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# Fowl Antibody: I. Some Physical and Immunochemical Properties

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**Summary.** Fowl antibodies to rabbit γ globulin, bovine serum albumin and to ε toxin were studied by means of their reactions with rabbit antisera to fowl globulin. Specific precipitates redissolved either in antigen excess or in solutions of lower salt concentration than those in which they had been formed were used to measure the sedimentation and diffusion coefficients and the electrophoretic mobilities of the soluble complexes of fowl antibody with antigen.

Fowl antiserum to any one antigen was found to contain two types of homologous antibodies, derived from different constituents of serum, having the same electrophoretic mobility but giving two distinct bands of precipitation with rabbit anti-fowl-globulin serum. The values obtained for the molecular weights of soluble complexes indicate that one type of antibody has a molecular weight of about 600,000 and the other 180,000, and also suggest that the latter type combined with only one molecule of antigen in antigen excess. Both types of antibody were precipitated by antigen in 0.9 and 8 per cent NaCl; the larger precipitates formed at the higher salt concentration contained more of the low molecular weight antibody, together with another component that was neither antigen nor antibody.

## INTRODUCTION

Double diffusion experiments in Ouchterlony plates using fowl antisera and homologous antigens led to the observation that the precipitation bands always formed nearer the serum well. This occurred regardless of the nature or concentration of the antigen, or of the salt concentration (0.85-8 per cent NaCl) in the agar, and was especially surprising when the antigen was rabbit γ globulin (molecular weight 160,000). Previous studies of the precipitation reactions of fowl antisera have been concerned mainly with the effects of salt concentration on the amount of precipitate (e.g. Goodman, Wolfe and Norton, 1951), with the specificity of the reaction at the higher salt concentrations (e.g. Deutsch, Nichol and Cohn, 1949) and with the valency of the antibody (Banovitz, Singer and Wolfe, 1959), but have all assumed that the molecular sizes of fowl and mammalian antibody were

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roughly the same. The following investigations are an attempt to determine some of the physical and immunochemical properties of fowl antibody to three soluble antigens of different molecular weights.

## MATERIALS AND METHODS

### Antigens

*Rabbit γ globulin (RGG)* was prepared from pooled normal rabbit serum by $Na_2SO_4$ fractionation (Marrack, Hoch and Johns, 1951). The material used for immunizing fowls was precipitated at 18 and 15 per cent $Na_2SO_4$ concentrations, dissolved in M/15 phosphate, pH 8.0 and finally dialysed against 0.85 per cent NaCl buffered with phosphate at pH 7.2. That used as test antigen was reprecipitated three times, dialysed against M/20 phosphate, pH 5.5 and centrifuged before the final dialysis as above.

*Bovine serum albumin (BSA)*. A crystalline preparation obtained from Armour & Co. was used.

Clostridium welchii ε *toxin* was prepared as a crude concentrate from culture filtrates of *Cl. welchii* type D (Orlans, Richards and Jones, 1960).

*Fowl globulin* was made by precipitating pooled fowl serum once with 17 per cent $Na_2SO_4$ and twice with 15 per cent $Na_2SO_4$, redissolving the precipitates in M/15 phosphate pH 8.0 each time and finally dialysing against 0.85 per cent saline, pH 7.2 for 3 days.

### Antisera

Fowl antisera to RGG and BSA were made by injecting 40-50 mg. of antigen/kilo body weight intravenously, either once or twice with an interval of 3-4 weeks, into young adult male birds (R.I.R. × L.S. for the RGG antisera and Br. Leg. for the BSA antisera). The birds were bled 8 days after the last injection.

Fowl antiserum to ε toxin was made by giving adult Br. Leg. birds of mixed sexes two intramuscular injections of alum-precipitated toxoid followed by eight injections of alum-precipitated toxin over a period of 5 months. A pool of the sera from two birds was used. Anti-BSA serum was similarly produced in two adult male Br. Leg. birds by giving three intramuscular injections of alum-precipitated BSA and one intramuscular injection of non-precipitated BSA, the total amount of antigen given being 160 mg. with weekly intervals between injections. The birds were bled 1 week after the last injection; this serum is referred to as 'anti-BSA i.m.'

Rabbit antisera to the fowl-globulin fraction were prepared by giving two rabbits (R1 and R2) intramuscular injections of alum-precipitated fowl globulin at weekly intervals. Antisera from each rabbit obtained at three different bleedings were used and, as they differed slightly, are labelled R1 or R2, III, IV and V. Antisera to hen and cock serum were kindly provided by Mr. J. Williams, Department of Biochemistry.

*Specific precipitates.* Optimum proportions for the fowl systems were determined by flocculation tests using constant antibody (Dean and Webb, 1926), as there were insufficient materials for quantitative precipitin curves. These tests were carried out in 0.9, 3 and 8 per cent NaCl concentrations. In 8 per cent salt, as flocculation was rapid and simultaneous in all but the lower antigen concentrations, it was not possible to determine the optimum. Flocculation in 0.9 per cent NaCl was slower and inhibited by antigen excess; that in 3 per cent NaCl was intermediate. The optimum concentration of antigen was taken to

be that in the mixture containing least antigen which flocculated in the least time; this was the same in both these salt concentrations and assumed to be the same in 8 per cent saline.

Specific precipitates were made in either 0.9 or 8 per cent NaCl by incubating antigen and antibody at optimum proportions for 3 hours at 37° (Wolfe, Mueller and Neess, 1959). After being washed three times in the appropriate saline solutions, the precipitates were used for the formation of soluble complexes or for protein analysis using the Markham modification of the micro-Kjeldahl method (Kabat and Mayer, 1948).

*Soluble antigen-antibody complexes* were made either by dissolving the precipitates in a solution of lower salt concentration than that in which they were formed or, by adding an amount of the respective antigen equal to twenty times equivalence. The tubes were left for 2 days at +4° with occasional tapping. Precipitates formed in 0.9 per cent NaCl solution dissolved more slowly and less completely than those formed in 8 per cent NaCl. Any undissolved material was removed by centrifugation.

