LIMITING SENSITIVITY OF THE METHOD OF PRECIPITATION IN AGAR

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Precipitation in agar has been widely used for determining the comparative characteristics of the numerous components of the antigenic systems [5]. On the one hand, it allows the number of individual antigens in a complex system to be determined from the number of precipitation bands; on the other hand, the presence or absence of an identity reaction between two antigens of the two systems to be compared makes it possible to recognize the identity or qualitative differences of their components.

In cases when the relationships between the antigens and the antibodies are not optimal, or when the system is near to the limit of sensitivity, the results cannot always be evaluated precisely. The effect may then be that quantitative differences in antigen content may be mistaken for qualitative differences.

In order to increase the scope of the method, the limits of sensitivity must be determined, as has been done in the present work.

In determining the number of antigens, the limit of the sensitivity of the method is set by the conditions under which the antigens and corresponding antibodies in the system will fail to give precipitation bands (limit of precipitation). When comparing antigens with each other, the limit of sensitivity is determined by the conditions under which the precipitation band between antigen and antibody in one system will fail to give an identity reaction with a similar antigen in the comparison system (limit of detection).

In the present work, we have studied the dependence of the limiting values on antigen-antibody relationships, and on their absolute concentrations; in addition, we have shown that the limit of precipitation may be increased considerably by the application of Cd^{++} salts [3].

METHODS

As antigen, we used rabbit γ-globulin, obtained from serum by electrophoresis in agar [4]. As antibody, we used immune donkey serum, obtained by multiple immunization of a donkey with the general globulin fraction of rabbit serum. Its content of antibodies to rabbit γ-globulin was 2 mg/ml. The serum was concentrated four times by precipitating the globulins with 50%-saturated ammonium sulfate, which fraction served as the starting point for the subsequent dilutions. The system γ-globulin—antiglobulin donkey serum gave only one precipitation band when tested by immunoelectrophoresis and double diffusion in gel.

The precipitation reaction in agar was carried out by A. I. Gusev and V. S. Tsvetkov's micromodification [2].

The limit of precipitation. To determine the precipitation limit of the antibody at a given dilution of serum, the serum was poured into the central reservoir, and into the peripheral holes twice-diluted antigen was added in all strengths up to a dilution of 1 : 32,768 (Fig. 1).

As can be seen from Fig. 1, the precipitation band corresponding to the initial antigen concentration is formed near the hole containing serum, which is acted upon by the excess antigen; then as the dilution increases, the band is displaced toward the holes containing antigen. It then passes through a zone of optimal relationship between antigen and antibody, where the band shows the greatest intensity, is most sharply delineated. After passing through this zone of optimal relationships, the precipitation band becomes displaced more and more toward the reservoir of antigens, on account of the excess of antibody, and at a certain concentration of antigen, appears to disappear into its hole. Evidently, with this arrangement of the experiment, only those concentrations

of antigen can be determined which give a precipitation band between holes, and concentrations higher or lower than this amount cannot be detected. To increase the limit of sensitivity, it is essential to "bring out" the precipitation band into the space between the holes, as can be done by diluting the serum (Table 1).

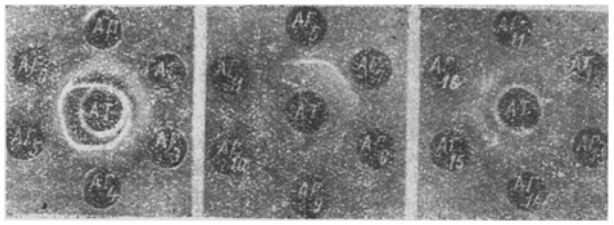

Fig. 1. Precipitation reaction in agar of a known concentration of antiserum with twice-diluted antigen. AT) Donkey antiglobulin serum concentrated four times; AG_1, AG_2 .. AG_{16}) double dilution of rabbit γ-globulin at strengths ranging from the initial concentration of 16 mg/ml of protein to a dilution of 32,768 times. АТ = AT, АГ = AG.

As can be seen from Table 1, the successive dilutions of the antibody enable ever lower concentrations of antigen to be revealed (relative limit of precipitation). However, when the serum was diluted, (in this case to 1 : 256) a moment occurred when despite the optimal relative amounts of the reagents, no visible precipitate was formed (absolute limit of precipitation).

