exclude the possibility of spreading "dust" infection via the air and thus infecting via the aspiration pathway other test animals in the vicinity.

If the test material contains massive numbers of tularemia bacteria, white mice die from tularemia within 3-4 days of the subcutaneous injection of the suspension prepared from this material, guinea pigs on the 4-6th day; when the infection of the injected material was less heavy, the disease may be protracted and death occur only after 7-9 or even 15-18 days (extremely rarely, up to 25 days for guinea pigs). The pathoanatomical alterations of organs and tissues, typical for tularemia, are detected on the dead animals, particularly guinea pigs, which, with a certain amount of practical experience, enables a tentative diagnosis to be established even during the autopsy. In the contact smears from the spleen, liver, local lymph node and the blood of the white mice which have died from tularemia, an enormous number of tularemia bacteria can be observed, while the bacterioscopy of smears from the tissues of the same organs from guinea pigs is frequently negative or doubtful. The cultures of tularemia bacteria from the dead biological test animals are isolated by inoculation of coagulated yolk

medium. When cultures are made from the organs of white mice which have died from tularemia, the growth on the medium appears within 18-24 hours of incubation in the thermostat and when the cultures are made from the organs of guinea pigs, within 2-3 days of later which is due to the difference in the degree of contamination of the organs of these animals with tularemia bacteria prior to their death. The animals can be killed as soon as they are in agony which makes it easier to obtain a pure culture of tularemia bacteria. The bacterioscopy of smears from the organs of the white mice, killed during the last days of the disease, is positive but with a lesser degree of bacterial contamination than with those that have died.

The surviving white mice are killed on the 15th day and the guinea pigs on the 25th day after the beginning of the test and control cultures are made of these organs on coagulated yolk medium.

Methods for rapid diagnosis by means of biological test. In special cases, in order to obtain the culture of tularemia bacteria more quickly, it is recommended to use several white mice for the analysis and to kill them during the first days after infection, leaving one mouse as a control. Careful inoculation of coagulated yolk medium is carried out with material from the dead mice or even better, on Yemel'yanova medium, using material from a lymph node (inguinal) nearest to the injection site and from the spleen. The triturated tissues suspended in a small volume of physiological salt solution, are applied to Yemel'yanova medium (in dishes or test tubes). When guinea pigs are used for the biological test it is recommended to take a specimen obtained by puncture of the tissue from the injection site and the nearest lymph node 1-2 days after the subcutaneous infection and to inoculate nutrient media with this specimen.

Detection of tularemia germs in animals and the physical environment. Wild vertebrates, particularly rodents, caught alive or found dead, remains of their cadavers in nests and the nest itself, bloodsucking arthropods, straw, corn, victuals and also water from natural reservoirs or from wells can be subjected to examination. The tularemia germ belongs among the group of germs which cause particularly dangerous infections, hence its isolation and identification can be carried out only in specially equipped laboratories of the wards for particularly dangerous infections of quarantine stations, antiplague institutes and scientific research institutes or in suitably equipped field laboratories and only by personnel suitably trained and vaccinated against tularemia. The material (rodents, bloodsucking arthropods, etc.) must be delivered to the laboratory for bacteriological examination with the observation of all the rules of precautions, prescribed for work with specially dangerous infections. When collecting rodents and other small mammals, facilities must be provided for carrying out autopsies on them on the site and the subsequent delivery to the laboratory only of the extracted organs (spleen, enlarged lymph nodes, etc.), immersed in a preservative, for example, in sterile vaseline-paraffin mixture (10 parts vaseline oil and one part paraffin). Only fresh, not yet putrified organs can be kept in this mixture, preferably in the cold; the higher the storage temperature, the more quickly die the tularemia bacteria in the organs.

Examination of cadavers of feral vertebrates which have died in their natural habitat. The collection and examination of rodents, particularly those belonging to group I (common field mice, domestic mice, water rats, rabbits, etc.) are the most effective methods of investigating epizooties of tularemia.

Bacterioscopy, cultures, precipitation reactions and biological

tests can be used for detecting tularemia among the collected dead animals. In order to establish a tentative diagnosis, all four enumerated methods are used simultaneously. When a tularemia epizooty has been established, the investigation can be limited during the further examination of the rodent cadavers of group I to cultures and microscopic examination of smears, while keeping part of the organs in cold storage until the results of the investigation are apparent. In doubtful cases (absence of typical pathological alterations, advanced decomposition of the cadavers), the method of biological test must be used. The cadavers of animals of group II and III must always be examined by means of the biological test. Thoroughly individual examination of the animal cadavers makes it possible to establish an accurate idea concerning the causes of death and the degree of dissemination of the infection.

Bacterioscopy. Contact smears are made on an object glass from the spleen, enlarged lymph nodes, liver and blood, well dried and fixed in Nikiforov mixture (ethanol-ether, ana) or in methanol and stained by the Romanovskiy-Giemsa method. In a correctly stained smear, the tularemia microbes have an intense lilac color. In contact smears from spleen, liver or lymph nodes and in the blood smears from animals of group I which have died from tularemia, provided that the organs are fairly fresh, the tularemia bacteria are normally detected in the form of a large number of large and small microbe clusters in each field of vision. The most accurate results are normally obtained by bacterioscopy of the spleen. Owing to the lysis of the bacteria, their observation in putrefied cadavers becomes more difficult. The bacterioscopy of smears from organs of animals of group II which have died from tularemia, often gives a doubtful or negative result.

By means of bacterioscopy (in combination with precipitation

ests) it is possible to obtain a rapid (within 2-3 hours) positive nswer during the examination of rodent cadavers brought to the laboratory, but this is merely tentative because the positive results of the acterioscopical examination (and the precipitation tests) should be onfirmed by isolation of a culture.

The contact smears made on glass lides from animal organs can be xamined by means of fluorescence microscopy in presence of the respective specific sera. The detection of bacteria with specific fluorescence in the smears provides a basis for a tentative diagnosis of tularemia. A final answer can be given on the basis of the isolation of a ulture of the pathogenic agent.

Inoculation. The most practical and at the same time fairly sensitive medium for the growing of tularemia bacteria under normal laboratory conditions is the coagulated yolk medium, proposed by MacCoy (see . 875).

Cultures are prepared primarily from the spleen, liver and enlarged lymph node.

It is most convenient to carry out the inoculation by the method of contact smears, for which purpose pieces of organ are pressed consecutively with the section surface against the medium, beginning with the lower part of the test tube. The contact method has the advantage, that even if the culture is contaminated by extraneous microflora, the typical growth of the tularemia microbe is nevertheless often visible in addition to the colonies of the extraneous microflora. The inoculation can also be carried out by carefully rubbing a piece of organ over the entire surface of the medium. In this case, the inoculation is also begun in the lower part of the test tube. The cultures are grown at 37°. With copious inoculation, the growth of tularemia bacteria on coagulated yolk medium appears within only 18-24 hours of incu-

bation in the thermostat and within 2-3 days attains a maximum. The grown culture has the appearance of a tenuous, shiny, meandering (shagreen) surface deposit, which has the same color as the medium and is visible to the unaided eye only upon careful examination. With sparse inoculation, discrete, small, shiny, round colonies become visible on the 3-5th day of growing and even later. Hence, the cultures must be kept in the thermostat up to 10 days.

When completely fresh cadavers of rodents are delivered to the laboratory (for example, in winter time), a culture of the tularemia bacteria can be obtained by inoculation of coagulated yolk medium within 24 hours. The isolation of the culture forms the basis for the final diagnosis.

Precipitation test. This test is usually positive when rodents of group I are examined which have died with intense dissemination of the organs with tularemia bacteria. When the number of bacteria in the organs is small, the precipitation test is negative. A nonspecific reaction may occur in some cases.

The precipitation test is carried out with agglutinating serum with a high titer (1:6000 - 1:8000). When dead rodents are to be examined, pieces of the spleen and liver are usually taken (with a weight of up to 1 g), ground up in a mortar, 3 ml of physiological salt solution added, mixed until a fairly uniform suspension is obtained and filled into a sterile test tube. The suspension thus obtained is boiled for 15-20 minutes on the water bath and filtered through asbestos wool until a completely clear extract is obtained. 0.2-0.3 ml of agglutinating serum with a high titer is filled into a small (agglutination) test tube and the same quantity of extract allowed to flow in carefully along the walls of the test tube by means of a Pasteur pepette. When the reaction is positive, a ring of turbidity of white

color which is clearly discernible with the unaided eye, appears at the interface of the two liquids within 3-5 minutes. In order to check the test result, the following control tests are carried out: 1) test extract plus normal serum; 2) physiological salt solution plus normal serum; 3) physiological salt solution plus agglutinating serum; 4) tularemia antigen (extract from tularemia diagnosticum) and agglutinating serum. A positive precipitation test in combination with positive microscopic examination serves as a tentative orientation for the diagnosis, but it must be confirmed by the isolation of a culture of tularemia bacteria.

Examination of feral vertebrates caught alive in their natural surroundings. The biological examination is the basis of diagnosis on live rodents and other animals. This method makes it possible to detect the tularemia germ in the organs of the animals in different stages of the disease or in healthy carriers, when the use of the conventional methods of culture and microscopic examination of the bacteria does not give any result. Killed animals, in which striking pathoanatomical changes are not detected during the autopsy, are examined by the group method, i.e., the organs of several animals are combined in a single analysis (5-10 of the same species and found in the same locality). The lymph nodes (cervical, submaxillary and inguinal) and pieces of spleen are taken for the examination. Cultures and smears from the organs are not prepared in this group analysis.

The culture and microscopic methods can be used in combination with the biological test in the investigation of killed animals, whose organs are pathologically altered. Individual biological tests are carried out with parts of these animals. Greatest attention is given in the epizootic investigation to the examination of species of animals of group I: field mice, domestic mice, water rats, etc. The probability

of the development of epizooty among the animals of group II (gophers, field mice, etc.) is not very great and the frequency of isolation of tularemia cultures from them is somewhat less than that of the isolation of the germ from animals of group I. The wild animals of group II, caught alive, for example, gophers, can be examined by using the agglutination test (using the volume method) and the allergy test. Tularin with a concentration of 1 billion microbial cells in 1 mℓ is used for the last mentioned test; 0.1 mℓ is injected intradermally and the result tentatively evaluated 24 hours later and again, finally, 48 hours later.

Examination of domestic animals. The domestic animals belong to species which are not very sensitive to tularemia; the agglutination test and the intradermal test with tularin (a preparation with a concentration of 1 billion microbial bodies in 1 mℓ is used) can be used for their examination. With pigs, the tularin is injected in a quantity of 0.2 mℓ into the skin at the base of the ear, with sheep into the skin in the area of the fold under the tail or the skin in the axillary region, etc. The reaction is evaluated first after 24 hours and again, finally, after 48 hours by examining the part of the skin into which the tularin had been injected. When the blood serum of domestic animals is examined for tularemia, a parallel agglutination test with brucellosis diagnosticum must be carried out. When the two test results are identical (with respect to the titer), impovering of serum according to the method of Castellani must be carried out. When dead animals or killed sick animals are to be examined, cultures from the organs and biological tests are used. The pathological changes in the lymph nodes and also the spleen are primarily investigated.

Examination of invertebrates. The detection of tularemia bacteria in the organism of invertebrates can be achieved quite reliably by the

biological method of investigation. A few cases have been reported in which isolation of cultures of tularemia bacteria by direct culture of a suspension of ground-up starving ticks on coagulated yolk medium had been successful but this method is not suitable for the examination of large numbers because of the considerable contamination of the ticks with various microbes which invade the culture.

Sexually mature blacklegged ticks are examined in groups of 50 by injecting a suspension into white mice or in groups of 100 and injection of the suspension into guinea pigs. The tick nymphs are combined into groups of 50-100 specimens depending on their degree of nutrition. Fleas, lice and gamasid ticks can be examined in groups of 100-200 in a single analysis, mosquitos in groups of 100, horseflies 25-50 specimens, etc. (with horseflies, the legs and wings must be clipped off first). The body surface of the sexually mature blacklegged ticks is first disinfected by washing twice with alcohol and then 3-4 times with sterile distilled water. Tick nymphs and also those of other forms of carriers are examined without preliminary treatment. Water animals are first carefully washed with several portions of pure water and sterile physiological salt solution

All species of invertebrates, with the exception of sexually mature blacklegged ticks, should be examined on the day on which they have been delivered and at most not later than a day after they have been removed from their natural habitat. When the suspension is prepared from the invertebrates, it must be remembered that a suspension of too high concentration can be toxic to the biological test animals causing their premature death, while an extreme dilution of the suspension and use of only part of it for the inoculation of the test animals may give a negative result lf the analyis. It is not recommended to carry out an examination of dead invertebrates which have died while

they were kept at the laboratory, in view of the fact that any tularemia bacteria contained in them, die off.

Examination of ambient objects. Water, grain or other food products, straw, chaff, contaminated by rodents, the excrements of the latter, including accumulations of frozen urine (in winter) can be used for the bacteriological investigation. The examination of samples from ambient objects is most successful during the cold season when the tularemia germ remains in a viable state for long periods. Samples of water can be examined with success in summer and winter.

The tularemia bacteria, present in ambient objects, can be detected only by the biological method. When water is to be tested, 0.5-1 ml of it is injected into a white mouse or 5-10 ml into a guinea pig. With grain, straw and othersubstrates, washes are prepared with physiological salt solution. 40 ml of physiological salt solution is used for preparing a wash from a grain sample with a weight of 100 g, and 50 ml for 25 g straw. The washes from rodent nests are made with a larger quantity of solution because they are more hygroscopic. In order to remove mechanical impurities from the washes, they are allowed to stand for 5-10 minutes and sucked up with a syringe through a cotton-wool swab. 0.5 ml of the wash is injected into white mice.

The examination of samples from ambient objects (particularly rodent nests) during hibernal epizooties in straw stacks is just as effective as the examination of rodent cadavers and is more effective than investigations on live animals caught among the same objects. In infection foci situated in the foothills near streams, the examination of the water of these streams is as effective as examination of the rodents.

Identification of the microbes, isolated during the bacteriological investigation

The identification of the freshly isolated tularemia culture is carried out on the basis of the coincidence of the following characteristics; 1) the morphology and color of the bacteria in the smears; 2) the nature of growth on the coagulated yolk medium; 3) the absence of growth on meat-peptone agar and broth; 4) the agglutination by specific serum; 5) the capacity to cause the death of white mice and guinea pigs when they are infected with a culture, exhibiting the alterations in the organs, typical for tularemia and the subsequent isolation of a pure culture.

Following this up with a verification of the virulence of the freshly isolated culture (or cultures) is carred out only when conditions warrant it and is carried out on at least two or three species of animals - white mice, guinea pigs and white rats. Additional testing of the virulence on domestic rabbits makes it possible to distinguish the Euro-Asian from the American strains. The former are not highly virulent for rabbits (The DCLM is 1 billion microbe cells and more), while the American strains are as virulent for rabbits as for white mice and guinea pigs (the DCLM is 1 microbe cell according to the standard of the State Control Institute).

Nutrient Media

Mac Coy's medium. Yolks from fresh hen's eggs are mixed in a sterile manner with physiological salt solution in a ratio of 3:2, the mixture poured into sterile test tubes in a quantity of 4-5 mℓ for each and coagulated in a canted position at 80° for one hour. The medium thus prepared is left in the thermostat at 37° for 24 hours to check its sterility and then kept in the cold to prevent drying out.

Yemel'yanova medium. Distilled water 100 mℓ. A hydrolysate of fish

flour or fresh fish 20 ml; Gelatine hydrolysate 10 ml. Yeast autolysate 2.5 ml. Sodium chloride 0.5 g. Glucose 1 g. Cystine 0.1 g. Agar 1.5-2.5 g, depending on quality, pH = 7,2-7.4.

The medium is melted for sterilization, cooled to 45°, 10 ml of defibrinated rabbit blood added, poured into test tubes and canted or poured into Petri dishes.

Francis medium. Meat-peptone 1.5-2.5% agar containing 1% peptone and 0.5% sodium chloride 100 ml. Cystine 0.1 g.

Glucose 1 g. pH = 7.2-7.4.

The medium is melted for sterilization, cooled to 45-50°, 5-10 ml defibrinated rabbit blood added and poured into test tubes which are canted or into Petri dishes.

Antsiferov medium. 100 ml of sterile Ukhalov-Mikhaleva medium is melted, cooled to 45° and 5 ml of hen's egg yolk added (diluted with physiological salt solution in a ratio of 3:2), poured into test tubes, which are canted.

Ukhalov-Mikhaleva medium: 100 ml of crude infusion of basic Hottinger solution, 250 ml of abomasum peptone, 4 g sodium chloride. The mixture is boiled for 3 minutes, after which the pH is measured, and again boiled for 3 minutes. The medium is passed through a paper filter, 20 g agar added which has first been steeped in water. Boiling must be avoided when melting the agar because this alters the properties of the medium.

The medium is sterilized at 120° for 20 minutes.

Preparation of the abomasum peptone: the abomasum from a freshly slaughtered cow is separated from the fat, carefully washed and passed through a meat grinder. To 400 g of the ground material, 1 liter of 1% hydrochloric acid is added. The further preparation is carried out by the same method as that used for the preparation of Martin peptone.

MALARIA

Sh. D. Moshkovskiy, Professor, Member of AMN USSR, and
N.A. Demina, Candidate of Medical Sciences

Development cycle of the malaria parasite. The malaria parasite is a unicellular animal (protozoa) Protozoa of the class Sporozoa, family Plasmodidae, genus Plasmodium.

4 species of malaria parasites are known to infect humans: Plasmodium vivax - causing tertian malaria, Plasmodium malariae, causing quartan malaria, Plasmodium falciparum (synonym Laverania falcipara) which causes tropical malaria, and Plasmodium ovale which causes a special form of tertian malaria. The lastmentioned species is found under natural conditions in Africa, Palestine, South America and the Philipines. In the USSR, the occurrence of P. ovale has not been established.

Under natural conditions, humans may be infected via mosquitos with Plasmodium cynomolgi bastianelli, which causes malaria in simians.

The development of the malaria parasites involves a change of host. In the carrier, the fertilization takes place with subsequent cell division, as a result of which the sporozoites are formed (see further on) - the sporogony. In humans, asexual reproduction, the schizogony, takes place.

Schizogony takes place:

a) in tissue cells - tissue schizogony; b) in the erythrocytes - erythrocytal schizogony.

The body of the malaria parasite consists of the cytoplasm and the nucleus. In some stages the parasites contain a pigment (see further on) which cannot be stained but has its own natural color: dark-brown, golden-yellow, brownish, almost black (varying with the species of parasite).

Infection of humans takes place as a result of the bite of an infected Anapheles mosquito. The sporozoites enter the human organism with the saliva of the mosquito.

The sporozoites settle down in the parenchymatous cells of the liver, become round, grow, and attain a diameter of 40-50μ and over. The nucleus divides several times and then the cytoplasm is segmented. As a result, a large number of small, about 1μ mononuclear bodies are formed, the tissue merozoites. Some of these merozoites invade the erythrocytes and give rise to the arythrocytal development cycle of the parasite.

Other merozoites penetrate into tissue cells in which the development of the tissue forms continues. The stages of the parasite which develop in the liver cell, are termed tissue or exerythrocytal forms. Preerythrocytal forms, developing in the organism prior to the invasion of the erythrocytes, and parterythrocytal forms, whose development in the tissue cells takes place in parallel with the development of the erythrocytal forms, are distinguished.

The penetration of the first merozoites into the erythrocytes takes place, depending on the species of parasite, within 6-8 weeks from the moment of infection.

The development of the tissue forms is absent in P. falciparum.

When infection with P. vivax takes place, the tissue forms of the parasite are present in the organism also during the infection periods when the proliferation of the parasites in the blood is temporarily stopped. The renewed proliferation of the parasites in the blood after these periods (late relapse) occurs as a consequence of renewed invasion of the merozoites from the tissue cells.

The erythrocytal schizogony of P. vivax, P. malariae and P. ovale takes place in the peripheral blood. With P. falciparum, only young

schizonts are found in the peripheral blood which are ringshaped. The other stages of schizogony up to the division of the parasite into merozoites and the development of the sexual forms (gamonts), takes place in the internal organs. Only in the case of severe comatose malaria can other stages of the schizogony cycle be observed in the peripheral blood. In some strains of P. falciparum all stages of schizogony can be detected in the peripheral blood in a large proportion of pateints and with relatively benign course of the disease.

The proliferation of the parasite in the erythrocytes takes place in the form of regularly alternating cycles. From the moment of invasion of the erythrocyte by a merozoite to the complete development of the parasite, terminating with the formation of new merozoites, 48 hours elapse with P. vivax, P. falciparum and P. ovale, and 72 hours with P. malariae. Some of the merozoites which have invaded the erythrocytes, are transformed into male and female sex cells, the gamonts (the male - microgamonts, the female - macrogamonts). The female gamonts attain the stage of full maturity - the gamete stage in human blood, while the male gametes become mature in the organism of the carrier. When the process of maturation is terminated, the process of exflagellation is observed in the stomach of the mosquito, the release of 4-8 male gametes by the male gametocyte, which are thin mobile cilia which, after separation, move around actively in the stomach contents and are capable of penetrating into the female gamete and of fertilizing it (sexual process).

In P. falciparum, the gametocytes first assume a spherical shape and only later form microgametes.

The fertilized female gamotes (zygotes) penetrate through the epithelium of the middle intestine (stomach) of the mosquito and form cocytes under its external wall. The cocytes grow and a large number of

sporozoites, mononuclear, fusiform bodies (length 11-15μ, width 1-1.5μ. The cysts burst open and the sporozoites emerging from them, spread with the hemolymph through the tissues of the mosquito, thus getting into the salivary glands after which the mosquito is capable of transmitting malaria to humans.

A malaria infection can occur not only via mosquito bites, but also through transfusion with blood from a donor in whose blood parasites are present, even if in very small numbers.

Laboratory Diagnosis of Malaria

The parasitological diagnosis of malaria is based on the detection of the parasites in stained preparations (large drop and smear) of blood.

The parasites can be present in the blood not only during the bouts of fever and the intervals between these attacks, when they follow one another, but also during the long periods, in which an elevated body temperature is not observed. The presence of parasites in the blood in the absence of fever bouts is termed the parasite-carrying stage. Persons in this state are parasite carriers. They are capable of infecting mosquitos. When wholesale examination is carried out, blood samples must be taken regardless of the presence or absence of fever.

In accordance with the instructions of the Ministry of Health of the USSR, the blood of all patients with fever must be tested for malaria.

Technique of preparing and staining smears. The smears are prepared as for conventional blood analysis. A small drop of blood is placed on a carefully washed and degreased object glass at some distance from the short end. The glass is placed on a horizontal surface, held with the left hand and with the right hand a ground glass with

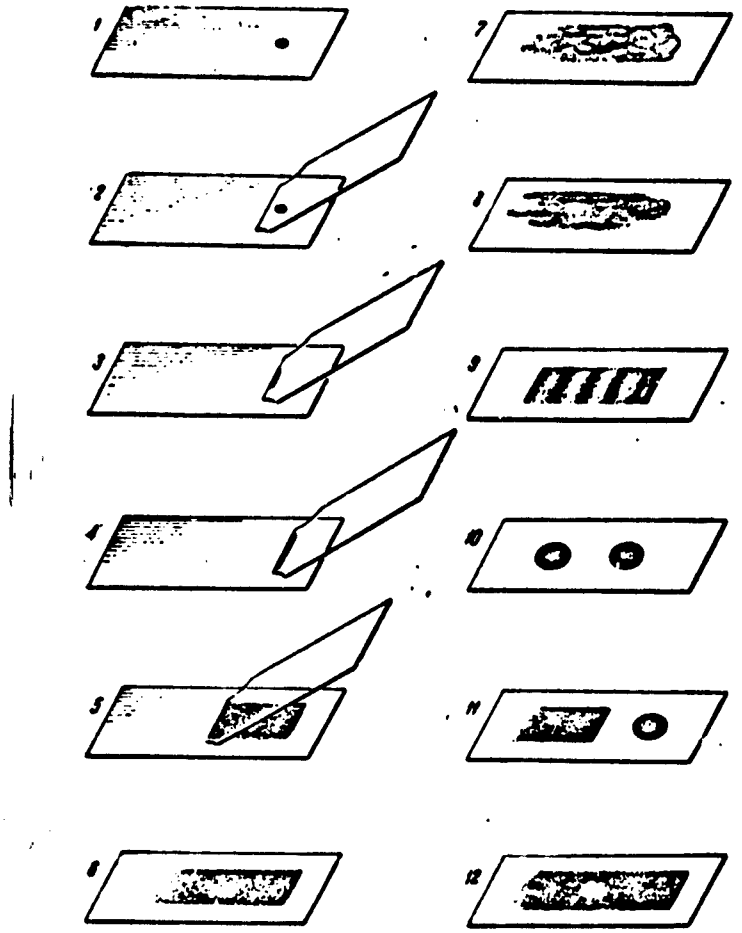


Fig. 99. Preparation of a smear and "large drop". 1-5) stages of the preparation of the smear; 6) correctly prepared smear; 7-9) incorrectly prepared smears; 10-12) various methods of preparing the "large drop".

cut-off corners (in consequence of which its edge is slightly narrower than the width of the object glass) inclined at an angle of 45° is pushed towards the drop from the opposite end of the glass. As soon as the drop has spread in the acute angle between the edges of the ground glass and the object glass, the ground glass is quickly pushed in the opposite direction (Fig. 99).

The smear must be prepared quickly to avoid clotting of the blood.

To mark the preparations, an inscription can be applied on a small

TABLE 66

Differential Diagnosis of Malaria Parasites in Humans

	Pathogenic agent			
	Plasmodium vivax	Plasmodium malariae	Plasmodium falciparum	Plasmodium ovale
Clinical form of the infection	Malaria tertiana	Malaria quartana	Malaria tropica	Malaria of the tertiana type
Duration of schizogony, hours	48	72	48	48
Stages of development of the parasites in the peripheral blood	All stages of schizogony and development of gamonts	All stages of schizogony and development of gamonts	Normally only rings and mature gamonts. In comatose cases schizonts of different age and morulas	All stages of schizogony and development of gamonts
Young schizonts (rings)	Regularly ring-shaped, occupying 1/3-1/4 of the diameter of the erythrocyte	The same as in Plasmodium vivax	The small rings occupy 1/6 of the diameter of the erythrocyte, the larger ones up to 1/3 of the erythrocyte diameter, often with two nuclei	The same dimensions and shapes as with Plasmodium vivax and Plasmodium malariae but with larger nucleus

Ameboid schizonts	Occupy more than 1/3 of the erythrocyte diameter. The pseudopodia are clearly visible. The pigment, which is uniformly distributed in the young schizonts, becomes more concentrated in the mature parasites	a) the same as in Plasmodium vivax, with different size depending on the maturity of the schizont. The pseudopodia are broad but not very distinct b) Ribbonlike schizonts: the parasite extends across the erythrocyte in the form of a ribbon with varying width. The nucleus is elongated along one edge. The pigment in the ameba-like schizonts is distributed in the same way as in the ameboid schizonts of Plasmodium vivax. In the ribbon-like schizonts, the pigment is arranged along one edge, opposite to the one at which the nucleus is situated	The ameboid nature is not very marked. Even in an early stage, the pigment is concentrated in a small area. Normally not found in the peripheral blood	Similar to the schizonts of Plasmodium malariae but have larger dimensions. The pigment distribution is the same as in the ameboid schizonts of Plasmodium vivax and Plasmodium malariae

Morula	12-18 merozoites are arranged in an irregular manner around the compact pigment cluster	8, more rarely up to 12 merozoites are arranged in a regular rosette along the periphery of the pigment which is concentrated in a cluster	12-24, normally about 16 merozoites, which are smaller than the other forms, arranged irregularly around the pigment which is concentrated in a cluster	8 merozoites are arranged irregularly around the pigment which is concentrated in a cluster
Gamonts [1]	Circular or elliptical cell without pseudopodia, and without vacuoles, occupying almost the entire erythrocyte, which is enlarged in size. The pigment is coarser than in the schizonts and uniformly distributed	The same as in Plasmodium vivax but with smaller size, being not larger than normal erythrocytes	Crescent shaped. In the female gamonts the pigment closely adjoins the nucleus which is in the center of the parasite. In the males, the pigment extends far from the nucleus, occupying more than half of the length of the gamont	The same as in Plasmodium vivax and Plasmodium malariae.

1) The differential diagnosis of female and male gamonts is described in the text p. 889.

TABLE 67

Alterations in Erythrocytes, Affected by Malaria Parasites

		Erythrocytes			Azurophilic Elements				
		Alteration of Shape	Increase	Alteration of Color	In which stage of the development of the parasite appear the azurophilic elements	Shape of the azurophilic elements	Number of azurophilic elements	Method of Detection	Designation
Plasmodium vivax		Inconstant	Increase considerably in size	Marked fading of the color	Young schizonts	Points	Abundant	Staining according to Romanovskiy at pH of about 7.5	Schüffner granularity
Plasmodium malariae		Not observed	No increase in size	Fading of the color hardly noticeable	Young schizonts	Small grains	Less than with Plasmodium vivax	Stained with difficulty under special working conditions	
Plasmodium falciparum	Agamonts (asexual forms)	• •	The same	In the fresh state the erythrocytes have a copper-red tinge	Rings	Blotches	Few	Staining according to Romanovskiy with a high azure content and at pH not under 7.5	Maurer's maculosity
	Gamonts (sexual forms – gametocytes)	The stroma of the erythrocyte is drawn tightly on the adult gametocytes, only on the concave side of the gametocyte, can erythrocyte residues sometimes be separated		When stained according to Romanovskiy, the whole erythrocyte sometimes assumes a slight abovementioned tinge	Mature gamonts	Spirochete-like patterns. Coarse grains	Single pattern. Several grains		Argutinskiy's grains
Plasmodium ovale		Numerous (about 25%) erythrocytes, containing adult schizonts, are drawn out and have an elliptical shape. Many have an irregular shape	Increase in size less than with Plasmodium vivax	Very marked fading of the color	Young rings	Points	Abundant	Under the same conditions as Schüffner's granularity, stain develops more strongly	James granularity.

area of the smear with a glass corner, a needle or a simple pencil. When the smear has dried, it is fixed in 96° ethyl alcohol for 15 minutes or in methyl alcohol for 3 minutes. The smears are stained after fixing with Romanovskiy's stain, diluted with distilled water (1-2 drops per 1 ml water of neutral or weakly alkaline reaction, pH = 7.0-7.2). The staining requires 30-50 minutes depending on the quality of the dye. After staining, the preparation is rinsed with water and dried in air.

Technique of preparing and staining the large drop. A smear is prepared on a glass slide and a drop of blood allowed to drop on the still wet smear. The drop spreads uniformly, forming a circle. As soon as the drop is dry, the smear is marked with a simple pencil. The preparation is stained without previous fixing as indicated above. Then the preparation is carefully rinsed with tap water (avoiding washing off the drop) and dried.

The smears and large drops are examined under the microscope using an immersion lens system.

Morphology of the malaria parasites in the blood smear (Fig. 100). A merozoite which has invaded an erythrocyte, is transformed into a young schizont which is visible as a ring (No. 1, 9, 17, 18). The parasite then increases in size and assumes an ameboid shape but retaining the lumen (vacuole) between the nucleus and the main part of its cytoplasm (No. 2, 3, 10). Pigment grains appear in the cytoplasm of the parasite. The ameboid schizont fills a considerable part of the volume of the erythrocyte, then begins to assume a circular shape (No. 4, 19), the vacuole disappears (stage of preparation of the cell division), and the pigment which is fairly uniformly distributed in the younger ameboid schizonts, begins to be concentrated into individual clusters. No. 11, 12, represent schizonts typical for P. malariae, the ribbonlike

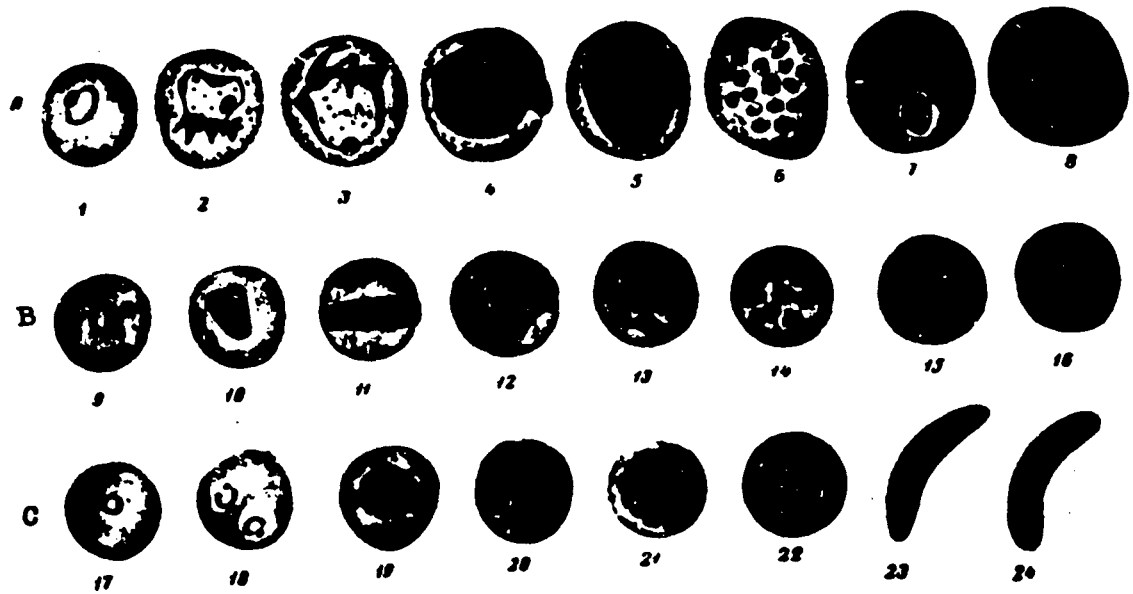


Fig. 100. The pathogenic organisms responsible for human malaria in the smear.

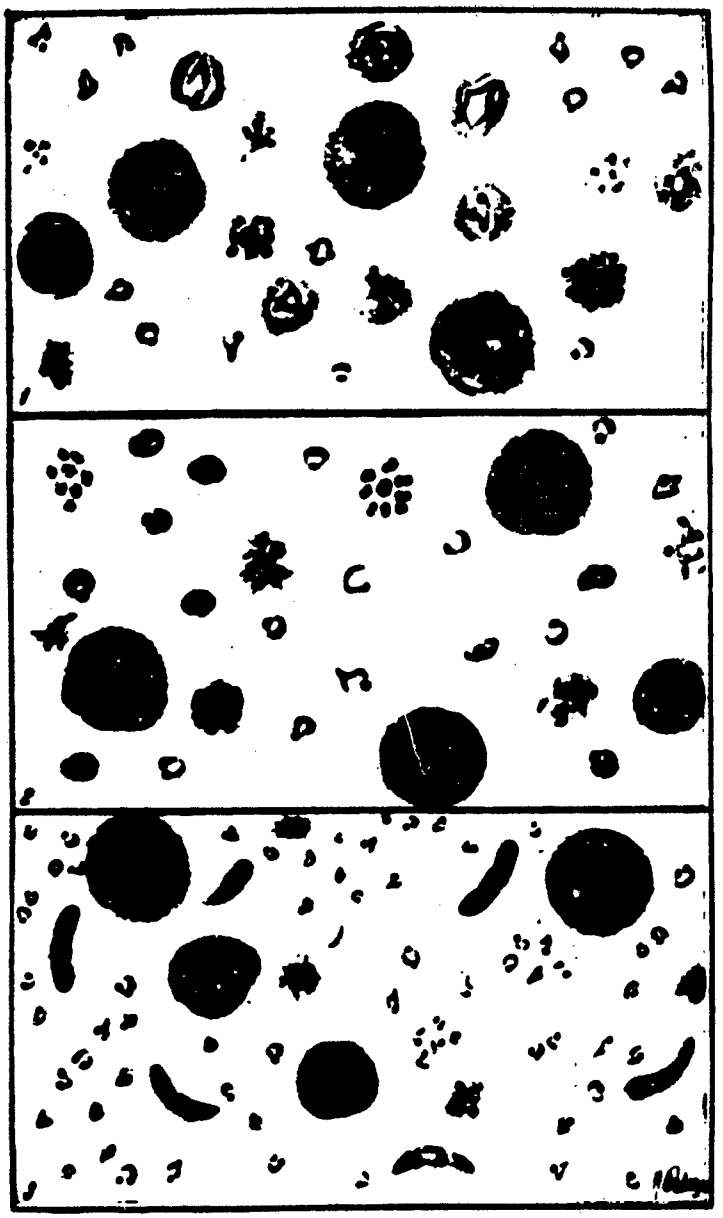


Fig. 101. Human malaria parasites in the large drop.

forms. The nucleus of the parasite begins to divide (No. 5, 13, 20, 21) and then the cytoplasm of the parasite divides in such a manner that a small clump of cytoplasm adjoins each daughter nucleus (morulation). As a result, a cluster of merozoites is formed, the morula (No. 6, 14, 22). During the morula stage, the pigment is concentrated into a single compact cluster. In P. falciparum concentration of the pigment sets in even before the complete morula formation. The merozoites are dispersed into the blood plasma, where part of them die, while some succeed in invading other erythrocytes.

Gamonts. The shape of the gamonts is circular or slightly elliptical in all malaria parasites, with the exception of P. falciparum, P. falciparum has elongated gamonts (crescent shape) with rounded ends.

The male and female gamonts differ with regard to the size and structure of the nucleus and the intensity of the color of the cytoplasm. Female gamonts (No. 7, 15, 23): small compact nucleus, the cytoplasm is stained intensely blue. Male gamonts (No. 8, 16, 24): large, diffuse loose nucleus, occupies a considerable part of the cell; the cytoplasm is stained a pale color, and part of the zone around the nucleus assumes a violet tinge.

The morphological characteristics which are typical for the development stages of the malaria parasites of different species, are presented in Table 66.

The morphological changes of the erythrocytes, which have been invaded by malaria parasites of different species, are presented in Table 67.

Morphology of the malaria parasites in the large drop. Because the large drop is stained without fixation, the erythrocytes are leached out and the malaria parasites undergo considerable deformation.

In P. malariae and P. falciparum, the infected erythrocytes in the

large drop are leached out completely like the normal erythrocytes.

In the large drop, the stroma of erythrocytes, infected with P. vivax, is sometimes preserved in the form of a delicate rose-red disc. In the latter case, the diagnosis of the species of malaria parasite in the large drop is facilitated.

Residues of erythrocytes, infected with P. ovale, are also partly preserved. Hence, when the blood of a patient coming from a country, where P. ovale is found (mainly from Africa), is tested, a differential diagnosis by means of a blood smear must be carried out when such types of erythrocyte damage are detected.

The rings rarely retain their shape in the large drop, being usually distorted, drawn out in the form of an exclamation mark, sometimes distorted into the form of a comma.

The ameba-like schizonts are often flattened, their cytoplasm sometimes being fragmented, while the ribbon-like schizonts of P. malariae assume a round shape in the large drop and are not distinguishable from the ameboid schizonts.

It is usually difficult and sometimes impossible to distinguish schizonts which are about to commence cell division, from a gamont (with the exception of P. falciparum).

The morula are easily recognizable in the large drop.

The morphology of the pathogenic parasites in the large drop is presented in Fig. 101. The cytoplasm of the parasites, as a result of the effect of the chemotherapeutic preparations, is less intensely stained, assumes a glasslike appearance, becomes vacuolized and break up into individual clumps. The nuclei are loosened or, conversely, more compact and dense. When certain preparations (for example, achrichin) act on them, the pigment collects into clusters and is ejected from the parasite. The recognition of parasites which have been subjected to

such deformations in the large drop becomes more difficult and sometimes impossible.

Diagnostic errors. Thrombocytes can be confused with malaria parasites in blood smears and large drops, a single plaque can be mistaken for a merozoite, an accumulation of plaques can simulate a morula. In the Romanovskiy-stained preparations, the plaques differ from the merozoites by the following characteristics: the merozoite consists of a red, compact nucleus and a clump of blue cytoplasm, the background of the plaque in the smear is sometimes stained a pale blue color, but its grains assume a red color of a lighter shade than the merozoite nuclei.

Extraneous microorganisms can also be observed in the preparations which are similar in shape to the malaria parasites: fungi, monocellular algae, freeliving protozoa, etc. These formations can get into the preparations from insufficiently carefully cleaned glasses, from the finger of the patient and also from the water with which the dye has been diluted, in particularly, when it has been taken from a container, which has not been cleaned for a long time.

Some monocellular organisms with a compact nucleus in the center and which are stained dark red by Romanovskiy dye, can simulate gamonts of P. falciparum. They can be easily distinguished by the absence of pigment.

Freeliving protozoa with one or several flagellae can be mistaken for the stage of the formation of the male gametes (exflagellation) of the malaria parasite.

Examination of mosquitos for infection with malaria parasites. The mosquitos are dissected and the presence of cocytes on the stomach and of sporozoites in the salivary glands (see Fig. 102) is determined.

The mosquito is paralyzed with chloroform or ether (a few drops on

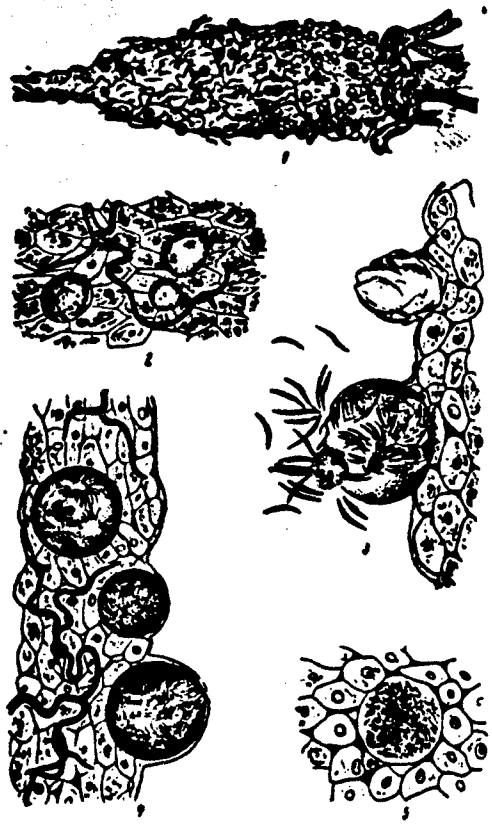


Fig. 102. Different stages in the development of the cysts on the mosquito stomach.

the cottonwool stopper of the test tube) or with tobacco smoke. The legs and wings are clipped. The mosquito is immersed for a few seconds into 50° alcohol by grasping it behind the proboscis and transferred to an object glass in a drop of physiological salt solution. The mosquito is transected across the thorax with needles, and the head, together with the anterior half of the thorax is transferred to another drop of physiological salt solution, slightly colored with methylene blue and, holding thus part of the thorax with one needle, the head is pulled off with the other and together with the digestive tract and other organs, the salivary glands are extracted. Having separated the clump of organs from the head, they are covered with a cover glass.

The chitin covering of the abdomen between the third and second segment from behind on the posterior half of the mosquito is then broken with the needle. Holding the thorax of the mosquito with needle on which it is stuck, one pulls off, by means of another needle, the end of the abdomen from which trail the sexual organs (they are removed) and the intestine with the Malpighian vessels, which issue from the posterior and of the stomach (they are also removed). The dilated posterior part of the stomach is carefully protected with a cover glass.

The cocysts are visible in the native preparations even under weak magnification with a dry lens but are easier to examine under strong magnification (objective 40; ocular 7 or 10). Immature cocysts appear in such preparations as round homogeneous formations containing a pigment which consists of several small lumps. Well formed sporozoites which are in close contact with each other, are visible in the mature cocysts. The palisade-like rows of sporozoites are positioned at different angles to each other with the result that the cocyst assumes a mosaic appearance. When a moderate pressure is exerted with the cover glass on the preparation, the sporozoites are squeezed out from the cocyst. When the pressure is weak, the sporozoites remain in place. In this case, the burst cyst, because of the sporozoites which stick out in different directions, resembles a hedgehog. When the pressure is greater, the sporozoites are completely liberated and enter the fluid around the stomach, in which they are easily noticed because of their typical movements.

In the salivary glands, the sporozoites are arranged in rows perpendicular to the duct of the salivary glands and also in the form of conglomerates in the secretion of the salivary glands. When the sali vary glands are compressed, the sporozoites enter the ambient fluid in which they are easily discerned on account of their mobility.

Permanent sporozoite preparations can be made from the infected stomachs and salivary glands. The smears from the compressed mosquito stomachs with mature cocysts and also the infected salivary glands are fixed with methyl or ethyl alcohol. It is best when fixing the preparation, to pour the fixing agent on the preparation to avoid washing out of the sporozoites. When the fixing agent has evaporated, the preparations are stained according to the Romanovskiy method in the same manner as the blood preparations. The stained preparations are examined microscopically, using an immersion lens system. Sporozoites in which the cytoplasm is stained a blue color, and the nucleus consists of several clumps of chromatin, a red-violet, are clearly visible in these preparations.

Total permanent preparations are made from the infected stomachs and also the salivary glands. The stomachs or salivary glands adhering to the object or cover glass are drawn through alcohols with ascending molecular weight and embedded in Canada balsam.

Trematode cysts (which can simulate the cysts of the malaria parasites) can be seen on mosquito stomachs in addition to the cysts present in malaria-affected mosquitos. The differences between the trematode cysts and the cysts of the malaria parasites are the following: the cysts of trematodes are attached to the wall of the anterior part of the stomach, often in direct vicinity to the anterior intestine. They are seen in the form of solitary specimens and sit on a pedicle. The cysts of the malaria-infected mosquitos cling to the posterior half of the mosquito stomach in varying, sometimes very large numbers (200-500), they have no pedicles, and sit directly on the stomach wall under the outer envelope.

SKIN INFECTIONS

STAPHYLOCOCCUS INFECTIONS

G. V. Vygodchikov, Professor, Member AMN USSR

Staphylococci are widely distributed in nature and are the pathogenic organisms responsible for many human and animal diseases, greatly differing in their manifestations, from simple local folliculitis to fulminant staphylococcal sepsis. Staphylococcus infections are most frequently observed in maternity homes in parturient women and the newborn (sepsis, mastitis, pyodermia and pneumonia of the newborn).

Most diseases with staphylococcus etiology arise as a result of an infection with pathogenic staphylococcus strains which are resistant to antibiotics.

Biology of staphylococci; culture properties

A number of special media has been proposed for growing, selective isolation and differentiation of staphylococci.

A correlation between the biochemical and culture properties of staphylococci and their pathogenicity can be established when they are grown on Chapman medium. This medium consists of aqueous 1.5% agar containing 5.5% sodium chloride, 1% mannitol, 0.25% yeast extract, 3% gelatin, 1% tryptone and 0.5% potassium diphosphate.

The ability of the staphylococci to ferment lactose and mannitol is used to determine their biochemical activity which is taken as a criterion of their pathogenicity. For this purpose, media containing these carbohydrates, either solid (3.5% agar) or liquid and an indicator (pH 8.6) are inoculated with the test material.

When pathogenic staphylococci are grown at 37° for 18 hours, they

decompose the carbohydrate and the indicator, thus changing the color of the medium. However, a considerable number of strains of nonpathogenic staphylococci (from 11 to 55%) are also capable of fermenting mannitol, which is the reason why this test cannot be considered to be an adequate criterion of pathogenicity.

Besides, when antibiotic-resistant staphylococcus strains were isolated, their capacity for fermenting mannitol was found to change considerably.

A number of other methods of identification and differentiation of staphylococci is known.

A certain correspondence exists between the formation of phosphatase and coagulase by staphylococcus strains. A phosphatase test in cultures on dishes with nutrient medium is recommended for excluding nonvirulent strains, in particular, cultures isolated from the noses of possible carriers of pathogenic staphylococci. Agar medium containing phenolphtalein diphosphate is used for this purpose. By producing phosphatase, the microbes liberate free phenolphtalein which is then detected when the Petri dish containing the culture is exposed to ammonia vapor.

Formation of toxins

Staphylococcus toxin. Different points of view exist with regard to the nature and principle of action of the staphylococcus toxin. Until now, discussion has centered on the problem, whether the numerous toxic properties of the staphylococcus toxin belong to a single toxic complex or whether each of them is due to a separate substance. However, all researchers acknowledge the hemolytic function of the staphylococcus toxin.

The connection between the hemolytic capacity of staphylococcus and its pathogenicity allows us to consider hemolysis a sufficient cri-

terion of pathogenicity.

Of importance for practical purposes is the determination of the hemolytic functions of the α and β toxins of staphylococcus. When pathogenic strains of staphylococcus are grown which secrete α toxin when grown at 37° on agar dishes, containing 5% sheep or rabbit erythrocytes, large hemolysis zones are observed around the colonies; this is an indication, that the staphylococci produce α toxin. When inoculation is carried out with staphylococcus strains isolated from animals, the hemolysis of rabbit erythrocytes does not take place. However, β hemolysin lyses sheep erythrocytes but only on condition that after growing at 37°, the cultures are placed in the cold for 24 hours. β hemolysin has been termed hot-cold hemolysin because of this property.

TABLE 67a

Pathogenicity and plasmacoagulation (according to G. V. Vygodchikov)

Years	Number of investigated pathogenic staphylococcus strains	% of strains giving a positive plasma coagulation reaction
1948	867	97.6
1949-1951	768	96.7
1960-1961	832	95.2
Total	2467	96.5

The data obtained in the investigation of pathogenic staphylococci with respect to their capacity of coagulating rabbit blood plasma are summarized in Table 67a.

