THE VALENCY OF ANTIBODIES.

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Received for publication March 20, 1951.

THE lattice theory of the precipitation and agglutination of antigens by antibodies is based on the assumption that antibodies are multivalent ; unless antibodies have at least two valencies* the large aggregates, which this theory predicates, cannot be built up. The theory and this underlying assumption have been criticized by Hooker and Boyd (1942) and Boyd (1947).

Heidelberger (1939) suggested that " incomplete " antibodies—that is, antibodies that combine specifically with the homologous antigen, but do not, by themselves, form a precipitate with the antigen—have only one valency and cannot therefore build up these aggregates. The matter has become less academic since the importance of incomplete antibodies in relation to erythroblastosis foetalis was discovered.

The bulk of the evidence bearing on the subject has been indirect. It has on the whole favoured the lattice theory, rather than the alternative theory that antigen molecules or particles become coated with antibody molecules that, as a result of their combination with antigen, lose their affinity for water.

However, the lattice theory supposes that antigen and antibody are closely packed in the aggregates in an orderly manner. The combining sites of the antibody molecules must not only be in close contact with the corresponding sites on the antigen molecules, but the molecules must be properly orientated in order that the binding sites may fit. It is difficult to see how the molecules of diphtheria toxin, again properly orientated, can be packed into the aggregate formed by diphtheria antitoxin and rabbit antibody to horse globulin (Smith and Marrack, 1930 ; Eagle, 1937) ; or how antigen-antibody floccules, already formed, can take up an amount of complement protein equal to about 72 per cent of the antibody present (Hiedelberger, Rocha e Silva and Mayer, 1941) ; still more, how the large molecules of ribonucleic acid and its breakdown products can diffuse in and out of ribonuclease-antiribonuclease aggregates so fast that the activity of the enzyme is reduced by only 20 or 30 per cent (Smolens and Sevag, 1942–43).

The most direct evidence is that of Eisen and Karush (1949). They measured the number of molecules of a haptene bound by a molecule of pure antibody at varying haptene concentrations ; it was between 1 and 2. By extrapolation to infinite haptene concentration they concluded that the maximum valency

* With regard to the use of the word valency in this connection, we may quote Hooker and Boyd (1942) : " Since the reaction appears to be essentially chemical, it is natural to use valence for the number of combining sites or active patches on the surface of reactants. There are many indications that the reactive groups are antigenically complex, i.e., made up of a number of different chemical groups, so the term valence as we use it, although correct etymologically, does not connote quite the same thing as in classical chemistry."

of antibody molecules is 2. It might be supposed that the affinity of protein antigens for antibodies is higher than that of a haptene, and that an antibody molecule could combine with two molecules of antigen at moderate concentrations.

The amounts of antigen combined with antibody in the soluble compounds formed in the presence of a great excess of antigen can be studied by electrophoresis or by ultracentrifugation, if the mobility or sedimentation constant of antigen differs sufficiently from that of antibody. Such studies cannot be made by adding excess of antigen directly to an antiserum, or even to a solution of whole serum globulin, as the great excess of non-specific proteins would hide the antigen-antibody compound. In many cases it is difficult to get a sufficiently pure preparation of anti-body. One method of getting over this difficulty is to form a precipitate of antigen and antibody by adding antigen to serum, to wash his precipitate free from non-specific proteins and to redissolve the precipitate in excess of antigen. This method was used by Tiselius and Kabat (1939), but they did not publish any estimates of the amount of antigen bound by the antibody. Heidelberger and Pedersen (1937) redissolved the precipitate, formed by egg albumin with antiserum, in excess of egg albumin and studied the solution in the ultracentrifuge. From the data given in one experiment (No. 10) we reckon that one molecule of antibody combined with only one molecule of antigen, when 4 times the equivalent amount of antigen was present. Pappenheimer, Lundgren and Williams (1940) studied both, mixtures of excess diphtheria antitoxin with globulin from antitoxic serum, and solutions of toxin-antitoxin precipitates in excess of toxin. They concluded that the valency of diphtheria antitoxin was more than 1 and not more than 2 ; this is the only direct evidence of a valency for actual antigen that exceeds 1. We have continued studies on these lines. In the earlier experiments we redissolved the precipitates formed by adding antigen to the whole globulin of antiserum in excess of antigen ; we assumed that all the precipitated antibody had the mobility of γ-globulin. In later experiments we isolated γ-globulin from antiserum ; we (1) added antigen, in approximately equivalent amounts, to the solution of γ-globulin, separated the precipitate and redissolved it in excess antigen, or (2) added the γ-globulin solution directly to excess of antigen. In the last type of experiment we found evidence that when a great excess of antigen was present, more γ-globulin combined with antigen than was carried down in precipitrates formed by antigen and antibody.

METHODS.

Antigens.

The antigens used in the experiments reported were :

1 (*a*). A preparation of crystalline horse serum albumin given us by Dr. E. R. Holiday. This contained 0·2 g. of tryptophane per 100 g. of protein. It was dissolved in 0·9 per cent sodium chloride and filtered through a Seitz filter. No evidence of α_1-globulin (Fig. 1) was found by electrophoresis in barbiturate buffer at pH 8·6 (Fig. 1).

1 (*b*). A solution of the same serum albumin which had not been passed through a Seitz filter. There was evidence of some α_1-globulin in this solution (Fig. 2).

(2) A preparation of crystalline ox serum albumin obtained from Messrs. Armour & Co. There was no evidence of α_1-globulin in solutions of this albumin (Fig. 3). However, Dr. H. Gutfreund tells us that he found that a sample of Armour crystalline ox serum albumin contained some 2 or 3 per cent of a constituent with a sedimentation

constant higher than that of albumin; and recently, Wallace, Osler and Mayer (1950) reported that a sample contained a trace, estimated at less than 5 per cent, of a component with a sedimentation constant of about 7.

Crystalline egg albumin and crystalline human serum-albumin were also used in experiments which are not reported in detail.

Antibodies.

Rabbits were immunized by Proom's (1943) method and bled 12 to 14 days after an injection of antigen. The sera used in these experiments were obtained by bleeding at the following times after immunization began:

Exp. 3	1 and 4 months.	Exp. 8	14 months.
,, 6	8 months	,, 9	5 weeks.
,, 7	1 year.	,, 10	6 and 7 months.

In the earlier experiments the globulin of the serum was precipitated by half saturation with ammonium sulphate. The precipitate was washed with half-saturated ammonium sulphate, dissolved and dialysed against 0·9 per cent sodium chloride solution to remove ammonium sulphate.

In later experiments γ-globulin was prepared by a modification of Kekwick's (1940) method. Serum was dialysed against 18 per cent sodium sulphate solution, repeatedly changed, at room temperature. The precipitate was centrifuged down or filtered off and washed twice with 18 per cent sodium sulphate. It was re-dissolved and dialysed against 15 per cent sodium sulphate solution, repeatedly changed, at room temperature. The precipitate was centrifuged down and washed twice with 15 per cent sodium sulphate. Precipitation and washing with 15 per cent sodium sulphate were repeated. A solution of the precipitate was cloudy and contained some β-globulin. It was dialysed at 4° C. against a buffer solution (pH approximately 5·4, μ = approx. 0·05) containing 96·5 ml. of M/15 KH_2PO_4 and 3·5 ml. of M/15 Na_2HPO_4 diluted to 135 ml. A precipitate formed which did not dissolve completely in phosphate buffer (pH 8·0, $\mu = 0{\cdot}1$). The supernatant was neutralized with sodium hydroxide and kept at 4° C. overnight; a slight precipitate formed which was centrifuged off. The supernatant fluid was almost water-clear and contained about three-quarters of the antibody of the original antiserum. In three preparations, S4 (Fig. 4), S6 (Fig. 5) and S7, no component faster than γ-globulin was detected. A third, S8, contained 5 per cent of β-globulin (Fig. 6). This β-globulin was not removed when all the precipitable antibody was precipitated by addition of an equivalent amount of antigen (Fig. 6A); none of the precipitable antibody was in the β-globulin fraction. Three other preparations contained some β-globulin; after further precipitation of two of these by 15 per cent sodium sulphate and dialysis against phosphate buffer (pH 5·4, $\mu = 0{\cdot}05$) preparations S9 and S10, free from detectable β-globulin, were obtained (Fig. 7 and 7A); the third (S11, Fig. 19) was used without further treatment.