The soluble complexes formed by dissolving, in antigen excess, precipitates which had been formed in 8 and 0.9 per cent NaCl are labelled 'x' and 'y' respectively. The solution obtained by extracting with 0.9 per cent NaCl solution a precipitate formed in 8 per cent NaCl solution is called 'z'. For ultracentrifugation analysis, a few drops of concentrated antigen solution were added to the 'z' solution to ensure antigen excess.

Increasing the salt concentration of the supernatant solution of the 'y' precipitate to 8 per cent produced further precipitation (without the addition of more antigen) in the RGG and BSA systems but not consistently in the ε toxin system. When this precipitate was redissolved in antigen excess, a solution 'w' was obtained.

*Precipitation in agar.* The agar for double diffusion precipitation (Ouchterlony, 1953) was made up as 1.5 per cent in a veronal buffered pH 7.4, 3 per cent NaCl solution, except when stated otherwise. This salt concentration provided a reasonable compromise for rabbit and fowl antibody precipitation.

*Diffusion coefficients* were measured by the method of Allison and Humphrey (1960) using agar similar to that above. After the development of bands, plates were dried and stained (Grabar and Burtin, 1960) and the tangent of the angle θ between the antigen trough and the precipitation band measured after projection through a photographic enlarger on graph paper. Values for diffusion coefficients of RGG, BSA and ε toxin were taken from Edsall (1953) and Orlans, Richards and Jones (1960).

*Immunoelectrophoresis.* Glass lantern slides 3¼ in. square were used as the base for the agar. Borate buffer pH 8.6, 0.05 M. was used for the baths and at half that strength in the agar; the potential was 5 V/cm. After the development of the bands of precipitation the slides were washed in saline, dried and stained with amido-schwarz (Grabar and Burtin, 1960). Some of the solutions were concentrated before electrophoresis by dialysis against 'carbowax' (Kohn, 1959).

*Sedimentation constants* were determined from ultracentrifuge analyses made in a Spinco Model E centrifuge at 59,780 rev./min., for 72 minutes at approximately 19°. Solutions for analyses were made up to contain approximately 0.8 per cent protein in 0.85 per cent NaCl, phosphate buffered at pH 7.2. Peaks were drawn and measured on graph paper after projection (enlargement factor of 5-7). The values obtained were not corrected for temperature or buffer solutions and were not determined at zero protein concentrations. Temperature during any given run rose no more than 2°, and from one run to another deviated by no more than 1° from 20°.

## RESULTS

### ANTIBODY CONTENTS OF SERUM POOLS

Table 1 shows the amount of precipitate formed by the different fowl anti-sera with appropriate amounts of antigen in 8 and 0.9 per cent NaCl. It also shows the additional precipitate obtained from the supernatant fluids, after removal of the precipitate formed in 0.9 per cent NaCl, by raising the salt concentration to 8 per cent without further

TABLE 1

AMOUNT OF SPECIFIC PRECIPITATE OBTAINED UNDER DIFFERENT CONDITIONS FROM 1 ML. OF EACH OF THE SERUM POOLS USED SUBSEQUENTLY

| | *System* | | | |
|---|---|---|---|---|
| | *anti-RGG i.v.* | *anti-BSA i.v.* | *anti-BSA i.m.* | *anti-ε-toxin i.m.* |
| | *mg. precipitate* | | | |
| Precipitate formed in 8% NaCl (1) | 1.94 | 2.35 | 1.37 | 1.02 |
| Precipitate formed in 0.9% NaCl (2) | 0.75 | 1.44 | 0.89 | 0.37 |
| Precipitate formed by raising NaCl conc. of supernatant (2) to 8% | 1.15 | 0.71 | 0.50 | no ppt. |
| Residue of precipitate (1) after extraction with 0.9% NaCl | 1.48 | 1.70 | not measured | 0.80 |

addition of antigen. This value probably depends on the precise ratio of antigen to antibody used in the initial precipitation, and hence is not very meaningful in the absence of quantitative precipitation curves. The portion of the specific precipitates formed in 8 per cent NaCl that redissolved in 0.9 per cent saline (residue shown in Table 1) was close to 25 per cent throughout.

TABLE 2

DIFFUSION COEFFICIENTS ($D_b$) OF FOWL ANTIBODY TO THREE DIFFERENT ANTIGENS

| *Antigen* | *No. of plates* | *Tan θ* | $D_g \times 10^7$ | $D_b \times 10^7$ |
|---|---|---|---|---|
| RGG | 17 | 1.41 ±0.03 | 4.1 | 2.1 |
| ε-toxin | 14 | 1.42 ±0.03 | 7.2 | 3.6 |
| BSA i.v. | 4 | 1.63 ±0.07 | 6.1 | 2.3 |
| BSA i.m. | 3 | 1.60 ±0.07 | 6.1 | 2.4 |

### DIFFUSION COEFFICIENTS OF FOWL ANTIBODIES

The values ($D_b$) obtained for the diffusion coefficients of fowl antibody to different antigens are shown in Table 2. The values for the diffusion coefficients of the antigen ($D_g$) used in the calculation are also shown.

With the rabbit γ globulin and ε toxin systems, measurements were at first made in both 2 and 8 per cent NaCl for each antigen/antibody ratio. The differences in tan θ values found at the different salt concentration were no greater than the variations between duplicate plates, that is of the order of 5 per cent, and were not considered significant. All subsequent measurements of diffusion coefficients were made in 3 per cent NaCl.