The method of plasma coagulation consists in the following: blood, taken from the heart of a rabbit in a quantity of 10 m*l*, is placed into a test tube containing 1 m*l* of 5% sodium citrate solution. The plasma thus obtained is diluted with physiological salt solution 1:4 (ex tem-

pore) and poured into sterile test tubes in a quantity of 0.5 mℓ in each. The test tubes containing the citrated rabbit plasma are then inoculated by means of a platinum loop with staphylococcus colonies, grown at 37° for 12-18 hours and placed into a thermostat at 37°. The pathogenic staphylococci coagulate the plasma within a period of 30 minutes to 10 hours, but usually within 2-4 hours.

The rabbit plasma can be replaced by human plasma for this coagulation test. The plasma of a person which has received a glucose injection shortly before the taking of the blood sample must not be used. Human plasma cannot be used if the test culture has been grown in a carbon dioxide atmosphere. The plasma-coagulation reaction is inhibited in both cases.

We have indicated earlier the contraindications existing with regard to the use of plasma from a donor. Individual series of human plasma used for blood transfusion, differ greatly with respect to the plasmacoagulation test, hence control test with coagulase-positive and previously known coagulase-negative staphylococcus cultures are necessary.

In this situation, blood plasma obtained by means of oxalates or heparin, which is not coagulated by enterococcus strains, must be used. It is also necessary to keep in mind that not all staphylococci which form part of a population, are capable of producing coagulase and, therefore, their ratio in the material taken for the test, may affect the plasmacoagulation reaction [Smith, Morrison, I. Lominskiy, 1952].

The reaction on glass can be used as a preliminary test. This method is based on the capacity of the coagulase-positive staphylococcus strain to be agglutinated by plasma. The coagulase-negative strains do not have this property [Kadnis, Grevs et al., 1943].

Ilek proposed to use the plasmacoagulation test for identifying pathogenic staphylococci on solid media. A strip of paper, moistened with

purified and concentrated antitoxic globulins from normal antitoxic antistaphylococcus serum from horses, immunized with staphylococcus toxin, is place on the surface of an agar dish, containing the blood plasma. The toxin is obtained by growing of strains of the toxigenic staphylococcus Wood-46. The staphylococci are used to inoculate the dish in a direction at right angles to the paper strip. The staphylococcus antitoxin inhibits the coagulation effect, while an inhibition of the plasma-coagulating effect is not observed in a control dish, in which the paper strip had been moistened with normal horse serum.

Antigen structure; serotypes; phagotypes

The pathogenic and nonpathogenic staphylococci can also be differentiated by means of the agglutination test.

The precipitation test, which has proved so valuable in the classification of streptococcus, has received a contradictory evaluation. Dudgeon and Simson could not arrive at any final conclusions with this method but Julianel and Vicard found it possible to differentiate the staphylococci by means of the precipitation test into two groups: pathogenic and saprophytic.

Using a large number of strains, Thomson and Corazo and Cowan found that each of these groups is made up of numerous subgroups. Among these groups, determined by means of the precitation test, different types can be distinguished by means of the agglutination test (Gowan).

The pyogenic staphylococci were subdivided by means of adsorbing sera into 7 specific types and 8 types with less specific reactions (Allison and Hobbs). Using these sera, Hobbs was able to identify 256 strains of staphylococci at his disposal, while only 67.5% of the strains were identified by means of bacteriophages.

The method of phage typing the staphylococci is of great importance. The staphylococci can be subdivided into different phagotypes by

means of specific staphylococcus phages.

This method makes it possible in some cases to confirm the identity of staphylococcus strains, isolated from carriers and from patients and thus to carry out the important epidemiological task of elucidating the epidemiological connections and the sources of infection in diseases with staphylococcus etiology.

The method of phage typing makes it possible to determine to some degree the pathogenicity of staphylococcus strains because only pathogenic staphylococcus strains can be phage typed.

For the purpose of staphylococcus phage diagnosis by means of specific lysis, phages are used belonging to the following five groups: to the first group belong the typing phages 29, 52, 52A, 79 and 80; to the second group 3A, 3B, 3C, 55 and 71; to the third group 6, 7, 42E, 73, 53, 54, 75 and 77; to the fourth group 42D and to the fifth group 187.

Observations of some foreign researchers indicate the existence of special socalled "epidemic" staphylococcus strains; to these belong the staphylococcus strain of phagotype 80, appertaining to the first group, and also the staphylococcus strain of phagotype 77 which belongs to the third group. These staphylococcus strains are most frequently isolated during flareups of staphylococcus hospital infections.

General recognition for the typing of staphylococcus has been accorded the method of Wilson and Atkinson (1945) who use the method of isolating phages proposed in 1942 by Fisk. Williams and Rippon (1952) described in detail the improved method of Wilson and Atkinson adopted by them.

For the purpose of typing, the staphylococcus bacteriophages are used in critical test dilution. The critical test dilution is defined as the greatest phage dilution which will still give complete lysis of a stephylococcus strain by the phage concerned. The method of phage

typing of staphylococcus cultures isolated from different materials consists in the following. Agar is filled into Petri dishes, slightly dried at 37° for 30-40 minutes and then a four-hour-old broth culture of the staphylococcus to be typed is poured over the surface of the agar. The excess culture is removed and the dishes dried for 20-30 minutes. For convenience, a grid is placed on the bottom of the dish, consisting of squares. A drop from each bacteriophage is applied to the dried agar surface, using one square of the grid pattern for each drop.

After slight drying of the phage drops, the dishes are left at 30° for 18 hours or at 37° for 5-6 hours. The results of the lysis are designated as confluent lysis (++++), confluent lysis with secondary growth (+++) and more than 50 negative colonies by (++).

The test result is considered positive only when the bacteriophage gives a lysis of the strain of not less than ++.

The nature of the sensibility of the staphylococci to the respective phage is very constant and isnot altered by repeated subcultures and subinoculations.

Up to 60% of the isolated staphylococci can be typed by means of the basic phages in critical test dilution (Matejovska, 1957; Maclean, 1956 and Vogelsang, 1959).

According to the data of Soviet authors, the percentage of isolated staphylococci which can be typed with phages is 73.7 (G. V. Vygodchikov, Akatov). If a staphylococcus culture is not lysed by the critical test dilution of phage, typing should be repeated with a concentration 100 × that of the critical test dilution.

Pathogenicity

Staphylococci can be divided into 3 groups on the basis of their pathogenicity and toxicity (Gross, G. V. Vygodchikov).

The staphylococci of the first group give considerable hemolysis

on 5% blood agar with rabbit or sheep's blood. When such a culture is injected subcutaneously, considerable necrosis is produced, sometimes followed by the death of the rabbit. The staphylococci of this group coagulate citrated rabbit plasma within 1-2 hours. Staphylococci having this property, as a rule, produce toxin and are isolated from patients with furunculosis, hydroadenitis, osteomyelitis, phlegmone, sepsis and several other purulent diseases. The staphylococci of the first group are to be considered as absolutely pathogenic.

The staphylococci of the second group cause slight hemolysis on 5% blood agar with rabbit or sheep's blood. When injected subcutaneously, hyperemia or infiltrations appear, rarely followed by necrosis. The staphylococci of this group coagulate citrated rabbit blood plasma within a period of 6 hours and over. The staphylococci with these properties are considered as conditionally pathogenic. They are often found on open skin surfaces, in folliculities, sometimes on wound surfaces.

The staphylococci of the third group do not cause hemolysis on 5% blood agar with rabbit or sheep's blood, do not cause necrosis when given to a rabbit subcutaneously and do not coagulate citrated rabbit blood plasma. These staphylococci must be considered as saprophytes; they are often isolated from the surface of healthy skin and also from various objects.

Laboratory Diagnosis of Staphylococcus Infections

Staphylococci can be relatively easily isolated from a human infection focus and it is thus not necessary to use a concentration method. In cases of staphylococcus pneumonia, the staphylococci are often isolated in the form of a pure culture. The same applies to staphylococcus enteritis of children. When the culture is made from the skin surface, the surface of the focus is carefully cleaned and the inoculation carried

out with material from the deeper layers using a platinum loop. The material taken up with the loop is carefully rubbed on the surface of an agar dish with a pH = 7.2-7.4.

To isolate staphylococci, Petri dishes containing milk-salt agar are inoculated. Meat-peptone agar with a pH of 7.2-7.4, containing 7.5% common salt, is kept at a temperature of 10° after sterilization and melted before use and 10% skim milk added. The latter is prepared by the usual method, poured into test tubes and sterilized at 100° for 30 minutes a day for 3 successive days.

Dishes with 5% blood agar with rabbit or sheep's blood are inoculated at the same time. After 18-20 hours of incubation at 37°, the colonies which have grown on the dishes are microscopically examined, smears made and gram-stained. The staphylococcus colonies are then subinoculated in test tubes containing nutrient medium and placed into the thermostat at 37°. After 12-18 hours of growth gram-stained smears from the cultures thus grown are microscopically examined, the plasma coagulation test is carried out, and a suspension is prepared from the remaining culture in physiological salt solution calculated to contain 2 billion microbial bodies in 1 ml of the suspension. 0.2 ml of this suspension is injected subcutaneously into a rabbit, the hairs being first removed by plucking or careful clipping. The reaction is read 24-48 hours later. It is not recommended to carry out more than 4-5 tests on one rabbit.

When blood cultures are made, the following method should be used. Following careful disinfection of the surface of the elbow joint with ether and alcohol, a blood sample is taken with a 10-gram syringe and, holding the needle, the tip of the syringe is sterilized in a flame and the blood squirted into a test tube containing 1 ml of sterile physiological salt solution. The test tube with the blood is placed into the

thermostat for 20 minutes and then into the refrigerator. Broth, containing 0.25% glucose and having a pH of 7.5, 50 ml of which is poured into test tubes with a diaemeter of 2 cm and a length of 30 cm, is used as the culture medium. Serum and part of the erythrocytes is sucked up with a Pasteur pipette and the test tubes with the medium are inoculated. The blood clot is used to inoculate another test tube. When a growth is observed in the broth after 12-18 hours, the microbes are microscopically examined and a subinoculation carried out in a test tube containing a sloping agar layer with pH = 7.5 and dishes with 5% blood agar made up with rabbit or sheep's blood.

On the following day, the culture is injected into a rabbit subcutaneously as indicated above (necrosis test) and then inoculation of citrated rabbit plasma is carried out (plasma coagulation test). 2 centrifuge test tubes filled with nutrient broth are then inoculated with the culture in order to determine the toxigenicity of the staphylococcus strain. The test tubes are placed into an exsiccator in which 20% of the air has been replaced by carbon dioxide. The test tubes are then centrifuged 48 hours later. 1 ml of the supernatant fluid is sucked off and 1 ml of antitoxic staphylococcus serum, containing 1 AYe/ml and a drop of rabbit erythrocytes, diluted in a ratio of 1:2 with physiological salt solution are added. The rabbit erythrocytes are previously washed three times in the centrifuge with physiological salt solution.

The degree of hemolysis is gaged after the test tubes have been in the thermostat for an hour. If the staphylococcus culture is toxigenic, hemolysis of the erythrocytes does not take place in the test tube to which the antitoxic serum has been added, while hemolysis should take place in the test tube to which no antitoxic serum had been added.

In order to determine the degree of toxigenicity of the isolated staphylococcus strain, the strain is grown on agar, prepared by the

method of Parish and Calrke (agar, diluted to half the concentration with Martin broth). The dishes are placed in an exsiccator in which 20% of the air has been replaced by carbon dioxide. 24 hours later, 10 mℓ Martin broth is added to each dish and the dishes placed into the exsiccator for 24 hours. The contents of the dishes are then filtered through gauze and Chamberlain candles. The filtrate thus obtained, if it contains toxin, should: 1) dissolve rabbit erythrocytes; 2) cause necrosis when injected subcutaneously into a rabbit; 3) kill a rabbit within 24 hours when given intravenously.

To determine the hemolytic capacity of the filtrate, it is diluted with physiological salt solution in dilutions of 1:100 to 1:1000, poured into test tubes in a quantity of 1 mℓ in each and a drop of rabbit erythrocytes, diluted 1:2 with physiological salt solution, added. The rabbit erythrocytes are first washed three times in the centrifuge with physiological salt solution. The hemolysis is gaged after the test tubes have been in the thermostat for an hour. The strains are considered to fairly toxigenic if the filtrate from their cultures dissolve rabbit erythrocytes in a dilution of 1:200 or higher.

To determine the skin necrosis effect, 0.2 mℓ of filtrate is given to rabbits subcutaneously in different dilutions. A toxin of medium strength causes the formation of necrosis in a dilution of 1:500.

To determine the lethal dose of staphylococcus toxin, 1.5 mℓ of the toxin is injected intravenously into a rabbit with a body weight of 2 kg. Observation time – 24 hours. In the overwhelming majority of cases the rabbit dies within 15 minutes after the injection. A toxin with medium strength kills a rabbit in a dose of 0.75 mℓ per 1 kg of body weight.

For the isolation and identification of the staphylococci which cause food poisoning, see p. 577 .

Manuscript Page No.	[Transliterated Symbols]
896	AMH = AMN = Akademiya meditsinskikh nauk = Academy of Medical Sciences
905	AE = AYe = aktivnaya yedinitsa = activity unit

GAS GANGRENE

K. I. Matveyev, Professor, and B. D. Bychenko, Candidate of Medical Sciences

Gas gangrene is a disease which usually appears as a result of deeply penetrating wounds in the muscles and other tissues under conditions, when the wounds are easily contaminated with anerobic pathogenic organisms stemming from environmental objects such as soil particles, clothes, etc., in the absence of modern surgical treatment involving the administration of antibiotics and chemotherapeutic substances. Typical for this disease are a rapidly spreading edema of the soft tissues and necrosis in them. Cases of gas gangrene were frequently encountered in wartime. In times of peace, they usually occur in connection with motorcar accidents, severe industrial and agricultural accidental injuries and also cases of abortus among women which have not been hospitalized. Gas gangrene is found as a complication after operations on the gastro-intestinal tract and after subcutaneous or intramuscular injection of various remedies.

The presence of pathogenic anaerobic bacteria in a wound does not always lead to the development of gas gangrene in the victim because the microbes responsible for gas gangrene have been detected in 18-40% of cases of combat injuries, while diseases resulting from them were recorded only in 0.3-1.9% of cases.

The classical pattern of gas gangrene with symptoms of myonecrosis, edema of the tissues, vigorous gas formation in them and also general intoxication and hemolytic anemia is caused mainly by Cl. perfringens

of type A. The possibility that other types of Cl. perfringens (B, C, D, E, and F) are capable of causing gas gangrene in humans, cannot be excluded, however, because the typical clinical pattern of this disease can be easily reproduced in laboratory animals by means of cultures of these microbes, producing lecithinase

Normally, gas gangrene is only rarely caused by any single species of clostridium. In this disease, different microbial associations are most often isolated from the wounds. Sometimes these are formed only by pathogenic clostridia (Cl. butiricum, Cl. tertium, Cl. bifermentans, etc.) or with proteolytic species such as Cl. sporogenes or Cl. histolyticum, which considerably aggravates the course of the infection. Cases of association of anaerobic bacilli with streptococci, staphylococci, E. coli and other aerobic bacteria are frequent.

Clostridium Perfringens

Morphology of Cl. perfringens

Cl. perfringens of the types A, B, C, D, E, and F are large, gram-positive bacilli which forms a capsule (Fig. 103, 104). They are devoid of cilia, immobile and form central or subterminal spores under certain

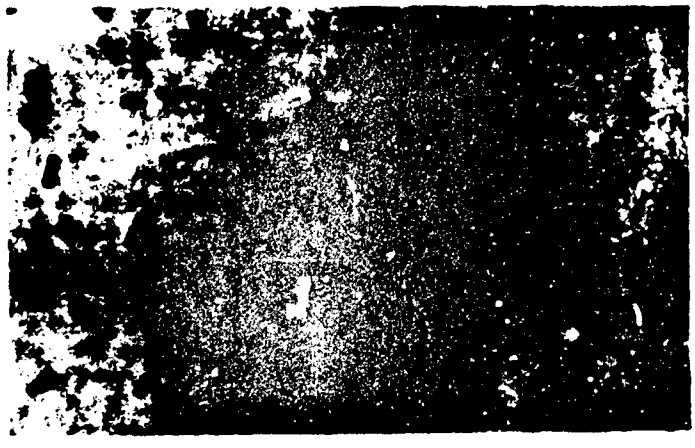


Fig. 103. Cl. perfringens A. Two week old culture on liver agar. 1800x.

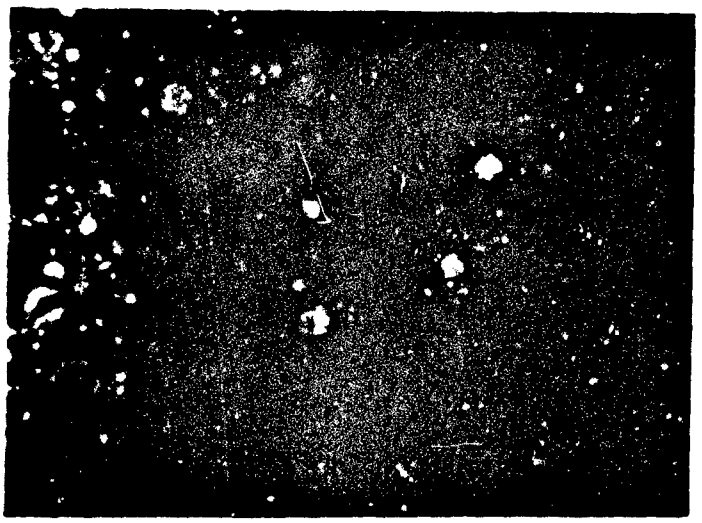


Fig. 104. Cl. perfringens. Smear from the liver of a guinea pig, which died of gas gangrene. A capsule surrounds the microbial cell in the form of a bright band. 1000x.

conditions (Fig. 105). the cells of different strains can be distinguished by their thickness and length. In some cases they are short, thick rods (0.6-1μ × 4-8μ, in others, long fibers with acuminate tips × 100-145μ).

The cells in 6-8 hour old cultures are gram-positive, well stained by methylene blue and other basic dyes. Old cells become gram-negative. They do not absorb methylene blue but can be stained with fuchsin.

Biology of Cl. perfringens; culture properties

In liquid nutrient media, prepared from meat or casein hydrolysates (pH = 7.4) at 37-43°, Cl. perfringens of all types grows rapidly (3-8 hours) with vigorous gas evolution and an alteration of the pH of the medium in the direction of lower values. A mixture of gases (hydrogen, carbon dioxide, ammonia, hydrogen sulfide, volatile amines, aldehydes and ketones) is evolved from large volumes of growing culture and an explosion can occur in presence of a flame.

Cl. perfringens is capable of forming spores on alkaline media,

Fig. 105. Spores of Cl. perfringens F. Oval bright formations at the end of long thick rods. Six day old culture in meat broth. 1900x.

rich in protein and free of fermentable carbohydrates.

Three stable variants of Cl. perfringens colonies exist: smooth (S), mucilaginous (M) and rough (R), but under certain conditions, colonies of the mixed variant (O) may appear.

The smooth forms of colony on the surface of agar resemble delicate dewdrops during their initial growth and then lose their transparency and assume a grayish or white appearance. They are circular, juicy, dome-shaped, with a smooth, shiny surface and even edges. In the depth of the agar in thin test tubes, the smooth forms of colony have the form of lenses or cotton wool clumps. If the edge of the colony is crenelated, its shape is cordiform. Sometimes several colonies, intersecting under different angles, have -aeroplane-like", "tricorn", etc. shapes.

The smears from S-forms of these colonies contain short cells without capsules.

The mucilaginous (M) colonies are similar to the smooth ones and differ from the latter by the greater height of the dome-shape and their

mucilaginous consistency. They consist of cells with capsules, which can form a thick slime when growing on liquid media.

The rough (R) variants on solid media have colonies of irregular shape with a crenelated "festoon-like" edge, sometimes with outgrowths in the form of spines and an uneven, tuberous surface. In the depth of the agar they resemble dense cotton wool clumps. Coarse, long cells or chains of short and medium-long elements are seen in the smears from these colonies.

Colonies of Cl. perfringens, growing on the surface of blood agar, are often surrounded by one or two hemolysis zones and assume a greenish color when kept in air (Fig. 106).

Around the colonies which have produced lecithinase toxin, a zone of nacreous precipitate formson the surface of yolk agar.

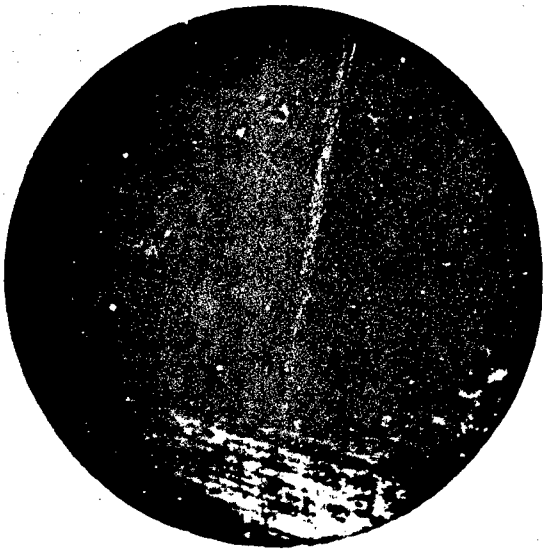


Fig. 106. Smooth colonies of Cl. perfringens A on blood agar, surrounded by a hemolysis zone. Natural size (according to Ye. A. Gil'gut)

Most strains of Cl. perfringens are only moderately proteolytic, liquifying coagulated serum or cooked pieces of meat slowly (on the 2-7th day). Many strains produce enzymes which liquify 7% gelatin within

24 hours. Typical for Cl. perfringens is its ability to coagulate litmus milk with formation of a clot of brickred color and complete clarification of the milk serum. All strains ferment glucose, galactose, lactose, levulose, maltose and saccharose with formation of acid and gas and do not ferment mannitol and dulcite. Some strains are capable of decomposing glycerol and inulin.

Formation of Toxins

The subdivision of Cl. perfringens into 6 types is based on the capacity of these microorganisms to produce lethal and nerotic toxins which differ in their antigen properties (Table 68).

Most of these substances are secreted into the ambient medium during the growth process of the microorganisms and are not retained in the cells, while some of them are elaborated in the form of inactive protoxins (epsilon and iota), which diffuse slowly through the cell walls and are capable of being transformed into powerful toxins by treatment with proteolytic enzymes, such as trypsin.

Different types of Cl. perfringens cause certain human and animal diseases (Table 69).

Cl. perfringens A, which produces α toxin in large quantity, is now considered to be the principal pathogenic organism of gas gangrene which is responsible for 70-80% of the cases of this disease. During the prior wars and the Second World War (1939-1945), the type of the strains of Cl. perfringens, isolated during the bacteriological diagnosis of gas gangrene from wounds, was determined by means of the therapeutic sera of type A, which can contain normal antitoxins for the other type of this microbe. Type-specific sera were not used. In this connection, the role of Cl. perfringens B, C, D, E, and F in the etiology of gas gangrene remained indeterminate.

Under experimental conditions, the strains of Cl. perfringens of

TABLE 68

Types of Cl. perfringens

Symbols:

+++ principal lethal and necrotic toxin, produced by all strains of the given type

++ toxin, produced by many strains

+ toxin or enzyme, produced in small quantities by all strains

± toxin or enzyme, produced by individual strains

Serial Number	Name of toxins and enzymes	Activity of the the toxins and enzymes	Type of Cl. perfringens A	B	C	D	E	F
1	Alpha α	Lecithinase C, lethal, necrotic, hemolytic factor	+++	±	±	±	±	±
2	Beta β	Lethal, necrotic factor	–	+++	+++	–	–	+++
3	Gamma γ	Lethal factor (minor)	–	±	±	–	–	±
4	Delta δ	Hemolytic lethal factor	–	++	–	+++	–	–
5	Epsilon ε	Lethal, necrotic protoxin, activated by trypsin	–	++	–	+++	–	–
6	Eta η	Lethal (doubtful)	±?	–	–	–	–	–
7	Theta θ	Hemolytic, oxygen-labile factor, whose activity is restored in presence of sodium thiosulfate or l-cysteine	±±	±	±	±	±	–
8	Iota ι	Lethal, necrotic protoxin, activated by trypsin	–	–	–	–	+++	–
9	Kappa κ	Collagenase (acting on pro-	++	±	++	±	+	–

		collagen and gelatin), lethal necrotic factor						
10	Lambda λ	Gelatinase, acting on denatured collagen and gelatin	−	±	−	±	+	−
11	Mu μ	Hyaluronidase	+	+	−	±	−	−
12	Nu ν	Desoxyribonuclease.	+	+	+	+	+	+

TABLE 69

Diseases, Caused by Cl. perfringens of the Types A, B, C, D, E, and F

Type of microbe	Name of disease
A	Gas gangrene of humans and animals, toxic food infections, pernicious anemia (?), chicken emphysema (?)
B	Dysentery of lambs, young goats, foals, farrows, enterotoxemia of sheep and goats
C	Hemorrhagic enterotoxemia ("Struck") of sheep, goats, farrows, calves
D	Infectious enterotoxemia of humans, sheep goats, rabbits, calves, "grass disease" of horses
E	Enterotoxemia of lambs and calves
F	Necrotic enteritis of humans.

any type, which produces a sufficient quantity of lecithinase α toxin), causes gas gangrene in laboratory animals.

The capacity for producing this toxin determines the role of the microbe in this infection. Cl. perfringens of the types B, C and F, which produce the lethal and necrotic β toxin, cause diseases, whose pattern of pathoanatomical alterations in the organs of humans and animals are similar. The most typical symptom of these alterations is a

hemorrhagic inflammation of the intestine mucosa, sometimes accompanied by ulceration and perforation of the walls of the small intestine. β toxin (up to 20-40 DLM for white mice) can be detected in these cases in the intestinal contents and the peritoneal fluid.

In cases of enterotoxemias caused by type D or E, the inflammation changes in the intestinal mucosa are never so pronounced in consequence of the fact that the necrotic properties of the lethal ε and ι toxins are weaker than those of the β toxin. Intestinal infections, caused by Cl. perfringens of different types, are often accompanied by pulmonary edema and blood extravasations in various internal organs.

The toxic factor of Cl. perfringens of type A, which is mainly responsible for the development of toxic food infections has not yet been discovered. The strains of type A, isolated from the products and excretions in cases of human food poisoning, are capable of forming heatresistant spores, produce small quantities of lecithinase and in most cases do not produce θ hemolysin.

esistance to physical and chemical factors

The toxins of Cl. perfringens are relatively quickly (in a few hours) destroyed under the influence of various ambient factors. The greatest stability is possessed by the prototoxins (ε and ι which can even withstand brief boiling (up to 30 minutes – 1 hour).

The toxins can retain their activity in the intestinal contents of cadavers for several hours and sometimes even days. Then they are decomposed or adsorbed on particles of the medium and the tissue.

The resistance of Cl. perfringens to the effect of various physical and chemical factors under otherwise equal conditions depends on the individual properties of the strain.

The vegetative forms of these microbes are normally quickly killed by the action of atmospheric oxygen, sunlight, high temperature, acids,

alkalis, alcohols, disinfectant liquids and antibiotics, which act on the gram-positive flora. The spores of Cl. perfringens of the types B, C, D and E are usually killed by 15-30 minutes boiling. Individual strains of type A and most strains of type F, however, form thermally stable spores which can withstand boiling or autoclaving for 1 to 6 hours. Such spores can get into raw material used for the preparation of various food products (including canned goods) from the environment and are capable of withstanding prolonged heating. Under certain conditions, viable spores can germinate. The cells of Cl. perfringens, which proliferate rapidly in the food product, when they enter the small intestine with the food, where they continue to proliferate at a tremendous rate, produce toxins and other active substances, which, by injuring the mucosa, interfere with its normal secretory-resorptive function and get into the blood. In some cases of toxic infections, caused by type A, septicemia is possible. In these cases, the microbes, which have penetrated the intestinal wall and entered the blood and the organs, proliferate rapidly, which is fatal to the patient.

Clostridium Oedematiens

Morphology of Cl. oedematiens

Cl. oedematiens is a large, straight or slightly curved gram-positive bacillus with a length of 4-10μ and a width of 1μ, which is strictly anaerobic, forms large elliptical subterminal spores, is mobile, peritrichate and has up to 20-25 cilia (Fig. 107).

Biology of Cl. cedematiens; culture properties

4 types of Cl. oedematiens (A, B, C, and D) are distinguished. Certain strains, when growing on the surface of solid media in the anaerostat, form round, juicy semitransparent, grayish colonies, sometimes with slightly grainy surface and a serrated edge, with a diameter of up to 3-4 mm within only 48 hours of incubation (Fig. 108). Colonies

Fig. 107. Day-old culture of Cl. oedematiens in meat broth. Stained by the method of M. A. Morozov. 1000x.

Fig. 108. Colonies of Cl. oedematiens A. Two-day old culture on liver agar. 32x.

of the strains of type A and B often have a tendency to creeping growth and formation of daughter colonies. The colonies of the strains of type D are usually very small, resembling dew drops.

The strains of type B and D have the least ability of developing on the surface of agar. On agar containing horse erythrocytes, colonies of these types, which produce β hemolysin, are surrounded by a hemolysis

zone which appears around colonies of type D after only 24 hours and is not present around colonies of type C.

Colonies of type A and B growing on blood agar containing benzidine as a result of the presence of hydrogen peroxide become black when they are exposed to the air for 30-60 minutes.

In the depth of glucose agar, colonies of Cl. oedematiens may resemble biconcave lenses, lumps of cotton wool, snow flakes, etc. They often have a compact yellowish or brownish center, surrounded by a corona of entangled threads. They break through the agar surface within 36-48 hours of the inoculation. 48 hours are often needed to obtain an intensive growth in a culture of Cl. oedematiens on liquid media. During the process of growth of Cl. oedematiens in liquid media, the pH of the medium becomes more acid, lactic, succinic, butyric and acetic acid and hydrogen sulfide being formed (particularly in cultures of type D).

The types A, B and C do not produce indole, while type D produces it in large quantities. Cl. oedematiens of types A, B and C ferment glucose, fructose and maltose, type D only glucose.

All types liquify gelatin but do not modify coagulated egg white.

Formation of toxins

Every type of Cl. oedematiens has the capacity of producing certain soluble antigens (toxins and enzymes).

The strains of the first two types which cause gas gangrane, produce lethal and necrotic α toxin, which is a powerful capillary poison and interferes with the permeability of the blood vessel walls, leading to an intensification of the exudation of fluid and protein. Some strains of type B produce this toxin in a quantity of up to 100,000 DLM (for white mice).

TABLE 70

Types of Cl. oedematiens and the Soluble Antigens Produced by Them

Name of antigen	Activity	Types of Cl. oedematiens			
		A	B	C	D
		Cl. novyi	Bac. gigas	Cl. bubalorum	Cl. haemolyticum
Alpha α	Thermally labile, lethal, necrotic, capillary poison	+	+	−	−
Beta β	Necrotic, hemolytic, lethal, lecithinass C	−	+	−	+
Gamma γ	Necrotic, hemolytic lecithinase	+	−	+(?)	−
Delta δ	Oxygen-labile hemolysin	+	−	−	−
Epsilon ε	Lipase, causing opalescence of hen's egg yolk solutions	+	−	−	−
Zeta ζ	Hemolysin	?	+	−	−
Eta η	Tropomyosinase	−	+	−	+
Theta θ	Enzyme causing opalescence of solutions of hen's egg yolk	−	? Traces	−	+
Diseases caused by it		Gas gangrene	Gas gangrene, "black disease" of humans and herbivorous animals	Chronic osteomyelitis of buffalo	Bacillary hemoglobinuria of calves.

The β and γ toxins belong among the hot-cold hemolysins. They cause hemolysis only when the erythrocytes treated with the toxin at 37° are then kept at low temperature (4°) for 1-2 hours; the β hemo-

lysin is produced only by strains of the types B and D, in larger quantity by the latter. This enzyme causes strong hemolysis of mouse and also erythrocytes of horses and sheep.

γ hemolysin is produced only by the strains of type A and is more potent with respect to erythrocytes of horses than those of sheep. The other soluble agents apparently play only a secondary part in the pathological processes, caused by Cl. oedematiens (Table 70).

Pathogenicity

Under natural conditions, Cl. oedematiens is found in the soil (in 30-50% of investigated samples), in the excretions of humans and animals, the water of lakes and rivers, etc. They can be isolated fairly frequently from wounds (from 5 to 50% of cases of all isolated anaerobes); they are responsible for a considerable proportion of the fatal issues in gas gangrene. They are normally encountered in association with Cl. perfringens, Cl. sporogenes and other microbes. Gas gangrene, caused only by Cl. oedematiens, is very rare. It has a longer incubation period (5-6 days) than the infections caused by Cl. perfringens (from some hours to 1-2 days). Typical symptoms of the disease are: a gelatinous edema, copious secretion of serous fluid from the wounds (sometimes several liters a day), the absence of gas bubbles in the tissues and the absence of a putrid odor.

Only types A and B are capable of causing gas gangrene in humans. The bacteria of both these types are highly virulent for laboratory animals.

When toxic culture fluids are given subcutaneously or intramuscularly to guinea pigs or rabbits under experimental conditions, patterns of alterations reminiscent of those in humans are easily reproduced in animal tissues.

When mice are injected subcutaneously with α -toxin they die within

24-48 hours. Weakly virulent cultures are preferably injected into animals intramuscularly with 2.5% calcium chloride solution. The strains used for the infection can be isolated from the edematic fluid and blood from the heart of the dead animals. The antitoxic serum against α toxin protects against the disease.

Cl. oedematiens of type C is nonpathogenic when given to laboratory animals subcutaneously, while a 24 hour old culture of Cl. oedamatiens of type D, when injected subcutaneously, causes the death of guinea pigs, rabbits and mice within 24-48 hours with symptoms of slight subcutaneous hemorrhage and hemoglobinuria. The latter is most easily reproduced in rabbits and mice. The initial strain can be isolated from the blood of the heart and spleen.

Resistance to physical and chemical factors

All types of Cl. oedematiens are highly sensitive to oxygen. Hence, colonies of Cl. oedematiens, which have grown on the surface of a medium, should be exposed to air as little as possible, otherwise all the cells may die quickly. The spores of Cl. oedematiens are fairly resistant to all environmental factors. They can even withstand boiling (up to 1-2 hours). The spores of type D remain viable in the bones of dead animals for up to 7-8 years.

The zonal distribution of Cl. oedematiens in the soil has not been investigated. However, there is a definite connection between the frequency of black disease of sheep (type B) and of bacillar hemoglobinuria of calves (type D) and the pasture regions where the animals are kept. Cases of transfer of bacillary hemoglobinuria from one region to another are known to have occurred, when sick calves or bacillus carriers were transported.

Clostridium Septicum

Morphology of Cl. septicum

Polymorphous gram-positive mobile bacillus with a length of 4-5μ and a width of 0.8μ with subterminal ellipitical spores (Fig. 109). Depending on the strain and the medium, it can be transformed into short, distended shapes or long threads. The latter are often detected in the cadavers of animals which have died of bradzot, on the surface of the liver adjacent to the diaphragm (Fig. 110).

Fig. 109. 18 hour old culture of Cl. septicum in meat broth. 1900x.

Biology of Cl. septicum; culture properties

Strictly anaerobic organism. On the surface of solid nutrient media it has a tendency to form shiny, semitransparent colonies with a diameter of up to 4 mm with uneven fringed edges within 48 hours of incubation and has a tendency to creeping growth (Fig. 111). The R-form of colony is the most frequent.

In the depth of 1% agar it forms colonies with a diameter of 1-2 mm with thickened center and radially outgoing entangled threads. In 2% agar, the colonies assume a lentil or heart shape, etc., sometimes

Fig. 110. Cl. septicum in the liver of a guinea pig, which has died from gas gangrene. 1800x.

Fig. 111. Incipient development of colonies of Cl. septicum on liver agar 10 hours after inoculation. 64x.

with protuberances. On blood agar, a narrow hemolysis zone appears around the colonies but not immediately, usually on the second day.

It ferments glucose, maltose, and lactose and does not break down glycerol, saccharose and mannitol, liquifies gelatin and coagulates litmus milk with formation of acid and gas. It does not digest coagulated serum and egg white, does not form indole, does not reduce nitrates and nitrites and does not produce large quantities of hydrogen sulfide.

Formation of toxins

Cl. septicum produces at least three soluble antigens; the α toxin (lethal, necrotic, hemolytic, oxygen-stable), the β antigen

(deoxyribonuclease) and δ toxin (oxygen-labile hemolysin).

The first toxin, when given to mice intravenously, depending on the dose, causes fairly rapid death of the animals with symptoms of convulsions and paralysis. Its effect on the heart is reminiscent of the effect of digitalis. Pointlike hemorrhages are found upon autopsy in the endocardium and epicardium and the different parenchymatous organs. It is capable of hemolysing sheep erythrocytes under condition that contact with the erythrocytes lasts at least 5-60 minutes and that saccharose is not present in the solution. The other antigens do not play an important part in the development of the pathological processes. Cl. septicum is serologically closely related to Cl. chauvoei (Cl. feseri) which entitles us to regard these clostridia as belonging to the same species. Cl. septicum causes gas gangrene in humans and various animals and also bradzot of sheep.

Pathogenicity

The cases of gas gangrene, produced by these microbes, have an incubation period of 2-3 days. The quantity of serous exudate from the wounds is considerably less than in the case of infection with Cl. oedematiens. The gas formation in the tissues is not very intense, and the putrid odor is absent if proteolytic clostridia do not participate in the infection.

Following intramuscular injection of 1 ml of a fresh 24-48 hour old culture of Cl. septicum, obtained on a medium of meat or casein hydrolysate, into the leg of a guinea pig, intense edema of the tissues develops at the injection site after some hours, and the animal dies within 24-48 hours.

Severe edema, extending from the injection site to the entire anterior part of the abdomen, is observed upon autopsy. The tissue has a red color and a bloody, foaming fluid can be pressed out from it. The

muscles are not liquified. The initial strain can be easily isolated from the blood of the heart and liver, where the proliferation foci are normally located.

Cl. septicum is very widespread in nature. This microorganism can be isolated from 8-21% of all investigated soil samples. The zonal distribution has not been sufficiently investigated, although data exist, indicating that cases of gas gangrene and bradzot disease of sheep are found most frequently in places where contamination of the soil with spores of Cl. septicum had been particularly severe.

Resistance to Physical and Chemical factors

The vegetative forms of Cl. septicum are fairly sensitive to oxygen, hence it is recommended not to leave dishes with colonies which have grown on the surface of agar, in contact with the air too long during reinoculation. The spores of Cl. septicum are resistant to the action of environmental factors and can even withstand boiling for brief periods. This crganism is capable of vegetating in the soil for several years.

Clostridium Histolyticum

Morphology of Cl. histolyticum

Gram-positive mobile bacillus with a length of 3-8μ, and a width of 0.5-0.8μ, forming elliptical subterminal spores (Fig. 112). Peritrichate, but strains are found in nature which do not have cilia.

Biology of Cl. histolyticum; culture properties

Amphimicrobian. Under aerobic growth conditions it does not form spores and develops poorly. On the surface of blood agar it grows well under anaerobic conditions. Within 24-48 hours, the colonies attain a diameter of 0.5-1 mm, they are semispherical, transparent, shiny, with even edge and a narrow hemolysis zone around them (Fig. 113). Later on, the colonies lose their transparency, becoming gray or white with un-

Fig. 112. Two day old culture of Cl. histolyticum on liver agar. 1900x.

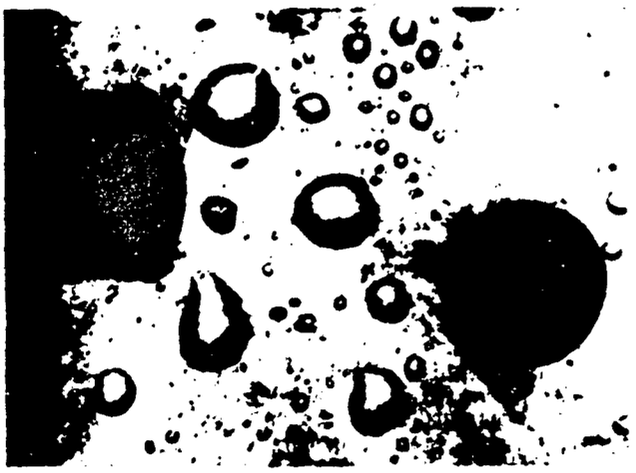


Fig. 113. Colonies of Cl. histolyticum on blood agar. Within 3-4 days, grayish, opaque daughter colonies appear within the colonies. 64x.

even edges. Their is no tendency to compact growth.

In the depth of agar, themobile strains often form flake-like colonies with compact center. The immobile strains form lensshaped colonies, sometimes with protuberances.

A peculiarity of Cl. histolyticum is the fact that this microorganism does not ferment a single sugar, but is strongly proteolytic,

decomposing gelatin and coagulated blood serum.

Pieces of meat, placed into liquid nutrient medium, containing a vigorously growing culture of Cl. histolyticum, are subjected to rapid proteolysis. Although hydrogen sulfide is produced in large quantities, indole not formed.

Formation of toxins

Cl. histolyticum produces several soluble antigens: α toxin which is lethal and causes necrosis; β toxin, which is a collagenase, decomposing azocoll and gelatin and kills mice when injected intravenously; γ antigen, a proteinase, which is activated by reducing agents, does not decompose native collagen but acts on gelatin, azocoll and casein; δ antigen, an elastase, which also decomposes azocoll and gelatin; and the ε toxin which is an oxygen-labile hemolysin.

Pathogenicity

Cl. histolyticum is detected in the soil, human and animal excreta, etc. It is isolated from wounds in 1-18% of cases. Infections caused exclusively by this microorganism in humans are extremely rare (single cases). Only the strains of the smooth form of Cl. histolyticum are pathogenic. Gas gangrene in humans takes a severe course with symptoms of rapid and profound liquefaction of soft tissues and often with fatal issue.

The typical pattern of tissue modification, caused by Cl. histolyticum can be reproduced when 0.5 mℓ of a fresh culture of the S-form of this microbe prepared on meat or casein hydrolysate medium is injected intramuscularly into a guinea pig. The death of the animal occurs, depending on the virulence of the strain concerned, within a period of several hours to several days.

If the strain produces much α toxin, severe tissue alterations cannot develop at the injection site, because the guinea pig may die

within a few hours. Strains of medium virulence kill the animals more slowly with symptoms of almost complete local digestion of the soft tissues due to the presence of proteolytic enzymes. Toxicosis symptoms are almost completely absent.

Resistance to physical and chemical factors

The vegetative forms of Cl. histolyticum are only slightly resistant to the action of physical and chemical factors. The spores can withstand brief boiling and are capable of remaining viable in the soil for several years. Their zonal distribution in nature has not been investigated.

Clostridium Sporogenes

Morphology of Cl. sporogenes

Gram-positive, mobile bacillus with a length of 3-6μ and a width of 0.5-0.8μ, which easily forms elliptical, subterminal spores which extend the cell.

Biology of Cl. sporogenes; culture properties

Strictly anaerobic; on the surface of solid nutrient media it forms within 48 hours shiny, semitransparent, whitish-gray colonies with a diameter of 3-5 mm with a flat surface and raised center. The edge of the colonies is smooth or festooned. A tendency to creeping growth is sometimes observed.

On blood agar, the colonies are round with a narrow hemolysis zone. In the depth of the agar, the colonies may have the appearance of flakes with dense center or are lensshaped, sometimes with modified edges.

On a medium consisting of meat or casein hydrolyzate, intense growth can be observed within only 24 hours, with partial digestion of pieces of meat on the bottom. A layer of fat normally accumulates on the surface of the medium and the cultures have a putrid odor (skatole).

It ferments glucose and maltose, but does not break down lactose and saccharose. Some strains can ferment salicin. It decomposes gelatin and peptizes milk, sometimes with formation of a clot. It liquifies coagulated blood serum and egg white, does not form indole.

It is widely disseminated in soils, human and animal excrements, taking an active part in the processes of putrescent decomposition of protein substances in nature.

It can often be detected in wounds (approximately in half the cases).

Pathogenicity

Some strains are pathogenic for laboratory animals. When a guinea pig is injected intramuscularly with 0.1 mℓ of a day old culture of such a strain obtained from meat hydrolyzate medium, the animal dies within 1-2 days with symptoms of local putrescent decomposition of the muscle tissue. The cultures of most strains of Cl. sporogenes are not pathogenic for laboratory animals and can cause only local alterations which heal spontaneously.

Cl. sporogenes strongly increases the virulence of species such as Cl. perfringens and Cl. septicum in mixed cultures, isolated from gas gangrene cases. The zonal distribution in nature has not been studied.

Resistance to physical and chemical factors

The spores of Cl. sporogenes are very resistant to the action of various factors. The spores of some of its strains can withstand boiling for 15 minutes to 6 hours.

Clostridium Sordelli

Morphology of Cl. sordelli

Gram-positive bacillus with a length of 2-4μ and a width of 0.6-1μ (Fig. 114) which readily forms elliptical, central and subterminal

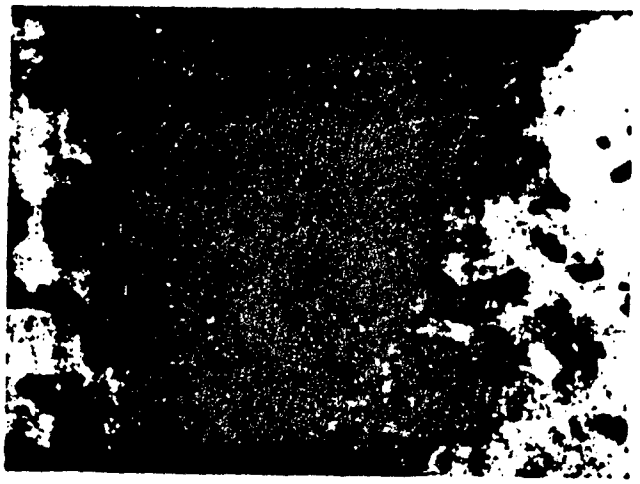


Fig. 114. Two day old culture of Cl. sordelli on liver agar. 1900x.

spores on normal media. Peritrichate, mobile in fresh cultures.

Biology of Cl. sordelli; culture properties

Not strictly anaerobic. On the surface of solid nutrient media it forms within only 24-48 hours slightly convex grayish-white colonies with irregular outlines (Fig. 115). On blood agar (horse erythrocytes) the colonies are surrounded by a narrow hemolysis zone. On agar with cocoa blood the colonies are surrounded by a narrow clear zone (owing to proteolysis). On agar containing hen's egg yolk, milk and lactose, a zone of opalescence appears around the colonies. This phenomenon can be partly or completely prevented by means of antitoxic serum active against perfringens of type A. In the depth of the agar, the colonies have a lens or heart shape, sometimes with outgrowths at the edges. In liquid meat nutrient media, intense growth of Cl. sordelli takes place within the first 24 hours, often with formation of slime.

It ferments glucose and fructose and does not decompose lactose and saccharose. It liquifies gelatin and coagulated blood serum but more slowly than Cl. histolyticum and Cl. eporogenes and produces hydrogen sulfide and indole.

It also produces lecithinase C, which has certain serological properties in common with the lecithinase of Cl. perfringens, although it is less active. This enzyme hemolyses mouse erythrocytes fairly efficiently, rabbit erythrocytes somewhat less so and has hardly any effect on sheep and horse erythrocytes.

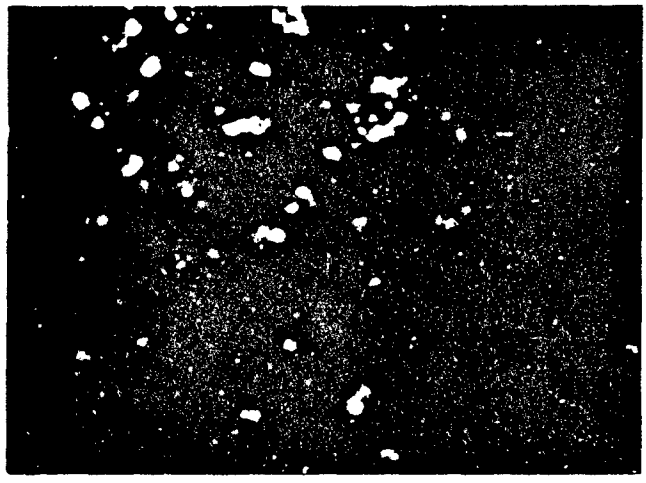


Fig. 115. Colonies of Cl. sordelli on blood agar 6-7 days after inoculation. 4×.

Formation of toxins

According to one point of view, Cl. sordelli is a virulent form of Cl. bifermentans. However, Brookes and Ipsen (1959) do not agree with this opinion, having shown by their researches, that these are two different species of bacteria. Their data are considered to be correct by many bacteriologists. The virulent strains secrete a highly potent lethal toxin, similar in properties to the α toxin of Cl. oedematiens. A specific antitoxic serum against it can be easily prepared.

Pathogenicity

Used experimentally on animals, it can cause a disease similar to gas gangrene with subcutaneous gelatinous edema, sometimes having a

rose-red color. Blood extravasations and gas bubbles are observed in the inouclated muscle. Some strains can cause local liquifaction of the muscle tissue similar to the weakly virulent strains of Cl. histolyticum.

The death of the animals usually occurs in consequence of the lethal toxin within 1-2 days after injection of the culture.

Cases of fatal intestinal disease of cattle have been described, caused by Cl. sordelli. Cases of food poisoning in humans, caused by Cl. sordelli, have also been observed.

Resistance to physical and chemical factors

The spores of Cl. sordelli are resistant to different environmental factors. They are able to survive in the soil for long periods.

Clostridium Fallax

Morphology of Cl. fallax

Gram-positive, straight, sometimes encapsulated bacillus with a length of 2.5μ and a width of 0.5μ with rounded ends, rarely forming elliptical central or subterminal spores. Peritrichate, mobile in young cultures.

