

UNCLASSIFIED

AD NUMBER
AD837905
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; JUL 1968. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Release Branch/TID, Frederick, MD 21701.
AUTHORITY
Army Biological Defense Research Lab ltr dtd 22 Oct 1971

THIS PAGE IS UNCLASSIFIED

AD837905

TRANSLATION NO. 503

DATE: 15 July 1968

DDC AVAILABILITY NOTICE

Reproduction of this publication in whole or in part is prohibited. However, DDC is authorized to reproduce the publication for United States Government purposes.

AUG 21 1968

STATEMENT #2 UNCLASSIFIED

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Dept. of Army, Fort Detrick, ATTN: Technical Release Branch/TID, Frederick, Maryland 21701

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

UNITED STATES ARMY
CHEMICAL CORPS BIOLOGICAL LABORATORIES
Fort Detrick, Maryland

Misc Tr
503

An attempt to analyze my own experimental work.

by J. Tomcsik.

Translated from the pamphlet (Basel, Benno Schwabe, 1960) by the
Technical Library Branch, Technical Information Division.

Introduction

I was asked early in June, 1960, to review my papers for an academy, to emphasize those that I consider most important, and to include reasons for my choice.

I was forced to reflect. My work may have illuminated some problems with novel concepts in the principal fields of my endeavor, the medical domain and that of general bacteriology and immunity, but they usually found application only among a small circle of specialists in these two fields. Should I offer a concise, tedious summary of details in the customary style, which once again would be addressed to a narrow group of specialists, depending on the subject? Have I not read enough of these correctly written, but boring compilations? Such summaries and reviews left many a question unresolved.

The number of scientific publications that constitute the world literature has become immense, even within a certain special area. Most reference journals are poor. Only a few specialists are so well informed that they can competently review not only the books, but also individual articles of various authors working in their field of interest. Other reviewers usually lack the time to study the literature profoundly enough to enable them to evaluate the entire work of even one single colleague with absolute competence.

Would it not be better to request a self-analysis from the author, in which he describes his results in a few chapters and with greater brevity than is customary, placing them within the framework of knowledge expected of a larger circle of readers? Every writer who has worked for years or decades in a certain special area knows best which observations and experiences in the literature prompted him to treat a specific problem. Why should he not get the opportunity to account for his actions in his own words? The reviewer would have an easier task; his principal duty would be to establish whether the author has assigned too much significance to his own work.

I believe that overstatement has been avoided in my past experimental work. Nevertheless, I am faced with some difficult problems in this compilation. Was it not my task to emphasize my most important papers? Is it permissible to cite books by other authors, some perhaps containing exaggerated evaluations? I tried to find a compromise solution. The customary printing technique was helpful in this connection: I had such a text printed in "petit." Later I listed my own opinion of a possible overstatement.

I succeeded in placing on 19 printed pages about 100 of my experimental papers in 8 chapters within the framework of current knowledge of bacteria, erythrocytes and various serological reactions. The critic must determine whether such an analysis was worded objectively, or whether I made occasional overstatements that require revision.

I called this undertaking an "attempt." I realize that every attempt can be made with improved methods.

Basel, 10 November 1960.

Analysis of scientific publications by J. Tomcsik 1923-1960.

Of 145 papers, 36 pertain to the field of "hygiene;" they will not be discussed here. One hundred and nine papers, nearly all dealing with experiments in the fields of bacteriology, hematology, and immunology-immunochemistry, are, with few exceptions, classified in one category. Many of these experimental papers can be grouped under the designation "immunocytology."

I. Miscellaneous papers not discussed in this report.

Experimental medicine	3 papers (1, 2, 3)
Diagnostic reactions	2 papers (5, 6)
Microbiology	3 papers (43, 66, 77)
Immunochemistry	1 paper (76)
Various immunizations	9 papers (44, 50, 67, 73, 81, 91, 92, 97, 101)
Epidemiology	4 papers (13, 15, 54, 90)
Environmental hygiene	3 papers (55, 57, 72)
Public health administration	12 papers (12, 48, 49, 51, 52, 53, 61, 62, 63, 65, 69, 130)
Public health care	4 papers (37, 58, 59, 60)
Reform of medical education	3 papers (45, 100, 139)
Medical biography	3 papers (56, 98a, 98b, 142)

II. Discussion of experimental work.

1. Diphtheria: Toxin production, immunity, prophylactic immunization, epidemiology (14 papers).

The production of diphtheria toxin was improved by addition of glucose and sodium acetate to a modified medium of the Pasteur Institute, Paris (22).

Tomcaik used such a toxin to produce diphtheria toxoid for prophylactic immunization of about 500,000 children; he organized immunizations and evaluated the epidemiological results. Aside from Ramon's flocculation reaction, a special ring precipitin reaction in gel was used to evaluate the antigen's effectiveness experimentally (25, 26). In addition, the immunizing activity of several toxoid preparations produced in various European countries was evaluated at the request of the League of Nations' Hygienic Section through numerous antitoxin analyses in the blood of immunized persons (28, 29). Comparative morbidity statistics were obtained with good results among about 100,000 immunized and 100,000 non-immunized children (30). Comparative studies of changes in the epidemic curve in immunized and non-immunized districts, the study of age distribution among diphtheric patients in immunized districts, as well as several epidemiological investigations, contributed toward clarification of the problem (14, 20, 23, 24, 32, 33, 36).

These endeavors injected little novelty into our experimental knowledge. The value of these studies is found in the epidemiological area and may be characterized by the following two circumstances:

1. Prophylactic immunizations mentioned here were carried out at a time when the periodically fluctuating curve of diphtheria morbidity was still rising.

