THE RESULTS OF SOME QUANTITATIVE EXPERIMENTS ON THE SERUM PRECIPITATION REACTION.

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CONTENTS.

INTRODUCTION.

The work described in this paper is based, very largely, on the work of Dean and Webb (1926), and the methods used in the various experiments are similar to those described by them. The results are discussed in two parts. The first is concerned with the mixture of different specimens of anti-horse serum, and with the relationship of the antibody in one specimen to the antibody in another. The second part deals with the nature of the reaction between antigen and antibody in the serum precipitation reaction. The two parts are dependent on each other, and the subject of the second suggested itself whilst the work on the first was in progress. The order in which the work is described is mostly that in which it was done. Nevertheless, conclusions arrived at in one part are, in one or two cases, of importance in the consideration of the other part, and *vice versa*. A question as to the possibility of strengthening the ratio of an antiserum by the addition of another antiserum led to the earlier experiments.

The work was carried out in the Department of Pathology at Cambridge. To Prof. H. R. Dean I am indebted for advice and criticism.

All the antisera with the exception of the anti-goat serum used in the experiments to be described were made by the injection of normal horse serum into rabbits. All reactions took place at room temperature. 0·85 per cent. saline was the diluent in all cases.

PART I. MIXED ANTISERA.

Dean and Webb (1926) showed that when horse serum is mixed with anti-horse serum, the most rapid particulation takes place when the ingredients are in definite proportions. The proportion of horse serum to antiserum yielding optimal results is expressed as the antigen-antibody ratio. An antiserum of which 20 c.c. react most rapidly with 1 c.c. of horse serum is said to have an antigen-antibody ratio of 1 to 20. Different specimens of antiserum have different ratios. This may be due to a qualitative or quantitative difference in the antibody content of the respective antisera. The following experiments show

that in the majority of cases a quantitative difference in the antibody content is responsible for these differences. Different antisera contain varying amounts of the same antibody. The ratio of an antiserum is entirely dependent on the antibody content, and is not influenced by the other constituents of the serum. The constituents of an antiserum, other than antibody, do seem to influence the speed of the reaction, but in no way affect the proportions of horse serum and antiserum necessary for optimal reactions (Part II). Two antisera of the same ratio may take widely varying times to react with horse serum, when the dilutions of the ingredients in the two experiments are alike. It might be thought that the differences in the ratios of antisera are due wholly, or in part, to the effects exerted by variations in the constituents of the respective sera. This would not appear to be so. The amount of antigen in 1 c.c. of horse serum always reacts with a definite amount of antibody, although varying volumes of different antisera may be necessary to provide that quantity of antibody. The term "antigen-antibody ratio" does not mean that 1 part of antigen reacts with so many parts of antibody; it means that 1 part of horse serum reacts with so many parts of antiserum. It is not known how many parts of antibody react with 1 part of antigen, using antigen and antibody in the strictest sense. However, antigen does react with antibody in definite proportions, although those proportions are not yet capable of determination.

Exp. 1 (Table I). Horse serum in falling quantities titrated against anti-horse serum 1192 ZA used in a dilution of 1 in 10.

Table I.

Tube	H.S. 1 in 500 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 12 min.
H.S.C.	1·0	1 in 500	—	—
10	1·0	1 in 500	1 to 50	—
9	0·95	1 in 526·3	1 to 52·6	—
8	0·9	1 in 555·6	1 to 55·6	2
7	0·85	1 in 588·2	1 to 58·8	1
6	0·8	1 in 625	1 to 62·5	3
5	0·7	1 in 714·3	1 to 71·4	—
4	0·6	1 in 833·3	1 to 83·33	—
3	0·5	1 in 1000	1 to 100	—
2	0·4	1 in 1250	1 to 125	—
1	0·3	1 in 1666·7	1 to 166·7	—
A.S.C.	—	—	A.S. only	—

Controls unaffected.

In such experiments we are in the habit of referring to the horse serum dilution in a tube as being the dilution of the 1 c.c. of horse serum dilution placed in the tube. Similarly with the antiserum dilution. Both these dilutions are doubled of course, when the 1 c.c. of each are mixed, but the relative proportions of the two remain unchanged.

This experiment is similar to Exp. 2 described in Dean and Webb's paper (1926, p. 477). The tabulation is simpler, and not so full. The series in this case is a close one; the amounts of horse serum dilution falling by 0·05 c.c. in the upper tubes to allow a nearer approximation to the correct result. A previous rough test had been performed. Horse serum in falling quantities was titrated

against a constant amount of anti-horse serum (1192 ZA). The first column describes the tubes in the series; H.S.C. and A.S.C. are the horse serum and anti-horse serum controls respectively. The second column shows the volume of a 1 in 500 dilution of horse serum delivered into the respective tubes. The antiserum control received none. The volume in each tube was made up to 1 c.c., where necessary, by the addition of the appropriate amount of saline; 1 c.c. of saline was added to tube H.S.C. and 1 c.c. placed in A.S.C. The third column gives the dilution of horse serum which each tube now contained. The fourth column shows the proportion of horse serum to antiserum in each tube after the addition of 1 c.c. of a 1 in 10 dilution of antiserum to every tube except H.S.C. Each tube now had a volume of 2 c.c. The time of the addition of antiserum was noted. The fifth and last column registers the order of the degree of particulation, and the time after which it was possible to determine this. Tube No. 7 was first, 8 second, and 6 third. No particulation occurred in the controls, even after many hours. It is seen that this antiserum reacted most rapidly with horse serum when the proportion of horse serum to antiserum was 1 to something between 55·6 and 58·8. If this antiserum is given an antigen-antibody ratio of 1 to 57, there is very little error.

The anti-horse serum from one rabbit may have a ratio of 1 to 20, that from another rabbit a ratio of 1 to 40. That is to say, with the same quantity of horse serum twice as much antiserum from one rabbit is needed to yield optimal results as from another rabbit. It might be thought that the antibody portion of one antiserum was different from the antibody portion of the other, or that the antigen was not the same in the two cases; one rabbit picking out one antigen and the other rabbit another. If the antibody in one antiserum is the same as the antibody in another, the difference in the ratios can be explained by saying there is more of this antibody in one than in the other. Assuming the antibody is the same:

If we mix equal parts of two anti-horse sera X and Y, of ratios 1 to x, and 1 to y, what should be the ratio of the resulting antiserum?

