

THE FORMATION OF PRECIPITATES ON MIXING ANTI-HORSE SERA

By G. R. E. NAYLOR

Department of Pathology, University of Cambridge

INTRODUCTION

It has been known for many years that a precipitate occasionally appears when two homologous antisera are mixed. If two rabbits be immunized with horse serum, by a similar series of injections, the antisera so produced will precipitate when mixed with suitable dilutions of horse serum, and a mixture of the two antisera will also occasionally develop a precipitate. Several possible suggestions have been put forward to account for this 'mutual' precipitation of antisera. Some workers think that one or both of the rabbit anti-horse sera contain remnants of horse serum, and that for some reason homologous antigen and antibody may co-exist side by side in the same antiserum without undergoing precipitation, whereas others think that horse serum is a mixture of antigens and that an anti-horse serum may contain some of the antigenic components of the injected horse serum together with antibodies for other antigens of horse serum, but not homologous antigen and antibody. Consequently such an anti-horse serum will give a precipitate on mixing with horse serum, and a precipitate will also result when this antiserum containing antigenic components of horse serum is mixed with another anti-horse serum containing antibodies for these particular antigens.

The experiments which help to elucidate this problem may be grouped into two categories. In the first category there is a series of experiments designed to investigate the equilibrium of precipitation reactions. In these experiments precipitinogen and precipitin have been mixed in various proportions and, after the formation of a precipitate, the supernatant fluid has been examined for the presence of antigen by adding antiserum and for the presence of antibody by adding antigen. The second category of experiments is concerned with searches for antigen in the blood of animals which have received injections of an antigen. In this case an animal, which has previously been immunized with an antigen, receives an injection of the same antigen and, at varying intervals following the injection, samples of blood are withdrawn and the sera tested for precipitins by mixing with antigen, and for antigen by mixing with the serum of the same animal obtained before the test injection or with the serum of another animal similarly immunized.

Linossier & Lemoine (1902) investigated the equilibrium of precipitation reactions using various animal sera (horse, human and bovine) as antigens, and concluded that when antigen and antiserum were mixed in various proportions there were three zones. At one end of the scale the supernatant fluid contained antigen, at the other end antibody and, in a broad intermediate zone, both antigen and antibody could be demonstrated in the supernatant fluid. They explained their results on a chemical basis, postulating that for complete pre-

precipitation of one reagent, the other must be added in great excess. Eisenberg (1902) obtained similar results and also explained the phenomenon on the basis of an equilibrium between the two components of the system, some of both reagents remaining in the supernatant fluid, so that the further addition of either reagent would lead to a disturbance of the equilibrium and a renewal of the reaction.

Von Dungern (1903), using various invertebrate bloods as antigens, found that he could seldom demonstrate the presence of both antigen and antibody in the supernatant fluid of a precipitation reaction; either alone might be present, but rarely both. However, in order to explain the results of other workers who demonstrated the simultaneous presence of antigen and antibody in the supernatant fluid, von Dungern postulated a multiplicity of antigens in the same antigenic serum. According to this view, horse serum consists of a number of antigens, and anti-horse serum contains a corresponding number of antibodies present in different proportions. Therefore, when horse serum and anti-horse serum are mixed, a number of reactions take place, the reagent present in excess being the antigen of some reactions and the antibody of others; so that on testing the supernatant fluid with horse serum and anti-horse serum, a precipitate is obtained with both, but the reagents present in the supernatant are not homologous antigen and antibody. Von Dungern (1903) was the first to search for injected antigen in the serum of immunized animals. He found antigen in the serum of immunized rabbits, which at the same time contained precipitins, and again explained this on the basis of a multiplicity of antigens. The work of Ascoli (1902) also demonstrated the multiplicity of antigens in animal sera. Uhlenhuth & Weidanz (1909) found that antigen persisted in the blood of immunized animals after the appearance of precipitins. Ionesco-Mihaesti (1911) also demonstrated the co-existence of antigen and antibody in the sera of rabbits immunized with horse serum. Gay & Rusk (1912) showed that antigen persisted in the circulation of rabbits highly immunized to horse serum for 24–48 hr. and sometimes for a much longer period, despite the large quantity of co-existing precipitin in the sera. These antisera containing antigen did not precipitate on storage or dilution and did not fix complement. Zinsser & Young (1913; Zinsser, 1923) confirmed the results of Gay & Rusk and sought to explain the co-existence of antigen and antibody in the same serum by a colloidal analogy.

These arguments were continued by Weil (1916), who showed that both antigen and antibody could be easily demonstrated in the supernatant fluid of reactions involving mixed antigens such as horse serum and egg white, and that precipitation frequently occurred on mixing antisera to these antigens. However, he never found antigen and antibody together in the supernatant fluid of reactions involving a purified antigen, crystalline egg albumin, and he never obtained a precipitate on mixing antisera to this antigen. On the other hand, Bayne-Jones (1917), working with purified antigens—edestin from hemp seed and crystalline egg-albumin—could demonstrate both antigen and antibody in the supernatant fluid of appropriate mixtures and in the same antiserum.

Opie (1923*a, b*) and Culbertson (1935) obtained results similar to those of Weil. Using purified antigens, they never found antigen and antibody in the same

serum. Culbertson also examined the sera of animals immunized against crystalline egg albumin after injection of small and large quantities of antigen. After the injection of small doses of egg albumin, the precipitin content of the serum was diminished and antigen could not be found. After large doses of egg albumin, the sera contained antigen but not antibody.

In the light of more recent work, the problem of the equilibrium of precipitation reactions rather loses its importance. The multiplicity of antigens in animal sera has been confirmed by many workers (Landsteiner, 1946), the difficulties involved in the preparation of pure proteins have been fully realized, and these facts, together with experiments on the equivalence zone (Heidelberger, 1939) and on the supernatant fluid of antigen and antibody mixed in optimal proportions (Duncan, 1932; Taylor, Adair & Adair, 1934), have explained the divergent results obtained by earlier workers. As a result of this work, it seems clear that an antigen and its antibody can be mixed in such proportions that, after the formation of a precipitate, the supernatant fluid may be shown to contain neither of the reagents or traces of both. The tests available are not usually sensitive enough to demonstrate both. If more than traces of antigen and antibody are found together in the supernatant fluid of a precipitation reaction, then a mixed system is being used. Kendall (1937) has applied this principle as a criterion of immunological homogeneity and clearly states that positive tests for both antigen and antibody are not given by the same supernatant fluid in a system containing a single antigen and its homologous antibody.

Although it has been shown that the co-existence of 'antigen' and 'antibody' in the same serum readily occurs with mixed antigens and rarely occurs with purified ones, the explanation of the phenomenon of 'mutual' precipitation of antisera on the basis of a multiplicity of antigens needs proof by demonstrating, in the sera of animals immunized with a mixed antigen, the presence of a particular protein component of that mixed antigen and the absence of its homologous antibody. In the present paper this proof has been supplied. Certain rabbit anti-horse sera have been found to contain horse serum crystalalbumin but no anti-crystalalbumin. These sera gave a precipitate on mixing with other anti-horse sera containing an anti-crystalalbumin.