2. The clear evidence of epidemiological results of this project led to the world's first law of mandatory immunization with respect to diphtheria prophylaxis.

The theoretical foundations of immunity to diphtheria and the results of prophylactic immunization were discussed by the author in the "Results of Internal Medicine and Pediatrics" in 1932 (31).

2. The role of polysaccharides in the theory of immunity (17 papers).

Until 1923 the general doctrine specified that only protein substances or their high-molecular derivatives are capable of reacting with antibodies. Zinsser (New York) was the first to produce protein-free bacterial extracts which gave a specific precipitin reaction with the homologous antibacterial immune serum; they did not, however, elicit

production of antibodies in experimental animals upon i.v. injection. Zinsser called the unknown specific substances of his bacterial extracts "residual antigens;" they were later designated haptens upon Landsteiner's recommendations. In 1924 Mueller and Tomcsik demonstrated (4) that the specific residual antigen of the yeast cell consists of polysaccharide. The same issue of the Journal of Experimental Medicine (New York) contained the world-famous paper by Avery and Heidelberger, according to which species-specific polysaccharides can be isolated from broth cultures of pneumococci.

The two papers listed above substantiate the study of cellular polysaccharides with the aid of specific antibodies.

Soon the possibility was raised that specific bacterial polysaccharides originate principally in the bacterial capsule. It was assumed as early as 1931 that all bacterial capsules consist of specific polysaccharides. The work of Tomcsik subjected this "doctrinal concept" to a certain modification. A few examples will be listed here. In contrast to *B. anthracis* (7), *Kl. pneumoniae* and *A. aerogenes* (7, 8) indeed contain only polysaccharides as specific capsular haptens. Polysaccharides show optimal linkage with their homologous antibodies both in the precipitin test and the complement fixation reaction (10).

On the other hand, the polysaccharide slime substance of *Rh. radicleae* does not act as a specific substance, since it is not organically tied to the surface of the living cell, and no polysaccharide antibodies are produced upon "immunization" with slime-containing cell suspensions (7). Recent discoveries about the chemistry, structure and serological reactions of the bacterial capsule are discussed in Chapters 3-5 of this analysis.

Mueller and Tomcsik (4) isolated specifically reacting polysaccharides from yeast cells without capsules. In studying numerous yeast cells with dissimilar serological activity, Tomcsik (21) found their polysaccharide's specificity to be identical with the behavior of intact cells in the agglutination reaction. Tomcsik's (7, 34, 132) assertion to the effect that the specific polysaccharide of *B. anthracis* is not contained in the capsule, but in the cell wall, was questioned by Heidelberger at the II. International Congress in London, and was vindicated later by several confirmations.

Even today, these observations validate the conclusion that those polysaccharides that are incorporated organically in the capsule or the cell wall of the living cell (probably as supportive groups of protein substances) may act as specific substances. They are designated complete antigens or haptens, depending on their isolation or "degree of purity."

In the early stages of polysaccharide research the important medical question was raised, whether pure polysaccharides as specific haptens could produce genuine anaphylactic shock or allergy. Tomcsik (9) found that the polysaccharide of *A. aerogenes*, which does not elicit toxic symptoms upon intravenous injection of 1 mg, kills a specifically sensitized guinea pig with typical anaphylactic shock within 3-5 minutes in amounts measured in γ . Avery and his associates confirmed this observation, which was expanded by Tomcsik and others in several papers (11, 16, 17, 18, 27, 93, 96). These experimental findings form the basis of many a concept in current allergic doctrine (Harkavi, New York, USA, 1st International Congress of Allergy, Zurich, 1952).

Soon after the publication of the first two papers on polysaccharides (9, 11), Prof. Dr. R. Doerr commented on the discovery of polysaccharide anaphylaxis in the *Manual of Pathogenic Microorganisms* by Kolle, Kraus and Uhlenhuth (1929), Vol. 1, pp. 951-953:

p. 951: "Recent experiments by Tomcsik and Kurotchkin (11) have brought the problem of bacterial anaphylaxis into a new phase.

"Tomcsik and Kurotchkin immunized rabbits with *Bacillus lactis aerogenes*, pneumobacilli or yeast; 1-4 cc of the resultant immune sera were used to sensitize guinea pigs passively and heterologously (intraperitoneal injection). However, the intravenous injection for effect was not made with extracts (with high primary toxicity) of the indicated microbes, but with chemically prepared products which gave no protein reactions and contained not more than 0.2-0.9% N, associated with a high concentration (66-83%) of carbohydrates. The animals reacted with absolutely typical, acutely lethal shock, even to 0.01-0.03 mg, while untreated controls tolerated the same or larger doses (up to 2 mg) without reaction; only aerogenes derivatives had primary toxicity at these high levels; they did not produce shock symptoms, however, but caused chronic intoxication leading to death on the second or third day.

"Experiments on the uterus of sensitized guinea pigs also gave clear results; concentrations of protein-free microbe derivatives inducing contraction were found to be 1:5-20 million by Dale; the uterus of normal guinea pigs was unaffected by concentrations 1000 times higher. It must be noted that shock-inducing protein-free preparations at remarkably high dilutions (1:500,000 to 1:2,000,000) yielded specific precipitations with passively prepared rabbit immune sera, which seems to indicate the validity of close relations between the precipitin reaction and anaphylaxis even in the area of bacterial antigens."

I cannot agree with the last sentence. Nor do I endorse the discussion on page 953. I shall cite the following sentence from the end of this chapter (p. 953): "It is impossible to assess the effect of further work in the direction set by Tomcsik on the negative evaluation of relationships between anaphylaxis and infection."

