QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

THE RÔLE OF MULTIPLE REACTIVE GROUPS IN ANTIGEN-ANTIBODY UNION AS ILLUSTRATED BY AN INSTANCE OF CROSS-PRECIPITATION*

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(Received for publication, January 9, 1934)

The writers have been studying an antigen-antibody system in which the antigen was the red dye, R-salt-azo-benzidine-azo-crystalline egg albumin[1] (1, 2), fractionated to the extent that it reacted only exceptionally with anti-egg albumin sera. However, as stated previously (2), the original specificity was not entirely abolished, since antisera to the dye protein yielded precipitates with crystalline egg albumin. A quantitative comparison of the dye-antidye and egg albumin-antidye reactions disclosed great differences, as will readily be seen from Table I and the corresponding Fig. 1. In the latter the solid curve (I) represents the amount of antibody ([total N − antigen N] × 6.25) precipitated per cubic centimeter from an antidye rabbit serum by varying amounts of dye protein; the broken curve (II) shows the amount of antibody precipitated by varying amounts of egg albumin from the same serum, while the dotted curve (III) represents antibody precipitated by egg albumin from anti-egg albumin of similar precipitin content. The conclusions regarding antigen-antibody interaction drawn from the data will be discussed below.

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

[1] Referred to throughout this communication as "dye protein" or "dye."

EXPERIMENTAL

When small quantities of antigen are added to antibody in the dye-antidye[2] system, or egg albumin (E.A.)-anti-E.A.[3] system, antigen cannot be detected in appreciable amounts in the supernatant from the specific precipitate until the equivalence point is reached; that is, the point at which the sum of the molar concentrations of both components is at a minimum. However, in the E.A.-antidye system small amounts of E.A. often fail to yield precipitates with antidye (although larger amounts do) but may be demonstrated by the addition of anti-

TABLE I

Analytical Data on Direct and Cross-Precipitin Reactions

Antigen added per cc. antiserum	Protein precipitated per cc.		Reaction of supernatant with anti-E.A.		Antibody precipitated per cc.		Reaction of supernatant with antidye
	Anti-E.A. Solution B. 157	Serum 7.42 antidye			Anti-E.A. Solution B. 157	Serum 7.42 antidye	
mg.	*mg.*	*mg.*			*mg.*	*mg.*	
E.A.							
0.007	0.10		−		0.09		
0.017	0.21		−		0.19		
0.033	0.44		−		0.41		
0.067	0.67		±		0.60		
0.075		0.07		++		0.06	
0.10	0.75		+±		0.68		
0.15		0.10		+++		0.09 (A)	
0.167	0.42		+++		0.36		
0.72		0.61		++++		0.55	
1.50		0.69				0.63	
Dye protein							
0.075		0.75				0.68	−
0.15		0.78				0.70 (B)	+
1.0		0.19				0.16	

E.A., which quickly forms a precipitate (see also Table I, Columns 4 and 7). This affords evidence for the dissociation or greater solubility of the E.A.-antidye complex and for the absence in the antidye serum of the characteristic antibody

[2] Antidye is defined in this article as the antibody produced in rabbits on injection of preparations of R-salt-azo-benzidine-azo-crystalline egg albumin which have been fractionated so as to remove substantially all material precipitating in anti-egg albumin sera.

[3] Anti-egg albumin is defined as the antibody produced in rabbits on injection of crystalline egg albumin.

to E.A. That solubility alone does not account for the observations summarized in the table and the figure is indicated by the continued increase in precipitation at concentrations of E.A. far higher than those necessary for combination with all of the antibody, just as addition of an ion in common drives back the dissociation of a sparingly soluble salt and increases precipitation.