Precipitation curves.

Progressively increasing amounts of antigen were added to a series of constant amounts of antibody solution. The mixtures were kept at room temperature until floccules formed and then at 4° C. for 2 days. The precipitates were centrifuged down, washed three times with cold saline and the protein estimated by the tyrosine method (Heidelberger and MacPherson, 1943; Heidelberger and Anderson, 1944); this was checked from time to time by Kjeldahl's method. The supernatant fluids were tested for excess of antibody and antigen.

Precipitation and resolution.

Pilot experiment.—A pilot experiment was made in order to estimate the amount of antibody precipitated with the antigen in the main experiment. To an antibody solution an equivalent or slightly less than equivalent amount of antigen was added. The ratio of antigen to antibody, length of time between mixing and centrifuging and other conditions of precipitation were the same as in the main experiment. The precipitate was centrifuged down, washed twice with cold 0·9 per cent NaCl solution and the protein estimated by Kjeldahl's method. The amount of antibody precipitated was equal to the total protein precipitated minus the antigen protein.

Main experiment.—All apparatus was sterilized by heat or by soaking in 70 per cent alcohol, followed by washing with sterile 0·9 per cent NaCl solution. All stages of the experiment, up to the dialysis before electrophoresis, were done with precautions against contamination. The ratio of antigen to antibody was the same as in the pilot experiment, but larger amounts were used. The precipitate was washed as in the pilot experiment and stirred with a few ml. of 0·9 per cent saline. A concentrated solution of antigen was added in measured lots. The mixture was periodically warmed to 38° C., and usually kept at room temperature during most of the day and at 4° C. during the night. When all or a considerable proportion of the precipitate had dissolved the mixture was dialysed against phosphate buffer pH 8·0, $\mu = 0{\cdot}1$, and examined by electrophoresis.

Direct addition of antibody to excess antigen.

In order to measure the maximum precipitable antibody, the equivalent amount of antigen was added to a sample of γ-globulin solution. After floccules had formed at room temperature the mixture was left for 2 days, with occasional shaking, at 4° C.; the precipitate was centrifuged down, washed three times with cold 0·9 per cent saline solution and estimated by Kjeldahl's method. In the main experiment a measured amount of the γ-globulin solution was added slowly with continual mixing to a large excess of antigen. The mixture was dialysed against phosphate buffer pH 8, $\mu = 0{\cdot}1$ and examined by electrophoresis.

Kjeldahl estimations.

The concentration of protein in the various solutions and in the precipitates was estimated by measuring the nitrogen content by Kjeldahl's method (Hoch and Marrack, 1945).

Electrophoresis.

In the examination of the samples of serum albumin (Fig. 1, 2, 3) the electrophoresis took place in barbiturate buffer of pH 8·6 and ionic strength 0·1. All other electrophoresis experiments were done in phosphate buffer of pH 8·0 and ionic strength 0·1. The patterns were analysed by the method used by Svedberg and Pedersen (1940). The estimates of the individual components were probably accurate to within ± 1 per cent of the sum of the components when the peaks were well separated, but often the conditions were less favourable and occasionally the error may have been ± 3 per cent. The error in the estimates of the two components of the complex, represented by two peaks in Fig. 9 (Exp. 6), may have been of this order. In this case the two components of the complex, expressed as per cent of the total, might be 11·6 and 18·5 or 17·6 and 12·5 instead of 14·6 and 15·5, the figures given in the account of the experiment.

Calculation of the composition of antigen-antibody complexes.

In experiments in which an antigen-antibody precipitate was re-dissolved the protein in the precipitate was calculated from the result of the pilot experiment; the amount

of antigen added to dissolve the precipitate was known. Hence the total protein was known; it was also measured by Kjeldahl's method in several experiments and the amount found agreed well with that calculated. In experiments in which γ-globulin solution was added directly to excess of antigen, the amount of *precipitable* antibody was known from precipitation curves.

Owing to the great differences between the mobilities of the antigens used and γ-globulin, the antigen-antibody complexes in solution usually showed in the electrophoresis patterns as peaks distinct from the free antigen and from any inert γ-globulin, if present. The ratios of free antigen, complex and inert γ-globulin (if present) to total protein were calculated from the ratios of the areas of the corresponding peaks to the total area of the peaks. As the total amount of protein was known the amounts of free antigen, complex and inert γ-globulin (if present) could be calculated. The combined antigen was the difference between the total antigen and free antigen. The antibody was known from the pilot experiment, and was also calculated as the difference between total complex and combined antigen. As the complex boundary in the descending side was free from excess of the faster-moving antigen, the calculations were based on the proportions of the components found on the ascending side.

The molecular ratio of antigen to antibody was calculated on the assumption that the molecular weights of albumin was 70,000, and that of the antibody 160,000.

RESULTS.

Three experiments other than those reported were done with horse serum albumin as antigen. In these the amount of residual precipitate was too large to be disregarded; as its composition was not known it was not possible to calculate the amount of antibody

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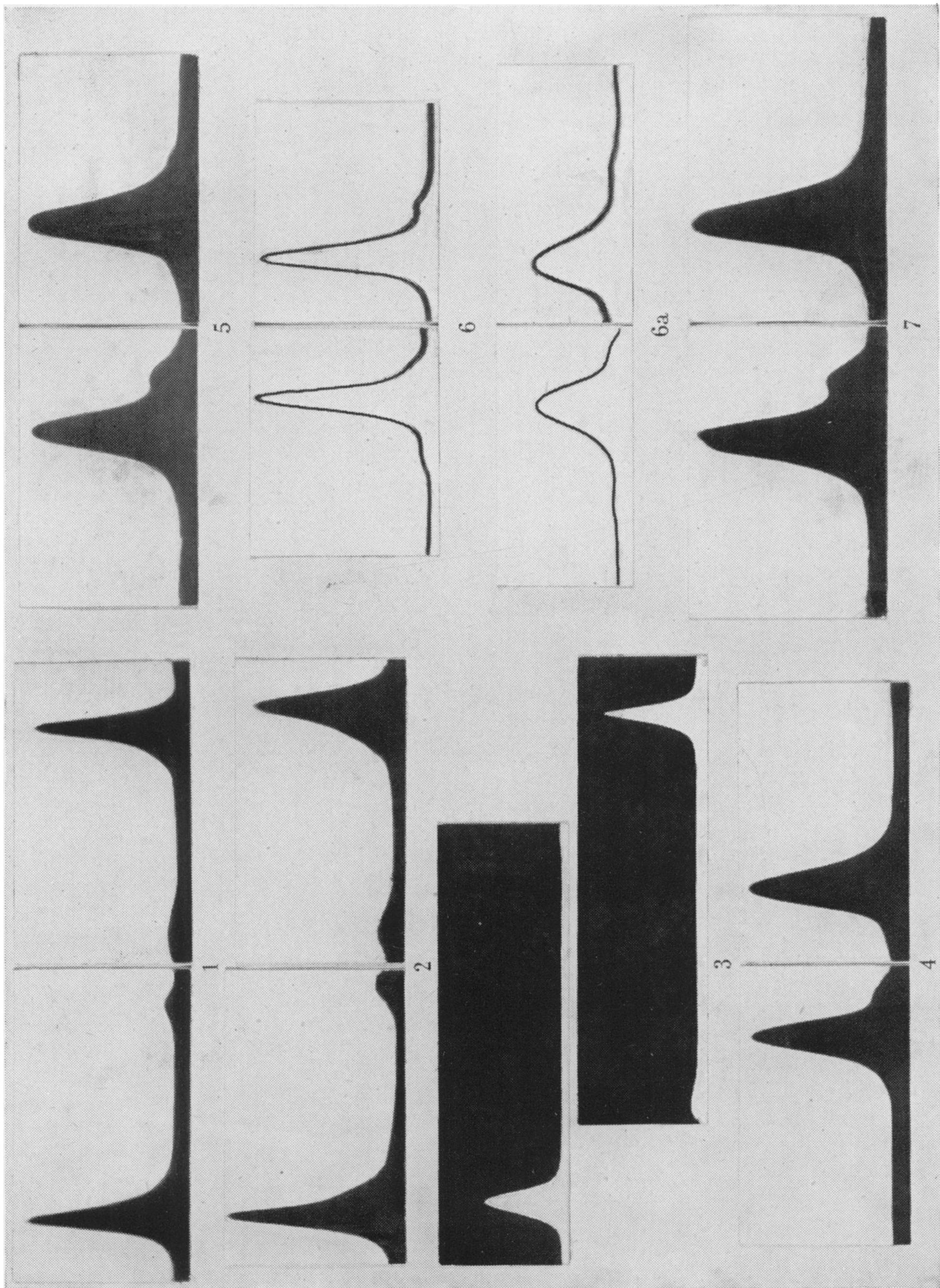
EXPLANATION OF PLATES.