INTRODUCTION TO THE EXPERIMENTAL METHOD

On the basis of the evidence available at the present time, horse serum may be regarded as consisting of a number of different proteins, each of which is antigenically distinct and capable of producing its own specific antibody on injection into an animal of another species (Landsteiner, 1946).

When rabbit anti-horse sera are titrated by the optimal proportions method of Dean & Webb (1926), multiple zones of rapid flocculation are frequently observed (Goldsworthy, 1928). Goldsworthy & Rudd (1935) investigated anti-horse sera exhibiting two zones when titrated against horse serum, and showed that one zone was due to an albumin-anti-albumin system and the other to a globulin-anti-globulin system. Naylor (1948, 1950) investigated a pool of rabbit anti-horse serum showing at least four zones, and showed that these zones were due to different

antigenic components in horse serum, but the detailed analysis of the antigens responsible for the individual zones was complicated by the fact that fractions of horse serum prepared by ammonium sulphate precipitation contained more than one antigen. It seems clear that multiple zones are due, in part at least, to the independent activity of multiple antigens and their homologous antibodies.

When anti-horse sera are titrated against preparations of horse-serum albumin and horse-serum globulin, multiple zones are also frequently observed (Taylor *et al.* 1932; Naylor, 1950), indicating that preparations of horse-serum derivatives contain a number of antigens.* Crystalbumin, a highly purified derivative of horse serum, shows two zones of reaction with the majority of anti-horse sera when titrated by the method of optimal proportions. (See experimental details later in the paper and Table 6.) One zone of reaction usually has an optimal ratio of 1:64 or 1:128 with 2.9% horse-serum crystalbumin and is referred to as the main zone of the reaction. The other zone occurs with the most concentrated solutions of crystalbumin used, i.e. 1:5 and 1:10 of a 2.9% solution. These double zones of reaction indicate that there are at least two antigens present in the crystalbumin preparation. Considerable evidence supports the view that the main zone is due to a reaction between crystalbumin and its homologous antibody, whereas the zone occurring with the high concentrations of crystalbumin represents a reaction between impurities present in the crystalbumin preparation and their homologous antibodies present in the anti-horse sera.

The evidence for these views includes the following. The difference in the optimal proportions of the two zones of reaction is probably more of a reflexion of the difference in concentration of the two antigens present in the crystalbumin preparation than a difference in the molecular weights of these antigens or in the concentrations of their homologous antibodies in the antiserum. Therefore the main zone of the reaction, occurring with far lower concentrations of the mixed antigen than the secondary zone, probably represents a reaction involving the major antigenic component of the crystalbumin preparation. Naylor (1950), working with preparations of horse-serum albumin and horse-serum crystalbumin of the same protein content and a pool of rabbit anti-horse sera, showed that

* Since the nomenclature of the proteins present in animal sera is capable of misinterpretation, definition of the terms used is necessary. In this paper the term albumin refers to a protein constituent of horse serum which, though not necessarily homogeneous, probably consists of very similar molecules and is considered to behave as a single antigen. The usual preparations of albumin are believed to consist largely of albumin but are also thought to contain other proteins which are very different from the albumin and which are present in small amounts as impurities. The term albumin does not refer to that fraction of horse serum not precipitated by half-saturation with ammonium sulphate.

The term crystalbumin refers to a protein constituent of horse serum also consisting of similar, but not necessarily identical, molecules and also considered to behave as a single antigen. The preparation of crystalbumin used in this work is thought to contain other entirely different proteins present in small amounts as impurities. Crystalbumin and albumin are regarded as similar proteins but do not necessarily embrace an identical range of molecules, as the technique used in the preparation of crystalbumin excludes less soluble proteins which may be present in albumin preparations and, from crystalline horse-serum albumin, Sørensen (1925, 1930) has prepared several fractions with similar crystal form but with different solubilities in ammonium sulphate solutions.

whereas the main zone of the reaction of each antigen with the antiserum had the same optimal proportions, the second zone, involving higher concentrations of the antigens, occurred with a much higher concentration of the crystalalbumin preparation than it did with the albumin preparation. Hence, the main zone represented the reaction of the major component in each preparation, and the percentage of impurity was much higher in the albumin preparation than in the crystalalbumin preparation, a result which was confirmed by electrophoresis. In the same paper it is also shown that absorption of the anti-horse serum in the proportions of the second or subsidiary optimum of the albumin preparation removes an antibody for an important antigenic constituent of a globulin preparation, and hence this second zone of the albumin-anti-horse serum reaction represents a reaction between a globulin and its antibody. Taylor *et al.* (1932) found that anti-crystalline-egg-albumin sera showed two zones of optimal particulation with crystalline egg albumin, one zone occurring with high concentrations of the antigen. This zone was probably due to impurities in the crystalline egg albumin reacting with the homologous antibodies in the antiserum. Hooker & Boyd (1936) used absence of a second zone as additional proof of the purity of their specimen of crystalline egg albumin.

Antisera produced by the injection of multiple antigens, such as horse serum, are frequently deficient in antibodies to one or more of the injected antigens, but react well with other antigens contained in the same mixture. Hektoen & Welker (1925) noted that injections of dog serum failed to call forth precipitins for dog pseudoglobulin and albumin. Goldsworthy & Rudd (1935) noted considerable variations in the precipitin response of rabbits to injections of horse serum, and mention antisera in which the sole antibody present was anti-albumin, and others in which it was anti-globulin. This failure of animals to produce precipitins for all the proteins in a serum is frequently shown in the results of optimal proportions titrations by the position or lack of zones of reaction. Taylor *et al.* (1932) showed that when some anti-horse sera were titrated against parallel dilutions of horse serum, horse-serum globulin and horse-serum albumin, particulation occurred in the horse-serum dilutions and in corresponding parts of both the albumin and globulin series, showing that these sera contained antibodies against both albumin and globulin. Other anti-horse sera showed a reaction with horse serum and in the corresponding part of the globulin series, but not in the corresponding part of the albumin series, thus showing that the antibody of the main zone was an anti-globulin and that the antisera probably did not contain an anti-albumin. They noted that such sera gave a faint reaction with high concentrations of the albumin preparation. Presumably this was due to the presence of globulin impurity in the albumin preparation. Goldsworthy & Rudd (1935) obtained similar results.

This evidence shows that if anti-horse sera are titrated against horse-serum albumin and a reaction occurs solely with high concentrations of the albumin preparation, it may be concluded that such antisera do not contain a precipitin for albumin but contain antibodies for the impurities. In the experimental part of this paper, such antisera are referred to as antisera which fail to show a main zone reaction with albumin. Antisera which contain an anti-albumin show two

zones in their reaction with the albumin preparation, the main zone being due to the albumin-anti-albumin system and the subsidiary zone to the impurities and their homologous antibodies. The results obtained with these two kinds of antisera are shown in Tables 3 and 6. It is clear that these two kinds of anti-horse sera are sharply divided. There was never the slightest difficulty in deciding whether an anti-horse serum did or did not show a main zone reaction with crystalbumin, as no sera were found which gave a zone of reaction between the usual positions of the two zones. The main zone, when present, always had an optimal ratio of 1:64 or 1:128 with 2.9 % crystalbumin.

