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Fowl Antibody: II. The Composition of Specific Precipitates Formed by Antisera to Serum Albumin, Haemoglobin and Myoglobin and some Properties of Non-Precipitating Antibody

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Summary. Specific precipitates formed in 0.9 per cent and 8 per cent NaCl and the precipitates formed by raising the salt concentration of '0.9 per cent' supernatants to 8 per cent were measured quantitatively. With antisera to haemoglobin and myoglobin the antigen in the precipitates was also measured. Except for some very high antibody/antigen ratios found in some cases in antibody excess, these ratios were the same as those found with rabbit antibody, and did not depend on salt concentration.

Non-precipitating antibody, prepared by serial absorption of antiserum with small portions of antigen, did not precipitate with antigen even in 8 per cent NaCl; it co-precipitated with homologous rabbit antiserum and delayed its flocculation, but produced no permanent inhibition.

Rabbit antiserum to washed specific precipitates made from fowl antisera was used to confirm the presence of two globulins, one a macroglobulin, in the precipitates, and to study their different properties when free in whole serum and when combined with antigen.

INTRODUCTION

Despite general agreement (e.g. Goodman, Wolfe and Norton, 1951; Makinodan, Gengozian and Canning, 1960) that the precipitation of fowl antibody by antigen is greater at salt concentrations of the order of 1.5 M (i.e. 8 per cent NaCl) than at the usual 0.15 M (0.85 per cent NaCl), there is as yet no explanation of this phenomenon. Makinodan *et al.* suggest that the increase is due to the co-precipitation of non-specific serum protein of macroglobulin character; earlier workers (Deutsch, Nichol and Cohn, 1949) thought that 'α globulin' was carried down at the higher salt concentrations. Banovitz, Singer and Wolfe (1959) considered that any non-specific precipitation that might occur was much too small to account for the increase in precipitation in 8 per cent NaCl, and found that fowl antisera to BSA contained antibodies that formed two electrophoretically different types of soluble complexes with excess antigen. Evidence for the presence of two kinds of antibody to a single antigen in fowl antisera was also found by Orlans, Rose and Marrack (1961) who showed that the two antibodies were derived from two different serum proteins, had different molecular weights, combined with different amounts of antigen, but were both precipitated (though to different degrees) by antigen in 0.9 per cent and 8 per cent NaCl.

The experiments described in this paper were intended to provide some more data on the composition of specific precipitates formed under various conditions by fowl antisera and homologous antigen.

MATERIALS AND METHODS

ANTIGENS. Crystallized bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co. Crystalline horse oxyhaemoglobin was prepared according to Keilin and Hartree (1935). Antarctic seal metmyoglobin (amorphous), obtained from Professor J. R. Marrack, was given to him by Dr. Kendrew.

Specific precipitates for the preparation of 'anti-floccule' sera were made by adding BSA to fowl antiserum (pool 496, see below) in 0.9 per cent NaCl. All the precipitates were made in the antibody excess zone and washed eight times in large volumes of 0.9 per cent NaCl.

ANTISERA. Fowl antisera were made in White Leghorns, Breed 505. Antisera to BSA were obtained by giving either one or two intravenous injections of 40 mg. antigen/kg. body weight, and bleeding 8 days after the last injection.

Antisera to haemoglobin and myoglobin could not be made by this method. After an initial intravenous injection, as above, the birds were given three to four intramuscular injections of 40 mg./kg. body weight on alternate days and bled 1 week later.

Rabbit antiserum to BSA was obtained after two intramuscular injections of alum-precipitated BSA.

Antisera to specific BSA-fowl-anti-BSA precipitates were made in two rabbits, on two occasions, with single injections of precipitate resuspended in saline and mixed with three parts of Freund's adjuvant (Difco). The four antisera differed only slightly from one another and are labelled R_1 or R_2 bleed 1 or 2.

Rabbit antiserum to seal metmyoglobin was provided by Professor J. R. Marrack.

All the fowl antisera used were pools from two or three birds and had, with one exception, been kept for at least 6 weeks, with repeated freezing, thawing and centrifugation of any insoluble material that formed, and are considered 'aged'. The one exception, 'fresh' anti-BSA pool 911, was less than 1 week old and had been kept frozen until used.

Before being pooled all antisera from individual birds were tested against several concentrations of homologous antigen in Ouchterlony plates. Antisera that gave more than one band (such as sometimes occurred with myoglobin) were not used for the quantitative precipitation work.

Precipitation in agar, Ouchterlony and L plates, immunoelectrophoresis, and measurement of diffusion coefficients were performed as before (Orlans *et al.*, 1961). Staining for haemoglobin was with o-dianisidine (Owen, Silberman and Got, 1958).

Specific precipitates were made from either 0.5 or 1.0 ml. of serum in total volumes of 1.5 or 2.0 ml. Antigens were added as concentrated solutions with an Agla syringe; volumes and salt concentrations were adjusted with NaCl solutions of suitable concentration. Unless otherwise stated, the mixtures were incubated for 3 hours at 37° in a water bath, centrifuged at room temperature and washed twice with 3 ml. of either 0.9 per cent or 8 per cent NaCl solution. The supernatant fluids obtained after precipitation in 0.9 per cent NaCl were carefully transferred to clean tubes and the salt concentration brought to 8 per cent with suitable amounts of 30 per cent NaCl. The resulting precipitates were treated in the same way as the original precipitates.

