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QUALITATIVE IMMUNO-CHEMICAL ANALYSIS: METHOD USING THE DIFFUSION OF ANTIGENS INTO PRECIPITATING IMMUNE SERUM WITH GELOSE

By Jacques Oudin

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QUALITATIVE IMMUNO-CHEMICAL ANALYSIS; METHOD USING THE DIFFUSION OF ANTIGENS INTO PRECIPITATING IMMUNE SERUM WITH GELOSE*)

(First Part)

By Jacques Oudin**)

(Pasteur Institute. Dept. of Microbial Chemistry)

Several immuno-chemical methods are available now for the detection, the identification, and the titration of antibodies by specific precipitation. But all these methods seem to be applicable in general to simple precipitating systems, i. e., to systems in which a single antigen meets within the immune serum antibodies capable of precipitating with it. This is true of Ramon's primoflocculation method (1), of Dean and Webb's very similar method of optimal proportions (2), of Heidelberger's quantitative method (3) and of the more or less approximate methods of titrating the antigen or the antibodies by diluting one or the other of the two reagents.

Thus the classical immuno-chemical methods have been used very rarely for the analysis of a solution having a biological origin, without assistance from chemical or physico-chemical fractionating methods, such as salting out. Such a panoply of means, combining fractionation by different techniques with immuno-chemical methods, and in particular with the exhaust of antibodies has been applied to different materials in a rather large number of investigations. Let us

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*) French society of microbiology, meeting of June 3, 1948.

**) This work was performed with the technical collaboration of Mlle Simone Le Lous, research assistant at C.N.R.S. The injections and blood-lettings of hyper-immunized animals were performed by M. Marcel Challeil; we thank them for their cooperation.

mention in particular those of Harris and Eagle (4) on horse and human serum, Kendall (5) on human serum, Marrack and D. Duff (6) on horse serum, P. Grabar and A. M. Staub (7) on carbuncle antigens, Treffers, Moore, and Heidelberger (8) on horse serum. More rarely, other authors (for example, Ando and his collaborators /9/) have considered serum as a natural mixture of antigens among which many are also gifted with the antibody function and have studied it by means of more strictly immuno-chemical methods[1].

Purely physical or physico-chemical methods - salting out or related methods of precipitation by organic solvents, ultrafiltration, ultra-centrifuging, electrophoresis - enable one to count the substances with large molecules contained in a mixture, and often to identify them by certain physical or chemical characteristics.

Immuno-chemistry must afford an analysis of the same order, differentiating substances thanks to their antigenic specificity.

Antigenic specificity cannot be expressed numerically, but its character is much more "personal" than the criteria used in physico-chemical methods. Moreover, it presents a greater biological interest, as was shown by Landsteiner's works, at least for protein molecules, since it carries so to speak the mark of origin of these molecules in the organism, and that of the place occupied among living beings by the species producing these molecules.

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1) One should indicate here also the method recently developed by Naylor (10) about which we heard while preparing the present note, thanks to the courtesy of its author and that of Dr. Coombs; this method combines Ramon's primo-flocculation with Dean and Webb's optimal proportions and leads by the study of "isochronous" curves (see Boyd /11/) to the count of the antigens contained in a mixture.

In any case, it is not certain that a purely immuno-chemical method of analysis could duplicate the results obtained by physico-chemical methods. It is possible that the classification imposed by immuno-chemistry on the substances of a natural mixture would not coincide exactly with the classification of the same substances obtained by physico-chemical methods. For example it is not impossible that two substances differentiated by electrophoresis or salting out may have the same antigen specificity or, vice versa, an apparently homogeneous electrophoretic or salting out fraction may contain antigens having different specificities[2]. On the other hand, it is not likely that one may find in immuno-chemistry a method of separation such as accompanies the physico-chemical methods of analysis.

Finally the physiological conditions of acidity and salinity proper to the very mild reagents used in immuno-chemistry seem to be well adapted to the fragility of the substances they will help us study.

The goals of qualitative immuno-chemical analysis ~~will be as follows~~, using antibodies as sole reagents:

1) Count as completely as possible the antigens contained in a mixture;

2) Try to identify the antigens contained in a mixture of given origin, using the homologous or heterologous specificity of the antigens found in mixtures having different origins, or in products of fractionation. ~~In this quest one~~

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2) For example, Gell and Yuill (12), working with albumin fractions from horse serum prepared by Kekwick (13) found by the method of optimal proportions that one of these fractions gave with several antisera, two zones of flocculation while it seemed to be homogeneous when subjected to ultra-centrifuging and electrophoresis. On the other hand, two substances having the same antigen specificity, may differ in one part of their molecule and be distinguishable by physico-chemical means.

may use also information relating to other characteristics of antigens besides their specificity, information that may be yielded by their reaction with antibodies.

We have attempted to develop a method that will lead to the realization of this program (14, 15). The present work is a critical expose of this method.

I. Principle of a Method of Immuno-Chemical Analysis.

1. Two liquid layers are poured into a tube, one above the other, without mixing; one contains a solution of antibodies, the other the single antigen of a simple precipitating system. We shall assume that it will be possible to prevent the operation of the force of gravity on the precipitate formed by the combination of the two reagents. This condition may be realized in practice by making both layers gel, or else only the one that is to be observed. The gelid state, the nature and concentration of the gelling substance modify the course of diffusion, but we think that their import may be regarded as negligible in the following reasoning where the quantitative aspect of the question is not considered.