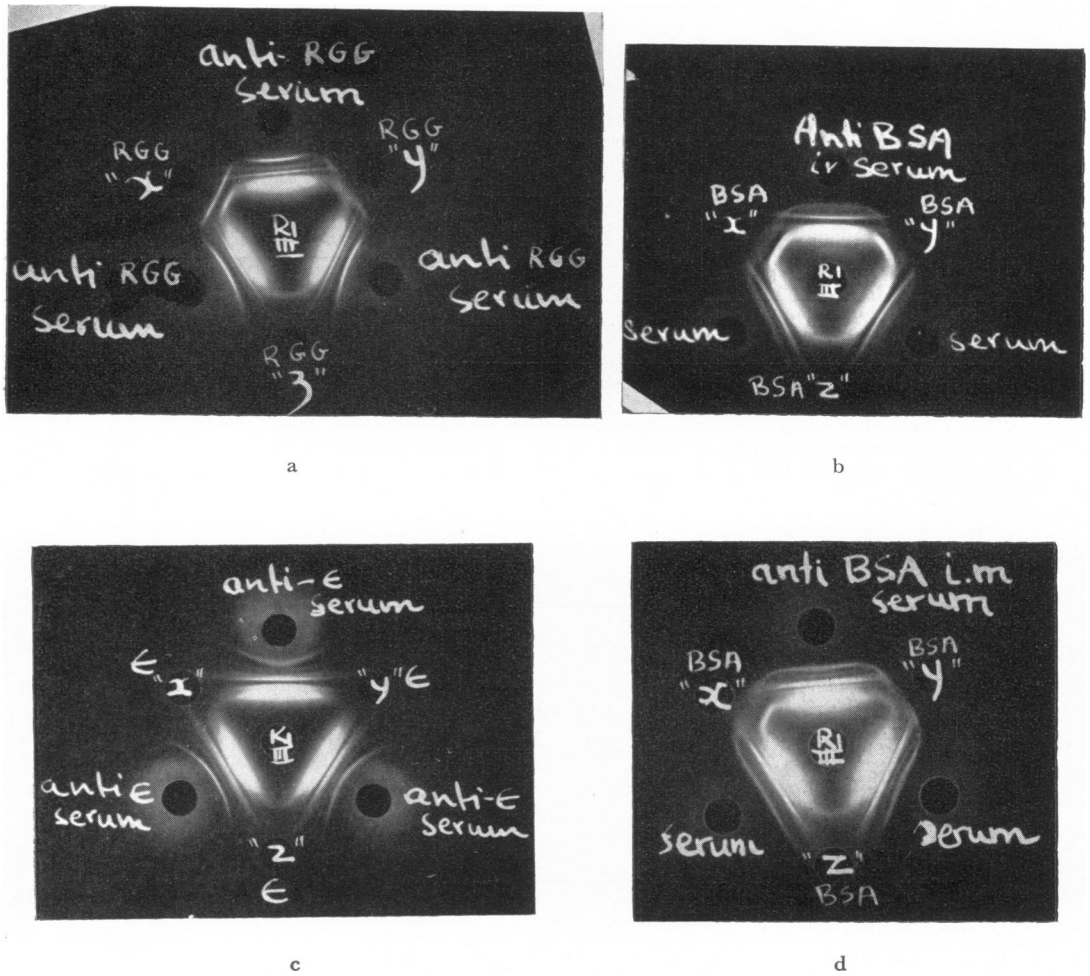



FIG. 1. Reactions of rabbit anti-fowl-globulin ($R_1$III, centre well) with soluble complexes 'x', 'y', and 'z' of various antigen-antibody systems, and with the fowl antisera from which the soluble complexes were derived.

## REACTIONS OF SOLUBLE ANTIGEN-ANTIBODY COMPLEXES WITH RABBIT ANTI-FOWL-GLOBULIN SERA

Antisera prepared against fowl globulin, made by $Na_2SO_4$ precipitation and apparently homogeneous by free boundary electrophoresis (kindly performed by Dr. A. E. Pierce), were found to contain antibodies to six or seven components when tested against whole

fowl serum (Figs. 1, 3 and 8). Immunoelectrophoresis and comparison with antisera to whole fowl serum (such as anti-hen W.9) showed that all the components were slow moving, and that antibody to albumin was virtually absent (Fig. 8).

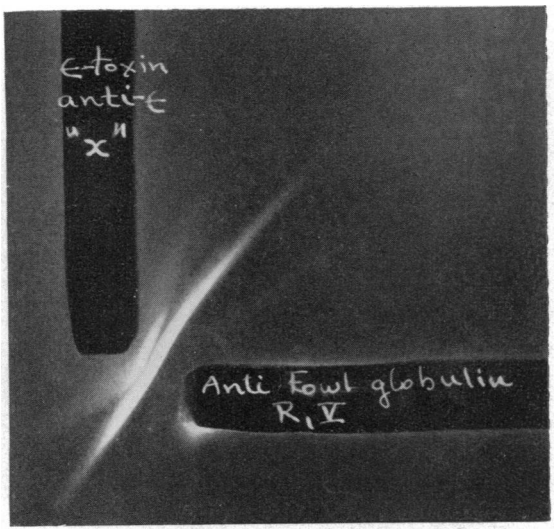



FIG. 2. Reaction of soluble complex 'x' of the ε-toxin-antitoxin system with rabbit anti-fowl-globulin showing the presence of a small amount of the slowly diffusing component.

Fig. 1 a,b,c,d shows the reactions of soluble complexes, and of the sera from which the specific precipitates were made, with the same rabbit anti-fowl globulin serum ($R_1$, III). These figures show that in the anti-BSA and RGG systems complexes 'x' and 'y', made

TABLE 3

DIFFUSION COEFFICIENTS OF THE CONSTITUENTS OF SOLUBLE ANTIGEN/FOWL ANTIBODY COMPLEXES, MEASURED AGAINST RABBIT ANTI-FOWL-GLOBULIN

Bands are numbered clockwise from the antigen well. The diffusion coefficient of rabbit antibody was taken as $D_b = 4.1 \times 10^{-7}$ cm²/sec.

| | *System* | *RGG i.v.* | *BSA i.v.* | *ε-toxin i.m.* |
|---|---|---|---|---|
| Band 1 | tan θ | 0.56 ± 0.03 | 0.59 ± 0.02 | 0.51* |
| | D × $10^7$ | 1.29 | 1.43 | |
| Band 2 | tan θ | 0.85 ± 0.06 | 0.87 ± 0.04 | 0.87 ± 0.07 |
| | D × $10^7$ | 2.96 | 3.10 | 3.10 |
| Band 3 | tan θ | 1.30 ± 0.14 | 1.37 ± 0.05 | 1.28 ± 0.14 |
| | D × $10^7$ | 7.1 | | |