Biology of Cl. fallax; culture properties

Strictly anaerobic. On the surface of agar it forms after only 24-48 hours of incubation at 37° flat, transparent colonies with irregular periphery (diameter 1.5-2 mm) which then become opaque, with a raised center.

On agar with horse blood, the colonies are surrounded by a narrow hemolysis zone. In the depth of agar, the colonies resemble small lenses, sometimes with outgrowths at the edges. It ferments glucose, maltose, lactose, saccharose, mannitol and salicin, does not break down starch and glycerol, does not liquify gelatin, coagulated blood serum or egg white. It curdles milk with formation of acid. It does not form

hydrogen sulfide or indole and does not reduce nitrates to nitrites. It is isolated from wounds in 1-4% of cases.

Pathogenicity

Fresh cultures isolated from wounds are pathogenic in the first generations for mice and guinea pigs. The virulence of these strains is lost quickly in the course of several passages through artificial meddia. Virulent cultures in a dose of 1-2 mℓ can kill guinea pigs (in the course of a week) with symptoms of hemorrhagic alterations in the injected muscle and with a gelatinous edema. It is possible to isolate the original strain from the blood of the heart and the site of injection of the culture of the dead animal. Bacteremia usually begins shortly before death. It obviously produces a lethal toxin, to which antibodies have not yet been produced.

These microorganisms are evidently widely distributed in nature. They have been detected in cases of appendicitis, wounds, chronic arthritis of humans and in cattle gangrene.

The zonal distribution has not been studied.

The vegetative forms and the spores are killed by brief boiling.

Among the other clostridia which are relatively often isolated from wounds, we must mention Cl. tertium, Cl. tetanomorphum, and Cl. cochlearium, which are not pathogenic for laboratory animals but can nevertheless exert some influence on the course of gas gangrene, when they are associated with pathogenic clostridia.

Laboratory Diagnosis of Gas Gangrene

In view of the fact that pathogenic clostridia are widely distributed in our environment (particularly Cl. perfringens) their isolation from wounds in itself is not of particular diagnostical significance, because their presence in a contaminated wound does not necessarily indicate the development of gas gangrene.

It is also necessary to take into account the fact that the incubation period of gas gangrene is usually a few hours to 2-5 days, i.e., the time, required for completing a fairly complex bacteriological analysis. Hence, the tests for pathogenic anaerobic bacteria need not be carried out in all cases of wounds but only in presence of certain clinical indications. Purpose of the bacteriological diagnosis in this case is the confirmation of the clinical diagnosis of gas gangrene.

The results of the bacteriological diagnosis can be of great importance for the timely selection of remedies for the antitoxic therapy, and also for treatment with antibiotics and chemotherapeutic remedies.

Materials for the test; selection of specimens

Exudate, pieces of altered tissue from the wound of the patient and also blood from a vein (5-10 m*l*) is taken for the bacteriological examination.

Material from a cadaver (wound exudate, pieces of altered muscles, blood from the heart, 10-20 g from the spleen and from the liver) must be taken as soon as possible after death (within a few hours) because various other pathogenic anaerobic microorganisms, which are always present in the gastro-intestinal tract, which are not the direct cause of the given disease, may penetrate into the tissues of the cadaver.

All the sample material is placed into a sterile, hermetically sealed glass or plastic container and immediately sent to the bacteriological laboratory.

The samples in which the pathogenic agents are most easily detected (material from wounds, blood, liver, spleen) are examined first.

Microscopic examination

All samples are subjected to microscopical examination. For this purpose, contact smears are taken and gram-stained.

The presence of a large number of large gram-positive rods serves as a preliminary indication for suspecting a clostridium infection.

Bacteriological Investigation

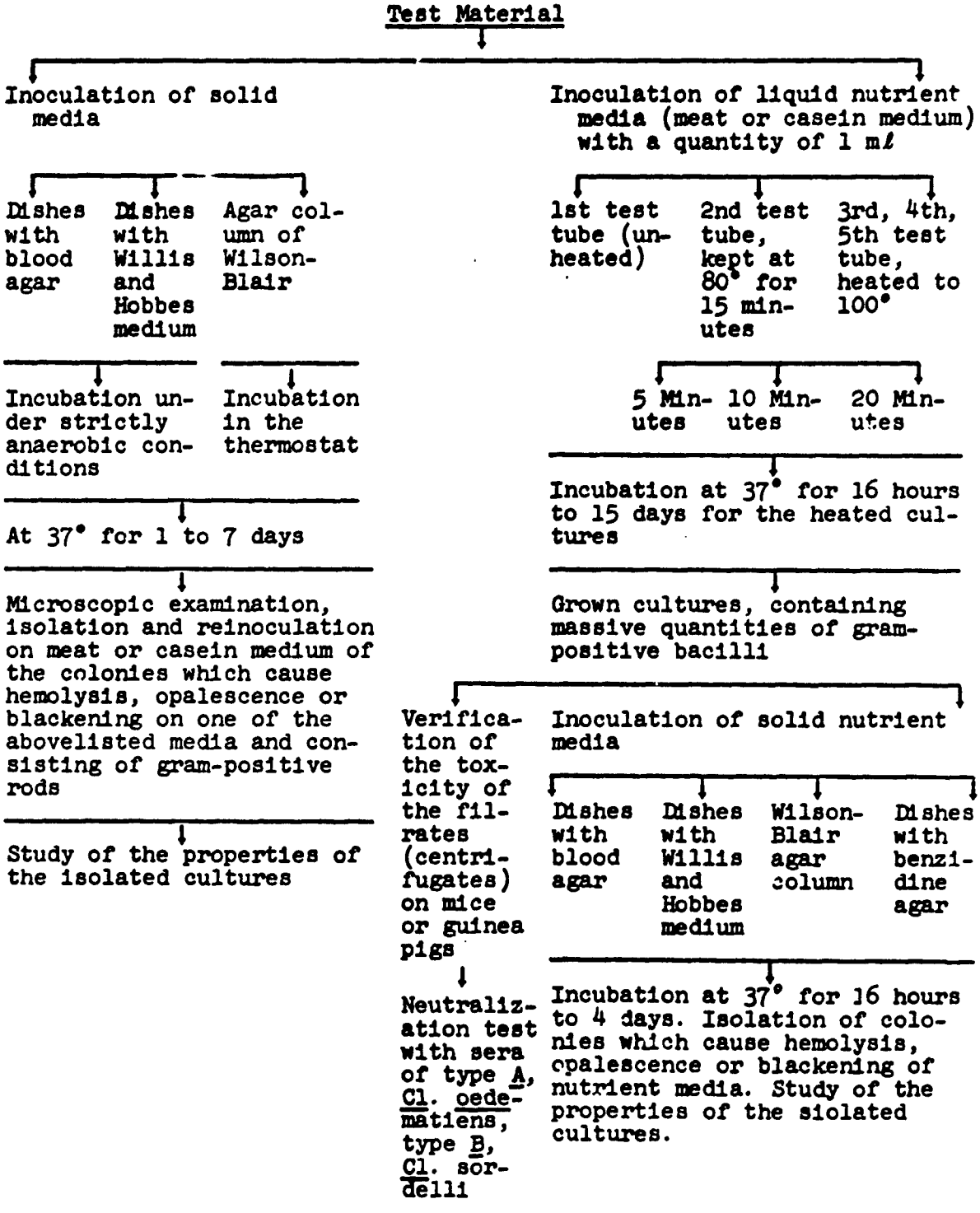
The solid materials are cut up in a sterile manner with scissors and ground in a mortar with sterile sand or glass in an equal volume of physiological salt solution. Blood or exudate is centrifuged at 3000 rpm for 30 minutes and the precipitate used for the inoculation of the medium. For nutrient media for culturing of anaerobic bacteria, see p. 958.

The suspension of the test material is used to inoculate solid nutrient media by means of a platinum loop – blood agar (see p. 970) and benzidine agar (p. 973). The cultures are incubated under anaerobic conditions at 37°, examined the next day and then every 2 days (up to 7 days) for isolating the suspected colonies. The grown colonies, which cause hemolysis on blood agar, opalescence, proteolysis or the appearance of a nacreous aureole on Wilson-Blair medium, are checked for purity and the presence of gram-positive bacilli (by microscopy) and then used to inoculate test tubes containing liquid casein-fungus medium (p. 965) under a layer of vaseline oil. These are then incubated in a normal thermostat for 24-48 hours and examined in accordance with the general testing scheme until pure cultures are obtained (Table 71).

1 ml of the original suspension of the test material is placed into each of 5 test tubes containing casein-fungus medium or a medium made of meat hydrolyzate under vaseline oil. The test tubes are then heated as indicated in Table 71 and incubated at 37° for 15 days, and examined daily for the presence of growth. When signs of the growth of an anaerobic culture are evident (cloudiness, gas evolution), the cultures are checked by microscopic examination for the presence of large

TABLE 71

Examination of Samples for the Presence of Pathogenic Clostridia



gram-positive bacilli.

Isolation of a pure culture. All grown cultures which contain a large number of gram-positive bacilli (including contaminated cultures) must be reinoculated on the solid media, indicated in Table 71, and this stage of investigation must be completed by isolation of pure cultures.

The isolated strains are studied in accordance with the general scheme (Table 72) for their morphology, mobility, capacity to ferment carbohydrates and to change litmus milk, to liquify gelatin and coagulated blood serum or egg white.

The toxic and virulent properties of the cultures are checked for their ability to modify blood agar, Willis medium and also in tests on laboratory animals (mice, guinea pigs).

Detection of the pathogenic agents of the gas gangrene by means of the biological test

Relatively pure cultures, in which an extraneous flora is either absent or met in the form of single cells in the field of view among dominant large numbers of cells of the basic culture, are tested for toxicity in vivo.

To this end, 0.2 ml each of the centrifugate of the cultures is injected intravenously or intraperitoneally into white mice or subcutaneously into guinea pigs. When the mice die during the first 24 hours or when skin necrosis appears in the guinea pigs, the neutralization test is carried out on mice or guinea pigs with the diagnostic sera of Cl. perfringens, type A, Cl. oedematiens, type A and B, Cl. septicum, Cl. histolyticum and Cl. sordelli (Table 73).

The toxin-antitoxin mixtures are kept at 20° in a dark place for 20 minutes and then 0.5 ml of it is injected into white mice (calculating 2 mice per test tube) or 0.2 ml subcutaneously into a guinea pig.

TABLE 73

Neutralization Test on Mice or Guinea Pigs

No. of test tube	Centrifugate of the test culture, ml	Antitoxic sera					
		Cl. perfringens, type A	Cl. oedematiens, type A and B	Cl. septicum	Cl. histolyticum	Cl. sordelli	Physiological salt solution
1	0.9	0.6 ml 50 AYe	-	-	-	-	-
2	0.9	-	0.6 ml 100 AYe	-	-	-	-
3	0.9	-	-	0.6 ml 100 AYe	-	-	-
4	0.9	-	-	-	0.6 ml 100 AYe	-	-
5	0.9	-	-	-	-	0.6 ml 100 AYe	-
6 control	0.9	-	-	-	-	-	0.6 ml

The results are evaluated 5-6 hours later and finally on the 3rd day. The species of the culture under examination is determined on the basis of the neutralizing effect of the homologous antitoxic serum.

When negative results are obtained, the presence of skin necrosis in guinea pigs or the death of the animals, the neutralization test is carried out with the specific diagnostic typing sera for Cl. perfringens A, B, C, D, and E.

Rapid Methods of bacteriological diagnosis

Because a complete bacteriological analysis for clostridium infection is fairly complex, numerous rapid and simple methods of determining some pathogenic anaerobes have been proposed.

1. In some cases of fulminant development of gas infection, when

TABLE 72

Basic Properties of Certain Pathogenic and Certain Nonpathogenic Clostridia, Isolated from Wounds

1 Название клостридий	2 Морфология: 6 размеры палочек	7 подвижность	8 появление на поверхности агара через 48 часов	9 появление в глубине агара	3 Споры: 10 форма, расположение	11 устойчивость к кипячению	4 Протеолитические свойства: 12 желатина	казеина	свернутый сывороточный белок	15 индол
Cl. perfringens типов A, B, C, D, E, F 33	4—8× ×0,5—1	—	2—4 мм S, SR, R, M	Чечевицы или комочки ваты 34	Овальные, субтерминальные, реже—центральные	От 5 минут до 6 часов 36	±	±	—	—
Cl. oedematiens типа A и B 40	5—10×1	+	1—3 мм SR, R	Пушинки с плотным центром, чечевицы 41	35	До одного часа 42	+	—	—	—
Cl. septicum	3—8× ×0,6	+	2—4 мм SR, R 47	Хлопьевидные с древовидными разветвлениями, сердце, чечевицы	То же 46	От 2 до 45 минут 48	+	—	—	—
Cl. histolyticum	3—5× ×0,5	+	1—2 мм SR	Мелкие плотные комочки 66	То же 46	От 30 минут до 9 1/2 часов 49a	+	+	+	—
Cl. sporogenes	3—6× ×0,5	+	3—5 мм SR	Комочки ваты с уплотненным центром 53	То же 46	От 1 до 3 часов 54	+	+	+	—
Cl. bifermentans	2—4× ×0,5—1	+	2—3 мм SR	Комочки ваты, чечевицы 56	То же 46	До 1 часа 57	+	+	+	+
Cl. sordellii	2—4× ×0,5—1	+	2—3 мм SR	Комочки ваты, чечевицы	То же 46	То же 46	+	+	+	+
Cl. fallax	2—5× ×0,5	+	1—2 мм SR	Мелкие чечевицы 59	То же 46	То же 46	—	—	—	—
Cl. tertium	3—5× ×0,4	+	1—2 мм S, SR	Мелкие чечевицы с уплотненным центром 63	Терминальные, овальные 64	То же 46	—	—	—	—

Сбраживание углеводов 5															
Лакмусовое молоко 16	Глюкоза 17	Левулеза 18	Галактоза 19	Галактоза 20	Сахароза 21	Мальтоза 22	Инулин 23	Манит 24	Дульцит 25	Салицин 26	Глицерин 27	Крахмал 28	Сероводород на кровяном агаре 29	Среда Вильсона и Хобс 30	Вакцинный агар 31	Патогенность для животных 32
Свернутый сгусток, КГ 3—16 часов 37	+	+	+	+	+	+	—	—	—	—	±	+	±	Покраснение, ореол опалесценции 38	—	Газовая гангрена 39
Медленное свертывание несколько дней 43	+	—	—	—	—	±	—	—	—	—	—	—	+ 44	Опалесценция, перламутровая зона (тип А)	Почернение 45	То же 46
То же 46	+	—	+	—	—	+	—	—	—	+	—	—	+	Покраснение 49	—	То же 46
Пептонизация 50	—	—	—	—	—	—	—	—	—	—	—	—	+	Зона просветления вокруг колоний 51	—	Газовая гангрена с расплавлением мышц 52
То же 46	+	—	—	+	—	+	—	—	—	—	—	—	+	Зона опалесценции и перламутрового блеска 55	—	Местные расплавления ткани, иногда убивает животных 67
» »	+	—	—	—	—	+	—	—	—	+	—	—	+ 58	Зона опалесценции и узкого просветления	—	Непатогенный 68
» »	+	—	—	—	—	+	—	—	—	—	—	—	+	То же 46	— 61	Газовая гангрена
Медленная пептонизация 60	+	—	±	+	+	+	±	—	—	±	—	+	±	Покраснение 49	— 62	Некоторые штаммы вызывают газовую гангрену
Медленное свертывание 65	+	—	+	+	+	+	—	—	—	+	—	+	—	Покраснение 49	—	Непатогенный 68

Название клостридий	Морфология: размеры палочек	подвижность	колонии на поверхности агара через 48 часов	колонии в глубине агара	Споры: форма, расположение	устойчивость к кипячению	Протеолитические свойства: желатина	свернутая сыворотка	свернутый яичный белок	индол	Сбраживание углеводов: лакмусовое молоко	глюкоза	левулеза	лактоза	галактоза	сахароза	мальтоза	инулин	маннит	дульцит	салицин	глицерин	крахмал	Гемолиз на кровяном агаре	Среда Виллиса и Хоббс	Бензидиновый агар	Патогенность для животных
Cl. tetanomorphum	Барабанные палочки 3—5 × 0,5	+	1—2 мм		Терминальные	До 1 часа	—	—	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—	—	—	—	Непатогенный
Cl. cochleаrium	Столовая ложка	+		Чечевица	Терминальные, овальные	То же	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	То же

1) Name of clostridium; 2) morphology; 3) spores; 4) proteolytic properties; 5) fermentation of carbohydrates; 6) rod dimensions; 7) mobility; 8) colonies on agar surface within 48 hours; 9) colonies in the depth of the agar; 10) shape, arrangement; 11) resistance to boiling; 12) gelatin; 13) coagulated blood serum; 14) coagulated egg white; 15) indole; 16) litmus milk; 17) glucose; 18) levulose; 19) lactose; 20) galactose; 21) saccharose; 22) maltose; 23) inulin; 24) mannitol; 25) dulcit; 26) salicin; 27) glycerol; 28) starch; 29) hemolysis on blood agar; 30) Willis and Hobbes medium; 31) benzidine agar; 32) pathogenicity for animals; 33) Cl. perfringens of types A, B, C, D, E, F; 34) lens-shaped or cotton wool clumps; 35) Elliptical, subterminal, more rarely, central; 36) from 5 minutes to 6 hours; 37) coagulated clot, KG 3-16 hours; 38) reddening, aureole, opalescence; 39) gas gangrene; 40) Cl. oedematiens type A and B; 41) flakes with dense center, lenses; 42) up to one hour; 43) slow coagulation, several days; 44) opalescence, nacreous zone (type A); 45) blackening; 46) the same; 47) flocculent with dendritic branching; 48) from 2 to 45 minutes; 49) reddening; 49a) from 30 minutes to 9½ hours; 50) peptization; 51) cleared-up zone around the colonies; 52) gas gangrene with liquefaction of muscles; 53) cotton wool clumps with thickened center; 54) from 1 to 3 hours; 55) zone of opalescence and nacreous lustre; 56) cotton wool clumps, lens shapes; 57) up to 1 hour; 58) zone of opalescence and narrow zone of clearing; 59) cotton wool clumps, small lens shapes; 60) slow peptization; 61) gas gangrene; 62) some strains cause gas gangrene; 63) small lens shapes with thickened center; 64) terminal, elliptical; 65) slow coagulation; 66) small dense clumps; 67) local liquefaction of tissues sometimes kills the animal; 68) nonpathogenic; 69) cylindrical rods; 70) terminal; 71) "Table spoon"; 72) lenses.

it is possible to obtain a large quantity of relatively pure wound exudate, the neutralization test is carried out on the centrifugate of the test liquid (Table 73).

To determine the presence of lecithinase, the test can also be carried out with the t st liquid *in vivo*, using lecithovitellin or erythrocytes of sheep, mice or rabbits. By the inhibition of the reac-

tion with sera of Cl. perfringens or Cl. oedematiens, the specificity of the detected lecithinase can be established.

2. The volume of work can be reduced by inoculating 2 Petri dishes containing Willis and Hobbes medium, having previously added to the melted and cooled (to 50°) agar of one of the dishes a mixture of antitoxin of Cl. perfringens of type A and of Cl. oedematiens, type A (10 AYe per 1 ml of medium), with the wound exudate and other materials.

Although accurate data on the different clostridia cannot be obtained by this method, it is nevertheless possible by means of it, to determine Cl. perfringens, Cl. sordelli, Cl. oedematis type A, Cl. histolyticum and also nonproteolytic strains of Cl. botulinum (Table 74).

TABLE 74

Behavior of Some Pathogenic and Nonpathogenic Clostridia on Willis and Hobbes medium (Lactose-Yolk-Milk agar, 1959)

1 Название микроба	Опалесценция 2		5 Перламутровый слой	6 Ферментирует лактозу (ярко-красный ореол вокруг колоний)	7 Протеолиз (узкая зона просветления вокруг колоний)
	3 вызывает	4 подавляется на чашке добавлением смесью антитоксинов Cl. perfringens и Cl. oedematiens			
Cl. perfringens типов A, B, C, D, E и F	+	+	−	+	−
Cl. oedematiens:					
тип A	+	+	+	−	−
» B	+	−	−	−	−
» C	−	−	−	−	−
» D	+	−	−	−	−
Cl. bifermentans	+	+	−	−	+
Cl. sordellii	+	+	−	−	+
Cl. sporogenes	+	−	+	−	+
Cl. histolyticum	−	−	−	−	+
Cl. septicum	−	−	−	+	−
Cl. cochlearium	−	−	−	−	−
Cl. tertium	−	−	−	+	−
Cl. sphenoides	−	−	−	+	−
Cl. fallax	−	−	−	+	−
Cl. butyricum	−	−	−	+	−

1) Name of microbe; 2) opalescence; 3) causes; 4) is inhibited on a dish to which a mixture of the antitoxins of Cl. perfringens and Cl. oedematiens has been added; 5) nacreous layer; 6) ferments lactose (red aureole around colonies); 7) proteolysis (narrow cleared-up zone around colonies); 8) type(s)

3. With the aim of determining the pure toxigenic cultures of Cl. perfringens of type A, Cl. oedematiens, Cl. septicum and Cl. tetani, one of the antitoxins is added in a concentration of 8 AYe per 1 ml to transparent nutrient agar on the basis of Hottinger or Martin broth. Thus, it is necessary to have 4 dishes with four different antitoxins, which are inoculated with the test culture. The colonies grown after 48-72 hours form a ring of precipitation around their periphery with homologous serum. The test is carried out more economically by dividing a dish into 4 symmetrical sectors and pouring the respective antitoxin on the agar (Petri and Stiben).

Deficiencies of the method are the low sensitivity (strains which are not highly toxic may not give a precipitate) and the long duration (over 48 hours).

4. One of the rapid in vitro methods of diagnosing the principal pathogenic agents of gas gangrene is based on the phenomenon of a change in the morphology and the nature of growth of anaerobic bacteria when they are cultured in semiliquid medium in presence of specific antitoxin sera (O. A. Komkova).

A special medium is used for this purpose, consisting of Pope broth with 0.1% agar, 0.4% gelatin and 0.5% glucose. The medium is poured into 10 ml test tubes and sterilized 2 times for 20 minutes in steam with an interval of one day.

The test material - the infected pieces of tissue - is distrib uted in 10 test tubes containing semiliquid medium; 5 of these test tubes are kept on the water bath at 80° for 20 minutes. To each pair of test tubes - a heated and an unheated one - different monovalent antigangrene sera are added in such a quantity that 1 ml of medium contains not less than 200 AYe of Cl. perfringens type A, not less than 300 AYe Cl. oedematiens and not less than 50 AYe Cl. septicum

and Cl. histolyticum antitoxic serum. No serum is added to the two last test tubes.

The contents of each test tube are carefully mixed and all test tubes placed into the thermostat at 37-38°. The result is evaluated 10 and 18 hours later.

The detection of the streptobacillus form and the growth of isolated colonies in the test tubes with a certain antitoxic serum and the absence of these phenomena in the other test tubes indicate the presence of pathogenic gas gangrene organisms in the test material which correspond to the given species of serum.

If the streptobacillus form is detected in test tubes with different sera, this indicates the presence of several species of gas gangrene microbes in the test material.

Another rapid method, proposed by O. A. Komkova, is a further improvement of the test in which the toxin is neutralized by antitoxin by means of subcutaneous injection into guinea pigs.

3-5 guinea pigs, depilated on one side of the abdomen, are needed for an analysis. One guinea pig is injected with 0.2 ml of the test liquid together with 0.1 ml of physiological salt solution, the others with 0.2 ml of some monovalent antigangrene serum. The guinea pigs are observed for 24 hours.

The early diagnosis of the pathogenic agents of gas gangrene by this method is based on the rapidly ensuing change of the skin color of the guinea pigs (violet, rose-red, bluish tinge) in consequence of the local disturbance of the blood circulation. The conclusions are based on the absence of a positive reaction in the guinea pigs which have been given the test material in combination with homologous antigangrene serum and the presence of a reaction in all other test animals.

This method sometimes enables toxic strains of gas gangrene organisms to be detected within a period of 30 minutes to 4 hours.

TETANUS

K. I. Matveyev, Professor, T. I. Sergeyeva and V. A. Sidorova, Candidates of Medical Sciences

Tetanus is an infection which appears in consequence of various traumatic and other wounds in connection with contamination of the wound with soil, pieces of clothing, and animal excrements containing the spores of Cl. tetani, the pathogenic organism which causes tetanus.

Morphology of Cl. tetani

Mobile bacillus with a length of 4-8μ and a width of 0.4-0.6μ with rounded ends (Fig. 116). It forms terminal, round spores, as a result of which it assumes the appearance of a cylindrical rod (Fig. 117). Peretrichately arranged cilia (Fig. 118). Easily stained by all aniline dyes. It is stained gram-positive; gram-negative individuals are also found in old cultures. The spores are difficult to stain. When the spores are stained with methylene blue, or gram-stained, they have an annular appearance. For staining of spores, see p. 41.

Fig. 116. Cl. tetani. 1900x.

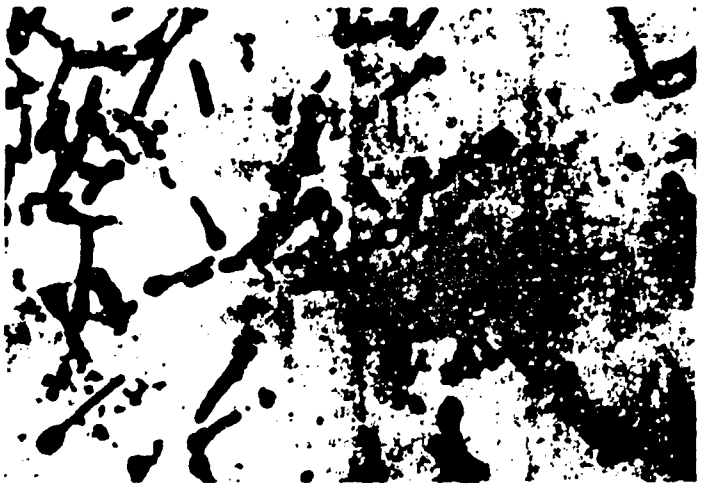


Fig. 117. Spore-forming Cl. tetani. 1900x.

Fig. 118. Cl. tetani, flagellate. 11,500x.

Biology of Cl. tetani; culture properties

The pathogenic microorganism of tetanus is strictly anaerobic and highly sensitive to oxygen. This microbe grows well in the depth of liquid nutrient media, filled into tall test tubes, flasks or cylinders in presence of reducing agents (glucose, pieces of liver or muscle, cotton wool, etc.). Martin broth, Weinberg medium in the TsIEM

modification, the medium developed by P. M. Gluzman and coworkers, the medium of K. I. Matveyev and coworkers, etc., (see p. 959) are used for culturing the tetanus bacillus. The nutrient medium should be neutral or slightly acid (pH = 6.8-7.4). The liquid media should be covered with a layer of vaseline oil and the oxygen removed from them prior to inoculation by boiling on the water bath for 10-15 minutes, after which they are quickly cooled to 40-50°. The optimum temperature for growth is 35-37°.

On solid nutrient media - blood and liver agar - the tetanus bacillus grows only when the oxygen has been completely removed. This is achieved by placing the Petri dishes into a micro- or macro-anaerostat, on the bottom of which an open Petri dish containing a 10% alkaline solution of pyrogallol has been placed, after which the air is evacuated.

After 2-4 days of growth on agar dishes at a temperature of 35-37°, individual transparent or slightly grayish colonies appear with a size of 2-5 mm and uneven granular surface, the edges of the colonies being ragged and dendritic. Older colonies have a yellowish dense center and completely transparent, colorless, ragged, dendritic fringes (Fig. 119). On blood agar, every colony is surrounded by a hemolysis zone. When the condensation liquid of canted agar in test tubes is inoculated with tetanus bacillus, the microbe grows in the form of thin, hardly visible threadlike ougrowths, which creep on the agar surface. This peculiarity of the microbe can be utilized for the isolation of a pure culture. The test tubes with canted agar must be placed into an anaerostat. Dense colonies in the form of lenticular grains or discs (R-form) or in the form of flakes with dense center (S-form) grow in a high agar column within 1-2 days. In a gelatin column, Cl. tetani grows within 5-6 days in the form of a herringbone pattern the gelatin being liquified at

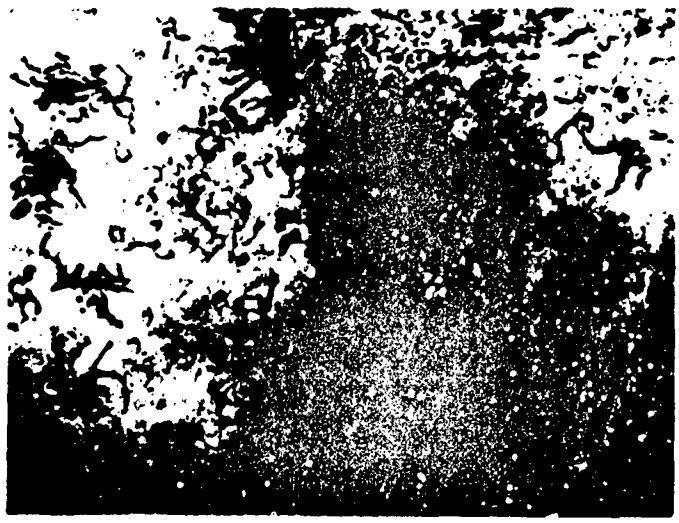


Fig. 119. Colonies of Cl. tetani on Martin agar after 5 days of culturing. 10×.

the same time.

The tetanus bacillus is only weakly proteolytic: when grown in a liquid medium containing a piece of liver or hardboiled egg white, it does not cause proteolysis of these materials even after long periods. The microbe does not ferment monosaccharides or polyvalent alcohols. Only individual strains ferment glucose. All strains ferment milk slowly with formation of very fine flakes.

The pathogenic microorganism of tetanus produces a powerful toxin consisting of two components: tetanospasmin or neurotoxin, and tetanohemolysin. The tetanospasmin is the principal lethal component, which affects the nerve system of humans and animals and causes tonic contractions of the striped muscles. The tetanohemolysin destroys the erythrocytes of many animal species.

Resistance to Physical and Chemical Factors

The vegetative form of the tetanus bacillus is not very resistant to the action of temperature and chemical agents, while their spores are highly resistant. In a moist medium, the spores can withstand a

temperature of 80° for 4-6 hours and more, while they die when boiled for 40-50 minutes. Dry spores withstand even higher temperatures: heating to 115° destroys them only after 20 minutes. The spores are totally insensitive to low temperatures. They can withstand a temperature of -40-60° for years. A 1% solution of corrosive sublimate or a 5% carbolic acid solution takes 10-12 hours to kill the spores. Under the action of diffuse sunlight, the spores die only after long periods of exposure. Being protected against light in the soil and on various environmental objects, the spores can survive for many years.

Laboratory Diagnosis of Tetanus

The examination for the presence of Cl. tetani is carried out:

a) to confirm the clinical diagnosis of tetanus on patients;

b) to check the sterility of bandages, catgut and silk and various preparations for subcutaneous injection;

c) to determine certain epidemiological data (environmental objects – soil in localities with a high tetanus incidence, dust and air of operating theaters and dressing rooms in hospitals).

Bacteriological examination

Examination of material from patients and cadavers

The tetanus bacillus is detected in most cases at the site where it has entered the organism of the patient. Hence the examination of material, taken from the site of the wound is the mostpromising. It is necessary to take samples of pus, pieces of tissue, foreign bodies, scraps of clothing, swabs, placed into the wound during dressing, bandaging material containing wound exudate, etc.

In the cases, when the pathway of infection is unknown, it is essential to examine the patient carefully for the presence of scratches, abrasions, catarrhal and inflammation processes and old wounds which have not healed for a long time. Once such foci are established, mate-

rial is taken from the place where they are located, and in some cases mucus from the bronchi, nose, pharynx and deposits on the tonsils is sampled. Attention must be directed to the presence of old cicatrizations from previous injuries in which tetanus bacilli can remain viable for long periods. In cases of postpuerperal or postabortus tetanus, vaginal and uterine secretions are taken for examination. When tetanus is suspected in a newborn, the secretions from the umbilical cord are examined.

It should be pointed out that cases of tetanus have occurred in recent years in humans in most cases as a result of slight injuries, scratches, punctures, abrasions and wounds which may have healed before the development of the clinical symptoms of tetanus.

When cadavers are examined, material is also taken from wounds, if any and also from various inflammation foci and old cicatrices. Owing to the fact that a generalization of the infection takes place in some cases of tetanus, the pathogenic microbe can also be detected in the internal organs, Hence blood (10 ml), pieces of liver and spleen with a weight of 20-30 g are taken from the cadaver.

The examination of material taken from tetanus patients or from corpses, can be carried out with the aim of detecting tetanus toxin and the tetanus bacillus.

Detection of tetanus toxin. The test material is ground in a sterile mortar with sterile quartz sand and double the volume of physiological salt solution added. Part of this material is used to inoculate 2 flasks with liquid nutrient medium and the remainder is left in the mortar for the extraction of the toxin which takes an hour at room temperature.

To carry out the neutralization test on animals, an extract from the test material is filtered through cottonwool-gauze or filter paper

and injected intramuscularly into the thigh of the hind legs of a mouse (filtering through a talcum filter must be avoided as this adsorbs the toxin). 0.5 or 1 ml each of extract is injected into two mice and the same doses of extract into two other mice together with antitetanus serum. The mixture of extract with antitetanus serum is prepared in such a manner that 0.5 ml of diluted serum with 200 AYe are added to 1 ml of extract. The mixture is kept at room temperature for 40 minutes for the neutralization of the toxin by the antitoxin. The mixture of toxin and antitoxin is then injected intramuscularly into 2 mice, using 0.75 ml or 1.5 ml for each (for guinea pigs, 1.5-3 ml each). The animals are then observed for 4-5 days.

Depending on the quantity of toxin, the symptoms of tetanus develop in the animals on the first or second day. The first symptom of tetanus in mice is a rumpling of the hairs and a certain rigidity of the tail and hind legs. A sudden sound stimulus (rapping on the jar or cage in which the mice are kept) causes a tensing of the tail, as a result of which it becomes erect. These symptoms in mice are designated as tetanus of degree I. Paralysis of the leg, into which the toxin had been injected, expressed in immobility and lateral extension of the leg, indicates tetanus of degree II. Later on, symptoms of tetanus develop in the animals, involving paralysis of the entire extremity and part of the body muscles. This is tetanus of degree III; degree IV is characterized by marked clinical symptoms of tetanus involving curvature of the animal's spine. If the animal is placed on its back, it is not capable of returning to the prone position.

The tetanus symptoms are absent in the animals which were given tetanus toxin in combination with antitetanus serum. This indicates, that tetanus toxin is present in the test material.

Detection of Cl. tetani. Simultaneously with the neutralization

test, 2 flasks or 2 test tubes with nutrient medium (Martin broth, Weinberg broth, etc.), covered with a layer of vaseline oil, are inoculated with the material which has been ground up with sand. Before the inoculation is carried out, the oxygen is removed from the medium by boiling for 15 minutes, followed by cooling to 40-50° and addition of 0.5% glucose. Pieces from the organs of the cadaver with a weight of 10-20 g are also ground in a sterile mortar with sterile sand, after which the medium is inoculated with them. 5-10 ml of blood from the cadaver is triturated with sand to disintegrate the clot and the medium inoculated with it.

Following the inoculation, one of the flasks or test tubes is heated on the water bath at a temperature of 80° for 20 minutes for the purpose of inhibiting or destroying the extraneous, nonsporogenic microflora present in the material from the patient. Following culturing at a temperature of 35°, smears taken from the cultures are examined microscopically on the 2nd, 4th, 6th and 10th day and the culture fluid examined for the presence of tetanus toxin. For this purpose, the neutralization reaction with antitetanus serum is carried out in accordance with the abovedescribed method.

When tetanus toxin has been detected in the culture and when gram-positive bacilli with round terminal spores are found to be present, it may be concluded that Cl. tetani has been detected in the test material.

If tetanus toxin is not detected in the first culture, but bacilli are observed during microscopic examination, which are morphologically similar to the pathogenic organism of tetanus, the original culture is used to inoculate solid nutrient media in a Petri dish or in a high agar column in test tubes. Blood or liver agar is preferred for the Petri dishes. The dishes with the cultures are placed into a micro-

or macroanaerostat and grown at a temperature of 35° under complete vacuum. In order to create satisfactory anaerobic conditions, an open Petri dish, containing a 10% solution of pyrogallol, is placed on the bottom of the anaerostat.

Within 1-3 days, transparent colonies of Cl. tetani grow in the dishes with blood agar in the form of dewdrops surrounded by a hemolysis zone. The colonies are examined under a powerful magnifying glass or the microscope MBS-1, or MBS-2. Individual colonies of this microbe on the surface of the agar are sometimes difficult to obtain because the microbes have a tendency to creep over the surface of the medium and to form a transparent, networklike film which is hardly noticeable to the unaided eye.

The seeding of the agar column in test tubes is carried out with the culture fluid, first kept on the water bath at 80° for 20 minutes in order to kill off the nonpathogenic extraneous microflora. For this purpose, 5-8 test tubes with agar are melted on the water bath and then cooled in water to 40-50°. The inoculation is carried out in the liquid agar by successive reseeding from one test tube of the primary culture into the next with the sealed end of a Pasteur pipette. After the inoculation, the test tubes are immediately lowered into a container with cold water for rapid solidification of the agar and then placed into the thermostat at 35°. Dense colonies in the form of lenses or flakes grow in the agar within 1-2 days. Smears are prepared from the colonies on dishes and a high column and examined under the microscope. When bacteria, morphologically similar to Cl. tetani, are present, liquid nutrient medium is inoculated with them and tested for the capacity to produce tetanus toxin. The latter property is a sufficient basis for a diagnosis of tetanus.

Examination of dressing material, catgut and surgical silk

Very strict conditions of sterility must be observed for the examination of dressing material with the aim of detecting Cl. tetani. In the rooms, where these tests are carried out, work with anaerobic bacteria, including the tetanus bacillus, must not be carried out. The room must be freed of all unnecessary objects and disinfected. The inoculations must be carried out in a special, thoroughly disinfected box. The staff, present during the bacteriological testing of dressing materials should don a second set of sterile gowns and also sterile gloves, caps and cotton wool-gauze masks over the nose and mouth. All inoculation media must be carefully checked for sterility by keeping them in the thermostat for 5-7 days prior to the inoculation. The instruments used in this work, the vessels, etc., should be sterilized twice in an autoclave. The seeding of material in the box must be carried out in sheet metal trays which have previously been thoroughly burnt off with an alcohol flame or a gas burner.

Prior to inoculation, the dressing material is carefully examined and samples taken for inoculation from different places in the depth and on the surface. Every sample is seeded into 2 flasks containing a previously boiled medium under a layer of vaseline oil and containing 0.5% glucose. After inoculation, one of the flasks is kept at a temperature of 80° for 20 minutes on the water bath. For inoculation with cotton wool, several small flakes per flask but not less than 5-10 g are used; gauze is first cut into small pieces, then 5-10 g seeded into each flask with nutrient medium. Other forms of dressing material are also placed into the flasks in small pieces. Test tubes with medium are not suitable for the examination of dressing material on account of their small volume.

Catgut (5-10 g per flask of medium) must be ground up in a sterile

mortar with sterilized sand before the inoculation. Surgical silk, also 5-10 g per flask with medium, is cut into small pieces and seeded out.

Culturing is carried out at 35° for 10-15 days. This is necessary because of the slow growth of some Cl. tetani spores. Smears from the culture flasks are examined under the microscope and the toxin neutralization test carried out on mice with the filtrate from the culture fluid by the abovedescribed method. Positive conclusions are derived if tetanus toxin is detected in the cultures.

Detection of the tetanus bacillus in the environment

A bacteriological analysis of soil, dust and the air is necessary to determine the distribution of the tetanus bacillus in the environment. Air samples from operating and dressing rooms, surgical wards and also from the rooms for the sterilization of dressing material, catgut and surgical silk are examined.

A definite relationship between the contamination of the soil with tetanus spores and the incidence of tetanus has been established in many localities of the USSR and periodic examinations of the soil for the presence of tetanus bacillus is thus necessary.

The testing of soil and dust for the presence of Cl. tetani can be carried out by two methods: the biological test and the neutralization test on animals and also by inoculating nutrient media with soil and dust samples. Soil samples in a quantity of 25-30 g are taken in population centers from roads, farmhouses, gardens, markets, from plowed fields, situated close to population centers, from playing fields, etc., and placed into sterile jars, test tubes or parchment bags. A soil core up to 20 cm long is taken with a sterile Nekrasov drill or a spatula or scoop; before taking samples from each section, the tools must be wiped with alcohol and heated with an alcohol flame. When large

numbers of samples are taken from a single section, they can be combined for examination.

The bacteriological testing of soil and dust should be carried out as soon as possible after the taking of the samples. If the testing must be postponed, the samples should be kept in a refrigerator.

The soil sample is first thoroughly mixed, 3-5 g taken from it and transferred to a sterile porcelain mortar and carefully ground, after which 6-8 mℓ of physiological salt solution is added, mixed and left for 3-4 hours at room temperature. 1 mℓ of the soil suspension thus obtained is injected into each of 2 mice into the muscles of the hind leg; 3-5 mℓ of the suspension can be used for guinea pigs. When tetanus bacilli are present in the soil, the clinical pattern typical for tetanus develops in the mice. Tetanus of animals is such a typical illness that it is easy to diagnose. For greater reliability, the neutralization test with antitetanus serum is then carried out with the soil samples, in which Cl. tetani has been detected, in accordance with the abovedescribed method.

Samples of dust, collected from different objects in the rooms of the surgical department, are placed into sterile test tubes stoppered with sterile cotton wool plugs and examined by the same methods as the soil samples.

Soil and dust can be used for inoculating nutrient media in flasks flasks. Much greater quantities of material, up to 10-15 g, can be taken for this than for the tests on animals. The soil and dust is first ground carefully in a mortar, physiological salt solution added, and divided into two parts, one of which is kept at 80° for 20 minutes on the water bath prior to inoculation and the other seeded out into the medium without heating. 2, 4, 6 and 10 days after growing in the thermostat, smears are prepared from the inoculated medium and when

bacteria, similar to Cl. tetani, are detected, the neutralization test with antitetanus serum is carried out on animals. When necessary, a pure culture is isolated by inoculating a high agar column, and its capacity for producing tetanus toxin is determined.

The testing of the air in operating theaters, dressing rooms and surgical wards is carried out by means of the bacteria traps kept for this purpose (see p. 266).

NUTRIENT MEDIA FOR THE CULTURING OF ANAEROBIC BACTERIA

K.I. Matveyev, Professor, T.I. Bulatova, Candidate of Medical Sciences, B.D. Bychenko, Candidate of Medical Sciences, and T.I. Sergeyeva, Candidate of Medical Sciences

Peptiized broth

Preparation of pepsin-peptone. 200 g of sausage meat is heated with 1 liter town water to 45-50°, 5 to 10 g pepsin which has first been dissolved in water and 1% hydrochloric acid are added. The mixture is poured into bottles, stoppered with a cotton wool plug and placed into the thermostat at 45-50°. 24 hours later the peptone thus obtained is heated to 80° and kept at this temperature for 5 minutes and at 100° for 10 minutes and then stored in the cold. Peptone which has been stored for 5-7 days is used in the work.

Preparation of liver decoction: one part liver with 4 parts water is boiled for 20 minutes and filtered through cotton wool.

Broth formula: 25 liters peptone are heated to 80°, neutralized with alkali, then boiled 5 minutes and filtered through linen. To the filtered peptone, 4 liters of liver decoction, 21 liters of meat extract and 0.5% sodium chloride (calculated on the total volume) are added. The whole mixture is boiled for 20 minutes the pH is adjusted to 7.3. The broth is then ready to be poured into previously prepared test tubes and flasks containing pieces of raw meat or liver and hygroscopic cotton wool, and sterile vaseline oil is poured on the broth in a 0.5 cm

thick layer and the whole sterilized for 30 minutes at 120°.

Casein media

(K.I. Matveyev, Ye. A. Schchekochikhina, G.S. Soykina, L.I. Mogil'nikova, N.V. Khatuntseva, T.V. Oreshkina, and L.N. Stovichek, 1953, 1960).

These media are based on acid and alkaline hydrolyzates of casein, fish flour and corn. To prepare the casein hydrolyzate, 8 kg of dry casein and 1 kg of chemically pure hydrochloric acid are taken for 100 liters of water. These are mixed and boiled for 20 minutes. The hydrolysis is carried out under a pressure of 1 atmosphere for 26-30 minutes. For the fish and corn hydrolyzates, 10 kg fish flour, 3.3 kg milled corn and 1.3 kg of hydrochloric acid are taken for 100 liters of water. The hydrolysis is carried out for 4 hours at a pressure of 1.5 atmospheres.

Fish-casein medium. 20% fish flour hydrolyzate, 5% casein hydrolyzate, and 71% water are mixed and heated to 80°. 3% corn extract and up to 0.5% sodium chloride are added, the mixture brought to ph = 7.2 with a 20% alkaline solution and boiled for 5 minutes. After boiling, the pH is again adjusted to 7.2 and 0.1% Na_2HPO_4 added, mixed and boiled for 10 minutes. The medium is then filtered or centrifuged in the hot state and sterilized under a pressure of 1.5 atmospheres for 30 minutes. Chemical characteristics of the medium: total nitrogen 200-250 mg-%, protein nitrogen 50-80 mg-%, amine nitrogen 100-150 mg-%. With a higher pH of 8.0-8.2, this medium is used for growing Cl. oedematiens. For growing Cl. botulinum type E, 1.6 times less casein is taken, the pH adjusted to 7.6-7.8 and the protein nitrogen to 35-69 mg%, the amine nitrogen to 70-130 mg-% (K.I. Matveyev, A.T. Kravchenko, T.I. Bulatova, 1953, 1959). The media are filled into flasks with cotton wool on the botton, 3-5 g of millet being added for growing of the

botulism bacillus.

Casein medium. 40% casein hydrolyzate is taken, 56% water added, mixed and heated to 80°. 3% corn extract and sodium chloride to 0.5% is added, the mixture brought to pH = 7.2 with a 20% alkaline solution and boiled for 5 minutes. After boiling, the pH is again adjusted to 7.2, 0.1% Na_2HPO_4 and KH_2PO_4 added, mixed and boiled for 10 minutes. The medium is then hot filtered and sterilized. Chemical characteristics of the medium: total nitrogen 250-270 mg-%, protein nitrogen 100-109 mg-%, amine nitrogen 90-100 mg-%. The ready medium is filled into test tubes and flasks with cotton wool.

Casein-vegetable medium for growing Cl. tetani and producing tetanus toxin (I.N. Vinogradova, V.A. Petrenko, F.F. Tsurikov, N.A. Palkina, N.G. Martinelli, 1958). Hydrochloric acid casein hydrolyzate is used as the basis for this medium.

To prepare the hydrolyzate, 800 g casein, 10 liters water and 360 ml chemically pure concentrated HCl are filled into a 20 liter flask. The hydrolysis is carried out in the autoclave at 127° for 2 hours. At 38-40°, the hydrolyzate is made alkaline, using universal indicator, to pH = 4.7, the precipitated protein filtered off by passing through a cloth, and the hydrolyzate diluted with water, adding 50 liters water to 100 liters of hydrolyzate, so that the peptone concentration in the diluted hydrolyzate does not exceed 3%. To clarify the hydrolyzate, activated charcoal is added (about 2 kg charcoal for 100 liters), the mixture boiled for 10 minutes and the hydrolyzate filtered through a dense linen filter. The clarified hydrolyzate contains: total nitrogen 450-470 mg-%, amine nitrogen 190 mg-%, peptone 3%.

Yeast extract and bran autolysate are used as growth factors.

The yeast extract is prepared in the following manner: 250 g of pressed baker's yeast is ground, suspended in 1 liter of distilled

water, boiled with constant stirring until the foam has settled (about 5 minutes) and filtered through linen. 1% chloroform is added and the extract shaken carefully and stored in the dark.

Bran autolysate: 2 kg wheat bran, 12 liters town water and 1% chloroform are filled into 20 liter carboys. The extraction and autolysis are carried out at a temperature of 45° for 12-14 hours. The mixture is stirred from time to time, the solution then decanted, filtered through cloth and used on the same day for the preparation of the medium.

To prepare the medium, 40 liters of yeast extract and 12 liters of bran autolysate are added to 100 liters of neutralized casein hydrolyzate. The concentration of amino nitrogen is brought to 100-120 mg-% by dilution. The mixture is heated to boiling, 0.05% Na_2HPO_4 and 0.05% KH_2PO_4 added, the pH adjusted (7.3), boiled 10 minutes and the pH again checked.

The broth is then filtered through cloth, poured into test tubes or flasks with cotton wool on the bottom and sterilized at 110° for 30 minutes. The pH of the ready-to-use broth is 7.1-7.2, the total nitrogen content 350-380 mg-%, that of protein nitrogen 5-8 mg-%, peptone 1.3-1.5 mg-%, amino nitrogen 110-120 mg-%.

Casein-fungus medium for growing Cl. botulinum of types A, B, C, and E (T.I. Bulatova, I.N. Vinogradova, K.I. Matveyev, 1959). Preparation of casein-fungus hydrolyzate. 8 kg casein and 100 liters town water are mixed. The mixture is gradually heated almost to boiling with constant addition of alkali until a pH of 8.3-8.4 is attained (this corresponds to a pale-rose color with phenolphtalein). During this procedure the casein forms a colloidal solution. The solution thus obtained is acidified with HCl to pH = 7.0, cooled to 45° and 2 kg fungus protease added. The process of hydrolysis requires a tem-

perature of 45-58° and a pH = 7.0. 3-4 hours later, when the concentration of amino nitrogen has attained 210-280 mg-% and that of the peptone 2.5-3%, the hydrolysis is terminated. 1 liter of glacial acetic acid is added and the hydrolyzate boiled 10 minutes and filtered through linen. The hydrolyzate is used on the same day or 1% chloroform is added and it is stored at 10°. The hydrolyzate contains: total nitrogen 750 mg-%, amino nitrogen 200 mg-%, peptone 2-2.5%.