Thirty-one years have passed since this sentence was written, but no additional clarity has been brought into the role of anaphylaxis in infectious diseases.

Polysaccharides as heterogenetic antigens were discussed in a Swiss paper (68), and immanopolysaccharides in general were treated in a British paper (102).

3. *Bacillus anthracis*: Bacteriology, cell structure, infection, immunity (21 papers).

Early in this century, Gruber as well as Preisz discovered an absolutely close correlation between the ability of anthrax bacilli to form capsules in the infected animal and the virulence of *B. anthracis*. In the initial era of polysaccharide research, the American school of Avery tried to clarify the role of capsular polysaccharides in pneumococcal virulence in a number of world-famous papers. These studies constituted the apex of microbiological research in the world literature. At that time pneumococci were most thoroughly explored with respect to immunochemistry.

Tomcsik's work with anthrax (17, 18, 19, 34, 35), begun in 1930, indicated that schematization is not permissible even in this area of biological research:

a. Anthracis strains attenuated in virulence by Pasteur's method may produce enormous capsules on ordinary agar medium under normal O₂ pressure.

b. The capsule of *B. anthracis* consists not of a specific polysaccharide, but of a specific protein derivative (P_p).

c. Specific anthracis polysaccharides (P_s) may be extracted from non-capsular anthrax bacilli without admixture of P_p.

d. Admixture of P_p and P_s (extract of encapsulating bacilli) yields the first substance upon precipitation with CuSO₄ in chemically pure form (34, 35).

Tomcsik's pupil, Ivanovics, subjected P_p substance to thorough chemical analysis; together with the chemist Bruckner he obtained only d(-) glutamic acid building blocks upon hydrolysis of this substance. Since, according to later reports, a similar glutamyl-polypeptide was isolated from cancerous tissue, the finer chemical structure of this substance was studied by numerous chemists (in Hungary, England, USA and Israel). The British biochemist Bevarnick modified the technique of Ivanovics and Bruckner for isolation of pure anthracis polypeptide. Apparently Bevarnick was unaware of Tomcsik's initial paper, in which purification of this substance was described in detail (Zeit. f.

Immunitätsforschung 77, No. 1/2); his "modification" with CuSO_4 -precipitation of the polypeptide is identical with the method described in 1932 by Tomcsik and Szongett (35).

Anthraxis polysaccharide and polypeptide were initially (34, 35) characterized chemically and with the aid of two antibodies devoid of cross reaction with the two anthracis haptens. These papers (34 and 35) formed the beginning of immunochemical research in anthracis polysaccharide and polypeptide.

The chemical nature of the polysaccharide, which reacted with anthracis polysaccharide antibodies prepared and defined by Tomcsik, was soon clarified (Ivanovics). British biochemists assumed the existence of a second anthracis polysaccharide, which was described as mannan and gave a completely different serological reaction. This obvious error was removed in paper (132). The "second anthracis polypeptide" corresponds to the specific yeast gum (4, 21) originating with a yeast hydrolysate added to the medium. Paper (133) demonstrated the conditions under which vegetative cells as well as bacterial spores may bind foreign antigenic substances from the nutrient with practically irreversible results, leading to false inferences from analyses of bacterial antigens carried out with immune sera produced in this manner. A single specific polysaccharide of anthrax bacilli is known; it shows the same serological specificity in numerous anthracis strains (34, 112, 132) and is extracted in chemically pure form from isolated and purified cell walls of anthrax bacilli (132). This polysaccharide shows a certain serological relationship with the polysaccharide in certain types of *B. cereus* (131). However, this polysaccharide is incorporated in the cell wall of *B. anthracis* in a manner different from that of *B. cereus*. The behavior of the cell wall of *B. anthracis* may be differentiated from that of *B. cereus* upon immune agglutination as well as by means of the peculiar forms of anthrax bacilli in the presence of penicillin (112, 131). Anthracis polysaccharide antibodies do not confer immunity to experimental anthrax infection.

Specific antibodies against the glutamyl acid polypeptide may be produced in rabbits with 10 or more i.v. injections of killed, encapsulated anthrax bacilli (35, 38, 39, 40, 41, 42, 46). These antibodies agglutinate only encapsulated anthrax bacilli (38). Only they can demonstrate the capsule of *B. anthracis*, which is invisible in native preparations (39). These antibodies were used to demonstrate that:

a. anthracis polypeptide liberated in the blood of infected animals is identical with anthracis polypeptide produced "in vitro" (40);

b. polypeptide is produced most intensely at the plane of bacillary division within the chain (112).

The greatest interest from the viewpoint of experimental pathology was produced by the observation that mice, which in the past could not be protected against a single lethal dose, were protected against 100 fatal doses by polypeptide antibodies (42, 47). Guinea pigs and rabbits were not protected by polypeptide antibodies (40, 47). At a later time, very intensive research on anthrax in the USA (Camp Detrick) and England (Porton) established that these animals can be effectively immunised with other (still imperfectly defined) antibodies. In his well-known manual ("Immunity, Hypersensitivity, etc."), Raffel (Stanford) discusses the reasons why it would be important for our knowledge of infection to clarify the marked differences in the protection conferred by polypeptide antibodies on various animal species. Certain references to this matter are made in papers (41 and 47).

Guinea pigs sensitized passively with homologous antibodies are killed by T-quantities of both anthracis polysaccharide and polypeptide (17, 18).

A report by Manninger and Mogradi on induced mutation prompted me to study this phenomenon. The pertinent papers (82, 83, 87) list results that are difficult to interpret, although they led to isolation of a very peculiar bacillus whose thorough investigation produced the impetus for closer study of bacterial immunocytology (cf. Chapter 5).