In order to simplify and schematize the extremely complex problem thus stated by reducing it to a better known problem, we shall assume also that the reaction between the two reagents gives rise to insoluble compounds at all the levels of the tube where, owing to diffusion, the concentrations of these compounds reach values such that a precipitate would be formed in an appreciable volume of the mixture containing the same reagents at the same

respective concentrations[3].

2. a) When the initial conditions required to form the precipitate in the antibody layer are realized, the zone of precipitation will recede from the antigen layer and will be separated from the interface by a region where the precipitation is less dense (inhibition zone due to an excess of antigen).

b) When the initial conditions required to form the precipitate in the antigen layer are realized, the precipitation will spread to an ever extending layer of antigen, without any inhibition zone[4].

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3) Reservations would have to be made which do not affect the qualitative aspect of the question, and one cannot pass so easily from observations made in the "static" state on a mixture with unchanging proportions, to the occurrences taking place at a given level of a tube where the proportions of the reagents are changing constantly because the concentration of one of them increases steadily. Thus one would have to consider perhaps the effects of the progressive addition of antigen, as in Danysz' phenomenon.

This simplified postulate neglects also the time of formation of the precipitate. Unless we consider this time to be zero, it is clear that the precipitate, first formed, then dissolved by an excess of antigen, will always carry a certain time lag; the time element will never be exactly proportional to the appreciable time in which a precipitate would be formed in a mixture containing fixed proportions of the two reagents at the same respective concentrations that they hold at a given moment in the layer under consideration. The time may be divided into the following successive elements: 1) the reaction between the two reagents; 2) the aggregation of the compounds formed to give insoluble complexes (second phase, specific according to Heidelberger (16) and Marrack (17), not specific according to Hooker and Boyd (18). The rate of compounding seems to be very great because in studying the reaction of pneumococcic polyosides of type III and VIII with their homologous antibodies from horse, Mayer and Heidelberger (19) found that the compounding was complete to within less than 10% after less than three seconds (minimum time that they could test). On the other hand, it has been observed repeatedly that a mixture antigen-antibody centrifuged after a much longer time (several minutes, for example) continues to form a precipitate.

The simplifying hypothesis above must evidently be least exact during the first moments of the reaction, while a given concentration of the diffusing reagent passes most rapidly from one level to another.

4) See p. 6.

c) A third eventuality intermediate between a and b would occur when the precipitation takes place in the neighborhood of the interface without appreciable displacement toward one or the other of the two layers.

One cannot expect to see a simultaneous diffusion of the two reagents in opposite directions, to see for example antibodies diffused toward the antigen layer when the precipitation occurs in the antibody layer[5]. In this case the antigen will neutralize and render insoluble on the spot the molecules of antibodies as soon as diffusion will carry it farther into their layer.

It may be foreseen that in a given precipitating system, the initial concentrations of the antibodies and the antigen will play a determining part in the conditions necessary to realize one of the three possibilities above. There will be, no doubt, a relation between the ratio of the initial concentrations of the two reagents compatible with the third possibility c, and the ratio in which the same reagents are combined when they are equivalent under the usual conditions of precipitation in a liquid medium.

3. In any case, a precipitating system containing a single antigen can give rise in the antibody layer only to a single zone of precipitation.

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4) This will be realized every time one uses rabbit antibodies and more generally antibodies such that an increase in their quantity mixed with a constant volume of the same antigen solution does not bring about a decrease in the amount of precipitate formed. It is thus true also of horse antipolyosides, but not of horse antitoxins.

5) We must recognize that this does not seem to be objectively evident since Adair (20), in his attempts to apply the theoretical laws of diffusion to the indicator method (case of NaCl diffusing in a gel containing $AgNO_3$), takes into account the diffusion of silver nitrate (p. 770). The fact (see later and Fig. 3) that the density of the precipitate (see next paragraph) seems to vary very little or not at all, when the zone recedes from the interface in the course of time, seems to be at least a presumption in favor of accepting in practice our viewpoint.

Heidelberger's quantitative method enables one to establish the curve of the precipitated weight of nitrogen[6] in function of the amounts of antigen mixed with a constant amount of antibody. We know of no example when such a curve evidenced more than one definite maximum in the case of an incontestably unique antigen.

The maximum would remain unique if, in drawing the curve, one were to carry in abscissa an increasing or decreasing function of the amounts of antigen, instead of these amounts proper. But there is no doubt that in this case the concentration of the diffusing antigen is a decreasing function of the distance from the interface.

If, when the precipitation occurs in the antibody layer, the antibodies are not diffused in opposite direction to the antigen through the precipitation zone, then, for the reasons stated above, the concentration of free or precipitated antibodies may be considered as being constant throughout the height of their layer.