*Band too weak and curved for reliable measurement.

by precipitating in 8 and 0.9 per cent NaCl respectively and redissolving in antigen excess, contained two components that gave reactions of identity with two different components of whole antiserum. The component that gave the band nearest the antigen wells was

absent in complexes 'z' made by extracting specific precipitates made in 8 per cent NaCl with 0.9 per cent saline. It was also absent from all the complexes of the ε toxin system, but small amounts of it could be demonstrated in the perpendicular trough arrangement used for the diffusion coefficient measurements (henceforth called L plates), Fig. 2. Sera from which the precipitating antibody had been removed showed a weakening or even disappearance of this band (Fig. 3 a and b), with the RGG and BSA systems but not with ε-toxin (Fig. 3b).

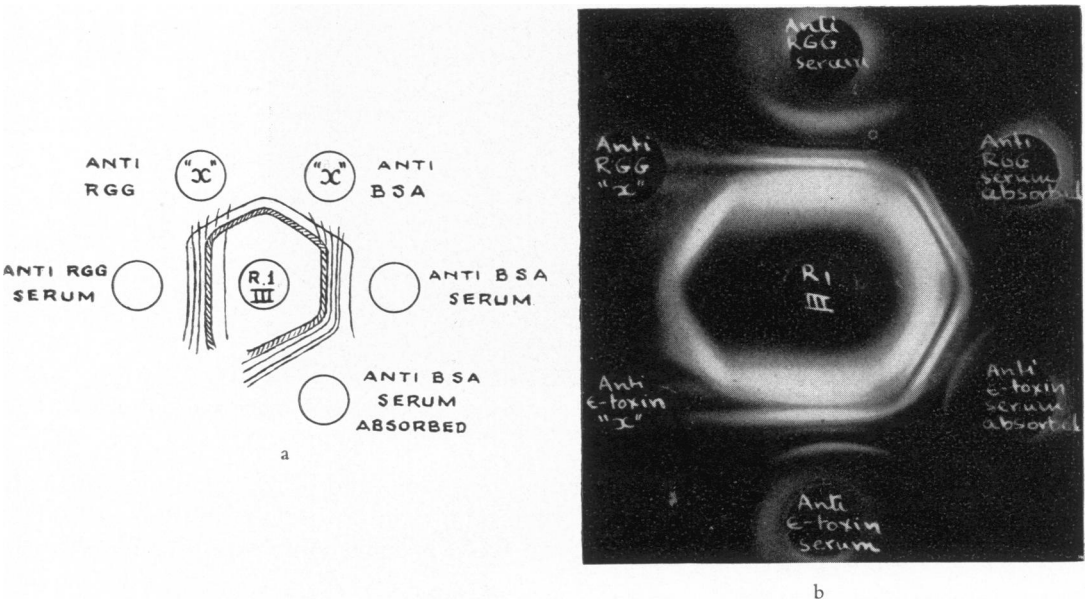



FIG. 3. (a) and (b). Reactions with rabbit anti-fowl-globulin ($R_1III$, centre well) of fowl antisera before and after removal of the specific precipitate (in 8 per cent NaCl); the corresponding soluble complexes 'x' are included.

The reactions of the 'x', 'y' and 'z' soluble complexes of the BSA i.v. system in L plates (agar in 3 per cent NaCl) are shown in Fig. 4 and the values of tan θ and $D_b$ for the bands found for the complexes of all four antigen antibody systems are given in Table 3. In addition to the two bands already seen in Fig. 1 (bands 1 and 2 in the table) a third faint band (visible in Figs. 4 a and c) was obtained with all complexes 'x' and 'z', that is, those derived from specific precipitates formed in 8 per cent NaCl. Because of the faintness of this band the diffusion coefficient for this component is subject to an error of ±12 per cent compared with variations of 5 per cent for the others (except band 1 of the ε toxin system), but shows that it is due to a very rapidly diffusing substance. The bands '3' of different antigen-antibody systems gave reactions of identity when tested against rabbit anti-fowl globulin serum in an Ouchterlony plate, showing that the substance responsible for it is derived from the fowl antiserum. More antibody to this component was present in antisera to whole hen or cock serum (e.g. W.9 in Fig. 6) than in the antiglobulin sera. The addition of more antigen to 'z' complexes did not change their diffusion coefficients, showing that the material extracted from specific precipitates formed in 8 per cent NaCl (at 'equivalence') by 0.9 per cent NaCl is antigen-antibody complex that cannot combine with more antigen.

SEDIMENTATION DATA

For ultracentrifugation excess antigen was added to all 'z' complexes. With complex 'x' of the anti-RGG system there were two distinct antigen-antibody peaks in addition to the slowest peak given by excess antigen (Fig. 5b). In eight runs of the anti-BSA and anti-ε-toxin systems, complexes formed in 8 per cent NaCl and redissolved in antigen excess gave a single peak corresponding to antigen-antibody complex although, as in the anti-RGG system, these preparations contained two types of complexes with different diffusion constants and immunological specificities (Fig. 1). In one case, however, an anti-BSA complex formed in 8 per cent NaCl gave a small faster peak corresponding to the heavier of the anti-RGG peaks. Anti-BSA complex formed in 0.9 per cent NaCl and redissolved in antigen excess gave an additional very fast (S = 21) peak, corresponding probably to a larger aggregate (Fig. 5a). The uncorrected sedimentation coefficients of the peaks seen in the various soluble complex preparations are listed in Table 4.