The quantitative determinations of precipitin were made by the method previously described (2). In the case of the E.A.-anti-E.A. system (in which the antibody solution contained the total globulin from three different anti-E.A. rabbit sera) the total amount of E.A. used was deducted from the precipitable protein as far as the equivalence point, beyond which E.A. first began to appear

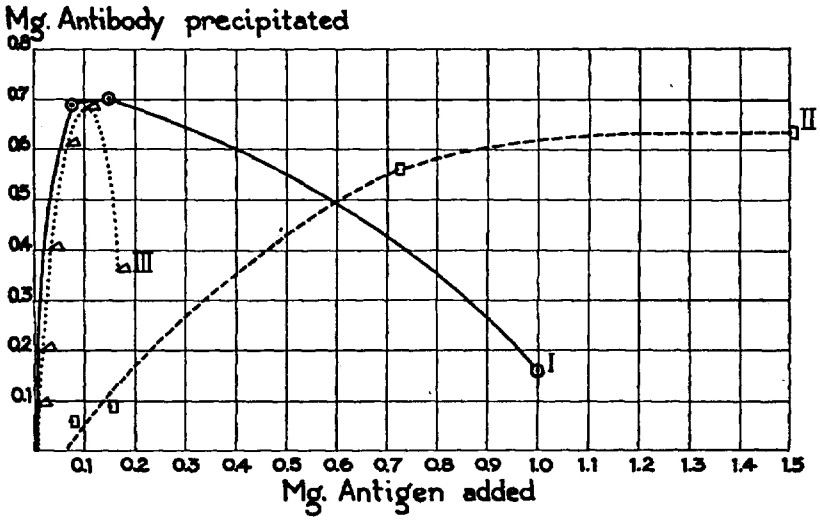

FIG. 1

in the supernatant. In the single determination in Table I and Fig. 1 in the region of the inhibition zone, the excess of E.A. in the supernatant was determined by setting up an aliquot with an excess of the same antibody solution and calculating the amount of E.A. from the total protein precipitated, as previously described in the case of a specific polysaccharide (3). From this value the amount in the original precipitate was obtained. In the case of the E.A.-antidye system the amount of antibody precipitated was calculated by deducting from the total protein precipitated one-twelfth of its value, it having been found in this laboratory that the equivalence point ratio of E.A. to anti-E.A. is 1 to 11 (*cf.*, however, Culbertson (4)), so that 12 parts of precipitate would contain 1 of antigen. In the E.A.-antidye system this method of calculation involves the

assumption that the composition of the precipitate is constant over the entire range. This would appear justifiable as a first approximation, since the E.A.-antidye cross-reacting system differs from the two homologous systems in showing no inhibition zone with excess antigen. There is thus at least one less compound possible in the cross-reaction, the writers having shown, in agreement with Arrhenius' belief, that a soluble compound is formed in the inhibition zone of homologous reactions (5, 1).

The supernatant from the precipitate marked (A) in Table I was divided into 2 aliquot portions and precipitated with a slight excess of dye. 0.60 mg. of antibody was recovered, calculated per cc. of the original serum. This, added to the 0.09 mg. precipitated by the E.A., equals 0.69, in excellent agreement with (B), the total found with the dye alone.

Similar results were obtained with other sera, although the form of the E.A.-antidye curve was somewhat different in each case. Serum 8.01, for example, contained 1.94 mg. of antidye per cc., but of this only 1.09 mg. was precipitable by E.A. With 25.6 mg. of E.A. 1 cc. of this serum precipitated 0.97 mg. of antibody, while the supernatant, with 0.05 mg. of the dye, yielded 0.85 mg. of antibody, calculated to the original volume, or a total of 1.82 mg. as compared with 1.94 mg. found directly. In this serum, then, even a large excess of E.A. failed to inhibit the dye-antidye reaction markedly, although greater inhibition was shown in other sera. In this serum, also, equilibrium in the E.A.-antidye system was established more slowly than in Serum 7.42, so that in subsequent work the E.A.-antidye mixtures were allowed to stand for 2 days before completing the analysis. The fact that the amount of antidye precipitated by both antigens in succession corresponds so closely to the amount of antidye found with the dye alone indicates that appreciable dissociation of the E.A.-antidye did not occur during the relatively short time consumed by the washings at 0°C. Supernatants from the maximum dye-antidye precipitate often yielded, when tested with E.A., an amount of additional precipitate equivalent to 0.05 to 0.11 mg. of anti-E.A. per cc. While these quantities fall within the limits of error of the method used, the occurrence of these precipitates is not inconsistent with the interpretation discussed below.