Electrophoresis patterns; pH 8·0 unless otherwise stated; ascending on the left, descending on the right (except in Fig. 13 which has been accidentally reversed).

FIG. 1.—Horse serum albumin, sample 1*a*; pH 8·6.
FIG. 2.—Horse serum albumin, sample 1*b*; pH 8·6.
FIG. 3.—Ox serum albumin; pH 8·6.
FIG. 4.—γ-globulin, S4.
FIG. 5.—γ-globulin, S6.
FIG. 6.—γ-globulin, S8.
FIG. 6A.—γ-globulin, S8, after precipitation of antibody.
FIG. 7.—γ-globulin, S9.
FIG. 7A —γ-globulin, S10.
FIG. 8.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 3.
FIG. 9.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 6.
FIG. 10.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 9A (6.i.50).
FIG. 10A.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 9A (11.i.50).
FIG. 11.—Antigen excess added to γ-globulin, Experiment 7A.
FIG. 12.—Antigen excess added to γ-globulin, Experiment 7B.
FIG. 13.—Antigen excess added to γ-globulin, Experiment 9B.
FIG. 14.—Antigen excess added to γ-globulin, Experiment 10A.
FIG. 15.—Supernatant fluid after precipitation of antibody, Experiment 8A.
FIG. 15A.—Supernatant fluid plus excess antigen, Experiment 8A.
FIG. 16.—Supernatant fluid (Fig. 6A) plus excess antigen, Experiment 9C.
FIG. 17.—Supernatant fluid after precipitation of antibody, Experiment 10B.
FIG. 18.—Supernatant fluid (Experiment 10B) plus excess antigen.
FIG. 19.—γ-globulin (S11) Experiment 11, ascending.
FIG. 20.—Inert γ-globulin plus ox serum albumin, Experiment 11.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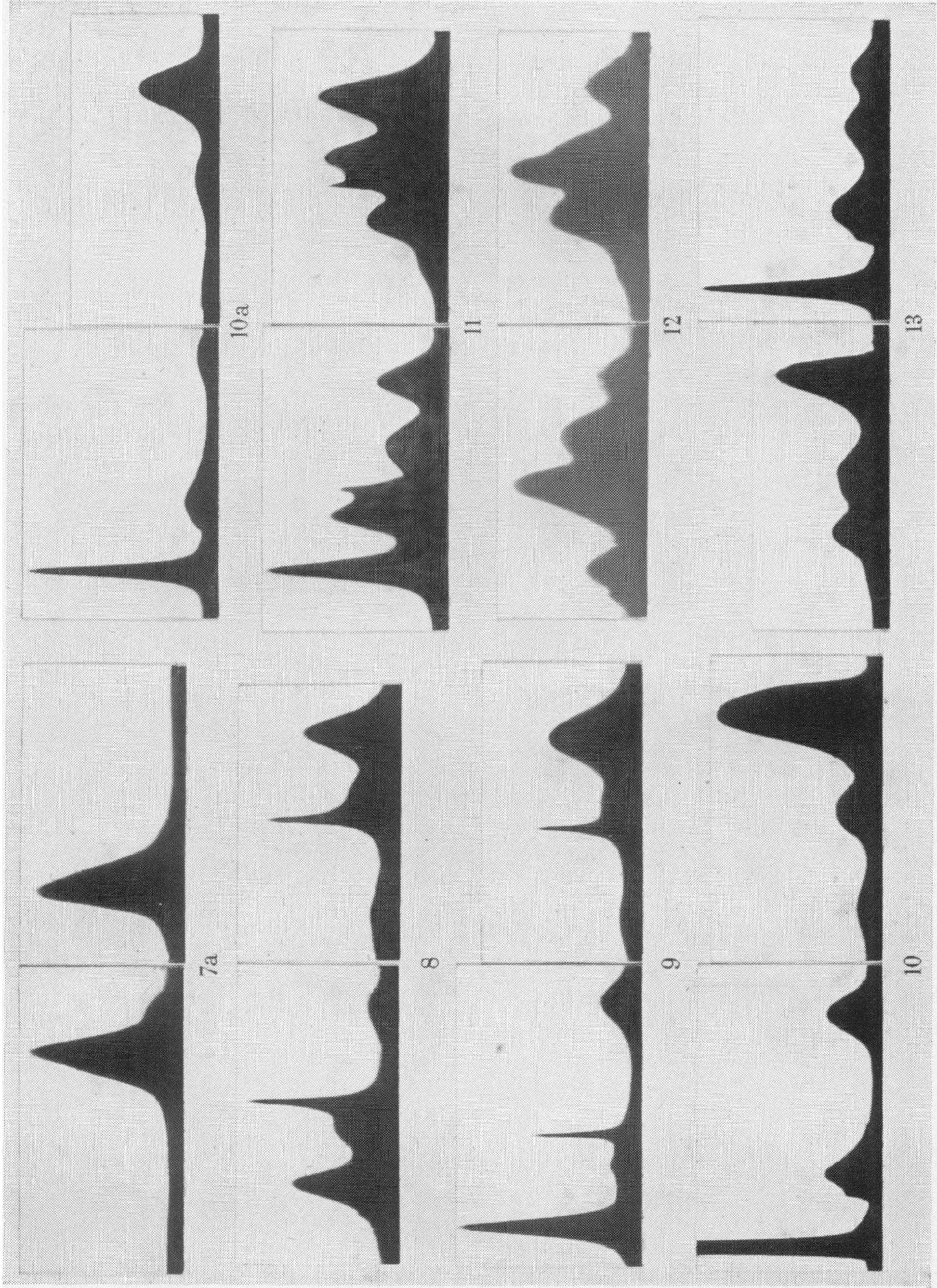
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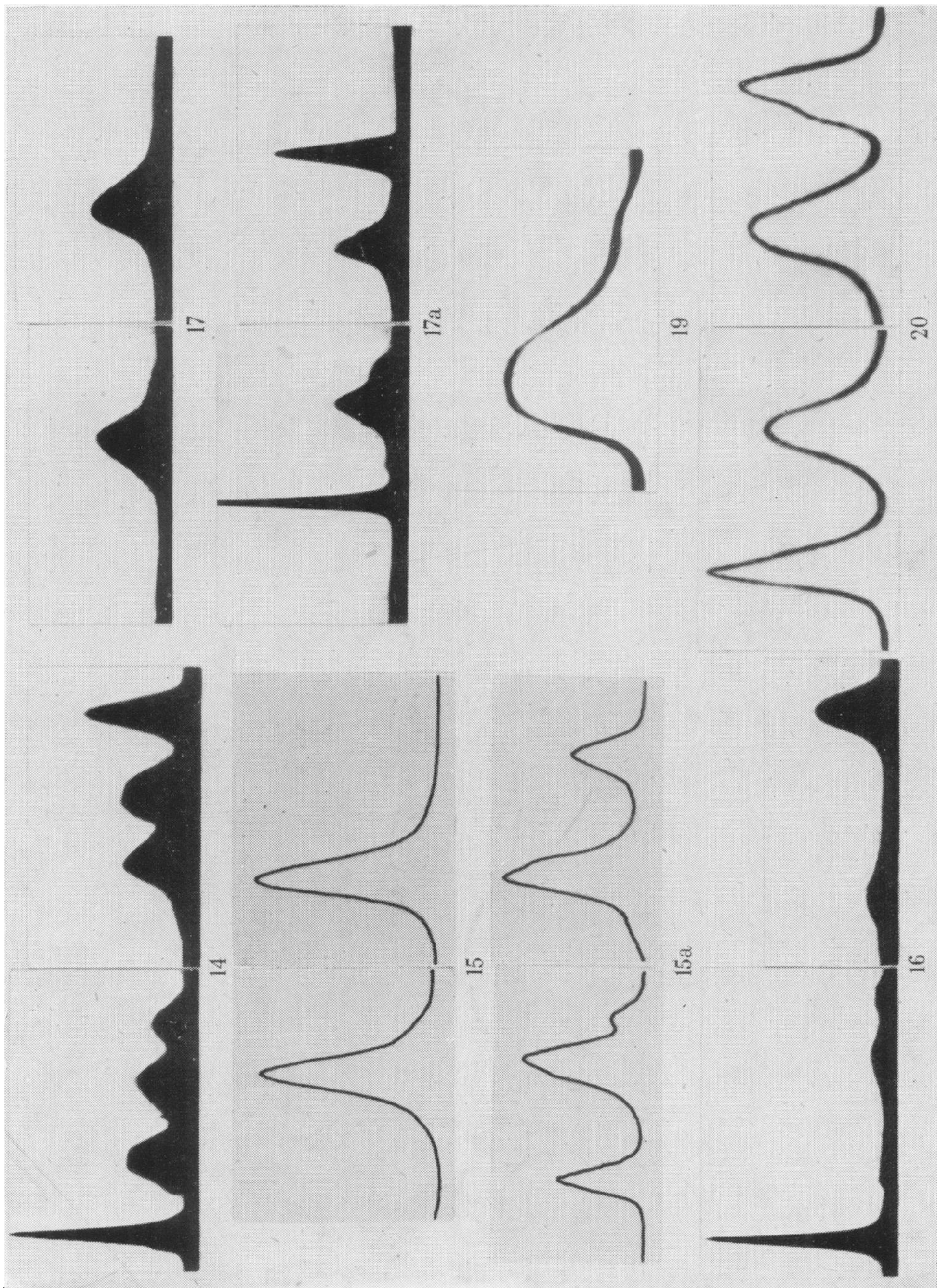