4. Electrostatic, pH-associated protein complexes on bacterial limiting surfaces (6 papers).

Based on a new principle (105), bacterial capsules invisible in aqueous suspensions are distinctly visualized under the phase contrast microscope in dependence on pH and the type of proteins added. Optimal pH is constant for different strains of the same species or type, provided the same protein is added. On the other hand, optimal pH varies:

- a. according to the type of microorganism (Cryptococcus, Streptococcus, Pneumococcus, Klebsiella and several species of genus Bacillus) and
- b. according to the isoelectric point of added proteins.

Tomczak calls this reaction "non-specific capsular reaction."

It develops due to an electrostatic, salt-like complex linkage between the bacterial cell surface and the added protein within a narrow range of pH. Proteins with high isoelectric points (e.g., protamines) show a wider effective range in this reaction; at sufficiently high concentrations they destroy the bacterial cell (105, 107, 117, 123).

The new principle led to development of a simple capsular stain reaction which gives positive visualization of the capsule after "mordanting" with serum proteins at a certain pH, even following Gram staining (104).

The non-specific capsular reaction may also serve as indicator in the demonstration, in body fluids, of basic protein substances whose significance in the course of infectious diseases still awaits clarification (107).

A "non-specific cell wall reaction" may be demonstrated in some bacteria with the same principle, provided the cell wall is separated enzymatically from the cytoplasm, or the cytoplasm is destroyed electively by enzymatic or mechanical means (117, 125).

5. Immunocytology of bacteria (25 papers).

Roger and, several years later, Neufeld reported that certain organisms or their capsules revealed "swelling" after addition of homologous immune serum. This reaction was designated as "capsular swelling reaction" throughout the world. Tomcaik demonstrated at a symposium of the Society of General Microbiology held at London (119) that this designation is false, since the bacterial capsule simply becomes visible in this reaction, without changing its form and without swelling. He recommended the name "specific capsular reaction" for this phenomenon. This new term has already been adopted in some recent bacteriological papers (e.g., Wilkinson, Amer. Review of Bacteriology).

The nature of this reaction consists of the specific linkage of antibody molecules with antigen molecules present on the cell surface. It is evident that the following premises must be met before a microscopically visible "specific" reaction can take place:

- a. Permeability of bacterial limiting structures for antibody molecules.
- b. Appropriate thickness of the antigenic layer (adequate number of antigen molecules reacting with antibodies).
- c. Optical contrast of the proper cellular layer vis-a-vis other limiting layers of the cell after completed reaction.

Ad a. The permeability of the bacterial capsule, the sporangium and the exosporium is very favorable for antibody molecules, that of the vegetative cell wall and the spore wall is limited or variable.

Ad b. Thin layers of capsules, cell walls and exosporia cannot be visualized.

Ad c. In order to produce specific visualization of the bacterial cell wall or the spore wall, the latter must be separated enzymatically (lysozyme) from the cytoplasm, or the cytoplasm is partially removed.

A number of papers dealing with experimental immunocytology pointed out that isolated, chemically defined and purified antigens may be used to identify antibodies chemically, and that the latter may serve to establish topographical localization of known antigenic substances in the cell's structure. The following reactions leading to morphologically distinct pictures were described:

- a. Specific capsular reaction.
- b. Specific cell wall reaction.
- c. Specific sporangium reaction with apparent swelling of the sporangium.
- d. Specific exosporium reaction.
- e. Specific spore wall reaction.

Of these reactions, a. and b. will be discussed briefly in this chapter, based on published papers, while c., d. and e. are described in Chapter 7.

In his book, "The Cytology and Life-History of Bacteria" (Livingstone Ltd., Edinburgh/London 1955, p. 17/18), K. A. Bisset (Birmingham) writes the following about this phase of Tomcsik's work:

"A revolution in the use of the phase-contrast microscope, comparable with the introduction of specific staining methods in classical microscopy, has resulted from the brilliant work of Tomcsik, who, by the use of enzymes, antibodies and other proteins has specifically demonstrated a variety of chemically definable materials and structures in the bacterial cell, and has revealed an entirely unsuspected complexity of structural details in the capsule. The most striking of Tomcsik's methods, which is possibly the greatest single advance in cytochemical technique in the last half-century, and which has potential application in all biological fields, consists in the preparation of antibodies against chemically defined fractions of the bacterial capsule. When these antibodies are allowed to react with bacteria which contain the appropriate chemical fraction, in the field of the phase-contrast microscope, the antigen-antibody combination shows clearly in dark contrast, presumably because of the coagulation of the antigen..."

The text quoted from Bisset could be criticized in the sense that, according to Tomcsik's papers, the hope for a "potential application in all biological fields" is not very great. There are many cells with surface structures that cannot be visualized microscopically with this technique.

On the other hand, the topography of chemically definable antigens built into the limiting surfaces of "suitable" cells is demonstrated with a clarity that exceeds that of preparations treated by Coons' (Boston) method with fluorescent antibodies. Numerous microphotographs of this type made by Tomcsik have been reproduced in recent books on bacterial cytology.