1 part of X reacts with $\frac{1}{x}$ parts of horse serum (H.S.).

1 „ Y „ $\frac{1}{y}$ „ „ „

$\therefore$ 2 parts of mixed antiserum (A.S.) react with $\frac{1}{x}+\frac{1}{y}$ parts of H.S.

i.e. 1 part of „ „ $\frac{1}{2x}+\frac{1}{2y}$ „

i.e. 1 „ „ „ $\frac{x+y}{2xy}$ „

$\therefore$ $\frac{2xy}{x+y}$ parts „ „ 1 „

So the H.S. to A.S. ratio of the mixed antiserum is 1 to $\frac{2xy}{x+y}$.

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Exp. 2 (Table II). Horse serum titrated against a mixture of equal parts of two anti-horse sera, 1192 ZA and 1056 ZH. 1 c.c. of each antiserum were mixed, and the volume immediately made up to 20 c.c. The mixed antiserum is, therefore, in a dilution of 1 in 10.

Table II.

Tube	H.S. 1 in 350 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 15 min.
H.S.C.	1·0	1 in 350	—	—
10	1·0	1 in 350	1 to 35	—
9	0·95	1 in 368·4	1 to 36·8	—
8	0·9	1 in 388·9	1 to 38·9	3
7	0·85	1 in 411·6	1 to 41·2	1
6	0·8	1 in 437·5	1 to 43·75	2
5	0·75	1 in 466·7	1 to 46·7	—
4	0·7	1 in 500	1 to 50	—
3	0·6	1 in 583·3	1 to 58·3	—
2	0·5	1 in 700	1 to 70	—
1	0·4	1 in 875	1 to 87·5	—
A.S.C.	—	—	A.S. only	—

Table II is an example of an experiment in which normal horse serum in falling quantities is titrated against a constant quantity of mixed antiserum, made by mixing together equal parts of two anti-horse sera, 1192 ZA and 1056 ZH. 1192 ZA is the antiserum used in Table I, Exp. 1. The ratio of this antiserum has been determined as 1 to 57 (Exp. 1). The ratio of the second antiserum 1056 ZH was determined in a like manner, and found to be 1 to 34. Exp. 2 was carried out in exactly the same way as Exp. 1; the quantities, of course, differ. Before the experiment the calculated value was found for the mixed antiserum from the ratios of the component antisera, and a series of horse serum dilutions arranged accordingly. The equal parts of the component antisera were mixed, and immediately the dilution of 1 in 10 was made; this dilution showed a slight degree of opalescence. 1 c.c. of the antiserum dilution was at once delivered into each of the tubes except H.S.C. The antiserum control showed only a faint trace of opalescence, and no attempt at particulation, even after 24 hours. Tubes 7 and 6 were the first and second respectively with ratios 1 to 41·2 and 1 to 43·75. The ratio is taken as 1 to 42. Substituting the values of the ratios for the component antisera, 1192 ZA and 1056 ZH (1 to 57 and 1 to 34), in the given formula:

$$\text{Ratio is 1 to } \frac{2xy}{x+y}, \text{ we have 1 to } \frac{2 \times 57 \times 34}{57+34},$$

i.e. 1 to 42·6 as the ratio of the mixed serum; which agrees with the experimentally determined ratio of 1 to 42.

Table III is self explanatory. Ten different mixtures of two antisera in each were made from a series of ten antisera. These ten different antisera came from nine different rabbits. 1103 J and 1103 K were different bleedings of the same rabbit; they had very different ratios, as it happened, 1 to 35 and 1 to 15. This liability of the ratio of a rabbit's serum to change from bleeding to bleeding

was pointed out by Dean and Webb. These nine rabbits had been in the laboratory for longer or shorter periods, had various numbers of courses of injections, and various numbers of bleedings. Various specimens of horse serum had been used in the production of these antisera. Each rabbit had had horse serum from more than one horse at one time or another. The periods from the bleeding of the rabbits to the time of experiment varied very much, the

Table III. *Particulars of ten experiments with mixtures of two antisera in equal parts.*

No. of test	Particulars of component antisera		Mixed A.S. H.S. to A.S. ratio	
	A.S.	H.S. to A.S. ratio	Experimental	Calculation
1	1056 ZH	1 to 34	1 to 42	1 to 42·6
	1192 ZA	1 to 57		
2	1058 Q	1 to 19	1 to 26	1 to 24·6
	1103 J	1 to 35		
3	1058 Q	1 to 19	1 to 29	1 to 29·1
	1195 F	1 to 62		
4	1103 J	1 to 35	1 to 47	1 to 44·3
	1195 F	1 to 62		
5	1196 F	1 to 20	1 to 29	1 to 30·2
	1195 F	1 to 62		
6	1105 I	1 to 37	1 to 44	1 to 46·3
	1195 F	1 to 62		
7	1103 K	1 to 15	1 to 20	1 to 19·5
	1398 A	1 to 28		
8	1308 D	1 to 41	1 to 46	1 to 47·7
	1192 ZA	1 to 57		
9	1196 F	1 to 20	1 to 27	1 to 26·9
	1308 D	1 to 41		
10	1103 K	1 to 15	1 to 21	1 to 21
	1103 J	1 to 35		

longest period was $4\frac{1}{2}$ months, the shortest 2 months, *i.e.* sera of different ages were used. In fact it may almost be said that the antisera used were the result of all manner of doses and specimens of normal horse serum. The series did not include antisera which were either unusually quick or slow in precipitating. For this purpose an antiserum is defined as quick if, when it is diluted 1 in 10 and mixed with an optimal proportion of horse serum, particulation is evident within 10 minutes, and as slow if particulation has not taken place within $1\frac{1}{2}$ hours.

It might be suggested that, in the case of two anti-horse sera X and Y, of ratios 1 to x, and 1 to y, the antibody of X is not necessarily the same as the

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antibody of Y; and that the antigen leading to the formation of the antibody of X is not necessarily the same as the antigen leading to the formation of the antibody of Y. If there were two antigens and two antibodies the following would result.