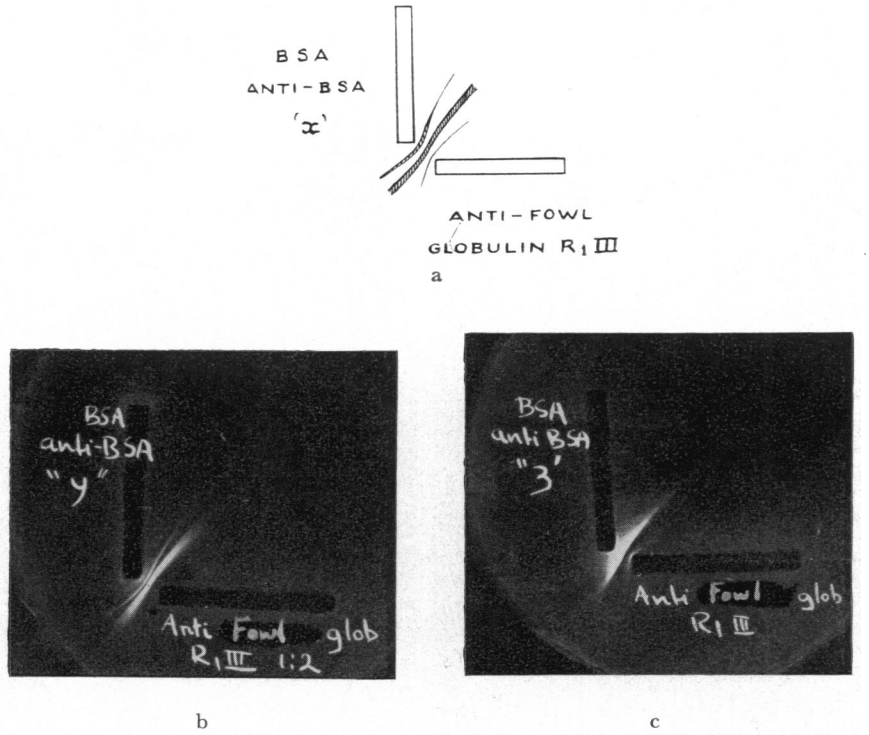


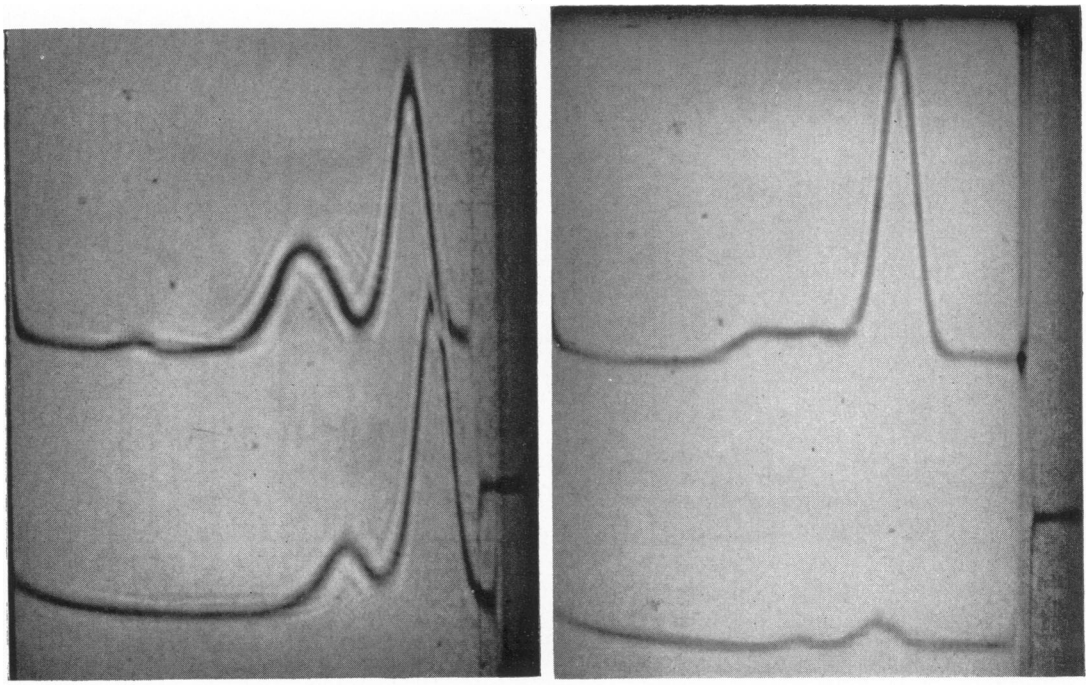

FIG. 4. (a), (b) and (c). Reactions in L plates of soluble complexes 'x', 'y' and 'z' of the BSA-fowl-anti-BSA system, with rabbit anti-fowl-globulin. The third band present in (a) and (c) is faint.

Calculation of the molecular weights of the complexes using these values of S and the corresponding values of D from Table 3 and 0.2523 for $(1-\bar{v}\rho)$ gave the values shown in Table 5. Subtracting the weight of one antigen molecule from the weight of the 'z' type (slow) complexes gives consistent values of about 185,000 for the molecular weight of this kind of fowl antibody; this suggests that it combines with only one molecule of antigen. As the heavier type of complex may contain both of the immunologically distinct kinds of antibody that form soluble complexes with different diffusion rates, a similar calculation

TABLE 4

SEDIMENTATION COEFFICIENTS OF THE CONSTITUENTS OF SOLUBLE ANTIGEN/FOWL ANTIBODY COMPLEXES

| *Soluble complex* | *Sedimentation coefficients of visible peaks* ($S \times 10^{13}$) | | | |
|---|---|---|---|---|
| Anti-RGG 'x' | 7.0 | 10.7 | | 13.7 |
| Anti-RGG 'z' | 7.0 | 10.6 | | |
| Anti-BSA 'x' | 4.3 | 9.4 | | |
| Anti-BSA 'y' | 4.3 | | 11.2 | 21 |
| Anti-BSA 'z' | 4.3 | 7.9 | | |
| Anti-BSA 'z' | 4.3 | 7.9 | 11.0 | |
| Anti-ε-toxin 'z' | | 7.1 | | |



a b

FIG. 5. Sedimentation diagram of four soluble complexes.

(a) Top. Anti-BSA 'y' at 32 minutes showing 21*S* peak in addition to complex and free antigen.
Bottom. Anti-BSA 'w' at 32 minutes.