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and antigen in the soluble complex. However, in one of these (Experiment 5B) 1·8 mg. of precipitate remained undissolved. Even if this precipitate consisted of antibody only, the molecular ratio would be 1·9; if the precipitate contained two molecules of antibody to one of antigen the molecular ratio would be 1·8.

Experiment 3.

Antigen : Crystalline horse serum albumin (sample 1*a*) (Fig. 1).

Antibody solution : Solution of globulin from antiserum precipitated by half saturation with ammonium sulphate and dialysed against 0·9 per cent NaCl solution; pH adjusted to about 7·5.

Pilot experiment : 2 ml. of antibody solution and 0·8 ml. of 0·039 per cent albumin solution (0·312 mg. of albumin) formed 2·99 mg. of precipitate. Antibody 2·68 mg., or 1·34 mg. per ml.

Main experiment : 12 ml. of antibody solution, 4 ml. of 0·9 per cent NaCl and 0·96 ml. of 0·198 per cent albumin solution (1·90 mg. of albumin).

Antigen added to washed precipitate: 1·2 ml. of 1·89 per cent albumin and a further 0·6 ml. on the following day.

Total albumin added . .	34·0 mg.
Total albumin in mixture .	35·9 mg. (19 times the equivalent amount).

The mixture was kept at 38° C. for a total of 8 hours on consecutive days and at 4° C. during two nights. The bulk of the precipitate had then dissolved and the mixture was dialysed against phosphate buffer, pH 8·0 for 1 day. A small precipitate (0·2 mg.) remained undissolved. Interval between first addition of excess antigen and electrophoresis 3 days.

Original precipitate (calculated from pilot experiment) .	= 17·9 mg.
Albumin added	= 34·0 ,,
Total protein calculated	51·9 ,,
Found in residual precipitate	= 0·2 ,,
Found in supernatant solution	= 51·1 ,,
Total found	51·3 ,,

Composition by electrophoresis (Fig. 8):

	Ascending.	Descending.
Albumin . . .	47·0 per cent .	52·0 per cent.
Complex (1) . . .	19·0 ,, .	18·0 ,,
Complex (2) . . .	34·0 ,, .	30·0 ,,

No component with the mobility of γ-globulin was detected. The complex formed 2 peaks.

Neglecting the precipitate in the calculation:

Total protein in solution	= 51·1 mg.
Total albumin in solution	= 35·9 ,,
Free albumin in solution 51·1 × 0·47	= 24·0 ,,
Combined albumin	= 11·9 ,,
Complex in solution 51·1 × 0·53	= 27·1 ,,
Antibody in complex, by difference	= 15·2 ,,
Antibody, from pilot experiment	= 16·1 ,,

Ratio of antigen to antibody in complex, $\frac{11\cdot 9}{15\cdot 2} = 0\cdot 78$.

Molecular ratio, 1·8.

Experiment 6.

Antigen : Crystalline horse serum albumin (sample 1*a*) (Fig. 1).

Antibody solution : Solution of globulin precipitated from antiserum by half saturation with ammonium sulphate, dialysed against 0·9 per cent NaCl, neutralized and Seitz filtered.

Pilot experiment : 2 ml. of antibody solution and 0·4 ml. of 0·302 per cent albumin solution (1·208 mg. of albumin) formed 10·3 mg. of precipitate. Antibody 9·1 mg., or 3·03 per ml.

Main experiment : 10 ml. of antibody solution and 2 ml. of 0·302 per cent albumin (6·04 mg. of albumin).

Antigen added to washed precipitate ; 6·5 ml. of 2·8 per cent albumin in 3 instalments.

Total albumin added	. 182 mg.	
Total albumin present	. 188 ,,	(31 times the equivalent amount).

Final volume 8·57 ml., protein concentration 2·73 per cent.

Original precipitate, calculated from pilot experiment	= 51·5 mg.
Albumin added	=182 ,,
Total protein	=233·5 ,,
Protein found 8·57 × 27·3 mg.	=234 ,,

The mixture was kept at room temperature, with repeated shaking, by day and at 4° C. at night, for 9 days, then dialysed, for electrophoresis, against phosphate buffer pH 8. The precipitate was completely dissolved.

Interval between precipitation and electrophoresis, 10 days.

Composition by electrophoresis (Fig. 9) :

	Ascending.	Descending.
Albumin . . .	66·7 per cent	71·4 per cent.
Faster complex . .	14·6 ,,	10·4 ,,
Slower complex . .	15·5 ,,	16·0 ,,
Material with mobility between slow complex and γ-globulin . .	3·2 ,,	2·2 ,,

No component with the mobility of γ-globulin was detected.