Suppose:

$\overset{A}{\underset{X}{}}\overset{G}{}$ is the antigen $\longrightarrow$ antibody in X

$\overset{A}{\underset{Y}{}}\overset{G}{}$ is the antigen $\longrightarrow$ antibody in Y

$\overset{A}{\underset{X}{}}\overset{B}{}$ is the antibody of X, and $\overset{A}{\underset{Y}{}}\overset{B}{}$ is the antibody of Y.

Then the $\overset{A}{\underset{X}{}}\overset{G}{}$ in one part of horse serum would react with the $\overset{A}{\underset{X}{}}\overset{B}{}$ in x parts of X, and the $\overset{A}{\underset{Y}{}}\overset{G}{}$ in the same part of horse serum would react with the $\overset{A}{\underset{Y}{}}\overset{B}{}$ in y parts of Y.

Therefore to react with the $\overset{A}{\underset{X}{}}\overset{G}{}$ and $\overset{A}{\underset{Y}{}}\overset{G}{}$ in one part of horse serum, x parts of X, and y parts of Y would be required, *i.e.* $x + y$ parts of antiserum in all, for there is no $\overset{A}{\underset{X}{}}\overset{B}{}$ in Y, and no $\overset{A}{\underset{Y}{}}\overset{B}{}$ in X (if they differ).

So no amount of mixed antiserum less than $x + y$ parts could satisfy the antigens in 1 part of horse serum.

$$x + y \text{ is greater than } \frac{2xy}{x+y},$$

$$\because \quad \frac{x^2 + 2xy + y^2}{x+y} = x + y,$$

and if anything less than $x^2 + 2xy + y^2$ is divided by $x + y$, something less than $x + y$ results; and $2xy$ is obviously less than $x^2 + 2xy + y^2$.

So actually the antigen in 1 part of horse serum is satisfied by a less amount of antiserum than could possibly contain the necessary antibody, if there were two antigens and two antibodies. Experimentally 1 part of horse serum reacts with $\frac{2xy}{x+y}$ parts of mixed antiserum. There cannot be two separate antigens and antibodies concerned in the reaction.

Experiments with mixture of antigens and mixture of antibodies.

Exp. 3 (Table IV). 0·5 c.c. of anti-horse serum 1196 F (ratio 1 to 20), and 1 c.c. of anti-goat serum 1295 B (ratio 1 to 40) were mixed, and the volume made up to 30 c.c. by the addition of saline, yielding a 1 in 20 dilution of mixed antiserum, a 1 in 60 dilution of anti-horse serum, and a 1 in 30 dilution of anti-goat serum. Twice as much anti-goat serum is needed to react with 1 c.c. of goat serum as anti-horse serum to react with 1 c.c. of horse serum. 0·5 c.c. of

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horse serum and 0·5 c.c. of goat serum were mixed and the dilutions shown in column 2 arranged.

Table IV.

Tube	Mixed antigenic serum dilution	Mixed antigen to mixed A.S. ratio	Order of degree of particulation after 20 min.
Antigen C	1 in 500	—	—
12	1 in 500	1 to 25	—
11	1 in 555·6	1 to 27·8	2
10	1 in 600	1 to 30	1
9	1 in 666·7	1 to 33·3	3
8	1 in 750	1 to 37·5	—
7	1 in 857	1 to 42·8	—
6	1 in 1000	1 to 50	—
5	1 in 1200	1 to 60	—
4	1 in 1500	1 to 75	—
3	1 in 2000	1 to 100	—
2	1 in 3000	1 to 150	—
1	1 in 6000	1 to 300	—
A.S.C.	—	A.S. only	—

Controls unaffected.

Exp. 3, the one represented in Table IV, was a titration of mixed antigens against mixed antibodies. The antigens were horse serum and goat serum. The antibodies, obtained in both cases from rabbits, were anti-horse and anti-goat sera. Equal parts of horse serum and goat serum were mixed. The dilutions shown in the second column were obtained by making a series of dilutions from a primary 1 in 500 dilution, falling 0·5 c.c. at each step, and a series from a primary 1 in 600 dilution, also falling 0·5 c.c. at each step. The tubes used in the experiment were taken from the two series and arranged appropriately. Tube 10 was the optimal one, and in this tube both horse serum and goat serum are in a dilution of 1 in 1200. Anti-horse serum is in a dilution of 1 in 60; the horse serum to anti-horse serum ratio is, therefore, 1 to 20. Anti-goat serum is in a dilution of 1 in 30; the goat serum to anti-goat serum ratio is, therefore, 1 to 40. That is to say, horse serum and anti-horse serum are present in optimal proportions; similarly goat serum and anti-goat serum are present in optimal proportions. Now 1 c.c. of mixed antigen serum contains enough antigen to react completely with the antibody content of 10 c.c. of anti-horse serum, and of 20 c.c. of anti-goat serum; since 1 c.c. of mixed antigen serum contains 0·5 c.c. of both horse serum and goat serum. If the antigen content of 1 c.c. of horse serum, and of 1 c.c. of goat serum were contained in 1 c.c. of mixed antigen serum, then this 1 c.c. of mixed antigen serum would react with 20 c.c. of anti-horse serum plus 40 c.c. of anti-goat serum. Such a mixed antigen serum would have a ratio of 1 to 20 + 40, *i.e.* 1 to 60. This confirms the hypothesis that two separate antigens in an antigen serum react optimally with a mixed antiserum containing two different antibodies, when the ratio between antigen serum and antibody serum is 1 to $x + y$. Table IV shows that the optimal tube has a mixed antigen serum to mixed antiserum ratio of 1 to 30. This is an apparent anomaly, but 2 c.c. of mixed antigen serum are needed to furnish antigen to unite with 20 c.c. of anti-horse serum plus 40 c.c. of anti-goat serum. In the discussion as to whether there were two antigens and two anti-

bodies concerned in the titration of a mixture of antisera X and Y, the point was whether there were two antigens in the same 1 part of horse serum. The mixed antigen serum in this experiment is an artificially produced serum containing two antigens, and two parts of mixed serum are necessary to contain the antigens of 1 part of each of the components. If the antigens from 1 c.c. of both antigenic sera were contained in 1 c.c. of mixed antigenic serum, the ratio would be 1 to 60. A little thought will clear up this apparent anomaly.