In the titration of a number of rabbit anti-horse sera by the method of optimal proportions (Dean & Webb, 1926), using horse-serum crystalbumin as antigen, a number of the antisera gave no main zone precipitate. Presumably, they did not contain an antibody for crystalbumin. These antisera all gave a marked precipitate on mixing with anti-horse sera which did give a main zone precipitate with crystalbumin. Therefore it seemed likely that the 'mutual' precipitation of these anti-horse sera was due to the presence of horse-serum crystalbumin and absence of anti-crystalbumin in some antisera, and the presence of an anti-crystalbumin in others. In order to test this hypothesis experiments were performed to demonstrate the presence of horse-serum crystalbumin in antisera which did not contain an antibody for this antigen.

MATERIALS AND METHODS

Preparation and storage of sera

The anti-horse sera were obtained from rabbits which had received numerous injections of horse serum by the intraperitoneal route for 2 or 3 years, courses of injections alternating with periods of rest. Immediately before bleeding, the rabbits had received a course of injections of horse serum, each rabbit receiving 2 c.c. intraperitoneally every 4 days for five injections. The rabbits were bled 10 days after the last injection and again on the following day. The two samples of serum obtained from each rabbit were mixed. The rabbits had all been treated in the same way, except that some of them had been receiving injections of horse serum for a slightly longer period than others, but, in the main, any differences noted in these antisera in the course of the experiments were due to differences in the response of the individual rabbits and not to differences in the method of preparation of the antisera.

The horse-serum crystalbumin was prepared by the method of Hewitt (1938) and recrystallized five times. Naylor (1950) notes that horse-serum crystalbumin prepared by the method of Hewitt contains little impurity as shown by the wide separation of the two zones of reaction obtained with a pool of rabbit anti-horse sera. All the sera and fractions were stored frozen at -20°C . without preservative. The diluent used throughout the experiment was 0.85 % saline.

Performance and observation of the precipitation reaction

All precipitation reactions were performed in the same way. 0.5 c.c. of an antigen dilution and 0.5 c.c. of a dilution of the antiserum were mixed in a long narrow

tube of internal diameter 0.85 cm. and length 15 cm., one of the reagents being added from an all-glass syringe to ensure rapid mixing. The tube was then placed in a water-bath maintained at 37° C., with one-third of the fluid column immersed, thus producing convection currents in the mixture. The water-bath was fitted with glass windows both in the back and front. A strip light behind the back window gave oblique illumination of the tubes, and the mixtures were observed through the front window using a hand lens. In some reactions the time elapsing between the mixing of the antigen and the antiserum and the first appearance of discrete particles of precipitate was determined. In others the presence or absence of a precipitate after a certain time was noted. No mixture was reported as failing to develop a precipitate until it had been observed in the water-bath for at least 1 hr. after mixing, and observed again in the water-bath after standing at +2° C. overnight.

Determination of optimal proportions

In determining the optimal proportions of an antigen and an antiserum, the approximate optimal proportions were first determined by a 'rough test' (Dean & Webb, 1926) using serial doubling dilutions of the antigen. The optimal proportions were then determined more accurately by 'fine tests'. In performing fine tests, the antiserum, suitably diluted, was added to 4/5-fold serial dilutions of the antigen, the antigen dilutions being spaced about the approximate optimal antigen concentration as determined in the rough test. The optimal tube was noted and the titration repeated twice.

Absorption

Precipitin was absorbed by mixing antigen and antiserum in optimal proportions, as done by Duncan (1932) and Goldsworthy & Rudd (1935). The reagents were mixed in a centrifuge tube in optimal proportions with attention to rapid mixing. Usually, a certain amount of saline was added as a diluent, sometimes sufficient, along with the antigen added, to make the final dilution of the antiserum in the mixture 1:10 or 1:20. A rubber bung was then inserted and the mixture left at room temperature for 1 or 2 hr., being gently inverted 2 or 3 times every 5–10 min. It was then left at +2° C. overnight, and on the following morning the precipitate was packed by centrifugation. The supernatant fluid was then separated.

Preliminary titration of the rabbit anti-horse sera

The rabbit anti-horse sera used in the experiments to be described all gave a marked precipitate with horse serum. In order to clarify the experimental details, these antisera are divided into two groups—A Sera and B Sera—and the identification number of each serum is prefixed by A or B to indicate its group. The A sera failed to give a main zone reaction with crystalbumin and hence did not contain an anti-crystalbumin. The B sera gave a main zone reaction with crystalbumin and hence contained antibodies for crystalbumin. To anticipate the experimental results, the A sera contained horse-serum crystalbumin, whereas the B sera did not. Both the A sera and the B sera contained antibodies for horse-serum proteins other than crystalbumin. The reagents present in these sera are

summarized in Table 1, together with the identification numbers of the sera in each group. A precipitate formed on mixing any one of the A sera with any one of the B sera. The A sera contained horse-serum precipitinogen and horse-serum precipitin (but not homologous antigen and antibody), and in the experiments to be described were sometimes used as antigens and sometimes as antisera.

Table 1. *Rabbit anti-horse sera*

A sera	B sera
0963	0964
0983	0988
0997	0990
1102	0992
1103	0993
	0995
Contain horse-serum crystalbumin	Do not contain horse-serum crystalbumin
Do not contain anti-crystalbumin	Contain anti-crystalbumin
<hr style="width: 50%; margin: 0 auto;"/> Contain antibodies for other proteins present in horse serum	

Titration of the A sera

The details of the titration of the A sera against horse serum by the method of optimal proportions are shown in Table 2. Each serum, diluted 1:10, was added to serial doubling dilutions of horse serum from 1:10 to 1:5120. The time taken for the formation of visible particles of precipitate was determined for the optimal tube and also for a few neighbouring tubes, but not for all of the more slowly particulating tubes. In the case of two of the sera, A0963 and A0983, multiple zones were observed. Table 3 shows the results of constant antiserum titrations of these same sera using horse-serum crystalbumin as the antigen. Each serum diluted 1:10 was added to serial doubling dilutions of 2.9 % horse-serum crystalbumin diluted from 1:5 to 1:40,960. The mixtures were examined after standing 2 hr. in the water-bath and again in the water-bath after standing at +2° C. overnight. As shown in Table 3, none of these rabbit sera gave a main-zone precipitate with horse-serum crystalbumin (i.e. they failed to give a zone in the region of tubes 7-10), but they did give a precipitate with high concentrations of crystalbumin from 1:5 to 1:40 or 1:80. In each case the precipitate formed first and was most marked in the mixture containing crystalbumin 1:5, i.e. in tube 1. No precipitate was formed with any dilutions of crystalbumin above 1:80. The precipitate formed with high concentrations of crystalbumin is regarded as the result of a reaction between impurities in the horse-serum crystalbumin and their homologous antibodies in the sera.