(b) Top. Anti-RGG 'x' at 40 minutes showing two peaks due to complexes (10.7*S* and 13.7*S*) and one due to free antigen.
Bottom. Anti-RGG 'z' at 40 minutes showing an antigen peak and a single peak (10.6*S*) due to complex.

for this kind of complex is probably unsound. The values obtained by subtracting two antigen molecules from such complexes are none the less included because the very low diffusion constants found for anti-BSA and anti-RGG (Table 2) made it seem likely that some of the fowl antibody has a much higher molecular weight.

TABLE 5

MOLECULAR WEIGHTS OF SOME SOLUBLE ANTIGEN/ANTIBODY COMPLEXES

| | *Constants* | | *Molecular weights* | | | |
|---|---|---|---|---|---|---|
| Complex | S $\times 10^{13}$ | D $\times 10^{7}$ | Complex | Antigen | Complex −Antigen | Complex −2×Antigen |
| Anti-RGG (slow) | 10.6 | 2.96 | 348,000 | 160,000 | 188,000 | |
| Anti-BSA (slow) | 7.9 | 3.10 | 248,000 | 65,000 | 183,000 | |
| Anti-ε-toxin (slow) | 7.1 | 3.10 | 222,000 | 38,000 | 184,000 | |
| Anti-RGG (fast) | 13.7 | 1.29 | 1,030,000 | 160,000 | | 710,000 |
| Anti-BSA (fast) | 11.2 | 1.43 | 760,000 | 65,000 | | 630,000 |

IMMUNOELECTROPHORESIS

Immunoelectrophoresis of the three types ('x', 'y' and 'z') of anti-BSA soluble complex, using for precipitation rabbit anti-sera to cock serum, fowl globulin and BSA gave the patterns in Figs. 6 and 7. Here again, precipitates formed in 8 per cent NaCl ('x' and 'z') were found to have an extra component which migrated to the anode somewhat faster

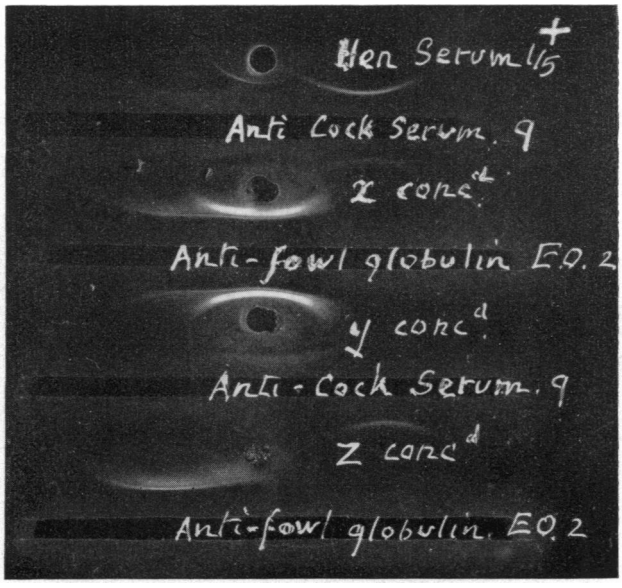



FIG. 6. Immunoelectrophoresis of hen serum 1 : 5 and of BSA-fowl-anti-BSA soluble complexes, using rabbit antisera to cock serum and to fowl globulin for precipitation, and showing the presence of the fast constituent in complexes 'x' and 'z', and of two electrophoretically distinct antibody components in both 'x' and 'y'.

than BSA (not shown) or fowl serum albumin (Fig. 6). Preparations 'x' and 'y' each had two distinct antigen-antibody complexes; the slower of these gave a long thin band with anti-fowl-globulin; the other (at the origin in Fig. 6) gave a shorter, thicker band. Preparation 'z' only had the slower of the two complexes. With rabbit anti-BSA similar

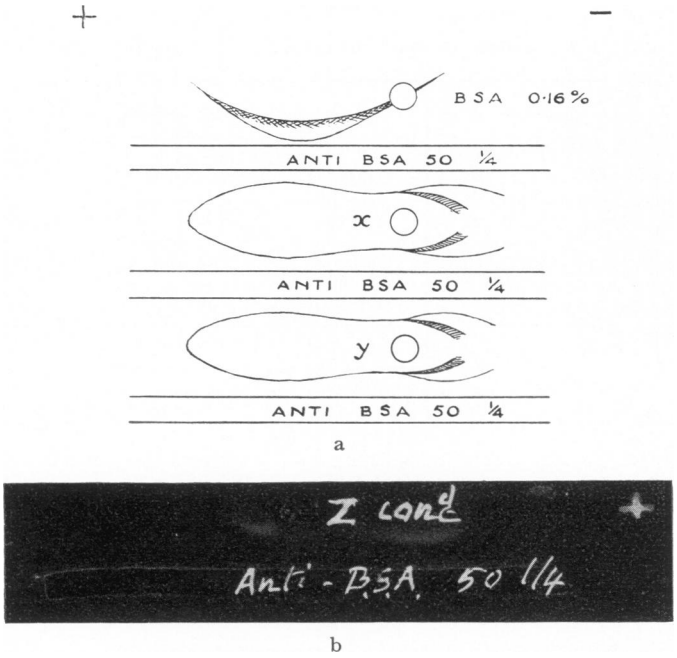



FIG. 7. Immunoelectrophoresis of BSA and of BSA-fowl-anti-BSA soluble complexes using rabbit anti-BSA for precipitation and demonstrating that both the antibody components shown in Fig. 8 also contain antigen.

results were obtained; the bands given by the slower complex component ('x' and 'y' in Fig. 7) were very thin compared with those given by the faster component, indicating that less antigen is bound in this kind of complex.