Protein nitrogen was measured by the Markham modification of the Kjeldahl method. Haemoglobin and myoglobin in specific precipitates were measured by dissolving the washed precipitates in 4.0 ml. of 0.3 N NaOH, adding $Na_2S_2O_4$ and measuring the absorption of the resulting haemochromogen at 424 mμ. for myoglobin and 423 mμ. for haemoglobin, in the Unicam SP 500 spectrophotometer using 1 cm. cells. Several standard solutions at different concentrations, also dissolved in 0.3 N NaOH but in the presence of BSA (to ensure excess denatured protein for haemochromogen formation), were read at the same time and checked against the original calibration curves of such solutions, based in turn both on protein N, and on haem values determined by the cyanide method of Drabkin (1945). With the haemoglobin preparation these two values agreed; with the myoglobin preparation the protein N value was 5 per cent higher than the haem value, which was taken as the correct one.

Non-precipitating fowl antibody was prepared from anti-BSA pool 496 by five additions of small and decreasing portions of antigen. The first three of these were made in 0.9 per cent NaCl; the third gave almost no precipitate until the salt concentration was raised to 8 per cent. The last two precipitations were in 8 per cent NaCl; further additions of BSA to small samples of the absorbed serum gave no more precipitation in 8 per cent NaCl, even after prolonged storage at 4°. The absorbed serum was then dialysed against 0.9 per cent NaCl solution.

Inhibition. The effect of this absorbed fowl anti-BSA serum on the flocculation rate of rabbit anti-BSA serum was examined by adding increasing amounts of the fowl serum to series of tubes containing constant amounts of rabbit antiserum and different amounts of antigen, chosen to include the flocculation optimum. The total volume was kept constant, and hence also the concentration of rabbit antibody.

Co-precipitation. The effect of the absorbed fowl anti-BSA serum on the amount of precipitate given by rabbit anti-BSA and antigen was measured by comparing precipitation curves obtained (*a*) with 0.3 ml. of rabbit serum and saline and (*b*) with the same amount of rabbit serum plus 0.3 ml. and 0.6 ml. amounts of non-precipitating fowl serum, all in the same total volume. The order of mixing was (1) antigen, (2) saline, (3) fowl serum, (4) rabbit serum. The tubes were kept 2–3 hours at room temperature and overnight at 4°.

RESULTS

FOWL ANTI-BSA SERA

Precipitation curves obtained in 0.9 per cent and 8 per cent NaCl with two different pools of sera (one was used both fresh and aged, the other only aged) are shown in Figs. 1a, 1b and 2. Also shown are the additional precipitates obtained by raising the salt concentrations of the supernatants of the low salt precipitates, together with the sums of these additional precipitates and of the low salt precipitates. Except in the ascending parts of the curves, these combined precipitates were consistently smaller than those obtained in 8 per cent NaCl, this was also found with the other antigen/fowl-antibody systems used. Losses of serum in the transfer would account at most for a 4 per cent difference, whereas the difference found was generally 10 per cent or more.

The changes found with ageing were small: a decrease of 0.2 mg. in maximum precipitation both in low and high salt, representing 17 per cent and 8 per cent decreases respectively and hence more noticeable in low salt. The precipitation peaks occurred at slightly lower antigen levels with the aged serum.

There are striking differences between the two anti-BSA serum pools, obtained by exactly the same immunizing procedure in the same breed of fowl and both with high antibody levels. With pool 496, the precipitate formed in 0.9 per cent NaCl was 72 per cent of that formed in 8 per cent NaCl, whereas with pool 911 the proportion was 48 per cent.

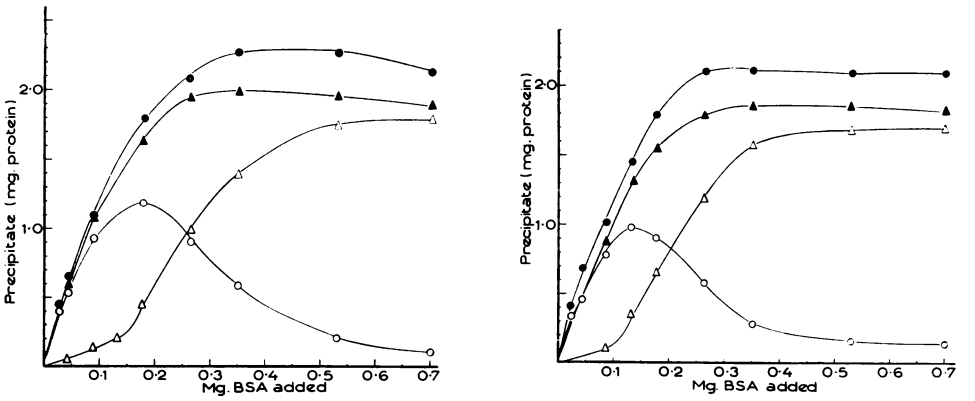

FIG. 1. Precipitation curves of anti-BSA fowl serum 911 fresh (a) and aged (b).
●——● Precipitate formed in 8 per cent NaCl.
○——○ Precipitate formed in 0.9 per cent NaCl.
△——△ Precipitate formed from supernatants of ○——○ raised to 8 per cent NaCl.
▲——▲ Sum of ○——○ and △——△.