As the A sera gave no main-zone precipitate with horse-serum crystalbumin, they were examined for the presence of non-precipitating antibodies for crystalbumin by an inhibition technique similar to that used by Pappenheimer (1940) (see Table 4). Each A serum, diluted 1:10, was added to a series of doubling dilutions of 2.9 % crystalbumin from 1:160 to 1:5120 and the mixtures incubated for 1 hr. at 37° C. After the period of incubation all the mixtures appeared perfectly clear, and 0.5 c.c. of serum B0964, diluted 1:10, which gave a main zone precipitate

Table 2. *Optimal proportions titrations of the A sera with horse serum as antigen*

Antiserum and dilution	Horse serum dilutions and tube numbers									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Serum A 0963 1:10	1	2	3	4	5	6	7	8	9	10
Serum A 0983 1:10	>30	24.0*	>30	12.0*	12.5	10.0*	12.0	15.5	30.0	>30
Serum A 0983 1:10	>20	>20	14.5*	>20	11.0*	13.0	15.0	>20	>20	>20
Serum A 0997 1:10	>25	>25	21.0	16.0*	18.0	20.0	21.0	>25	>25	>25
Serum A 1102 1:10	>15	>15	>15	>15	11.0	8.5*	9.5	14.0	>15	>15
Serum A 1103 1:10	>15	>15	>15	14.0	8.5*	9.0	15.0	>15	>15	>15

The times taken for the development of discrete particles of precipitate are recorded in minutes.
* Indicates the optimal mixtures.

Table 3. *Constant antiserum titrations of the A sera with horse-serum crystalbumin as antigen*

Interval after mixing before reading	Antiserum and dilution	Dilutions of 2.9 % horse-serum crystalbumin and tube numbers														Anti-serum control
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960	
2 hr.	Serum A 0963 1:10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	—
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Overnight	Serum A 0983 1:10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	—
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
2 hr.	Serum A 0997 1:10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	—
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Overnight	Serum A 1102 1:10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	—
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
2 hr.	Serum A 1103 1:10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	—
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p

— = Clear; 0 = Opalescent; p = Discrete particles or precipitate.
Antiserum control consists of 0.5 c.c. of antiserum under investigation, diluted 1:10, plus 0.5 c.c. saline.
Antigen control consists of 0.5 c.c. of crystalbumin, diluted 1:5, plus 0.5 c.c. saline.

with crystalbumin (Table 6) and therefore contained anticrystalbumin, was added to each mixture and the particulation times determined. At the same time a control experiment was carried out using normal rabbit serum in place of the A serum. On comparing the particulation times of each of the series containing an A serum with the control series containing normal rabbit serum it was apparent that in no case did an A serum delay the formation of a precipitate by serum B 0964; hence, by this technique, there was no evidence of non-precipitating antibodies present in the A sera. Examination of the particulation times in Table 4 shows that the A sera hastened the formation of discrete particles of precipitate in the region of relative antiserum excess (relative antigen deficiency). As shown later, the A sera contained horse-serum crystalbumin, and hence this result was to be expected. The ability of the A sera to hasten the formation of discrete particles of precipitate in the region of relative antiserum excess (relative antigen deficiency), as shown by the particulation times of mixture 6 in each series, where the effect is most marked and the differences in the particulation times are the greatest, was directly proportional to the crystalbumin content of these A sera as determined by the method of optimal proportions (see p. 67). Thus, placing the A sera in decreasing order of their ability to hasten the formation of particles of precipitate in the region of antiserum excess, the list is A 1103, A 0983 and A 0997, A 0963 and A 1102.

Table 4. *Tests for presence of non-precipitating antibody to crystalbumin in the A sera*

Sera added to crystalbumin dilutions	Dilutions of 2.9 % horse serum crystalbumin and tube numbers					
	1:160	1:320	1:640	1:1280	1:2560	1:5120
Normal rabbit serum 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	17	10.5	15	24	50
Serum A 0963 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	16.5	10	12	15.5	22
Serum A 0983 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	21.5	12.5	12.5	15	19
Serum A 0997 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	20	11	13	15.5	19
Serum A 1102 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	16.5	10	11.5	18	33
Serum A 1103 1:10. Incubate 1 hr. All clear. Particulation times after adding serum B 0964 1:10	> 60	21.5	10	11	12	12.5

The times taken for the development of discrete particles of precipitate are recorded in minutes.

The particulation times of serum B 0964 1:10 shown in this table are not comparable with those shown in Table 6 as the total volume of the reacting mixture is 1 c.c. in Table 6 and 1.5 c.c. in this table.

This list corresponds with the list of sera placed in order of their content of crystalbumin as determined by the method of optimal proportions.

Titration of the B sera

The results of the titrations of the B sera are shown in Tables 5 and 6. Table 5 shows the optimal proportions titrations of the B sera against horse serum. The

Table 5. *Optimal proportions titrations of the B sera with horse serum as antigen*

Antiserum and dilution	Horse-serum dilutions and tube numbers									
	1:10 1	1:20 2	1:40 3	1:80 4	1:160 5	1:320 6	1:640 7	1:1280 8	1:2560 9	1:5120 10
Serum B0964 1:10	> 30	> 30	> 30	> 30	> 30	9.5	7*	8	15	> 30
Serum B0988 1:10	44	18*	50	12	2.5*	4	3*	5	9.5	22
Serum B0990 1:10	> 100	98*	> 100	43	19	17*	18	21	34	64
Serum B0992 1:10	25†	> 60	18*	22	17	15*	17	19	26	58
Serum B0993 1:10	> 60	> 60	38	34*	> 60	4	3*	7	17	39
Serum B0995 1:10	> 60	> 60	> 60	> 60	> 60	27	22*	29	54	> 60

The times taken for the development of discrete particles of precipitate are recorded in minutes.

* Indicates the optimal mixtures.

† Indicates that mixture 1 developed discrete particles of precipitate before mixture 2

method of titration and recording is identical with that used for the A sera in Table 2. Multiple zones were observed with sera B0988, B0990, B0992 and B0993. Table 6 shows the titration of these sera using horse-serum crystalbumin as antigen. Each serum, diluted 1:10, was added to serial doubling dilutions of 2.9 % horse-serum crystalbumin from 1:5 to 1:40,960, and the particulation times determined. Particulation times over 1 hr. were not determined. The table also shows the appearance of each mixture after 1 hr. The particulation time of the optimal mixture is shown for several dilutions of serum B0988, as this serum was used in Exps. 1 and 2. These sera all showed two zones of reaction with horse-serum crystalbumin. One zone had optimal proportions of 1:64 or 1:128, and this is referred to as the main zone. The second zone occurred with high concentrations of crystalbumin, 1:5, 1:10 and 1:20; and in each case the precipitate of this zone formed first in the mixture containing crystalbumin 1:5, i.e. in tube 1. These sera reacted with both crystalbumin and the impurities contained in the crystalbumin preparation, in contrast with the A sera, shown in Table 3, which reacted solely with the impurities. For reasons explained earlier, the main zone is considered to represent the reaction of crystalbumin with its homologous antibodies and the zone occurring with high concentrations of the crystalbumin preparation is regarded as the reaction of the impurities in the crystalbumin preparation with their homologous antibodies in the antiserum.