To extend the precipitation limits, we have used the property of cadmium salts to increase the precipitation reaction in agar, and so to reveal bands which could not otherwise be discerned visibly [3]. The absolute limit was then increased four times, and there was a considerable extension of the relative amounts at the limit (see Table 1).

From the experiments described above it can be seen that to determine the number of antigens in the system, they must be titrated with various dilutions of serum, up to threshold values. Otherwise, antigens outside the relative sensitivity limits for the given dilution of serum cannot be revealed (Fig. 2). The results obtained correspond to the quantitative theory of the method of double diffusion in a gel [6].

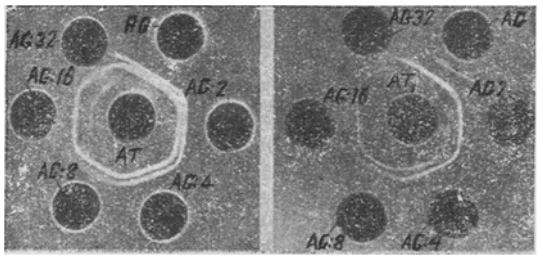

Fig. 2. Analysis of three-component antigen systems. AT) Donkey antiglobulin serum; AG_1...AG_{32}) two-fold dilution of the rabbit serum fraction.

Sensitivity limits. The relative amounts of the components affect not only the presence of precipitation bands, but also the identity reaction; consequently a large quantitative difference in antigen content in two systems under study may appear as a qualitative difference. It is therefore important to establish the sensitivity limits of the identity reaction, i.e. the limits for detection. For this purpose we have used several test systems made up of known dilutions of serum and of γ-globulin in optimal amounts (in Table 1 the optimal relative amounts of antigen and antibody are enclosed in a double-framed square). The first system was made up of the initial serum, and the antigen diluted 16 times; the second and subsequent test systems were made up by twice diluting the first. As comparison systems, we used successive dilutions of this test system.

The experiments were carried out according to a checkerboard arrangement [1]. One of the holes of the figure (Fig. 3) was filled with a known concentration of antibody of the test system, and the diagonally opposite hole with the corresponding antibody; in the remaining two holes were placed the antigen and the antibody of the systems to be compared.

As can be seen from Fig. 3, when two systems of the same concentration were compared, the precipitation band is a straight line which lies between the holes of the antigens and antibodies. On dilution of the systems to be compared, the precipitation band changes from a straight to a bent line, whose ends approach the holes of the antigen, and of the antibody present in the least concentration. At a certain minimum concentration of the components, the precipitation band ends in the holes of the reagents, i.e. the limit of detection has been reached. Such a test system failed to reveal the comparison system in all the subsequent dilutions, which were below this limit. Dilution of the test system, just as in the case of the limit of precipitation, led to an increase in the sensitivity of the reaction (Table 2).

TABLE 1. Sensitivity Limits of the Precipitation Reaction in Agar and Its Dependence on the Serum Dilution

Dilution in serum	Antigen (in mg/ml of protein)													
	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	0.015	0.007	0.003	0.0015
I (initial)			+	+	+[I]	+	+	+	+	±	Cd+			
1:2				+	+	+[II]	+	+	+	+	—			
1:4			Cd+	+	+	+	+[III]	+	+	+	±	Cd+		
1:8			Cd+	Cd+	+	+	+	+[IV]	+	+	+	Cd+		
1:16				Cd+	+	+	+	+	+[V]	+	+	Cd+		
1:32					Cd+	Cd+	+	+	+	+[VI]	+	±	—	—
1:64					Cd+	Cd+	+	+	+	+	+[VII]	+	Cd±	—
1:128						Cd±	Cd+	Cd+	Cd+	Cd+	+	+[VIII]	Cd+	—
1:256											Cd+	Cd+	Cd+[IX]	—
1:512											Cd+	Cd+	Cd+	[X]
1:1024										—	—	—	—	—

Designations: square with double outline indicates a reaction of optimal (equivalent) relative amounts of antigen and antibody; I-X) optimal relative amounts of serum, ranging from initial concentration to the dilution of 1:1024; Cd+ band of precipitation is seen after treatment of the solution with cadmium salts; +) presence of precipitation reaction; —) absence of precipitation reaction; ±) doubtful precipitation reaction.