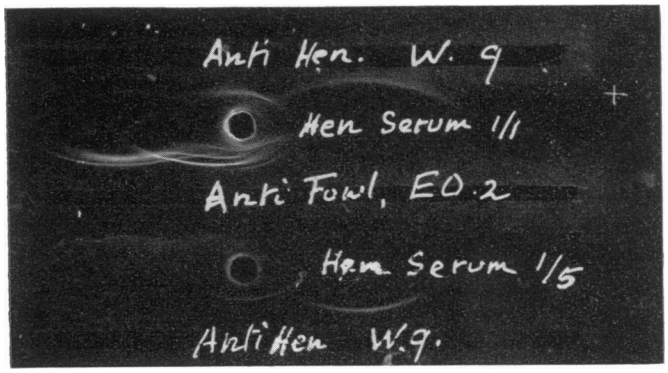



FIG. 8. Immunoelectrophoresis of hen serum, undiluted and 1 : 5, using rabbit antisera to hen serum and to fowl globulin for precipitation and demonstrating the differences in the antibody contents of the two rabbit sera.

Immunoelectrophoresis of fowl anti-sera, in the usual manner, with the homologous antigen to demonstrate the antibody-containing globulin fraction gave poor results because the low ionic strength needed for electrophoresis was not suitable for the precipitation of fowl antibody. Moreover, as can be seen in Fig. 8, fowl serum contains two γ globulins (or slowest components) with the same mobility, but giving two parallel bands with rabbit anti-fowl globulin. In order to identify the antibody-containing fraction and also the fast extra constituent present in precipitates formed in 8 per cent salt, the rabbit antisera to fowl globulin and cock serum were absorbed with washed specific fowl anti-BSA precipitates. Specific precipitates (a), (c) and (e) were formed in 8 per cent NaCl; precipitate (c) was washed in 0.9 per cent NaCl, the other two in 8 per cent NaCl;

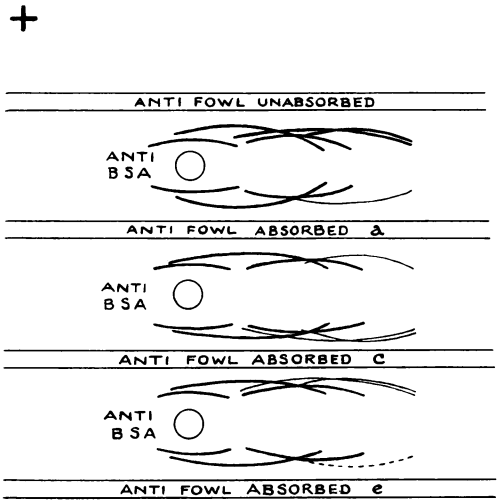



FIG. 9. Immunoelectrophoresis of fowl anti-BSA serum using for precipitation rabbit anti-fowl-globulin absorbed with specific precipitates (BSA-fowl-anti-BSA) (a), (c) and (e) and unabsorbed.

(e) was larger than (a). The immunoelectrophoretic patterns given by the anti-BSA fowl serum with rabbit anti-fowl globulin serum, unabsorbed and absorbed with each of the precipitates, are shown in Fig. 9. Of the two bands seen in the γ-globulin position with unabsorbed antiserum, the inner one is removed completely and the outer one weakened by absorption with (a) and more so with (e). Absorption with (c) weakened both bands. No fast component other than serum albumin was seen either with the anti-fowl-globulin or with the anti-cock-serum.

## DISCUSSION

Fowl antiserum to a single antigen has been shown to contain two types of antibody. They differ from one another in specificity against rabbit-anti-fowl-globulin, in molecular weight and in the type of complex they form with antigen, but possess the same electrophoretic mobility. They will be referred to as 'heavy' and 'light' antibody in this discussion. Both antibodies are precipitated specifically in 0.9 per cent NaCl. At the higher salt concentrations, viz. 8 per cent NaCl, more of the light antibody is added to the specific precipitate together with an unidentified substance possessing a higher electrophoretic mobility and diffusion constant than serum albumin. This additional precipitation of light

antibody at the higher salt concentration occurs only when sufficient antigen is present, since precipitation curves at different salt concentrations coincide in the antibody excess region (Goodman, Wolfe and Norton, 1951). It can be interpreted as the salting out of soluble antigen-antibody complex and, as such, also occurs with some horse antitoxins (Raynaud and Relyveld, 1959; Pope, 1955). Conversely, when a precipitate formed in 8 per cent in the region of maximum precipitation* is extracted with a dilute salt solution, some, but not all, of the light antibody (combined with antigen) is brought back into solution; the unidentified third component also appears in such solutions.

A recent study of soluble BSA-fowl-anti-BSA complexes by free boundary electrophoresis (Banovitz, Singer and Wolfe, 1959) also showed the presence of two distinct soluble complexes in BSA excess, which the authors attributed to the existence of two types of antibody in fowl serum. These authors also found that the molecular ratio of antigen to antibody in these complexes was never above 0.9, in contrast to a limiting ratio of 2 for similar complexes made with rabbit antibody. They concluded that this was due to the weaker binding of BSA by fowl antibody and resulting dissociation, rather than to the presence of univalent antibody. This conclusion was based in part on their finding that 'the antibody in the slower of the two complex peaks is preferentially precipitated at 1 per cent NaCl whereas that in the faster peak is concentrated in the 8 per cent NaCl fraction'. We found on the contrary, that the slower peak contained only the light antibody and that precipitates formed in 1 per cent NaCl contained both types of antibody, thus satisfying the postulate of Banovitz *et al.* that bivalent (heavy) antibody should (a) be more readily precipitable and (b) bind more BSA and hence form complexes with the higher electrophoretic mobility. Immunoelectrophoresis made it possible to demonstrate that both antigen and antibody were present in both the fast and slow complexes (Figs. 6 and 7), showing that any dissociation, if it had occurred, was not complete. This discrepancy in the experimental results may be caused by the fact that Banovitz *et al.* used about three times more antigen for precipitation in 8 per cent NaCl than in 1 per cent — calling both concentrations 'slight antigen excess'. As they found it necessary to add more antigen to produce precipitation when raising the salt concentration from 1 to 8 per cent, it seems likely that their initial precipitation was in antibody excess.

Evidence for the univalence of the light antibody is provided by diffusion and sedimentation data found with the complexes and the molecular weights of complexes deduced from these. The validity of this conclusion depends on there being no dissociation.