Exp. 4 (Table V). Horse serum titrated against a mixture of 0·5 c.c. of anti-horse serum 1196 F (ratio 1 to 20), and 1 c.c. of anti-goat serum 1295 B (ratio 1 to 40). The volume was made up to 30 c.c., so the anti-horse serum is present in a dilution of 1 in 60, and the anti-goat serum is present 1 in 30.

Table V.

Tube	H.S. 1 in 600 (c.c.)	H.S. dilution	H.S. to anti-H.S. ratio	Order of degree of particulation after 40 min.
H.S.C.	1·0	1 in 600	—	—
10	1·0	1 in 600	1 to 10	—
9	0·9	1 in 666	1 to 11·1	—
8	0·8	1 in 750	1 to 12·5	—
7	0·7	1 in 857	1 to 14·3	—
6	0·6	1 in 1000	1 to 16·7	2
5	0·5	1 in 1200	1 to 20	1
4	0·4	1 in 1500	1 to 25	3
3	0·3	1 in 2000	1 to 33·3	—
2	0·2	1 in 3000	1 to 50	—
1	0·1	1 in 6000	1 to 100	—
A.S.C.	—	—	A.S.	—

Controls unaffected.

In this experiment Tube 5 was the first. It contained horse serum in a dilution of 1 in 1200, and in the mixed antiserum dilution anti-horse serum is present 1 in 60, so the horse serum to anti-horse serum ratio is 1 to 20, showing that horse serum and anti-horse serum reacted in optimal proportions in spite of the presence of anti-goat serum. A corresponding experiment was performed in which goat serum alone was titrated against the mixture of anti-horse and anti-goat sera. The result was similar, giving goat serum to anti-goat serum ratio of 1 to 40.

Exp. 5 (Table VI). Horse serum titrated against a mixture of equal parts of three anti-horse sera 1192 ZA, 1056 ZH, and 1103 K. 0·5 c.c. of each antiserum were mixed, and the volume immediately made up to 30 c.c. The mixed antiserum is therefore in a dilution of 1 in 20.

Exp. 5, Table VI, is an example of an experiment carried out on the same lines as the experiment of Table II. In Exp. 5, however, the antiserum is made up of equal parts of three different antisera, 1192 ZA, 1056 ZH and 1103 K. The ratios for these three antisera were respectively 1 to 57, 1 to 34, and 1 to 15. Here, again, the equal parts of antisera were mixed, and the volume immediately made up to 30 c.c. As there were 1·5 c.c. of mixed serum, the mixed anti-

Table VI.

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 25 min.
H.S.C.	1·0	1 in 400	—	—
10	1·0	1 in 400	1 to 20	—
9	0·95	1 in 421·0	1 to 21	—
8	0·9	1 in 444·4	1 to 22·2	—
7	0·85	1 in 470·6	1 to 23·5	—
6	0·8	1 in 500	1 to 25	2
5	0·75	1 in 533·3	1 to 26·7	1
4	0·7	1 in 571·4	1 to 28·6	3
3	0·6	1 in 666·7	1 to 33·3	—
2	0·5	1 in 800	1 to 40	—
1	0·4	1 in 1000	1 to 50	—
A.S.C.	—	—	A.S. only	—

serum was in a dilution of 1 in 20. 1 c.c. of this dilution was at once delivered into each of the tubes (except H.S.C.) which already contained appropriate amounts of horse serum dilution. There was a faint opalescence in the antiserum dilution, but the antiserum control showed only the faintest trace, and did not particulate even after many hours.

Again assuming that the antibody is the same in these three antisera, if we mix equal parts of antisera X, Y, Z of ratios 1 to x, 1 to y, and 1 to z respectively, what is the ratio of the resulting antiserum?

1 part of A.S. X reacts with $\frac{1}{x}$ parts of H.S.

1 „ A.S. Y „ „ $\frac{1}{y}$ „ „

1 „ A.S. Z „ „ $\frac{1}{z}$ „ „

Then 3 parts of mixed A.S. react with $\frac{1}{x}+\frac{1}{y}+\frac{1}{z}$ parts of H.S.;

i.e. 1 part „ „ „ $\frac{1}{3x}+\frac{1}{3y}+\frac{1}{3z}$ „ „

i.e. 1 „ „ „ „ $\frac{xy+xz+yz}{3xyz}$ „ „

$\therefore$ $\frac{3xyz}{xy+xz+yz}$ parts of mixed A.S. react with 1 part of H.S.

$\therefore$ H.S. to A.S. ratio of mixed A.S. is 1 to $\frac{3xyz}{xy+xz+yz}$.

In Exp. 5 the ratios of the three best tubes were, in order, 1 to 26·7, 1 to 25, and 1 to 28·6. The ratio of 1 to 26 was given to the mixed antiserum.

Substituting the ratio values of the component antisera (57, 34, and 15) in the formula, H.S. to A.S. is

$$1 \text{ to } \frac{3xyz}{xy+xz+yz},$$

we have

$$1 \text{ to } \frac{3\times 57\times 34\times 15}{57\times 34+57\times 15+34\times 15},$$

i.e. 1 to 26·7, which agrees with the experimentally determined ratio.

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Table VII is self-explanatory. Five mixtures, each containing three antisera, were titrated, as in Table IV. These mixtures were made from amongst

Table VII. *Particulars of five experiments with mixtures of three antisera in equal parts.*

No. of test	Particulars of component antisera: A.S.	Particulars of component antisera: H.S. to A.S. ratio	Mixed A.S. H.S. to A.S. ratio: Experimental	Mixed A.S. H.S. to A.S. ratio: Calculation
1	1192 ZA	1 to 57	1 to 26	1 to 26·7
	1103 K	1 to 15		
	1056 ZH	1 to 34		
2	1058 Q	1 to 19	1 to 33	1 to 30·8
	1195 F	1 to 62		
	1103 J	1 to 35		
3	1105 I	1 to 37	1 to 31	1 to 32·2
	1195 F	1 to 62		
	1196 E	1 to 20		
4	1056 ZH	1 to 34	1 to 36	1 to 36·3
	1192 ZA	1 to 57		
	1398 A	1 to 28		
5	1103 J	1 to 35	1 to 25	1 to 25·1
	1103 K	1 to 15		
	1308 D	1 to 41		

the ten antisera included in Table III; in addition another antiserum, 1196 E appears in Test 3 in Table VII. The previous remarks on this group of antisera apply equally whether they be used in mixtures of two or three.