Total protein in solution	=234 mg.
Total albumin	=188 ,,
Free albumin (234 × 0·667)	=156 ,,
Combined albumin	= 32 ,,
Total complex (234 × 0·333)	= 78 ,,
Antibody in complex, by difference	= 46 ,,
Antibody, from pilot experiment	= 45·5 ,,

$$\text{Ratio of antigen to antibody, } \frac{32}{45 \cdot 5} = 0 \cdot 70.$$

Molecular ratio, 1·6.

In this experiment the division of the complex into two peaks is clearly seen. The composition of the whole complex agrees fairly closely with that of a mixture in which the two main components had the composition AG_2 and A_2G_3, and the slowest component the composition AG.

Experiment 7.

Antigen : Crystalline horse-serum albumin. This preparation, unlike that used in Experiments 3 and 6, had not been passed through a Seitz filter ; it contained a small

amount of α_1-globulin, as shown by asymmetry of the peak in the electrophoresis pattern at pH 8·6 (Fig. 2).

Antibody : S4, solution of γ-globulin (Fig. 4) prepared from antiserum. The precipitable antibody in this solution found by precipitation with an equivalent amount of antigen was 5·15 mg. per ml.; the total globulin was 13·1 mg. per ml.

(A) 5 ml. of S4 (total protein 65·5 mg., precipitable antibody 25·75 mg.) were added slowly with thorough mixing to 1·5 ml. of 4·58 per cent albumin solution (68·7 mg. of albumin, 23 times the equivalent amount) and 0·5 ml. of phosphate buffer pH 8·0. $\mu = 0{\cdot}1$. The mixture was dialysed against phosphate buffer pH 8·0 for 2 days.

Composition by electrophoresis (Fig. 11):

	Ascending.	Descending.
Albumin	34·5 per cent	37·4 per cent
Complex	44·9 ,,	43·3 ,,
γ-globulin	20·6 ,,	19·3 ,,

Total protein	=134·2 mg.
Total albumin	= 68·7 ,,
Free albumin (134·2 × 0·345 mg.)	= 46·4 ,,
Combined albumin	= 22·3 ,,
Complex (134·2 × 0·499)	= 60·4 ,,
Globulin in complex	= 38·1 ,,
Precipitable antibody	= 25·75 ,,

Ratio of antigen in complex to *precipitable antibody*, $\frac{22{\cdot}3}{25{\cdot}75} = 0{\cdot}87$.

Molecular ratio, 2·0.

Ratio of antigen in complex to total globulin in complex, $\frac{22{\cdot}3}{38{\cdot}1} = 0{\cdot}586$.

Molecular ratio, 1·34.

(B) After electrophoresis 5 ml. of this solution, containing 45·85 mg. of protein (22·4 globulin, 8·8 precipitable antibody, 23·45 albumin), were taken and 2 ml. of solution S4 (26·2 mg. globulin, 10·3 mg. precipitable antibody) were added, the antigen in the mixture was 11 times the equivalent amount. The mixture was dialysed further and examined after 5 days. 2·25 mg. of precipitate formed which are assumed to have contained 0·4 mg. of albumin and 1·85 mg. of antibody.

Composition of electrophoresis (Fig. 12):

	Ascending.	Descending.
Albumin	15·5 per cent	15·8 per cent
Complexes	53·0 ,,	54·4 ,,
γ-globulin	31·5 ,,	29·8 ,,

Total protein in solution (72·05 − 2·25)	= 69·8 mg.
Total albumin (23·45 − 0·4 mg.)	= 23·05 ,,
Free albumin (69·8 × 0·155 mg.)	= 10·8 ,,
Combined albumin	= 13·25 ,,
Complex (69·8 × 0·53 mg.)	= 37·0 ,,
Globulin in complex, by difference	= 23·75 ,,
Precipitable antibody	= 17·25 ,,

Ratio of antigen in complex to *precipitable antibody*, $\frac{13{\cdot}25}{17{\cdot}25} = 0{\cdot}77$.

Molecular ratio, 1·76.

Ratio of antigen in complex to total globulin in complex $\frac{13{\cdot}25}{23{\cdot}75} = 0{\cdot}56$.

Molecular ratio, 1·28.

In the first part (A) of this experiment the separation of two complex peaks was conspicuous, the slower peak was steep and narrow. The great difference between the amount of precipitable antibody and the amount of globulin found in the complexes will be discussed later.

Experiment 8.

Antigen: Crystalline horse serum albumin. This preparation had been passed through a Seitz filter, but still contained a little α_1-globulin.

Antibody: S6, solution of γ-globulin prepared from antiserum. The precipitable antibody in this solution found by precipitating with an equivalent amount of antigen was 11·5 mg. per ml.; the total globulin was 45·2 mg. per ml.

1·5 ml. of S6 (total globulin 67·8 mg., precipitable antibody 17·3 mg.) were added slowly with thorough mixing to 5·5 ml. of 0·61 per cent albumin solution (total albumin 33·5 mg. = 17·7 times the equivalent amount). The mixture was opalescent and after it had been kept at 4° C. for 3 days 4·0 mg. of precipitate separated.

Composition by electrophoresis:

	Ascending.	Descending.
Albumin . . .	18·7 per cent .	20·1 per cent.

The complex peaks could not well be separated from the γ-globulin and therefore no estimate was made of the relative proportions of complex and free γ-globulin.

In the calculation it is assumed that the precipitate formed had the composition A_2G and contained 0·7 mg. of albumin and 3·3 mg. of antibody.

Total protein (101·3 − 4·0 mg.)	= 97·3 mg.
Total albumin (33·5 − 0·7 mg.)	= 26·5 „
Free albumin (97·3 × 0·187 mg.)	= 18·2 „
Combined albumin	= 8·3 „
Precipitable antibody (17·3 − 3·3 mg.)	= 14·0 „

Ratio of antigen to *precipitable antibody*, $\frac{8\cdot3}{14\cdot0} = 0\cdot59$.

Molecular ratio, 1·26.

In view of the presence of a large amount of globulin which combined with antigen when a large excess antigen was present in solution, but was not carried down in the precipitate formed with antigen at the equivalence point, a further experiment was made with solution S6.

(A) To 6·5 ml. of a 1·55 per cent solution of S6 (total globulin 100·6 mg., antibody 27 mg.) the equivalent amount of albumin (2·93 mg.) was added in solution in 0·60 ml. The mixture was left at room temperature until it flocculated and kept at 4° C. overnight. The precipitate was then packed by centrifuging. The supernatant was dialysed against phosphate buffer pH 8·0 and examined by electrophoresis; no component with mobility faster than that of γ-globulin was detected (Fig. 15). To this supernatant, after electrophoresis, 0·5 ml. of 4·58 per cent albumin (22·9 mg.) was added; this was 7·8 times the equivalent of the antibody present in the original 6·5 ml. of S6 used: 2 mg. of precipitate formed. No complex was found in the electrophoresis pattern of the supernatant fluid (Fig. 15A); but a slight shoulder appeared on the γ-globulin peak which can be seen if Fig. 15 and 15A are compared.

Experiment 9.

Antigen: Ox serum albumin (Armour); no evidence of α_1-globulin or slower components (Fig. 3).

Antibody: Solution of γ-globulin S8 containing 5·7 per cent of a component with the mobility of β-globulin (Fig. 6); total protein 25 mg. per ml.; precipitable antibody

5·7 mg. per ml. After the precipitable antibody had been removed by an equivalent amount of antigen the faster component made up 8·5 per cent of the total protein (Fig. 6A); this component, therefore, contained no precipitable antibody.

This preparation was examined in the ultracentrifuge by Dr. Kekwick of the Lister Institute and found to be homogeneous.

(A) *Solution of specific precipitate in excess antigen.*

Pilot experiment: 0·9 ml. of S8, 0·9 ml. of 0·9 per cent NaCl and 0·18 ml. of 3·95 per cent albumin solution (0·710 mg.) formed 5·85 mg. of precipitate; antibody 5·14 mg. (5·7 mg. per ml. of S8).