We shall call precipitation density the amount of precipitation per unit of volume; it may be assumed that this density is constant in a cylindrical tube at all points equidistant from the interface. A more precise definition of this density is that it is the derivative of the total amount of precipitation contained in the tube between the interface and a given level, taken with respect to the distance between this level and the interface. As in the case of the

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6) The weight of the precipitate is sensibly proportional to the precipitated weight of nitrogen only in the case of holoprotein antigens; nevertheless, it seems legitimate to generalize the following, extending it even to non-nitrogenous antigens.

curves mentioned above representing the weight of the precipitated nitrogen, the precipitation density will not exhibit more than one maximum when a single antigen is involved.

If more than one maximum occurs, one must conclude that the system in question is not simple; the number of antigens contained in one of the layers and capable of precipitating with the antibodies contained in the other layer is not less than the number of maxima occurring in the precipitation density curve. There may be more antigens than maxima: a) if the precipitation due to one of the antigens takes place totally within the antigen layer, an eventuality to be foreseen under certain conditions of concentration; b) if two or more maxima are so close together that they cannot be distinguished. For these reasons, the number of observed maxima must be considered as the lower limit of the number of antigens present.

4. The possibility of a phenomenon similar to Liesegang's, where the precipitation progresses periodically (or rhythmically) rather than continuously, may nevertheless vitiate our conclusion.

Phenomena of this type relating to the precipitation of mineral reagents, especially in a gelled medium, have been the object of very numerous investigations. [See for example Hedges (21), S. Veil (22), M. F. Taboury and F. J. Taboury (23)]. Among the authors who have studied up to now and for different ends, either theoretical or applied to bacteriology, the diffusion of antigens in gels containing antibodies [Bechhold (24), Reiner and Kopp (25), Petrie (26), Sia and Chung (27), Kirkbride and Cohen (28), R. Brown (29), Petrie and

D. Steabben (30), Elek (31)7, and Ouchterlony (32) in his still unpublished works[7], most have noted the appearance of phenomena related according to them, to Liesegang's (24, 25, 26, 29, 30, 31). The darkness still surrounding the mechanism of such phenomena does not enable us to foresee the conditions necessary and sufficient for their appearance in reactions of the type we are studying. For this reason the objections aroused by these phenomena cannot be considered any further in the theoretical part of this work; they will be studied in the light of experimental facts (see below: causes of error, p. 22).

We will have to indicate also the experimentally discovered exceptions to our theoretical conclusions.

Let us note immediately, moreover, that it is not easy to ascertain directly the maximum precipitation density in a column of gel; for this reason it will be sufficient in practice to observe the maximum diffused light by lighting the precipitate obliquely on a black background (this is the type of lighting we used for our photographs), or else the maximum absorbed light, the precipitate being seen by transparency on a lighted background. In practice also, the complete immune serum will be used instead of the antibodies, and controls will be prepared in order to reveal possible non-specific precipitations.

II. Techniques

We have selected agar-agar for a gel instead of gelatin, because the low melting point of the latter makes its use inadvisable.

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7) O. Ouchterlony's work (from the Statens Bakteriologiska laboratorium of Stockholm), undertaken with bacteriological ends in mind, with techniques different from ours, may be applied to immuno-chemical analysis as we have defined it; we thank him for having imparted to us spontaneously the text of his work before its publication.

1. Preparation of the gel. - A known weight of agar-agar, cut fine, is washed several times in twice distilled water, then dissolved in twice distilled water, and mixed with fresh rabbit serum, so that the serum represents approximately 10% of a 4% solution of agar-agar; the solution is heated for fifteen minutes in the oven to about 115°. On removing from the oven and before the gel has set by cooling, the solution is centrifuged at about 7,000 r.p.m., until a perfectly clear liquid is decanted. Pasting with serum seems to increase the limpidity of this liquid. The approximate content of dry gel in this medium, decreased by centrifuging, is determined by weighing the dry residue of a gel prepared with the same original concentration and under identical conditions, except that serum was not added; the dry weight differs little from that of the pasted gel. To all the decanted liquid, water and sodium chloride are added until the salt concentration is about 0.85% and the dry gel content about 2.4%.

The liquid is poured into glass tubes that are sealed with an open flame and sterilized by heating twice to 100° for thirty minutes each time in an open oven, with a twenty-four hour interval in between.

2. Preparation of the tubes and vats of gelled immune serum. - We have used in the present work a final gel concentration of 0.3% of the mixture and, in almost all cases, a 50% concentration of total rabbit serum. If necessary, the immune serum is brought to a concentration twice its final concentration in the mixture, by dilution with fresh rabbit serum, both sera having been carefully centrifuged. The serum is mixed over a bath at 46-48° with a superfused gel solution concentrated to 0.6% by dilution with salt water at 0.85%.

An appropriate antiseptic in sufficient quantity has been added beforehand to the constituents of the mixture in order to prevent the later appearance of any microbial cultures; we use in general sodium ethyl-mercuro-thio-salicylate in the conc. of 1/10,000 (Merthiolate from Eli Lilly and Co., or Thiomersalate from B. D. H.).