With pool 496 the amount of precipitate formed at the two salt concentrations was the same almost to the point of maximum precipitation in 0.9 per cent NaCl; the highest ratios of precipitate to antigen were found at the lower salt concentration in antibody excess. With the other pool, precipitates were slightly larger in high salt even in the

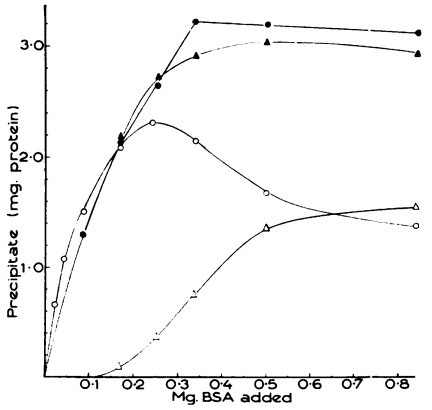

FIG. 2. Precipitation curves of anti-BSA fowl serum 496 after ageing only. Symbols as for Fig. 1.

ascending part of the curve. Antibody/antigen ratios in the antibody excess region (the only region in which antigen precipitation can be assumed to be complete) were much higher with pool 496, reaching a maximum of 30, compared with one of 18. Ab/Ag ratios

calculated at comparable antigen levels, namely at one-fourth of the optimum in each case, were 20 and 15 respectively. The highest ratio found for BSA and rabbit antibody in great antibody excess was 17.

The supernatants from precipitates formed in 8 per cent NaCl were tested for antigen in Ouchterlony plates with dilutions of rabbit anti-BSA. Antigen was found as soon as the plateau part of the precipitation curve was reached; but whether all or part of this antigen was free or combined with antibody in soluble complexes is not known. No antigen was found in the high salt supernatant before the inhibition zone of the low salt curve. Incomplete precipitation of antigen in 0.9 per cent NaCl was demonstrated more sensitively by adding salt to the supernatants.

FOWL ANTI-HAEMOGLOBIN SERA

Precipitation curves in 0.9 per cent and 8 per cent saline together with the antigen found in the precipitates are shown in Fig. 3. The additional precipitates formed by adding salt to the 0.9 per cent NaCl supernatants are also shown; these increase linearly with the

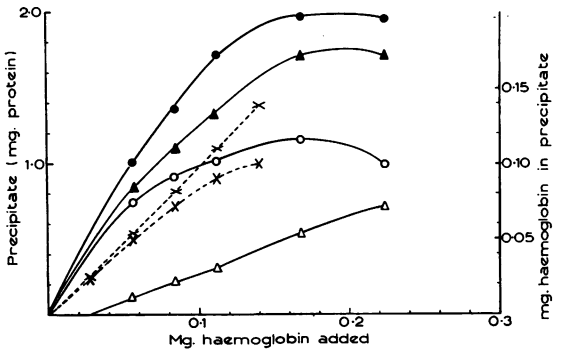

FIG. 3. Precipitation curves of anti-horse-haemoglobin fowl serum.
●——● Precipitate formed in 8 per cent NaCl.
○——○ Precipitate formed in 0.9 per cent NaCl.
△——△ Precipitate formed from supernatants of ○——○ raised to 8 per cent NaCl.
▲——▲ Sum of ○——○ and △——△.
×---× Mg. haemoglobin found in precipitate formed in 8 per cent NaCl.
X---X Mg. haemoglobin found in precipitate formed in 0.9 per cent NaCl.

antigen concentration, unlike the BSA precipitates in Figs. 1 and 2 which were sigmoidal. Precipitation of antigen in high salt was complete at all the levels measured, and incomplete in low salt.

Unfortunately these data cannot be used for antibody/antigen ratio calculations because washed and redissolved specific precipitates were found to contain a component that was neither antigen nor antibody. The band given by this extra constituent could be stained with o-dianisidine, showing that it contained haemoglobin; in immunoelectrophoresis (Fig. 4) it was in the position usually shown for haemoglobin-haptoglobin complex (human).

FOWL ANTI-MYOGLOBIN SERA

Because of the surprising results obtained with the first anti-myoglobin pool (991) another batch of sera was prepared and the strongest of these (924) investigated in the

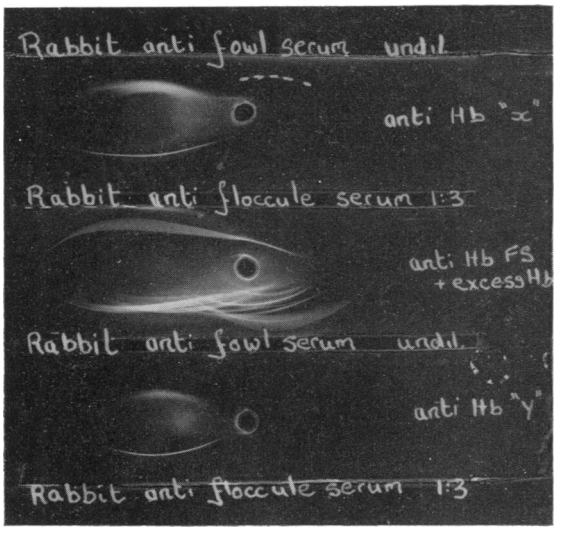

FIG. 4. Immuno-electrophoresis of fowl antiserum to haemoglobin with excess Hb; also of specific precipitates redissolved in antigen excess; anti-Hb 'x' and 'y' were specific precipitates made in 8 per cent and 0.9 per cent NaCl respectively. The dotted line shows the position of the haptoglobin band which was too faint for photographic reproduction.