Technology of the preparation of fungus protease. The process of preparing fungus protease consists of two stages: the production of a culture of Aspergillus terricola for inoculation and the proliferation of this culture under production conditions.

Preparation of inoculation material under laboratory conditions in the form of a culture of Aspergillus terricola on wort agar in test tubes (stock fungus). The culture used for mass inoculation is a strain of the fungus Aspergillus terricola, kept on wort agar.

Preparation of wort agar. The process of preparing wort agar consists of the three following stages: germination of barley or oats, preparation of wort from the malt and of a solid medium from the wort.

Preparation of malt. 1. The barley or oat grains are soaked for 24 hours in town water, the water is then pressed out and the grain left to germinate in a dark place for 8-10 days, mixing it from time to time.

2. The germination is complete when the length of the shoots attains 3/4 of the length of a grain.

3. The green malt thus obtained is passed through a meat grinder, dried in the thermostat until it is air dry and used as needed.

Preparation of the wort. 1. One part by weight of malt is mixed with four parts town water.

2. The saccharification is carried out on the water bath with con-

stant stirring and observation of the following temperature conditions:

1) The mixture is heated to 45° and allowed to stand at this temperature for 30 minutes.

2) The temperature is raised to 52° the mixture left for 30 minutes at this temperature.

3) The temperature is raised to 58° and the mixture left for one hour at this temperature.

4) The temperature of the mixture is raised to 62° and this temperature maintained until the saccharification of the starch is complete (about 3 hours). The process of saccharification is considered to be terminated as soon as a violet no longer appears upon mixing of the test solution with Lugol solution or M/100 iodine solution.

3. The wort thus obtained is heated to boiling and boiled for 20 minutes, then town water is added to restore the original volume and filtered hot through a layer of gauze and cotton wool. After cooling 1% chloroform is added and the solution left to stand for 24 hours.

4. The remaining wort is decanted and used for preparing wort agar. When it is necessary to store the wort for some time, it is sterilized at 110° for 30 minutes.

Preparation of wort agar. 1. The wort is diluted with town water in such a manner that the sugar concentration is equal to 4-5% as determined by the Balling method (the best undiluted wort contains normally 12-15% sugar).

2. 2.5-3% agar-agar is added, heated until completely dissolved, boiled, filtered and filled in a quantity of 5 ml into sterile test tubes or flasks.

3. It is sterilized 3 times in steam or 1 time at 110° for 30 minutes.

Proliferation of the fungus culture. 1. Slanted wort agar is inoc-

ulated once a month with the yeast culture and grown for 6-8 days at a temperature of 28-30°. By this time, the culture has assumed an orange or light-brown color because it is copiously covered with spores.

2. The spore culture is transferred to storage at room temperature and used as needed for mass inoculation of bran (suing the agar culture from two test tubes for one tray).

Preparation of yeast protease (by the method of I.N. Vinogradova).

1. Wheat bran is moistened with water, using 320 mℓ per 400 g of bran, carefully mixed to avoid the formation of clumps, sterilized in the autoclave at 120° for 15 minutes and transferred to stainless steel or galvanized iron trays with dimensions of 700 × 25 × 10 cm.

400 g of dry bran is placed on each tray and inoculated with the suspension of spores, obtained after washing out the culture on wort agar (from two test tubes) with 100 mℓ of physiological salt solution. The inoculated bran is then carefully levelled in such a manner that it does not touch the walls of the tray. The thickness of the bran layer is 1-1½ cm. The trays are covered with gauze and sheet metal.

2. The trays are placed into a well aerated thermostat at a temperature of 26-28° for 18-20 hours, then transferred on the 5-8th day to a thermostat held at a temperature of 18°.

The following conditions must be observed during growing. The yeast is transferred to a room with a temperature of 18° when copious mycelium appears (down to the bottom).

The culture should have a grayish-white appearance by this time. During the further growth after the transfer of the mycelium, the culture becomes white. In proportion to the development of the fungus, drops of water accumulate on the underside of the sheet metal.

To prevent them from dropping into the trays, the cover sheets are turned over from time to time. On the fifth day, the cover sheets

are removed. On the seventh day, the process of fungus growing is considered to be terminated.

By this time the culture is permeated by crange spores down to the bottom. It is left another 3-4 days at 18° for drying out until it is transformed into a practically dry mass which contains the complex of proteolytic enzymes. It is used as fungus protease.

3. The yield of fungus protease is 50% of the weight of the dry bran, its activity according to Fuld-Gross being 10,000 units.

4. For the purpose of storage, the fungus protease is filled from the trays into paper bags and transferred to a dry, refrigerated room. It can be kept under these conditions for 2-3 years.

To prevent the spores from being dispersed and getting into the organism of the personnel, the following precaustions are taken:

a) the workers wear gas masks which protect them from dust during the transfer of material;

b) before transporting the fungus protease to the reaction vessel or boiler where the hydrolysis is carried out, it is moistened with water.

Preparation of the broth. To prepare 100 liters of broth, 50 liters of casein-fungus hydrolyzate, 35 liters of 10% yeast extract, and 15 liters of 20% corn extract are taken. The pH is adjusted to 7.8. The mixture is boiled for 20 minutes, the pH checked and, if necessary, adjusted by addition of alkali. The medium is then filtered through linen, filled into test tubes and flasks with cotton wool or millet and sterilized at 110° for 30 minutes. After the sterilization, the pH is 7.6.

Medium from acid casein hydrolyzate for growing Cl. botulinum type C (K.I. Matveyev, T.I. Bulatova, Ye. K. Petrova, 1952, 1955). To prepare this medium, 20% hydrochloric acid casein hydrolyzate and 76%

water, are carefully mixed and heated to 80°, 3% corn extract and 0.4% NaCl added, the mixture adjusted to pH = 7.2 with 20% NaOH solution, 0.1% each of Na_2HPO_4 and KH_2PO_4 added, mixed and boiled for 10 minutes. It is hot filtered and sterilized at 110° for 30 minutes.

Meat media

Medium of P.M. Gluzman, M.P. Chervyakov and G.M. Starobinets for producing tetanus toxin. To 1 kg of ground horse meat or beef 2 liters of water are added and allowed to soak for 24 hours. The meat suspension in water is then boiled 30 minutes and pressed out in a linen bag. To the meat which remains in the bag water is added in a ratio of 1:3 and 1-3% chemically pure hydrochloric acid added to it, after which it is autoclaved for an hour under a pressure of 2 atmospheres. The paste-like mass is then boiled for another 10 minutes and filtered hot through cotton wool. The slightly turbid liquid obtained after filtration is mixed in a ratio of 1:1 with aqueous meat extract, 0.5% common salt added and boiled for 10-15 minutes. After adjustment of the pH to 6.8-7.0, the medium is filled into test tubes and flasks, to which boiled meat in small pieces or boiled sausage meat is added. The sterilization of the medium is carried out 2 times for 30 minutes: the first time at a pressure of 2 atmospheres, the second time at a pressure of 1½ atmospheres. 0.5% glucose is added to the medium before the inoculation.

Martin broth. 1. Fresh pig's stomachs are freed of the fatty tissue, washed with warm town water, and ground up together with the submucosa and muscle layer. 500 g of stomach tissue, 10 ml hydrochloric acid and 1 liter of water heated to 50° are mixed, allowed to stand at this temperature for 12-15 hours, mixing it from time to time in the earthenware or porcelain vessel. The mixture is then heated to 80°, the fat removed, decanted with a siphon, heated to 80°, neutralized

with concentrated caustic soda solution and filtered through paper. The neutral, clear liquid which should contain about 4% peptone, is bottled and sterilized in steam for 30 minutes on each of 3 successive days.

2. A mixture of equal parts of veal and beef is soaked for 20 hours in the ice box in an equal quantity of water (by weight). The aqueous meat extract is kept at 45° for an hour, then boiled 30 minutes and filtered.

3. Equal volumes of meat extract and Martin peptone are mixed, 10 g glucose per 1 liter of medium added, made slightly alkaline by addition of soda solution, boiled 10 minutes, filtered through paper, bottled and sterilized for one hour at 100° on each of 3 days.

Veynberg broth for growing anaerobic bacteria and producing toxins. To 1 kg of beef heart which has been passed through a meat grinder, 1 liter water is added, slowly heated to boiling, cooled and the fat removed. 400 g of pig's stomach, 400 g liver, 40 g hydrochloric acid, 4 liters water, heated to 50°, are mixed, allowed to stand at this temperature for 18-24 hours, kept at 100° for 10 minutes, decanted, filtered, 0.2% disodiumphosphate added and the pH adjusted to 7.4.

1 liter of aqueous extract from beef heart and 2 liters peptone are mixed, the pH adjusted to 7.8-8.2, and sterilized at 120° for 30 minutes.

The broth is filled into test tubes and flasks. Pieces of cooked liver are added to the broth in the test tubes and flasks, covered with a 0.5 cm thick layer of vaseline oil and then sterilized at 120° for 30 minutes. 0.5% glucose is added to the broth prior to inoculation.

Pope and Smith tryptic digestion broth for culturing anaerobic bacteria and producing toxins. 900 g of sausage meat made from fresh meat is mixed with 1 liter water, heated to 80°, 2 liters cold water added,

brought to pH 8.0 by addition of anhydrous soda and placed into the thermostat at 50°. Every half hour for 6 hours one adds for each liter of decoction 2 ml of trypsin emulsion, prepared in the following manner: to 800 g pancreas gland which has been freed of the connective tissue and ground up, 2.4 liters of distilled water and 1 liter of methanol are added; 3 days later, 0.1% concentrated hydrochloric acid is added to this mixture. A week later the suspension is ready for use (it should be stored in a dark, cool place).

The digestion of the sausage meat is carried out at 50° with constant stirring, maintaining the pH of the liquid at 8.0. After 6 hours, 1% glacial acetic acid is added and the mixture boiled 30 minutes and filtered through cotton gauze. It is placed into a refrigerator at 5-6° overnight; the following day it is heated to 30° and a pH of 7.0 established by means of a 40% caustic soda solution. 2 g dry yeast is made into an emulsion with 1 liter medium, the temperature held at 30° for an hour, the pH adjusted to 8.0, heated to 60°, 0.3% maltose added and filtered through paper. It is then poured into test tubes and flasks, a 0.5 cm thick layer of sterile vaseline poured over it, and sterilized in steam for 30 minutes on each of 3 successive days. The best toxin is obtained when the broth contains 140 to 160 mg-% of amine nitrogen.

Hottinger broth: a) Preparation of meat digest according to Hottinger.

Recipe:

65 kg lean sausage meat
100 liters town water
10 kg ground pancreas (pickled pancreas may be used)
2 liters chloroform
20% NaOH

1. The raw sausage meat is dropped into hot water, stirred, made

alkaline to pH 8.6-8.4 with mixing, boiled 10 minutes and filtered and the sausage meat pressed out.

2. The filtered meat infusion is cooled to 45° (to prevent inactivation of the enzyme), then made alkaline to pH 8.4-8.6 using phenolphtalein and 10 liter portions poured into 18-20 liter carboys, thus filling them to not more than 2/3 of their volume.

3. 6.5 kg of boiled pressed-out sausage meat is put into each carboy, 1 kg pancreas gland (100 g per 1 liter of liquid) and thoroughly mixed.

4. 200 mℓ chloroform is added to each carboy (20 mℓ per 1 liter of liquid), then they are closed with rubber stoppers and again shaken carefully. The stoppers are then taken out, the chloroform vapor allowed to escape, then closed again and placed into the thermostat at 37-45° for 5-10 days.

5. 1-2 days later, a sample is taken (a mixture from all carboys) to determine the amine nitrogen and tryptophan concentration. The pH is checked every day in each carboy and when this is found to have decreased, it is adjusted to 8.4-8.6 (using phenolphtalein, until a weak rose-red color appears or with bromothymol blue until a distinct blue color appears).

6. 5-10 days later, the meat pulp is transformed into a homogeneous grayish precipitate, with a clear supernatant liquid, which has a straw color when the digestion has been carried out correctly. At about this time, the pH is stabilized around 8.4-8.6 and the amine nitrogen concentration is 900-1200 mg-%. The free tryptophan concentration, attaining a maximum of 200-300 mg-%, begins to decrease. This indicates that the peptone is ready for use because further protein decomposition reduces the quality of the medium. The hydrolysate is then transferred to a cool room (+8 to +10°) for storage (the enzyme is not inactivated).

b) Preparation of the broth.

Hottinger hydrolyzate 20 liters

Aqueous meat extract 1:2, 40 liters

20% NaOH solution

Hydrochloric acid, 300-400 ml for clarifying the medium

The mixture is heated to boiling, pH 7.6. 0.5% NaCl is added. It is filled into test tubes and flasks containing pieces of boiled meat and sterilized at 120° for 30 minutes.

Semiliquid medium. For growing Cl. botulinum, Hottinger broth or pepsine-peptone may be used as a base, for Cl. perfringens, Veynberg or Pope broth.

0.1% agar and 0.4% gelatin is added to the medium. The medium in the test tubes is covered with a 0.5 cm thick layer of vaseline oil, and 3-5 g of boiled meat placed on the bottom. The medium is then sterilized for 20 minutes at 120°.

Blood sugar agar. 100 ml of 2% agar made up with Hottinger broth (for Cl. botulinum) or with Veynberg broth (for Cl. perfringens) is melted, cooled to 45° and then 10-15 ml of freshly taken sterile defibrinated rabbit, sheep's or bovine blood and 10 ml of 20% sterile glucose solution added. The mixture is shaken, avoiding the formation of foam and bubbles and poured into Petri dishes. The dishes are dried in the thermostat or in a box containing an ultraviolet lamp until the condensate is removed.

This medium can be used for isolating pure cultures of anaerobes.

Agar for inoculation of a high column. To Hottinger, Veynberg or Pope broth, 1.5-2% agar and 1% glucose are added, the pH adjusted to 7.4-7.6, poured into narrow (20 x 0.8-1 cm) thinwalled test tubes and sterilized for 20 minutes at 115°.

Lecithovitellin medium. 40 g heart extract or peptic liver hydro-

lyzate, 5 g disodium phosphate, 2 g sodium chloride, 0.01 g magnesium sulfate, 2 g glucose and 25 g agar are added to 1 liter water; the medium is brought to pH 7.6. Following sterilization and cooling to 50°, 0.1 part of a suspension of egg yolk is added to this medium. Preparation of the suspension: the yolk is aseptically removed from a fresh egg and a suspension of it made with an equal volume of sterilized physiological salt solution.

The colonies of Cl. perfringens on this medium have a diameter of 3-5 mm, are elevated, smooth and surrounded by a precipitation zone.

Wilson-Blair medium. 10 ml of 20% sodium sulfite solution and 1 ml ferrous chloride are added to 100 ml of melted and then cooled (to 80°) alkaline agar made up with Veynberg or Pope broth and 1% glucose. The ferrous chloride solution is made up with distilled water and the sodium sulfite solution is sterilized for an hour in steam. The medium is filled into test tubes in a quantity of 7 ml for each; the anaerobes grow in black colonies, often causing a complete blackening of the medium. Black colonies are formed by some anaerobes (S. tuphi, S. schottmuelleri, etc.) because of the reduction of the sodium sulfate to sodium sulfide, which by reacting with the ferrous chloride, form a black precipitate of iron sulfide. The sodium sulfite can be replaced by sodium thiosulfate and the ferrous chloride by ferrous sulfate. Cl. perfringens, Cl. septicum, and Cl. sporogenes have the property of reducing the sodium sulfite in this medium. They give colonies with an intense black color. Cl. tetani, and Cl. histolyticum form greenish-black colonies; Cl. perfringens forms black colonies very quickly (within 4 hours of inoculation).

Combined medium consisting of Wilson-Blair medium and blood agar (B.D. Bychenko, 1961). To obtain iron-sulfite agar, a 10% Na_2SO_3 solution is made up first (sterilization with live steam for an hour), 8%

$FeCl_3$ solution in distilled water and 8% lead acetate emulsion. 10 ml of the hot Na_2SO_3 solution is added to 100 ml of 2% melted nutrient agar, made up with Veynberg or Pope broth, and then, after cooling to 56°, it is mixed carefully with 0.025 ml of $FeCl_3$ solution and 0.5 ml of lead acetate suspension. The finished medium is poured into Petri dishes in a thin layer (thickness 1 mm).

Not more than 3-4 ml agar is required for one dish. The inoculation material is applied gently in streaks on the solidified agar plate by means of a platinum loop or the end of a Pasteur pipette. With one loop, material is applied to 3 dishes. The cultures are then quickly covered with a thin layer of blood agar and placed into an anaerostat at 37° under complete vacuum. Most bacteria develop in the depth of the blood agar. Only 6-8 hours after fresh cultures of Cl. perfringens have been grown, the growth of toxigenic colonies is apparent by the hemolysis of the upper plate and the blackening of the lower agar plate. If the Cl. perfringens colonies do not secrete hemolysin, they can be still readily detected by the blackening of the lower agar plate. If a vacuum apparatus is not available, another layer of simple 2% agar is poured into the dishes (in a layer of not less than 2 mm thickness to create anaerobic conditions) and after solidification of the agar, the dishes can be incubated at 37° in a conventional thermostat, the dishes with the cultures being inverted; 16 hours later, the colonies of the toxigenic strains of Cl. perfringens are visible in strong transmitted light in the form of smoke-gray or black formations which are surrounded by a hemolysis zone.

Medium for determining the saccharolytic properties of anaerobes. 0.5% agar is added to 100 ml of peptone water, sterilized at 120° for 20 minutes, then 1% of the test carbohydrates is added and 1 ml Andrede indicator and the pH brought to 7.2-7.4. The medium is filled into test

tubes and sterilized at 115° for 15 minutes. The same medium can be used without agar but in that case, a 0.5 cm layer of vaseline oil must be poured over the medium in the test tube before sterilizing.

Willis and Hobbes medium. The mixture (400 ml Hottinger broth, 4.8 g agar, 4.8 g lactose and 1.3 ml of a solution of neutral red) is autoclaved, cooled to 50-55° and 15 ml of a yolk suspension (hen's egg yolk, mixed with an equal volume of physiological salt solution) and 60 ml of sterile skim milk are added.

Benzidine agar. Benzidine agar (0.25 g benzidine, 0.3 ml 1 N hydrochloric acid, 50 ml water) are sterilized by boiling and the solution added to sterile nutrient agar, using 10 ml of the solution for 100 ml of agar.

It is absolutely essential to pour a layer of liquid vaseline oil with a thickness of 0.25-0.5 cm over all liquid media in test tubes and flasks prior to sterilization. Before the inoculation, all liquid media must be regenerated by boiling for 15 minutes in order to remove the oxygen.

LEISHMANIASIS

Sh. D. Moshkovskiy, Professor, Associate Member of the AMN USSR, and Ye. A. Pavlova, Candidate of Medical Sciences

The pathogenic agents are parasites from the class of Flagellata, Leishmania tropica and L. donovani.

Test material; selection of samples

When nonulcerous nodes of patients with the skin form of Leishmaniasis are to be examined (pathogenic agent L. tropica), the skin is rubbed with alcohol and pricked so as to hit the center of the node. Smears are prepared on object glasses from the serosanguinous drop which emerges, dried, fixed and stained by the Romanovskiy method.

When ulcers with copious secretions are examined, it is not recom-

mended to take material for the investigation from the surface, because the profuse bacterial flora and the large number of decomposed cells obscures the picture. The raised nonulcerous edge of the ulcer should be pricked with a needle or thin scalpel in the direction of the base of the ulcer. Smears are prepared from the emerging serosanguinous drop, dried, fixed and stained by the Romanovskiy method.

In presence of the internal form of leishmaniasis (pathogenic agent L. donovani), a specimen of bone marrow obtained by puncture (the sternum is usually punctured at the level of the 3rd-4th rib) or from an enlarged lymph node is subjected to examination. A smear is made from the puncture fluid on an object glass, dried, fixed with alcohol (methyl or ethyl) and stained by the Romanovskiy method.

Microscopic examination

The stained smears are examined using an immersion lens system. The leishmania forms of the parasite can be detected in the smears. These stages of the parasite have a spherical or, more frequently, an oval shape. Dimensions 2-4 × 1.5-2.5 μ. The single nucleus is in the center of the cell, close to it there is the kinetoplast (blepharoplast) in the form of a short rod or large coccus. Romanovskiy staining imparts a blue color to the cytoplasm, a red color to the nucleus, while the blepharoplast is stained a cherry red which is darker than that of the nucleus.

The parasites are seen in the preparation, partly free and partly enclosed in the cytoplasm of large mononuclear cells of the type of macrophages and other reticulo-endothelial cells.

Bacteriological examination

The serosanguinous fluid from a papula or ulcer is used to inoculate NNN medium.

The inoculation with the test material is carried out into con-

densation liquid, part of which is then distributed over the agar surface. The seeded test tubes are kept at a temperature of 22-25°. To prevent drying out of the medium, hot paraffin is poured over the cotton wool stoppers of the test tubes. A growth in the form of small transparent colonies which then fuse, are seen on the 3-4th day. A large number of leishmania is also observed in the condensation liquid. The culture can be kept for 3-4 weeks and longer without reinoculation (provided the stoppers are properly sealed with paraffin). Reinoculation is carried out by taking individual colonies from the agar surface with a loop and placing them into test tubes with fresh medium on the agar surface together with a small proportion of the condensation liquid.

The leishmania are present in a culture in the Leptomonas form. They have an elongated, lanceolate body with a size of 10-14 × 4-5 μ, a nucleus in the central part of the body and a blepharoplast near to the anterior end of the body of the parasite. Starting out from the blepharoblast and issuing from the anterior end of the cell body are cilia whose length is 1½ that of the body of the parasite. Part of the Leptomonas forms are often collected into a rosette (cilia directed inwards), formed of 10-15 individuals. Forms without cilia, in the form of elliptical, sometimes almost circular formations with a size of 4 × 5 μ can be observed in old cultures.

Serological examination

The serological tests with specific antigen are carried out in accordance with the method of the complement-fixing test. The antigen for this test is represented by disintegrated bodies of L. donovani from a culture or from the spleen of an infected animal. Owing to the difficulty of preparing the antigen, this test has found only limited application.

Nonspecific serological reactions. Formol reaction: a drop of commercial formalin is added to 1 mℓ of the patient's serum. This is mixed and left at room temperature. When the reaction is positive, the serum becomes turbid and coagulates. The test result is evaluated after an interval of 30 minutes. In cases of malaria, syphilis or leprosy, gelatinization also takes place but much later, within an hour or even later.

Bramahari test: 2-3 mℓ distilled water is added to 1 mℓ of the patient's serum. When the reaction is positive, a milky turbidity appears.

Detection of Leishmania by means of the biological test

The pathogenic agent of the skin form of Leishmaniasis (weeping form) of humans can be transmitted experimentally to white mice, gerbils and hamsters (Cricetulus migratorius, Cricetulus transcaucasicus). Leishmania from an ulcer or a culture serve as infection material. The leishmania from the culture are injected subcutaneously. When vaccination with leishmania from an ulcer is carried out, a piece of granular ssue containing leishmania is injected into a skin pouch, usually at ne base of the tail, more rarely the auricle or the thigh.

10-20 days later, an infiltrate develops at the site of injection of the leishmania, which contains the leishmanial stage of the parasite.

Detection of the pathogenic agent in the environment

Canine leishmaniasis. Cases of canine leishmaniasis are found in foci of internal leishmaniasis. The skin form of leishmaniasis is observed on dogs in the form of small papulae and ulcers situated at the edge of the lips, the auricles, in the region of the eyebrows, and the visceral form which invades the entire organism, including the skin, where alopecia and ulceration may appear in different places. The sick dogs sometimes fail to show any external symptoms of the disease, par-

ticularly when the animal is in the early stage of the disease.

When examination for internal leishmaniasis is carried out, the liver, spleen, bone marrow and lymph nodes of the animal are investigated. In presence of the skin form of leishmaniasis, biopsy specimens (scrapings) and smears are prepared, dried and stained by the Romanovskiy method.

The leishmanial forms of Leishmania canis, the pathogenic agent of canine leishmaniasis, are detected in the preparations.

Proliferation on the NNN medium takes place in the leptomonas form.

The leptomonas and leishmanial forms of L. canis are morphologically indistinguishable from the corresponding forms of L. tropica and L. donovani.

Examination of carriers. The females of Phlebotomus which are the carriers of skin and internal leishmaniases, are subjected to examination. The winged phlebotomus must be sought in human habitations and also in rooms where domestic animals and birds are kept, and in various other buildings. A test tube with flat edge can be used to catch phlebotomus. The insect, sitting on a wall, is covered with the test tube and the insect then pushed to the bottom of the test tube by means of a cotton wool plug. Up to 10 live phlebotomus can be collected with one test tube. The test tube with the phlebotomus must be kept in a dark, not too dry, cool place.

The female phlebotomus in the test tube are killed with tobacco smoke, sulfuric ether or chloroform. The phlebotomus is taken with pincers, the wings and legs being torn off and then placed on an object glass into a drop of physiological salt solution. With two prepared needles, the chitin cover is broken between the second and third abdominal segment (counting from the tail end). One needle is inserted

into the thorax and the other into the posterior end of the abdomen behind the tear. By carefully pulling, the needles are slowly moved apart. By pulling the needle, which has been inserted into the rear end of the abdomen, the internal organs are exposed. The stomach is retained and the other organs removed from the glass. The head is transferred to another glass.

The stomach is opened and examined in the living state or a stained preparation is made. The tongue is removed from the head of the insect. The tongue is ground up with a drop of physiological saline solution and examined in the vital state or in the stained form.

Staining: the stomach contents and the tongue in a drop of physiological salt solution are spread on an object glass in a thin layer, dried in air, fixed with alcohol (methyl or ethyl) and stained by the Romanovskiy method.

The leishmania in the carrier are present in the leptomonas form.

Nutrient Media

The NNN medium is prepared in the following manner: agar (14 g agar, 6 g common salt and 900 mℓ distilled water) are poured into test tubes (approximately 3-4 mℓ into each test tube), and sterilized in the autoclave. Approximately 30% by volume of defibrinated rabbit blood is added to the test tubes containing melted agar heated to about 45-50°. The medium is carefully mixed (by rolling the test tubes between the palms). The test tubes are then placed in a slanting position. Following the solidification of the agar, the test tubes are placed into the thermostat at 37° for 24 hours in order to check their sterility. During culturing it is essential to take care to prevent bacterial contamination.

ANTHRAX

Ye. I. Silant'yev, Candidate of Medical Sciences

Anthrax is a severe acute infectious disease of humans and animals, caused by the specific rodshaped spore-forming microorganism Bacillus anthracis. Depending on the place of primary penetration and the final location of this microorganism, cutaneous (anthrax, carbunculus anthracis, pustula maligna), visceral (pulmonary, intestinal) and general visceral clinical forms of the disease develop.

Anthrax can appear in all continents and all countries in connection with the production, consumption and treatment of animal raw materials (meat, wool, hides, bristles, etc.) and caring for sick animals.

Morphology of B. antracis

The anthrax bacillus is a relatively large (1-2 x 6-10 μ) bacilliform, immobile microorganism which is readily gram stained and with all aniline dyes. It forms short chains in the organism, which are surrounded by a capsula. Capsules are sometimes formed on artificial media as well when animal protein (blood, serum) is added to the medium. The capsule can cover individual cells or several microbial cells simultaneously. In liquid nutrient media it is usually arranged in long chains.

Biology of B. anthracis; culturing properties

The pathogenic agent of anthrax is not very demanding and is capable of developing on different laboratory media, meat-peptone agar and broth, gelatin, milk, plant seed extracts, different carbohydrate media and also in hay infusion.

During the growth of anthrax bacillus, the broth remains clear with a precipitate being formed on the bottom which resembles a piece of cotton wool. On dense media, relatively large, dull colonies with a

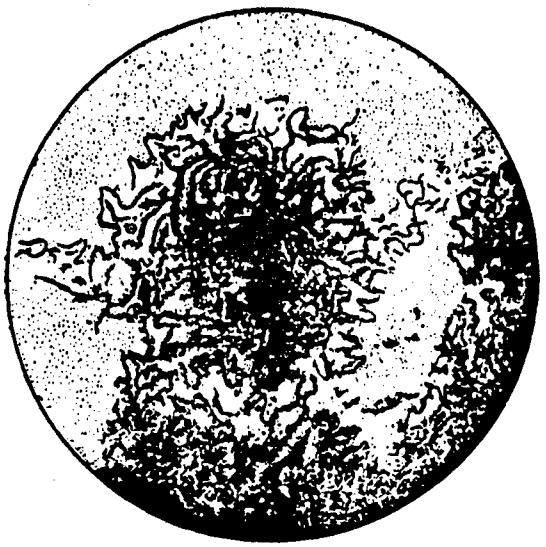


Fig. 120. Typical anthrax colonies on agar under low magnification. Formation of tangled fibers.

fibrous structure (with formation of threads from the center of the colony to the periphery: "fimbria", "strands of hair", "lion's mane", etc.) (Fig. 120).

The capsular form of this microbe is the virulent R-form. The growth optimum occurs near 36°.

When the medium is exhausted or dried out, the vegetative forms of the microbe are transformed into the spore forms. A single central spores is then formed in each bacillus. A necessary condition of spore formation is access of oxygen and a temperature within the range of 12-42°. The spores are not formed in the patient's organism or in a nondissected cadaver.

In absence of oxygen the vegetative forms grow only very slowly. When an agar column or gelatin is inoculated by puncture, it grows like an inverted treetop; the upper part of the gelatin is liquified ("button") by a proteolytic enzyme (Fig. 121).

On blood agar, growth takes place without hemolysis. Milk is coag-

Fig. 121. Anthrax culture on meat-peptone gelatin.

ulated on the 2-4th day with subsequent peptization. Most strains break down galactose, glucose, trehalose, maltose, saccharose (slowly), levulose, dextrin, glycerol and salicin (variably) with formation of acid and without gas formation. Hydrogen sulfide is not always formed. Indole is not formed.

When the alkalinity of a broth culture is increased, turbidity may appear. Individual strains, in addition to the formation of the typical precipitate on the bottom of the vessel with liquid medium, can cause moderate turbidity of the liquid medium.

In addition to the typical R-form, smooth S-colonies, dwarf G-colonies and intermediate Q-forms (pigmented, slimy, aerosive, etc., colonies) can be isolated from different materials. The shape of the microbes, the culturing and biochemical properties, the virulence, etc., are variable. For example, growth may be absent in gelatin, while in broth there may be a diffuse turbidity with an amorphous precipitate. On solid media, the colonies may differ in their color, transparency and density.

New properties of the anthrax bacillus have been described in the last ten years such as phosphatase formation; variability on media containing penicillin ("pearlshell necklace" test); fluorescence under the microscope when the test materials are treated with special luminescent anthrax sera; lysis of the anthrax microbes under the influence of specific bacteriophage.

Resistance to physical and chemical factors

The vegetative forms of the microbe die relatively quickly in absence of oxygen, particularly under the influence of putrefactive microorganisms. Sometimes, a pure culture can no longer be obtained from

a cadaver a few days after the death of the animal. The vegetative forms also killed quickly by heating to 75-100° and the action of various disinfectants. In contrast to this, the spore forms are stable. In the soil and on the bottom of water reservoirs the spores keep practically indefinitely, for tens of years. They can withstand different disinfectants for hours. Most effective against spores are disinfectant combinations (formalin, corrosive sublimate, bleaching powder, etc.) and also 10% caustic soda solution and solutions of calcium hypochlorite, containing not less than 4% active chlorine. The effectiveness is increased in proportion to the temperature.

In liquid medium (broth, water, etc.), anthrax spores are killed by boiling at 100° for not less than 10-20 minutes. The survival rate of the spores may vary, depending on the temperature and the kind of environmental object.

Dry heat kills spores at 140° within 3 hours.

Direct sunlight acts very slowly and practically never kills anthrax spores present in the soil.

The elucidation of the specific properties and the relationship of the pathogenic agent to the environment is of great importance in connection with the difficulties presented by differential diagnosis. Pseudoanthrax (B. pseudoanthracis) and anthraxlike bacilli (B. Anthracoides), which cannot be differentiated morphologically are widely distributed in nature. Besides, there are other sporogenous aerobes such as the hay bacillus (B. subtilis), the potato bacillus (B. mesensricus), the cabbage bacillus (B. megatherium) and the rhizomorphous bacillus (B. mycoides) which may be present in the various environmental objects, subjected to medical and sanitary investigation. In the Berdzhi (1957) classification, special attention is given to the differentiation of B. cereus.

Laboratory Diagnosis of Anthrax

The laboratory diagnosis of anthrax consists in producing a pure culture and in its identification. The aim of the investigation depends on the clinical or sanitary-hygiene tasks in a given situation.

Test material; sampling

Tissue exudates from under a scab and from the base of an ulcer, blood (in cases of high body temperature, particularly with the generalized forms), sputum (with the pulmonary form), and excreta (with the intestinal form) may serve as the objects of the laboratory tests.

The pathological material is taken from the depth of the ulcer or from under the scab. The serous exudate or tissue exudate is collected with a syringe or Pasteur pipette and put into a test tube of small glass flask. The sampling must be done carefully to avoid tissue injury. Some difficulties may arise in this connection because in typical cases of anthrax, pathological excretions are present only in small quantity and pus is entirely absent.

This is also the limitation in typical cases of the cutaneous form. Indications for sampling of blood are rare, only when a generalized process is suspected. Blood can be taken from a vein or sometimes from the ear lobe. 3-5 mℓ is taken from a vein; 2 thick smears are prepared which are dried in air without fixation, put together and sent to the laboratory; the remaining blood is used to inoculate meat-peptone broth or some other suitable medium.

Sputum and feces are collected in presence of the corresponding indications in a sterile vessel.

The manipulation of the materials, their transport, storage, etc., are carried out in accordance with the rules governing the work with particularly dangerous infections. When large quantities of pathological fluids have to be dispatched to the laboratory, lumps or fragments

of some adsorbent may be soaked in them (chalk, sugar, charcoal, thread, etc.) which are also placed into a sterile vessel and sent to the laboratory with all necessary precautions.

If little pathological material is available, this may be limited to two smears, one of which is gram-stained for microscopy and the other is used to prepare scrapings for inoculation of broth and for infecting laboratory animals. The autopsy of anthrax cadavers is forbidden.

Microscopic examination

The microscopic examination of the pathological material makes it possible to determine the pathogenic agent, to establish the morphology and staining properties of the microbe and to make visible the capsules and spores.

It is only rarely possible to detect anthrax bacilli in native smears from tissue exudates. Hence, microscopy is usually carried out on the cultures which grow after inoculation with the contaminated material. The microscopic examination may then be part of the identification of the culture.

Microscopy with a hanging drop reveals the immobility of the anthrax microbe.

To obtain a stained smear, a drop of test material is placed on an object glass, covered with another object glass and the two glasses drawn in opposite directions. Smears are thus obtained on the two glasses which are suitable for microscopic examination. The smears are dried in air (protected against flies). Following drying, the smears are fixed by the normal method in a burner flame. Contact smears from organs, blood and exudates are fixed with formaldehyde vapor or with osmium.

Development of the capsules. Rebiger method of development of

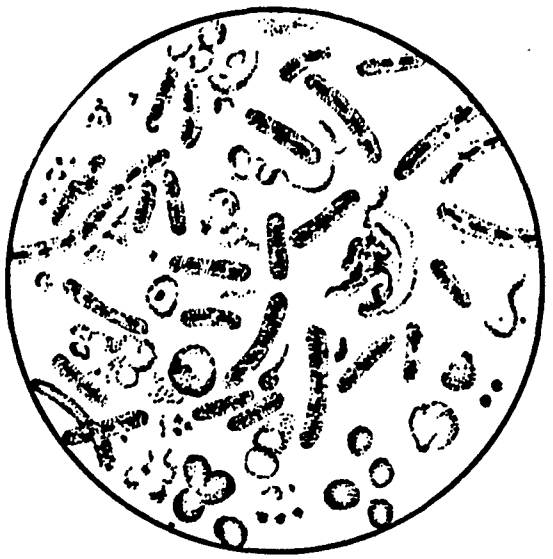


Fig. 122. Anthrax bacilli with capsules. Smear from spleen.

capsules: the preparation is fixed with formalin and stained at the same time; 15-20 g gentian violet is dissolved in 100 mℓ of common formalin; the solution is allowed to stand several hours at room temperature and then filtered; without prior fixation, the smears are stained with this solution for 15-20 seconds, washed and dried. The capsules are stained red-violet, the bacteria dark-violet (Fig. 122). Development of spores (see p. 41).

Bacteriological tests

The material obtained from the patient, remaining after preparation of the smears for microscopy, is used to inoculate broth using one dish with meat-peptone agar and one dish with blood agar. The cultures are placed into the thermostat (37°) and left overnight. The next day the cultures are examined. Pure cultures, isolated from an organism in this manner, are usually present in the typical R-form. A moderate quantity of precipitate forms on the bottom of the test tube in the form of small flakes, while the broth, as a rule, remains clear. When smears from this broth are examined under the microscope, long

threads of gram-positive microorganism are detected. Dull, fibrous colonies without hemolysis zone form on agar. With the skin forms of the disease, inoculation is successful only in 50% of cases.

Penicillin test. A decomposition of B. anthracis cells is observed on 2% meat-peptone agar containing penicillin (1 dish contains 0.5 yed/ml; the second 0.05 yed/ml; the third one is a control and does not contain penicillin). 1 drop of a 3 hour old broth culture of the test strain is applied to each of the dishes. The cultures are placed into the thermostat for 3 hours at 37° and then examined under the microscope with a highpower dry or immersion system (spherical "pearl-shells" of B. anthracis can be seen; the other sporogenic microbes have their normal unaltered cell shape; in the control dish, the cells also retain their normal unmodified shape, appearing as long or short chains).

Bacteriophage test. An anthrax culture is lysed by its specific bacteriophage. This is made apparent by applying a drop of phage to a -hour agar culture. The examination is carried out after the culture as been in the thermostat for 6-18 hours. The same on canted agar in test tubes. Following inoculation, the culture is placed into the thermostat for 30 minutes at 37° and 1 drop of specific bacteriophage applied. The test and control test tubes are then returned to the thermostat. Intense lysis can be observed after 16-18 hours (where the drop of phage has dropped on it).

Detection of anthrax bacillus by means of the biological test

In cases when normal cultures do not provide sufficient information for a diagnosis and also for greater reliability, infection of laboratory animals with the test material is carried out at the same time as the culturing.

To this end, a 10% suspension of the test material in sterile

physiological salt solution or sterile water is prepared and injected subcutaneously in a quantity of 0.1-0.2 ml into white mice or guinea pigs. In order to accelerate identification of the culture, some of the animals are killed within a few hours or on the day after they had been infected.

When virulent microbes are present in the test material, the animals normally die within 2-4 days from generalized infection. The animals are observed for up to 10 days. If the animals have not died within this period, the presence of anthrax microbes in the injected material can be excluded.

An autopsy is performed on the animals, contact smears and cultures made from the blood of the heart and from organs (spleen, liver, kidneys, infiltrate at the point of infection, etc.) to determine the cause of death and to identify the isolated microbes. In presence of anthrax infection, the normal capsular forms of the pathogenic agent of anthrax are isolated.

Testing procedure

First day: 1) the smears which have been gram-stained and which have been stained to reveal the capsules, are examined; 2) test tubes with broth, a dish with meat-peptone agar and a dish with blood agar are inoculated; 3) 3-5 guinea pigs or 5-10 white mice are infected with a 10% suspension of the test material, normally subcutaneously, or intraperitoneally if the infection process is to be accelerated.

When the smears are examined on the first day of the investigation, preliminary data can be obtained confirming the diagnosis.

Second day: the growth in broth and on agar is investigated. The isolated culture is examined under the microscope. Data for a preliminary answer are provided when a typical growth and morphology of the isolated microbes is present. In doubtful cases one must wait for the

results of the biological test and also examine the cultures which have been grown in broth and agar. The biological test is decisive. It is characteristic for the pathogenic agent of anthrax that it kills nonimmune white mice and guinea pigs in 100% of cases and, as a rule, not later than within 5-6 days. Negative results of the biological test and doubtful data of the preceding investigations exclude the presence of the anthrax microorganism in the test material. The culture of the anthrax bacillus can be isolated from the blood or organs of the dead animals and identified (see Table 75 and 76).

Allergy test

The intradermal allergy test has been proposed to reveal the specific alteration of the organism (Instruction approved by the Committee of Vaccines and Sera of the Public Health Ministry of the USSR, 20th February 1960). The specific allergen anthraxin is required for carrying out this test (it is stored at 4-10° under sterile conditions and in the factory packing for not more than 1 year). The test must be carried out intradermally, the allergen being injected into the skin of the forearm in a quantity of 0.05-0.1 ml. A control liquid of the same volume isinjected into the other forearm. In persons who have never had anthrax and have not been vaccinated against it, the test is negative. In presence of an anthrax process, hyperemia and an infiltrate with a diameter of not more than 5 mm appears at the point where the allergen has been injected. The reaction is observed twice, after 24 and 48 hours.

Detection of the pathogenic agent in the environment

Various environmental objects - meat, hides, wool, soil, different objects, air and water - can be subjected to examination. Under natural conditions the pathogenic agent of anthrax is primarily found in products of animal husbandry obtained from animals with anthrax; on

objects, contaminated by these products; in the soil - where animals which have died of anthrax have been buried and also in the dust and air from the premises in which the corresponding raw materials are processed (wool, bristles, hair, hides, etc.).

The need may sometimes arise to prepare cultures from samples of water, swabs from objects or air samples. Solid particles are first ground as far as this is possible and a 10 fold quantity of sterile water or physiological salt solution added and allowed to stand for 1-2 hours. After the sample has been prepared in this way, it is divided into 2 equal parts (preferably in 2 test tubes).

The material from one test tube is collected with a Pasteur pipette and broth, meat-peptone agar and blood agar inoculated with it, while the remainder is used for the biological test (infection of mice, guinea pigs or rabbits, as usual, subcutaneously). The material in the other test tube is heated 70-75° and kept at this temperature for 30 minutes to kill the vegetative forms of the microbes (or at 80° for 10 minutes).

The heated material is used for inoculating the same kind of media and infecting a similar group of animals. In the presence of anthrax spores, cultures also develop after 1-2 days in the thermostat at 37°, and the infected animals die within the normal periods. The microscopic examination and the observation of the growth of the culture sometimes do not provide sufficiently reliable information. In these cases the decisive data are obtained from the infected animals because the pathogenic agents of anthrax are invariably pathogenic organisms.

Positive results are usually obtained by the culturing of samples of animal materials, swabs from parts of organs and tissues, sometimes from cultures of soil from places where cadavers of animals which were

killed by anthrax are buried.

In order to detect the anthrax microbe in the mud of water reservoirs or in the water, filtration of the suspended mud and the water samples through special filters (see p. 257) is essential to concentrate any spores or individual vegetative forms of the microbes, which might be present.

The methods of air sampling are selected on the basis of the consideration that the anthrax microbe is relatively large and can thus be detected in aerosols of relatively large particles. Hence, to detect the anthrax microbe in air, many methods which make it possible to concentrate large aerosol samples in a small volume of water or physiological salt solution, are suitable (see p. 266). After this concentration has been effected, the liquid through which the air has been passed, the membrane or gelatin filters, etc., are subjected to the usual tests (cultures, infection of animals, identification of the cultures). The direct microscopic examination of environmental materials, treated with fluorescent sera, can give preliminary, tentative results. The accumulation and proliferation of the microbes in the cultures on media or in test tubes with the corresponding identification of the cultures makes it possible to obtain reliable, positive results. Up to 200 DLM of B. anthracis have sometimes been detected in 1g of the dust from factory bins.

The pathoanatomic alterations are also taken into account in the diagnosis of anthrax and the identification of the culture. In animals which have died from anthrax, rigor mortis is either very slight or absent. The blood is thick, darkred and copious infiltrations and extravasations are found: the spleen is flaccid, and gives tarlike scrapings. The internal organs and lymph nodes are hyperemic.

Rapid methods of bacteriological diagnosis

Precipitation test. Owing to the thermal stability of the precipitinogen this test is termed thermoprecipitation test and can be used for rapid diagnosis. The test is convenient because in presence of old, decaying test material, when the pathogenic organism of anthrax is difficult or quite impossible to isolate, it can give completely reliable positive results. In these cases, the test should also be used to detect the specific anthrax antigen in the test material. The precipitation test is widely used in the hide and wool processing industries.

The following is required for carrying out the precipitation test: precipitating serum (for the Ascoli test); an antigen extract from the test material; standard anthrax antigen for the control test; a tissue extract from a healthy animal and normal serum, also for the control test.

Antigen: pieces of skin, wool, muscles, soil, etc., are ground and physiological salt solution added in a quantity of 100 ml for 1 g of material, boiled 10 minutes, filtered until completely clear through calcined asbestos wool, talcum filter or paper, moistened with physiological salt solution. In addition, a method of preparing antigen in the cold is known. This is more reliable and can be used for the control of the thermoprecipitation test. The test material can be sterilized in the autoclave before the isolation of the antigen. Then the material is also ground and 1:10 of common salt solution (0.85%) containing 0.3% crystalline phenol is added. This is left for 20 hours at room temperature (or at 4-10° for 30-40 hours). The extract thus obtained is filtered through asbestos (the asbestos should give a neutral reaction with litmus; when the asbestos has an acid or alkaline reaction, it should be carefully washed). The filtrate should be clear.

0.2-0.3 ml of clear precipitating serum is run into narrow test

tubes or test tubes with tapering bottom, then the same quantity of serum is run in carefully with a Pasteur pipette so that it flows down along the wall and forms a layer on the test antigen. A white, cloudy precipitation ring (++) should appear at the interface of the two liquids (usually within 15 seconds to 2 minutes) not later than 15 minutes. When the test is negative, the ring does not form, whereas in doubtful (±) and negative tests it may appear after an interval of more than 15 minutes. In the testing of hides, two doubtful tests are considered as positive.

The following control tests are carried out in similar test tubes:

1) precipitating serum and an extract from material known to contain anthrax; this should give a marked precipitation (with standard anthrax antigen);

2) precipitating serum and extract from a healthy animal; there should be no reaction;

3) precipitating serum and physiological salt solution or water the same liquid which was used for producing the antigen extract); there should be no precipitation;

4) normal serum and standard anthrax antigen; there should be no precipitation.

In all difficult cases of anthrax diagnosis in humans, the clinical data and the epidemiological connection of the patient with the environment are given primarily consideration, because the laboratory methods may not give reliable results in certain cases.

Of all the laboratory data, the most decisive are the data of the biological test and the results of the bacteriological cultures with subsequent identification of the isolated cultures.

Serological-fluorescence method. The principle of the method consists in the detection of antigens by means of specific sera with fluor-

escent dyes (Descriptions of the method of examining water, soil and swabs from objects for the presence of the anthrax bacillus, 1963, Ye. A. Levina et al.). The test material is grown at 37° for 3 hours, then preparations are made, fixed and treated with special fluorescent sera. The preparations are examined under the fluorescence microscope ML-1 or ML-2 fitted with the fluorescence apparatus ON-17 and a system of violet color filters. The method of work is described in detail on p. 74. The method is considered suitable only for detecting the vegetative forms of the anthrax bacillus.

Method of detecting capsule formation (GKI, V.R. Arkhipova). The test suspensions are cultured (swabs from various objexts; swabs from membrane and other filters used for the concentration of bacteria during their isolation from water, soil and air) on the special GKI medium. The cultures (several drops) are placed into the thermostat at 37°. It is assumed that capsule formation of B. anthracis begins within 1-2 hours and that most of the microbial cells present in the test material, have formed a capsule within 16-18 hours. Normal smears are prepared for microscopy to reveal their presence. The smears are fixed with methanol for 15 minutes, then stained with Löffler blue and examined under 900 fold magnification. The capsula is stained rosered, the microbes blue.

When microbial cells with capsules are found, this is a preliminary positive result and the isolated culture is subjected to further full identification.

Method of detecting capsule formation (according to Shlyakhov and Gruz). 0.1-0.2 of a swab (from membrane and other filters, from various environmental objects) is injected into white mice intraperitoneally, then 2 mice are killed each time after 30-60-120 minutes after the injection. Two mice are left as controls (they are not killed until

the investigation is terminated). An autopsy is performed on the dead mice and smears made from the prepared exudate and the organs in order to reveal the presence of capsule microbes. Positive results enable preliminary tentative conclusions to be made. Later on, cultures must be isolated in the pure form and identified (see Tables 75 and 76).

Nutrient Media

GKI medium. The GKI medium (State Control Institute) is prepared in the following manner: 60 mℓ Hanks solution is run into a sterile flask and (in a sterile manner) 40 mℓ of bovine serum, inactivated for 30 minutes at 56°, is added. After careful mixing, the pH of the medium is brought up to 7.2 by adding soda solution and 2 mℓ lots placed into test tubes. The test tubes are closed with sterile rubber stoppers. The medium keeps for long periods at a temperature of 4°.

Hanks solution consists of the following ingredients:

Sodium chloride 8.0 g
Potassium chloride 0.4 g
Calcium chloride 0.14 g
Magnesium sulfate 0.1 g
Magnesium chloride 0.1 g
Disodium phosphate 0.06 g
Monopotassium phosphate 0.06 g
Sodium bicarbonate about 0.15 g
Glucose 1.0 g
Phenol red 0.02 g
Doubly distilled water 1000 mℓ

Nutrient medium for producing a spore culture of B. anthracis. Digested casein medium, pH 7.2-7.4. The basis of the medium is tryptically digested casein to which yeast extract diluted with water and various salts which play a part in the nutritional requirements of the

anthrax bacillus is added (Gladstone-Field medium). Spore formation is obtained with this medium in 90-95% of cases.

