SPECIFIC PRECIPITATION REACTION IN AGAR BY THE OUCHTERLONY METHOD

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It has been known for some time that addition of antigens to agar containing the appropriate antiserum is accompanied by formation of lines of precipitate. Oudin [8, 9] carried out detailed studies on diffusion of antigens in the gel and conditions of formation of precipitates upon interaction of antigens and antibodies. He showed that the number of precipitation lines formed corresponded to the number of antigen-antibody systems participating in the reaction. In Oudin's method the specific precipitation reaction is carried out in test tubes with agar in which the antigen and antibody diffuse towards each other. Ouchterlony [6] modified this reaction suggesting that instead of one diffusion center for antigen two such centers be used, thus enabling direct comparison of two antigen preparations. The reaction was carried out in Petri dishes, 90-96 mm in diameter, with a thin layer of 1.5-2% agar (6-8 ml) poured on the bottom, dies were then placed in position, another layer of agar (16-18 ml) was poured over them after which the dies were removed carefully and the resulting hollows of 0.3-0.5 ml capacity were filled with antigens and antiserum. The dies were made of plastic in the shape of parallelepipeds measuring 12 × 12 × 12 × 3 mm; sometimes stoppers from penicillin vials were used for this purpose. The placement of the holows can vary depending on the number of participating ingredients. Experiments can be carried out in dishes with three and more hollows. Figure 1 shows the approximate placement of hollows in an experiment with 5 ingredients.

The agar used in the reaction must be transparent and it was therefore filtered, while hot, through cotton wool after precipitation by 0.5% calcium chloride. The solidified agar was cut into small pieces (about 1 mm^3) and washed in running water for 72 hours. Prior to pouring the molten agar into the dishes, NaCl was added to it (final concentration 0.8%), as well as merthiolate 1:10,000 and methyl orange 2:100,000. Merthiolate prevents bacterial growth, while methyl orange increases the contrast properties of the medium. The course of the reaction is affected by concentration of agar, its electrolyte content, thickness of the layer, placement of the hollows, pH, temperature of the reaction [13]. The reaction may be carried out at room temperature, at 36.5° and in the cold (at 4-6°). Temperature does not affect the distribution of precipitation lines. Addition of NaCl to the agar is necessary to prevent nonspecific precipitation even though the reaction proceeds more smoothly in the absence of salt [13].

The optimal conditions are: temperature 36.5°, pH 7.5 and sodium chloride concentration 0.8% [13]. It is very important for the success of the reaction that the antiserum have a high precipitation titer. In order to prevent rapid evaporation of the antigens and sera it is recommended that the dishes be covered and sealed hermetically by putting gauze dipped in molten paraffin around their edges; this obviates the need for adding ingredients to the hollows during the experiments.

In Ouchterlony's experiments precipitation lines appeared on the 7th-10th day [6]. In our experiments the agar concentration was lowered to 1% and concentrated antisera were used. This enabled us to observe the

appearance of precipitation lines at room temperature 18-20 hours after the beginning of the experiment.

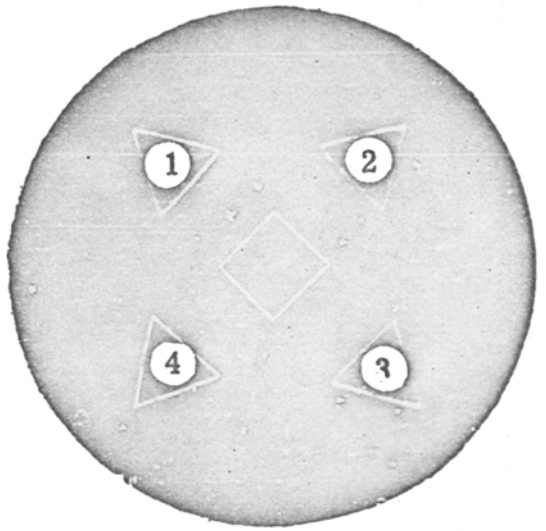

Fig. 1. Photograph of an experiment on specific precipitation reaction in agar with intracellular antigens. In the hollows: in the center – spleen cytoplasm antigen; in 1-4 – antiserum to whole tissue of leukemic spleen before and after depletion by nuclear antigen.

Halbert et al [2] used 0.6% agar and carried out the reaction at 4°. The results were available after 3 days. Jennings and Malone [3, 4] developed a more rapid method of precipitation reaction giving results in 2-4 hours.

Analysis of the reaction. When antigens diffuse against antiserum in agar, limited zones are formed in which the antigens and antibodies are found in optimal concentrations. Precipitation lines form in these zones. Figure 2, a, presents three variants of the reaction on comparison of various antigens. If the antigens being compared are identical, the precipitation lines cross; if only partial fusion of precipitation lines occurs with the formation of an appendage ("spur") it indicates that the antigens are related but are not identical [6, 7]. Reaction of this sort takes place in those cases in which the molecule of one antigen being compared is a carrier of several active groups whereas the molecule of the other one carries only some of these active groups.