It follows from Table 2 that each successive test system gives an identity reaction to dilution of the comparison system which is lower than the previous one. Then, in each column, the test system gives an identity reaction with the comparison system, diluted 16 times.

When comparing the limits of precipitation and the limit of detection, it was found that with the four-cornered arrangement subthreshold concentrations of antigens which were below the precipitation limit could be detected. Antigen at a concentration of 0.003 mg/ml of protein gave no visible precipitate with a dilution of the serum (see Table 1). Test systems in low concentrations (V and VI—Table 2) revealed this antigen.

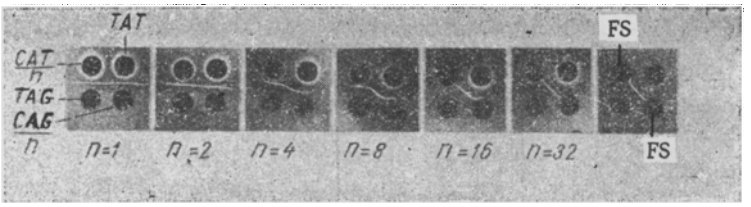

Fig. 3. Identity reaction of test systems of known concentration with twice-diluted comparison serum (4-cornered arrangement). TAT, TAG) Antibody and antigen of the test system; CAT, CAG) antibody and antigen of the comparison system; n) degree of dilution of the components of the comparison system; FS) physiological saline.

Similar systems for determining the limit of detection were carried out with the normal arrangement, with the antibody in the central hole, and the compared antigens in the peripheral holes (or vice versa). In these experiments, approximately the same relationships obtained as were established for the four-cornered arrangement, i.e. the limit of detection was increased by dilution of the test system. However, the relationship broke down at a certain dilution of the test system, when the compared component was present in subthreshold concentration, and gave no precipitation reaction.

TABLE 2. Limits of Detection of Successive Dilutions of the Test System (γ-Globulin —Antiglobulin Serum) with a Four-Cornered Arrangement

Test system	Comparison system											Physiological saline
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
I	+	+	+	+	+	±	—	—	—	—	—	—
II	+	+	+	+	+	+	—	—	—	—	—	—
III	+	+	+	+	+	+	+	—	—	—	—	—
IV	+	+	+	+	+	+	+	+	±	—	—	—
V	+	+	+	+	+	+	+	+	+	±	—	—
VI	±	+	+	+	+	+	+	+	+	+	±	—
VII	—	—	+	+	+	+	+	+	+	+	+	—

Designations: I-XI) Systems taken in relative amounts optimal for the initial serum, and for sera diluted by 1:2, 1:4, etc., as in Table 1; +) identity reaction; —) difference reaction.

Therefore, the four-cornered arrangement makes it possible to determine a lower concentration of antigen.

Determination of the relationship both for the limit of precipitation and for the limit of detection concerns not only the γ-globulin system with its corresponding antibody, but also the system of rabbit albumin with donkey antiserum containing the antibody to it.

SUMMARY

In order to increase the utilization of the method of precipitation in agar, a study was made of the relationship between its sensitivity and the absolute concentrations of antigens and their antibodies. The sensitivity of the precipitation reaction, as measured by either the relative or absolute quantities, may be increased by choice of the corresponding antigen and antibody concentration, and by the use of cadmium salts.

LITERATURE CITED

1. G. I. Abelev, Byull. éksper. biol. i med., No. 3 (1960) p. 118.
2. A. I. Gusev and V. S. Tsvetkov, Lab. delo, No. 2 (1961) p. 43.
3. A. Crowle, J. Immunol., Vol. 81 (1958) p. 194.
4. P. Grabar, in: Methods of Biochemical Analysis, Vol. 7 (New York, 1959) p. 1.
5. O. Ouchterlony, in: Progress in Allergy, Vol. 5 (Basel, 1958) p. 1.
6. J. R. Preer, Jr., J. Immunol., Vol. 77 (1956) p. 52.

All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.