In Table II are given the data resulting from the addition of E.A. in successive portions to the antidye Serum 1.14 and a mixture of Sera 1.15 and 1.46. It was shown (2)[4] that Serum 1.14 contained 3.33 mg. of specifically precipitable antibody per cc. at the time of bleeding, and 3.06 mg. 6 months later. When used in the present experiment, 1½ years after the bleeding, the antidye content was unchanged; namely, 3.19 mg. per cc. The mixture of the other two sera, which originally contained 1.53 and 1.56 mg. of precipitable antibody, yielded 1.69 mg. The sera contained 0.01 per cent of merthiolate. To 5.0 cc. of Serum 1.14 and 10.0 cc. of the mixture of Sera 1.15 and 1.46 were added 0.20 cc. of saline and

[4] Heidelberger, Kendall, and Soo Hoo (2), p. 150.

0.20 cc. of a 1:1000 solution of crystalline egg albumin, with thorough mixing. In the first three or four runs the tubes were allowed to stand for 24 hours at room temperature and then 24 hours at 0°, but in the later runs a period of 48 hours at 0° was allowed in order to minimize the risk of bacterial growth in spite of the antiseptic present. The tubes were whirled in the refrigerating centrifuge[5] and the precipitates were analyzed as described in a previous article (2), using 4 and 3 cc. of chilled saline for the first and second washings, since the runs were carried out in 15 cc. centrifuge tubes. 5.0 and 10.0 cc. of the respective supernatants were then treated as before, with a final increase in the amount of E.A.

TABLE II

Serial Additions of Egg Albumin to Antidye

Total antidye N: 2.57 mg.				Total antidye N: 2.70 mg.		
Amount of E.A. nitrogen added	Serum 1.14			Mixed Sera 1.15 and 1.46		
	Total N precipitated	Antibody N precipitated (calculated)	E.A. N precipitated (calculated)	Total N precipitated	Antibody N precipitated (calculated)	E.A. N precipitated (calculated)
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.031	0.314	0.288	0.026	0.380	0.348	0.032*
0.031	0.280	0.257	0.023	0.294	0.269	0.025
0.031	0.210	0.192	0.018	0.256	0.235	0.021
0.062	0.226	0.207	0.019	0.264	0.242	0.022
0.155	0.134	0.123	0.011	0.138	0.126	0.012
0.62	0.258	0.236	0.022	0.308	0.282	0.026
2.98	0.144	0.132	0.012	0.164	0.150	0.014
2.98	0.144	0.132	0.012	0.148	0.136	0.012
2.98	0.054	0.049	0.005	0.064	0.059	0.005
0.015 dye N	0.076	0.068		0.132	0.117	

* Actually a trace of E.A. was found in the supernatant with anti-E.A., indicating that this figure should not have been >0.030. These sera, or one of them, possibly contained small amounts of anti-E.A.

added. Even the first supernatants reacted with anti-E.A., again showing the E.A.-antidye precipitate to be dissociated. The nitrogen values found by the micro Kjeldahl method were calculated as in Table I, assuming a constant 1:11 antigen-antibody ratio for the precipitate. When only traces of antibody were finally precipitated by the E.A. added, final runs were made with small amounts of dye. In both cases the total amount of antidye precipitated was within 10 per cent of the total indicated by the original determination with the dye itself, which may be taken as a satisfactory agreement considering the large numbers of dilution factors and analyses involved.

[5] Manufactured by the International Equipment Co., Boston.