This mixture is spread up to the desired height (usually 3 to 6 cm) in tubes or in vats that have been subjected to a preliminary treatment for the following reason: gelose does not stick to the glass like gelatin and may be easily unmolded. Probably for this reason, when an immune serum gel poured into the tube in direct contact with the glass, is topped with antigen solution, especially if the latter is dense, a thin layer of antigen solution often penetrates between the tube wall and the column of gel and causes there a specific precipitate. In this case, because the diffusion of the antigen does not take place only through the upper part of the column of gel, the borderline of the precipitate, instead of becoming rapidly flat, assumes irregular shapes; this makes it sometimes difficult to take a reading, and in any case the appearance of the tubes is untidy. We have remedied this inconvenience by the following empirical means: the tubes and vats, heated to 60 or 70°, are filled with an about 1% gel solution heated to the same temperature: they are emptied at once and plunged into a bath at 0°; their inside surface is thus covered with a layer of gelose which is then dried by placing the tubes in a vacuum in the presence of sulfuric acid. This coating shows itself by an opalescent and watered sheen which disappears when the gelled serum is poured. Thanks to this precaution the almost constant inconvenience mentioned above, occurs rarely.

The antigen solutions are then poured into the tubes and the vats containing the gelled immune serum; they are carefully stoppered; the vats are stoppered with paraffin near its melting point, or, preferably, with wax softened by moderate heating and topped with a thin layer of canadian balm.

We shall see that the tubes and vats must be kept at a constant temperature, or at least kept from rapid temperature changes; it is desirable to bring the gel and the antigen to the final temperature before placing them in contact. This temperature was 22.5° in most of the present experiments. The antigen solutions are poured into the tubes and the vats to a height of from 2 to 3 cm.

3. Length of observation. - The length of observation rarely exceeds eight or ten days; when the delay is longer, secondary parasitical effects must take place, because we have occasionally observed inconsistent and incoherent results in tubes evolving for a much longer period of time.

4. Photography. - For ease in photographing, we used vats with parallel sides on which light fell, illuminating sections 1.5 mm x 5 mm. One of the advantages of the vats is that under appropriate lighting, the intensity of images along a perpendicular to the axis of the vat is constant, and therefore microphotometric tracings can be made. Moreover, the small inside thickness of the vats has allowed us to differentiate between very close-lying borderlines better than inside thicker cylindrical tubes.

The vats may be photographed by direct contact with the sensitive plate, light coming from a faraway source, or in a parallel beam; the positive of such a picture gives dark zones of precipitation against a light background. But all the photographs accompanying this article have been made by lighting

the vats and tubes slantwise against a black background. Our lighting was provided by two strings of light that could be placed either vertically, laterally and, as much as possible, symmetrically, at a variable distance from the vertical intersecting the axis of the objective and from the center of the object, or horizontally above and below this axis, and, in both cases, at a variable distance from the object. This lighting, although appreciably symmetrical, is unfortunately imperfect, because the light should be preferably parallel and should give constant lighting over the whole field of photography.

The pictures are in general directly enlarged while being taken (1.5x for the photographs published in this note, unless otherwise specified). The published pictures are developed positives and the precipitation zones appear on them in light against a black background; the microphotometric tracings, on the contrary, are taken from negatives and the light absorbed by the negative is an increasing function of the light diffused by the precipitation zones.

In order to discuss the principles of our method stated above, we shall consider now the results obtained when this method is applied to precipitating systems where the number of antigens placed in presence of antibodies with which they react under favorable conditions, is known.

This will lead us to study successively simple precipitating systems, complex precipitating systems, and multiple precipitating systems, that we shall define in their proper place. The study of complex and multiple precipitating systems will be reported upon in the second part of this note.

All our observations have been made with rabbit immune sera.

III. Simple Precipitating Systems

A. Standard Simple System: Chicken Ovalbumin in Homologous Reaction

Our standard of simple precipitating systems, i.e., a precipitating system in which a single antigen (or haptene) reacts with homologous or heterologous antibodies, is crystallized chicken ovalbumin, a convenient type of well-defined antigen, reacting with the homologous immune serum.

Later on we shall rapidly pass in review several other precipitating systems that are either simple or appear to be so.

Selection of the diffusing reagent. - 1) When the ovalbumin diffuses into a gelled medium containing antibodies (Fig. 1), the precipitating zone is bordered below by a very sharp line and topped by a region in which the high antigen concentration causes the partial or total dissolution of the precipitate.

2) When the antibodies diffuse, starting from the liquid serum into a gelled medium containing the antigen at a sufficiently low concentration (for example 3 γ of ovalbumin nitrogen per cc of gel, Fig. 2), the "precipitation density" first grows rapidly up to a low elevation, then continues to grow along a gentler slope up to the interface.

The first of these two techniques is the more interesting one for the ends that we are pursuing presently; indeed, in case several antigens were to react in the same tube, there would be in the first technique an inhibition zone that would be invisible in the second method every time rabbit antibodies were used, and this inhibition zone would insure with greater probability that one or more possible zones lying close to the interface would not be masked by another zone.

GRAPHIC NOT REPRODUCIBLE

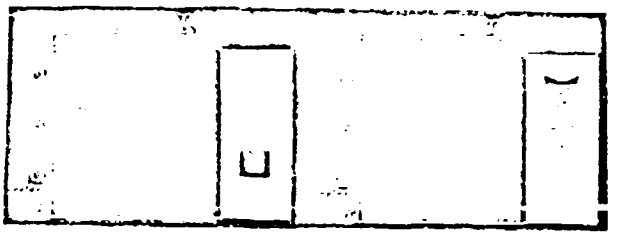

Fig. 1 Fig. 2

Fig. 1. Reaction of chicken ovalbumin (upper, liquid layer) with homologous rabbit immune serum (lower, gelled layer); the antigen is the diffusing reagent. The microphotometric tracing (*) has been made from the negative. The abscissas are assumed to be proportional to $T = I/I_0$, where I is the light transmitted at the microphotometer cell, and I_0 is the emitted light. The ordinates are the distances h from the different levels to the interface (which corresponds to zero on the scale). The tracing has been reduced by photography to the same scale as the picture.