Exp. 6 (Table VIII). Horse serum titrated against a mixture of equal parts of ten anti-horse sera. 0·5 c.c. of each antiserum were mixed, and the volume was immediately made up to 100 c.c., *i.e.* 5 c.c. of the mixed antiserum in 100 c.c. The mixed antiserum is, therefore, in a dilution of 1 in 20.

Table VIII.

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation: H.S. *A*	H.S. *B*	H.S. *C*	Mixed H.S.
H.S.C.	1·0	1 in 400	—	—	—	—	—
12	1·0	1 in 400	1 to 20	—	—	—	—
11	0·95	1 in 421	1 to 21	—	—	—	—
10	0·9	1 in 444·4	1 to 22·2	—	—	—	—
9	0·85	1 in 470	1 to 23·5	—	—	—	—
8	0·8	1 in 500	1 to 25	—	—	—	—
7	0·75	1 in 533·3	1 to 26·7	1	1	1	1
6	0·7	1 in 571	1 to 28·6	—	—	—	—
5	0·65	1 in 616	1 to 30·8	—	—	—	—
4	0·6	1 in 666·7	1 to 33·3	—	—	—	—
3	0·5	1 in 800	1 to 40	—	—	—	—
2	0·4	1 in 1000	1 to 50	—	—	—	—
1	0·3	1 in 1333·3	1 to 66·7	—	—	—	—
A.S.C.	—	—	A.S. only	—	—	—	—

Controls unaffected.

In Table VIII are shown the results of four experiments, in each of which the antiserum used was a mixed one, made up of equal parts of ten antisera.

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These ten antisera are the ones from which the antiserum mixtures were made in the earlier experiments. An eleventh antiserum, 1196 E, was used in Test 3, Table VII, but at the time of these final experiments this antiserum was used up, and so could not be included. The first three experiments (Table VIII) were titrations of three different specimens of normal horse serum *A*, *B*, and *C* against the antiserum mixture. In the fourth experiment a mixture of the horse sera *A*, *B*, and *C*, in equal parts, was titrated against the antiserum mixture. 1 c.c. of each horse serum were mixed, and immediately the volume made up to 60 c.c.; *i.e.* 3 c.c. in 60 c.c., representing a dilution of 1 in 20 of the mixed horse serum. The dilution of 1 in 400 was made from this. The results of the titrations are shown in the last four columns of the table. The tube with a ratio of 1 to 26·7 was the first in each experiment. The series was so close that further classification of the tubes was doubtful. This tube was the first in every case, whilst those next to it, on either side, were always ahead of the rest. If the ratio is given as 1 to 27, there is little error.

In order to work out a calculated value for the ratio of the mixed antiserum made from ten antisera, the following method was adopted:

1195 F (1 to 62) 1058 Q (1 to 19) 1103 J (1 to 35)	1 to 30·8		
1192 ZA (1 to 57) 1103 K (1 to 15) 1056 ZH (1 to 34)	1 to 26·7	1 to 27·9	
1196 F (1 to 20) 1105 I (1 to 37) 1398 A (1 to 28)	1 to 26·6		
1308 D (1 to 41)			

The sera were arranged in groups of three, leaving the tenth one over. Dealing with each group in turn, and substituting values in the formula 1 to $\frac{3xyz}{xy + xz + yz}$, the value of the ratio of each group was obtained. This yielded three values, which in turn were treated in a like manner. A value for the ratio of the first nine antisera was the result. The above illustration explains the method. The tenth antiserum had to be brought in: If *A* parts of antiserum *X* (ratio 1 to *x*) are added to *B* parts of antiserum *Y* (ratio 1 to *y*), what is the ratio of the resulting mixed antiserum?

1 part of *X* reacts with $\frac{1}{x}$ parts of H.S.

A ,, *X* ,, $\frac{A}{x}$,, ,,

1 ,, *Y* ,, $\frac{1}{y}$,, ,,

B ,, *Y* ,, $\frac{B}{y}$,, ,,

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Therefore $A + B$ parts of mixed A.S. react with $\frac{A}{x} + \frac{B}{y}$ parts of H.S., *i.e.* with $\frac{Ay + Bx}{xy}$ parts of H.S.

Therefore $\frac{(A + B)\,xy}{Ay + Bx}$ parts of mixed A.S. react with 1 part of H.S.

$$\text{Ratio of the A.S. is 1 to } \frac{(A + B)\,xy}{Ay + Bx}.$$

We have nine parts of mixed antiserum, with a ratio of 1 to 27·9, and one part of the tenth antiserum, ratio 1 to 41. Substituting these values in the above formula we get:

$$\text{Ratio is 1 to } \frac{(9 + 1)\,(27{\cdot}9 \times 41)}{9 \times 41 + 1 \times 27{\cdot}9}, \text{ } i.e. \text{ 1 to 28·8.}$$

The experimentally determined ratio of 1 to 27 agrees well with the value found by the above calculation. The difference between the two figures is very slight; the calculation was made from ten ratios, which were not absolute in any case, although very nearly so. These four experiments again demonstrate that the normal serum from any horse gives the same results. Together with the experiments in which goat and anti-goat serum were concerned they show that when horse serum and anti-horse serum are present in optimal proportions, the reaction is not affected by the very various other constituents of the participating sera. The constituent substances, protein and otherwise, of horse and goat serum must be different in many ways, and the constituents of the serum must vary from horse to horse, and from rabbit to rabbit. A mixture of many sera, therefore, must contain an assortment of constituents differing very materially in many points from the constituents of a relatively simpler mixture of a single specimen of horse serum and one anti-horse serum. It is worthy of note that from amongst the very numerous constituents of the sera of nine rabbits and three horses, antigen and antibody continued to seek out each other and react in definite proportions. And when goat and horse serum together with their corresponding antisera were mixed, or when only one of these antigenic sera was present with both antisera, the individual antigens reacted with the corresponding antibodies in proportions which were unaffected by the presence of a complexity of other substances. These definite proportions were unaffected by any of the changes which were made in the constitution of the sera in the surrounding medium.