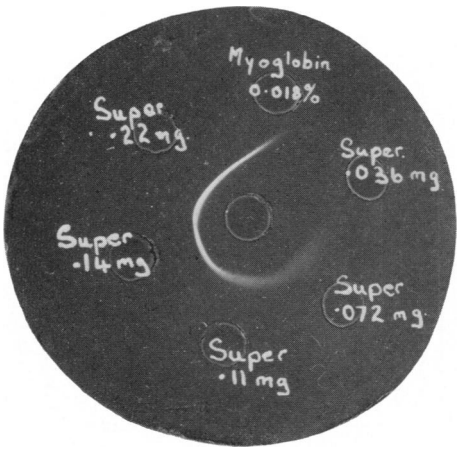

FIG. 5. Supernatants of precipitates formed in 8 per cent NaCl with seal metmyoglobin and fowl antiserum (corresponding to the curve in Fig. 6) tested for excess antigen with rabbit antiserum (dil. 1 : 3) to the same antigen in centre well.

same way as the first. Serum 924 contained three times as much antibody as pool 991, when estimations were done in 8 per cent NaCl.

When antigen was added to pool 991 in 0.9 per cent NaCl, flocculation occurred quite rapidly (4 minutes at the optimum which was 0.144 mg. myoglobin/ml. undiluted serum) in all tubes. After 2 hours at 37° these precipitates had disappeared almost completely. When the experiment was repeated and the precipitates watched continuously the beginning of re-solution became apparent after 25 minutes at 37°. Raising the salt concentration to 8 per cent produced immediate re-precipitation at all antigen concentrations. Table 1 shows these effects quantitatively at two antigen concentrations, chosen as being the flocculation optimum and antibody excess; they are in fact both in antigen excess (Fig. 5) if the criterion is the presence of antigen detectable with rabbit anti-myoglobin

TABLE 1

THE EFFECT OF INCUBATION TIME AND SALT CONCENTRATION ON THE AMOUNT AND COMPOSITION OF SPECIFIC PRECIPITATES FORMED BY 1 ML. OF FOWL ANTI-MYOGLOBIN SERUM 991 WITH ANTIGEN

Myoglobin added (mg.)	*NaCl conc. %*	*Incubation times*	*Precipitate (mg.)*	*Myoglobin in precipitate (mg.)*	*Precipitate from supernatant +NaCl (mg.)*	*Myoglobin in precipitate from supernatant +NaCl (mg.)*	*Total precipitate (mg.)*
0.072	0.9	20 min.	0.46	Not measured	0.80	Not measured	1.26
0.072	0.9	180 min.	0.09	Not measured	Not measured	Not measured	
0.072	0.9	180 min.	Precipitate not removed	Not measured	1.18	Not measured	1.18
0.072	8.0	20 min.	1.41	Not measured	—	—	—
0.072	8.0	180 min.	1.33	0.066	—	—	—
0.072	8.0	24 hr.	1.29	Not measured	—	—	—
0.144	0.9	20 min.	0.41	0.026	0.95	0.058	1.36
0.144	0.9	180 min.	0.09	Not measured	Not measured	Not measured	
0.144	0.9	180 min.	Precipitate not removed	Not measured	1.28	Not measured	1.28
0.144	8.0	20 min.	1.42	0.076	—	—	—
0.144	8.0	180 min.	1.37	0.086	—	—	—
0.144	8.0	24 hr.	1.30	Not measured	—	—	—

serum, in the supernatant, after precipitation in 8 per cent NaCl. Whereas in 0.9 per cent NaCl the disappearance of the specific precipitate was dramatic and almost complete, there was only a very slight decrease (similar to that found by Wolfe, Mueller and Neess, 1959) when 8 per cent NaCl was used. Total precipitates obtained by raising the salt concentration to 8 per cent either after re-solution or removal of the precipitates were also slightly smaller than those obtained by adding antigen in 8 per cent NaCl and incubating for 20 minutes. Judged by the few tubes in which myoglobin was also measured, antigen/antibody ratios were not affected by salt concentration or incubation time.

The precipitation curve obtained with pool 991 in 8 per cent NaCl is shown in Fig. 6; those obtained with the strong serum (924) both in 0.9 and 8 per cent NaCl in Fig. 7.

Specific precipitates formed with serum 924 in low salt did not redissolve; hence all antigen/antiserum mixtures were incubated for 3 hours at 37°. With this serum the largest precipitate formed in 0.9 per cent NaCl was only 33 per cent of the largest formed in 8 per cent NaCl. The flocculation optimum in low salt corresponded to the peak of the precipitation curve. With both sera, in 8 per cent NaCl, precipitation of antigen became incomplete and antigen detectable in the supernatant with rabbit anti-myoglobin serum, before the peak of the curve had been reached. Antibody/antigen ratios calculated at the experimental points of the high salt curves in Figs. 6 and 7 are shown in Table 2. Myoglobin was also measured in the precipitate obtained in 0.9 per cent NaCl with 0.085 mg.

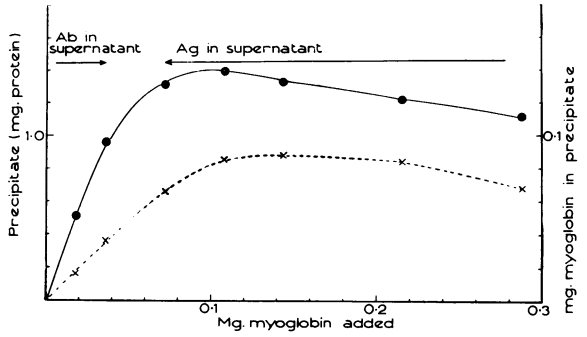

FIG. 6

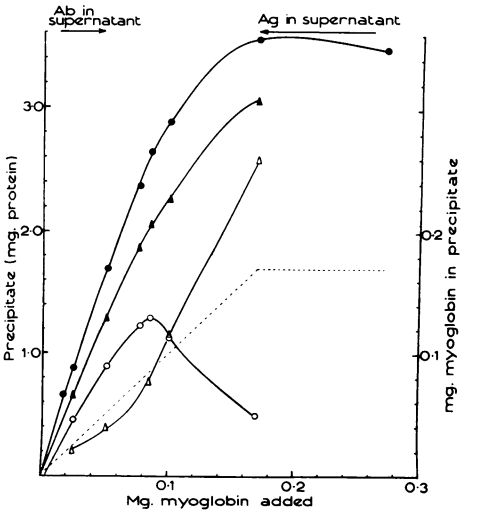

FIG. 7

FIG. 6. Precipitation curve of fowl serum 991 with seal metmyoglobin, in 8 per cent NaCl, using 1.0 ml. of serum. Crosses show amounts of antigen in precipitates.