Fig. 2. Same reagents as in Fig. 1, but here the antibodies diffuse starting from the upper, liquid layer (pure immune serum) into the lower, gelled layer (chicken ovalbumin with 3 γ of nitrogen per cc of gel). The picture was taken eight days after the beginning of the reaction.

For this reason we shall discuss henceforward in this memoir only the results of the first method (antibodies in the gelled medium).

Principal factors influencing the course of the reaction.

1. We shall only name them here, stopping only to the extent that these factors influence the qualitative aspect of the question.

The distance between the borderline and the interface increases with time; in the general case where a substance contained in a column of gel reacts

(*) We are grateful to Mme Guilmart (from Mme Ramart-Lucas' laboratory to whom we owe the microphotometric tracings presented in this memoir.

visibly with another substance diffusing in the gel, theory indicates that the distance between the interface and the level reached by the reaction is proportional to the square root of the time, when the concentration of the diffusing substance remains constant in the solution in contact with the gel [see J. Duclaux (33) II-87; although this condition is obviously not realized here as regards antigen, we shall retain this law as a sufficient indication of the influence of time on the distance from the interface to the borderline of precipitation.

The other principal factors may be divided into two categories, depending upon whether their growth causes: a) an increase, or b) a decrease, in the distance covered at the same time by the borderline. a) The initial concentration of the antigen, the concentration of substances other than the antigen that may be in solution with it, the temperature, belong to the first category. b) The concentration of antibodies, the concentration of substances other than the antibodies that may be in solution with them in the gelled layer, the concentration of agar-agar in this layer, belong to the second category. The experiments proving the truth of this classification will be described in later publications.

If we did not limit ourselves to a given antigen, other factors would have to be considered, varying with the nature of the antigen: its coefficient of diffusion, the ratio antibodies/antigen in the precipitate formed when the two are equivalent (both these variables are functions of the molecular weight of the antigen[10]), the more or less great solubility of the formed complexes...

We shall say nothing more about some of these variables; we shall retain only the time and the concentrations of the two reagents in order to study

rapidly their influence, less upon the space covered by the borderline than upon the precipitation density at different levels of the zone. We shall go over the influence of temperature when we consider the sources of error in the counting of antigens.

2. Effect of time. - One may gather an idea of what it is from the three tracings shown in Fig. 3 and from the three pictures represented in

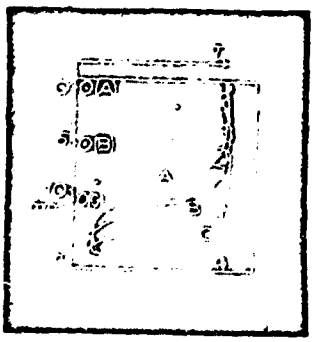

Fig. 3. Evolution of the tracing in the course of time, in the reaction of chicken ovalbumin with the homologous rabbit immune serum. The three tracings are from the negatives of three vats containing the two reagents in the same initial concentrations (ovalbumin: 1.58 mg of nitrogen per cc; immune serum: 1/2 dilution in the gel). The reaction has developed for nine days (A), four days (B), one day (C).

plate I, accompanying the second part of this article. The three vats contained the same anti-ovalbumin immune serum concentrated to 1/2 in the gel, and the same ovalbumin solution in the same initial concentration of 1.58 mg of nitrogen per cc; the contents of the vats were placed there one day, four days, and nine days, respectively, before the photograph was made, so that the distances between the borderlines and the interface would be in the approximate ratio of 1/2/3.

It should be noted that in spite of the probable imperfection of our lighting, the maxima of the three tracings are almost equal. This would tend

to confirm the opinion that the diffusing antigen meets approximately the same concentration of free antibodies at all the levels of the column of gel in proportion as the borderline reaches these levels. One could also observe both on the graph and on the photographs, that the image seems to develop in time as if the distance from the interface to some level with a constant precipitation density remained roughly proportional to the distance covered by the borderline.

3. Influence of the concentration of antibodies. - We used three different vats, and kept the concentrations of the antibodies in the gelled media of these vats in the ratio of 1 to 3.16 to 10; the medium with the greatest concentration of antibodies contained in 3/4 of its volume the same

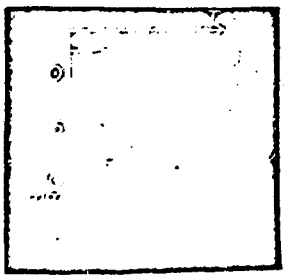

Fig. 4. Influence of the concentration of antibodies on the tracing in the reaction of chicken ovalbumin with the homologous rabbit immune serum. The concentration of antibodies varied proportionally to 1, 3.16, and 10 (A, B, C, respectively) in the gelled layers of the three vats whose negatives were used the tracings. The initial antigen concentration was the same in all three vats. The times were calculated so that the distance from the borderline to the interface remained roughly the same in all three vats.

immune serum as above; the media of the two other vats contained in 3/4 of their volumes the same immune serum diluted to the proportion of 1/3.16 and 1/10, respectively, with fresh rabbit serum. The antigen was poured into the three vats at the same concentration (1.58 mg of ovalbumin nitrogen per cc), but at different times, calculated[8] so that the distance between the interface

8) This computation was performed with the help of quantitative laws that will be the object of a later publication.

and the borderline would be about the same in all three vats at the moment when the picture that is to appear in the second part of this paper (plate I) was taken.