Table 6. *Optimal proportions titrations of the B sera with horse-serum crystalbumin as antigen*

Antiserum and dilution	Observation made	Dilutions of 2.9 % horse-serum crystalbumin and tube numbers													
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960
Serum B 0964 1:10	Particulation times	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Appearance after 1 hr.	50	55	>60	>60	>60	>60	8.5	6.5*	7.5	14	33	>60	>60	>60
Serum B 0988 1:10	Particulation times	<i>p</i>	<i>p</i>	0	—	—	0	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	0	0	—
	Appearance after 1 hr.	24	29	57	>60	>60	>60	25	2*	3	9	22	40	>60	>60
Serum B 0988 1:20	Particulation times	<i>p</i>	<i>p</i>	<i>p</i>	0	0	—	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	0	—
	Appearance after 1 hr.									6.5*					
Serum B 0988 1:40	Particulation times										15*				
	Appearance after 1 hr.	41	50	>60	>60	>60	>60	>60	48*	52	>60	>60	>60	>60	>60
Serum B 0992 1:10	Particulation times	<i>p</i>	<i>p</i>	0	—	—	—	—	<i>p</i>	<i>p</i>	—	—	—	—	—
	Appearance after 1 hr.	29	38	59	>60	>60	>60	>60	52	28*	58	>60	>60	>60	>60
Serum B 0993 1:10	Particulation times	<i>p</i>	<i>p</i>	<i>p</i>	—	—	—	0	<i>p</i>	<i>p</i>	<i>p</i>	0	—	—	—
	Appearance after 1 hr.	34	50	>60	>60	>60	>60	6	3*	7.5	19	45	>60	>60	>60
Serum B 0993 1:10	Particulation times	<i>p</i>	<i>p</i>	0	—	—	0	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	0	0	—
	Appearance after 1 hr.	45	55	>60	>60	>60	>60	>60	>60	50*	>60	>60	>60	>60	>60
Serum B 0993 1:10	Particulation times	<i>p</i>	<i>p</i>	0	—	—	—	—	—	<i>p</i>	0	—	—	—	—
	Appearance after 1 hr.														

Times taken for the development of discrete particles of precipitate are recorded in minutes.

* Indicates the optimal mixtures.

— = Clear; 0 = opalescent; *p* = discrete particles of precipitate.

The particulation times of the optimal mixtures for several dilutions of serum B 0988 are included because this serum was used in Experiments 1 and 2.

EXPERIMENTS

Experiment 1. Determination of the horse-serum antigen present in the A sera.

Optimal proportions titrations of serum B0988 against all the A sera followed by absorption of serum B0988 with each of the A sera and titration of the absorbed sera against crystalbumin.

As noted previously, a precipitate forms on mixing any one of the A sera with any one of the B sera. The reactions between the A sera, acting as antigens, and serum B0988 were investigated by the method of optimal proportions. Serial doubling dilutions of all the A sera were made from 1:1 to 1:32. Serum B0988, diluted 1:40, was added to each series of dilutions, and the time taken for the development of discrete particles of precipitate in the optimal tube and neighbouring tubes determined. The results are shown in Table 7. In these titrations

Table 7. *Experiment 1. Optimal proportions titrations of serum B0988 diluted 1:40 with sera A 0963, A 0983, A 0997, A 1102 and A 1103 as antigens*

Antigen serum	Antigen dilutions					
	1:1	1:2	1:4	1:8	1:16	1:32
A 0963	> 30	19.0	11.0*	17.0	29.0	> 30
A 0983	> 45	> 45	25.0	12.0*	15.0	24.0
A 0997	> 120	> 120	24.0	11.0*	17.0	27.0
A 1102	21.0	> 30	> 30	> 30	> 30	> 30
A 1103	> 20	> 20	> 20	14.0*	14.5	20.0

Times taken for the development of discrete particles of precipitate are recorded in minutes.

* indicates the optimal mixtures.

Serum B0988, 1:40 was added to every tube.

Serum B0988 was selected for use in this experiment because it rapidly gave a precipitate in the main zone with crystalbumin and hence could be successfully used at a high dilution for the determination of its optimal proportions against fluids which contained only small quantities of crystalbumin.

the same antiserum has been added to identical serial dilutions of five different sera, each acting as an antigen. In each case the reaction was essentially similar to any precipitation reaction as regards the occurrence of an optimal tube and the inhibitory effects of relative antigen excess and relative antiserum excess. With serum A1102 as antigen no optimal tube was apparent, tube 1 of the titration showing visible particles of precipitate before the others. It is obvious that this serum contained insufficient of the antigen to show a zone of relative antigen excess, even when the serum A1102 was undiluted, and the antiserum B0988 was diluted 1:40. From Table 7 the approximate optimal ratio of the antiserum (serum B0988) with regard to each of the A sera used as antigens can be deduced. For example, the optimal ratio of serum B0988 with regard to serum A0963 as antigen is 10:1. For purposes of absorption in optimal proportions the optimal ratios were determined more accurately by 'fine tests'. Serum A1102 contained insufficient of the antigen for an absorption to be carried out. The optimal ratios between serum B0988, as antiserum, and sera A0963, A0983, A0997 and A1103 as antigens were 10.24:1, 5.12:1, 5.12:1 and 4.1:1 respectively.

Serum B0988 was then absorbed, in optimal proportions, with the antigen contained in sera A0963, A0983, A0997 and A1103. In each case, sufficient of the antigen-containing serum was added to 0.5 c.c. of serum B0988 to make the proportions of the two reagents optimal, and then sufficient saline was added to make the total volume of the mixture 10 c.c., thus giving serum B0988 a dilution of 1:20 in the final mixture. In addition, two control mixtures were made consisting of 0.5 c.c. of serum B0988 and two different volumes of normal rabbit serum. The composition of these absorption mixtures was as follows:

1. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	4.38 c.c.	
Serum A 0963	5.12 c.c.	
2. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	6.94 c.c.	
Serum A 0983	2.56 c.c.	
3. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	6.94 c.c.	
Serum A 0997	2.56 c.c.	
4. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	7.45 c.c.	
Serum A 1103	2.05 c.c.	
5. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	4.5 c.c.	
Normal rabbit serum	5.0 c.c.	
6. Serum B 0988	0.5 c.c.	} Total volume 10 c.c.
Saline	7.0 c.c.	
Normal rabbit serum	2.5 c.c.	

These mixtures were made and treated as described in the section on materials and methods. A precipitate formed in mixtures 1, 2, 3 and 4, but not in the control mixtures 5 and 6.