FIG. 7. Precipitation curve of fowl serum 924 with seal metmyoglobin, using 0.8 ml. of antiserum.
●——● Precipitate formed in 8 per cent NaCl.
○——○ Precipitate formed in 0.9 per cent NaCl.
△——△ Precipitate formed from supernatants of ○——○ raised to 8 per cent NaCl.
▲——▲ Sum of ○——○ and △——△.
- - - - - Mg. myoglobin in precipitate.

of antigen (the peak of the low salt curve, Fig. 7) and in the precipitate formed by raising the salt concentration of the corresponding supernatant; Ab/Ag ratios in these two precipitates were 22 and 23 respectively. Ratios at the peaks of the high salt curves were also about 20 with both antisera (Table 2). The limiting ratio for precipitates formed in antigen excess was 15. In antibody excess the highest value found was 38. With rabbit antibody to the same myoglobin, the limiting value in antibody excess was 30, corresponding to a molecular ratio Ab_3Ag. Because of the small size of the myoglobin molecule a higher ratio than this seemed unlikely, unless the myoglobin itself had become aggregated. Estimation of the diffusion coefficient of the myoglobin (Fig. 8) gave a value of 10.5, showing that it was not aggregated.

TABLE 2

RATIOS OF ANTIBODY TO ANTIGEN IN SPECIFIC PRECIPITATES FORMED IN 8 PER CENT NaCl BY FOWL ANTI-MYOGLOBIN SERA 991 AND 924

Antibody/antigen ratios of precipitates formed in 8 per cent NaCl with anti-myoglobin sera 991 and 924

Serum 991	*Myoglobin added (mg.)*	0.018	0.036	0.072	0.11	0.14	0.22	0.29
	Ab/Ag	28	26	19	16	15	15	15
	Maximum precipitation ↑						↑ Flocculation optimum	
Serum 924	*Myoglobin added (ug.)*	0.017	0.025	0.051	0.077	0.085	0.10	0.17	0·27
	Ab/Ag	38	34	32	30	30	28	20	19.5
	Flocculation optimum ↑							↑ Max. precipitation	

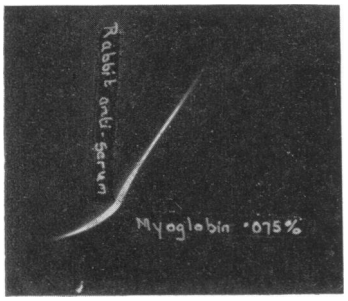

FIG. 8. Determination of diffusion coefficient of myoglobin preparation used: tan θ = 1.60.

NON-PRECIPITATING FOWL ANTI-BSA SERUM

The amount of non-precipitating antibody recovered from 1 ml. of original antiserum was estimated from the co-precipitation data below as being 1.7 mg./ml. or 30 per cent of the total antibody. Antibody precipitated by the serial absorptions was measured in a pilot experiment and found to be 3.5 mg./ml. or 60 per cent of the total. Both these estimations are subject to large experimental errors which may account for the 10 per cent discrepancy.

The effects of 0.3 and 0.6 ml. amounts of the non-precipitating fowl antiserum on the amount of precipitate given by 0.3 ml. of rabbit anti-BSA serum with different amounts of BSA, are shown in Fig. 9. In another experiment different amounts of non-precipitating fowl antiserum were tested at a single antigen level, 0.21 mg.; the amounts of protein added by 0.3, 0.5, and 0.7 ml. of the fowl serum were 0.33, 0.41 and 0.45 mg. respectively. The results in Fig. 9 show that the amounts of protein added by 0.3 and 0.6 ml. of absorbed antiserum were 0.33 and 0.43 mg. and that the point of maximum precipitation shifted from 0.175 mg. BSA (for rabbit serum by itself) to 0.235 and 0.264 mg. BSA respectively.

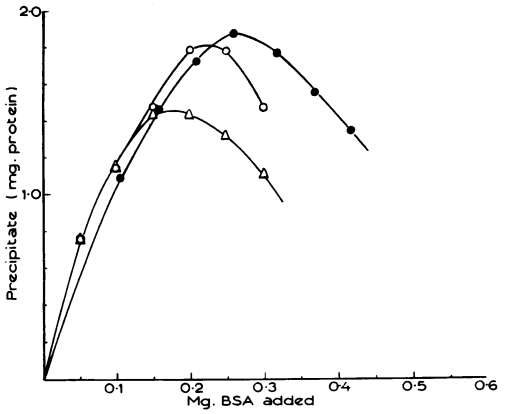

FIG. 9. Precipitation curves of rabbit antiserum to BSA by itself, and mixed with two different amounts of non-precipitating fowl anti-BSA.
△——△ 0.3 ml. rabbit antiserum alone.
○——○ 0.3 ml. rabbit antiserum +0.3 ml. fowl antiserum.
●——● 0.3 ml. rabbit antiserum +0.6 ml. fowl antiserum.