An examination of the results of the experiments shown in the foregoing tables shows that mixtures of antisera react with horse serum according to the formulae. These results are consistent with the idea that the antisera contain the same antibody. The calculated and experimental figures agree very well. The greatest difference between the two being 2·7 (in Test 4, Table III). No ratio assigned to an antiserum is absolute, although obviously it is very nearly so. The antiserum dilutions in the experiments varied; sometimes the dilution was 1 in 10, at other times 1 in 20. Different specimens of horse serum

were used in the course of the experiments. One specimen did not seem to differ from another. Dean and Webb pointed out that all normal horse sera behaved alike in experiments of this kind.

As they have become available other specimens of antiserum have been examined and have given confirmatory results.

Exp. 7 (Table IX). A mixture of a quickly acting and a normally acting antiserum titrated against horse serum. Horse serum, in falling quantities, titrated against a mixed antiserum consisting of equal parts of anti-horse sera 1396 C (ratio 1 to 14) and 1193 G (ratio 1 to 35). The mixed antiserum dilution is 1 in 20.

Table IX.

Tube	H.S. 1 in 300 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 25 min.
H.S.C.	1·0	1 in 300	—	—
11	1·0	1 in 300	1 to 15	—
10	0·9	1 in 333·3	1 to 16·7	—
9	0·8	1 in 375	1 to 18·75	—
8	0·7	1 in 428	1 to 21·4	—
7	0·65	1 in 461	1 to 23·0	—
6	0·6	1 in 500	1 to 25	2
5	0·55	1 in 545	1 to 27·3	1
4	0·5	1 in 600	1 to 30	2
3	0·4	1 in 750	1 to 37·5	—
2	0·3	1 in 1000	1 to 50	—
1	0·2	1 in 1500	1 to 75	—
A.S.C.	—	—	—	—

Controls unaffected.

In the description of Table III it was stated that no very quick, or very slow antisera had been used. Occasionally an unusually quick acting antiserum is obtained. Two such antisera were on hand at one time, and the opportunity was taken of testing their behaviour in mixtures. Anti-horse serum 1396 C had a ratio of 1 to 14. When this antiserum in a dilution of 1 in 20 was titrated against falling quantities of horse serum, particulation was very advanced in many tubes in less than 5 minutes; so advanced as to be past accurate reading. Used in a dilution of 1 in 40, particulation took place in the optimal tube in about 15 minutes. This quickly acting antiserum was mixed with anti-horse serum 1193 G (ratio 1 to 35) in equal parts, and titrated against horse serum, as shown in Table IX, Exp. 7. Antiserum 1193 G had been previously used in mixtures with other antisera which were like those used in the earlier experiments, neither unusually quick, nor slow; it had behaved according to the formulae. Antiserum 1193 G was quite a normal antiserum. Substituting the values of the ratios of these two antisera in the formula, ratio is

$$1 \text{ to } \frac{2xy}{x+y}; \text{ we have } 1 \text{ to } \frac{2 \times 14 \times 35}{14+35}; \textit{ i.e. } 1 \text{ to } 20.$$

A tube with a horse serum to mixed antiserum ratio of 1 to 27·3 was the first in the experiment, so this mixed antiserum was not obeying the formula. 0·5 c.c. of each of the component antisera was present in the 20 c.c. of mixed antiserum dilution. They were present in an individual dilution of 1 in 40. The ratio of

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antiserum 1396 C, the quick one, was 1 to 14. 1 c.c. of a 1 in 40 dilution of this antiserum would be in optimal proportions when mixed with 1 c.c. of a 1 in 560 dilution of horse serum. The tube which particulated first in Table IX, Exp. 7, had a horse serum dilution of 1 in 545. It would appear that antiserum 1396 C was itself satisfying the antigen in that tube. Similar results were obtained when antiserum 1396 C was mixed with another antiserum, which was a normally acting one. Anti-horse serum 1308 F, with a ratio of 1 to 24, was the second very quickly acting antiserum. When mixed with normally acting antisera, it behaved as did antiserum 1396 C, and seemed to be itself satisfying the antigen. When the two very quickly acting antisera, 1396 C and 1308 F, were mixed in equal parts, and titrated against horse serum, the ratio of the optimal tube agreed with the ratio obtained by using the formula. This would seem to show that there is a difference between the antibody in an unusually quick acting antiserum and the antibody in the antisera which behave normally. It would appear that the antibody of the quickly acting antisera has a greater affinity for antigen than has the antibody of the normally acting antisera.

Exp. 8 (Tables X and X A). A mixture of a slowly acting and a normal antiserum titrated against horse serum.

Horse serum in varying amounts titrated against a mixed antiserum, consisting of equal parts of anti-horse sera 1196 G, a normally acting antiserum (ratio 1 to 29), and 1419 C, a slowly acting antiserum (ratio 1 to 10). Mixed antiserum dilution 1 in 10.

Table X. *Primary zone.*

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 45 min.
H.S.C.	1·0	1 in 400	—	—
10	1·0	1 in 400	1 to 40	—
9	0·9	1 in 444·4	1 to 44·4	—
8	0·85	1 in 470	1 to 47	—
7	0·8	1 in 500	1 to 50	—
6	0·75	1 in 533·3	1 to 53·3	1
5	0·7	1 in 571	1 to 57·1	2 Very close
4	0·65	1 in 615	1 to 61·5	—
3	0·6	1 in 666·7	1 to 66·7	—
A.S.C.	—	—	A.S. only	—

Controls unaffected.

Table X A. *Secondary zone.*

Tube	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 50 min.
H.S.C.	1 in 10	—	—
1	1 in 10	1 to 1	—
2	1 in 20	1 to 2	—
3	1 in 30	1 to 3	—
4	1 in 40	1 to 4	2
5	1 in 50	1 to 5	1
6	1 in 60	1 to 6	—
7	1 in 70	1 to 7	—
8	1 in 80	1 to 8	—
A.S.C.	—	A.S. only	—

Controls unaffected.