The results of the diffusion experiments using fowl antibody with homologous antigen on the one hand, and with anti-globulin on the other (Tables 2 and 3) are difficult to interpret. The antisera to BSA and RGG both contain substantial amounts of an antibody with a diffusion constant greater than 3, since 3 is the value obtained (against rabbit antibody) for the smaller soluble complex. Hence the value of $D_b = 2.1$ to 2.4 found for antibody (against BSA and RGG, Table 2) must be determined by the slowly diffusing heavy antibody; this leads to the conclusion that the light antibody which diffuses ahead is unable to precipitate with antigen in the absence of the heavy antibody, regardless of the salt concentration. This explanation, however, does not account for the value $D_b = 3.6$ found for the fowl antitoxin, despite the presence (though in much smaller amounts) of heavy antibody complex. Possibly the decreased amount of heavy antibody in the anti-ε-toxin sera

* The term 'equivalence zone', by definition that in which no antigen or antibody is detectable in the supernatant, does not seem applicable to any system in which soluble antigen-antibody complexes are formed over a great range of antigen-antibody ratios.

is accompanied by an increase in the third unknown component of soluble complexes; larger amounts of this component were found in the antitoxic sera than in the other, but its role in specific precipitation is as mysterious as its identity. McDuffie, Kabat, Allen and Williams (1958) found that rabbit antisera to human specific precipitates also contained a third antibody not directed at either the 7*S* or 20*S* components of human γ globulin.

Buxton (1952) demonstrated non-agglutinating antibodies to *Salmonella pullorum* and *S. gallinarum* by means of a modified anti-globulin (anti-livetin) test in the sera of immunized hens. These antibodies were present in large amounts in the later stages of immunization and were transferred to yolk and chick. Investigations, still incomplete, of the proportion of non-precipitating or non-agglutinating antibodies in various fowl sera indicate that the length of immunization, rather than the route, or the nature of the antigen, is the principal factor.

Recent investigations of the specificity of the precipitation reaction of fowl antisera in 8 per cent NaCl (Banovitz and Wolfe, 1959; Goodman and Ramsey, 1957), have shown that α globulin is not involved, and that specific precipitates consist mainly of antibody and antigen. Goodman and Ramsey (1957), however, mention that some non-specific precipitation occurs in 1 per cent NaCl. This is confirmed by the difficulty encountered by Banovitz *et al.* (1959) and by ourselves in completely redissolving specific precipitates formed in 0.9 per cent NaCl with excess antigen. The use of agar made in 0.9 per cent NaCl in Ouchterlony plates often leads to the formation of heavy precipitates around the fowl serum wells; these precipitates can be washed away with 3 per cent NaCl solutions but not with 0.9 per cent NaCl, and appear to be non-specific. The presence in specific precipitates formed in 8 per cent NaCl of material that is neither antigen nor antibody has already been discussed.

The following hypotheses are offered to account for our results and to explain the behaviour of fowl antibody in the precipitation reaction:

1. That fowl antisera contain a higher proportion of non-precipitating antibodies than mammalian antisera, and that these non-precipitating antibodies may well be univalent.

2. That in 0.9 per cent NaCl a portion of these is co-precipitated in the lattice formed by multivalent antibody with antigen, in the same manner as non-precipitating rabbit antibodies (Heidelberger and Kendall, 1935).

In a critical and quantitative study of this kind of co-precipitation (with rabbit antibody) Fiset (1956) has shown that the amount of incomplete antibody that is co-precipitated depends on the ratio of antibody to antigen, of precipitating antibody to incomplete antibody and on the molecular size of the antigen, and is strictly limited by these conditions. With BSA and anti-BSA up to 1/3 of the total antibody could be non-precipitating, whereas with the ovalbumin system the maximum was 1/5.

3. That the additional precipitate obtained in 8 per cent NaCl consists mainly of antigen-non-precipitating-antibody complexes which are soluble in 0.9 per cent NaCl, and that the binding of these to the more insoluble part of the precipitate is qualitatively different from (and weaker than) that occurring normally between antigen and antibody. This extra precipitation may be due not only to a salting-out effect but also to the intervention of another serum component. Weigle and Maurer (1957) attributed the precipitation of soluble rabbit antibody-antigen complexes by fresh guinea-pig and human serum, to the interaction between the complexes and the $C_1$ component of complement.

Experiments now in progress on the Danysz effect in fowl antisera and on quantitative precipitation with labelled antigen should test the validity of these tentative hypotheses.

Another study of the precipitation reaction of fowl anti-serum to BSA has appeared since this work was completed (Makinodan, Gengozian and Canning, 1960). Some similar techniques were used, but led to some different results and to different conclusions. Increased precipitation in 1.5 M NaCl (8 per cent) was attributed to the co-precipitation of a normal serum macroglobulin ($S_{20,w} = 21$) which was found to be present in soluble complexes (prepared by redissolving washed specific precipitates in excess BSA) regardless of the conditions in which the specific precipitates were made; the one relevant exception being specific precipitates made from fresh serum in 0.15 M NaCl (0.9 per cent). In our work, out of twelve soluble complex preparations analysed in the ultracentrifuge, only two contained a 21*S* component and both of these were prepared from anti-BSA precipitates formed in 0.9 per cent NaCl and redissolved in excess BSA. The component responsible for forming the 21*S* peak may well be a normal serum macroglobulin, but since it did not appear consistently in our soluble complex preparations and has not been reported in other ultracentrifugal studies of fowl sera (Nichol and Deutsch, 1948) it may equally well be a larger antigen-antibody complex.

The electrophoretic mobility of the macroglobulin postulated by Makinodan *et al.* and its reaction with rabbit anti-chick plasma seem very similar to that of our heavy fowl antibody. As Makinodan *et al.* have not demonstrated that the component forming the 21*S* peak, seen in the ultacentrifuge pattern is the same as the electrophoretic component called macroglobulin are the same thing, it is possible that the latter is in fact the heavy antibody.

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