The tracings (Fig. 4) represent more precisely than does the picture the difference between the luminosity (by sideways lighting) of the specific precipitation zone in all three cases; the distances between the maxima of the three tracings and the points with the same abscissa corresponding to zero transmitted light, are very roughly proportional to the concentration of antibodies in the gel (1.3/3/8.7 and 1/3.16/10, respectively).

4. Influence of the initial antigen concentration. - We placed into three different vats gelled media having the same antibody concentration (the same immune serum as before, occupying 1/2 the volume of the gel); the initial concentrations of ovalbumin in the three vats had values proportional to 1, 3.16, and 10, the most concentrated of the three solutions containing 5 mg of ovalbumin nitrogen per cc; the time (different for the three vats) elapsed between the beginning of the reaction and the picture-taking (simultaneous for all three vats) was calculated[8] so that the distance separating the interface from the borderline would be about the same for all three vats at the moment of picture-taking (this picture will be reproduced on plate I to be published with the second part of the present memoir.

It is apparent from the tracings (Fig. 5) that the maxima reach very similar heights[9]; one may conclude therefrom that the initial antigen

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[9] The imperfect light shed by two vertical, lateral, and approximately symmetrical strings explains the slight difference between the maximum in tracing B (center vat) and the maxima A and C (symmetrical side vats) which are almost equal.

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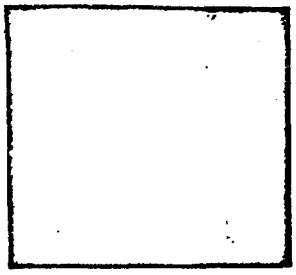

Fig. 5. Influence of the initial antigen concentration on the tracing in the reaction between chicken ovalbumin with the homologous rabbit immune serum. The antigen concentration varied proportionally to 1, 3.16, and 10 (C, B, and A, respectively) in the upper liquid layers of the three vats whose negatives were used to make the tracings[9]. The concentration of antibodies in the gel was the same for all three vats. The time has been calculated so that the distance separating the borderline from the interface would be roughly the same in the three vats.

concentration exerts very little or no influence on the density maximum of the specific precipitation zone.

On the contrary, the slope of the tracing immediately above the maximum is stronger when the initial antigen concentration is higher: keeping the same distance between the interface and the borderline, when the initial antigen conc. grows so does also the extent of the inhibition zone at the cost of the precipitation zone.

Conditions under which the specific precipitation zone appears in the gel. -

1. - Outside of the effect of time, which seems to be zero on the maximum precipitation density, we shall remember the following from the four preceding paragraphs: 1) the distance covered by the borderline in a given time is an inverse function of the antibody concentration, and a direct function of the initial antigen concentration; 2) the maximum density reached by the specific

zone is a direct function of the antibody concentration and does not seem to vary with the initial antigen concentration.

This enables us to understand that when the two principal factors in a given simple precipitating system (antibody concentration within the gel, and initial antigen concentration above the gel) vary beyond certain limits, there can be no precipitation within the gel, or else it cannot be detected:

1) Whatever the initial antigen concentration, there is a value of the antibody concentration below which the maximum density of the zone is so weak that the zone is not visible.

2) As we have hinted when explaining the principle of the method, there exists, for each concentration a of the antibodies, a minimum value g_o of the initial antigen concentration below which the precipitate does not appear in the gel, but is formed rather in the liquid layer of antigen solution. It is likely that the ratio between these two values varies for different antigens in the same direction as the ratio between the equivalent masses of the two reagents.

In practice, when the initial antigen concentration is sufficiently high, the precipitation borderline, which in the beginning had the shape of the interface (meniscus), becomes rather rapidly flat and perpendicular to the axis of the tube or vat (as on the photographs). When the antigen concentration assumes on the contrary the lowest values compatible with the formation of a zone within the gel, then the displacement of the borderline as a function of time is almost zero. The borderline never becomes flat; in the middle of the meniscus, it remains very close to the interface and separates itself only slightly on the circumference where it is sometimes hard to be seen. In such a case, and if

the antibody concentration is sufficiently removed from the minimum to which we were referring above, the borderline may be made more easily observable by diluting in another experiment the antibody concentration while the initial antigen concentration remains the same.

2. The resolving power of this method cannot be specified independently of the nature of the antigen. In the case of ovalbumin, an initial concentration of 0.01 mg per cc is enough to make the borderline penetrate into the gel, provided one uses an immune serum sufficiently diluted while still giving a discernible precipitation zone. But ovalbumin is a protein with a low molecular weight; the minimum detectable concentration for an antigen having a greater molecular weight would likely be higher, because the coefficient of diffusion of the antigen and the ratio of the equivalent masses antibodies/antigen would both be lower[10].