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Some antisera, used in a dilution of 1 in 10, react very slowly with horse serum. They will take from about 3 hours upwards to show particulation when mixed with horse serum in optimal proportions. Such slowly acting antisera usually have ratios less than the ratios of the antisera, which react within a more normal time. The ratio of a slowly acting antiserum is usually 1 to something less than 12. Anti-horse serum 1419 C was one of these slowly acting antisera, and its ratio was 1 to 10. When anti-horse serum 1196 G (ratio 1 to 29), a normally acting one, was mixed, in equal parts, with antiserum 1419 C, the resulting titration against horse serum showed a "double zone," such as those described by N. E. Goldsworthy (1928). There was an optimal tube with a ratio in the region of 1 to 50, and a second optimal tube with a ratio of 1 to something less than 10. Further tests showed that the ratios of the optimal tubes in the two zones were respectively 1 to 55, and 1 to 5. Tables X and X A outline the final tests on the two zones. The optimal tube of the zone in Table X particulated shortly before the optimal tube of the zone in Table X A.

In the primary zone, the one in Table X, if antiserum 1196 G were alone satisfying the antigen present, a tube containing a horse serum dilution of 1 in 580 would contain antigen and antibody in optimal proportions; for antiserum 1196 G is present in the mixed antiserum dilution in an individual dilution of 1 in 20. Experimentally the optimal point lay between Tubes 6 and 5. Tube 5 contained horse serum in a dilution of 1 in 571. It would appear that antiserum 1196 G, the one which reacted within a normal time, was itself satisfying the antigen in the primary zone.

At the moment I have no satisfactory explanation of the ratio of the optimal tube in Table X A. In the tubes of this experiment there is present such a large amount of horse serum that the content of adsorbable matter in the tubes cannot be regarded as constant, although the quantity of antiserum is constant. No doubt horse serum contains adsorbable matter, and where such a large amount of horse serum is present, it is likely that the concentration of adsorbable matter is varying considerably from tube to tube (see Part II). In the rest of the experiments in this part, and in Part II, the dilution of horse serum used is so great as to make the amount of adsorbable material furnished by the horse serum negligible. In all other experiments where antiserum is constant it has been quite proper to regard the adsorbable matter as constant. It is probable, therefore, that the figure for the ratio of the optimal tube in the secondary zone, viz. 1 to 5, is inaccurate to a fairly considerable extent. No "double zones" were found in the individual titrations of the constituent antisera, so it appears probable that the appearance of the secondary zone in the titration of the mixed antisera is due to the individual action of the antibody of antiserum 1419 C, the slowly acting one.

Exp. 9 (Table XI). A mixture of a degenerated and a normal antiserum titrated against horse serum.

Horse serum titrated against a mixture of equal parts of anti-horse serum

1308 E (ratio 1 to 50; previous ratio 1 to 40), and anti-horse serum 1196 F (ratio 1 to 20). Antiserum dilution 1 in 20.

Table XI.

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation	
H.S.C.	1·0	1 in 400	—	—	
10	1·0	1 in 400	1 to 20	—	
9	0·9	1 in 444·4	1 to 22·2	—	
8	0·8	1 in 500	1 to 25	3	
7	0·7	1 in 571·4	1 to 28·6	1	Zone. After 1 hour
6	0·6	1 in 666·7	1 to 33·3	2	
5	0·5	1 in 800	1 to 40	—	
4	0·4	1 in 1000	1 to 50	—	
3	0·3	1 in 1333·3	1 to 66·7	2	
2	0·2	1 in 2000	1 to 100	1	Zone. After 1¼ hours
1	0·1	1 in 4000	1 to 200	2	
A.S.C.	—	—	A.S. only	—	

Controls unaffected.

The ratio of an antiserum may change after a time. This alteration is always in one direction; the ratio changing to a greater figure, the antiserum becoming weaker. Thus within three weeks the ratio of anti-horse serum 1308 E was found to have changed from 1 to 40 to 1 to 50. This "degenerated" antiserum was mixed in equal parts with anti-horse serum 1196 F, and titrated against horse serum. Antiserum 1196 F (ratio 1 to 20) had maintained its ratio; it was one of the ten included in Table III. Table XI gives the results of the titration of the mixture of these two antisera. According to the formula, ratio is 1 to $\frac{2xy}{x+y}$, this mixed antiserum should have a ratio of 1 to 28·6. At the end of 1 hour, Tube 7, with a ratio of 1 to 28·6, was the leading tube. After 1¼ hours another zone was apparent. Tube 2 was the optimal one in this secondary zone. The horse serum dilution in this tube was 1 in 2000, and the individual dilution of antiserum 1308 E (ratio 1 to 50) was 1 in 40; so Tube 2 contained horse serum and antiserum 1308 E in optimal proportions. It is possible that in Tube 2 the antibody of 1308 E was alone satisfying the antigen present. That the optimal tube of the primary zone was in agreement with the formula shows that the antibodies in the two antisera were reacting with the same antigen. The explanation of the appearance of a second zone, in which the antibody of antiserum 1308 E might be acting alone, is not certain. It may be that the antibody of 1308 E had a greater affinity for the antigen than had the antibody of 1196 F. In the optimal tube of the primary zone there was not enough of the antibody from 1308 E to satisfy the antigen present, and the antibody of 1196 F made up the deficiency. This experiment was repeated and the results confirmed. An experiment with another degenerated antiserum gave similar results.

No such secondary zones were found when mixtures of normally acting antisera, included in Table III, were titrated against horse serum. There was

no evidence that the antibodies of these antisera had different affinities for antigen.

The experiments with very slowly and very quickly acting antisera, and with degenerated ones, have not been extensive enough to justify the formation of definite conclusions. They do seem to indicate that the antibodies in these types vary in some way from the antibody in the majority of antisera (the normally acting ones). It is possible that the antibodies in the very slow and quick ones may be very different from that in the normal antisera. In the degenerated specimens the antibody appears to differ from the normal antibody only as regards affinity for antigen, which points to a slight difference in the structure of the two antibodies. The appearance of "double zones," such as those described by N. E. Goldsworthy, suggests the thought that zones appearing in the titration of a single specimen of antiserum may be due to the presence in that antiserum of more than one antibody; either of closely similar antibodies or of very different ones.

Conclusions.

1. The antibody in all the normally acting antisera produced in rabbits by the injection of normal horse serum reacts optimally in definite proportions with some antigen in normal horse serum, and these proportions do not appear to be influenced by other serum constituents.