Preparation of the medium: tryptic casein digest 50 mℓ

Aqueous yeast extract 200 mℓ

7 Potassium diphosphate (K_2HPO_4) 5 g

6 + Potassium monophosphate (KH_2PO_4) 1 g +

5 + Sodium sulfate (Na_2SO_4) 0.3 g +

4 + Calcium chloride ($CaCl_2$) 0.1 g +

3 + Manganese sulfate ($MnSO_4$) 0.03 g +

2 + Magnesium sulfate ($MgSO_4$) 0.05 g +

1 Iron sulfate ($FeSO_4$) 0.01 g

Agar 30.0

Doubly distilled water - to 1000 mℓ

Remarks:

1. Instead of the yeast extract, 0.5% yeast solution "Bakto" is often used.

2. The salts designated by a + sign, may be omitted.

3. The Arabian figures from 7 to 1 indicate the sequence in which the salts are to be dissolved.

4. Sodium phosphate should not be used as a substitute for potassium salts.

By means of phosphate buffer, the medium is adjusted to pH 7.2.

LEPROSY

A.I. Togunova, Professor

Leprosy is a disease caused by a microbe of the genus Mycobacterium, M. leprae Hansen. Two clinical forms of the disease and their intermediate stages are distinguished: the cutaneous or torulose and the neural or anesthetic. On the 4th International Congress of Leprology at Cairo (1938), a definition was given of the malignant (lepromatous)

TABLE 75

Differential characteristics of bacillus anthracis and other aerobic gram-positive spore-forming microorganisms

Name	Culturing properties			
	Agar	Broth	Gelatin	Litmus serum
Anthrax	Young colonies, white, downy; under low magnification, the fibrous structure is clearly visible	Growth noticeable within 7-8 hours; within 18-24 hours, the broth becomes clear with a flocculent, finely divided precipitate. A film is not formed The ring near the wall is present, but slightly diffuse	When inoculation by puncture is carried out, a whitish rod with fine horizontal outgrowths is formed: "inverted tree pattern". Gelatin is liquified from the surface downwards in the form of a stocking within 3-4 days with flocculent precipitate	Reddening
Pseudoanthrax bacillus	Round, whitish, firm colonies; along the edges twisted threads, divided into individual sections	Cloudy with crumbly, tenacious precipitate; forms films on the surface and a nondiffuse ring near the wall	Intense growth upon puncture with large number of branches and severe liquefaction of the gelatin	Blue colora tion
Anthracoid	The same	The same	Thickened nodes along the puncture (not branches); gelatin is liquefied in form of a funnel	The same
Hay bacillus	Mat grayish-white deposit; film on the condensation fluid	First becomes cloudy then forms a film and becomes transparent	On gelatin the colony is surrounded by a corona of rays; on the surface of liquified gelatin it forms a film	" "
Potato bacillus	Mat-white puckered thick deposit; a film forms on the condensation fluid	Forms a puckered film; the broth remains almost clear	Round colonies with liquified gelatin	" "
Cabbage bacillus	Forms of gray slimy deposit	Slight precipitate without film	Funnelshaped liquefaction	" "
Rhizomorphous bacillus	Forms moderate felt-like deposits	Broth clear; on the bottom a precipitate resembling a piece of cotton wool, not disintegrated by stirring	Quickly liquified	" "

Egg medium with malachite green	Mobility	Capsule formation	Hemolysis	Pathogenicity: Mice	Pathogenicity: Guinea pigs	Pathogenicity: Rabbits
No growth, coagulation or cloudiness for the first 70-166 hours	-	+	-	Die within 24-36 hours	Young guinea pigs die on the 2nd day, old ones on the 3rd day	Die on the 3-5th day
Within 11 hours, the yolk is coagulated and the medium becomes clear	Weak +	-	+	Sometimes pathogenic when injected in large quantities into the abdominal cavity	Nonpathogenic	Nonpathogenic
The same	The same	-	+	The same	The same	The same
	Lively	-		Nonpathogenic	Nonpathogenic	Nonpathogenic
	The same	-		The same	The same	The same
-	Weak	-				
-	The same	-		" "	" "	" "

TABLE 76

Differential Characteristics of the Pathogenic Organism of Anthrax and Some Related Spore-forming Aerobes (according to Berdzhi, 1957)

Name of microbe	Morphology and dimensions	Mobility	Capsule Formation	Growth on gelatin	Growth in broth	Formation of acetylmethyl carbinol	Formation of lecithinase	Pathogenicity
B. anthracis	Rods 1.0-1.3x 3.0-10.0	Immobile	+	Slow liquefaction of the type of inverted tree	Growth on bottom of test tube in flakes, possible a loose ring near to the wall, slight cloudiness (opalescence)	Forms	Forms	For humans, cattle, pigs, sheep, rabbits, guinea pigs and mice
B. cereus	Rods 1.0-1.2x 3.0-5.0	Mobile	-	Rapid liquefaction	During growth, uniform cloudiness with precipitate and film or without film	Forms	Forms	
Variants	0.8-1.3x 2.0-6.0	Immobile	+	The same				Large doses of a 24 hour old culture are fatal to guinea pigs
B. cereus var. Mycoides	Very fine[1)]							
B. Mygatherium	Rods 1.2-1.6x 2.0-4.0	Mobile		Slow liquefaction	During growth, uniform cloudiness with precipitate or without precipitate	Nonformed	Nonformed	
Variants	0.9-2.2x 1.0-5.0	Immobile						

[1)] The other characteristics are similar to that of B. cereus.

type of leprosy and of the neural form, which comprises all cases of benignant leprosy with disorders of the polyneuritic type, spotty-anesthetic and tuberculoid. The lepromatous (nodal) form is characterized by the growth of a granulation tissue which forms the basis of the so-called lepromes the latter contain a considerable ("enormous") number of acid-resistant mycobacteria and are present in the skin, lymph nodes and the mucus of the nose and mouth.

Morphology and biology of M. leprae

The pathogenic agent of leprosy is similar in its morphology to the tubercle bacillus. It is a straight or slightly curved mycobacterium, dense or grainy and is stained more easily than the tubercle bacillus with fuchsin (without heating) and more easily decolorized by acids and alkalis. Its gram-positive. In the leprosy nodes (lepromas) the Hansen bacillus is found in enormous numbers, arranged like "bundles of cigars" (Figs. 123 and 124). Numerous attempts to grow the leprosy macobacterium on nutrient media compel us to doubt whether it is possible to obtain cultures of leprosy mycobacteria outside of human tissues. However, some authors have obtained slowly growing cultures from lepromas in the form of a grayish, wrinkled, dry or moist deposit (glycerol, placenta agar) or films with uniform cloudiness of the broth. Cultures have also been obtained colored yellow or orange by a pigment. However, it has not yet been possible to confirm that these cultures are identical with the leprosy bacillus because the basic criterion, the possibility of infecting experimental animals with the grown cobacteria, is absent. The isolated cultures can be classified (V.I. Kedrovskiy) according to the nature of the bacteria into three types: 1) acidresistant mycobacteria which are not pathogenic for any animal species; 2) acidresistant mycobacteria which grow only in the primary culture; 3) mycobacteria which are not acid or oxygen-

resistant.

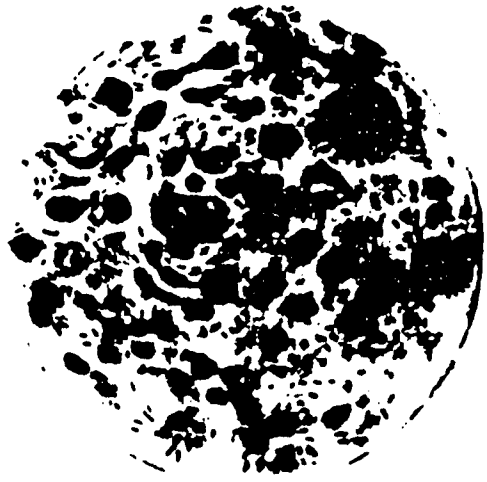


Fig. 123. Skin leprosy. Stained according to Ziel-Nielsen. 900x.

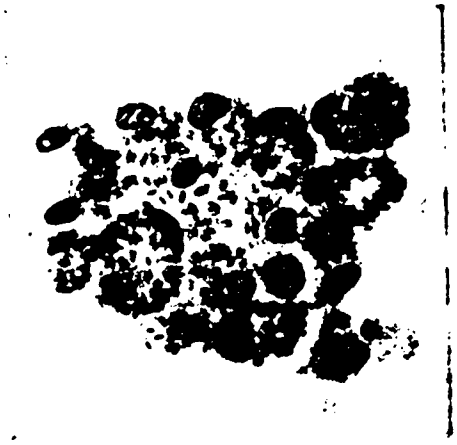


Fig. 124. Infiltrate in the pia mater in experimental leprosy. Typical arrangement. Stained with Ziel fuchsin-hematoxylin. 800x.

Laboratory Diagnosis of Leprosy

Microscopical examination

The diagnosis is established on the basis of the bacterioscopic examination of the nasal mucus or the tissue fluid (juice) from the skin lesions. Positive findings confirm the clinical diagnosis. When

the result is negative, a biopsy is carried out on the lesions or examination of smears or histological sections from them is carried out. Highly typical "leprous cells", elliptical or circular, filled with acidresistant bacteria, are present in leprous lesions. The large, greatly inflated cells containing the bacteria are termed "leprosy globules". These giant cells are not observed in the lepromas. The leprosy mycobacteria must be differentiated from tubercle bacilli on the basis of their property to stain more easily and their easier decoloration by alcohol and acids after staining.

One of the most widely used methods is that of Baumgarten: 5-6 drops of alcoholic solution of basic fuchsin is mixed with 5-6 mℓ of water. The smears are stained for 6 minutes without heating, and decolorized 30 minutes in nitric acid and alcohol (one part of nitric acid for 10 parts alcohol), washed in water and again stained with an aqueous solution of methylene blue. Sections of lepromas are stained 12-15^{m} in the fuchsin solution, decolorized half a minute in alcohol with nitric acid, washed with water and again stained with methylene blue. In old lepromas the leprosy bacillus is stained red; in fresh lesions, the mycobacteria are partly blue and partly red. Tubercle bacilli normally are not stained by this method.

Allergy test

To confirm a diagnosis of leprosy, the allergy test with lepromin * (Mitsuda test) is used; 8-24 hours after intradermal injection of lepromin, an infiltrate . yperemia develop. The Mitsuda test, however, although widely used, is not sufficiently specific.

GLANDERS

Ya. Ye. Kolyakov, Professor, Honored Worker in Science of the RSFSR

Glanders is an infectious disease of solid-ungulates (horses, donkeys, mules) which mainly takes a chronic form and can be transmit-

ted to humans. Changes arise in the organs in which the lesions are present (lymph nodes, lungs, liver, etc.) in the form of the typical glanders nodes of varying size. The pathogenic agent of glanders is Actinobacillus mallei (previously termed Bacillus mallei).

Morphology of A. mallei

Immobile, nonsporeforming rods with a length of 1-5 μ and typical grain structure. The bacterium is polymorphous: it can have a coccus-like or tympanous shape; the microbes are frequently arranged in threads and assume the form of rods with irregular outlines. The threads composed of bacteria and with average length, normally consist of 4-8 members.

A. mallei is gram-negative. It can be stained with Pfeiffer fuchsin or Loeffler blue within 5 minutes. The typical graininess of the bacterial cell is then clearly visible.

Biology of A. mallei; culturing properties

The microbe is not demanding with respect to the nutrient medium. It can be grown on meat-peptone agar and in broth, as well as on potatos. The growth on these media is considerably intensified when up to 5% glycerol is added (glycerophilicity). It is aerobic. The temperature optimum is 37°; below 20° and above 45° the glanders bacillus cannot develop. The microbe grows well when the medium is weakly acid, neutral or weakly alkaline.

Potato medium serves for purposes of differentiation. The growth of A. mallei on slices of potato, made alkaline with 1% soda solution and moistened with 5% glycerol solution, is particularly typical: towards the 3rd day, a uniform slimy, dark-amber honeylike deposit which is mat or often shiny, is formed. Towards the 6-8th day, the amber, pellucid culture assumes a reddish color and the transparency of the deposit disappears.

On meat-peptone agar containing 2% glycerol, the growth of the microbe normally begins within one day. A glanders culture on agar first takes the form of a transparent, grayish-white deposit with a nacreous lustre, which is slimy and viscous.

In meat-peptone broth containing 2-4% glycerol, a uniform cloudiness appears first, then a slimy grayish-white precipitate is formed, which rises like a corkscrew when the test tube is slightly shaken. Towards the 10th day, a gray, slimy film appears on the surface of the broth, particularly when it is grown in flasks.

The glanders microbe coagulates milk slowly, usually on the 6-8th day. It does not liquify gelatin, and exerts a proteolytic effect only at low gelatin concentrations. The glanders bacillus also develops on protein-free synthetic media in which ammonium salts of organic acids and carbonic acid serve as sources of nitrogen and carbon. Indole is not formed. Lactose and glucose are broken down with formation of acid but without gas evolution.

Pathogenicity and virulence

A. mallei is highly pathogenic for solid ungulates. Horses can be infected subcutaneously and by feeding with a tiny quantity of glanders culture. Horses usually have the chronic form of glanders. Cases of glanders disease in wild animals as a result of eating meat obtained from horses suffering from glanders have been described (lions, leopards, tigers, panthers, lynxes, wildcats, etc.). Cattle, sheep and goats do no contract glanders under natural conditions. Highly resistant to glanders are pigs, birds and rats.

Of the laboratory animals, guinea pigs can be infected with glanders; when they are infected subcutaneously, they die within 10-15 days but sometimes it takes 2-3 months.

Cats, which are particularly sensitive to this infection, can be

used as laboratory animals. In cats, glanders takes the form of septicemia.

Rabbits are not very susceptible, young rabbits being more sensitive. White mice cannot be infected with glanders.

Resistance to physical and chemical factors

A. mallei has a moderate degree of resistance with regard to environomental factors. Boiling kills a culture within a few minutes, a 60° it takes 2 hours or even longer to kill it. Drying out for 1-2 weeks is fatal to this microbe; on silk threads a culture could be kept for more than 3 months. In putrefying material the bacteria retain their viability for up to 24 days. In frozen materials the glanders bacillus is highly stable.

The following antiseptics and chemical disinfectants kill the microbe: corrosive sublimate in a dilution of 1:1000 in one minute, a 5% solution of bleaching powder, 2% formalin, 2% phenol solution, 3% creolin, 1% caustic soda solution, within an hour. Corrosive sublimate, bleaching powder and formalin are used in practice in the indicated dilutions.

Laboratory Diagnosis of Glanders

Test materials; sampling

Nasal discharges, putrescent secretions from ulcers and puncture specimens from subcutaneous abscesses, taken under sterile conditions, can serve as test materials. Individual nodes and infected parts are cut for the purpose of the examination in a sterile manner from whole cadavers from the lungs, liver, and lymph nodes. The material can be preserved with 30% sterile glycerol.

Microscopic examination

In view of the absence of specific staining methods for glanders

bacteria the microscopy of smears from pathological material has only limited diagnostic value but is important for the exclusion of other pathogenic organisms. The smears are gram and Romanovsky-Giemsa stained.

Bacteriological investigation

Potatoes with glycerol added, meat-peptone agar and meat-peptone broth are inoculated with the pathological material.

Serological investigation

The complement-fixing test forms the basis of the serodiagnostic method of detecting glanders. Identical results can be obtained by the conglutination test. In some cases it is recommended to use theagglutination test.

The preliminary and main complement-fixing test is carried out in accordance with the usual method (see page 154).

Components required for the test:

1) test serum. A serum which is known to be positive and normal serum, inactivated for 30 minutes at 58° on theday of the test, is necessary as a control;

2) glanders antigen, an extract prepared by shaking a suspension of dead glanders microbes;

3) complement serum from a guinea pig;

4) hemolysin;

5) a 2.5% suspension (from the precipitate) of sheep erythrocytes in physiological salt solution. The glanders antigen, hemolysin and glanders control serum are prepared in industrial biological laboratories.

Titration of glanders antigen. The working titer of the antigen is fairly constant (usually 1:100 or 1:200). A check is carried out on the titer indicated by the biological laboratory, when a new batch of antigen is received and then 1-2 times a year. A difference of at

most 25% between the result and the tiber indicated by the suppliers is permissible.

The antigen is tested on 2 glanders sera with different activity, one of which gives complete inhibition (++++) and two - normal. The titration is carried out by the so-called square method, in which the zonal activity of the antigen is studied in presence of active, weakly active and strongly diluted glanders sera.

The antigen titer is determined on the basis of the dilution which gives the maximum hemolysis inhibition with strongly diluted and weak glanders sera. Naturally, this antigen dose in itself must not have an inhibiting effect and an inhibition of hemolysis in presence of any normal serum with this antigen dose is not permissible.

Main test. The test serum is examined in a dose of 0.05 cm^3 and a control on a dose of 0.1 cm^3 is carried out at the same time in another test tube without antigen. The inactivation period is 30 minutes at a temperature of 58°.

Glanders antigen is used for establishing the titer.

The complement is taken in a dose determined by titration in a bacteriolytic system (see above).

The complement fixing is carried out on the water bath at a temperature of 37° for 20 minutes.

Hemolysin with working titer (i.e., double the nominal titer).

Erythrocytes 2.5% (from the precipitate from a suspension of sheep erythrocytes in physiological salt solution).

Duration of the test on the water bath 20 minutes.

The results of the main test are evaluated 2 times: 20 minutes after the test tubes with the main test have been placed on the water bath and a second time after the stand with the test tubes has been left overnight at room temperature.

The results of the main test are evaluated in the following terms: positive, doubtful (indicated by plus signs) and negative result. A positive result is inhibition of hemolysis by ++++, +++ and ++± and doubtful by ++ and +.

Conglutination test. The conglutination test has several technical advantages over the complement-fixing test, the possibility of carrying out the test without guinea pig complement and without the hemolytic system.

The guinea pig complement in the conglutination test is replaced by horse serum; instead of rabbit hemolysin, bovine serum is used. These two components can be obtained on any animal farm or abattoir and are easily preserved without losing their activity for long periods of time.

By conglutination is meant the agglutination of sensitized erythrocytes by inactivated serum (bovine conglutinin) in presence of a complement (horse serum).

In principle, conglutination is a modification of the complement-fixing test in which the indicator is not the hemolysis of the erythrocytes but their agglutination. When glanders antigen and inactivated glanders serum are added to the conglutination system (inactivated bovine serum, fresh horse serum and erythrocytes), the conglutination phenomenon does not take place, but if the horse serum to be tested does not contain the specific glanders antibody, the combination of the antigen with the antibody does not take place, and, as a result, the complement which is not fixed by this complex (antigen-antibody), is effective in the conglutination system and conglutination occurs.

In the absence of conglutination, which indicates the presence of glanders antibodies in the test serum, the precipitation of erythrocytes is observed in the test tube; this precipitate is loose and easi-

ly disintegrated and when the test tube is shaken, it is transformed into a homogeneous suspension of erythrocytes.

In presence of the conglutination phenomenon, which indicates the absence of glanders antibody in the test serum, a precipitate of erythrocytes is formed relatively quickly on the bottom of the test tube in the form of a filmy mass which is easily disintegrated by shaking into separate compact clumps of aggultinated erythrocytes. These clumps of erythrocytes cannot be broken even by energetic shaking and settle again quickly to the bottom of the test tube.

The total volume of the reaction components in the test tube is 2 cm^3. The components react in the following volumetric proportions: 0.3 cm^3 of the test serum in a dilution of 1:6; 0.3 cm of antigen in the working dose, 0.3 cm^3 of complement with the corresponding titer, 1 cm^3 of conglutinin of the corresponding titer and 0.1 cm^3 of a 3% suspension of sheep erythrocytes in physiological salt solution.

The titer of the conglutinin is determined in a conglutinating system with a constant dose of 0.1 cm^3 of complement while the working dose of conglutinin is 5 times larger.

The testing of the complement (horse) is carried out with a constant working dose of conglutinin in presence of glanders antigen. The next step in the investigation is the titration of the complement in a bacteriolytic system. The working dose of the complement is determined by the maximum quantity required for the conglutination of the erythrocytes in presence of a known normal serum with and without antigen with a correspondingly marked inhibition of conglutination (absence of any traces of conglutination) in presence of a known positive glanders serum; in itself, the positive serum in the test tube without antigen causes marked conglutination with a double dose of complement.

The titer of the glanders antigen in the conglutination test is

normal. The fixing time in the bacteriolytic system in the main test is 15 minutes at room temperature. The reaction of the main test lasts an hour on the water bath after which the test result is evaluated for the first time and the second evaluation is carried out on the next day (the test tubes are left in a cold place).

The absence of conglutination is considered as confirmation of a diagnosis of glanders (+); the conglutination of the erythrocytes is interpreted as a negative reaction (-).

The complement and the conglutinin are easily preserved with prolonged retention of activity by the method of Sobernheim (addition of 10% sodium acetate and 4% boric acid).

The most commonly used working titer of the conglutinin is 0.5-0.01 cm^3 and that of the complement 0.02 cm^3.

Agglutination test. This test is of the greatest importance for the identification of the isolated culture of A. mallei.

Normal agglutinins, whose titer does not exceed usually 1:400, are present in the blood of healthy humans and horses. Indices from 1:500 to 1:1000 are evaluated as a doubtful result. The blood of horses suffering from glanders agglutinates glanders microbes in serum dilutions of more than 1:1000 to 1:8000 and over.

TABLE 76a

Reaction Scheme of Agglutination

№ проб-1	Основное разведение сыворотки 1 : 100 2	Стандартная взвесь бактерий, мл 3	Конечное разведение сыворотки 4
1	0,5	2	1 : 500
2	0,25	2	1 : 900
3	0,2	2	1 : 1100
4	0,15	2	1 : 1400
5	0,1	2	1 : 2100
6	0,05	2	1 : 4100
7	0,025	2	1 : 8000

1) No. of test tube; 2) basic dilution of serum; 3) standard bacterial suspension, ml; 4) final dilution of the serum.

A suspension of killed glanders bacteria of a certain agglutinable strain is used as antigen. The culture is grown on 2% glycerol agar for 2-3 days, washed off with physiological salt solution containing 0.5% phenol and killed by keeping at 60° for 2 hours with subsequent control of the sterility. Prior to use, the antigen is brought up to the visual standard corresponding to 500 million microbial cells in 1 ml. The test serum is not inactivated. When diluting the serum it must be remembered that 2 ml antigen must also be added to each test tube (Table 76a).

The test tubes with the liquid are shaken thoroughly and then placed into the thermostat at 37° for 24 hours, then kept for 12 to 24 hours at room temperature, after which the reaction is evaluated.

When the agglutination test is carried out for the purpose of identifying a glanders culture, a suspension of killed microbes is prepared from the latter in accordance with the above-described method, which is then tested by means of agglutinating rabbit serum with a high titer (2000 and over). The agglutinating glanders serum is prepared by immunizing a rabbit with increasing doses of killed glanders culture.

Detection of glanders bacteria by means of the biological test

Guinea pigs and cats are susceptible to A. mallei.

Guinea pigs. The material taken under sterile conditions from closed abscesses is injected into the abdominal cavity of a male guinea pig. Within 2-4 days, often even later, edema of the scrotum, symptoms of orchitis, pyoderma and skin ulcers develop in the animal (scrotal phenomenon). The guinea pigs usually die within 15 days, sometimes later. Some animals survive.

In the presence of material contaminated by extraneous microflora (from open abscesses, ulcers, nasal discharges) it is preferable to infect the guinea pigs subcutaneously in the abdominal region. The dura-

tion and issue of the disease are the same as with intraperitoneal infection. The guinea pig is usually killed at the climax of the disease in order to carry out inoculations (see above). Allowance must be made for the fact that the scrotal phenomenon is not sufficiently specific because orchitis may also develop through inoculation with B. pyocyaneum, B. pseudotuberculosis, etc.

The cats in which the pattern of glanders septicemia is observed after infection, usually die on the 8-15th day, rarely later. The material (pus, etc.) is introduced under the skin of the nect in the occipital region by means of a swab into a skin pocket previously made by incision. To prevent being scratched by the cat, this operation is usually carried out in a specially adapted box. The infected cats can spread microbes by sneezing and spitting. As a precaution, the cage in which the cat is kept, is covered with gauze which is periodically moistened with a solution of corrosive sublimate. The cat is killed on the 5-8th day after infection. For safety reasons, the cadaver is ...t copiously wetted with carbolic acid solution. In view of the ...ecial hazard of working with cats, infection of guinea pigs is pre...rred because they can be manipulated more easily and with less dan...er.

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[Footnotes]

1000 Lepromin is prepared by biopsy of lepromas of leprosy patients.

SYPHILIS

Prof. N.M. Ovchinnikov

Morphology and Biology of Tr. pallidum

The pathogenic agent of syphilis, treponema pallidum (pale treponema) is a microorganism with corkscrew shape, an average length of 6 to 14 μ and a diameter of 0.25 to 0.35 μ. The number of turns of the helix varies between 8 and 12. The helices are uniform, with rounded tips, the distances between them are uniform, the height of the windings decreases towards the ends. The pale treponema is devoid of flagella. Treponema grows well on a number of liquid media, some cases of growth on solid media are also reported (Fig. 125, which differ morphological-

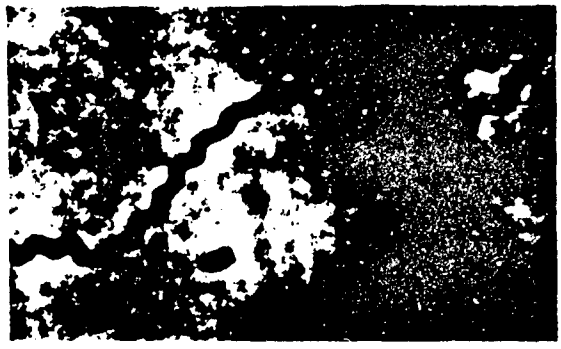

Fig. 125. Treponema pallidum from a culture.

ly only slightly from treponema present in tissues.

Antigen Structure

The antigen composition of treponemas (in cultures and tissues) is so closely similar that the antigens, prepared from cultured treponemas are being increasingly used recently for the complement-fixation test for syphilis. The pale treponema does not produce a toxin. There is not

a single species of animal which contracts syphilis under normal conditions. Infection of monkeys and rabbits is possible under artificial c conditions, and chancres develop in rabbits and secondary symptoms (usually papules) containing treponemas are found.

Resistance to Physical and Chemical Factors

The resistance of the pale treponema to ambient influences is relatively low. Keeping it at a temperature of 55° for 15 minutes kills it. The pale treponema is very sensitive to acids. In 0.3-0.5% hydrochloric acid it loses its mobility instantly. In 50-56° alcohol treponema loses its motility immediately. Penicillin in a concentration of 5 to 5000 YeD per 1 ml does not stop the movement of treponemas for 4 hours and 20 minutes and their viability is not effected. Penicillin in a concentration of 40,000 YeD in 1 ml quickly stops the movement of treponemas. Synthomycin, levomycetin, biomycin and streptomycin do not have a marked effect on the pale treponema.

LABORATORY DIAGNOSIS OF SYPHILIS

Laboratory diagnosis is used for detecting syphilis in humans and also for the control of the effectiveness of syphilis therapy.

Different methods of diagnosis are used depending on the stage of syphilis. In cases of a hard chancre, the content of the ulcer is analyzed for the presence of treponema. When ulcers are absent, in secondary and tertiary syphilis, serological examination of the blood serum and spinal fluid is carried out.

Culturing has not been used to establish a diagnosis of syphilis, because a method which gives a culture of treponema every time has not yet been developed.

Test Materials; Sampling

All erosions and ulcers on the urogenital organs are examined for the presence of the pale treponema. Testing of soft chancres for the

presence of streptobacillus is carried out simultaneously with the examination for pale treponema. In view of the different incubation periods of syphilis and soft chancre, the soft chancre bacillus is found first in cases of mixed chancres and the pale treponema later. When a single negative result is obtained, the examination is repeated several times. When the results of the examination of discharges from ulcers on the external sex organs of female patients are negative it is necessary to examine the cervix and, in cases of suspected erosions, the discharges from the latter. If treponema is not found in a patient who has been given local therapy, douches with physiological salt solution are prescribed and the examination repeated the next day.

The pale treponema can also be detected in syphilitic blotches and papules. The detection of treponema is more difficult during the stage when these formations have been reabsorbed.

The results of the examination depend to a considerable degree on the care taken in sampling. It is important to remember that the tonsils must also be examined for presence of pale treponema. When material is taken from the lips, the contamination of the test material with saliva and with this, of spirochaetas present in the teeth, must be avoided.

The surface of erosions or ulcers is freed of pus with cotton wool and moistened with physiological salt solution, taking care not to chafe the surface of the ulcer to avoid hemorrhage. Then, using a metal spatula which has been heated and allowed to cool, the surface of the ulcer is slowly pressed out; the discharge does not appear immediately. The dense part of the chancre must be pressed out, trying to obtain the fluid from deeper layers because the pale treponema is found there in the pure form and saprophytic treponema is hardly ever present.

If the tissue fluid is difficult to extract, the edges of the ul-

cer can be compressed with forceps or with the fingers. By carrying out this manipulation several times it is possible to obtain a sufficient quantity of fluid for examination.

A small drop of the tissue fluid is applied to a thin, clean object glass and a cover glass put over it. When there is too little fluid, it can be added to a drop of physiological salt solution which has first been applied to the object glass. Large drops are to be avoided, because the treponemas swim around in it and are then difficult to detect.

If the erosion is situated in a difficultly accessible site and also when the ulcers are severely contaminated with extraneous microflora, puncture of the lymph nodes is carried out. The puncture is carried out with the patient in a supine position in the following manner. The hairs on the surface of the gland which is to be punctured, are shaven off and the skin is disinfected with alcohol and painted with iodine. A thin needle, not a thick one, with a blunt point which fits well into the syringe is used for the puncture. The lymph node is firmly held with two fingers of the left hand and the syringe held in the right hand with the needle fitted and the puncture carried out in stages, first in the skin and then in the gland. Having made sure that the needle is in the lymph node, it is moved parallel to the capsule in the direction of the opposite end and then slowly withdrawing the needle, the contents of the node are sucked out. Before withdrawing the needle from the node, the piston of the syringe must be held to prevent the contents from running out. 0.2 ml sterile physiological salt solution may be injected into the gland and by sucking it out and removing it from the syringe, a considerable quantity of test material may be obtained.

Microscopic Examination

The best method of detecting the pale treponema is examination with dark field illumination. The advantage of the method is the rapidity of the examination and the presence of mobile treponemas which are more easily detected because of their movement and more easily differentiated by the nature of the movements from other types of treponema.

Viewing in the dark field is carried out by means of special condensers (cardioid condenser, paraboloid condenser, lamellar condensers, etc.). In the absence of special condensers a satisfactory image can be obtained by attaching a small circular piece of black photographic paper to a normal condenser. For this purpose, the Abbe condenser is unscrewed and the circle of black photographic paper with a size approximately that of a 15-kopik coin placed accurately into the center of the upper surface of the lower lens. A free annular area with a width of 2 mm should remain on the periphery of the lens. The both halves of the condenser are joint, and the field of view is controlled. If the field is still too bright, a circle of larger size is used and if it is too dark, then a smaller circle is made. A fairly good dark field can be obtained by means of this adjustment.

The object glasses for dark field illumination should not be thicker than 1.-1.2 mm and should be free of scratches and grease. The cover glasses should also not be too thick. For viewing with dark field illumination, the microscope stage with the condenser is placed into a horizontal position. A drop of water is applied to the upper surface of the condenser and the object glass with the test material placed on top. Microscopy is usually carried out with an objective No. 40 and ocular No. 10. When an immersion system is used, the objective must be fitted with a special diaphragm and immersion oil put on the object glass.

In the dark field, the pale treponema is seen in the form of a

thin helix, which shines with uniform brightness. Typical for the pale treponema are the smooth movements; particularly typical is a pendulum-like motion. The treponema is sometimes bent at an angle. When materials, obtained from the urogenital pathways, are examined for pale treponema, the latter must not be confused with Borellia refringens, which is present in particularly large numbers, when the material has been taken from the surface of an ulcer. The spirochaeta of the type refringens is much thicker than the pale spirochaeta, the windings are coarser, wider, more irregular and the tips are acuminate. They move quickly across the field of vision, stop and then continue to move.

When material from the oral cavity is examined, it is necessary to differentiate from the spirochaetas which are often found in the oral cavity, particularly in presence of various teeth (Borellia buccalis, Borellia Vincenti, treponema macrodentium, Treponema microdentium) and the treponema which causes rabbit spirochaetosis (treponema cuniculi).

In tropical countries one finds species of treponema which cause yaws (treponema pertenue) and pinta disease (treponema carateum). These treponemas, like the treponema of rabbit syphilis, are so similar to the pale treponema that they cannot be distinguished from it by microscopic examination. These diseases are not found in our regions, however, and rabbit syphilis is not infectious for humans.

Great difficulties for diagnosis are caused by Tr. microdertium, but it is shorter and usually thicker than the pale treponema. When viewed in the dark field, the dental treponema show some bending motions and it appears brighter than the pale treponema.

Viewing in the dark field is the only method which can be recommended for practical purposes; examination with phase contrast or by means of an anaptral device does not have any practical advantage over the dark field. In preparations, stained by different methods, the num-

ber of treponema is less and their morphology is also altered in a considerable degree. If the positive results obtained with dark field examination of treponema are taken according to Elze as 100%, only 15% are detected by examining Romanovskiy-Giemsa-stained preparations and when they are treated with India ink, only 7%.

Nonetheless, recourse to staining of preparations must be had at times. For this purpose, staining with Romanovskiy-Giemsa dye is the most suitable method. The air-dried preparations are fixed methyl alcohol or a mixture of equal part of ethyl alcohol and ether for 10-15 minutes. The staining method is the same as with blood preparations. The straining time depends on the staining capacity of the dye. With this method, the pale treponema are stained a darker color with a violet or blue tinge.

Of the methods of silver-staining, the simplest method and one which gives satisfactory results is that of M.A. Morozov. With this treatment, the treponema appears brown or almost black.

Serological Tests

With all forms of syphilis, serological tests are used in the absence of clinical symptoms. A complex of seriological tests is used for the serodiagnosis of syphilis: the Wassermann test and two precipitation tests, that of Kahn and the cytochol test. In insufficiently equipped laboratories or under field conditions, the complement-fixation test may be replaced by a modification of it (Grigor'yev-Rapoport test, Weinstein-Reznikova test, Ginzburg-Kalinin activin test) but these simplifications are to some degree reflected in the quality of the test results. All the simplified modifications on glass (Maksimov, TsKVI, Mandula-Ferenc, Izrael'son test, etc.) give only tentative conclusions. When positive results are obtained with them, another serological examination based on the above-mentioned complex is necessary.

A quantity of 5-7 ml of blood is taken for the examination with a needle and dry syringe or by free flow from the ulnar vein of the patient on an empty stomach, with children from an incision in the heel or from the cranial vein. Blood should not be taken from patients with fever, after the consumption of alcoholic beverages or two weeks before or after birth.

The blood is transferred from the syringe to a clean, dry test tube. The test tube with tn· blood is left for 2 hours at room temperature, then the blood clot is carefully removed from the walls of the test tube and the test tube placed into the refrigerator. The supernatant serum is sucked off on the next day.

When it is not feasible to examine the serum on the spot, it may be dried. For this purpose, 1 ml of serum without any admixture of erythrocytes is collected with a syringe or pipette and put on wax papaer, cellophane, praffined glass or simply writing paper in the form of two circular spots of 0.5 ml each. To each portion of serum spread for drying, 3 drops of 40% sucrose solution is added. The sugar solution is added to prevent denaturation of the proteins. The serum is then dried at room temperature in a dry place protected against flies; the drying is accelerated by ventilation. The dried serum is not suitable for use longer than 5 days from the moment of preparation in the southern regions and 10 days in the other parts of the USSR.

It is not recommended to use dried serum for the testing of blood donors. Dried serum is suitable for carrying out the Wassermann and the precipitation test and is not suitable for the determination of active modifications. In places where facilities for examining the native serum exist, the dried serum need not be used.

At the laboratory, the dried serum is removed from the cellophane, wax paper or glass and placed into a test tube, and when it has been

dried on writing paper, the circules are cut out together with the paper and physiological salt solution is added to it in the same volume as that of the serum originally used and dried.

Wassermann Test (Complement-Fixation Test)

Test serum, antigen, complement, hemolytic serum and sheep erythrocytes are needed to carry out this test.

The complement-fixation test has been described in principle on page 154. Here we shall mention certain peculiar features which are specific for the Wassermann test.

Serum. Before the Wassermann and precipitation test can be carried out, the test serum is inactivated by keeping at 56° for 30 minutes.

Antigens. Three antigens are used, one of which should be a cardiolipin antigen. The antigens are prepared from the organs of healthy animals. Antigens from cultures of treponema and their fractions and antigens from pale treponema, obtained from orchitis of syphilitic rabbits, are currently also used. The antigens from treponema are highly sensitive and specific. The most valuable of the culture antigens are the "sonicated" antigen and the treponema protein fraction, which differ from the cardiolipin antigen by given fewer postive reactions with patients who have received proper treatment and which detects the antibodes earlier. The antigen from tissue treponema is also valuable. When these three antigens are used (particularly the cardiolipin, protein fraction and the sonicated antigen), the maximum number of syphilis cases can be detected and the treating physician can be given a better idea of the immunological state of the patient.

The nonspecific antigens should be kept at room temperature in a glass vessel, sealed tightly with a cork stopper. The treponema antigens are kept in a refrigerator. For the test, the antigens are required in a dilution corresponding to their titer which is indicated on the

label. The titer gives the quantity of pure antigen in milliliters of the physiological salt solution. A 0.85% sodium chloride solution in distilled water which has been recently boiled to remove the carbon dioxide, is required for the dilution of the antigens and other ingredients for the Wassermann test. The method of dilution, slow or rapid, is also given on the label.

Complement (see page 163).

Main test. The test is carried out with a volume of 2.5 ml but it may also be carried out in a volume of 1.25 ml.

The test result is evaluated on the basis of the hemolysis taking place in the test tubes.

Every test must be accompanied by a control test carried out with previously known negative and positive sera.

The main Wassermann test is shown schematically (in half doses) in Table 77.

Complete hemolysis takes place in the test tubes when the result is negative, with positive results there is a varying degree of hemolysis inhibition. The degree of hemolysis is designated by plus signs: ++++ (complete inhibition of hemolysis, supernatant fluid colorless); +++ (strong inhibition of hemolysis); ++ (partial inhibition of hemolysis); + (traces of hemolysis); ± (doubtful result); − (complete hemolysis, negative result).

In the cases in which the hemolysis inhibition may primarily be due to faulty technique and also in cases where there is a marked difference between the test results obtained with different antigens, the examination should be repeated with the same portion of serum and, if necessary, with a new portion.

Quantitative Wassermann test method. For the purpose of comparing different methods of syphilis therapy, for the control of the results

of the therapy, in cases when positive results are obtained on patients which had not syphilis, etc., the quantity of reagins or antibodies in the syphilitic sera is determined, for which purpose the so-called quantitative method is used. Several methods have been proposed. The simplest and most convenient is the method of Boas using decreasing doses of test serum. The quantitative method is carried out only with positive sera giving +++, ++ at the usual serum dilution. For this purpose, the serum is diluted with physiological salt solution from 1 : 5 to 1 : 320 (1 : 5, 1 :10, 1 : 20, 1 :40, etc.) and the Wassermann test carried out with each serum dilution with one antigen in a volume of 1.25 ml. The result indicates the minimum serum dilution at which complete hemolysis inhibition can be obtained.

The dynamic quantitative method is of great assistance to the treating physician. A lowering of the titer during treatment attests to the efficacy of the therapy used in the particular case. A certain titer is typical for every stage of syphilis. For example, in secondary syphilis, the highest titers are found (1:160-1:320); titers of 1 : 80 are most frequently found in cases of latent syphilis, etc.

A much greater number of positive results and higher titers are obtained when the complement fixing test is carried out in the cold. By means of this method it is also possible to obtain earlier results in cases of primary syphilis. The difference between the usual method and the complement-fixation in the cold is only that after addition of the antigen and complement to the test serum (first reaction phase), the test tubes are left for 12-20 minutes at room temperature and then placed into a refrigerator at a temperature of 4-8° for 18-20 hours. The next day the test tubes are transferred to the thermostat (37°) for 15 minutes, then the hemolytic system is added and the test tubes again placed into the thermostat for 20-30 minutes (until hemolysis in the

control test is complete). The results are evaluated by the usual method.

TABLE 77

Main Wassermann Test (with a volume of 2.5 ml)

Ингредиенты, мл 1	№ пробирок 2			Контроль 3
	1	2	3	
4 Инактивированная испытуемая сыворотка	0,1	0,1	0,1	0,1
0,85% физиологический раствор	0,4	0,4	0,4	0,9
5 Антиген серии № 1, разведенный по титру	0,5	—	—	—
6 Антиген серии № 2, разведенный по титру	—	0,5	—	—
7 Антиген серии № 3, разведенный по титру	—	—	0,5	—
8 Комплемент, разведенный согласно рабочей дозе	0,5	0,5	0,5	0,5
9 В термостат на 45 минут				
10 Гемолитическая система (сенсибилизированная)	1,0	1,0	1,0	1,0
11 В термостат на 40—60 минут в зависимости от наступления гемолиза в контролях				
12 Регистрация опыта после наступления гемолиза во всех контролях				
13 Результат:				
14 1. С сывороткой больного сифилисом	—Г	—Г	—Г	+Г
15 2. С нормальной сывороткой	+Г	+Г	+Г	+Г

Remark: —G complete inhibition of hemolysis; +G complete hemolysis.

1) Ingredients, ml; 2) no. of test tube; 3) control; 4) inactivated test serum in 0.85% physiological salt solution; 5) antigen series No. 1, diluted to a titer of: 6) antigen series No. 2, diluted to a titer of; 7) antigen series No. 3, diluted to a titer of; 8) complement, diluted in accordance with the working dose; 9) in the thermostat for 45 minutes; 10) hemolytic system (sensitized); 11) in the thermostat for 40-60 minutes depending on the beginning of hemolysis in the control tests; 12) recording of the test results after hemolysis has begun in all control tests; 13) result; 14) with serum from a syphilitic patient; 15) with normal serum.

Active Methods of Serodiagnosis

In cases when it is impossible to carry out the Wassermann test, it may be replaced by its active modification with two simultaneous precipitation tests. The grigor'yev – Rapoport test (Table 78) or the

Weinstein-Reznikova test (Table 79) are used. The former is based on the use of the complement of the test serum and the second, on that of complement and hemolysin. Unheated serum is required for either test.

The recording of the test results is carried out immediately after hemolysis has begun in the control test tubes. If hemolysis inhibition is observed in the control serum, the examination is repeated with addition of 0.2 negative active serum.

TABLE 78

Reaction Scheme of the Grigor'yev and Rapoport Test

Ингредиент, мл 1	2 № пробирки		
	1	2	3
3 Активная сыворотка	0,2	0,2	0,2
4 Физиологический раствор	0,5	0,5	0,8
5 Антиген первой серии	0,3	—	—
6 Антиген второй серии	—	0,3	—
Встряхивание 7 25 минут при комнатной температуре			
8 Гемолитическая система	1,0	1,0	1,0
7 Встряхивание 25 минут при комнатной температуре Учет результатов 9			

1) Ingredient, ml; 2) no. of test tube; 3) active serum; 4) physiological salt solution; 5) antigen of the first series; 6) antigen of the second series; 7) shaking for 25 minutes at room temperature; 8) hemolytic system; 9) evaluation of results.

TABLE 79

Reaction Scheme of the Weinstein-Reznikova Test

№ пробирки 1	Активная сыворотка, мл 2	Разведенный антиген 3	Физиологический раствор (мл) 4		1% дефибринированная баранья кровь (мл) 5	
1 (основная)	0,2	$0,3_1$	—	Встряхивать 3 минуты 8	0,75	45—60 минут при температуре 24—28° 9
2 (основная) 6	0,2	$0,3_2$	—		0,75	
3 (контрольная) 7	0,2	—	0,3		0,75	

1) No. of test tube; 2) active serum, ml; 3) diluted antigen; 4) physiological salt solution (ml); 5) 1% defibrinated sheep's blood (ml); 6) main; 7) control; 8) shaking 3 minutes; 9) 45-60 minutes at a temperature of 24-28°.

PRECIPITATION TESTS

In addition to the Wassermann test, the Kahn test and the cytochol test form part of the recommended test complex for the seriodiagnosis of syphilis. Inactivated test serum and Kahn antigen are required for the Kahn test. The antigen is diluted in accordance with the titer indicated on the label. To dilute the antigen, the required quantity of physiological salt solution is measured into a test tube and a certain quantity of antigen into another. The physiological salt solution is quickly added to the antigen and the mixture transferred 6 times from one test tube to another and then left at room temperature for 10 minutes for maturation. To carry out the test, 0.025 ml of the antigen suspension is first placed into a test tube with a micropipette which reaches down to the bottom of the test tube. 0.15 ml of the test serum is then added. The stand is shaken energetically for 3 minutes, 0.5 ml physiological salt solution added and again shaken. A single control test is carried out: instead of serum, the corresponding quantity of physiological salt solution is added to the antigen suspension and after shaking for 3 minutes, another 0.5 ml of physiological salt solution is added and again shaken. The results are evaluated by means of an agglutinoscope or magnifying glass after addition of physiological salt solution and shaking. A flocculent precipitate is seen in positive sera, while the flakes are absent in negative sera.

Cytochol test. Inactivated serum and Sachs - Vitebskiy antigen are required for this test.

For the dilution, one part of antigen is quickly run into 2-3 parts physiological sodium chloride solution (depending on the titer) and left 10 minutes at room temperature to mature.

To carry out the test, 0.05 ml of diluted antigen and 0.1 ml serum are mixed, shaken for 3 minutes, left at room temperature for 30 minutes,

0.5 ml salt solution added and again shaken. A single control test is again carried out by adding to a test tube physiological salt solution instead of the serum and the other ingredients as in the main test. The results are evaluated by means of a magnifying glass or an agglutinoscope after addition of physiological salt solution and shaking. Flakes are precipitated in positive sera, while the precipitation of flakes is absent in negative sera.

All the above-listed tests are recommended in accordance with the instructions for the test complex for syphilis diagnosis and are used in most laboratories of the USSR. In addition, microprecipitation tests on glass have been proposed which are of relative importance only. They are very simple and differ only with regard to the antigen requirement (for the Schirwindt and Maksimov tests, Sachs-Vitebskiy cytochol antigen is needed, for the Izrael'son test, a special alcoholic extract from bovine gray matter and for the TsKVI test, the universal antigen TsKVI).

The principle of all these tests consists in the addition of an antigen suspension to a certain quantity of test serum; after mixing of the two ingredients, the results can be evaluated a few minutes later. When the test is positive, flakes are precipitated, when it is negative, the flakes are absent.

All these tests are only tentative. When positive results are obtained with any of them, the examination must be repeated in accordance with the usual complex.

In addition to the serological examination of the blood serum in syphilis, particularly in late forms of syphilis, the spinal fluid is tested in cases of syphilis of the nervous system and for determining whether a cure has been achieved. The Wassermann test is carried out with three doses of spinal fluid: undiluted and diluted with physiolog-

ical salt solution in a ratio of 1 : 1 and 1 : 5. In view of the fact that the spinal fluid does not have any anticomplement properties, the complement is used in titrated dose without addition. The examination is carried out with each dose of serum. The results are given separately for each dilution.

In addition to the Wassermann test, cytosis, and the protein concentration are determined in the spinal fluid and globulin tests (Nonne-Apelt, Weichbrodt and Pandi) and colloid tests (Takata-Ara, with colloidal gold - Lange) and some other tests, described in the manuals on clinical laboratory methods of examination, are carried out.

By using the Wassermann and the precipitation tests, the serologist cannot always distinguish the so-called pseudopositive reactions from the specific reactions. In latent syphilis, negative results are sometimes obtained and, conversely, positive results in persons which have been propertly treated for long periods for syphilis and who are now obviously free of syphilis. Difficulties also arise in cases of so-called unknown syphilis. New serological tests which are currently used, can be of use in a number of such cases: immobilization, agglutination, adhesion, fluorescence and complement-fixation with antigens derived from pathogenic treponema.

Immobilization test

Particularly widely investigated and recommended is the pale treponema immobilization test (Nelson-Meyer test). This test, as a rule, is highly specific. A positive immobilization test, however, is not very significant for persons which have received proper and prolonged treatment, because in spite of the cure, it remains positive for life. The principle of the test consists in the fact that after addition of the serum to pale treponema, obtained from early orchitis of rabbits, the movements of the spirochaete stop if the serum is from a syphilis

patient and that they continue to move when serum from a person not afflicted with syphilis is added. To carry out this test, early syphilitic orchitis of a rabbit is required containing a large number of treponema. The orchitis of a rabbit, infected with Nichols strain 7-8 days previously is normally used. In order to prevent accumulation of immune antibodies, the rabbit is first irradiated with x-rays and cortisone is given daily for the entire period before the test.

The rabbit is exsanguinated, the testes are removed, freed of the epididymis, cut into 10-15 pieces under sterile conditions and placed into a preservative solution.

Nelson medium:

0.4% gelatin solution, 20 ml

Phosphate buffer* 6.2 ml

1.2 ml of 1.5% sodium thioglycollate in distilled water

2.52 ml of 1.23% glutathione in physiological salt solution

1.96 ml of 0.63% cysteine hydrochloride in distilled water

0.62 ml of 1% sodium pyruvate

2.26 ml of 1.26% sodium bicarbonate

6.76 ml of 0.85% sodium chloride solution, and

2.5 ml of ultrafiltrate of bovine serum.