DISCUSSION

Although the antigen used in these experiments, R-salt-azo-benzidine-azo-crystalline egg albumin, was fractionated until it was practically non-reactive with antisera to crystalline egg albumin over a wide range of concentration, antisera produced with the dye can be largely deprived of their antibody content by suitable additions of crystalline egg albumin. There is no *a priori* reason why the antigen could not be split in the animal body so as to form the same antibody as does egg albumin itself, but it is believed that the experiments described show conclusively that practically all of the antibody which reacts with the egg albumin is the antibody formed as a result of the specific antigenic stimulus due to the dye protein itself. There is thus no necessity for assuming the presence of antibody other than antidye. This conclusion is based on (1) the totally different quantitative aspect of the egg albumin-anti-egg albumin and egg albumin-antidye reactions (Fig. 1); (2) the similarity of the egg albumin-anti-egg albumin and dye-antidye reactions (Fig. 1); (3) the additivity of successive egg albumin-antidye and dye-antidye precipitations; and (4) the fact that at all concentrations of egg albumin in the egg albumin-antidye cross-reaction free egg albumin may be detected in the supernatant by addition of anti-egg albumin.[3] This result would be expected if the combination between egg albumin and antidye were a loose, highly dissociated one. Consistent with this interpretation are the large amounts of egg albumin required to force the egg albumin-antidye complex out of solution—amounts far in excess of those required to throw an homologous reaction completely into the inhibition zone, whether one is dealing with the egg albumin-anti-egg albumin or the dye-antidye system. Moreover, in the two homologous reactions antigen cannot be detected in the supernatant until after the equivalence point has been reached, showing that in these cases the antigen-antibody complex is almost undissociated.

The occurrence of this one-sided cross-reaction may be explained by the assumption that in the dye protein there still remain minor antigenic groupings common to egg albumin itself. These may be somewhat masked by the dominant azo groupings which determine the homologous specific reaction, thus preventing the *in vitro* union of dye and anti-egg albumin. However, in the animal, these minor

antigenic groups might become reactive and occasion, in the antidye molecule, the formation of correspondingly minor reactive groups, through which antidye could react with egg albumin as well as with the dye itself.

The following diagrams may make this interpretation clearer:

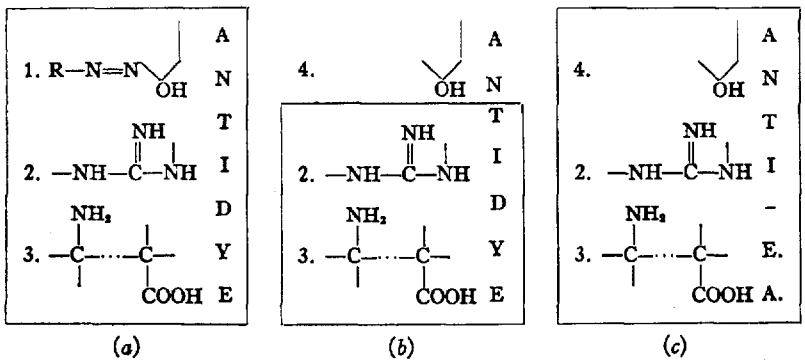

FIG. 2

Grouping 1, in accordance with Landsteiner's findings, is the dominant antigenic group in the dye protein, while 2 and 3 are other arbitrarily chosen groups of which the steric configuration is characteristic for egg albumin. The dominant reactive group on the antidye molecule would be one evoked in the animal by virtue of the dominant antigenic group (*cf.* Breinl and Haurowitz (6), Mudd (7)). The resulting dye-antidye combination might therefore be represented by Fig. 2 *a*, and would be expected to be relatively firm and undissociated. Other less important groups on the antibody molecule might be expected to result from Groupings 2 and 3, characteristic of the egg albumin portion of the dye molecule. Thus crystalline egg albumin, with its dominant antigenic Group 4, arbitrarily chosen by analogy with Group 1, might be expected to react with antidye by virtue of Groupings 2 and 3. The union could reasonably be expected to be a relatively weak, highly dissociated one, since there would be no anti-4 in the antidye, and the egg albumin contains no Grouping 1 to react by virtue of the anti-1 present. This is represented in Fig. 2 *b*. Again, crystalline egg albumin, with its dominant

antigenic Group 4, would be expected to form the observed firm, undissociated union with anti-egg albumin, containing anti-4, as represented in Fig. 2 *c*.