Main experiment: 5 ml. of S8, 5 ml. of 0·9 per cent NaCl and 1 ml. of 0·395 per cent albumin (3·95 mg.). Albumin added to the washed precipitate 1·8 ml. of 7·90 per cent solution (142 mg.). Total albumin 145·95 (37 times the equivalent amount).

The mixture was left overnight at room temperature. The precipitate dissolved completely. The volume was made up to 8 ml. with phosphate buffer, pH 8·0, $\mu = 0 \cdot 1$; protein concentration 2·20 per cent.

Protein in precipitate, from pilot experiment	= 32·5 mg.
Albumin added	=142 ,,
Total albumin	=146 ,,
Total protein	=174·5 ,,
Protein found (8 × 22·0) mg.	=172·0 ,,

Dialysed against phosphate buffer pH 8·0 for 2 days. Composition by electrophoresis on 3 occasions; the concentrations of the solutions inevitably differed on these 3 occasions:

	Ascending.	Descending.
6.i.50 (2 days after re-solution, Fig. 10):		
Free albumin	78·6 per cent	83·2 per cent.
Complex and fast component of S8	22·4 ,,	16·8 ,,
11.i.50 (7 days after re-solution, Fig. 10A):		
Free albumin	75·0 per cent	82·6 per cent.
Complex and fast component of S8	25·0 ,,	17·4 ,,
17.i.50 (13 days after re-solution):		
Free albumin	74·5 per cent	79·3 per cent.
Complex and fast component of S8	25·5 ,,	20·7 ,,

As the proportions of the fractions did not change appreciably between the 7th and 13th day after re-solution, we assume that final equilibrium had been reached by the 7th day.

In this case it is more satisfactory to calculate the combined albumin from the difference between the complex formed and the precipitable antibody found in the pilot experiment, rather than from the difference between the albumin added and free albumin found, which is a small difference between two large quantities.

6.i.50		
	Total protein	=174·5 mg.
	Complex (174·5 × 0·224 mg.)	= 39·1 ,,
	Antibody from pilot experiment	= 28·5 ,,
	Combined albumin, by difference	= 10·6 ,,

Ratio of antigen to antibody in complex, $\frac{10 \cdot 6}{28 \cdot 5} = 0 \cdot 37$.

Molecular ratio, 0·85.

11.i.50	Complex (174·5 × 0·25 mg.)	= 43·6 mg.
	Antibody	= 28·5 „
	Combined albumin, by difference	= 15·1 „

Ratio of antigen to antibody in complex, $\frac{15\cdot 1}{28\cdot 5} = 0\cdot 53$.

Molecular ratio, 1·2.

(B) *Addition of antibody solution directly to excess of antigen.*

7 ml. of a more dilute solution of S8, containing 60·9 mg. of globulin, 18·2 mg. of precipitable antibody and 3·5 mg. of inert fast component were added slowly, with thorough mixing, to 0·8 ml. of 7·95 per cent solution of ox serum albumin (63·6 mg.). The solution S8 contained 39·2 mg. of γ-globulin that was not precipitable by antigen.

The mixture was dialysed against phosphate buffer pH 8·0. A precipitate, 2 mg., formed ; this differed from the usual antigen-antibody precipitate, as it was not readily soluble in dilute acid.

Composition by electrophoresis (Fig. 13) :

	Ascending.	Descending.
Albumin	42·3 per cent .	47·8 to 50·4 per cent.
Complex	34·2 „ .	30·8 to 28·2 „
γ-globulin	23·5 „ .	21·4 per cent.

Total protein (60·9 × 63·6) mg.	124·5 mg.
Total albumin	63·6 „
Free albumin (124·5 × 0·423 mg.)	52·8 „
Combined albumin, by difference	10·8 „
Complex plus fast component of S8 (124·5 × 0·342 mg.)	42·5 „
Complex (42·5–3·5 mg.)	39·0 „
Globulin in complex	28·2 „

Ratio of antigen to *precipitable antibody* in complex $\frac{10\cdot 8}{18\cdot 2} = 0\cdot 595$.

Molecular ratio, 1·4.

Ratio of antigen to total γ-globulin in complex, $\frac{10\cdot 8}{28\cdot 2} = 0\cdot 38$.

Molecular ratio, 0·87.

(C) *Addition of antigen to supernatant after precipitable antibody had been removed.*

7 ml. of diluted supernatant from Experiment 8A containing 0·42 per cent of globulin (of which 8·5 per cent was fast inert component, Fig. 6A) were added slowly with thorough mixing to 0·8 ml. of 7·95 per cent ox albumin solution (63·5 mg.). The mixture was dialysed against phosphate buffer, pH 8·0.

Composition by electrophoresis (Fig. 16) :

	Ascending.	Descending.
Albumin	66·9 per cent .	71·4 per cent.
Intermediate component .	9·3 „ .	5·8 „
γ-globulin	23·8 „ .	22·8 „

Total protein	93·0 mg.
Intermediate component (93 × 0·093 mg.)	8·6 „
Inert fast component of S8 (29·4 × 0·085 mg.) . .	2·5 „
Complex, by difference	6·1 „
Total albumin	63·6 „
Free albumin (93 × 0·669 mg.)	62·0 „
Bound albumin	1·6 „
γ-globulin in complex	4·5 „

In Fig. 16 there is evidence of a small but definite peak between the peaks corresponding to albumin and γ-globulin.

Experiment 10.

Antigen: Ox serum albumin (Armour); no evidence of α_1-globulin or slower component (Fig. 3).

Antibody: Solution of γ-globulin S9 + 10, which contained no detectable faster component (Fig. 7 and 7A); total protein 10·7 mg. per ml.; precipitable antibody 3·24 mg. per ml.

(A) *Addition of antibody directly to excess of antigen.*

7 ml. of the solution S9 + 10 containing 22·7 mg. of precipitable antibody were added slowly, with thorough mixing, to 0·7 ml. of 8·25 per cent solution of ox serum albumin (17 times the equivalent amount); the mixture was slightly opalescent. On dialysis against phosphate buffer pH 8·0, 0·4 mg. of precipitate formed. This, as in Experiment 9B, was partly insoluble in dilute acid.

Composition by electrophoresis (Fig. 14):

	Ascending.	Descending.
Albumin	32·8 per cent	33·0 per cent.
Complex	35·3 ,,	36·1 ,,
γ-globulin	31·9 ,,	30·9 ,,

Total protein	136·65 mg.
Total albumin	57·75 ,,
Free albumin (132·25 × 0·328 mg.)	43·5 ,,
Combined albumin, by difference	14·25 ,,
Complex (132·25 × 0·353 mg.)	46·8 ,,
Globulin in complex, by difference	32·65 ,,

Ratio of antigen to *precipitable antibody* in complex, $\frac{14 \cdot 2}{22 \cdot 7} = 0 \cdot 63$.

Molecular ratio, 1·45.

Ratio of antigen to total globulin in complex, $\frac{14 \cdot 25}{32 \cdot 45} = 0 \cdot 44$.

Molecular ratio, 1·0.

(B) *Addition of excess antigen to the supernatant after removal of precipitable antibody by precipitation with an equivalent amount of antigen.*

Albumin solution, 0·6 ml., containing 3·3 mg. of albumin were added with stirring to 7 ml. of S9 + 10. After floccules formed the mixture was put in a refrigerator, mixed by inversion on the following day, centrifuged on the 3rd day, and the precipitate was washed 3 times with cold saline. It contained 26·0 mg. of protein (22·7 mg. of antibody).

The supernatant fluid was dialysed against phosphate buffer pH 8. No component faster than γ-globulin was detected by electrophoresis (Fig. 17); all the albumin was precipitated and no soluble complex formed.

Ox serum albumin, 0·50 ml. of 8·25 per cent solution (41·25 mg. of albumin) as added to 7 ml. of the dialysed supernatant, which contained 35 mg. of γ-globulin. The mixture was dialysed against phosphate buffer pH 8. A small precipitate (about 0·05 mg.) settled out.