TABLE 3

THE EFFECT OF NON-PRECIPITATING FOWL ANTIBODY TO BSA ON THE FLOCCULATION TIME OF HOMOLOGOUS RABBIT ANTISERUM (RS). FS (a) AND (b) ARE TWO DIFFERENT PREPARATIONS OF NON-PRECIPITATING FOWL ANTIBODY. TOTAL VOLUMES WERE 1 ML. THROUGHOUT

	Flocculation time in minutes						*R Ab/F Ab*
BSA μg.	8.4	16.8	25.2	38.6			
RS ml. 0.05 FS ml. none	11	*4*	6	17			—
RS 0.05 FS (a) 0.2	25	*6*	13	38			1 : 0.5
RS 0.05 FS (a) 0.5	22	9	*8*	10			1 : 1.3
BSA μg.	5	10	15	20	25	30	
RS ml. 0.03 FS ml. none	20	*10*	*10*	19	23	54	—
RS 0.03 FS (a) 0.3	30	27	*26*	29	36	—	1 : 1.3
RS 0.03 FS (a) 0.6	—	27	26	*25*	29	—	1 : 2.5
RS 0.03 FS (b) 0.4	49	30	29	*28*	30	—	1 : 2.9
RS 0.03 FS (b) 0.7	50	32	29	*28*	30	31	1 : 5

These results show that co-precipitation does not increase in proportion to the amount of incomplete antibody present, but depends on the nature and the amount of the precipitating antibody. In ascending parts of the curves the precipitates obtained with rabbit serum alone and rabbit serum plus 0.3 ml. of fowl serum were exactly the same; with 0.6 ml. of fowl serum there was a slight decrease of precipitation. Increased precipitation in the presence of the fowl serum was found only in the region of maximum precipitation for the rabbit serum by itself. Zones of inhibition by antigen excess were displaced towards higher antigen levels to roughly the same extent as the precipitation maxima.

The effect of non-precipitating fowl antibody on the flocculation rate of rabbit antibody was studied with mixtures containing much higher proportions of fowl serum than those used in the quantitative work. The results are shown in Table 3. The delaying effect of the fowl antibody can be seen in all mixtures of the rabbit and fowl antisera. With the larger amounts of fowl serum, however, this effect is obscured by the higher total antibody concentration which leads to more rapid flocculation and to a broadening and flattening of the optimum. None the less three facts are clear: (1) That the flocculation optimum becomes displaced from 12.5 μg. to 20 μg. of BSA, and the flocculation time increased 2–3-fold, when the ratio of fowl to rabbit antibody is 2.5. (2) That raising this ratio to 3 and 5 has no further effect either on the position of the optimum or on the optimal flocculation time. (3) That relatively large amounts of this type of non-precipitating antibody, even in antibody excess, cannot produce any significant inhibition of precipitation. Rough estimation (optical density at 280 mμ. of precipitates dissolved in NaOH) of the precipitates formed from 0.03 ml. of rabbit serum alone and mixed with 0.7 ml. of absorbed fowl serum showed a small reduction of precipitation in antibody excess and a small increase in all the other tubes containing fowl serum.

ANTI-FLOCCULE SERA

These rabbit antisera had been made by giving rabbits the very thoroughly washed precipitates obtained from fowl anti-BSA pool 496 in 0.9 per cent NaCl in the course of preparing non-precipitating antibody. They contained some antibody to BSA, and gave two main bands when tested against whole fowl serum (Fig. 10). The band that is always nearer the fowl serum well is due to a macroglobulin component and disappears at the higher serum dilutions. With rabbit antiserum 2 the other band appears double at some fowl serum concentrations. It corresponds to the long γ-globulin band in immunoelectrophoresis (e.g. Figs. 4 and 11) and also appears complex there, because of the faint spurs coming out of the γ-globulin band. These spurs were only seen when whole serum (as opposed to redissolved specific precipitates) was used for immunoelectrophoresis and their position and character differed from serum to serum as can be seen in Fig. 12, which shows the bands given by three different anti-BSA fowl sera with anti-floccule rabbit serum and BSA. Precipitation bands formed even by strong fowl antisera with homologous antigen, after electrophoresis, were always faint and diffuse and appeared only after the plate was soaked in 8 per cent NaCl. The failure to obtain bands under these conditions was not due to the low ionic strength alone, since much stronger bands were formed when sera and antigen reacted together in the same agar, under the same conditions, but without electrophoresis.

In immunoelectrophoresis of whole fowl serum the macroglobulin component that reacts with anti-floccule serum gives a very curved band at the origin (Figs. 11 and 12).

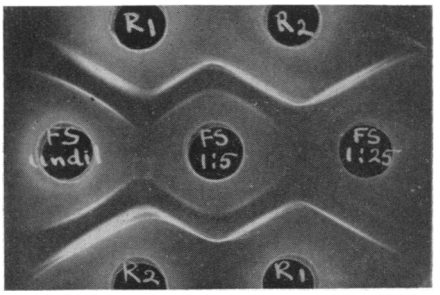

FIG. 10

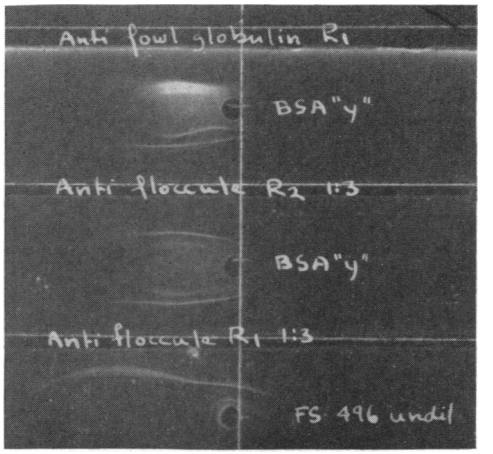

FIG. 11

FIG. 10. Reactions of anti-specific-precipitate (anti-floccule) rabbit sera R_1 and R_2 with fowl serum undiluted and diluted 1 : 5 and 1 : 25.