Sources of error and their elimination. - The value of our method depends on the condition that one antigen may not give rise to more than one zone, or more than one borderline; in other words, it should not yield more than one maximum of the precipitation density, unless other possible maxima, whatever their cause, may be characterized and recognized.

1. Non-specific precipitation. - In spite of the physiological conditions of acidity and salinity to which is subjected any antigen solution that is studied, one may not exclude a priori the possibility of non-specific precipitation zones. A control containing fresh rabbit serum instead of immune

10) See Boyd and Hooker (34) for the relations between the molecular weight of antigens and the ratio of equivalent masses antibodies/antigen.

serum must help detect them. It is theoretically possible that a non-specific precipitation would occur in one serum, but not in the other, because of their different compositions, but this is very unlikely and we have never observed this to happen. Moreover, we have noted only by way of exception a non-specific precipitation giving rise, for example, to more than one zone in a simple system.

A gross source of error would be a microbial culture in a medium that would be very favorable to it, if it did not contain antiseptics.

2. Bands due to changes in temperature. - If a tube developing at a constant temperature (e. g., 22. 5°) is suddenly cooled (e. g., placed in an icebox at 4 or 5°), a narrow band of less dense precipitate appears at the level occupied by the borderline when the temperature changed. If, on the contrary, the tube is heated (e. g., from 22. 5° to 37° in an oven), then a narrow band of denser precipitate will appear.

In both cases the band appears at the exact place occupied by the borderline when the temperature changes, and remains there. It will be seen at the same level until it is lost because of the progression of the precipitation zone, and inhibition, even when apparently complete, does not erase it always.

When the temperature change is only temporary (this occurs most frequently when the change is accidental) as when a tube developing before and after at 22. 5°, is cooled for 10 minutes to 4°, then a less dense band appears, immediately followed below by a denser band, and in direct contact with it. These two contiguous bands are obviously in reverse order when they are the result of a temporary rise in temperature.

Photographs of vats representing these four types of accidents will be represented in plate I that will be published with the second part of the present memoir.

Even when the temperature changes are far smaller than the examples given above, they may suffice to give still visible bands, especially when dealing with very dense bands.

These accidents may be avoided if sufficient precautions are taken, either to keep the temperature constant within the enclosure where the tubes are kept, or, more simply, to make the accidental, limited temperature variations sufficiently slow. Moreover, the particular appearance of these bands and their fixed position in space at all times makes them so distinctive that provided each tube is examined more than once, they cannot be mistaken for anything else. These non-specific accidents may be recognized if the same tube is examined twice with a sufficient time interval in between, or else, if two tubes containing the same initial solution, but developing for a different length of time, are examined simultaneously.

3. Liesegang's phenomenon. - We have not encountered it so far with ovalbumin. We cannot explain its absence by the need for a special ratio between the initial concentrations of the two reagents, or for certain absolute values of these concentrations, because, with another goal in mind, we have varied these concentrations (in steps of 3.16) almost to the materially possible limits. Thinking that the weak concentration of agar-agar in our gels may have been unfavorable to the development of Liesegang's phenomenon, we have vainly attempted (in fragmentary and non-systematic tests, it is true) to

make it appear in gels containing 2% agar-agar, or 1% agar-agar and 4.1% of gelatin (these latter were left to develop in the icebox).

Let us add immediately that we have not observed any Liesegang type phenomenon with any of the precipitating systems, simple, complex, or multiple, that we have studied, where the number of antigens was known. This was because, outside of the sources of error already mentioned, the number of zones or borderlines was never larger than the number of antigens.

If Liesegang's phenomenon were to occur under the methodological conditions adopted by us, our method of counting antigens would be invalidated just as it is invalidated by accidents due to temperature variations. Liesegang's phenomenon is characterized by the periodicity and the immobility of the bands marking the successive stages in the progress of the precipitate. Periodicity is likely to be a treacherous criterion: 1) because it may happen that several not interrelated zones due to different antigens assume the appearance of Liesegang's phenomenon, in spite of their uninterrupted progress; 2) because the stacking of several connected and unconnected zones one above the other may hide their periodicity or else make it very difficult to detect. The immobility of the bands should on the contrary, by contrast with continuous progression, make them easily recognizable by the same means that the bands due to temperature variations are recognized.

It seems, moreover, that these two series of phenomena may be rather closely connected and it is not unlikely that they have been confused in some cases (see Hedges (21), p. 19 and 20).

It remains to be explained why phenomena of Liesegang's type, recorded by many authors /Bechhold (24), Reiner and Kopp (25), Petrie (26), R. Brown (29), Petrie and D. Steabben (30), Elek (31)/ as occurring during specific precipitation in a gelled medium, have never been observed by us up to now. Can this divergence be due to differences in technique?

B. Other Examples of Simple Precipitating Systems

1. Albumins from horse serum. - Two fractions made available to us by M. P. Grabar have been studied and already mentioned in an earlier work (15); we made them react with a mixture of sera from rabbits immunized with injections of horse serum.