Simpler media can also be used: the fluid from the anterior chamber of the eye of an ox, sheep or rabbit serium, diluted 1 : 1 or 1 : 2, 0.2% solution of food-grade gelatin with addition of a solution of 5% dry human albumin and rabbit serum.

The pieces of testes are transferred to a 250 ml Erlenmeyer flask into which 10 ml of the medium had previously been placed and the flask filled with a mixture of nitrogen (95%) and carbon dioxide (5%). These gases should be free of impurities which are harmful to spirochaetas and contain a minimum concentration of oxygen. The flask is then shaken

20-30 minutes on a shaking apparatus and the number of mobile treponemas determined; if at least 10 spirochaetas are present, the medium is centrifuged for 10 minutes at 1000 rpm to remove coarse particles. Test method: 0.05 ml inactivated serum, 0.1-0.2 ml undiluted complement and 0.3 ml antigen (medium with treponema) are placed into the test tubes of the main test.

The same ingredients are placed into the test tubes of the control but inactivated complement is used. In addition, controls are carried out with serum known to be positive and negative.

The ingredients are added in a sterile manner. All test tubes are then placed in a microanaerostat, the air exhausted and the anaerostat filled with the above-mentioned gas mixture. The whole is placed into the thermostat at 35° for 18 hours; the number of mobile and immobile treponemas as compared with the control is counted. The test result is evaluated on the basis of the ratio of mobile and immobile treponemas in the main and control test tubes. If the number of immobile treponemas is less than 20%, the test is negative, if up to 30%, the result is doubtful, from 31 to 50% weakly positive and only when the number of immobile treponemas exceeds 50%, is the test result considered to be positive. The technique of this test is very simple but the large amount of preliminary work involved and a number of conditions required in carrying it out and also the complex nutrient medium which is required, impede the wider use of this valuable test.

Simple method of carrying out the immobilization test with pale treponema, which can be done without the use of a gas mixture or complex apparatus and which makes it possible to carry out the test in all large serological laboratories (N.M. Ovchinnikov). The test is carried out in blood count pipettes. Blood serum, kept at 56° for 30 minutes, is collected up to mark I in the blood count pipette, the treponema sus-

pension with active complement to mark II and the same with inactive complement in the other pipette; these mixtures are prevented from flowing out by a rubber ring as in the transport of blood. All the necessary control tests are also carried out. The whole is placed on a special stand and into the thermostat for 18 hours. The test is evaluated in the usual manner. The medium used is a 0.2% solution of food grade gelatin to which rabbit serum and a solution of dried human serum albumin has been added. A comparative investigation of this method with the classical method showed a high percentage of agreement in the results.

Other serological tests

Agglutination test. A suspension of pale treponema, obtained from an early orchitis of a rabbit infected with syphilis, usually of the Nichols strain, is used as antigen for the agglutination test.

To prepare the antigen, the treponema are washed several times in physiological salt solution or sodium citrate and then killed by heating. The test technique is the same as with other infections. The test result is evaluated by examination under the microscope in the dark field. It is generally accepted that two kinds of agglutinins exist: nonspecific and specific. In order to eliminate the former, Hardy and Nell first exhausted the test serum with the antigen, used for the DRL test, after which only the specific agglutinins remained. The test has no independent importance and is used only as an accessory to the other serological tests.

Adhesion or disappearance test. The principle of the test is that when one adds to the blood serum from a syphilis patient rabbit or better human erythrocytes and also a suspension of treponema from an early orchitis of a rabbit (dead ones may be used) and examines the serum in the dark field after a certain time in the thermostat, it is found that

the number of spirochaete in the upper part of the serum is many times less than in the serum of a healthy person, having almost completely disappeared.

The explanation for this is that the treponema stick to the erythrocytes and are carried along by them into the precipitate. A difficulty with this test is that treponemas from animals with early orchitis which have previously been irradiated with x-rays, are required. An advantage is that the test is simple and can be carried out with dead treponema. The results of this test agree largely with those of the treponema immobilization test.

The fluorescence test with treponema antigens does not differ in its technique from those used with other infections; the indirect method of Kuhns and Kaplan is normally used: a small drop of a suspension of treponema of the Nichols strain from a 7 day old orchitis in physiological salt solution is applied to a thin object glass (the methods of infection and of producing early orchitis are the same as for obtaining treponema for the immobilization test) and a smear with a diameter of 1 cm is prepared from it by circular movements with the pipette. Two smears should be prepared for each test serum for greater reliability. The smears are dried in the thermostat, fixed in the flame of a burner and placed into acetone for 15 minutes. The smears are then dried in air, placed into a moist chamber and the test sera which have first been kept at 56° for 30 minutes, and diluted 1 : 10, are applied to them. 30 minutes later, the serum is washed off the smears, and the preparations washed carefully with physiological salt solution, placed into a fresh portion of physiological salt solution for 10 minutes and dried in the thermostat. The preparations are then again placed into the moist chamber and antihuman serum labeled with fluorescein isocyanate or fluorescein isothiocyanate diluted 10 times is applied to the

smears and left for 30 minutes. Then the preparation is washed for 10 minutes in physiological salt solution, dried and small drops of buffered glycerol applied to them, a thin cover glass put on and examined in the fluorescence microscope with immersion system, equipped with filters SS4 and SS8, using a special immersion oil.

In the preparations which were treated with serum from syphilis patients, treponemas which shine with a greenish-yellow light are visible. The degree of brightness of the treponemas depends on the quantity of antibodies in the serum of the patient and is designated with crosses (from + to ++++). In the preparations which had been treated with sera which do not contain antitreponema antibodies, the treponema do not fluoresce, being usually invisible or only their shadows visible.

This test is simple and not time-consuming and even exceeds the immobilization test for pale treponema in sensitivity.

In spite of several positive features, all the enumerated new serological reactions (even more of them have been proposed) cannot replace the normal complex of serological tests used at the present time in the daily work of the serological laboratories all over the world: the complement-fixation test with cardiolipin and treponema antigen and the Kahn and cytochol precipitation tests. These tests, as compared with all others, are simple to carry out when carried out correctly, and with correct interpretation of the results, can satisfy the needs of the clinic in the overwhelming number of cases. All other tests are only of auxiliary importance. Of these, the immobilization test and the immunofluorescence test deserve particular attention, but even they cannot replace the normal complex of serological tests and should be used only in presence of special indications.

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[Footnote]

1027 Phosphate buffer: 40 ml of 0.1M Na_2HPO_4 solution and 10 ml of 0.15M KH_2PO_4 solution.

SOFT CHANCRE

N.M. Ovchinnikov, Professor

The pathogenic agent of soft chancre is Haemophilus ducreye (previously termed Streptobacillus ulceris mollis).

Morphology of H. ducreyi

H. ducreyi is a short, gram-negative bacillus with a length of 0.5 to 2.5 μ, which tends to form chains and is immobile.

Biology of H. ducreyi; Culturing Properties

It grows under aerobic and relatively anaerobic conditions. It does not form a toxin. As all microorganisms of the genus Haemophilus, it requires the X-factor when growing on nutrient media, it develops on blood media.

Fine (0.5-1 mm), grayish-white, semispherical, isolated, shiny colonies similar to streptococcus colonies, grow on solid nutrient media within 24-48 hours. A craterlike depression is formed on them within 48 hours. In the condensation fluid it grows in the form of flakes or granules, sometimes settling on the bottom. Broth remains clear but some strains cause a slight cloudiness. The bacilli in the smears from colonies grown on solid media have the shape of short chains, those from liquid media, that of long chains.

The reinoculations are carried out after 2-3 days. The percentage of positive results varies depending on the material used for the inoculation and the nutrient medium within the limits of 6 to 79.

Resistance to physical and chemical factors

The soft chancre bacillus is resistant to low temperatures and is very sensitive to high temperatures. A temperature above 40° causes the death of the microbe. In the human organism, the soft chancre bacillus is characterized by a great resistance to disinfectant preparations, while it is highly sensitive to them in cultures. Thus, a 1% solution of carbolic acid kills the bacillus within a minute, a 0.5% solution of quinosol within 5 minutes, 0.25% formaline solution within 50 minutes. Dusting the ulcers with streptocid album or peroral administration quickly cures the patient. At the present time, there are no cases of soft chancre in the USSR.

Laboratory Diagnosis of Soft Chancre

The aim of the laboratory examination of human patients is solely the diagnosis of the disease. The diagnosis of soft chancre, as a rule, is established by means of a single bacteriological examination of the ulcer contents. No matter how much the ulcer resembles the soft chancre type it is always necessary to examine it also for pale treponema. When a mixed infection is suspected, both pathogenic agents must be looked for, taking into account that the pale treponema appears later. The detection of the soft chancre bacillus in mixed ulcers is not often successful. With female patients it must not be forgotten to examine the ulcers in the vagina and on the cervix.

Test material, sampling

The soft chancre bacillus can be detected in fresh as well as in old ulcers. To obtain material for the bacteriological examination, the surface of the ulcer is carefully cleaned with a swab, moistened with physiological salt solution. The material is taken by means of a platinum spatula (the same as for spirochaeta) from under the protruding edges of the ulcer, trying to obtain pieces of tissue, in which the soft

chancre bacillus has its most typical arrangement. In view of the great painfulness of this manipulation, the surface of the ulcer may be painted with 1% novocaine solution. When the results of the investigation are negative, the sampling of material is repeated 2-3 times. When the ulcer is contaminated by extraneous microflora, it is advisable to paint the surface of the ulcer after cleaning with a 10% iodine tincture in collodium and to leave it on for a day. 24 hours later, the film is removed and the test material taken from under the edges of the ulcer.

Morphological investigation

The material thus obtained is placed on an object glass and carefully spread on it, taking care not to disturb the characteristic arrangement of the bacilli. The smears are dried in air and fixed by passing over a flame.

The examination of the content of the bubos for the presence of the soft chancre bacillus rarely gives a positive result.

The smears are gram-stained. They can also be stained with Ziehl's fuchsin, diluted 1 : 10 with distilled water or with a solution of methylene blue. The best method which is useful for differential diagnosis, is gram-staining. In pus, the soft chancre bacillus is usually found singly. The typical arrangement in the form of long, parallel chains can be observed in the smears from ablated tissue (Fig. 126). The bacilli have the shape of a figure eight of weaver's shuttle. When the bacterioscopic examination of smears for the soft chancre bacillus is carried out, its polymorphism must be kept in mind. Of similar microorganism, the enterococcus should be mentioned, which is often found in the urether and has a chainlike arrangement, but is gram-positive. The saprophytic bacillus described by Pfeiffer, which is arranged in chains, but is also gram-positive, is also of importance.

Fig. 126. H.ducreyi in a smear from pus.

Bacteriological examination

The bacteriological examination of soft chancre is very rarely used for diagnositc purposes because the percentage of positive results is insufficient. The soft chancre bacillus is hemophilic, hence it is grown on media to which 20-30% blood has been added. To prevent drying out, the test tubes are sealed with a rubber cap. Martin broth, also with addition of defibrinated blood, can be used. Pieces of tissue, pus from ulcers, puncture specimens from bubos or material from pustules after inoculation are used for seeding.

The agglutination test is used to identify the cultures thus obtained. The serum is obtained by immunization of a rabbit with a suspension of soft chancre bacillus from a culture.

Detection of the soft chancre bacillus by means of the biological test

When the results of the bacteriological investigation are negative, autoinoculation is sometimes carried out. For this purpose, the skin on the abdomen or the extremities is carefully cleaned with alcohol, and ether, then with sterile distilled water. Then a few incisions are made distant from each other not less than 1 cm so that later the lesions cannot merge and avoiding hemorrhage as in smallpox vaccination. Pus from an ulcer of the patient is rubbed into these incisions with a

spatula. Physiological salt solution is applied to a control incision. The entire surface is covered with a watch glass which is stuck on with a strip of adhesive plaster. In positive cases a small pustule is formed within 2-3 days in whose contents the soft chancre bacillus can be detected. When it is no longer needed, the ulcer is cauterized with carbolic acid and dusted with streptocid. Vaccination with material obtained from another patient is not permitted. Autoinoculation should be used only in the exceptional cases when it is not possible to diagnose the soft chancre by other methods.

Allergy test

Some researchers recommend the use of the intradermal test with soft chancre vaccine for the purpose of diagnosis. The soft chancre patients display a heightened reactivity to the injection of the vaccine, which appears during the second week of the illness and remains for several years. The vaccine is injected intradermally in a quantity of 0.1 ml. The reaction takes place within 3-6 hours but becomes most marked within 48 hours. A papula and an erythema and infiltrate around it with different size and intensity appear. The reaction then begins to decrease in intensity and finally disappears within 10-15 days. The reaction is particularly intense in persons which have previously had soft chancre, complicated by bubos.

Nutrient Media

Medium for preserving the viability of a culture for a month at room temperature: 0.25% agar, 1% starch, 20% defibrinated rabbit blood.

GONOCOCCUS INFECTIONS

N.M. Ovchinnikov, Professor

Morphology of Gonococcus

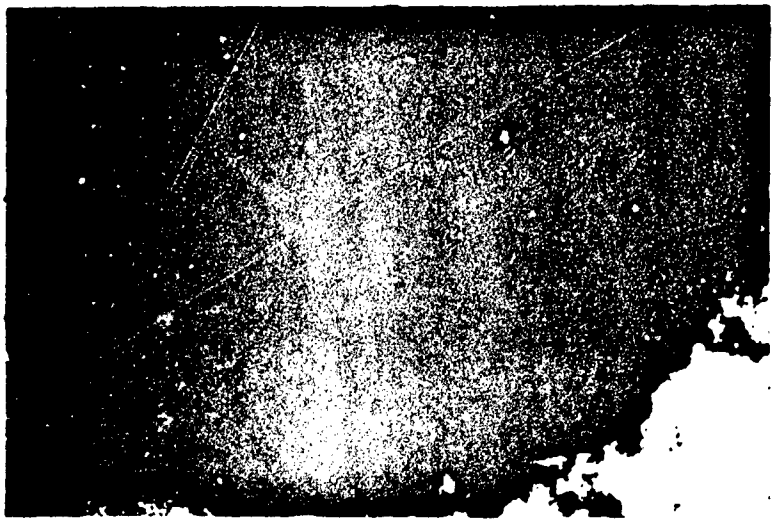

Fig. 128. Ultrathin section of gonococcus. Electron microscope.

Fig. 129. Gonococcus in discharge from patient with acute gonorrheal urethritis. [illegible].

Fig. 130. Gonococcus colony on ascites agar. Day-old culture.

The pathogenic agent of gonorrhea, gonococcus (Neisseria gonorrhoeae) is a diplococcus which is usually compared with coffee beans or kidneys, combined in pairs with the concave sides turned inwards; the gonococcus is pinched in in the middle forming two halves. Its size varies, depending on age, nutrition conditions and the treatment of the test material. The average length is 1.25 μ, the width 0.7-0.8 μ. Around the gonococcus in the test material, capsule-like slimy substances can be seen (Fig. 127, 128).

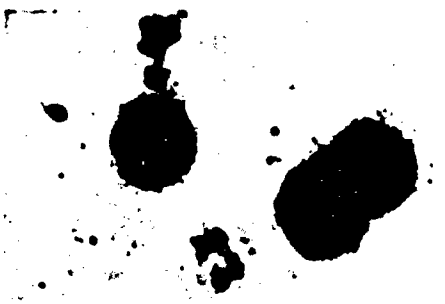

Fig. 127. Gonococcus. Electron microscope. 6000×.

The double-contour is clearly visible in the electron micrographs of ultrathin sections of gonococcus (Fig. 127). The most characteristic properties of gonococcus are its morphology, the gram-negative staining and the fact that it is found inside the leucocytes in the pus from patients (Fig. 129).

Biology of gonococcus; culturing properties

Gonococcus grows on ascitic agar usually within 24, more rarely 48 hours in the form of transparent colonies resembling dewdrops, with even edges and a smooth, shiny surface. The gonococcus colonies are similar to those of meningococcus but the latter are less transparent (Fig. 130). The size of the colonies depends to a large extent on the composition of the nutrient medium and its reaction. Daughter colonies may be formed during prolonged culturing.

Of the carbohydrates, media containing dextrose, levulose and maltose are used for differentiation. These media may be liquid or solid with addition of 30% ascitic fluid.

The catarrhal micrococcus does not break down any of the above listed sugars, gonococcus only breaks down glucose and meningococcus, dextrose and maltose. The biochemical charactersitics of gonococcus are not strictly constant and sometimes gonococcus does not ferment any of these sugars. Of the otner diplococci, from which the gonococcus must be differentiated, we must mention Neisseria flava, which forms coarse dry colonies on simple agar and diplococcus crassus, which grows slowly at room temperature in the form of grayish-white colonies and can break down saccharose, maltose, lactose, and levulose.

Gonococcus does not grow on milk, gelatin, or potato medium. It does not form ammonia, hydrogen sulfide or indole and does not alter blood media.

The agglutination test is also employed for the identification of the cultures. Antigonococcus sera from rabbits are normally used. It is preferable to use serum from rabbits which have been immunized with a large number of different strains. When immunization is carried out with a small number of gonococcus strains, the test often gives negative results because of the existence of a large number of gonococcus types

and the absence of the corresponding agglutinins in the serum. Low serum dilutions are used (1 : 10, 1: 50, rarely greater). A day old culture, grown on ascitic agar and diluted according to the billion microbe standard is added in a quantity of 1 drop per 1 ml. Old laboratory strains are not suitable, because they often given spontaneous agglutination. The test tubes are placed into the thermostat at 37° for 2 hours, then they are taken out and left overnight at room temperature.

The agglutination test does not always give reliable results and group agglutination may occur.

Conococcus does not form a true toxin, its toxicity being connected with the body of the gonococcus (endotoxin).

Resistance to physical and chemical factors

Gonococcus has little resistance outside the human organism, being killed by drying out of the material. In the human organism it is very resistant. A temperature of 39-40° reduces its viability and a higher temperature kills it. Silver nitrate in a silution of 1 : 10,000 kills gonococcus within 1 to 10 minutes, potassium permanganate in a dilution of 1 : 50 in one minute, penicillin in a concentration over 0.5 YeD in 1 ml is bactericidal for gonococcus, levomycetin 1-2 γ/ml, synthomycin 2 γ/ml, biomycin above 1 γ/ml, tetracycline 1-2 γ/ml, streptomycin above 4-5 YeD in 1 ml. Sulfanilamide preparations in vitro have only a weak bacteriocidal effect on gonococcus.

Laboratory Diagnosis of Gonorrhea

The basic method of laboratory diagnosis of gonorrhea is microscopic examination and once a cure has been effected, particularly by treatment with sulfanilamide preparations or antibiotics, also bacteriological investigation.

Test materials; sampling

In an early stage of the disease in men, the discharge from the

urether is examined for the presence of gonococcus and in females discharges from the urether, vagina, cervix and rectum, in children the discharges from the vagina, urether and rectum.

In chronic cases, biopsy specimens are taken with a blunt scoop from the mucosa. In men with chronic gonorrhea and also towards the end of treatment of acute gonorrhea (when there is little discharge), the fibers and flakes in the urine are examined.

When different glands, joints, etc., are infected, material is taken from them. During the post-partun period, the cleansings are examined and in cases of eye disease, the purulent discharges from the conjunctiva.

The material for the investigation should be taken by the treating physician and the sampling of material should not be entrusted to medium-level personnel.

When material, taken by the usual methods (loop, scoop, swab) from the recta mucosa is tested, the number of positive results is less.

In cases of diseases of the prostate gland, the fluid from it is examined.

The material from joints and inflamed adnexes is obtained by puncture, carried out by the usual method. With virgins, the material is mainly taken from the vulva, vagina, urether and rectum by means of thin swabs or a small, blunt scoop.

The smears from the initial test material are prepared by spreading it on 1/3 of an object glass with the edge of a second object glass, scoop, dressing forceps, swab, etc. Smears are prepared on 2 glasses.

The irrigation fluids, urine, puncture fluids, etc., are immediately sent to the laboratory and examined. The percentage of positive results depends to a considerable degree on the time elapsed between [illegible] and the examination. The longer the time elapsed between

sampling and examination, the lower is the percentage of positive results.

The discharge of the vagina is taken with a swab loop or scoop introduced into the vagina. The examination of vaginal secretion for gonococcus rarely gives a positive result.

Material from the cervix is taken after introduction of a Kusko speculum into the vagina. The cervix is wiped dry and the external mucus and pus plug (the vaginal end) is removed as carefully as possible and with a dressing forceps or vaginal forceps the viscous slime is taken from the cervical aperture. The purulent lumps are taken from the mucous material for the examination, spreading them on an object glass in a thin layer.

Material from the Bartholin's gland is taken only when the corresponding clinical indications warrant it.

Material from the rectum is usually taken from irrigation fluids. Irrigation is carried out with warm physiological salt solution by means of a Janet syringe 3-4 hours after defecation, using a tip with two nozzles which is introduced into the rectum to a depth of 5-6 cm.

For the examination of the fibers and flakes in the urine, the first portion of urine in a quantity of 10-15 ml, secreted after long urine retention, is brought to the laboratory as quickly as possible. The smears are prepared either from the pus lumps which have been taken from it (the larger lumps which have settled to the bottom of the test tube should be taken) or from the centrifugate.

When material is taken from the female urether and the paraurethral pathways, the index finger is introduced into the vagina and the contents of the urether pressed out by a slight movement along the rear wall of the urether.

Microscopic examination

Unstained preparations from the discharge of the urether, cervix, vagina and other organs are not examined for gonococcus. Only the secretion of the prostate gland is first microscopically examined in the unstained condition: a drop of the fluid is covered with a cover glass and examined under a microscope with dry system. Attention is paid to the number of pyogenic cells, their arrangement (accumulations, uniformity of distribution over the entire field of vision), the presence of number of lecithin granules, amyloid bodies, etc.

Following the microscopic examination of the unstained preparation, the cover glass is taken off, the smear dried and stained. The entire detected microflora can be observed in the stained preparation.

The smears from the initial material (urether, cervix, etc.) are gram-stained or stained with eosin-formalin and methylene blue. When eosine-formalin staining is used, the preparation is dried in air; fixation over a flame is not essential because with this method the staining takes place simultaneously with the fixation of the preparation.

The preparations stained with eosine-formalin and methylene blue or with methylene blue along, can be gram-stained if necessary. For this purpose, the oil is removed with filter paper or cotton wool, moistened with xylene or benzene and the smear stained again.

The gram-staining is of fundamental differential-diagnostic importance for the identification of gonocuccus and is an obligatory method. Special attention must be given to the correct decoloration.

The staining with eosine-formalin and methylene blue is used for investigating the cytological pattern of the smear, mainly to determine the eosinophiles; for the staining of bacteria, this method gives only tentative results. If cytological examination of the smear is not required, staining with methylene blue alone is sufficient. When diploco-

ci or similar microbes are detected, gram-staining is obligatory.

The sequence of staining with eosine-formalin and methylene blue is the following.

1. The preparation is stained for 2 minutes with alcoholi eosine-formalin (1% solution of eosin in 60% alcohol and 0.5 ml formalin).

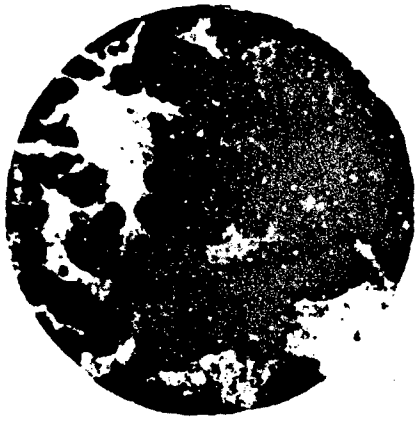

Fig. 131. Gonococcus in the discharges from a case of acute gonorrheal urethritis 3 hours after administration of penicillin. 900×.

2. The dye is allowed to run off.

3. The preparation is additionall stained with 0.5% aqueous solution of methylene blue for 30-60 seconds.

4. The preparation is rinsed with with water and dried.

Microscopic pattern: in the repo given by the laboratory, the number of pyocysts and epithelial cells and the quantity of mucus and microflora is given. If it is difficult to count the leucocytes, the report should state 'entire field of vision covered," "the whole field of vision densely covered," "half the field of vision," "single leucocytes, embedded in mucus," etc.

The degree of alteration of the leucocytes is indicated: "in a dis integrated state," "well preserved," "nucleus pycnotically altered" and the shapes of the leucocytes and particularly the number of lumphocyte and eosinophiles are recorded.

The presence of large numbers of lymphocytes compels a suspicion concerning a possible tuberculous nature of the disease. A large number of eosinophiles indicates a gonorrhea infection, particularly in chron ic cases. With other diplococcus and coccus infections of the urether, cervix or prostate, eosinophilic cells are either not found or only in

small numbers. An increase in the numbers of monocytes serves as an indication of an effective reactivity of the organism.

The examination of the cytological pattern is only significant in a dynamic study, because it may vary, depending on the stage of the disease, its duration, the reactivity of the organism, etc. The same eosinophilic cells may be considered as a favorable or unfavorable symptom for the prognosis, depending on the time of their detection, early or late in the disease.

Bacteriological smear. In acute processes, the gonococci are mostly found inside and outside of the leucocytes (Fig. 131).

The gonococci present on epithelial cells, are often arranged in straight lines in a characteristic manner. Not one of these characteristic of the gonococcus is absolutely constant. The diagnosis can be established only on the basis of a combination of these symptoms.

If intra- and extra-cellular typical gonococci are found, the question is settled. When a small number of gram-negative diplococci, a considerable number of pyocysts and eosinophilic cells are observed, a diagnosis of gonorrhea may be made. If isolated extracellular, gram-negative diplococci with a small number of pyocysts and also gram-positive diplococci are found in the material from patients which have been treated with sulfidine, streptocid or antibiotics, the examination and culturing must be repeated. Particular care is necessary in such cases when a diagnosis is to be made on children. When the rectal discharges are examined, the epithelium of the deeper layers with the lymphocytes and gonococci must not be confused with coli bacteria. When the patients are treated with penicillin, the morphology of gonococcus and its staining characteristics are markedly modified. In such cases one finds in the smears unusually large diplococci with spherical halves, with halves of unequal size, cocci of different sizes (in addition to the large

specimens, very small cocci and diplococci are found) (Fig. 131). These modified gonococci may be intra- or extracellular, some of them being stained gram-positive, others gram-negative. When treatment with other antibiotics is carried out, very small specimens can be observed. In such cases, too, culturing gives rarely a positive result because these microbes may be already dead or may have only little viability. Furthermore, in cases when the penicillin treatment is successful, the gonococci disappear within 24 hours.

Fig. 132. Gonococcus from a day old culture on ascitic agar. 900×.

Bacteriological examination

Culturing is carried out in all cases in which it is impossible to establish a diagnosis by means of microscopical examination (presence of extracellular gram-negative diplococci in patients with a non-typical clinical pattern, absence of gonococcus in the smears from suspected gonorrhea cases and also in laten gonorrhea, when determining the results of the treatment, etc.).

Before the taking of sample material, the use of local antiseptics and administration of antibiotics must be avoided for at least 1-2 days or when they are used, the culture must not be made during the period for which their presence in the organism must be assumed. Material from the urether is sampled after prolonged urine retention. The patient must not be given a douche with disinfectant solutions or given antiseptic substances perorally before culturing from the urinary pathways. Cultures from the cervix are best made during the menstruation period.

It is recommended to inoculate meat-peptone agar, prepared from

rabbit meat or placenta (pH = 7.4-7.6) with addition of ascitic fluid or Bailly medium from ox heart (not from spleen), also preferably with ascitic fluid. Ascitic agar and Bailly medium with ascitic fluid are usually prepared by addition of up to 1/3 of ascitic fluid to melted which has been cooled to 56°. The ascitic fluid should contain fair proportion of protein, be free of bile and traces of disinfectant substances (chloroform is normally used for preserving ascitic fluid; to remove it, the liquid is kept at 55-56° for half an hour and then combined with the warn agar).

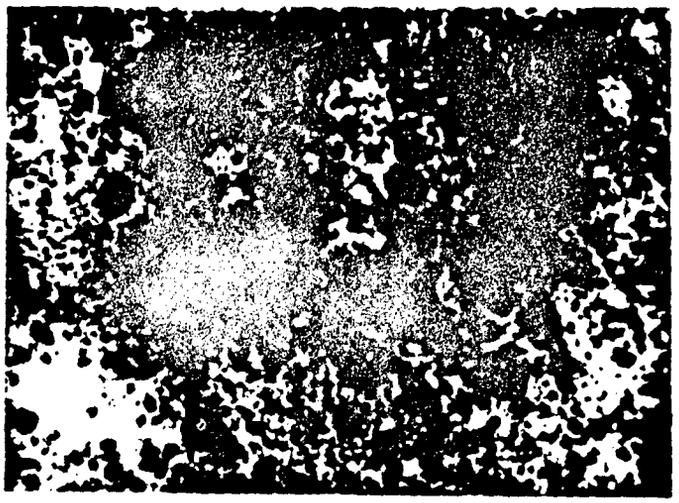

Fig. 133. L-forms of gonococcus.

When Bailly medium without ascitic fluid is inoculated with material obtained from patients, a less copious growth is obtained than on ascitic agar. Bailly medium and a medium with pieces of liver is suitable for inoculation with laboratory strains and for preparation of vaccines. When culturing from hidden foci is carried out (puncture specimens, secretion the prostate gland, sperma, etc.), the material is seeded out on ascitic agar and ascitic broth at the same time. Part of these two materials is kept under normal conditions and part of it covered with vaseline oil. The growing of gonococcus is also carried out

in a carbon dioxide-containing atmosphere (up to 10%).

Essential conditions for good growth of the gonococcus are the following: 1) a suitable fresh moist medium with pH = 7.4-7.6; 2) the thermostat temperature must be exactly 36.5-37°; correct and timely taking of the specimens for culturing. The inoculation is carried out immediately after taking of the test material.

In the microscopical examination of the smears prepared from colonies which have grown on ascitic agar for 24 hours, the gonococci are approximately of the same size and gram-negative (Fig. 132); later on (within 48 and particularly 72 hours) very large and small diplococci and cocci are found. A fairly irregular pattern is observed in the gram-stained smears. In the cultures made from patients, treated with streptocid, sulfidine or penicillin, gram-positive gonococci are sometimes also detected and further reinoculation gives a uniformly gram-negative culture. When colonies, typical for the L-form of gonococcus, are examined, gonococci with the most diverse shapes, sizes and colors are observed (Fig. 133). The gonococci do not cause cloudiness in ascitic broth, forming a tenuous film on its surface, which grows gradually and attains its maximum development on the 3-5th day; some of it settles on the bottom and forms a crumbled or flocculent precipitate.

A grown culture is identified on the basis of the external appearance of the colonies, the bacterioscopic examination of the smears prepared from these colonies, and on the basis of agglutination tests and the breakdown of sugars. To distinguish gonococcus from other microorganisms in mixed cultures, the application of a 1% solution of paraphenylene diamine (oxidase test) on the culture by means of an atomizer is also used. Within 3-4 minutes, the gonococcus colonies turn black, while colonies other microorganisms remain unchanged.

Gonococcus, meningococcus and catarrhal micrococcus are gram-nega-

tive. These differ among themselves by the following features: 1) with respect to their occurrence: meningococcus and the catarrhal micrococcus are extremely rarely found in the urinary pathways but are present in the oral and nasal mucosa; gonococcus is not found in the oral cavity but is present in the discharges from the urogenital pathways: 2) with regard to their biochemical activity.

Serological examination

The agglutination test with serum from patients is hardly ever used for the diagnosis of gonorrhea, because it does not give reproducible results, because the agglutinin titer of the blood is generally low and the agglutinins are absent in acute diseases. The titer of the agglutination test increases only in cases of gonorrheal complications (arthritis, epididymitis, prostatitis, etc.) but in these cases, better results are obtained with the complement-fixation test (Bordet-Gengou test) which is fairly widely used, particularly in gonorrheal complications. Antigens, obtained by treatment of gonococcus cultures either by changing the temperature or with antiformin, are used. It is very important to keep in mind that the antigen must be prepared from a large number of gonococcus strains of different origins, in order to reveal the presence of antibodies as reliably as possible. For the method of the complement-fixation test, see page 154.

The gonococcus antigen determination test which has been fairly widely used at one time (the so-called Lisovoskiy urine test and the Feigel test on cervical discharges) is little used at present because the fairly high percentage of nonspecific positive reactions reduces its value.

Detection of gonococcus by means of the biological test

Vaccination of animals with test material is not used because not any animal species, with the exception of man, is susceptible to gonor-

rhea infection. It is possible, however, to produce septicemia in mice by injecting them intraperitoneally with gonococcus culture (250,000-500,000 microbial bodies) in combination with a 5% mucin and 1% dextrose solution. This model is used for testing the effectiveness of various antigonorrhea preparations.

Allergy tests

The diagnostic value of the skin tests on gonorrhea patients is reduced by the fact that the test remains positive for many years after recovery from the disease. The intradermal test gives a considerable percentage of correct results, if fresh gonococcus vaccine in physiological salt solution, killed by keeping at 56° for half an hour, is used as antigen; a preservative is not added. The standard is approximately 100 million microbe bodies in 1 ml. The suitability of the vaccines for the skin test is first checked. A vaccine is used which does not cause erythema in healthy persons.

The same vaccine, but killed by keeping it in the autoclave at 120° for half an hour, is used as a control. The control liquid is first checked: it should not give a reaction either on patients or on healthy persons. The vaccine and control liquid are injected intradermally in a quantity of 0.1 ml. The evaluation is carried out within 24 hours. A hyperemic spot, sometimes with a swelling in the center, is observed in gonorrhea patients at the injection site within 24 hours, if the dose of vaccine has been correctly chosen. In the control, the hyperemia should either be completely absent or be considerably less severe. With healthy persons, hyperemia is not observed either in the control or at the point of injection of the gonococcus vaccine. When the same hyperemia is present at the point where the control liquid and the vaccine have been injected (this is often the case with certain patients, for example, those suffering from eczema), the test is considered to be negative. The hyperemic spot decreases considerably in size within 72 hours but in gonorrhea patients the hyperemia remains

tenaciously at the point, where the swelling occurred. The test may also be negative in gonorrhea patients in a very severe condition. Good results gives the test with gonoccoccus polysaccharide and gonoprotein. The polysaccharide and gonoprotein are used for the intradermal tests in dilution with physiological salt solution of 1 : 1000 in a dose of 0.15 ml. The antigen test on healthy persons should give the same result as that with the control liquid.

MIXED INFECTIONS CAUSED BY BACILLUS PYOCYANEUS

B. D. Bychenko, Candidate of Medical Sciences

The bacillus Pseudomonas aeruginosa (synonym Bact. pyocyaneum) belongs to the genus Pseudomonas of the family Pseudomonadaceae. This is the only microbe of this genus which is conditionally pathogenic for humans. The other numerous species of the genus Pseudomonas are capable of causing disease in certain mammals, fishes, insects, plants and fungi (Ps. fluorescens, Ps. salmonicida, etc.) or lead a saprophytic form of existence, mainly in the soil.

Ps aeruginosa is widely distributed in nature. It has been isolated from the intestine of humans and various animals, from pus, from the surface of the skin and mucosa of warmblooded animals, from food products and also from the soil, from river, lake and stagnant water, etc.

The virulent strains of this microbe are capable of causing, particularly in organisms with low resistance (babies, old people, young animals) various diseases: otitis, meningo-encephalitis, pneumonia, pyelitis, typhoid infection, and liver abscesses. Besides, Ps. aeruginosa causes destructive lesions of the skin and mucosa: pustules, gangrenous ecthyma, necrotic and ulcerous lesions of the mucosa of the digestive tract, diarrhea of the newborn with a high percentage of fatal issues, etc.

Morphology of Ps. aeruginosa

Ps. aeruginosa has dimensions of 1.6-3 × 0.4-0.6 μ, a straight axis, and slightly rounded tips. In the smears from cultures the cells are solitary or arranged in parallel short chains. It is gram-negative. It is readily stained with the usual aniline dyes. It moves by means of 1-3 flagellae issuing from the same end. It is surrounded by a thin slimy capsule. It does not form spores.

Biology of Ps. aeruginosa; culturing characteristics

The microbe grows well only under aerobic conditions on standard media and on suitable synthetic media, consisting of mineral salts, sources of nitrogen and carbon (ammonium salts, citrates, acetates, etc.). When nitrate is added to the medium, Ps. aeruginosa is capable of growing under anaerobic conditions. The development of the microbe takes place within the temperature range of 5 to 42°. Optimum growth occurs at 30-37°.

On the surface of meat-peptone agar, Ps. aeruginosa forms within 1-2 days at 25-37° round hyaline, colonies with yellow-green fluorescence and a diameter of up to 1-3 mm with straight or slightly wavy edge, a low dome and a smooth, moist, shiny surface. The size of the colonies increases slightly within 5-6 days, their center clears up and the peripheral surface becomes streaked in a radial direction, the edge becomes wavy, festooned or fluffy. The medium below the colonies assumes a greenish-yellow coloration.

On slanted agar, there is copious growth after only 2 days, the consistency of the microbe mass is oily, homogeneous, the surface is corrugated with translucent radia strands; the edge is irregular. The color is greenish-yellow and fluorescent. The medium acquires the same color. A few days later (5-6 days), clear spots sometimes appear in the growth centers as a result of spontaneous lysogeny which resemble the

spots appearing in presence of bacteriophage (phenomenon of pseudobacteriophagy). These regions are covered with a crystalline deposit which gives them a shiny appearance. Numerous decomposed cells can be detected in the smears from these spots.

The colonies of some strains when grown on the surface of blood agar, are surrounded by a marked hemolysis zone which makes it possible to distinguish them from other fluorescent bacteria of the genus Pseudomonas, which do not produce hemolysins. The microbes partly digest coagulated serum within a period of 14 days. A stab cluture in gelatin gives a moderate fibrous growth on the surface of the medium. A slow craterlike partial digestion of the gelatin takes place within a period of 14 days. The liquified gelatin becomes cloudy because of the proliferation of the microbe and assumes a yellow-greenish color.

In liquid nutrient media, vigorous growth with intense clouding develops within 2 weeks. The medium assumes a yellow-greenish color, a thin, homogeneous or thick wrinkled film is formed on its surface and a powdery precipitate of cells which is easily disintegrated by shaking, settles on the bottom. A few days later, the preceipitate becomes slimy, flocculent and copious and is only partially homogenized by shaking. Liquid cultures of Ps. aeruginosa give off an unpleasant odor of trimethyl amine.

Ps. aeruginosa is not very active with respect to carbo-hydrate breakdown. On media containing 0.5-1% peptone, it ferments only glucose with formation of acid but without gas evolution. In aqueous peptone extract, containing not more than 0.07% peptone, it also decomposes arabinose, xylose, mannose and galactose. It peptizes litmus milk and completely decolorizes it in 5 days at 30-37°; the medium then assumes a greenish color. Sometimes, the milk is first partly coagulated. The microbe fails to produce indole and skatole. The culture fluid of

Ps. aeruginosa may give a false indole reaction with Böhme reagent, the acid of the reagent combining with the pigment pyocyanin giving rise to a compound with red color. It should be pointed out that the false reaction does not take place when a reagent, containing oxalic acid is used for the indole test. The microorganism reduces nitrates with eveolution of free nitrogen. All strains of Ps. aeruginosa produce considerable quantities of ammonia and hydrolyze urea but not sodium hippurate or starch. The bacillus produces a catalase. It does not form hydrogen sulfide. One of the interesting peculiarities of the microbe is its capacity to form hydrocyanic acid in broth cultures and in the organism of an infected animal.

Resistance to Physical and Chemical Factors

Ps. aeruginosa is not very resistant to the action of environmental factors. It is quickly killed by the influence of sunlight. A temperature of 55° kills the bacillus within an hour. It is readily destroyed by the usual antiseptics but has considerable resistance to many antibiotics (levomycetin, gramicidin, microcid, furacilin, penicillin, sanosin, ecmolin, albomycin).

Antigen structure; serotypes

The antigen structure of Ps. aeruginosa has not been studied extensively. According to the data of Van den Ende (1952), Ps. aeruginosa has 6-10 separate types of O-antigens and according to the data of A.B. Chernomordik (1959), 10 serotypes. Serological reactions are not yet used for the identification of Ps. aeruginosa.

Pathogenicity

The highly virulent strains of Ps. aeruginosa kill guinea pigs and rabbits within 24 hours. Massive doses of cells, injected intravenously, can cause the death of the animals with symptoms of acute intoxication. In addition to generalized infections as a result of the introduction

of the microbe into the vascular system of laboratory animals, it is also possible to produce local inflammation processes, for example, abscesses by subcutaneous injection of a culture. This is not always successful, however, because strains with low virulence and even avirulent strains of Ps. aeruginosa are often found in nature.

When autopsy is performed on the dead animals, one finds hemorrhagic edema of the subcutaneous adipose tissue at the site where the culture had been injected, small, point-like blood extravasations in the gastric and intestinal mucosa and under the serosa of various internal organs. Renal inflammations (nephritis) are sometimes detected. The microorganism can be isolated from the blood, liver, spleen, kidneys and other internal organs.

Pigment formation

One of the important diagnostic characteristics of Ps. aeruginosa is its capacity of forming a pigment. The pigment, produced by this microbe, consists of two different substances: pyocyanin ($C_{26}H_{20}N_4O_2$) which is a blue-green substance without fluorescence easily obtained from peptone extract and soluble in chloroform, nitrobenzene, pyridine, phenol and hot water; and fluorescein ($C_4H_7O_2N$), a green-yellow fluorescent substance, formed only in presence of phosphates and soluble in water, phenol and acetic acid, but not in chloroform and other organic solvents.

The characteristics of pigment is not constant in many strains of Ps. aeruginosa. Its intensity depends greatly on the medium, in which the microbe is cultured. Mg, K, Fe, SO_4 and PO_4 ions, the presence of glycerol in the medium and other factors exert an important influence on the process of pyocyanin formation.

Pyocyanin has the properties of an antibiotic, respiration enzyme and indicator. It is resistant to acids, forming red salts with them.

In alkaline medium, pyocyanin has a blue color and can decompose relatively quickly. Fluorescein is more readily produced by the bacillus on media containing glycerol and asparagine. In acid medium it is colorless, but in alkaline medium it has a greenish and reddish color and gives an intense green fluorescence. In old cultures of this microbe, t the active yellow enzyme α-oxyphenase is often present, which is a potent bactericide and has the same effects as the flavins.

When the capacity of a certain strain to produce pyocyanin is to be determined, 2 to 5% glycerol or mannitol is added to the medium on which the microbe is being grown, in order to stimulate the formation of this pigment. The test for the detection of pyocyanin is very simple and can be used for the rapid identification of the Ps. aeruginosa bacillus. A small quantity of chloroform is added to a fresh liquid culture of the Ps. aeruginosa bacillus. The mixture is made alkaline, adjusting to pH = 7.8-8.0 by means of 0.1 N caustic soda solution and careful shaking. The chloroform layer assumes a blue color. The same test can be carried out with a thick cell suspension in physiological salt solution, obtained by washing a culture off the surface of nutrient agar. However, the microbe material must first be triturated with sand or glass powder in a sterile mortar, adding a small quantity of chloroform to it.

The presence of pyocyanin in the culture fluid can also be demonstrated by varying its pH. The strongly acidified fluid should assume a red color, and when made alkaline, a blue color. This test is less sensitive, than the preceding test, however.

LABORATORY DIAGNOSIS OF DISEASES CAUSED BY PS. AERUGINOSA

To isolate the microbe from various contaminated substrates (soil, excrements, pus, etc), media containing inhibitors for many other microorganisms, are used. One of the convenient media on which the growth of

bacteria and of Proteus vulgaris is inhibited, is alkaline (pH = 7.8) nutrient agar, containing 0.1% cetyl trimethyl ammonium bromide (citramide).

A combination of various antibiotics in small doses (penicillin, levomycetin, etc.) can also be used as an inhibitor of the aerobic microflora.

DISEASES CAUSED BY PATHOGENIC PROTOZOA OF THE FEMALE UROGENITAL SYSTEM

S.D. Astrinskiy, Professor

To the pathogenic protozoa of the urogenital system belong the Flagellata of the family Trichomonadae. Three species of tetra flagellate trichomonadae are found in humans:

1) the vaginal - Trichomonas vaginalis (Donne, 1837);

2) the intestinal - Trichomonas intestinalis hominis (Davaine, Leukart, 1875);

3) the stomatic - Trichomonas tenax (Müller, 1773; Dobell, 1939) (synonym T. elongata, T. buccalis).

Morphology of Trichomonas

The vaginal trichomonas is very large, attaining a length of 20-36 μ. It is predominantly found in the lower sections of the male and female urogenital system.

The intestinal trichomonas is much smaller than the vaginal type, attaining a length of 10-17 μ and having a long, strongly convoluted, undulatory membrane, extending a great distance beyond the end of the body in the form of a free flagellum. This species of parasite lives in the large intestine of humans.

The stomatic trichomonas is also much smaller than the vaginal species, attaining a length of 10-17 μ and has only a short undulatory membrane. It is found mainly in elderly persons with bad teeth, suffering from paradontosis.

The vaginal trichomonas has an elliptical shape (Fig. 134) with a

cluster of flagellae going out from a group of parabasal granules at the anterior end of the body. Another flagellum goes out from the same point, to the rear of the body along the edge of the undulatory membrane, bordering the body of the protozoon on one side. This marginal flagellum may extend beyond the acuminate posterior end of the body. At the base of the undulatory membrane, along the line of its attachment, extends another solitary flagellum. Along the axis of the body extends an elastic skeletal strand, the axostyle, which projects slightly beyond the acuminate end of the body. Adjacent to it, behind the group of parabasal bodies, is the nucleus. Proliferation takes place via longitudinal cell division, the flagellae being partly distributed among the daughter cells, partly formed anew. By means of these flagellae and the undulatory membrane to the side of them, the trichomonas is capable of locomotion.

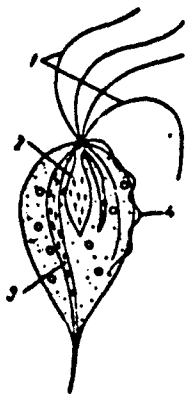

Fig. 134. Schematic view of the structure of the vaginal trichomonas. 1) flagellae; 2) nucleus; 3) axostyle; 4) undulatory membrane.

Resistance to chemical and physical factors

The resistance of trichomonas to heating is slight, they die instantly at a temperature of 60° and within 10-15 minutes at a temperature of 45°. They are more resistant to low temperatures: at a temperature of −5° their viability is retained for 1-1 1/2 hours and at −10°, for up to 20-45 minutes.

Solutions of corrosive sublimate (1 : 1000), carbolic acid (1%) and chloramine (1%) kill trichomonas in a few seconds. Infection of white mice is possible with cultures containing actively moving trichomonas.

LABORATORY DIAGNOSIS OF TRICHOMONOSIS

Test Material; sampling

A diagnosis of trichomonosis is made on the basis of a microscopic examination of the discharges from the vagina, cervix, urether, the paraurethral pathways and in males, by analysis of the urine discharged from the urether and the fluid from the prostate gland. Smears on object glasses are prepared from these discharges.

Native preparations can be used for rapid diagnosis: the discharge is extracted with a blunt scoop or surgical forceps and placed into a drop of physiological salt solution, heated to a temperature of 35-37°. The drop is covered with a cover glass and then examined under the microscope. The native preparations reveal immediately the presence of mobile trichomonas but they should be supplemented by stained smears, on the basis of which conclusions can be drawn regarding the entire microbial biocoenosis and the degree of purity of the vaginal secretion.

Microscopical examination

The method of the dry smear has several advantages over the method of the native preparation. The stained smears give a larger percentage of positive results as compared with the native preparations (2.6% more) and permit the examination of the material long after sampling. The stained smears enable an examination to be carried out on very small quantities of secretion.

The smears can be stained by the method of Pick-Jakobson (4 ml 1% alcoholic solution of methylene blue and 1 ml Ziehl's fuchsin and 35 ml distilled water) within an hour without and by the Romanovskiy-Giemsa method.

Both staining methods give a typical pattern. With the first method, the trichomonas with their blue-violet color and eccentrically arranged small, dark-violet, nucleus are clearly visible against the

background of squamous epithelium cells and also leucocytes with pink protoplasm, violet nucleus and blue-stained microbes. When Romanovskiy staining is used, the trichomonas are stained blue and the elliptical, eccentric nucleus violet. Depending on the pH of the secretion and the pH of the dye, staining of the flagellae, axostyle and the undulatory membrane may also be achieved. Their staining, however, is of no practical importance, because trichomonas is readily differentiated in stained smears even without the staining of these formations (see Figs. 135, 136).

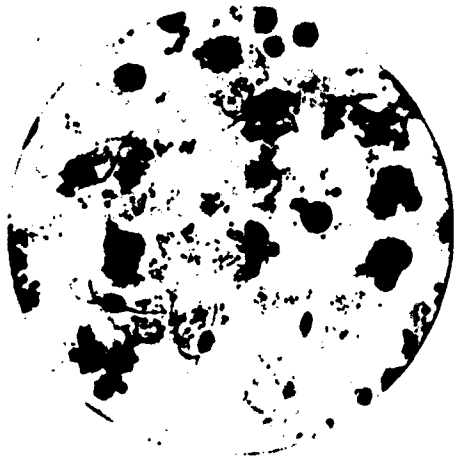

Fig. 135. Vaginal discharge. trichomonas, coccus microflora (according to N.A. Tsagikyan).