FIG. 11. Immunoelectrophoresis of anti-BSA fowl serum and of soluble antigen-antibody complex (BSA 'y') made from washed specific precipitate formed in 0.9 per cent NaCl and redissolved in excess BSA. The top trough contains rabbit anti-fowl globulin serum (used in Orlans *et al.*, 1961), the others rabbit anti-floccule serum.

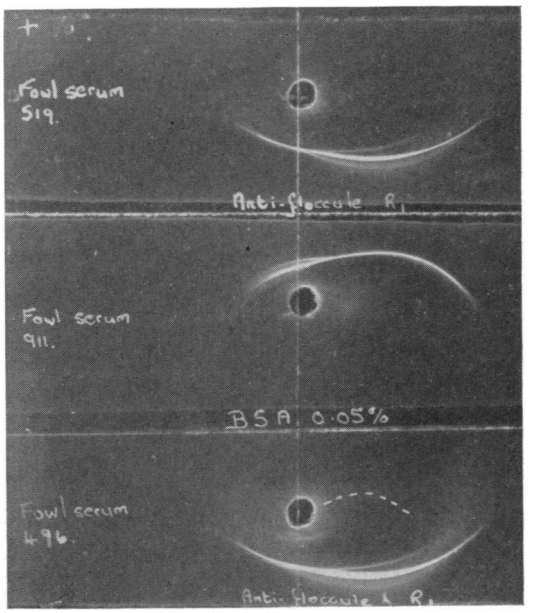

FIG. 12. Immunoelectrophoresis of three different anti-BSA fowl sera. Alternate troughs contain 0.05 per cent BSA and rabbit anti-floccule serum. The position of the very weak and diffuse band formed with BSA is shown by the broken line.

When derived from washed specific precipitate redissolved in antigen excess it migrated more slowly, regardless of whether the antigen was BSA (Fig. 11) or haemoglobin (Fig. 13).

The anti-floccule serum was also used to investigate the presence of the macroglobulin component in specific precipitates formed with different amounts of antigen and at different salt concentrations. Fig. 14 shows an example of this; the redissolved precipitates correspond to points in the precipitation curves in Fig. 1a; macroglobulin is present in precipitates formed at 'equivalence' and antigen excess both in 0.9 and 8 per cent NaCl, and to a greater extent than in the original serum; it is absent from the supernatant and from the precipitate 'w' made by raising the salt concentration of the supernatant.

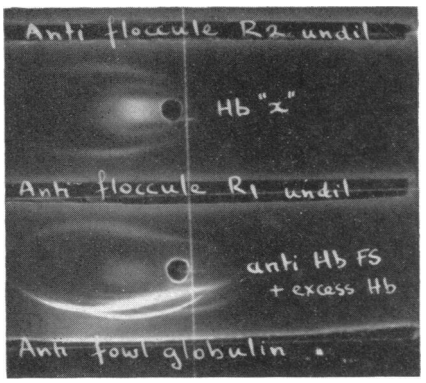

FIG. 13

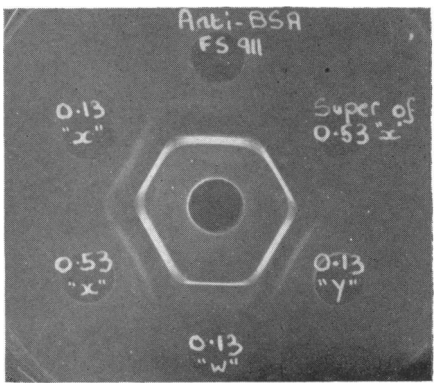

FIG. 14

FIG. 13. Immunoelectrophoresis of anti-haemoglobin fowl serum with excess antigen, and of a washed specific precipitate redissolved in antigen excess. Troughs contain undiluted anti-floccule sera R_1 and R_2 and rabbit anti-fowl serum.

FIG. 14. Reactions of rabbit anti-floccule serum with redissolved specific precipitates made from fowl antiserum 911 (see Fig. 1) using the amounts of BSA shown, with a supernatant and with the original serum; 'x', 'y' and 'w' were derived from precipitates made in 8 per cent NaCl, in 0.9 per cent NaCl and from a supernatant brought to 8 per cent NaCl, respectively.

The diffusion coefficients of the two components of fowl serum that react with anti-floccule serum were measured in L plates. The rabbit antiserum was used undiluted, with fowl serum diluted 1 : 20 or 1 : 25 to give a straight γ-globulin band of precipitate and undiluted for the macroglobulin band. The values found for D_{20} were 3.60 and 1.95 respectively. Values of D_{20} for these serum constituents combined with excess BSA in redissolved specific precipitates were 3.10 and 1.43 respectively (Orlans *et al.*, 1961).

DISCUSSION

The experiments with antisera to specific floccules confirm that specific precipitates made from fowl antiserum contain in addition to antigen two immunologically distinct globulins. Both these change their electrophoretic properties and diffusion coefficients on combination with antigen. That which is present in much larger amounts in serum gives a typical long γ-globulin band in immunoelectrophoresis but because of the unusual branching, seems less homogeneous than human or rabbit γ globulin.