1) Fraction T5. - This fraction seems to hold a single antigen, and behaves qualitatively like chicken ovalbumin, giving a single zone with a sharp lower borderline.

2) Fraction L3b. - This fraction seems to hold at least two different antigens; it presented the appearance of a simple system and yielded a single zone with a sharp lower borderline when the immune serum with which it reacted had been exhausted beforehand by treatment with fraction T5.

2. Duck ovalbumin in heterologous reaction. - We used an amorphous preparation reacting with a rabbit serum that was a specific against chicken ovalbumin.

We obtained a single zone whose appearance was qualitatively similar to that of the homologous zone (that of chicken ovalbumin) with an equally sharp lower borderline.

3. Somatic antigen O from "Eberthella typhosa". - This antigen extracted from microbial bodies (strain O-901) by the method of

Boivin, I. Mesrobeanu, and L. Mesrobeanu (35) yields a zone with a blurred lower borderline (plate I, to be published with the second part of the present memoir). In spite of a strong antigen concentration the zone does not penetrate deeply into the gel. even after a considerable time. This may be explained by the large size of the antigen particles which has been proved for example by means of ultrafiltration (36), and by the very low ratio antibody nitrogen/antigen in the precipitate formed when the reagents are in equivalent amounts (according to the results of Hornus and Grabar (37), this ratio is from 0.17 to 0.21, instead of 3.6 for the polyoside).

4. Polyoside O from "Eberthella typhosa". - The polyoside obtained by acetic hydrolysis of the somatic antigen O above has never yielded a sharp borderline like the four preceding protein antigens, but rather a zone without definite lower limit[11], as may be seen on the tracing and the photograph (Fig. 6).

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11) In order to see well the precipitation zone of the polyoside, the eye (or the objective) must be placed inside the very acute-angled cone formed by diffused rays (the angle of the cone is much more acute than with ovalbumin, for example). This leads one to think that the particles of the precipitate formed in the gel are smaller in this case than in the case of albumins, since the gel is the same in all cases. In the case of albumins, the precipitate appearing between the maximum precipitation density and the interface seems for the same reason to be formed by particles whose size decreases as the level approaches the interface (this could be foreseen, since the precipitate is in process of being dissolved in an excess of antigen).

GRAPHIC NOT REPRODUCIBLE

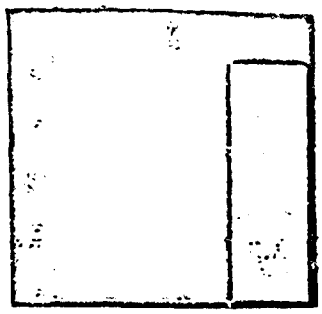

Fig. 6. Reaction of the C polyoside from E. typhosa (upper layer, initial liquid concentration: 1 mg/cc) with an immune serum specific against the somatic antigen (1/2 the volume of gel). Photograph taken after 12 days.

5. Pneumococcus polyoside (type VIII). - The specific zone of this polyoside is similar to that of the typhus haptene, because it also lacks a sharp lower limit.

The seven precipitating antigens discussed above behave identically in one respect toward antibodies in a gelled medium: they yield a single precipitation zone. The principle of our method, temporarily accepted as being the result of a theoretical reasoning, is thus confirmed experimentally. The conditions selected by us are such that the principle may be generalized.

The behavior of the seven studied antigens differs in other respects, among which we shall remember mostly the sharpness or blurring of the lower limit of the precipitation zone, i. e., the presence or absence of a sharp borderline.

1) The borderline is sharp in the case of the four antigens having a protein nature, of animal origin.

2) The lower limit of the zone is blurred in the case of one complete antigen and two haptenes having a bacterial origin and a partially or totally

polyosidic nature.

The antigens of the second type are products of trichloracetic extraction, acetic hydrolysis, or bacterial autolysis. By contrast, antigens of the first type have been prepared by fractionating techniques using salts, techniques that are not likely to degrade the antigens; these antigens are supposed to have a very definite molecular weight. We shall admit therefore as probable the hypothesis according to which a sharp borderline is characteristic of homogeneous diffusing antigens, while the absence of sharp borderline is characteristic of polydispersion, although objections may be raised against this hypothesis.

This duality in the behavior of diffusing antigens carries with it practical consequences: supposing that several simple systems held together in a tube behave as if each one were alone, a small distance between two sharp borderlines of the "ovalbumin type" will be sufficient to prove the presence of two antigens, but if the two zones are of the "polyoside type" without sharp lower limits, the distance between their maxima need be much larger. In other words, the method will probably be much more effective if the mixed antigens whose number is to be determined belong to the first type rather than to the second. If, the two types being present, a sharp borderline tops a precipitation region that degenerates at the bottom, the preceding observations permit us to conclude that two antigens are present, even when the maximum density of the "polyoside type" zone is hidden behind the superimposed "ovalbumin type" zone.

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In the second part of this memoir we shall study to what extent the presence of several antigens within the same solution and their simultaneous

precipitation by antibodies within the same gelled medium (complex and multiple precipitating systems) modifies the reaction with respect to what it would be, had the same reagents formed simple systems.

We shall consider also in the light of this study the practical application of our method to the counting and identification of antigens present in unknown numbers in a biological liquid that reacts, forming a multiple precipitating system.

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