The most remarkable microphotographs were made with a bacterial strain (Bacillus M) isolated during projects (82, 83 and 87). This bacterium shows morphological reactions resembling those of *B. megaterium*, although its cytochromic system is different, according to Weibull (Stockholm) from that of "typical" *B. megaterium* strains which have been used most extensively in recent studies of bacterial cytology. This strain, as a rule, produces capsules consisting of alternating layers of genus-specific glutamyl polypeptide and a type-specific polysaccharide. The latter form transverse capsular septa in direct continuation of the transverse wall and occur as polar caps at both ends of the chain (94, 95, 99). It is most remarkable that strongly developed polysaccharide transverse septa of the capsule form a direct continuation of the transverse wall; there are no analogous observations in botany which show growth of the transverse wall beyond the limits of the cell wall. The peculiar structure of this bacterial capsule, consisting of polypeptides and polysaccharides, was termed "complex capsular structure" by Tomcsik, who pointed out that this bacterium's capsule incorporates polysaccharides similar to those contained in the cell wall, in contrast to other bacteriological observations. This claim has been challenged by Ivanovics in several papers. Wilkinson (Bact. Rev. USA, 78, 423, 1958) also assumed that capsular polysaccharides of *Bacillus M* are slime substances held by electrostatic forces of the polypeptide (105).

These authors apparently failed to consider papers (108 and 109), in which the genesis of the complex capsular structure was described in great detail, and where the production phases of chemically definable substances on a cellular surface were identified by their characteristic topography. Since modifications in the composition of nutrient made it possible to equip several strains of *B. megaterium* with capsules consisting solely of specific polysaccharides without polypeptide (134), Wilkinson admitted his erroneous interpretation (personal communication). Forty-five strains of *B. megaterium* were used to demonstrate variations in the complex capsular structure within this species (125). A special technique allowed demonstration of the peculiar capsular structure of *Bacillus M* under the electron microscope, solely with the aid of antibodies discussed above (127).

Usually no changes can be expected under the phase contrast microscope, when homologous cell wall antibodies are added to an intact bacterial suspension. At most, certain grampositive bacilli may seem to gain in size after such treatment.

The behavior of some grampositive bacteria is quite different when their cell wall is partially depolymerized with enzymes and separated from the cytoplasm by loosening of the rigid structure. At this stage the cell wall is barely visible under the phase contrast microscope. However, as soon as homologous cell wall antibodies are added, it becomes distinctly visible (99). Tomcsik called this new specific reaction "specific cell wall reaction." This reaction is seen even more readily when bacteria

are shaken with tiny glass beads in Mickle's electromagnetic vibrator, causing the removal of cytoplasm through barely visible fissures in the cell wall (121). The wall of the evacuated bacterial cell is on the margin of microscopic visibility. It is shown distinctly by addition of homologous, type-specific polysaccharide antibodies (106, 111, 115, 131, 134). Ultrasound cannot replace the Mickle vibrator satisfactorily for this purpose (136).

Attempts were made to substitute enzymatic proteolysis for mechanical removal of cytoplasm. Contrary to the general findings in the pertinent literature, trypsin digested all intact strains of *B. megaterium* quite distinctly, whereas anthracis strains were attacked moderately. None of the cereus strains were affected by this ferment (123). Extensive experiments revealed that the removal of a very thin lipid layer from the cell's surface is necessary for rapid and thorough digestion of cytoplasm by trypsin, even in the case of *B. megaterium* (135). Crystalline trypsin acts only in the presence of the customary preservative, chloroform, or in conjunction with a number of other organic solvents. The latter may be replaced by the enzyme lipase, which is an impurity of commercial trypsin preparations (135). The specificity and effectiveness of another ferment, lysozyme, were studied quantitatively with numerous strains of the "bacillus" group (124).

A combination of methods for the isolation of the cell wall was employed in taxonomical work with numerous strains of the species cereus, anthracis and megaterium; cross tests were based on the criterion of the new specific cell wall reaction. Generally speaking, the same classification was established as had been secured with the agglutination reaction of intact bacilli (131, 134). According to findings by Tomczak and his associates, *B. megaterium* and *B. cereus* have the greatest number of types (even on the basis of cell wall polysaccharides) of all known bacteria. In contrast to biochemically related strains of *B. cereus*, non-capsular strains of *B. anthracis* are not agglutinated by their homologous immune sera (36), although they do give a distinct specific cell wall reaction with anthracis polysaccharide antiserum (112, 131). Different serological types of *B. anthracis* were not demonstrable, either with the specific cell wall reaction or with serological analysis of anthracis cell wall polysaccharides from various anthracis strains (131, 132).

Chemical (principally chromatographic) studies characterized the following purified specific substances in the course of investigations discussed here:

a. *B. megaterium*: polypeptide, capsular and cell wall polysaccharide, cell wall mucoprotein (103, 118);

b. *B. anthracis*, *B. cereus*: cell wall polysaccharide (132).

A summation of immunocytological papers discussed here leads to the conclusion that a system was developed which, with the aid of immuno-chemically defined antibodies, permitted investigation of the topographical distribution of individual antigens on the limiting surface of intact or enzymatically/mechanically pre-treated bacterial cells (110). Reports on this field were compiled in:

- a. The structure of bacterial limiting surfaces (117);
- b. Antibodies as indicators for bacterial surface structures (122);
- c. Fine structure of bacterial limiting surfaces (129).
6. Bacterial protoplasts (6 papers).

Fleming discovered lysozyme, a ferment which can digest some grampositive bacteria in their entirety.

It was soon discovered that lysozyme is a polysaccharidase. The action of lysozyme on the structure of the bacterial cell was described by Tomcsik and Guex-Holzer (99). When egg albumin at 1:200 or crystalline lysozyme at 1:10,000 is added to a live suspension of *Bacillus M* (or, in appropriate concentrations, to any strain of *B. megaterium*), the phase contrast microscope reveals, even at room temperature, a chronological sequence of changes that permits a profound insight into the fine structure of bacterial cells. These changes include:

- a. Dilatation of the cell wall, which is rigid prior to treatment and becomes progressively more depolymerized in the course of lysozymic action. The dilated cell wall is invisible at a certain stage of depolymerization; it may be demonstrated under the phase contrast microscope by addition of homologous, type-specific antibodies.