Precipitation bands formed by fowl antiserum, after electrophoresis, with homologous

antigen are in a position that corresponds roughly to the faster third of the γ-globulin band situated behind the origin; no band is formed with an antigen in a position corresponding to the macroglobulin band formed with anti-floccule serum. This may only be a quantitative effect; on the other hand, it may mean that the macroglobulin, once separated from the γ globulin, cannot combine with antigen, and therefore is not an antibody. Evidence is being obtained from work (to be published later) with decomplemented fowl antisera and with fractions prepared on DEAE cellulose that the macroglobulin behaves in many ways like a component of complement. This suggestion has also been made by Makinodan *et al.* (1960).

Experiments with non-precipitating fowl antibody prepared by serial absorption with antigen from a precipitating anti-BSA serum showed that raising the salt concentration to 8 per cent did not suffice to precipitate soluble complexes of fowl antibody (of this type) and BSA and, therefore, that increased precipitation in 8 per cent NaCl is not due to a simple salting-out process. The behaviour of these non-precipitating antibodies in the presence of precipitating rabbit antibody and homologous antigen is exactly the same as that of non-precipitating rabbit antibody prepared in the same manner (Fiset, 1956).

The quantitative precipitation curves obtained with fowl antisera display disconcerting differences in behaviour between individual sera or serum pools to the same antigen. These differences may reflect the different proportions of precipitating and non-precipitating antibodies in various sera, but it is important to remember that, although all the sera but one were old and had been frozen and thawed repeatedly, they had not been decomplemented. Little is known of the role of fowl complement in precipitation. Gengozian and Wolfe (1957) found that the addition of fresh normal serum and of normal serum that had been kept frozen increased specific precipitates formed by aged antiserum in 0.9 per cent NaCl; also that EDTA reduced precipitation in 0.9 per cent NaCl but not in 8 per cent NaCl; and that heating had no appreciable effect except in occasionally increasing precipitation. Aitken and Mulligan (1962), who kindly showed me their manuscript before publication, found that EDTA decreased specific precipitation also in 8 per cent NaCl. In the absence of any method for measuring even haemolytic fowl complement it is impossible to say whether specific precipitates, including those made in the presence of EDTA, are free of protein material contributed by complement.

The amount of specific precipitate obtained in 0.9 per cent NaCl as a proportion of that formed in 8 per cent NaCl ranged from almost none with one of the anti-myoglobin sera to over 70 per cent with one anti-BSA serum. With fowl antisera to human γ globulin (Goodman *et al.*, 1957) and to rabbit γ globulin (Orlans *et al.*, 1961) the proportions were 55 per cent and 40 per cent respectively, so that there seems to be no correlation between these values and the molecular sizes of the antigen. One is tempted to attribute these variations to the presence of incomplete antibodies in varying amounts; but here again non-specific factors are also involved; removal of complement from an aged antiserum by specific methods (Rose and Orlans, to be published) decreased specific precipitation in 0.9 per cent NaCl but not in 8 per cent; the subsequent addition of fresh normal serum increased precipitation in 8 per cent NaCl slightly; greatly increased precipitation in 0.9 per cent NaCl; and reduced the precipitate obtained by raising the salt concentration of the supernatant. Thus fresh normal serum not only added complement protein to the specific precipitate, but also led to more complete precipitation of antibody in 0.9 per cent NaCl. Precipitation of soluble antigen-antibody complexes by complement has been demonstrated by Weigle and Maurer (1957).

The re-solution of specific precipitates formed in 0.9 per cent NaCl by myoglobin and a fowl antiserum can only be explained by the presence of large amounts of incomplete antibody which compete for antigen. The reappearance of the precipitates on the addition of salt shows that there has been no destruction of antigen or antibody by enzyme action. It is difficult to see, however, why a precipitate should have formed in the first place; or why incomplete fowl anti-BSA could not inhibit the precipitation of rabbit anti-BSA. It is possible that the small size of the myoglobin molecule, with fewer antigenic determinants than BSA, makes such inhibition easier to demonstrate. The unusual position of the flocculation optimum of this serum, in much greater antigen excess than the point of maximum precipitation (Table 3), also lends some support to the explanation given.

The quantitative precipitation curves provide little evidence for the presence or function of a macroglobulin constituent (whether antibody or non-specific) in the specific precipitates. Unusually high values for Ab/Ag were found in antibody excess both with antiserum to BSA and to myoglobin, and did not seem to depend on salt concentration. Abnormally high values were also found by Aitken and Mulligan (1962) but in antigen excess, and only in 1 per cent NaCl. Antibody/antigen ratios at the peaks of anti-BSA curves were the same, i.e. 9 to 11, regardless of salt concentration, as already found by Goodman *et al.* (1951), and no different from those obtained with rabbit anti-BSA. With the myoglobin system, too, Ab/Ag ratios were the same as for rabbit and would correspond to Ab_2Ag at the peak, and Ab_3Ag_2 in antigen excess.

A firm conclusion that can be drawn from the precipitation curves, including those obtained with haemoglobin, and also from the data of Aitken and Mulligan (1962), is that incomplete precipitation of antibody at low salt concentrations is always accompanied by incomplete precipitation of antigen; that the composition of the soluble complexes, that must be formed, is not sufficiently different to alter the composition of precipitates formed in 8 per cent NaCl to a degree detectable by the methods used.

As increased precipitation by fowl antisera at high salt concentrations is due neither to a simple salting-out process of soluble complexes, nor to increased precipitation of a macroglobulin (as suggested by Makinodan *et al.*, 1960) the explanation must be sought in a more complicated interaction either between two types of antibody, or between antibody and a non-specific constituent of serum.

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