Each absorbed serum and the control mixtures were added to serial doubling dilutions of 2.9 % horse serum crystalbumin from 1:5 to 1:81,920. The appearance of the mixtures 1 or 2 hr. after mixing and again after standing at +2° C. overnight are shown in Table 8. The control mixtures of serum B0988 with normal rabbit serum gave two zones of rapid flocculation. This result is essentially similar to that shown in Table 5, and the presence of normal rabbit serum did not alter the precipitation appreciably. But the absorbed specimens of serum B0988 showed an entirely different result. In each case, after absorption, serum B0988 showed no main-zone reaction but gave a precipitate only with high concentrations of crystalbumin. Hence absorption completely removed the antibodies for crystalbumin from serum B0988. The reaction with high concentrations of crystalbumin was due to the presence of antibodies for the impurities which consisted of those originally present in serum B0988 together with those added in the A sera used for absorption. Since sera A0963, A0983, A0997 and A1103 absorbed anti-crystalbumin from a serum containing it, they contained crystalbumin. Thus a mixture of two anti-horse sera, in certain proportions, may fail to give a precipitate with horse-serum crystalbumin, although one of them, by itself, precipitates with that antigen.

Table 8. *Experiment 1. Constant antiserum titrations of serum B 0988 after absorption with sera A 0963, A 0983, A 0997 and A 1103 and serum B 0988 mixed with normal rabbit serum using horse-serum crystalbumin as antigen*

Interval after mixing before reading	Dilutions of 2.9 % horse serum crystalbumin and tube numbers															Anti-serum Control	Antigen Control							
	1:5	1:10	1:20	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100	1:120	1:150	1:200	1:250			1:320	1:400	1:5120	1:6400	1:81920	15	16
Serum B 0988 after absorption with serum A 0963	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Serum B 0988 after absorption with serum A 0983	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Serum B 0988 after absorption with serum A 0997	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Serum B 0988 after absorption with serum A 1103	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Serum B 0988 in half-strength normal rabbit serum	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Serum B 0988 in quarter-strength normal rabbit serum	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p

—=Clear; 0=Opalescent; p=Discrete particles of precipitate.
 Antiserum control consists of 0.5 c.c. of antiserum under investigation with 0.5 c.c. saline.
 Antigen control consists of 0.5 c.c. of horse serum crystalbumin, diluted 1:5, with 0.5 c.c. saline.

Table 9. *Experiment 1. Comparison between the participation times of two series of mixtures, one series containing crystalbumin as the antigen and the other containing serum A 0983 as the antigen*

Tube no. ...	Crystalbumin series										Serum A 0983 series							
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Dilution of 2.9 % horse-serum crystalbumin	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480
Dilution of normal rabbit serum	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64
Dilution of serum A 0983	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Participation time in min.	>40	35-0	14.5*	19.0	30.5	>40	>40	35-0	14.5*	19.0	30.5	>40	>40	30-0	14.0*	19.0	30.0	>40

Serum B 0988, 1:40, as antiserum, was added to every tube in both series.
 * Indicates the optimal mixtures.

Table 10. *Experiment 2. Constant antiserum titration of serum B 0988 absorbed with 2.9 % crystalbumin in the proportions of 1:50 with horse-serum crystalbumin as antigen*

Antiserum and dilution	Dilutions of 2.9 % horse-serum crystalbumin and tube numbers																						
	1:5	1:10	1:20	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100	1:120	1:150	1:200	1:250	1:320	1:400	1:5120	1:6400	1:81920			
Serum B 0988 absorbed with 2.9 % crystalbumin in the proportions of 1:50 and diluted 1:10	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p

The times taken for the development of discrete particles of precipitate are recorded in minutes.
 —=Clear; 0=Opalescent; p=Discrete particles of precipitate.

From the optimal ratios of serum B 0988 against sera A 0963, A 0983, A 0997 and A 1103 acting as antigens, the quantity of crystalbumin contained in these A sera can be calculated, provided that the optimal ratio of serum B 0988 against a standard solution of crystalbumin is also known. The optimal ratio of serum B 0988 against 2.9 % horse-serum crystalbumin was found to be 1:50. The quantities of horse-serum crystalbumin per c.c. contained in the A sera were:

Serum A 0963	0.000057 g.
Serum A 0983	0.00011 g.
Serum A 0997	0.00011 g.
Serum A 1102	Insufficient to estimate
Serum A 1103	0.00014 g.

In the course of this experiment two further questions were investigated.

(i) The first question was to confirm that in the absorption of serum B 0988 with sera A 0963, A 0983, A 0997 and A 1103, the antibodies for crystalbumin present in serum B 0988 had really been removed in the precipitate and were not simply inhibited from reacting with crystalbumin by some mechanism, such as incomplete antibodies present in the A sera. To investigate this question, the mixtures of absorbed serum B 0988 and crystalbumin shown in Table 8 were allowed to stand for 24 hr., after which 0.5 c.c. of unabsorbed serum B 0988 at a dilution of 1:20 was added to tubes 5-14 in each series, in which tubes no precipitate had formed. In each series, a main zone reaction occurred, visible particles of precipitate forming in tube 8 in 11 min. Therefore the crystalbumin in these mixtures was free and could combine rapidly with added anti-crystalbumin, thus confirming that the absorption of serum B 0988 with the A sera removed the anti-crystalbumin.

(ii) The second question was to compare the times taken for the development of discrete particles of precipitate when serum B 0988 was added to serial dilutions of crystalbumin and to serial dilutions of the crystalbumin-containing A sera. To control the influence of protein concentration on particulation time, the protein content of the crystalbumin dilutions had to be made comparable with that of the crystalbumin-containing sera. Equal volumes of horse-serum crystalbumin, diluted 1:320, and normal rabbit serum were mixed, giving a fluid containing crystalbumin 1:640 and normal rabbit serum 1:2. Serial doubling dilutions of this fluid produced serial dilutions of crystalbumin and of normal rabbit serum as shown in Table 9. Serial dilutions of serum A 0983 were prepared from 1:2 to 1:64. Serum B 0988, diluted 1:40, was then added to both series of dilutions. The times taken for the development of discrete particles of precipitate in each series are shown in Table 9. The dilutions of crystalbumin had been chosen so that the optimal tube was tube 3 in each series. As far as could be determined, there were no appreciable differences in the particulation times of corresponding tubes in each of the two series, and the later appearances of corresponding mixtures, as regards increase in the size of the particles, were the same. Similar results were obtained with sera A 0963 and A 0997 as antigens. Crystalbumin was apparently present in sera A 0963, A 0983 and A 0997, and the physical state of the crystalbumin did not apparently differ in any way from that of normal crystalbumin; it was evidently not present as a crystalbumin-anticrystalbumin complex.

Experiment 2. Confirmation of the horse-serum antigen present in the A sera.

Absorption of serum B0988 with crystalbumin followed by titration of the absorbed serum against all the A sera.