The experiment on diffusion of hen and duck egg albumins against hen egg albumin serum is usually cited as an example of this reaction. This experiment is demonstrated in Fig. 2, b. Hen and duck egg albumins are related but not identical: antiserum to hen egg albumin reveals that two active groups are present in it, and only one of them in duck egg albumin. A fused precipitate line is formed by virtue of the active group common to both, while the appendage to this line indicates the incomplete similarity of the antigens being compared. Ouchterlony [7] called such a reaction "reaction of incomplete identity". This name has been retained although Oudin [11] criticized it on the grounds that identity excludes all differences and the name "reaction of incomplete identity" was incorrect.

It should also be pointed out that the name "identical reaction" is relative to some extent: formation of an arc-like fused precipitation line does not necessarily indicate that two antigens being compared are identical: it may be connected with the absence in the antiserum used of antibodies capable of demonstrating their differences. It is therefore essential to use antisera to both antigens when two antigens are being identified by specific precipitation reaction.

When two antigenic complexes are compared a number of precipitation lines is formed corresponding to the number of separate antigens taking part in the reaction. By removing a particular antigen from the complex or depletion by it of the antiserum it is possible to exclude that particular precipitation line and find out with which antigen-antibody system it is associated. As can be seen from Fig. 2, c, removal of antigen B from the complex led to the disappearance of the middle precipitation line, whereas removal of antigen A led to the disappearance of the line nearest to the antiserum reservoir. This permits the conclusion that the latter line is associated with antigen A and antibody anti-A, and the middle line with antigen B and antibody anti-B. Other methods have been proposed for identification of antigens in complexes by suppression of one or other precipitation line; these methods include depletion in the medium (addition of excess antigen to the agar), addition of excess antigen to the serum reservoirs etc. [1].

The distribution of precipitation lines with respect to the diffusion centers depends on the initial concentration of the ingredients, the rate of diffusion, the time needed for the formation of visible precipitate and on the solubility of the antigen-antibody complex. Depending on the concentration of antigens the points of intersection or fusion of precipitation lines dispose themselves either symmetrically about the antigen reservoirs or are displaced away from the reservoir containing excess antigen (Fig. 3, a).

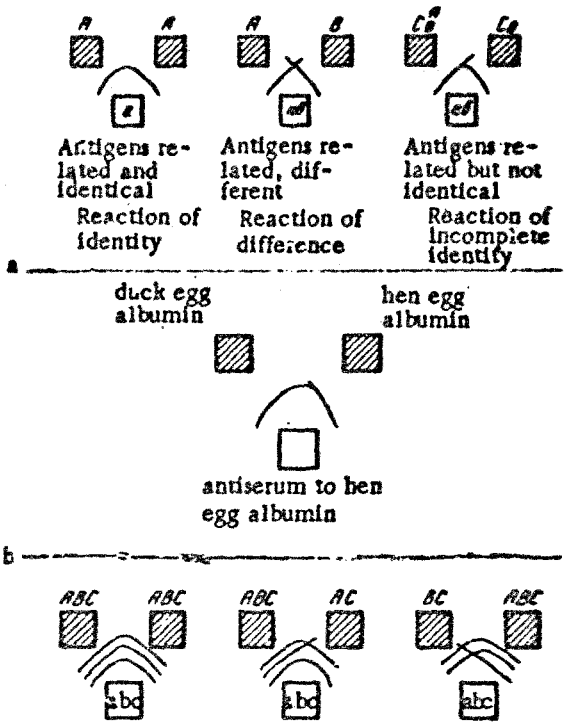

Fig. 2. Schemes of specific precipitation reaction in agar with simple and complex antigens.
a) Comparison of two antigens: b) example of reaction of incomplete identity; c) comparison of two antigenic complexes. Antigens are present in the shaded squares, antisera in the white ones.

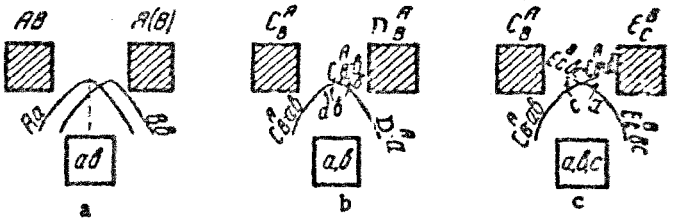

Fig. 3. Specific precipitation reaction in agar.
a) Comparison of two antigenic complexes. Precipitation line Bb displaced away from left reservoir in which antigen B is present in excess; b) comparison of a complex and a simple antigen with one common active group; one "spur" can be seen; c) comparison of two complex antigens with one common active group; two "spurs" can be seen. Antigens are in shaded squares, antisera to them in white squares.