- b. In parallel progress with cell wall dilatation, the cytoplasm separates from the cell wall, first at the poles, then laterally; ultimately it assumes an absolutely uniform spherical form.

- c. Spherical units of cytoplasm are liberated as uniform, round elements after complete dissolution of the cell wall.

- d. Liberated cytoplasmic spheres appear black under the phase contrast microscope; their luminosity is soon restored in physiological saline, with the exception of a peripheral layer that corresponds to the long-suspected cytoplasmic membrane of bacteria. This membranous layer also disintegrates after a few hours in physiological saline.

e. Spherical transformation of cytoplasm does not occur if bacteria are heated prior to subjection to lysozyme. Bacilli treated with heat are changed to thin, coagulated cytoplasmic rods without cell wall in the presence of lysozyme.

These observations (99) are highly important with respect to our knowledge of the fine structure of the bacterial cell. They indicate that lysozyme acts electively on the cell wall, that the dilated cell wall may be visualized microscopically with type-specific polysaccharide antibodies up to a certain stage of lysozymic depolymerization, and that the cytoplasmic mass, surrounded by an elastic cytoplasmic membrane, assumes a spherical shape when freed of the rigid cell wall, due to its extraordinarily high surface tension and minute volume. The second part of this observation (spherical transformation of cytoplasm due to the activity of lysozyme) was confirmed a year later by Weibull in an important and universally known paper. He called the spherical elements protoplasts, a term already in use by botanists during the last century as a designation of components without cell walls present in plant cells (gymnoplasmata). Weibull made the novel observation that lysozymic protoplasts of *B. megaterium* may be preserved in isotonic saccharose solution.

The two papers discussed here gave rapid impetus to intensive research in protoplasts. Participating biochemists demonstrated that lysozymic protoplasts synthesize protein and enzyme, and even show signs of growth and rudimentary cell division. A rapidly growing number of papers soon revealed confusion of concepts. Are lysozymic protoplasts identical with the spherical microorganisms that are formed in the presence of penicillin? (Paper 112 showed that "string-of-pearls" forms of anthrax bacilli produced with penicillin possess a heavy cell wall layer and that these forms have nothing in common with protoplasts.) What are the differences between protoplasts and L-forms of microorganisms? Twelve investigators collaborated in clarifying the concept of "protoplasts" in the journal "Nature" (126).

Tomcsik and Guex-Holzer (113) noted that lysozymic protoplasts possess an antigenic activity quite different from that of isolated and purified cell walls. Antibodies produced with these cell fractions showed no trace of cross reaction with two different cell fractions. The paper caused a few laboratories to review the production of bacterial vaccines in this sense.

Various serological reactions of protoplasts isolated from numerous types of *B. megaterium* were thoroughly developed and reported in paper (120).

Formation of protoplasts may be used in determining the active mechanism of genuine disinfectants (116). *Bacillus M* was brought into contact with different concentrations of tested disinfectant for 30 minutes at room temperature. The disinfectant was subsequently removed

by three-fold washing, and the lysozyme experiment was carried out. In these tests, Ringer solution was used with great success in place of isotonic saccharose solution for preservation of protoplasts. Under genuine disinfectants we included carbol, the quaternary ammonium bases and several other substances that neutralized spherical transformation at concentrations which inhibited culture. Another group included the mercury salts which showed excellent inhibition of culture at high dilutions, but did not neutralize spherical transformation even at low dilutions. Since mercury salts react with sulfhydryl groups, and their propagation-inhibiting effect is reversible, they are not genuine disinfectants. Experiments published in paper (116) may be interpreted in the sense that neutralization of spherical transformation of bacilli sensitive to lysozyme is suitable as a criterion to establish whether a chemical substance is a genuine disinfectant. The effect of a genuine disinfectant consists of irreversible denaturation of cytoplasm, preventing spherical transformation due to surface tension after enzymatic removal of the bacterial cell wall. Either the cytoplasm is coagulated irreversibly, or the elasticity of the cytoplasmic membrane is lost during denaturation.

7. Sporulation. Structure and serology of bacterial spores (5 papers).

The ability to produce spores among both pathogenic and a-pathogenic bacteria depends on the genetic factors of the species or on individual bacterial strains. In the presence of genetic factors, sporulation is induced by environmental conditions. Recent observations have clarified the role of trace elements and other inorganic and organic substances, but Buchner's (1890) old concept remains valid, according to which the physiological cause of sporulation is found principally in the critical shortage of nourishment. While Foster and his school (USA) challenged this old view energetically, it was reintroduced by Grelet in several biochemically oriented papers with modern formulation and precision. Paper (128) analyzes the individual factors involved in the question why Gladstone-Fields medium, so rich in amino acids, promotes vegetative growth of bacteria, but is wholly unsuited for sporulation. We identified the constituents which inhibit or promote sporulation in this highly complex medium. Grelet's data were supplemented (137).