Composition by electrophoresis (Fig. 17A):

	Ascending.	Descending.
Albumin	52·5	54·5
Complex	5·1	4·4
γ-globulin	42·4	41·7

There was a definite peak between those corresponding to albumin and γ-globulin as in Fig. 16.

There was also a shoulder on the γ-globulin peak as in Experiment 8A (Fig. 15). There is direct evidence of the formation of a small amount of complex. The amount is of the same order as the amount in Experiment 9C.

Experiment 11.

There is evidence in the preceding experiments that ox and horse serum albumin form a complex with the γ-globulin of a homologous antiserum from which all the precipitable antibody has been removed. An experiment was therefore made in which ox serum albumin was mixed with γ-globulin (S11) prepared from the sera of 2 rabbits which had not been immunized against ox serum albumin. One of these had received injections of Bence-Jones protein, but had formed no antibody detectable by this antigen; the other had been immunized with crystalline urease and had formed a small amount of precipitable antibody. This γ-globulin (Fig. 19) contained a small amount of a faster component. Of the solution S11 3 ml. (81 mg.) were added, with stirring, to 0·8 ml. of a 8·0 per cent solution of ox serum albumin (64 mg.). The mixture was dialysed against phosphate buffer pH 8·0. The composition by electrophoresis (Fig. 20) was:

	Ascending side.
Albumin	44 per cent.
Globulin	56 ,,

A hump on the slow side of the albumin peak suggests the formation of a small amount of a complex, with a mobility slightly less than that of albumin and therefore containing a high proportion of albumin to globulin. This complex would amount to about 5 per cent of the total protein present. The small fast component of the S11 appeared unchanged; there is no evidence of an intermediate peak comparable to those in Fig. 16A and 18.

DISCUSSION.

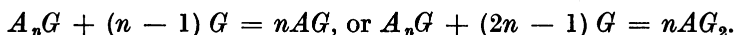
In these experiments two matters not directly related to the valency of antibodies may be noted. In the first place, in all the attempts at re-solution, except that of Experiment 9A, the precipitates dissolved much more slowly, and much more antigen was needed to effect solution than in the experiment of Heidelberger and Pedersen (1937). In other experiments which we have not reported we were not able to re-dissolve a considerable proportion of the original precipitate. This difficulty of re-solution was not a characteristic of the antigen concerned, as we had the same difficulty in experiments in which the antigens were human serum albumin and crystalline egg albumin, the antigen used in the experiments of Heidelberger and Pedersen (1937).

In the second place, the antigen-antibody complex persisted in the upper part of the descending limb of the electrophoresis apparatus, although it was left behind by the antigen, which was the faster component; that is, the complex remained, although it was not in presence of excess antigen. In all cases the

amount of complex (calculated from the electrophoresis pattern) was slightly less on the descending side than on the ascending side. It appears that the dissociation of the complex on removal of the antigen was slow. Samples of the complex, with no free antigen, were removed from the descending limb and kept frozen for several weeks ; no precipitate formed while the solutions were frozen, but formed in the course of a few days when the solutions were thawed and kept at 4° C. One such sample examined in the ultracentrifuge shortly after thawing by Dr. Kekwick of the Lister Institute was found to be polydisperse ; the excess antigen was probably dissociating from the complex and precipitable complexes were being formed.

Although Heidelberger and Pedersen (1937) found that their precipitates dissolved much more readily than ours did, they found some evidence of a progressive change after solution. This can be compared with the change found in our Experiment 9A, in which the amount of antigen in the complex increased between the 2nd and 7th day after re-solution. These slow changes are comparable to the Danysz effect, and contrast with the rapid reversibility, in the region of antibody excess, found by Marrack (1938).

According to the lattice theory the solution of the antigen-antibody precipitate in excess of antigen involves the breaking of large complexes A_mG_n, through intermediate stages, to the final compound AG_2 with possibly some larger compound such as A_2G_3. If, on the other hand, antibodies are univalent, the process of solution only involves the dispersal of the precipitate and the change :



$$A_nG + (n - 1)\,G = nAG, \text{ or } A_nG + (2n - 1)\,G = nAG_2.$$

In either case no process of dispersal or break-up is involved when antibody solution is added to excess of antigen.

In the experiments of Heidelberger and Pedersen (1937) final equilibrium was not reached in 3 days, although the precipitate dissolved readily in excess of antigen ; in our Experiment 9A in which the precipitate dissolved overnight there was some change between the 2nd and 7th day after solution, though none between the 7th and 13th days. It might be argued that in Experiments 3 and 6 the final stage of breakdown of large complexes with formation of small compounds containing a higher ratio of antigen to antibody had not been reached. If this were so, there should be evidence of a series of compounds of intermediate composition, and with mobilities intermediate between those of the main complex and of γ-globulin ; the complex peak should slope off gradually on the slow side. Actually the slope of the complex peak (Experiment 3 and 6, Fig. 8 and 9) was steep on the slow side, and if anything it was less steep in Experiment 9A (Fig. 10A), in which solution was rapid and no change appeared between the 5th and 11th days after re-solution. Also, when antibody solution was added directly to excess of antigen the break-up to large complexes would not be involved. But the peaks of the soluble complex found in the direct Experiments 7A and 9B (Fig. 11, 13) were very similar to those found in the re-solution experiments ; in both there were two peaks, and the demarcation on the side of slower mobility was sharp. In Experiments 3 and 6 (Fig. 8 and 9) there was evidence of a small amount of material of mobility between that of the soluble complex and that of γ-globulin. We suggested in discussion of Experiment 6 that this had the composition AG. It may have consisted of compounds of intermediate composition which would have disappeared if final equilibrium had been reached.

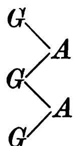
In the Experiments 7, 8, 9B, 10, in which antibody solution was added directly to excess of antigen, the amount of globulin found in the soluble complexes was considerably more than the precipitable antibody found in the antibody solutions. This complicates the calculation of the ratio of antigen to antibody in the complexes and these experiments will be discussed later. In Experiment 3, 6 and 9A in which the precipitate was re-dissolved the molecular ratio of antigen to antibody in the complex was more than 1 and less than 2. The ratios in Experiment 2, 4, 5 and 8, as far as it was possible to calculate by making allowance for the undissolved precipitate, were also between 1 and 2.

A remarkable feature of all the electrophoretic patterns—except Fig. 14 (Experiment 10A)—was the presence of two peaks corresponding to the antigen-antibody complex. In Fig. 8, 9 and 11 these peaks were sharply demarcated. The presence of these 2 distinct peaks suggests that 2 distinct compounds were formed. The most obvious explanation is that the two components were AG_2, formed by bivalent antibody, and AG, formed by univalent antibody. In Experiment 3 the molecular ratio in the whole complex was 1·7 or 1·8; to fit this ratio the ratio of molecules of AG_2 to molecules of AG would be 70 to 30 and the ratio of the corresponding areas of fast and slow components would be 210 to 69; the ratio of the corresponding areas (Fig. 8) is 19 to 34. In Experiment 6, again, the molecular ratio in the whole complex was 1·6; the corresponding ratio of molecules of A_2G to molecules of AG would be 60 to 40 and the ratio of the corresponding areas would be 180 to 92; the ratio calculated from Fig. 9 is 14·6 to 15·5. The differences in these two instances, in which the two peaks were clearly separated, is greater than the expected error of the estimates. The slower component of the complex must therefore have a composition A_mG_n, intermediate between AG_2 and AG.

In Experiment 6 a mixture of 10 molecules of AG_2, 6 of A_2G_3 and 1 of AG would fit the ratio of fast and slow components and the ratio of antigen to antibody found in the total complex; but this is not the case in Experiment 3 (Fig. 8), in which the area of the peak corresponding to the slower component of the complex is considerably larger than that corresponding to the faster component. Two peaks were found in Experiment 9A (Fig. 10A) in which final equilibrium was reached, and in Experiment 7A (Fig. 11) in which the breakdown of the large complex was not involved. So the presence of the slower peak with the composition A_mG_n cannot be ascribed to incomplete breakdown of larger molecules.