If an antiserum, which contains antibodies to many antigens, is absorbed by a mixture of antigens, which exhibits multiple zones, in the proportions of the optimum occurring with the lowest concentration of the mixed antigen, the antibody responsible for this zone is completely removed without the complete removal of other antibodies (Naylor, 1950). The optimal ratio of serum B0988 to 2.9 % horse-serum crystalbumin, in the main zone, was 1:50, so serum B0988 was absorbed in these proportions with crystalbumin. The absorbed serum, diluted 1:10, was titrated against serial doubling dilutions of 2.9 % horse-serum crystalbumin from 1:5 to 1:81,920, as shown in Table 11. The absorbed serum showed a single zone with high concentrations of crystalbumin and did not precipitate with dilutions of crystalbumin from 1:100 to 1:81,920, whereas the unabsorbed serum showed two zones (see Table 6). The absorbed serum B0988 did not give a main-zone reaction with crystalbumin. The anti-crystalbumin had been completely removed, and the serum reacted solely with the impurities present in the crystalbumin preparation.

The absorbed serum B0988 diluted 1:10 was then tested against serial doubling dilutions of all the A sera from 1:1 to 1:512. No precipitation occurred in any of the mixtures even after standing overnight at +2° C; except that mixtures containing serum A0963 at 1:1 and 1:2, although they showed no precipitate after 1 hr., developed a very slight but definite precipitate overnight, which may well have been due to some reaction not involving crystalbumin. Serum B0988 absorbed with crystalbumin did not precipitate on mixing with the A sera, whereas unabsorbed serum B0988 gave a marked precipitate with all these sera as shown in Table 7. This confirmed that the A sera, which did not give a main-zone reaction with crystalbumin, contained horse-serum crystalbumin.

Experiment 3. Determination of whether a precipitate forms on mixing two sera both containing anti-crystalbumin.

Serum B0988 diluted 1:20 was added to serial doubling dilutions of each of the sera B0964, B0990, B0992, B0993 and B0995 from 1:1 to 1:32. In each series a control consisting of 0.5 c.c. of the serum and 0.5 c.c. of saline was included. The mixtures were observed after 1 or 2 hr. in the water-bath and again in the water-bath after standing at +2° C. overnight. The results are not shown in tabular form, but there was no precipitation in any of the mixtures.

Serum B0988, which gave a main-zone reaction with crystalbumin, did not precipitate when added to five other B sera, all of which likewise showed a main-zone reaction with crystalbumin. No precipitation was obtained on mixing sera which both contained anti-crystalbumin, and there was no evidence that sera containing an anti-crystalbumin also contained crystalbumin.

DISCUSSION

The experiments described in this paper demonstrated the presence of horse-serum crystalbumin in rabbit anti-horse sera which did not contain antibodies for this antigen, and they apparently solve the problem of 'mutual' precipitation of anti-horse sera, for the antisera which contained crystalbumin precipitated on mixing with other antisera which contained anti-crystalbumin.

The co-existence of horse-serum precipitinogen and precipitin in the same specimen of anti-horse serum was not due to the presence of homologous antigen and antibody in the same antiserum, but to the presence of horse-serum crystalbumin in antisera which contained antibodies to horse-serum proteins other than crystalbumin. The interpretation of 'mutual' precipitation of antisera as due to a multiplicity of antigens agrees with the original theory of von Dungern, supported by the work of Weil & Opie.

Anti-horse sera which do not contain antibodies for albumin are fairly common. They have been described by Taylor *et al.* (1932) and by Goldsworthy & Rudd (1935). Antisera also occur which lack antibodies for globulin (Taylor, 1935; Goldsworthy & Rudd, 1935). It seems likely that such antisera might contain globulin and so precipitate with anti-horse sera which contain antibodies to globulin. Horse serum almost certainly contains more than two antigens (Landsteiner, 1946), and most probably 'mutual' precipitation could be demonstrated with anti-horse sera which fail to precipitate with any horse-serum antigen. The solution found in the present work is probably one example of a general principle, that rabbit anti-horse sera may contain antigens of the injected horse serum, but do not at the same time contain their homologous antibodies. Work with crystalbumin is convenient because anti-horse sera which do not contain anti-crystalbumin are fairly common, and crystalbumin can be prepared chemically in a comparatively pure state.

The lack of antibodies for some of the antigens of an injected serum may be accounted for by the removal of small amounts of antibody from the circulation by the injected antigen, but it seems unlikely that the rabbits used in the present experiments were bled in the 'negative phase', because Dean & Webb (1928) found that, although there was a sudden fall in the antibody content of anti-horse sera following an injection of horse serum, recovery was rapid, and began almost at once. By the third day there was a definite rise and the response was greatest 5-8 days after the injection. In experiments on the duration of the 'negative phase', multiple antigens, such as horse serum, may mislead by showing a short 'negative phase', because the antibody chosen for measurement may correspond to an antigen with a low concentration in horse serum. With crystalline egg albumin, Opie (1923*b*) found that the antibody titre rose to its pre-injection level or higher within 5 days, except in two experiments, in one of which no determinations were made between the second and eleventh days, while in the other antibodies had returned to considerable strength 4 days after the injection of 3 c.c. of a 5% solution of crystalline egg albumin, but no determination beyond the fourth day is recorded. An antigen disappears more quickly from the circulation

of an immunized than of a non-immune animal (Glenny & Hopkins, 1922, 1923; Opie, 1923*b*; Culbertson, 1935). In most experiments reported by these workers, injected antigen disappeared from the circulation of immune animals within 2 or 3 days and was never detectable in the serum of well-immunized animals unless large doses were given. Absence of antibody and presence of antigen in the serum of a rabbit 10 days after the last injection of antigen is probably due to failure of the rabbit to respond to the antigen and not to removal of circulating antibodies by the injected antigen. On the other hand, antigen found in the serum of immunized animals 24–48 hr. after the last injection may be the excess left after complete neutralization of all the antibody in the plasma. Later, as more antibody is produced, antibody but not antigen can be detected in the serum.

The combination and neutralization of antigen and antibody in the circulation of an immunized animal following an injection of antigen probably resemble the *in vitro* reaction, complicated by the role of tissue-bound antibody and by the metabolism and excretion of the antigen. After immunization of an animal with a mixed antigen, several antibodies appear in the blood stream, some abundantly, others in small amount, and some antigens fail to stimulate any antibody production. Later injections of the same antigen induce a number of reactions; some antigens are present in excess and some antibodies are present in excess. Therefore both precipitin and precipitinogen can be demonstrated in the serum, but they are not homologous. Probably homologous antigen and antibody do not co-exist in the circulation of an animal, and experimental results that appear contradictory are due to the use of impure or multiple antigens.

The method which has been used in these experiments may be applied to the study of single antigens and single antibodies when the only reagents available are mixtures. Few protein preparations contain one antigen alone, and antigenic impurities may confuse the significance of precipitin tests if mere formation of a precipitate is used as the indicator of an antigen-antibody reaction. The correlation of an α -procedure optimum with an antigen-antibody reaction permits the use of this optimum as the indicator of the reaction, even in the presence of other antigen-antibody reactions.