These two papers (128, 137) resulted in a new observation, according to which the sporangium shows pronounced swelling at a certain stage of sporulation after addition of homologous, vegetative polysaccharide immune serum. It was established that this swelling is not due to an antigen-antibody reaction, but is caused by enzymes of sporulation (128, 137, 144). This reaction was used in establishing the sporulation phase in which the most "sporangiolytic" ferments are produced. These ferments depolymerize the wall of the mother cell in the course of the antigen-antibody reaction with such speed that the

wall is loosened and, since it is visualized microscopically by the reaction with antibodies, simulates a "swelling reaction." This reaction was designated "specific sporangium reaction" and led to the study of "sporangiolytic" ferments (144). In the cytological study of these ferments, a distinction was made between "parietolysis" and "cytoplasmolysis." The primary effect is parietolysis (144). When concentrated sporulation ferment is added to non-sporulating chains of the same bacterium, the chain is soon divided into individual bacilli due to enzymatic cleavage of transverse walls. The lateral cell wall is attacked later, although addition of cell wall antibodies (but not polypeptide antibodies) protects the cell wall for a few hours against the destructive effect of enzymes. Following destruction of the cell wall, the cytoplasm, apparently unimpaired, goes into solution. Certain sera (but not polysaccharide and polypeptide antibodies) are able to protect the cytoplasm of sporangia for long periods against sporangiolytic ferments. In this case the cytoplasm of individual cells assumes a spherical shape after accomplished parietolysis. These spherical masses of protoplasm show a certain similarity to protoplasts, but are fundamentally different, since:

a. they can be preserved only in certain sera, but not in saccharose or in Ringer solution;

b. spherical elements of protoplasm have a tendency to confluence in moist preparations, forming giant spheres about 20 microns in diameter.

Numerous strains of *B. megaterium*, *B. cereus* and *B. anthracis* were examined for spore antigens in papers (137) and (138). Preparation of pure spore sera was based on following considerations:

a. Vegetative cells of *B. megaterium* are completely dissolved by lysozyme, whereas spores of this bacterium possess a high resistance to lysozyme. Spores of *B. megaterium* may therefore be freed completely from vegetative cellular residues by treatment with lysozyme.

b. Although vegetative cells of *B. cereus* and *B. anthracis* are resistant to lysozyme, vegetative cellular remnants may be removed from their surface with the aid of sporangiolytic enzymes.

We succeeded in producing such spore sera which gave no traces of serological reaction with vegetative cells, even without differential absorption. Such spore sera served in specific agglutination of spores at high titers. Particularly distinct titers were achieved by means of centrifugation introduced to spore agglutination. This technique permitted detailed serological typing of spores in the species *B. megaterium*, *B. cereus* and *B. anthracis*. In contrast to the high number of vegetative cell wall types, spores of *B. megaterium* and *B. cereus* showed but a few serological types which, however, always deviate from those of the vegetative cell wall. The spores of various *B. anthracis* strains were identical. However,

their specific polysaccharide was quite different from the cell wall polysaccharide of the vegetative form of *B. anthracis*.

A surprising observation was made during phase contrast microscopy of the effect exerted by spore antibodies on spores. Those sera that agglutinated a certain spore produced an unexpected capsule-like layer on the spore's outer wall. The most prominent experts in American electron microscopy, who have been studying the ultrastructure of bacterial endospores for years, describe a so-called "exosporium" on the surface of the cereus spore, but were unable to discern a similar structure on the surface of *B. megaterium*. A much simpler examination under the phase contrast microscope revealed exosporium on *B. megaterium* in utmost clarity in form of a capsule-like layer after treatment with spore immune serum (138). Tomcsik called this reaction "specific exosporium reaction." Contrary to current opinion, this reaction proved categorically that exosporium is not a remnant of the vegetative cell, but that its substance is synthesized at the time of sporulation.

Treatment with the Mickle vibrator leads to lesions in the spore wall and to evacuation of internal cell components. When spore immune serum is added to a "spore wall preparation" prepared in this manner, the result is a "specific spore wall reaction" (138).

Since treatment of purified spores with ultrasound separates the exosporium in about one hour without injuring the spore wall (136), the bacterial exosporium and the spore wall are accessible to chemical and serological studies.

8. Erythrocytes: The foundations of hemagglutination. Isolation, nature and topography of membrane antigens. Serological diagnosis of mononucleosis infectiosa (16 papers).

All serological and diagnostic laboratories of the world employ ovine and, occasionally, bovine erythrocytes in hemagglutination or hemolysis in connection with certain serological reactions, although it was not clear prior to 1945 which antigenic substances on the limiting surface of these erythrocytes participate in the various reactions. Conclusions were drawn indirectly, according to which both the "heterogenetic" antibodies present in the serum of healthy persons or produced in serum disease or in infectious mononucleosis, and isogenetic antibodies react with different antigens on the surface of erythrocytes. The proposed antigens were designated F, S, M or I. Such conclusions were based on cross absorption reactions usually familiar only to specialists. Tomcsik's work was aimed at the isolation of proposed F, S, M and I antigens, and at the definition of their nature and incorporation in the erythrocytic membrane.

In one paper (68) Tomcsik discussed polysaccharides as heterogenetic antigens and criticized a study by Rennaux and Thomas, who claimed that Forssman antigen from horse kidney has a chemical form different from that derived from horse heart. Although the horse heart is a very poor source of Forssman antigen, the substance was isolated in a form (70) that exceeded the material procured by Rennaux and Thomas from bovine heart several times in degree of activity. This substance was serologically and chemically identical with purest and most active Forssman antigen previously isolated by Brunius from horse kidney. Forssman antigen with nearly similar properties was extracted with cold ethanol from sheep erythrocyte stromata, when serologically inactive substances were first removed from the stromata by extraction with acetone (71). The same paper proposed methods suitable for large-scale production of erythrocytic stromata. A sensitive method for the evaluation of serologic activity of stromata and their fractions was developed (71).