These views derive from those expressed by Landsteiner and van der Scheer (8) and are somewhat analogous to those of Bergmann on the combination of an enzyme with its substrate (9).

In accordance with this interpretation egg albumin would react with the antibody to the dye protein by virtue of the minor antigenic groupings common to egg albumin and dye protein. The antibody would, however, be "antidye" as a result of the stimulus exerted on the animal by the dominant antigenic dye grouping, and it is shown in this communication that "antidye" has totally different reactivities from those of "anti-egg albumin." For example, egg albumin and anti-egg albumin yield a firm, undissociated combination (Fig. 1, Curve III; Table I). With small additions of egg albumin to anti-egg albumin it is impossible to detect antigen in the presence of excess antibody, and it is only on increasing the amount of egg albumin beyond that required to reach the equivalence point that antigen appears in the supernatant. The interaction of dye protein and antidye is of the same type (Fig. 1, Curve I; Table I). While it is possible that antisera to the dye contain minimal amounts of true anti-egg albumin resulting from undetected traces of egg albumin in the antigen, the reaction between egg albumin and antidye is qualitatively and quantitatively so different from the homologous reactions that it must be concluded that the reactive antibody is the antidye itself. The differences between the homologous and cross-reactions would seem therefore be due to the firm union brought about by the dominant antigenic and antibody groups in the first instance and to the loose, highly dissociated union resulting in the latter case from the reaction of minor groupings alone.

In the foregoing discussion "antidye" has been considered as a single entity since the experimental data can be most simply treated and presented on this basis. One may, at the other extreme, conceive of a series of "antidyes," all possessing the grouping anti-1 but differing, some by the absence of groupings anti-2 and anti-3, some in their relative proportion. This would perhaps account for the differences in the dissociation constants calculated for different

sera, but it might also be true for the antidye molecules in a single serum. However this may be, it does not affect the conclusion that the reacting antibody in the egg albumin-antidye system is antidye, and that the complex is relatively highly dissociated. It should not be difficult, by the quantitative method, to test the validity of the underlying principle in other cases of cross-precipitation. The partial precipitation of Pneumococcus III antipolysaccharide by partial hydrolysis products of the specific carbohydrate is being studied, as well as an instance of cross-precipitation due to species interrelationships.

A close analogy suggests itself between the instance studied and that observed by Landsteiner and van der Scheer with azo proteins derived from *o*-aminobenzenesulfonic acid and *o*-aminobenzoic acid (10), and also to the relation between Type II pneumococcus and Type V (Subgroup II *a*) (11). The egg albumin-antidye system, however, is evidently simpler than that studied by Avery, Goebel and Babers with α- and β-glucosido-azo proteins (12), or the cross-reactions between the Type B Friedländer bacillus and Type II pneumococcus (13).

The writers believe that their data can be qualitatively explained most simply on the basis of the laws of classical chemistry, assuming antidye as the sole reactive antibody, with a low dissociation constant for the homologous reaction and a high dissociation constant for the egg albumin-antidye reaction. The quantitative formulation of the cross-reaction data in terms of these laws leads to definite values of the dissociation constants which vary from serum to serum, but the calculations involve the making of assumptions which the writers prefer to test more fully.

SUMMARY

1. Antisera to R-salt-azo-benzidine-azo-crystalline egg albumin give precipitates with crystalline egg albumin by virtue of their antidye content.

2. The quantitative course of the reactions with increasing amounts of antigen is very similar for the dye-antidye and egg albumin-anti-egg albumin systems, but differs markedly for the cross reaction between egg albumin and antidye.

3. A possible explanation for the occurrence of this one-sided cross-reaction is given in terms of reactive groupings on the antigen and antibody.

4. A qualitative expression of the course of the cross-reaction is given in terms of the laws of classical chemistry.

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