SUMMARY

1. Five anti-horse sera which did not contain antibodies for horse-serum crystalbumin have been shown by absorption experiments to contain horse-serum crystalbumin.
2. All five sera precipitated when mixed with other anti-horse sera containing anti-crystalbumin, precipitation being due to the presence of horse-serum crystalbumin in one antiserum and of anti-crystalbumin in the other.
3. Antigen and its homologous antibody were never found together in the serum of an animal, and contradictory results in other experiments are probably due to impure multiple antigens.
4. It is concluded that anti-horse sera may contain some of the antigenic components of injected horse serum together with antibodies to other antigens of the horse serum, but not homologous antigen and antibody. Consequently, the 'mutual' precipitation of anti-horse sera is due to the presence of a number of

antigens in horse serum, one or more of which, present in one anti-horse serum, in the absence of its homologous antibody, may precipitate when mixed with another anti-horse serum which contains the homologous antibody.

5. The use in these experiments of a single α -procedure optimum as the indicator of an antigen-antibody reaction illustrates a method enabling the investigation of problems involving single antigen-antibody reactions, even though the available reagents consist of mixtures of antigens and mixtures of antibodies.

I am very grateful to Mrs M. E. Adair for providing me with horse-serum crystalbumin.

REFERENCES

- ASCOLI, M. (1902). Zur Kenntnis der Präzipitinwirkung und der Eiweisskörper des Blutserums. *Münch. med Wschr.* **49**, 1409.
- BAYNE-JONES, S. (1917). Equilibria in precipitin reactions. The coexistence of a single free antigen and its antibody in the same serum. *J. exp. Med.* **25**, 837.
- CULBERTSON, J. T. (1935). The role of the precipitin antibody in the removal of intravenously injected antigen. *J. Immunol.* **28**, 279.
- DEAN, H. R. & WEBB, R. A. (1926). The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. *J. Path. Bact.* **29**, 473.
- DEAN, H. R. & WEBB, R. A. (1928). The determination of the rate of antibody (precipitin) production in rabbit's blood by the method of 'optimal proportions'. *J. Path. Bact.* **31**, 89.
- DUNCAN, J. T. (1932). The use of equivalent proportions of antigen and serum in absorption of precipitin. *Brit. J. exp. Path.* **13**, 489.
- VON DUNGERN, F. (1903). Bindungsverhältnisse bei der Präzipitinreaktion. *Zbl. Bakt. Orig.* **34**, 355.
- EISENBERG, P. (1902). Untersuchungen über spezifische Präcipitationsvorgänge. Teil I. *Zbl. Bakt. Orig.* **31**, 773.
- GAY, F. P. & RUSK, G. Y. (1912). The persistence of a soluble antigen in the serum of immunized rabbits. *Univ. Calif. Publ. Path.* **2**, 59.
- GLENNY, A. T. & HOPKINS, B. E. (1922). Duration of passive immunity. Part I. *J. Hyg., Camb.*, **21**, 142.
- GLENNY, A. T. & HOPKINS, B. E. (1923). Duration of passive immunity. Part IV. *J. Hyg., Camb.*, **22**, 208.
- GOLDSWORTHY, N. E. (1928). The occurrence of multiple zones in the serum precipitation reaction. *J. Path. Bact.* **31**, 525.
- GOLDSWORTHY, N. E. & RUDD, G. V. (1935). Complexity of antigens in relation to zones in the precipitation reaction. *J. Path. Bact.* **40**, 169.
- HEIDELBERGER, M. (1939). Quantitative absolute methods in the study of antigen-antibody reactions. *Bact. Rev.* **3**, 49.
- HEKTOEN, L. & WELKER, W. H. (1925). Precipitin reactions of serum proteins. *J. infect. Dis.* **35**, 295.
- HEWITT, L. F. (1938). Preparation and properties of a globulin present in the albumin fraction of serum. *Biochem. J.* **32**, 26.
- HOOVER, S. B. & BOYD, W. C. (1936). The existence of antigenic determinants of diverse specificity in a single protein. III. Further notes on crystalline hen- and duck-ovalbumins. *J. Immunol.* **30**, 41.
- IONESCO-MIHAIESTI, C. (1911). Sur la coexistence de l'antigène et de l'anticorps dans le sérum des lapins préparés avec le sérum de cheval. *C.R. Soc. Biol., Paris*, **70**, 429.
- KENDALL, F. E. (1937). Studies on serum proteins. *J. clin. Invest.* **16**, 921.
- LANDSTEINER, K. (1946). *The Specificity of Serological Reactions*. Revised edition. Cambridge, Mass: Harvard University Press.
- LINossier, G. & LEMOINE, G. H. (1902). Sur les substances précipitantes des albumines (précipitines) contenues dans certains sérums spécifiques. *C.R. Soc. Biol., Paris*, **54**, 85

- NAYLOR, G. R. E. (1948). Some observations on the reaction between horse serum and a pool of rabbit anti-horse serum . I. *J. Hyg., Camb.*, **46**, 129.
- NAYLOR, G. R. E. (1950). Some observations on the reaction between horse serum and a pool of rabbit anti-horse serum. II. *J. Hyg., Camb.* (in the Press).
- OPIE, E. L. (1923*a*). The relation of antigen to antibody (precipitin) *in vitro*. *J. Immunol.* **8**, 19.
- OPIE, E. L. (1923*b*). The relation of antigen to antibody (precipitin) in the circulating blood. *J. Immunol.* **8**, 55.
- PAPPENHEIMER, A. M. (1940). Anti-egg albumin antibody in the horse. *J. exp. Med.* **71**, 263.
- SØRENSEN, S. P. L. (1925). *Proteins*, pp. 40 et seq. Lectures given in the United States of America in 1924. Published by the Fleischmann Company.
- SØRENSEN, S. P. L. (1930). The constitution of soluble proteins. *C.R. Lab. Carlsberg*, **18**, 5, 1.
- TAYLOR, G. L. (1935). The demonstration of the increase of globulin in diphtheria antitoxins by the precipitation reaction. *J. Hyg., Camb.*, **35**, 174.
- TAYLOR, G. L., ADAIR, G. S. & ADAIR, M. E. (1932). The estimation of proteins by the precipitation reaction. *J. Hyg., Camb.*, **32**, 340.
- TAYLOR, G. L., ADAIR, G. S. & ADAIR, M. E. (1934). The precipitation reaction. Optimal proportions, neutrality and maximal precipitation in mixtures of albumin and anti-serum. *J. Hyg., Camb.*, **34**, 118.
- UHLENHUTH, P. & WEIDANZ (1909). Quoted by Uhlenhuth, P. & Seiffert, W. (1928). *Handbuch der pathogenen Mikroorganismen*, **3**, 421 et seq. Founded by W. Kolle and A. v. Wassermann. Edited by W. Kolle, R. Kraus and P. Uhlenhuth.
- WEIL, R. (1916). Studies in anaphylaxis. XV. Equilibrium in precipitation reactions. Equilibrium in combination. *J. Immunol.* **1**, 19.
- ZINSSER, H. (1923). *Infection and Resistance*, 3rd ed. New York: Macmillan Company.
- ZINSSER, H. & YOUNG, S. W. (1913). On the possible importance of colloidal protection in certain phases of the precipitin reaction. *J. exp. Med.* **17**, 396.

(*MS. received for publication 11. I. 50.*)