Note. The scheme is based on the assumption that anti-A, anti-B and anti-C antibodies are independent molecules.

In model experiments with purified egg albumins Ouchterlony [7] determined more precisely the relation of the type of reaction to the nature and concentration of antigens. These experiments again confirmed that the appearance of multiple lines indicated multiplicity of antigens in a complex and that formation of appendages ("spurs") was connected with the fact that the composition of the molecule of one of the antigens included two active groups each evoking the formation of an independent antibody, while the molecule of the other antigen contained only one of these groups.

Investigation of mixture of homologous and heterologous antigens in various concentrations showed Ouchterlony that the reaction depended, to a certain extent, on the ratio of concentrations of the separate components of the mixture. Fusion of precipitation lines could occur in the presence of certain ratios. This is observed extremely rarely and simple dilution leads to separation of the lines and elucidation of the structure of the mixture or complexes.

"Pseudo spurs" may be formed when there is an excess of antigens. These, however, unlike the true ones, quickly dissolve and disappear. Nonetheless suitable concentrations of antigens must be chosen so that the precipitation lines would be symmetrically disposed, and trial runs with different concentrations are indicated.

Korngold [5] makes a detailed analysis of conditions under which true "spurs" are formed:

1) spurs are not formed when the antiserum contains only antibodies to those groups which are common to the antigens;

2) one spur is formed when the antiserum contains antibodies to both the group common to the two antigens and to the active group pertaining only to the homologous antigen (Fig. 3, b);

3) a double spur is formed in those cases in which the antiserum reacts with two antigens each of which has a common and a different active group and contains antibodies to all the active groups (Fig. 3, c).

In differentiating double "spurs" from "reaction of difference" it is essential to examine the nature of the appendages: the spurs are less solid than the main precipitation line, the change occurring abruptly; in the case of intersecting precipitation lines the decrease in solidity is gradual in the direction of the ends. Moreover, the precipitation lines fuse before the formation of "spurs" and remain "fused" after the formation of "spurs".

Differentiation of Antigen Complexes by Precipitation Reaction in Agar

Antiserum	Depletion by antigen	Test of depleted serum			
		Antigen	Reaction	Antigen	Reaction
Anti-(A+B)	B	B	Negative	A + B	Positive
Anti-C_B	C_B	C_B	"	C_B^A	"

The method of specific precipitation in a gel permits not only direct comparison of two antigens, determination of antigenic spectrum in an antigenic complex, identification of definite antigens in a complex, but also differentiation of complexes of independent antigens from complex antigens consisting of molecules with several active groups each of which is capable of evoking the formation of independent specific antibodies. This constitutes an important advantage of the given method over other serologic methods used for differentiation of antigens in antigenic complexes. For example, if the structure of antigenic preparations AB and C_B^A is studied by agglutination reaction then, as can be seen from the table, using appropriate sera and with their depletion by antigens B and C_B respectively the final reaction with the initial antigens will be the same in the case of the complex and the "mixed" antigen. In both cases the reaction demonstrates the presence in the serum of two antibody molecules with different specificities, but does not provide evidence on which it could be decided whether they are evoked by two separate antigens or by one antigen with two determinant groups; specific precipitation reaction, however, makes it possible to resolve this problem.

The method of specific precipitation in agar is simple, inexpensive and provides a practical means of differentiating any number of antigen components; it is almost as sensitive as other serologic methods. Very promising is the combination of this method with immunoelectrophoresis [12] which gives an electrophoretic characterization of each antigen participating in the formation of the precipitation line in the gel.

The method of specific precipitation in the gel has been used extensively in recent years for the study of problems in infectious and noninfectious immunology. It was used successfully (alone and in combination with immunoelectrophoresis) in the studies on the antigen structure of various infectious agents and products of their vital functions (staphylococci, streptococci, meningococci, tularemia and diphtheria bacilli, anerobic bacteria and their toxins, Hemophilus influenzae, small-pox virus, pig plague virus etc.), as well as in studies of antigenic structure of various proteins (snake venom, egg albumins, serum globulins in the normal and in cases of myeloma), antigens of normal and tumor cells in animals and man and antibodies in allergic and other diseases.

SUMMARY

Reaction of specific precipitation in agar is one of the methods of immunological analysis which have future prospects.

This method provides an opportunity to reveal the structure of complex combined antigens, to identify their separate components and to conduct a direct comparative analysis of various antigenic preparations. Special advantage of this method over other serological methods lies in the fact that it permits differentiation of the complex antigen, which is a molecule with two determinative groups from the combination or mixture of two antigens, each one of which represents a separate molecule.

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