Isolation of a culture

The routine isolation of pure cultures of vaginal trichomonas became possible only through the use of antibiotics for inhibiting the associated microflora. The medium for isolating trichomonas cultures consists of meat broth (pH = 6.0) with 0.1% dextrose, 10% equine or human serum, 300 YeD penicillin per 1 ml and 200 YeD streptomycin. The culture is developed at 36°; the precipitate is examined 3 and 6 days later for the presence of trichomonas.

The medium of Jones and Trassel, modified by B.A. Teokharov, consists of 20% ascitic fluid, 20% liver extract, 2% peptone, 1% cysteine hydrochloride, 0.3% maltose, 0.3% glucose and about 57% Tirode solution, the medium having a pH of 5.8-6.0. Before use, 200 YeD each of penicillin and streptomycin is added for each 1 ml of medium. If a semiliquid consistency is desired, 0.1% agar must be added. Cultures of vaginal trichomonas can also be obtained on a solid medium, which differs from the Jones and Trassel medium by addition of 3% agar and omission of the

cysteine from the composition. After one or two reinoculations, further addition of antibiotics is not necessary. In the above described liquid and semiliquid media, the trichomonas population attains 2-5 million within 3-4 days and in some cases even 6 million individuals in 1 ml.

The saline nutrient medium of P.A. Voloskov can also be used. Composition of the medium (per 100 ml); 0.7 g sodium chloride; 1 g trisodium citrate; 1 g medicinal glucose; 90 ml distilled water, 10 ml fresh milk (unboiled), freed of the entire fat content by centrifuging or separation (centrifuging 20-30 minutes at 3000 rpm, separation twice). After dissolution of the salts and glucose in distilled water and with addition of the milk, 50,000 YeD penicillin and 100,000 YeD streptomycin are added, 5-10 ml of the prepared medium or physiological salt solution is usually added to the penicillin and streptomycin in a flask and then the calculated quantity of antibiotics added to the medium. 10 ml portions of the medium are filled into clean test tubes and pure vaseline oil poured on top of it (0.75-1.0 ml into each). The culturing of trichomonas can also be carried out without the oil.

The medium can be made up in advance and stored in a refrigerator or at room temperature (in a cabinet) for 4-7 days. In view of the presence of antibiotics in the medium, further sterilization is not necessary.

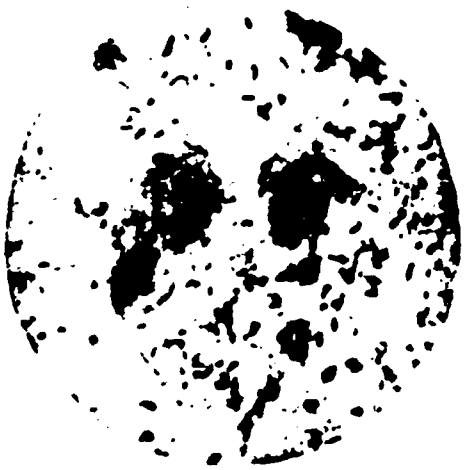

Fig. 156. Vaginal discharge; trichomonas and bacillary microflora (according to N. [illegible]).

The inoculation of media with the test material is carried out with a quantity of 0.3-0.5 ml from the precipitate of the specimen. The test material is applied to the bottom of each test tube with a separate pipette. The inoculated test tubes are placed into the

thermostat at 37°. The cultures are observed for 10 days and examined on the 3rd, 5th, 7th and 10th day.

When the growth is copious, the trichomonas can be detected by microscopic examination within only 24-48 hours. Trichomonas proliferate most intensely in the lower layer of the medium in the test tube, hence when the cultures are checked, the material should be taken by means of a Pasteur pipette from the bottom of the test tube.

The drops are examined in the dark field of the microscope at 280-600 fold magnification. When growth is copious, 5-50 and more trichomonas can be seen in one field of vision.

DISEASES CAUSED BY FUNGI

P.N. Kashkin, Professor, Honored Worker in Science, of the RSFSR

Fungus diseases (mycoses) infect humans, different animals and plants; their clinical manifestations differ and the patnogenic agents are numerous. Some fungus diseases are met in the form of sporadic cases which are not interconnected, others spread in the form of an epidemic and some are of an endemic nature.

Among the fungi which are pathogenic to man, we find monocellular and multicellular microorganisms, some of which grow on artificial media and some which do not develop outside of the patient's organism.

Morphology of fungi

The cell dimensions of fungi vary from a few microns (yeasts, dermatophytes) to tens and hundreds of microns (molds). Their cells contain protoplasm, a nucleus, vacuoles and several inclusions. The cell membrane of many molds is double-contoured and colorless, in others, conversely, it is more intensely stained than the protoplasm. The protoplasm of the young cells is usually homogeneous, that of more mature cells is granular. The fungus cell contains one or more nuclei with nucleoli; the shape of the nucleus is spherical. hondriozomes have been found in some fungi; they are particularly clearly visible in yeast cells. Inclusions which are invariably present in the fungus cell are fat, volutin, glycogen, and more rarely, crystals of salts, organic acids, and pigments.

The most frequent shape of young fungus cells is ovate or elongated in the form of tubules, giving branched fibers, the mycelium. In more

nature cultures, one can observe polymorphous, piriform, clubshaped, fusiform, ameboid cells.

The mycelium is a cylindrical tubule with a diameter of 1 to 10 μ, divided by transverse partitions into cells, whose length varies from 4-5 to 50-70 μ. The mycelium is branched, the branches arise in the form of lateral outgrowths which are situated in some fungi on the same side at regular intervals. by interlacing and forming anastomoses with each other, the fibers create a loose or dense mycelium, which is tightly connected with the nutrient substrate in some kinds of fungi, while it is easily separated from it in others. In some fungi, a characteristic branching is formed at the end of the fibers, which is reminiscent of reindeer horns, chandeliers, combs, ameboid mycelium, etc. The tips of the fibers are also very diverse, having the sahpe of helices and thin spirals or terminating in a swelling reminiscent of an acorn, crozier, club, spindle, and similar formations.

Instead of a true mycelium, a pseudomycelium is found in the cultures of yeast fungi, consisting of elongated cells with lateral branchings, similar to a true mycelium. This similarity, however, is superficial; the individual cells are not connected in any way, each of them being covered by their own membrane; instead of true branching there is only a dendritic arrangement of the cells.

The spores of the fungi serve for their proliferation and propagation in the environment. They are usually formed in large numbers and appear either inside the mycelium, the endospores, or outside of it, on the mycelium, the exospores. To the endospores belong the tissue forms of the pathogenic agent of coccidial mycosis, rhinosporidiosis, which develop in large spherical formations, the sphaeruli. The external spores are formed either directly from the mycelium, on its branches, or on characteristic sporophoric hyphae.

According to their origin, they are classified (Langeron) into: 1) arthrospores with angular or round shape, a size of 2-4 × 4-8 μ, formed by dissection of the mycelium; 2) blastospores, round or ovate

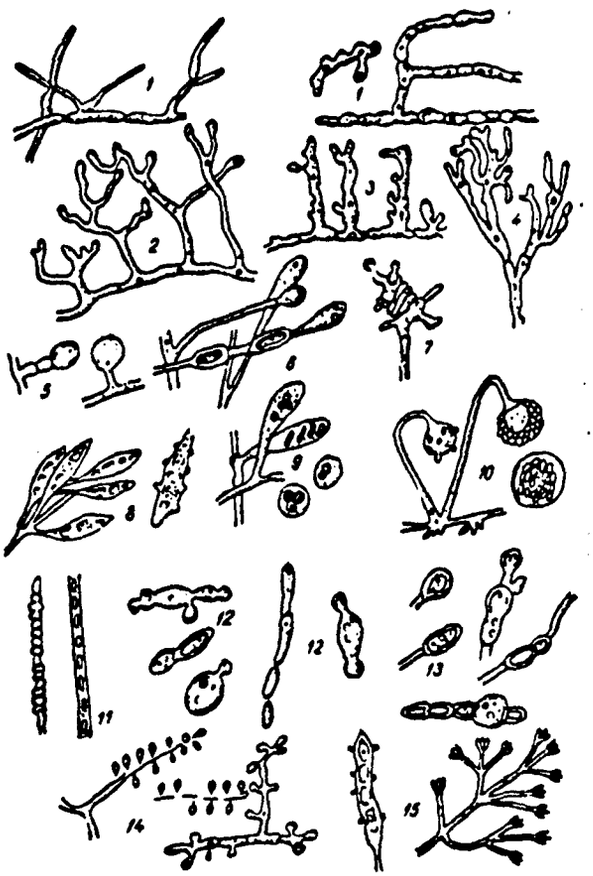

Fig. 137. 1) Mycelium; 2) branched mycelium in the form of reindeer horns; 3) cristate organs, mycelium tips of dermatophytes; 4) chandeliers, terminal branching of mycelium; 5) clubshaped mycelium tips; 6) mycelium tips in the form of acorns; 7) nodulose organs, the mycelium network of dermatophytes; 8) fusiform spores on the mycelium tips; 9) ascospores; 10) sporangiospores; 11) arthrospores; 12) blastospores; 13) terminal and intercalated chlamidospores; 14) aleurias; 15) macro- and microconidia.

with a size of 1.5 to 7-8 μ, formed by budding of a material cell; 3) chlamidospores, round, large, 15-20 μ in diameter with clearly visible, rough membrane, situated along the mycelium (intercalated, interjacent) or at the tips (terminal) of the mycelium; 4) aleurias, aleurospores with round or piriform shape and a size of 2-5 × 3-7 μ, formed in large

numbers from the mycelium contents, and arranged in clusters or singly; b) conidia or conidiospores with round or ovate shape, a diameter of 3-5 × 5-8 μ, formed on special conidiophores at the sides or the tips of the mycelium, being attached to it either directly or through thin stems (sporotrichium, etc.). The membranes of the conidia are smooth or rough, colorless or colored with different shades. To the conidia belong also the large fusiform shapes with a length of 6-8-40 μ, a width of 3-12 μ with transverse partitions; these are terned macroconidia or spindles (Fig. 137).

In infection foci and the pathological material from patients, the fungi are fairly similar, entirely different from those in cultures.

Biology of Fungi; culturing characteristics

The pathogenic fungi are aerobic. They use various proteins and carbohydrates for their nutrition, while some break down fats and keratin and can assimilate mineral compounds. The degree of decomposition of the proteins and carbohydrates varies also with the species of fungus. Some of them decompose proteins only to amino acids, others, conversely, produce ammonia and hydrogen sulfide. Some fungi form only acids from carbohydrates, others break them down to carbon dioxide and water.

Most pathogenic fungi develop best in acid media (pH = 6.0-6.5) while alkaline nutrient media are optimum for actino-mycetes (pH = 7.2-7.8).

The temperature optimum for most pathogenic fungi is 28-33°, but they grow fairly well at room temperature (16-20°) and at 35-37°.

The culture characteristics of some pathogenic fungi, grown at different temperatures, differ in their details; thus, for example, the pathogenic agents of coccidoidal mycosis, blastomycoses and histoplasmosis give mycelial forms at 30-33°, and at 35-37°, yeastlike forms,

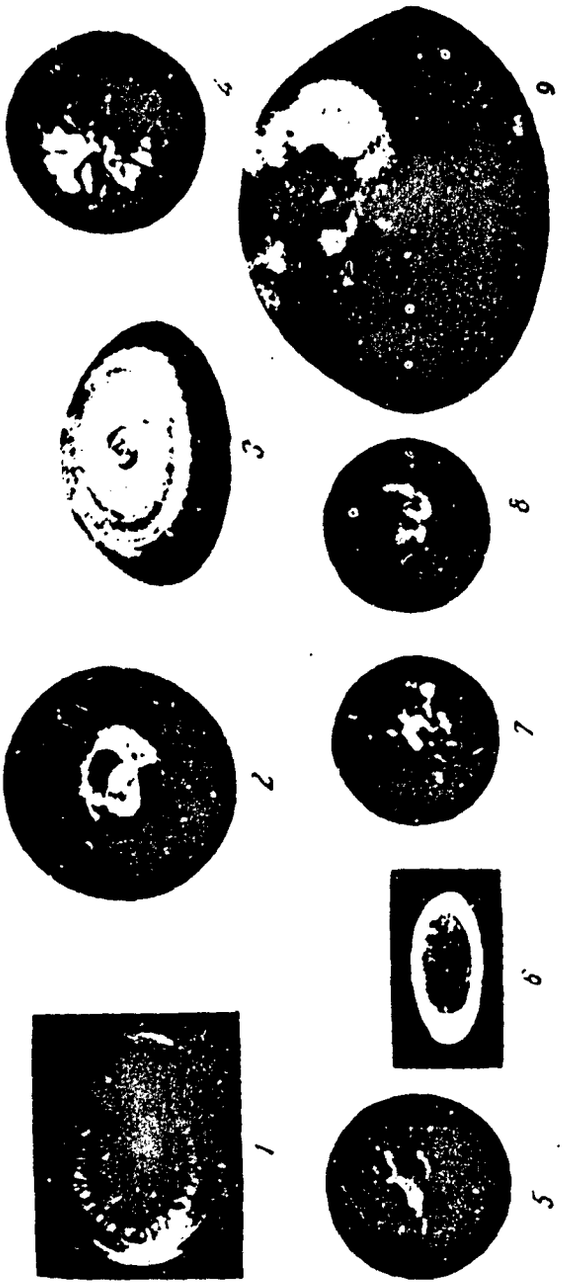

Fig. 138. Cultures of dermatophytes on Saburo medium. 1) Trichophiton violaceum; 2) trichophiton crateriforme; 3) trichophiton gipseum: 4) trichophiton faviforme; 5) achorion schonleini; 6) microsporum lanosum: 7) microsporum ferrugineum; 8) epidermophyton inguelnale; 9) epidermophyton Kaufmann-Wolf.

almost wholly without mycelium.

The cultures of pathogenic fungi are characterized by great diversity (Fig. 138). The size of the colonies varies from 0.5 to 3 cm and over in diameter. The colors of the colonies of different fungi also vary greatly; in addition to colorless cultures, white-yellowish, yellow, brown, pink, red, greenish, orange, violet and black colonies are found. Fungus colonies are sometimes flat and smooth, sometimes plaited, tuberous, wrinkled, sometimes craterlike or cupolashaped. The surface of the colonies of some fungi is smooth, leathery, sometimes shiny with a greasy appearance, in others downy, velvety or powdery.

The consistency of the cultures is also different: with some, it is leathery, solid, with others soft, pasty, slimy-viscous, in some friable.

The behavior on the substrate is also different; some penetrate deeply into the medium, others are easily separated from the nutrient medium.

The variability of the colonies is determined by the species and age peculiarities of the fungus, the composition of the nutrient medium and the growing conditions.

Resistance to physical and chemical factors

The environment, and various physiochemical and biological factors exert an exceptionally great influence on the development and viability of the pathogenic fungi.

Heating of the fungi in clutures and in pathological material to 60° and over causes their death and this the more rapidly, the higher the temperature. Boiling for 2-15 minutes kills all cellular forms of pathogenic fungi in clutures as well as in pathological material.

Fungi are fairly resistant to freezing; when they are vacuum-dried in the frozen state, they retain their viability for 18-24 months. Re-

peated freezing and thawing under normal conditions kills fungus cultures.

Irradiation with ultraviolet light has a fatal effect on fungi. X-rays, even in very large doses, are not harmful to them; diffuse sunlight favors the formation of pigment in some dermotophytes.

Of chemical factors, various antiseptic remedies are harmful to pathogenic fungi: phenol, chloramine, antiformin, formalin, etc. Mineral and organic acids such as hydrochloric, sulfuric, nitric, salicylic acid in 5-10% solution kill the fungi within 15-20 minutes. Much less active are hydrogen peroxide, boric acid, thymol, silver nitrate, protargol, chloroform, ether, ethanol, etc.

Mycocidal effects have the antibiotics nistatin, amphotericin, and griseofulvin, which have proved efficient in the therapy of superficial and deep fungus diseases.

LABORATORY DIAGNOSIS OF FUNGUS DISEASES

Test Material

Pus, skin scales, scrapings from the nails, deposits on the mucosa, sputum, spinal fluid, blood, urine, biopsy tissues, etc. are examined.

Microscopic Examination

The unstained preparations of the pathological material are examined in a drop of 10-20% caustic (KOH, NaOH), which has been slightly warmed or in a mixture of glycerol and ethyl alcohol (equal volumes).

Fungi can be stained in fixed preparations of pathological material by the methods of Gram, Ziehl-Nielsen, Romanovskiy-Giemsa, etc.

Very useful are the methods of concentrating the fungi in the test material (skin and nail scales, pus, urine, sputum) in 10% alkali with boiling (express method) or for several hours in the cold with subsequent washing by centrifuging.

The unstained preparations are first examined under the microscope

at small (10×5) and then at high (20-40×5-15) magnification; the immersion objective is used mainly for stained preparations.

The presence of mycelium, budding cells, different spores and, which is particularly important, of molds with external (conidia) and internal (sporangia, spherules) fruit bodies is a strong indication of the fungus nature of the given infection.

An abundance of fungoid elements and their increase during repeated examinations in the course of the disease are important indications of the fungus nature of the disease.

Culturing characteristics

When cultures are made from pathological material, this is sometimes treated with antiseptics, acids or antibiotics in order to eliminate any extraneous microbes.

Solid nutrient media are usually inoculated with small lumps of pathological material. The most suitable media for fungus culture are: Saburo medium, brewing wort agar 7% according to Balling, meat-peptone sugar agar, Czapek medium, and carrot agar.

The surface of the medium is inoculated by placing very small lumps of test material on it at several points at a distance of 1-2 cm from each other. Addition to the nutrient media of wide-spectrum antibiotics, naturally without harmful effect on pathogenic fungi, in a quantity of 50-100 YeD per 1 ml of medium makes it easier to obtain pure cultures of pathogenic fungi. The nutrient medium should be slightly acid, pH = 6.0-6.5. Dermatophytes and yeastlike fungi of the genus Candida are best grown at 30°, the pathogenic agents of deep, rare mycoses at separately 25 and 37°.

The growth of the fungi appears on the 3-5th day but if not, it is recommended to keep the cultures in the thermostat for up to 30 days, when fungus growth can be definitely considered to be absent.

The identification of fungi is always carried out on the basis of their peculiarities in the pathological material and taking into account their culturing characteristics, pathogenicity and biochemical activity.

Serological examination

To the serological examination belong the agglutination, complement-fixation and precipitation tests. Specific antigens are used to reveal the presence of the antibodies; homogeneous suspensions of pathogenic fungus cells for the agglutination tests; alcoholic-aqueous extracts and polysaccharides from mycelium for the complement-fixing and precipitation test.

Allergy test

The skin tests are carried out with sterile filtrates of liquid cultures, with extracted polysaccharides or with killed fungus suspensions (in cases of candidosis).

Dermatomycoses

To the dermtomycoses belong: ringworm, microsporia, favus and epidermophyty.

Ringworm

According to localization, superficial and deepseated, and acute and chronic ringworm is distinguished.

The skin and hairy part of the head are effected.

Pyodermal, impetiginous, deepseated inflammation forms, and also erosive, and chronic forms with hardly visible foci of alopecia and isolated damage to the hairs have been described.

Allergic forms of lesions often develop in the form of lichenoid, erythematous-squamous or pustulous trichophytosis on exposed parts of the body.

Visceral forms with lesions of the lymph nodes, lungs, kidneys, brain and bone tissue, usually with fatal issue, have been described.

The pathogenic agents of trichophytoses are the numerous trichophytons (Figs. 139, 140).

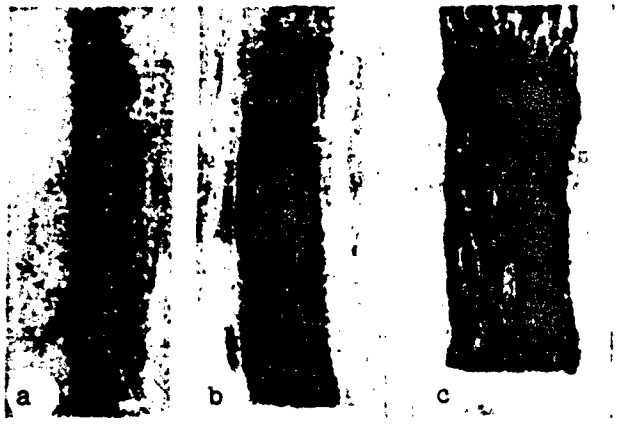

Fig. 139. Dermatophytes in hair. a) Trichophyton; b) microsporum; c) achorion.

They are subdivided in accordance with their culture characteristics into:

Type I - leathery, smooth, dense, slowly growing, colorless or colored colonies, seen under the microscope to consist of a dense network of partitioned mycelium, fibers consisting of cylindrical or polyhedral cells with chlamidospores of different size.

The following cultures belong in this group.

1. Trichophyton violaceum - an endotrix with large spores, forming violet, leathery, wrinkled colonies, which are striated or corrugated with short processes extending into the depth of the medium.

2. Trichophyton faviforme, an ectotrix with large spores. The colonies are pale-yellow, more rarely brown, sometimes shiny, smooth or slighly striated, tightly adhering to the nutrient medium.

Type II, down, luxuriating, white-gray colonies; their aerial mycelium attains a height of 2-5 mm. Under the microscope, they show a fairly even mycelium, aleurias, more rarely, chlamidospores; spindles are occasionally found. As an example Trich. niveum must be mentioned,

an ectotrix with small spores, whose snow-white cultures have a loose, uniform, downy surface; they do not grow into the medium.

Fig. 140. Trichophyton in a nail scale.

Type III, velvety-powdery, wrinkled colonies with a brittle, paper-like feel, pale-yellow color and outgrowths which extend into the medium from the rim of the colony.

Under the microscope, abundant aleurias are seen, arranged in lumps along the sides of the mycelium; terminal and intercalated chlamidospores, and more rarely, chains formed by them, are encountered.

1. Trichophyton crateriforme, an endotrix with large spores. The colonies are velvety, with a regular craterlike depression and small concentric folds along the periphery.

2. Trichophyton rasaceum, endo-ectotrix with large spores, forming flat, velvety colonies with few folds in mature cultures. The color of the colonies is initially pure white, then whitish, later on pink. It causes a disease in humans and cattle.

Type IV, mealy, rapidly growing, large surface colonies with white-yellow and brown tinge.

Their powdery appearance may be caused by: 1) A dense, uniform deposite; 2) regular, concentric accumulations of different density; 3) radial denticles which are denser along the periphery.

When examined under the microscope, round aleurias predominate, 4-5 spindle cells are found, helices, spirals, more rarely, chlamidospores.

Most widespread among the mealy fungi are the following.

1. Trichophyton gypseum asteroides, whose whitish powdery appearance is due to the presence of stellate clusters; it is pathogenic for

humans, causes diseases of horses, cattle, domestic and field mice, and can easily be transmitted by inoculation to rabbits, guinea pigs and rats, causing acute inflmmation reactions in them.

2. Trichophyton radialis, an endo-ectotrix. Its colonies are first velvety, then mealy, in the center with coarse wrinkles, cupolashaped, mealiness around the periphery, regular, short denticles surrounding the fairly large colonies of the fungus. The color of the colonies is whitish, pinkish or creamy.

Type V, faviform, tuberous-corrugated, yellow-brown colonies with powdery consistency. Under the microscope, abundant polymorphous chlamidospores with different sizes, patches of mycelium, reminiscent of chandeliers, rudiments of spindles and rocketshaped large cells are seen in the mature colonies. As an example, the following may be mentioned: Trichophyton faviforme discoides, an ectotrix with large spores, whose slowly growing colonies are fungus-like and waxy in the center, pale-yellow with powdery consistency. The central zone is leathery, mealy, grayish, the periphery ray-like.

Labcratory diagnosis of trichophytoses. The pathological material is microscopically examined in a drop of caustic solution, usually in the form of unstained preparations.

The fungus is found in the hairs in the form of spores and mycelium. Their dimensions and arrangement are taken into account in the differentail diagnosis of fungus diseases. The trichophytons are divided into three groups in accordance with their relationship with hair: endothrix, whose spores are only in the hairs; ectothrix, whose copious spores cover the hair and its base and neoendothrix, a type of fungus which is present inside as well as on the hair. With regard to spore size, large spore (diameter 5-7 μ) and small spore (diameter 3-5 μ) trichophytons are distinguished.

The hairs which are infected with trichophytons are short (1-2 mm and less), white, dry, sometimes appearing as a black point or comma, enclosed by a thin scale and visible under the magnifying glass or the microscope with low magnification.

The large spherical spores of the endothrix type fill the inside of the hair completely but are not found outside of it; the hair looks like a bag filled with nuts. With the ectothrix type, the large or small spores form several layers around the base of the hair, thus creating a wide (large spores) or narrower (small spores) sheath.

In addition to the spores, particularly in the peripheral parts of the hair, short or long chains, formed of round spores and sometimes fragments of mycelium which has formed spores, are found.

In the skin scales among the epidermal cells, partitioned mycelium fibers are seen, which are subdivided by partitions into square, rectangular and circular segments with dimensions of 3-5 × 2-3 μ, and chains and clusters of round or ovate spores with a size of 4-5 μ are also found.

In the nail scales, the trichophytons have the shape of a branched mycelium, chains consisting of round and polyhedral spores and also individual, fairly large (5-8 μ), double-contoured cells.

Microsporosis

Microspores effect the skin, hairs and only very rarely the nails. Microspore lesions of the beard, eye brows, and eye lashes have been described, but penetration of the fungus into the hairs does not always take place, the process beginning with a lesion of the bare skin.

The microspore lesions of the nails and the allergic exanthemas (microsporidia) are rarely encountered; they resemble the trichophyton infections.

The pathogenic agents of microsporosis infections are:

1. Microsporum lanosum, the most commonly found pathogenic agent of microsporia infection of humans, cats and dogs. The colonies of the primary clutures are large, grayish-white, in the center powdery, towards the periphery friable-downy. The stock cultures are uniformly downy, white or gray.

The mycelium is bamboo-like, consisting of rocketshaped cells, thickened at one end; large monocellular spindles, enlccsed in a serrated, fluffy membrane; round chlamidospores and mycelium with terminal "comblike organs;" not very numerous aleuria.

The fungus is pathogenic for guinea pigs and rabbits.

2. Microsporum ferrugineum, colonies fairly large, flat or tuberous, leathery; in the center domeshaped, subdivided by fine grooves into a number of convex sections. The color of the colonies is white, brown, more rarely reddish. Yellowish, funguslike waxy and also white-powdery, finely tuberous or wrinkled variants are found which are quite similar to the cultures of other dermatophytes.

Laboratory diagnosis. In mature cultures, the microscope reveals a large mycelium, large intercalated and terminal chlamidospores with a size up to 30 μ, and chains consisting of polymorphous cells. Spindles are absent, large aleurias rare, being found mainly in the cultures of the powdery variants. It causes disease only in humans (Fig. 141).

Fig. 141. Microsporum in skin scale.

When the hairs affected by microsporia are examined under the microscope, the fungus has a characteristic appearance: numerous small round (2-3 μ) spores surround the base of the hair densely, being closely adjacent in the form of a mosaic. Inside the hair, in its follicular region, partitioned mycelium can be seen, which fills the peripheral part of the

hair fairly densely. Sometimes a loose mycelial fringe is visible which projects beyond the peripheral end of the hair. In the scales, the fungus has the shape of fairly thin, branched fibers with sparse but clearly visible partitions. Chains of mycelium which decomposes into spores, can be seen. The spores of the fungus in downy hair are arranged in the form of a mosaic, either on the surface of the hair or like the endothrix type, filling the hair densely with parallel chains consisting of small spores.

The elements of the fungus in the nails are somewhat larger but basically similar to those in the skin scales.

Favus

Favus effects the smooth skin, the hairs and nails. A typical element are the scutulas, round, flat or saucer-shaped formations with yellow color, clinging tightly to the skin, with crumbly consistency, consisting almost entirely of fungoid elements and effected epidermis cells.

Favus of the smooth skin is found in the scutular, vesticular-impetiginous or seborrheal form and also in the form of blotches on the surface of a reddened base.

Favus of the hairy part of the head is found in humans mainly in three varieties: 1) scutellar or scaly form; 2) pustulous, scabby or impetiginous; 3) desquamative or pityroidic. The fungus affected hairs remain for a long time long, dry, dull, gray, tightly embedded in the thick part of the scutula.

In addition to the typical forms of favus, unusual forms are found, for example, pityriastic or seborrheal. Lesions of the lumph glands, lung tissue, intestinal mucosa and also of the bones and brain tissue have been described.

The most frequent pathogenic agent of favus in man is Achorion

Schönleini. The young cultures of the fungus are smooth, naked, pale-yellow, later they increase in thickness, and become wrinkled, similar to saddle fungus or Greek sponges, with crumbly consistency. The old cultures are dry, wrinkled, thick, dark-brown, sometimes surrounded by a white raylike rim along the periphery. Craterlike, gypsumlike cerebriform, exsiccated, velvety-downy variants and also cultures with outgrowths which penetrate deeply into the nutrient medium at the edges and under the colony itself are found.

In typical cultures, an extensive clearly partitioned mycelium with branches in the form of reindeer horns or chandeliers can be seen under the microscope. Large, round chlamidospores and also blunt-tipped, long and broad formations, similar to large spindles, are seen at the tips of the mycelium. Club-shaped swellings and broad, slightly oblate cells, resembling the head of a nail- "favus nails" - are often seen. In the powdery variants, large piriform aleuria are seen, which sit on the sides or ends of the mycelium or are arranged on it clusters.

When laboratory animals are infected with typical cultures, the disease develops in 15-20% of the cases. The powdery variants are more pathogenic, causing illness in most animals and also adult humans when rubbed into the skin.

Laboratory diagnosis of favus. Typical for the pathogenic agent of favus in hair is the polymorphism of the fungus elements: in addition to the thin (2-3 μ) mycelium with few partitions, a thicker mycelium (4-6 μ) consisting of rectangular cells with double-contoured membrane are found. At the same time, chains and clusters consisting of round and polyhedral spores are found in the hair, and air bubbles and fat droplets are visible. It should be pointed out that the hairs in cases of favus infection are never completely filled with fungus elements, the healthy layers of the hair being partially preserved, in consequence of

which the hairs remain long, dry and white for a considerable length of time.

Typical for the mature scutula is an abundance of large, polyhedral and spherical spores, arranged irregularly in clusters or, more rarely, in short chains; the cell elements of the skin are hardly visible.

In favus onychomycosis, the fungus elements are irregularly arranged in the scales, in the form of single cells or short branchlets of mycelium or very copiously in the form of a branched mycelium with chains of polymorphous spores and individual spore clusters.

Fig. 142. Epidermophyton in skin scale.

Epidermophytia

The epidermophyton infections are localized in the equinal and other skin folds on the feet and hands, the nails also being affected.

The fungus-affected nails, mainly on the toes I and V, are dirty-gray, deformed and brittle.

The pathogenic agents of epidermophytosis are:

1. Epidermophyton inguinale, the colonies are round, powdery, first dome-shaped and raised, and smooth, later wrinkled and tuberous, sometimes with irregular crater-like depression.

When young cultures of Epid. inguinale are examined under the microscope, a uniform, clearly partitioned mycelium predominates, while in mature cultures, large intercalated chlamidospores are found, sometimes chains formed by them. The piriform aleurias are not always present. Very typical spindles consisting of 4-5 cells and having a size of 20-35 × 6-8 μ are visible at the tips of the mycelial fibers; the spindles are usually arranged in clusters of 4-7 elements, similar to a

bunch of bananas (Fig. 142).

2. Epidermophyton Kaufmann-Wolf – rapidly growing colonies, velvety-powdery, surrounded at their periphery by a narrow rim consisting of young shoots which creep over the substrate. The mature cultures are crowded and powdery, their center is dome-shaped, wrinkled or tuberous.

The cultures of this fungus show great diversity. Downy, gypsumlike powdery, velvety-powdery smooth and wrinkled cultures are found, sometimes cerebriform variants, often isolated from the same patient.

The color of the colonies is usually white, more rarely, yellowish, pinkish, brown and even red cultures being found.

Under the microscope in cultures: long, branched, partitioned mycelium, long helices and spirals being fairly frequently seen on the tips of the mycelium; polymorphous, intercalated chlamidospores. Numerous aleurias with a diameter of 2-3 μ sit on the sides and the ends of the mycelium. The aleurias are often arranged in clusters, the latter most often in gypsum-like-powdery cultures.

The spindles of Epid. Kaufmann-Wolf are fairly typical: the length is 20-30 μ, the width 5-7 μ, they are subdivided by transverse partitions into 5-6 cells, the central ones being the largest. In old cultures, spindles are rarely found, but chlamidospores are much more frequent.

Inoculation of animals with the cultures is not always successful.

Laboratory diagnosis. In the scales from the infection foci, the fungi are seen in the form of partitioned, branched mycelium with a diameter of 3-5 μ and separate chains consisting of polymorphous cells with a size of 2-3 × 6 μ, solitary fungus elements being rarely found.

Methods of detecting dermatophytes. The dermatophytes enter the environment from infection foci of patients in affected hairs, skin and nail scales. They are most conveniently collected by repeated brushing

with a moist hair brush. It is essential to cover the greatest possible surface area of the test objects: floor, clothes, books (between the leaves and the corners of the pages in the lower right), carpets, various objects.

The brush is then carefully washed by repeated immersion in a sterile centrifuge test tube containing physiological salt solution and 50 YeD each of streptomycin and penicillin in 1 ml to check the associated microorganisms.

The liquid is centrifuged, examined under the microscope in a drop of aseptically prepared fresh physiological salt solution and solid and liquid nutrient media inoculated with it (Saburo medium, brewing wort-agar) with antibiotics to obtain cultures of the corresponding dermatophytes.

The detection of dermatophytes during the microscopic examination of the precipitate is of great importance; it attests to the fact that the test material is infested with dermatophytes.

Method of detecting dermatophytes in the soil: the soil sample which is to be examined, is placed into a Petri dish, preferably in a layer with 1 cm thickness, moistened with sterile water (10-15 ml), human or animal hairs with a length of 3-4 cm and sterilized at 120° placed on the soil sample, and the Petri dishes kept in the thermostat at 25-30°. They are examined every 2-3 days for 10-15 days. Hairs which are covered with a grayish-white (mycelial) deposit of nodules are taken out for microscopic examination and reinoculation of antibiotic-containing media and for intradermal infection of guinea pigs.

The species is then described on the basis of a study of the pure fungus cultures and their peculiarities in the pathological material from the infected animals.

To detect epidermophytons on objects from the environment of pa-

tients, basically the same methods are used as for the detection of the dermatophytes. Animals are not infected in view of the low pathogenicity of epidermophytons for them.

When baths, swimming pools or shower installations are to be investigated, samples are taken from the floor and walls of the basins or bathing utensils with a hair brush or they are scraped off with a scalpel. To detect epidermophytons in soap water, 10-15 liters of the water must be passed through a layer of gauze; the slimy mass is taken off the gauze with the scalpel and washed by repeated centrifuging in physiological salt solution containing antibiotica. The precipitate is examined microscopically and seeded out on solid media containing antibiotics.

The detection of the fungi in the scales during the microscopic examination of the precipitate is of great diagnostic importance; the species characteristics of the fungi are determined by preparing cultures which must be observed for 25-30 days.

Candidoses

The clinical manifestations of candidoses are very diverse. The fungi effect the skin and mucosa, various tissues and organs: the digestive tract, the respiratory pathways, the urogenital pathways and the central nervous and cardio-vascular system.

The habitats of the yeast-like fungi of the genus Candida are the skin and mucosa of humans, domestic fowl, some mammals, but they are also frequently detected on various fruits and berries, in milk products and fruit preserves. They are also found on objects used by patients, on the inventory of the sickrooms and dressing rooms, childrens homes, maternity homes, child clinics, and rooms of mothers and children.

The pathogenic agents of candidoses are the yeast-like fungi of

the genus Candida, which have a pseudomycelium, blastopores and in a few species also chlamidospores.

The young fungus cells have a circular or ovate shape, the mature ones are elongate or spherical with dimensions of 2 to 5 μ; the mature cells of the pseudomycelium are much larger, attaining 12-16 μ in length. The diameter of the young budding cells (blastophores) varies between 1 and 3 μ.

The chlamidospores, whose diameter may attain 10-20 μ, have larger dimensions; their shape is round, the membrane thick and double-contoured.

Budding-off is the sole form of proliferation of the yeast-like fungi. The buds are the youngest cells; their shape is round or slightly piriform.

The yeast-like fungi of the genus Candida develop fairly well and rapidly on solid agar media containing carbohydrates.

The dimensions of their colonies are much larger than those of bacteria, sometimes attaining a diameter of 1 cm; their consistency is creamy, in some species viscid or mealy. Growing into the nutrient substraute is common to many species. Concerning color, pure white or pale-yellow, cream colored colonies are found. The surface of the colonies is sometimes entirely smooth (S), mat or shiny, sometimes striated, with fine or coarse corrugations or rough (R) (Figs. 143, 144, 145).

The edges of the colonies are even, with sharp outlines, sometimes festooned, wavened or even sloping, merging gradually into the surface of the nutrient medium.

The antigen properties of the yeast-like fungi of the genus Candida have not yet been sufficiently investigated.

Culturing characteristics of the most widely occurring yeast-like fungi of the genus Candida:

1. Candida albicans. On brewer's wort-agar round, viscous, white, convex, shiny, usually smooth, with even edges and branched, dendritic pseudomycelium which grows deeply into the medium. The blastospores are arranged irregularly on both sides of the pseudomycelium. The chlamidospores are round, large, double-contoured. Candida albicans ferments glucose, maltose, levulose and partly galactose; it does not affect milk. It is pathogenic for laboratory animals.

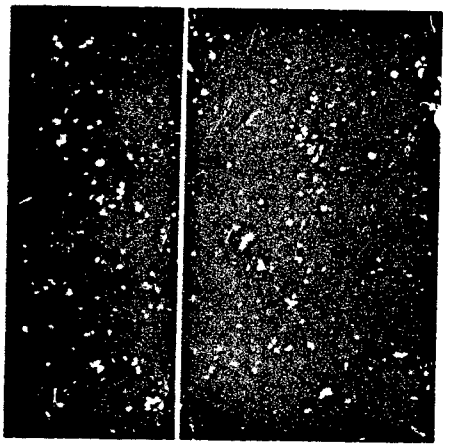

Fig. 143. Smooth (S) and (R) rough cultures of yeast-like fungi of the genus Candida.

2. Candida tropicalis. The colonies are round with serrated edges, grayish white, initially of creamy consistency, later on filmy, compact, with raised irregularly tuberous center and smooth or radially corrugated uneven edges. Growing into the substrate is moderate. On liquid media it forms a film, a thin ring and a copious precipitate on the bottom.

The pseudomycelium is highly developed, branched, with an abundance of blastospores and pseudoconidia; chains consisting of ovoid cells are found. Chlamidospores are absent. Fermets glucose, galactose, saccharose, maltose and levulose. It does not effect milk. The pathogenicity for laboratory animals is variable.

3. Candida pseudotropicalis. The colonies are round, flat, with grayish color and dull. On liquid media they produce a precipitate. The pseudomycelium is poorly developed. Chlamidospores are absent; the blastospores are not numerous. Ferments glucose, saccharose, lactose, raffinose and levulose. The pathogenicity is variable.

4. Candida Krusei. The colonies are white or gray, dry, with viscid consistency, dull, smooth or with fine wrinkles, the edges uneven

with substantial outgrowths into the substrate. The pseudomycelium consists of elongated blastopores; chlamidospores are absent. Candida krusei ferment only glucose and partly levulose. The pathogenicity for animals is low.

Laboratory diagnosis of candidoses. The skin and nail scales, the discharge from the ulcerous lesions of the mucosa, pus, spinal fluid, blood, bile, feces, urine, pieces of biopsy tissue and material from cadavers are examined.

The pathological material is microscopically examined in the unstained form in 10% caustic, a mixture of alcohol and glycerol (glycerol 2 parts, alcohol 2 parts, water 4 parts) or Lugol solution (iodine crystals 1 g, potassium iodide 2 g, water 150 ml). The last mentioned is the most suitable.

The detection of pseudomycelium or of chains consisting of round and elongate yeast-like budding cells is sufficient for a positive result.

The preparations which have been fixed by heating or with a mixture of alcohol and ether, are gram-stained or by the method of Ziehl-Nielsen and Romanovskiy-Giemsa. They are gram-positively stained, with Ziehl-Nielsen, they assume a blue color with pink-yellow, finely granular inclusions of lipoids. When they are stained by the Romanovskiy-Giemsa method, the cells of the yeast-like fungi are pink-violet, the chromatin substance is red, the volutin dark-violet.

The detection of yeast-like fungi during the microscopic examination of pathological material from an open infection focus does not have any decisive diagnostic importance. Confirmation by culturing, and serological and allergic test methods is essential. The detection of Candida in a closed infection focus, in the spinal fluid and bile, aseptically sampled urine, and also in histological sections from a

granulated disease focus is different tissues and organs is very valuable for establishing the candidosis nature of the disease.

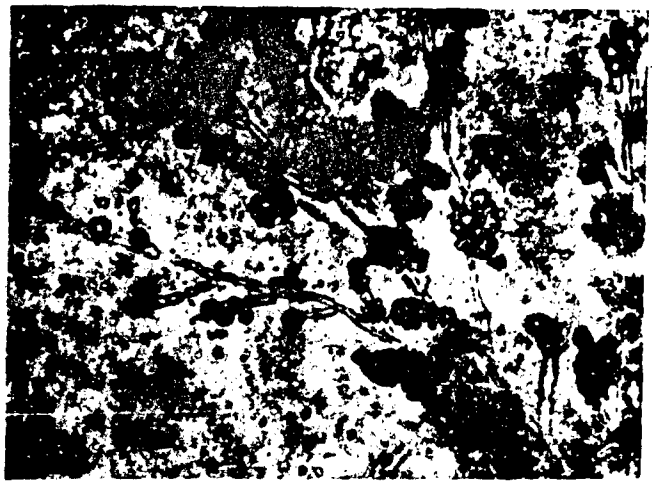

Fig.144. Microscopic pattern of Candida albicans in cultures (chlamidospores, pseudomycelium, blastospores).

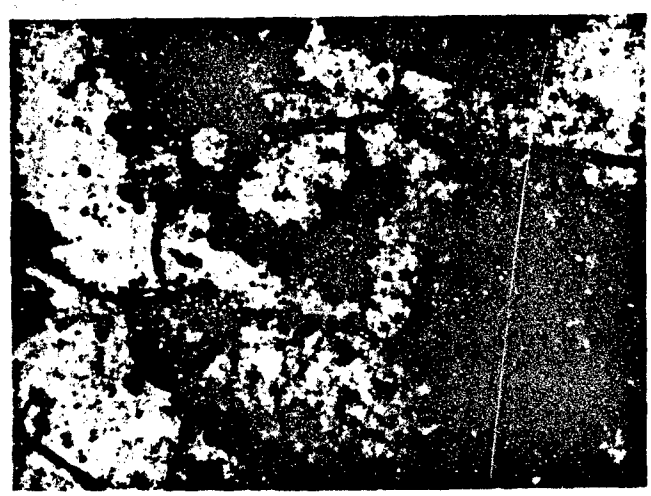

Fig. 145. Candida albicans in sputum (pseudomycelium and yeast cells.

Solid and liquid sugar media are widely employed for producing cultures of the yeastlike fungi.

In order to avoid contamination with bacteria, it is recommended to treat the culture material first with sulfuric or hydrochloric acid (7%) and also to carry out seeding on media which contain antibiotics:

penicillin or biomycin in a quantity of 50-100 YeD per 1 ml of nutrient medium.

The cultures are kept in the thermostat at 30-35° for 15 days.

In positive cases, usually on the 2-3rd day, small shiny, dome-shaped colonies of yeast-like fungi appear first, then large, often confluent colonies with outgrowths extending into the substrate.

The degree of pathogenicity (virulence) of the yeast-like fungi is determined on rabbits with a weight of 1.8-2.2 kg which are injected intravenously with 500,000 cells from a pure culture of yeast-like fungus in 1 ml of physiological salt solution. The rabbits are killed 9 days later and the degree of pathogenicity of the strain concerned is determined on the basis of the pathological alterations. Nonvirulent strains do not cause any alterations in the kidneys of a rabbit which is killed 9 days after infection. Fungi with low virulence cause the formation of nodes and microabscesses in the kidneys. The virulent strains cause the death of the rabbit within 9 days, causing specific changes in the kidneys (extensive multiple eruptions of grayish-white nodes and microabscesses in the cortical layer of the kidneys.

The serological diagnosis of candidoses is carried out by means of agglutination and complement-fixation tests. These tests are carried out by the usual method (see pages 108 and 154).

The antigen for the agglutination test is a suspension of a two day old agar culture in physiological salt solution with a density of 200 million Candida cells in 1 ml, which corresponds approximately to the optical density of the standard, containing 2 billion microbe bodies in 1 ml.

The positive agglutination tests with yeast-like fungi of the genus Candida, particularly, when they are obtained at low serum dilutions (1 : 50-1 : 100), are devoid of diagnostic significance owing to the

fairly frequent detection of agglutinins in the serum of healthy carriers. Only the dynamics of the agglutinins and their increase during the illness (1 : 160-1 : 2560) can be useful for confirming the candidosis nature of the disease.

A suspension of a two day old agar culture of Candida in 30% alcohol with a density of 500 million cells in 1 ml can serve as antigen for the complement-fixation test. The suspension is kept 48 hours at 37° and 10 days in the refrigerator at a temperature of 4-5°. The supernatant liquid, whose anticomplementarity and hemotoxcity is determined in accordance with the normal rules of complement titration is used as antigen for this work. Polysaccharides extracted from the cultures can also be used.

The positive test is most marked during the infection and disappears, as a rule, during reconvalescence.

Methods of detecting yeast-like fungi. To detect the yeast-like fungi of the genus Candida on environmental objects in contact with patients, on containers, linen, toys, on the floor and in dust, the test material is collected in the same manner as for the detection of dermatophytes, treated with antibiotics, the precipitate examined microscopically and solid and liquid media containing antibiotics or synthetic medium of Rolain inoculated. 10-15 days later, solid Saburo medium brewing wort agar or carrots are inoculated with the culture on the Rolain medium. The cultures of yeast-like fungi thus obtained are identified by the usual method.

To detect yeast fungi on vegetables, fruit, and berries, swabs with antibiotic-containing physiological salt solution are prepared, the precipitate examined under the microscope and seeded out on media containing antibiotics and on acid Rolain medium.

To detect yeast fungi of the genus Candida in milk products,

pickled vegetables and fruits, in jam and fruit juices, etc., the test material is diluted with physiological salt solution containing antibiotics (1 : 10, 1: 100, 1: 1000), centrifuged, the precipitate examined under the microscope and solid and liquid media containing antibiotics, Rolain medium and carrots inoculated with it. The species characteristics of the yeast cultures are determined in the usual manner.

The detection of yeast fungi in the precipitate during microscopic examination is not of great significance in view of the morphological similarity of their cells with those of other yeasts. The pathogenicity is then determined with pure cultures, obtained from the same or another sample of test material.

The detection of yeast fungi in wastes, animal excreta and also in effluents and soap water of baths and swimming pools, and in various soil samples is carried out basically by the same method. Double the quantity of antibiotics must be taken and the treatment time of the precipitate doubled, which is then seeded on solid media containing antibiotics, on Rolain medium and on carrots.

CRYPTOCOCCOSIS

Cryptococcosis is a deep-seated and severe disease which effects predominantly the central nervous system. Affections of lump nodes, lungs, and bronchial glands, skin, subcutaneous tissue, spleen, liver, kidneys and also of other organs in the abdominal cavity have been described. The proportion of skin lesions with this mycosis is not more than 5%.

The pathogenic agent Cryptococcus neoformans (Torula histolytica) is found in the natural environment, the soil, and in pigeon droppings.

The fungus has also been detected in fermented fruit juices, in tin milk, butter, grass, etc. (Langeron and Van Bruisegem).

The pathogenicity of the cultures is variable; completely reliable indications concerning fungus infections from the natural environment are not yet available.

The fungus grows on nearly all laboratory media. The cultures on solid media are shiny, greasy, of viscid consistency, dome-shaped, first smooth, later slightly wrinkled or finely granular. The young cultures are pale yellow, the mature and old cultures become brown and dark-brown.

Morphology: round cells with different size and small round or slightly elongated buds and slimy capsule. The protoplasm contains numerous granules, similar to endospores, and several small vacuoles (Fig. 146).

Fig. 146. Cryptococcus neoformans in a culture on agar (according to Conant).

The capsules of Cryptococcus neoformans contain mucopolysaccharides, and glucuronic acid.

The pathogenic agent of cryptococcosis has a low antigen activity. Antigen properties are absent in the strains with large capsules.

The fungus is pathogenic for laboratory animals; mice and rats are susceptible to infection with cultures and pathological material. Rabbits, guinea pigs, dogs and sheep are less suitable for infection.

The most successful method of infection is injection of the material into the brain or the anterior chamber of the eye. The mice die on the 5-8th day after intracerebral infection; death occurs earlier when the fungus is injected intravenously. Death occurs within several (3-6) weeks when intraperitoneal or subcutaneous injection is used.

The experimental infection is manifested locally or is of a general nature, taking a course similar to that of pseudo-tuberculosis or septicemia, with development of metastatic suppurations.

Laboratory diagnosis. Pus from fistulas, decayed material and pieces of tissue from the focal lesions, pus from absecesses and from under the edges of ulcerous lesions on the skin and mucosa, and the centrifugation precipitate of spinal fluid serve as test material.

In unstained preparations (in 10% caustic solution, physiological salt solution, mixture of alcohol and glycerol, etc.), the fungus appears in the form of round or, more rarely, ovate cells, coated with a clearly discernible double-contoured membrane. The size of the cells is 2-5 to 10-15 and even 20 μ with a single, slightly elongated bud. The fungus is nearly always surrounded by large capsules which are several times larger than the fungus and can be clearly seen with special staining methods.