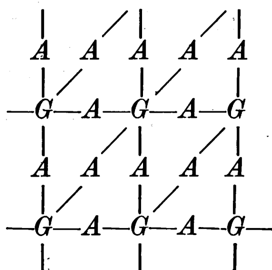
In Experiment 6 the molecular concentration of the free antigen is about 8 times the molecular concentration of the antibody. If no other factor besides the specific valencies of antibody and antigen were involved it would be expected, on grounds of probability, that most of the molecules of antigen would be combined with one molecule of antibody only; and that if antibody molecules have two valencies the bulk of the compounds formed would be AG_2 or AG, the ratio of the two concentrations, depending on the dissociation constant; there is, however, evidence of molecules of the composition A_mG_n. Another factor involved may be a non-specific attraction between antibody molecules when these are brought into close apposition by specific combination with antigen, as suggested by Boyd (1947). It is not necessary to regard compounds such as A_2G_3 as a chain. It is not improbable that antibody molecules may have two valency groups placed asymmetrically, as they seem to be on the molecules of diphtheria

antitoxin (Pappenheimer *et al.*, 1940). The compound A_2G_3 may be more appropriately represented by—

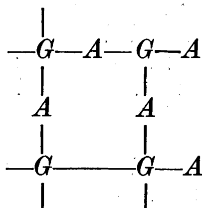


When the lattice theory was first suggested (Marrack, 1934) it was pointed out that, when antibody is in great excess, all the valencies of each antibody molecule are satisfied without any antibody molecules combining with two antigen molecules; and that, consequently, large complexes cannot be built up by specific valencies. It was then suggested that "when antibody molecules are attached to the antigen, the polar groups, on which the solubility of the antibody globulin normally depends, are brought into close apposition with each other and attract each other instead of water molecules."

We may therefore suggest that the second valency of antibody molecules is weak and is reinforced when antibody is in excess, and to a lesser degree, when antigen is in excess, by this non-specific attraction. In the neighbourhood of the equivalence zone the force holding a pair of molecules of antigen to one molecule of antibody is also reinforced by the attractions between other molecules of antigen and antibody. The stability of the structures depends on the fact that antigens are multivalent, so that structures such as—



are possible. Structures such as—



would be relatively unstable. Hence the formation of a precipitate is inhibited when antigen is in excess. The stability of the complex depends on the formation of a framework with several ties. The term "lattice" may be unsuitable, as the word has other connotations, and might be replaced by "framework." But "alternation" is unsuitable, as it suggests a linear structure.

The antigens used in these experiments were not homogeneous ; even the ox albumin may have contained some 2 per cent of a component which had a sedimentation constant above that of albumin. In all cases the antibody solutions were tested by the optimum proportions method of Dean and Webb (1923), with antigen solutions in amounts up to 5 times and down to one-third of the optimum. No zone was found other than that due to the major constitunet of the antigen ; this zone corresponded to the equivalence zone of the precipitation curve. It is possible that the reaction between a minor antigen and a small amount of the corresponding antibody might be hidden in the major zone. No precipitate was formed when the supernatant fluids at the equivalent points were tested with further antigen.

Solution S6 (S4 and S6 were prepared from the serum of the same rabbit, bled at different times) was tested by Oudin's (1947) method. When a solution of horse serum albumin (Fig. 2) was put on the agar column, a faint zone appeared above the main zone. When horse serum was superimposed a faint zone appeared below the main zone. It appears, therefore, that solution S6 contained a small amount of antibody to an antigen of which there was much less in the albumin solution than in the whole serum. In Experiment 7A, 7B, 8, 9B and 10A, in which antibody solutions were added directly to excess of antigen, the amount of globulin found in the complexes was 37 to 50 per cent more than the precipitable antibody. The precipitable antibody in these experiments was estimated by adding the equivalent amount of antigen to the antibody solution in one lot ; it included any incomplete antibody, such as that demonstrated by Heidelberger and Kendall (1935), which would be carried down with the precipitate formed by the complete antibody present. A minor constituent (G') of the antigen may have contributed towards this excess. The contribution cannot, however, account for the excess found. In Experiment 9B, 63·6 mg. of antigen (ox serum) albumin was used ; at 2 per cent this would contain about 1·3 mg. of G'. If *all* this was combined with antibody to form a soluble compound (which is improbable) and the composition of this compound was AG_2, this would amount to 3·9 mg. ; whereas the excess of globulin found in the complex was 10 mg. Also, if the excess of globulin found in the complexes in these experiments were included in soluble compounds of G' and anti-G', these complexes should also be found in experiments in which excess of antigen was added to supernatants after the precipitable antibody had been removed. No such complex was found in Experiment 8A, but definite peaks were found in Experiment 9C and 10B. In neither case were these peaks large enough to account for the excess found in 9A and 10A, although the accuracy of the measurements involved may not be enough for a conclusion to be based on this difference. The main reason for not attributing the excess of globulin in the complexes in these experiments to the presence of antibody to minor constituents of the antigens is that if a sufficient amount of such antibody had been present it should have been detected in the supernatant fluids, formed at the equivalence point, and by the optimum proportions method. In the experiments, other than 7A, in which antibody was added directly to excess of antigen, some precipitate, ranging from 0·4 mg. in Experiment 4 to 4 mg. in Experiment 8, was formed. These precipitates may have been formed by G' and anti-G', though the relative insolubility of these precipitates in dilute HCl suggests that they were not genuine antigen-antibody precipitates.

It seems, therefore, that the antibody solutions contained globulin that was not carried down in the precipitate formed at the equivalence point, but would combine with antigen when this was added in excess. It is quite possible that the amount of incomplete antibody that can be precipitated with the complete antibody is limited and the amount in an antibody solution may exceed the amount that can be carried, down in this way.

The estimates suggest that this excess globulin, that combined with antigen when whole antibody was added to excess of antigen in Experiments 9B and 10A, was more than the amount of globulin that combined when the supernatant fluids were added to

excess antigen. If so, some of this incomplete antibody was incorporated in complexes formed by precipitable antibody with excess of antigen, but did not combine with antigen when precipitable antibody was not present. This incorporation may have depended on the non-specific attractions between antibody molecules, as well as on a weak specific affinity for antigen. When the amount of antigen was less all the combining sites of antigen could be occupied by precipitable antibody, and this antibody of weak affinity would be excluded.

The excess globulin found in the soluble complexes in the experiments in which antibody solutions were added to excess antigen complicates the calculation of the ratio of antigen to antibody in these complexes. In Experiment 7A the molecular ratio of antigen to precipitable antibody was 2·0, but the ratio of antigen to total globulin was 1·34. If the excess globulin was incomplete antibody, with which some of the antigen was combined, it should be taken into account and conclusions on the valency of the antibody should be based on the lower ratio.

SUMMARY.

(1) The composition of the compounds formed by antibodies with excess of the homologous antigens has been studied by electrophoresis.

(2) Evidence has been found of the formation of two compounds of different composition.

(3) Antibodies appear to be bivalent.

(4) It is suggested that the formation of large complexes by antigens and antibodies depends on the formation of a framework in which a pair of antigen molecules are held together by several links; also that non-specific attractions between antibody molecules may increase the stability and insolubility of the complexes.

(5) Evidence has been found of the presence in solutions of γ-globulin, that contain antibodies, of some globulin that can be incorporated in soluble antigen-antibody complexes, but is not carried down with precipitable antibody in the equivalence zone.

The authors wish to thank Dr. Kekwick for investigations with the ultracentrifuge. This research was aided by a grant from the Thomas Smythe Hughes Medical Research Fund towards the purchase of parts of the electrophoresis apparatus.

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