Extraction and separation of S and M fractions succeeded after many fruitless efforts described in paper (75). Stromata of bovine erythrocytes without F antigen are the best sources of S and M antigen (78, 79). The same extraction procedure destroyed a considerable portion of M antigen in the stromata of ovine erythrocytes (78). The chemical nature of F, S and M antigens was discussed in paper (80). Extracted and purified M antigen of bovine erythrocytes absorbed 2 units of hemagglutinin of mononucleosis serum electively up to a dilution of 1:1,000,000, whereas it remained inactive during absorption with other hemagglutinins.

Separation of I (isophilic) antigen from other antigens of ovine erythrocytic stromata failed. We established that this antigen is extremely thermolabile and that its serological activity is completely neutralized with trypsin and papain (74). This behavior of I antigen is fundamentally different from that of F, S and M antigens which are still thermostable at 100°C and resist the proteolytic effect of trypsin and papain (74).

The same paper (74) points out that the agglutinates of sheep erythrocytes produced under the influence of I serum may be differentiated morphologically from those obtained with mononucleosis serum. I-agglutinates are compact; they consist of flat, deformed, almost "agglomerate" erythrocytes in close contact, while erythrocytes in M-agglutinates form large, loose aggregates, take a spherical form and are in actual contact only in one sector of the spherical surface. Tomcsik considers hemagglutination and hemolysis identical in nature, caused by the same antibody. He ascribes the quotient of "hemolysis/hemagglutination titer," which changes in accordance with the hemagglutinin system, to the variable topography of individual antigens on the cell surface (71). If the antigen is located on the extreme outer surface of the erythrocytic membrane, hemagglutination is favored (mononucleosis antigen of sheep erythrocytes). If the antigen lies deep within the semipermeable membrane of the same cell, agglutination is impeded sterically, while

hemolysis must be promoted, since the complement reacts with an antibody-antigen complex and the deeply incorporated antigen is an integral component of the erythrocytic membrane (74, 141, 143, 145, 146). The semipermeable membrane of erythrocytes consists primarily of a species-specific, thermolabile protein (I antigen) which is separable from the membranes (stromata) only by careful extraction with NaHCO_3 . Carefully extracted I antigen retains its serological activity, but it incorporates F, S and M antigens which cannot be separated without denaturation of the labile protein. These experimental results lead to the conclusion that the semipermeable membrane of erythrocytes is a thermolabile protein with supporting groups consisting of hapten-like substances deep within the structure as well as on the surface (141, 146).

Differences in the morphological aspect of agglutinates according to the topography of surface antigens indicate that Coca's concept of the definition of genuine agglutination and pseudoagglutination, although widely accepted in the literature, is incorrect.

The first stage in Tomcsik's research in erythrocytes was closed with the following 5 papers: Clinical diagnosis of mononucleosis infection (84), Antigenic structure of ovine and bovine erythrocytes (85, 86), Significance of the serum albumin-lipoid quotient in the determination of morphological types of hemagglutination (88, 89).

The papers discussed above were not always interpreted correctly in the literature. Contrary to the entire world literature, Blumenthal found no difference in the absorptive powers of the guinea pig kidney and human mononucleosis antibodies or other hemagglutinins. Lippelt and Nogalski still question the distinctiveness of so-called F and S antigens. Moellner apparently did not understand the essence of Tomcsik's work when he reported that he inactivated M antigen with the proteolytic ferment papain, but was unable to accomplish the same in the case of I antigen defined in paper (74). It is obvious that careful proteolytic treatment of the erythrocytic surface fails to reach the more deeply positioned I antigen of the erythrocytic membrane. Two detailed reports by the American researchers Simmons and Markovits are most peculiar. Although they confirm isolation and separation of F, S and M antigens from erythrocytic membranes by the method of Tomcsik and Schmarzweis, they cite some of the above-mentioned papers with erroneous sources and misleading data. They also claim to have worked simultaneously with Tomcsik and Schmarzweis, despite the fact that their first paper was published four years after the American release of papers (78, 79 and 80). The most disturbing factor concerned complete inactivation of M substance with trypsin and papain on the part of Simmons and Markovits, contrary to findings in paper (74).

In view of the circumstances described above, Tomcsik and his new associates resumed experimental work in this area after an interval of 10 years. Paper (140) gave solid confirmation of the effect of trypsin and papain on S and M antigen, as described in paper (74). Moreover, it was demonstrated with highly active, crystalline enzyme preparations that trypsin does not inactivate M antigen either in the membranes of bovine and ovine erythrocytes or in purified M fractions. Papain inactivates M antigen in erythrocytic membranes of sheep, but not in those of cattle. This constitutes the first report of a substance derived from two different cells, which has an identical serological reaction, but shows a variable resistance to a certain proteolytic (plant) enzyme. This result was not altogether unexpected, since earlier tests had shown (78) that the same method of chemical extraction which permitted isolation of highly active M antigen from bovine stromata, destroyed M antigen from ovine stromata.

Paper (141) is the most thorough compilation, supplemented by recent experimental data, of Tomcsik's ideas about hemagglutination and topographic distribution of heterophilic and isophilic antigens in erythrocytic membranes. Observations made in this paper led to the assumption of a loose, serologically inactive second protein layer on the extreme outer surface of erythrocytes, which covers haptan micelles exposed to variable degrees either partially (ovine erythrocytes) or completely (bovine erythrocytes), and may partially inhibit hemagglutination sterically or prevent this phenomenon completely, depending on its potency. After careful enzymatic removal of the loose protein layer, bovine erythrocytes are eminently suited for hemagglutination. Based on this observation, a new reaction was developed for the diagnosis of mononucleosis infectiosa (141, 143, 146), which is considerably simpler than absorption tests currently in use.

Experimental work now in progress is aimed at additional clarification of the structure of the erythrocytic membrane.