Cryptococcus cultures can be isolated fairly easily from pathological material.

The serodiagnosis of cryptococcosis has not yet been adequately developed. The agglutination and complement-fixation tests are not of great diagnositc importance. Allergy tests are not used for diagnosis.

The method of detection of cryptococcus in the environment is basically the same as that for yeast fungi of the genus Candida.

Very useful is the biological method, the infection of white mice with test material with subsequent identification of pure fungus cultures, isolated from the focal lesions in the experimental animals.

The method of soil flotation with subsequent inoculation of mice can be used for the isolation of the fungus from soils: a suspension of soil in 100 ml physiological salt solution is made up in glass cylinders with constant stirring of the liquid with a sterile wooden applicator. 2-3 hours later, 5-10 ml of this suspension is sucked off from the top layer of the liquid with a pipette and 1 ml each injected into mice, which are killed in the 4-6th week after the injection. Pieces of liver

and spleen are then seeded on Saburo medium; the cultures in the positive specimens grow fairly rapidly.

Histoplasmosis

Histoplasmosis is a deep-seated mycosis mainly of the reticulo-endothelial system of humans and some animals.

The pathogenic agent of histoplasmosis is the fungus Histoplasma capsulatum, which has been described in two forms, a tissue and a culture form.

The tissue is monocellular, fairly small, usually round, more rarely pyriform, with a size of 2-4 μ and even less. The cell has a nucleus with a volume which is nearly half that of the cell itself, the membrane and capsule; the protoplasm usually sticks to the cell wall. Typical for the tissue form is the intracellular location of the fungus in the macrophages, giant cells in the reticulo-endothelial system: spleen, liver, lymph nodes and in the white blood cells.

The arrangement of the chromatin substance in the form of a crescent with slightly enlarged tips, the small size, the location in the cells and color, which are basically the same as with leishmania, is responsible for the great similarity of these microorganisms.

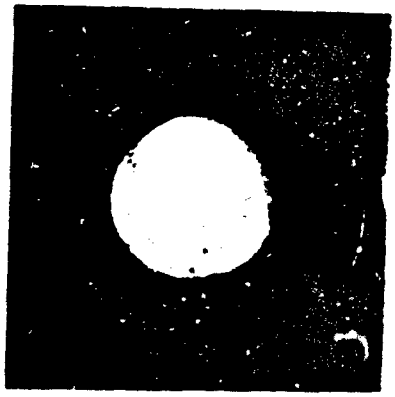

Fig. 147. Colony of Histoplasma capsulatum on agar.

Histoplasma capsulatum is dimorphous in cultures and is found in the yeast or fibrous form (Fig. 147), depending on the conditions of development. The yeast form on solid media grows in the form of fine, round, greasy-shining colonies like the yeasts. On some media, the colonies are wrinkled or tuberous. When such colonies are examined under the microscope, budding yeast cells pre-

dominate, which are arranged in chains and fibers (pseudomycelium). The cells have different sizes: the maternal yeast-like cell has a diameter of 2-4.5 μ, the bud is smaller, 1.5-2 μ; it remains connected with the maternal cell for a considerable time by means of a thin stalk. The yeast form resembles the tissue form of the fungus parasite.

Fig. 148. Microscopic pattern of the Van Bruisegem histoplasma in a culture.

The cultures with fibrous, mycelial form are velvety, flocculent, gray-brown with a dark, sometimes wrinkled membrane, with some degree of penetration into the the depth of the nutrient substrate. On Czapek medium, the colonies are downy. On liquid media it grows in the form of a velvety, white film with a rocketlike thickening of some cells. The mycelium in the cultures is branched, partitioned and has a diameter of 2-3 μ. The conidia are thick-walled, smooth or rough (Figs. 148, 149).

Fig. 149. Van Bruisegem histoplasma in tissues.

The chlamidospores are lateral or terminal; their shape is spherical or piriform, the surface rough or tuberous, the diameter varies from 7 to 33 μ; the contents are usually granular (some authors take such large cells as asci, others deny this). Histoplasma capsulatum grows well on solid and in liquid meat-peptone media on blood agar, on vegetables, farinaceous and egg media and on the synthetic medium of Capek with carbohydrates; addition of thiamine promotes the development of the fungus. It develops fairly well on Francis medium: veal broth 100 ml, rabbit or horse blood 80 ml, peptone 10 ml, glucose 10 g, sodium chloride 5 g, cystine or cystine hydrochoride 1 g.

The mycelium phase appears readily and develops fairly rapidly at a temperature under 30°, with improved aeration, on acid media (pH =

= 6.5), with low protein content, in nutrient media with low moisture content or when they dry out and during the aging process of cultures. The growth of the primary cultures of the fungus from pathological material can be observed on the 7-12th day, more rarely on the 25th day. Reinoculated cultures grow more quickly.

The pathogenic agent of histoplasmosis is pathogenic for white mice and rats, guinea pigs, dogs, particularly puppies and for monkeys, infection being carried out with either the yeast or the mycelium form of the fungus.

Laboratory diagnosis of histoplasmosis. Discharges from ulcers, taken from under their edges, pus, sputum, meningeal fluid, blood, puncture specimens from bone marrow, spleen and liver, contact smears from affected organs, urine, and more rarely, feces, serve as test material.

In view of the small size of the fungus, which is normally present in phagocyte cells, the microscopic examination of unstained preparations does not give good results. The preparations are stained by the Gram, Ziehl-Nielsen, Giemsa, Leishman, etc., methods.

Small, spherical, budding yeast-like cells are visible in the stained preparations. They appear to be surrounded by a bright aureole; their central part is much darker. The fungi are situated inside the cytoplasm of the microphages or free in the exudate. Their dimensions are 1-3 μ in diameter.

Examination by culturing has considerable advantages over the microscopic method; the isolation of the fungus culture attests to the presence of histoplasmosis disease, because healthy carriers of the fungus have not been detected. To make culturing more successful, antibiotics are added to the media; to avoid drying out and to limit the oxygen supply, the test tubes should be sealed.

Blood is first added to solutions of citrates or oxalates, then spread over the surface of agar media in Petri dishes. 5 ml blood can be mixed with 1 ml sodium citrate (C_6H_7Na) and the mixture left at room temperature for 2 weeks; the mycelium appears under the leucocyte layer in the erythrocyte precipitate. The procedure with oxalate blood is the same.

The most suitable materials for inoculation are puncture specimens from bone marrow, spleen, liver and also blood. Blood agar-agar and Saburo medium with glucose are best for obtaining cultures. The pathological material for inoculation should be used when completely fresh, otherwise the cultures do not grow.

Sputum which may contain mold spores and cells of Histolysma, is injected intraperitoneally into mice which are killed within 4 weeks; cultures are then prepared from the liver and spleen of the killed animals.

Of the serological reactions, the agglutination, precipitation and complement-fixation test is used in histoplasmosis. The agglutination test is carried out with serum from the patient and a suspension of killed yeast cells. Positive reactions in the patients are observed at low serum dilutions, 1 : 40, 1 : 80, rarely higher; their diagnostic value is not great.

The agglutination of histoplasmin, adsorbed on colloidal particles, has been proposed in recent years.

The precipitins appear early in the disease, during the 2-3rd week, when the complement-fixation test is still negative. The precipitation test is carried out by the usual method. Histoplasmin is used as antigen. This test gives more exact results which are useful in the early stages of the disease.

The complement-fixation test is considered to be the most specific.

It is positive only 2 months after the beginning of the disease, when the precipitins have already disappeared. The yeast phase of the fungus, triturated cells of Histoplasma capsulatum or histoplasmin are used as antigens. The titers of the positive tests are sometimes achieved with serum dilutions of 1 : 128. The test remains positive for many months.

The positive allergic histoplasmine tests appear earlier than the serological tests (agglutination, precipitation, complement-fixing) and remain positive in the cured patients for many years.

Methods of detecting H. capsulatum in the environment.

1 mg soil is placed into a sterile test tube, 9 ml physiological salt solution is added and the test tube shaken, then 8000 YeD each of penicillin and streptomycin per 1 ml is added. 1 ml of the suspension is injected subcutaneously into each of seven 4-week old mice. The mice which die within the first week, are not counted (histoplasmosis is not the cause of their death). The other animals are killed after different intervals and cultures made from their liver and spleen. The percentage of cultures obtained from the mice which are killed within 2-4 weeks, is 96-98 (Larsh, Hinton, Furkulov).

To detech H. capsulatum directly in the soil, mice are placed for 7 days in a vessel containing the soil, from which it is desired to isolate the histoplasmosis organism. The mice are then kept for 4 weeks in clean jars and then killed. Cultures are made from the liver, spleen and lungs (Hinton, Larsh and Silberg). In this manner it has been possible to detect Histoplasma capsulatum in many soil samples from endemic areas and also from samples which had been artificially contaminated.

North American Blastomycocis

The North American blastomycosis is a deepseated chronic disease which is encountered in two forms, cutaneous and visceral.

North American blastomycosis is a chronic disease; sometimes it is

difficult to diagnose, particularly with the cutaneous form of the disease. The immunological changes consist in an accumulation of agglutinins and complement-fixing antibodies, which is most obvious in the generalized and active forms of the disease.

A specific allergy develops in North American blastomycosis, which is detectable by intradermal injection of vaccine from the yeast phase of the fungus or of blastomycin.

Lung infections have been found in dogs as well as in humans; infection of humans through them is considered a distinct possibility.

The pathogenic agent of North American blastomycosis is Blastomyces dermatidis. A peculiarity of the fungus is its dimophism. In the tissues it is found in the form of yeast-like cells and in cultures in the mycelium and yeast form. Blastomyces dermatidis in tissues has the form of circular and elliptical cells with a diameter of 8-10 μ; sometimes they attain 20 μ; it proliferates by budding, producing a single bud. Sometimes, instead of forming a bud, the cell is stretched and assumes the shape of a bell clapper. The dimorphism of the fungus in cultures is determined by the culturing conditions and the composition of the nutrient media (Figs. 150, 151, 152).

When the fungus is grown at 37°, it develops in the yeast form; on the same media at room temperature it gives mycelial cultures. The yeast form on solid media gives white, shiny, greasy-looking circular colonies with creamlike consistency which do not adhere tightly to the nutrient medium.

When the colonies mature, they become striated and corrugated, very similar to the cultures of yeast fungi. Under the microscope they show almost exclusively round budding cells of different sizes (3-7 μ); in addition, but by no means regularly, elongated budding cells with a size of 3 × 6 μ are found, a kind of suggestion of the possibility of form-

ing a mycelium (Fig. 153).

The mycelium form is obtained on the same media at room temperature. The colonies are first round and smooth, and yeast-like. Later on, they are covered, first with a white, then yellow-brown light down, with short coremias which give the culture a thorny appearance. Under the microscope such cultures show, in addition to the round budding yeast cells, a profusion of long and short partitioned fibers with numerous lateral conidia with round, ovate, sometimes piriform shape and a diameter of 3-5 μ. In old cultures one finds large numbers of chlamidospores with a size of 7-18 μ.

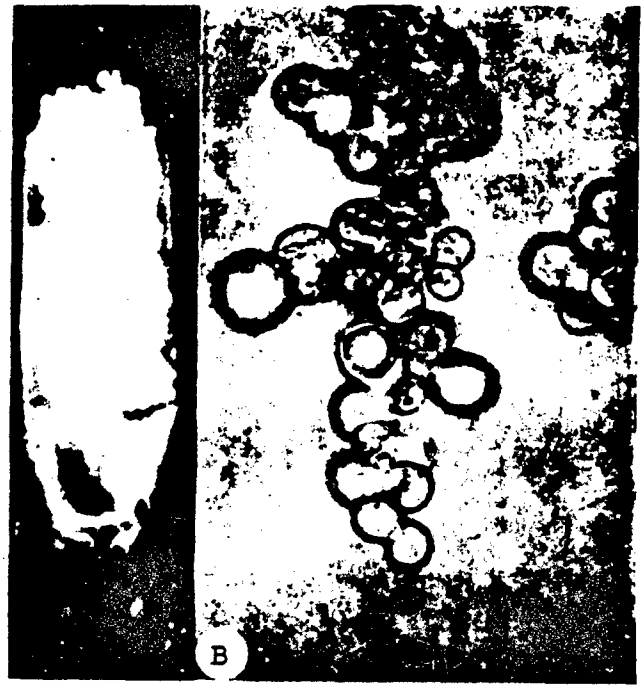

Fig. 150. Blastomyces dermatidis. A) Culture on agar; B) microscopic appearance.

The mycelium form is easily transformed into the yeast form when grown at 37° and vice versa. The more acid media (pH = 5.5-6.5) favor the mycelium and the more alkaline media (pH = 7.5-8.5), yeast form; the fibrous form predominates in liquid media; in the tissues of exper-

imentally infected animals, conversely, the yeast form predominates.

The fungus is pathogenic for laboratory animals and also for monkeys and dogs. When mice are infected intraperitoneally, granulomatous eruptions are seen within 3 weeks on the peritoneum, nodes in the liver, spleen, lungs and lymph nodes and the fungus can be detected by culturing and microscopically.

Laboratory diagnosis. Pus, scrapings from under the edges of ulcers, puncture specimens from soft subcutaneous abscesses and lymph glands, necrotic material and biopsy specimens from infection foci, spinal fluid, sputum, urine, puncture specimens from bone marrow or prostate discharge serve as test materials.

The material is examined under the microscope unstained in

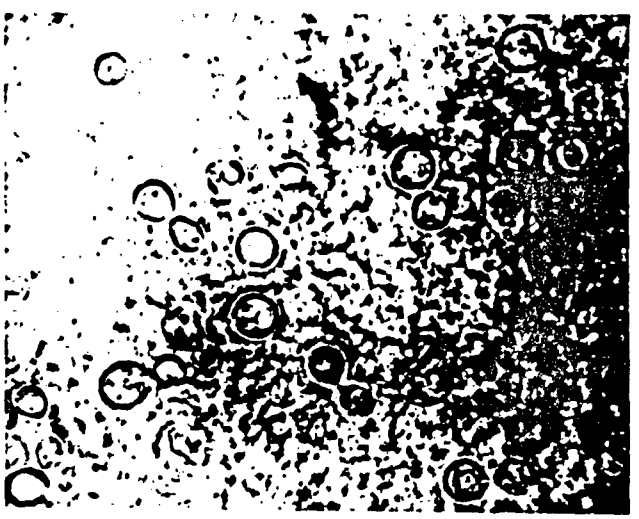

Fig. Blastomyces dermatidis in pus.

drops of 10% caustic heated on a glass slide at high magnification. The smears are stained by the Romanovskiy-Giemsa, Wright and Gram method; the microscopic examination of the stained preparations is carried out with an immersion system.

In pathological material and in tissue section, the fungus appears in the form of fairly large, budding cells, surrounded by a thick,

chitin-containing, sort of double-contoured membrane. The size of the cells is 3 to 25 μ, but the most frequent size is 8-15 μ. The buds, as a rule, are single, attached to the maternal cell by a wide base. Cells which are puffed up at one end are sometimes found. The fungus elements are either solitary or arranged in groups, sometimes in the form of short chains.

Fig. 152. Culture of Bl. brasiliensis on agar.

The cells are more clearly visible in the stained preparations with the immersion system; their color varies depending on their age; they are gram-positively stained, while gram-negative forms are only rarely seen.

The tissue forms of Bl. dermatidis differ greatly from those of other deepseated mycoses.

Thus, they differ from the yeast fungi of the genus Candida by heir circular shape and the absence of a pseudomycelium; from Crypto-

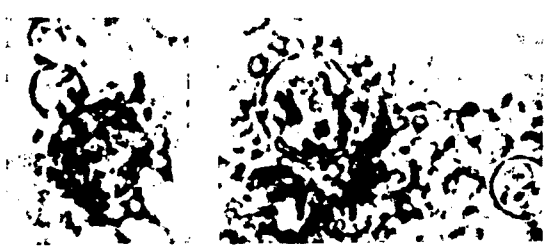

Fig. 153. Microscopic appearance of Bl. brasiliensis in culture.

coccus neoformans by the absence of the broad slimy capsules; from Blastomyces brasiliensis by the presence of a single bud on a fairly wide base (in Blastomyces brasiliensis the budding is multiple) from Histoplasma capsulatum by the large dimensions and the extracellular position; from Coccidioides immitis by the absence of spherules with

endospores.

For obtaining cultures, blood media, Saburo medium, brewer's wort-agar, or sugared meat-peptone broth are used. The cultures are kept in the thermostat and at room temperature. The growth appears sometimes fairly early, on the 3-4th day; sometimes, however, the fungus develops slowly, a noticeable culture being visible only in the 2nd or 3rd week.

Rapid growth of the fungus is observed on media, containing 5-10% fresh animal serum, 1% starch, 1.5% agar at pH = 7.2-7.3 and growing at a temperature of 35.5°.

By intraperitoneal inoculation of white mice with pathological material with subsequent inoculation of liquid and solid media with a piece of granulating growth on the peritoneum, pure cultures are more easily obtained.

The serological diagnosis is achieved by means of agglutination and complement-fixation tests; the latter gives the most specific results.

To detect Bl. dermatidis in the environment, the same methods are used as for the detection of the yeast fungi of the genus Candida. The cultures must be maintained at 25-30° to detect the mycelium and at 37° to detect the yeast phase of the fungus. The identification of the isolated fungi is carried out by the usual method.

South American Blastomycosis

The South American blastomycosis is subdivided into several clinical forms: 1) skin-mucosa affections; 2) lymphangoitic type; 3) visceral affections; and 4) mixed forms of the disease. The pathogenic agent of South American blastomycosis is the fungus Blastomyces brasiliensis. Tissue and culture forms are distinguished; the latter, depending on the growth conditions, are subdivided into yeast-like and mycelial forms.

The tissue forms of the fungus have the appearance of large, round yeast-like cells with clearly visible thick walls; their diameter in various stages of maturation varies from 10 to 30-60 μ. The fungus proliferates by budding which is multiple in contradistinction to that of Blastomyces dermatidis. The buds are ovate or round and of different sizes, with diameters of 0.5 to 6-10 μ. The fungus grows on normal media containing carbohydrates, on blood and serum agar, on glycerol carrots and also on synthetic media containing carbohydrates and amino acids.

In cultures, the fungus is found in the yeast-like and mycelial form. The yeast form is obtained by growing of the fungus at 37°, the colonies are smooth or cerebriform, similar to of yeast fungi cultures. Under the microscope they appear as elliptical or circular cells of farily large size, with single or multiple buds and without them. They are very similar to the tissue forms of the fungus. The mycelium form appears when the fungus is grown at 25°. Month-old cultures are rinkled, covered in the center with a grayish-white, later yellow down. Under the microscope, a branched mycelium and oval conidia, sitting on the sides of the mycelium fibers are seen; the size of the conidia varies within the limits from 3 to 5.5 μ in diameter; clusters of round spores are found.

The viability in clutures is similar to that of Blastomyces dermatidis.

Both forms of the fungus are pathogenic for guinea pigs and white mice. When guinea pigs are infected in the testes, orchitis with subsequent suppurating softening and involvement of the local lymph nodes develop. White mice are infected intraperitoneally. 4-5 weeks later, small blastomycetic nodes develop on the walls of the peritoneal cavity, on the mesenterium, the diaphragm, and in the liver and spleen.

Laboratory diagnosis of South American blastomycosis. Pus, sputum, scrapings from the infection foci on the skin and mucosa and biopsy and autopsy tissue from the infection foci and puncture specimens from the spleen serve as material for examination.

When the pathological material is examined under the microscope in unstained preparations (10% caustic, alcohol and glycerol), single cells with thick walls and buds of different size are observed.

The pathological material is used to inoculate sugared meat – peptone media, Saburo medium and blood and serum agar. Addition of antibiotics protects the cultures against bacterial contamination. The cultures are grown at room temperature and at 37°. Typical cultures which are pathogenic for laboratory animals, confirm the nature of the disease.

The infection of animals with the pathological material leads to the development of pathological processes within 4-5 weeks; the detection of the typical fungus forms in the tissues and the growing of cultures which correspond in appearance to Blastomyces brasiliensis, are valuable aids for the diagnosis of mycosis.

Of the serological tests, the complement-fixation test can be used but its positive results are sometimes of a collective nature, typical for all deepseated mycoses. The allergy tests are fairly specific, particularly with allergens from the tissue forms (pus from experimental orchitis).

For determining the presence of Blastomyces brasiliensis in the ambient medium, in objects in close contact with the patient, basically the same methods are used as for the detection of yeast fungi of the genus Candida. The cultures are grown at room temperature to develop the mycelium form and at 37° in the thermostat for the yeast phase of the fungus. The identification of the isolated cultures is carried out

by the usual method.

Coccidial Mycosis

Coccidial mycosis occurs in two forms: a primary or acute and a secondary or chronic form.

The primary form is characterized by a relatively short incubation period (7-14 days); it begins with indisposition, headache, symptoms of a slight cough, pharyngitis and a feverish condition. Pains are noted in the knee and the small joints. Pulmonary symtoms are often observed, reminiscent in some patients of tuberculosis. The primary form of coccidial mycosis usually lasts 2-3 weeks, an allergy developing in 76% of cases, whereas in the others this appears later; different antibodies can be detected.

The secondary form is characterized by a prolonged and severe course, profound disorders of the affected tissues and organs with lysis of the infection focus, formation of fistulas, asthenia and different degrees of emacitation.

The pulmonary lesions are marked, the infiltrations of these organs resembling tuberculosis. The lymph nodes are nearly always involved: the mediastinal and regional lymph nodes. The lesions of the bone tissue are highly typical: the ribs, clavicles, seapulae, vertebrae and the small bones of the hands and feet. Lesions of the meningae and the gastrointestinal tract have been described.

The skin form of coccidial mycosis appears in the form of a nodular infiltrate which often becomes necrotic, forming ulcers with papillomatous growth. The skin sometimes hardens as with sclerodermia, and the resemblance is all the more striking because the foci can retain this clinical pattern for long periods without important changes.

During this morbid process, agglutinins, precipitins, and complement-fixing antibodies are formed. The most obvious specific sensitiza-

tion of the organism is the allergy. It appears fairly early, within 3-4 weeks of the infection and remains noticeable almost for life but disappears in some patients with profound exhaustion of the organism. The allergy which occurs in cases of coccidial mycosis is considered to be protective, preventing the development of the secondary disease and of new infections.

Characteristic chronic forms, similar to coccidial granulema, have been detected in the USSR (A.N. Araviyskiy); their pathogenic agents differ from the typical Coccidioides immitis by their morphological and histological attributes and their low pathogenicity.

The soil in endemic areas is considered to be the source of the infection, the dust, the medium through which the fungus is spread. Cultures of Coccidioides immitis have been obtained from the soil by several authors (Mayer, Davis, Smith, Emmons, etc.).

Small rodents are the natural reservoir of the coccidial fungus.

Spontaneous cases of coccidial mycosis have been found in cattle, sheep, pigs and also cats and dogs. Squirrels, kangaroos and monkeys can be affected.

The pathogenic agent of coccidial mycosis is Coccidioides immitis.

The fungus displays dimophism, occurring in two forms, the tissue and culture form (Fig. 154).

Fig. 154. Colony of Coccidioides immitis on agar (cultured at [illegible]°, mycelial phase).

In the infection foci of animals and humans and in pathological material the fungus has the form of spherules with a size of 20-40 to 120 μ and over in diameter.

The spherules are of spherical shape, their wall is double-contoured, with a thickness of 2-3 μ, containing large num-

bers of small spores, which enter the tissues when the walls of the mature spherule burst. The endospores are round or slightly ovate, their walls are thin, hardly visible, and they contain a nucleus, vacuoles and granular inclusions (Fig. 155).

Fig. 155. Microscopic appearance of Coccidioides immitis in a culture.

During the process of successive division of the nucleus and protoplasm, the endospores are transformed into young spherules, which grow to large size, with an abundance of endospores in them (Fig. 156).

On Saburo medium the young colonies have a yellow-cream color, the consistency of soft leather, being covered fairly soon with an initially loose, later more dense short aerial mycelium, their surface becomes velvety, with a light-brown shade and during the maximum spore formation of the aerial mycelium, the colonies are covered with a downy deposit.

The surface of the colonies is frequently even or slightly striated by shallow furrows, more rarely corrugated or craterlike. The aerial mycelium does not always cover the culture with a dense gason, sometimes being present only at the margins or in foci in different areas of the colonies.

The mycelium with intercalated chlamidospores predominates in young cultures growing on dense media. The old cultures are rich in chlamidospores, arthrospores and patches of mycelium.

When the coccidial fungus is grown in the thermostat at 37°, smooth, leathery cultures with little aerial mycelium develop. Conversely, at room temperature, downy and powdery cultures with copious aerial sporophores develop.

The fungus is aerobic but retains its viability under anaerobic

conditions. It is pathogenic for mice, guinea pigs and rabbits, infected with the tissue or culture forms, particularly with intratesticular infection. Mice are suitable for intraperitoneal infection. Of the culture forms, the arthrospores are particularly pathogenic, inhalation of a few cells being sufficient to cause infection.

The viability of coccidial fungi as fairly great: they can withstand freezing and thawing, prolonged cold storage (110 days) and heating to a maximum temperature of 60-65°.

Boiling of cultures and pathological material for 20 minutes and drenching with a 2% solution of bleaching powder or 10% formalin for 2-3 hours kills the fungus.

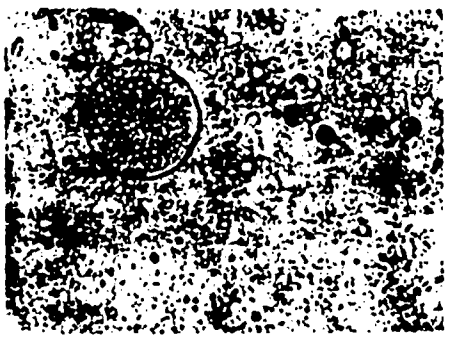

Fig. 156. Coccidioides immitis spherule in pus.

Laboratory diagnosis. The material for examination is pus, sputum, blood, spinal fluid, and biopsy tissue from the infection focus. The microscopic examination is carried out with unstained preparations, in a drop of 10% caustic, a mixture of alcohol and glycerol or in Lugol solution of twice the normal concentration. The microscopic diagnostic is based on the spheruli which are typical for the coccidial fungus. They are visible at low and high magnification, their numbers varying with the intensity of the pathological process and the nature of the test material. They are spherules with regular spherical shape of dif-

ferent size with a clearly visible membrane, and containing endospores in different stages of maturity. The fungus nature of the spherules is very convincingly demonstrated by the germination of the endospores in physiological salt solution on glass slides within a period of 8-12 hours.

With the usual staining methods (Gram, Ziehl-Nielsen, Romanovskiy-Giemsa, etc.) the spherules are weakly stained, sometimes only their outlines being visible.

The growing of a culture is considered to be the most valuable diagnostic method. Protein media, Saburo medium, meat-peptone agar or broth with glucose (pH of the medium 7.2-7.4) are most suitable for obtaining cultures of coccidial fungus. The growth of the fungus in the primary cultures is slow, appearing sometimes within a period of 3 weeks to 2 months.

The work with cultures requires the observation of particularly strict precautions in order to avoid the possibility of inhalation infection. Inoculations are carried out in test tubes; Petri dishes cannot be used because of the possibility of spores being dispersed in the air. During the examination of cultures in test tubes care must be taken to avoid dust development and inhalation of arthrospores. Prior to examination, the cultures should be drenched aseptically with sterile physiological salt solution and the culture material transferred in a drop of this from one test tube to another (to avoid contamination of the air with spores). Before carrying out the microscopic examination, it is recommended to pour 10% formalin solution over the culture, leaving it for 10-15 minutes and then to examine it only under a cover glass; the formalin does not alter the morphological structure of the fungus.

To obtain pure cultures of coccidial fungus, the biological method

of infecting first male guinea pigs or mice (intraperitoneally) or chick embryos with the pathological material is used. Pure cultures of the fungus can then be isolated more easily from infection foci.

The serological diagnosis is achieved by means of agglutination, precipitation and complement-fixation tests.

The agglutination test is preferably carried out with killed suspensions of cultures; the precipitation test with polysaccharide antigens and the complement-fixation test with coccidioidin which is also suitable for the precipitation test.

The allergy skin tests are carried out with coccidioidin in a dilution of 1: 1000, using a stronger dilution of 1 : 100 when the test is negative. The reactions are observed for 48 hours; intense reactions (erythema, swelling, papula at the injection site) which sometimes last for several days, attest to a specific sensitization of the organism.

Detection of coccidiosis fungus in the environment. To detect coccidiosis fungus in air, dust, or dry wastes, inhalation infection of mice which are the most susceptible animals is employed, using a special device for this purpose.

The inhalation device consists of a glass cylinder with a diameter of 5.5 cm with 4-5 glass tubes with a diameter of 3 cm connected to it at a height of 4 cm. The test sample of soil, dust, dry excrements, hay, etc., is spread on the bottom of the cylinder and the mice are placed into the lateral tubes with the head downwards into the main cylinder; all the openings, those of the main cylinder and the side tubes are sealed with cottonwool plugs.

The mice, being unable to turn their heads sideways in the test tubes, are compelled to inhale the dustladen air for several hours. After this, the mice are returned to their usual cages; after different intervals, they are killed and the foci of infection in the different

organs subjected to mycological examination (microscopy, culturing, passages through mice).

To isolate the coccidiosis fungus from soil, 1 g of the test material is suspended in 9 ml of physiological salt solution and shaken energetically. Dilutions of 1 : 100 and 1 : 500 are then prepared and 0.5 ml of the liquid seeded into each of 2 identical Petri dishes with solid nutrient medium. The cultures are kept at room temperature (18-20°) for 10-12 days; the suspected colonies are seeded on solid media (Saburo) in test tubes to obtain a pure culture for the further identification of the fungus and for determining its pathogenicity on mice.

Sporotrichosis

Several types of sporotrichosis lesions are known in which the lymph glands, the skin, mucosa, the bone system and the internal organs are involved. As a rule, these lesions are of a severe nature, which are accompanied by various general symptoms and are manifested in a different degree in different individuals.

The sporotrichoses are subdivided into primary, focal and secondary infections. Lesions of the lymph nodes are the most frequently occurring form of sporotrichosis. The visceral forms of sporotrichosis, according to Conant, are rarely encountered. Pyelonephritis, orchitis and epididymis is observed.

The pathogenic agents of sporotrichosis belong to the thread fungi, the hyphomycetes. They do not have complete fruiting organs, the sexual process being absent in them.

Sporotrichum is dimorphous. In the pathological material they are found in the form of yeast-like, round, spindle- or clubshaped formations and sometimes in the form of yeast-like budding cells with a diameter of 1-2 to 6-8 μ (Fig. 157). The tissue forms of sporotrichum-particularly the spindleshaped ones, are spindle shapes which appear in

the stained preparations (Gram, Leishman, etc.) surrounded by a bright ring of slime.

Sporotrichum is aerobic, grows well on normal nutrient media, on various vegetables, particularly when they have previously been treated with an aqueous solution of glycerol (4%), peptone (1%), glucose (3%), tartaric acid (0.1%) (100 ml).

Of carbohydrates, sporotrichum can assimilate various organic acids, di- and monosaccharides, polyhydric alcohols and some decompose starch. Normally they do not coagulate milk and do not liquify gelatin.

Fig. 157. Sporotrichum in pus.

The resistance of sporotrichum to the action of physicochemical factors and disinfectants differs little from that of other pathogenic fungi (yeast-like, dermatophytes); boiling kills them fairly quickly in cultures and in pathological material.

The antigen properties of different sporotrichum species and their relationships have not yet been adequately investigated. When animals are immunized with killed cultures of the yeast-like and mycelium phase of sporotrichum, agglutinins, precipitins and complement-fixing antibodies are formed.

Sporotrichum grows well at 37° in the media normally used for pathogenic fungi (pH = 6.5). Different strains begin to grow at different times, usually on the 4-6th day after inoculation. Mature cultures develop within 20-25 days. They are fairly large, sometimes covering

the entire surface of the nutrient medium in the test tube or flask.

White, grayish yellow, brown and black colonies are found, sometimes with a greenish or rusty shade. The color of the colonies is a farily constant attribute of some species of sporotrichum. The surface of the colonies is velvety-fluffy or powdery, more rarely, downy cultures are found. Sporotrichum has a branched, partitioned mycelium whose diameter varies from 1-2 to 6-8 μ and over. The conidia of sporotrichum also varies in size, their length being 2 to 6-8 μ, the width 1.5-3 μ. Elliptical, circular, piriform, rodshaped and fusiform cells., arranged at the sides of the mycelium or in clusters of 3-5-10 spores on the tip or along the sides of the mycelium, are found. In mature cultures of sporotrichum, one encounters intercalated chlamidospores with clearly visible membrane and granular contents. Mice and rats and, less often, guinea pigs and monkeys are used for experimental infection with sporotrichum.

The most frequently occurring pathogenic agents of sporotrichosis are:

1. Sporotrichum (Rhinocladium) Gougeroti. The young cultures on Saburo medium are black, strongly striated, convex, shiny and yeast-like. When they grow older they lose their glossiness along the margins appear spots of gray down, which covers the entire colony densely in old cultures. In subcultures, the young cultures are black, shiny and yeast-like, the old ones dark-gray and downy. Ovate and elongated cells predominate in the cultures of the yeast phase with a size of 1-2 × 4-8 μ, round cells with a size of 3.5-4 × 4-7 μ, which are budding and sometimes connected with each other by a lorg cell (Fig. 158) are also seen. Fibers with a diameter of 2-2.5 μ with numerous oblong spores 2-2.5 × 7.5 μ predominate in the downy colonies, which are solitary or collected into racemose clusters situated on short side growths of the aerial

mycelium.

2. Sporotrichum (Rhinocladium) Schenki-Beurmanni. On Saburo medium the colonies are elevated, dull, pale yellow; later on they become

Fig. 158. Microscopic appearance of Sp. Schenleinii in culture.

wrinkled, cerebriform, slightly downy, with chocolate-brown color. On broth it forms a wrinkled film. On gelatin, the type Schenk grows well, while the type Beurmann does not grow. The type Schenk ferments lactose, the type Beurmann does not ferment it.

A branched, colorless mycelium with a diameter of 2 μ is seen under the microscope. The spores are ovate, brown, 3-5 × 2-4 μ, on thin sterigmas, mainly at the tips of the fibers. The cultures are pathogenic for laboratory animals.

3. Sporotrichum (Rhinocladium) Jeanselmei. The colonies are initially white, later brown, smooth, sometimes downy. On broth it forms a white film. On potato, a white or dark deposit with powdery fluff. Under the microscope, a branched mycelium with a diameter of 2-2.5 μ, oval spores with a size of 3-4 × 2-3 μ on sterigmas are grouped around the tips of the mycelium. The cultures are pathogenic for white mice.

Laboratory diagnosis. Examination is carried out on pus from fistulas and ulcerous lesions, scrapings from under the edges and the base of ulcers, puncture specimens from occluded nodules, biopsy tissue and

autopsy material from focal lesions.

The examination of unstained preparations in 10% caustic or alcohol and glycerol is not always successful and only the presence of the mycelium forms of the fungus and the characteristic conidia is sufficient grounds for suspecting the presence of sporotrichum.

The fungi are much easier to detect in gram-stained smears. Most often the fungus appears in the form of small conidia with a size of 2-5 μ, which have distended centers and acuminate tips. They are gram-stained with a pinkish-violet color, covered with a thin layer of slime and situated within giant cells and polynuclear cells.

The mycelium is stained a pinkish-violet; the pigmented strains are more difficult to stain.

The pathological material from exposed surface lesions should first be treated with antibiotics. Blood agar, Saburo medium, brewer's wort-agar, glycerol meat – peptone agar, carrots with glycerol and tartaric acid are inoculated. The growing of the culture is carried out room temperature (20-25°) and in the thermostat at 37°.

Intraperitoneal or subcutaneous injection of the pathological material causes inflammation of the peritoneum and produces orchitis. The typical cigar-shaped cells of the fungus are found in large numbers inside and outside of the phagocyte cells. They are also clearly visible in the gram-stained preparations. The fungus is found inside and outside of cells.

Of the serological tests, the agglutination and complement-fixation test with serum from patients and sporotrichum antigen are used for diagnosis. The value of the serological tests in sporotrichosis has not yet been adequately evaluated. The quality of the antigens is variable; standard preparations are not available.

The positive skin allergy tests do not have any diagnostic import-

ance. The detection of sporotrichum in the environment (soil, plants, air, etc.) is achieved by inoculation of solid media containing antibiotics and subsequent identification and verification of the pathogenicity of the isolated cultures.

ACTINOMYCOSES

P.N. Kashkin, Professor, Honored Worker in Science

The actinomycoses are caused by ray fungi, the actinomycetes (Actinomyces).

Actinomycosis effects all tissues and organs; its primary forms are usually connected with lesions of the subcutaneous tissue, the oral mucosa and, more rarely, the lungs, pleura and intestine.

Morphology of the actinomycetes

All actinomycetes have a true, branched mycelium, growing in the substrate or aerial, most of them with fruiting branches and spores in the aerial parts of the colonies.

The fibers of the mycelium are straight or undulatory, with a length of 50 to 600 μ and a diameter of 0.2 to 1-2 μ. Transverse partitions are normally not observed in actinomyces; in contrast to the threadlike fungi, the mycelium is not partitioned.

The mycelium of actinomyces is branched, the branches developing from a small bud which is gradually drawn out into a rod and then a thin thread with side branches.

The proliferation of the actinomyces is achieved by spores, decomposition of the mycelium and budding.

Biology of actinomyces; culturing characteristics

The culture characteristics of actinomyces are very diverse. This diversity concerns the sizes and shapes of the colonies, their relation with the nutrient substrate and particularly the coloring of the

colonies.

The young colonies are thinner, flat and circular, and can often be lifted off completely from the surface of the medium with a loop or scoop. The mature colonies are smooth or striated, wrinkled or tuberous, with a soft, waxy or mealy consistency; they are more tightly connected with the nutrient medium by their long or short dense or loose outgrowths. Actinomyces colonies attain a size of 1-1.5 mm. The surface of the colonies varies: in some, it is smooth, in others velvety, downy or powdery. The latter is connected with the formation of the aerial mycelium and the spores. The pigmentation of the colonies is a highly typical characteristic which can be used for differentiating the actinomycetes. Dark and light-violet, blue, purple, red, orange, yellow, green, brown, black, gray and white colonies with different shades are encountered.

The microorganisms of the class actinomyces use different mineral and organic substances containing nitrogen and carbon. The actinomyces are divided on the basis of the type of respiration into aerobic and anaerobic.

The actinomycetes are pathogenic for animals, humans and plants.

S.F. Dimitriyev, on the basis of his comprehensive observations, described the following species of pathogenic actinomycetes.

1. Actinomyces albus with several variants, formed as a result of lysis processes. According to N.A. Krasil'nikov, this species is the most widespread and has the greatest number of subspecies. The mycelium is thin, straight, rarely branched; the sporangia are helical, single or in clusters; the helices have 5-7 turns, the spores are spherical. They are efficient in assimilating organic and inorganic nitrogen compounds. They do not form pigments.

The colonies are white or even snowy white, smooth, wrinkled or

tuberous with cartilaginous consistency, clinging to the substrate. The aerial mycelium is downy or powdery, forming a dense or patchy cover on the colonies.

2. Actinomyces violaceus with violet and pink variants. The mycelium grows in the substrate, is not partitioned, slightly curved, branching is rare, in old cultures it is decomposed into fragments of different length and a granular mass. The aerial mycelium is short and does not develop on all media. The sporangia are helical with 2-3 wide, counterclockwise turns. The spores are spherical or elliptical and are formed by fragmentation.

The young colonies are reddish in color, later on turning blue-violet or purple. The pigment is excreted into the medium which assumes the corresponding color. The aerial mycelium is developed to a different degree depending on the strain and is absent in some. It liquifies gelatin slowly, does not coagulate milk, inverst saccharose rapidly, reduces nitrates to nitrite and has an antogonistic activity to many ungi (N.A. Krasil'nikov).

3. Actinomyces madurae vincent (Berestnev, 1897). The threads of the mycelium are thin (0.4-0.5 μ), decomposing in old cultures into fragments of different length. The sporangia are usually straight, long, helical; the spores are cylindrical, elongated or elliptical. The colonies are red, pink or red-brown, wrinkled or tuberous, bare or covered with a pinkish-white aerial mycelium. It grows well on the usual media and on decoctions of straw or hay. It liquifies gelatin slowly, does not invert saccharose, does not curdle milk, forms ammonia. It is aerobic.

4. Proactinomyces Israeli (Kruse) (N.A. Krasil'nikov, 1941). Threadlike branched mycelium, decomposing fairly soon into rodshaped cells with different length – 3-10 × 0.6 μ; short rods and cocci, bul-

bous and spherically inflated cells are encountered; it does not form spores. The cells are gram-positive and not acid-resistant.

The colonies have a viscous consistency, are colorless, smooth or slightly tuberous. It does not peptize milk, does not liquify gelatin, ferments glucose and lactose only weakly. Conditionally aerobic.

LABORATORY DIAGNOSIS OF ACTINOMYCOSIS

Material for Examination

The pus from fistulas in various tissues and organs, sputum, urine, puncture specimens of softened infiltrations, spinal fluid, and more rarely, biopsy tissues and pieces from the focal lesions of cadavers are examined. Typical for actinomycosis is the presence of nodules, whose examination makes possible a correct laboratory diagnosis of actinomycosis.

Microscopic examination

Suspected clumps from the pathological material are transferred to an object glass in a drop of caustic (10-20%), slightly warmed, then covered with an object glass and examined under the microscope. Stone-like lumps, which crunch under pressure like sand, are very characteristic for actinomycosis.

Raylike formations with a dense, homogeneous center, surrounded by numerous raylike growths are usually seen under the microscope at low magnification. At high magnification of the microscope, the structure of the nodes is seen more clearly: in their center there is a matted accumulation of thin (1-3 μ) mycelium, along the periphery there are strongly striated club shapes with thin fibers or chains consisting of elongated fragments. Chains consisting of spores, fragments of threads and spores without clubs and nodes are found in pathological material.

The methods of Gram, Ziehl-Nielsen, etc., are used for the staining of actinomyces in pathological material. The actinomyces threads

are usually gram-positive, more rarely, pinkish-violet. The spores of the actinomycetes are stained more intensely, becoming pink, the mature spores being stained with difficulty and the largest of them are nearly always washed off from the object glass during the repeated rinsing of the preparations. Granules and nodules are not always detected in the gram- or Ziehl-Nielsen stained smears.

In the smears, stained by the method of Ziehl-Nielsen, the actinomyces threads are acidophilic, blue and the spores pink. Entirely acid-resistant strains of actinomycetes with red threads are sometimes found.

The nodules cannot always be detected in actinomyces lesions. Furthermore, they do not develop in all stages of the disease and are not typical for every subspecies of the ray fungus.

Nodules are not always found in the early stages of the disease and in acute forms with abscesses; very valuable for diagnosis is the detection of a thin, unpartitioned, branched mycelium. Gram-stained threads are dark-violet and clearly visible.

Isolation of cultures

The following is essential if actinomyces cultures are to be isolated successfully: 1) aseptic sampling of pathological material, preferably from deepseated and hidden focal lesions; 2) centrifuging of pus, urine, sputum in antibiotic solutions (100-200 YeD in 1 ml); 3) washing of the precipitate with physiological salt solution.

Blood and serum meat-peptone agar, glycerol agar and broth and Saburo and Czapek medium are inoculated with the pathological material. The cultures should be grown under aerobic as well as anaerobic conditions.

To obtain anaerobic actinomycetes cultures, the washed clumps of pus, sputum and nodules are seeded into 6-7 cm high columns of melted 1 and 2% sugar agar at a temperature of the medium of 45°. The culture

material is introduced in layers: close to the bottom of the test tube, in the middle and on the surface of the column and the medium then sealed with a 1-2 cm high layer of sterile vaseline oil or paraffin oil; growing is carried out at 37°. The growth appears within 1-2 weeks; the colonies are irregularly striated, polyhedral, sometimes coarsely granular with pale-yellow color.

Infection of animals with pathological materials and cultures is by no means always successful. A generally valid model of the actinomycetic process with nodules in laboratory animals has not yet been obtained.

Serological examination

The complement-fixation test gives the least ambiguous results; polyvalent actinolysate is used as antigen. The percentage of positive results in cases of actinomycoses is 80.

Nutrient Media for Fungi

Selective (experimental) Saburo medium: 4 g maltose (glucose), 1 g peptone; 1.8 g agar-agar; 100 ml distilled water. 18 g finely cut agar is placed into the flask, 1000 ml distilled water added and left to stand for 30 minutes to let the agar swell; 40 g maltose or glucose and 10 g peptone are added, and the whole boiled for 30 minutes. The medium is filtered through paper, then poured into sterile flasks and test tubes and sterilized at 110° for 15 minutes. Saburo medium is a good medium in which the fungus colonies develop in the most typical and luxurious manner.

Saburo peptone agar. 3 g peptone, 1.8 g agar-agar, 100 ml water; boiling for 30 minutes. The hot mixture is filtered through several layers of cotton wool and gauze or (preferably) paper, filled into test tubes and flasks and sterilized at 120° for 30 minutes. This medium with high peptone content is recommended mainly for growing Achorion Schönleini, which forms typical and well developed colonies on it.

Saburo medium "for preservation of dermatophytes": 1 g of peptone, 1.8 g agar-agar, 100 ml water. Sterilized at 120° for 30 minutes. Being free of carbohydrates and having a low peptone concentration, it deserves special attention because pleomorphic alterations occur much more rarely in this medium and when they are encountered, the pleomorphous down spreads more slowly.

Brewing wort-agar: 1000 ml brewing wort, 18 g agar-agar. Brewing wort from the brewery is diluted with distilled water to 7% carbohydrate content according to Balling, 1.5% agar in the summer and 1.8% agar in the winter is added, sterilized in the autoclave at 110° for 10 minutes, filtered through gauze and cotton wool in the autoclave, filled into a sterile container and again sterilized at this temperature. This nutrient medium is very cheap and easily obtainable. It is entirely suitable for culturing fungi: their growth begins fairly early and the colonies are luxuriant and typical.

Brewer's wort is a complete substitute for Saburo medium for obtaining cultures as well as for studying the morphological peculiarities of the dermatophytes.

Media from animal and human organs: pieces of organs (liver, spleen, brain tissue, kidneys, etc.) taken from freshly killed healthy animals, are passed through a meat grinder or ground in a mortar and weighed. To one part ground organ tissue, 2 parts water are added, passed through a sieve and boiled 2 hours in a Koch apparatus (100°). The next day, 2% agar-agar, is added, the mixture boiled 30 minutes, filtered through gauze and cotton wool, filled into test tubes and sterilized for 20 minutes at 110°.

Carrot-potato agar (according to Langeron): 200 g carrots, 200 g potatos, 18 g agar-agar and 1000 ml water. The carefully washed carrots and the properly cleaned potatos are passed through a meat grinder, al-

lowed to soak in water for an hour and then boiled for 10 minutes, then filtered through cotton wool, the agar-agar added and again boiled for 30 minutes. The medium is filtered through gauze and cotton wool, filled into test tubes and flasks and sterilized for 15 minutes at 115°.

Blood meat-peptone agar, a meat-peptone broth with 3% agar. To 2 parts agar, melted at 100° and cooled to 45°, one part of aseptically taken defibrinated blood of humans or laboratory animals is added; the blood agar is filled into Petri dishes or chilled in a slanting position in test tubes. The faviform dermatophytes grow well on blood media.

Hairs, shavings of horn, birds feathers, animal bones, liver, spleen, brain tissue, horse manure, etc., are used as natural media of animal origin. They are usually placed on a piece of cotton wool, which has been soaked in physiological salt solution or water; sterilized at 110° for 15 minutes (twice) or at 120° for 20 minutes.

Vegetables with high carbohydrate content, particularly acid ones, are very suitable for the culturing of dermatophytes.

Carrots, more rarely turnips, beets, potato and fruits with the skin peeled, are cut into 4 × 1, 5 × 2 cm pieces, soaked for 4-10 hours in a solution of glycerol and tartaric acid (40 g glycerol, 2 g tartaric acid, 1000 ml distilled water), then a plug of cotton wool with a size of 1.4-2 cm is placed on the bottom of a test tube and the liquid in which the carrots have been soaked, run in until the whole cotton wool is completely soaked with the solution. The media are sterilized at 110° for 15 minutes and tested for sterility in the thermostat for 1-2 days.

The following liquid nutrient media are used.

Sugar broth: 100 g meat-peptone broth, 1-2 g maltose (glucose). To 500 g finely ground meat without fat or tendons, 1000 ml water is added and left to soak for 10-12 hours in the cold, then the meat is pressed

out through a piece of flannel and 1% peptone and 0.5% sodium chloride added to the liquid; the mixture is kept at 120° for 15-20 minutes, cooled, filtered through a paper and 1% carbohydrate added to the slightly heated filtrate; it is then filled into sterile flasks and test tubes and sterilized for 10 minutes at 110°.

Brewing wort: brewing wort, 7% according to Balling, is boiled for 30 minutes, cooled, filtered through paper, filled into a sterile container and sterilized at 110° for 10 minutes.

Czapek-Dox medium: 3 g sodium nitrate, 1 g potassium monophosphate, 0.5 g potassium chloride, 0.5 g magnesium sulfate, 0.015 g ferrous sulfate, 50 ml yeast extract, 1 liter town water; the pH of the medium need not be adjusted, being usually 6.9-7.0. The medium is sterilized at a temperature of 120° for 30 minutes. Prior to inoculating the medium, sterile 40% glucose is added in such a quantity that the final glucose concentration in the medium is 4%.

The optimum pH of the nutrient media is slightly acid (6.0-6.8).

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