2. There has been no evidence that the antibodies in different normally acting antisera have varying affinities for antigen.

3. It would appear, therefore, that the antibody in the normally acting antisera is always the same.

4. The antigenic content of different specimens of normal horse serum is apparently constant, qualitatively and quantitatively.

5. Abnormally acting antisera seem to contain antibodies differing from those present in the other types of antisera. The degree of difference may possibly be great or small.

6. The presence of more than one type of antibody in the same specimen of antiserum may explain the phenomena of "Zones."

7. The titration of mixtures of antisera against horse serum is a useful method of comparing the antibodies in the antisera.

PART II. THE NATURE OF THE REACTION BETWEEN ANTIGEN AND ANTIBODY IN THE SERUM PRECIPITATION REACTION.

The nature of the reaction between antigen and antibody has been a matter of contention since Ehrlich and Bordet propounded their different theories to explain this phenomenon. Ehrlich conceived the reaction as one between two substances following the laws which govern the ordinary chemical behaviour of crystalloids. Bordet (1903) sought to explain the quantitative discrepancies,

which occurred in experiments, by putting forward the adsorption theory. He maintained that the reaction between antigen and antibody was in every way analogous to colloidal reactions, which are governed by the laws of adsorption. The phenomena observable in the serum precipitation reactions seem to indicate that in the explanation of this reaction, at least, both theories contribute their part.

The experiments described in this paper appear to show that the serum precipitation reaction, as performed *in vitro*, consists of two different stages. The first stage is a reaction between antigen and antibody resulting in the formation of an antigen-antibody complex. This complex is adsorbent, and the second stage is the adsorption by the complex of some of the serum elements other than antigen and antibody. The first stage follows the laws of chemical equivalents, the second follows the laws of adsorption.

It would appear that when we follow the course of flocculation in a precipitation experiment, such as those described by Dean and Webb (1926), we are witnessing the adsorption of non-specific serum constituents by the antigen-antibody complex. The increase in size of the particles follows from the adsorption of more and more of the adsorbable material in the mixture, and from the fusion of the particles. The observation of the speed of particulation, and so of adsorption by the complex, serves as an index of the reaction between antigen and antibody. The speed of particulation is dependent on the amount of the complex, and upon the amount and nature of the adsorbable matter present.

THE FIRST STAGE.

The reaction between antigen and antibody.

Dean and Webb (1926) found that, when horse serum and anti-horse serum were mixed in optimal proportions, neither antigen nor antibody could be found in the supernatant fluid in appreciable quantity. When antibody was in excess the equivalent proportion of antibody was present in the supernatant fluid. If antigen were in excess only a small amount, if any, remained in the supernatant fluid; an amount bearing no relation to the equivalent amount. These workers state that the formation of a precipitate in a mixture of horse serum and anti-horse serum is delayed or inhibited, if either ingredient is in excess. Their experiments were performed by titrating a constant amount of antiserum against falling quantities of horse serum. If the reverse procedure is adopted, and the amount of antiserum is varied, whilst the horse serum is kept constant, a most interesting and instructive result follows. The tubes in which antibody is in excess do not, in this case, particulate after the one in which the ingredients are in optimal proportions, but the one containing the largest amount of antiserum is the first to show particulation.

Exp. 1 (Table I). "Reverse" Experiment. (Horse serum constant.)

Anti-horse serum 1193 E (ratio 1 to 34), in falling quantities titrated against a constant amount of horse serum in a dilution of 1 in 340.

Table I.

Tube	A.S. dilution	H.S. to A.S. ratio	Order of particulation
10	1 in 5·0	1 to 68	1 (after 7 min.)
9	1 in 5·5	1 to 61·2	2
8	1 in 6·25	1 to 54·4	3
7	1 in 7·14	1 to 47·6	4
6	1 in 8·33	1 to 40·8	5
5	1 in 10·0	1 to 34·0	6
4	1 in 12·5	1 to 27·2	7
3	1 in 16·7	1 to 20·4	8
2	1 in 25·0	1 to 13·6	9
1	1 in 50·0	1 to 6·8	10

Controls with saline and horse serum; and with saline and antiserum were unaffected.

Exp. 1, Table I, gives the particulars of such a "reverse" experiment. It was performed in the same way as the usual antigen-antibody titration, described by Dean and Webb, save that, in this case, the amount of antigen was constant, whilst the antibody was in falling quantities. The ratio of the antiserum, as determined by the usual method of titration, was 1 to 34; so that the tube in which antigen and antibody were in optimal proportions was No. 5. Tube 10 showed particles in the shortest time; it was followed by the ninth and eighth tubes in order, and so on down the series. Tube 5 took its place in this orderly progression of particulation. Tubes in the antigen excess region took a much longer time to show the reaction, and the further from the optimal a tube was placed, the less did the reaction proceed to completion. The fact that, in the tubes containing excess of antigen, the reaction does not go to completion, was still more apparent in an experiment in which the amount of antiserum was doubled in each succeeding tube; after 3 days the tubes containing excess of antigen had not precipitated completely, whilst those containing excess of antibody all reached complete precipitation in 2 hours.

Exp. 1 shows that antibody in excess has not delayed or inhibited the reaction. As the amount of antigen is constant in every tube the tubes vary from each other only in antiserum content. It might be suggested, as the speed of particulation has increased with the increase in the antiserum content, that increasing amounts of antibody have reacted with the antigen. That this is not so is proved by the excess of antibody, in equivalent amount, being traceable in the supernatant fluid. Dean and Webb (1926) pointed out that such excess could as a rule be found. With a view to confirming this observation the remaining experiments, described in this paper, were undertaken, and ample confirmation was obtained. The particular antiserum 1193 E used in Exp. 1 was used in experiments designed to demonstrate the excess of antibody in a supernatant fluid. Therefore, in all the tubes of Exp. 1, from 5 to 10 inclusive, a constant amount of antigen has reacted with a constant amount of antibody, yielding a constant amount of antigen-antibody complex.

The reaction between antigen and antibody does not seem to be an adsorption for the following reasons:

(1) If antigen were the adsorbent and antibody the adsorbable material, then, with a constant concentration of adsorbable antibody, we should expect

the amount of antibody adsorbed to increase logarithmically with increase in the amount of the adsorbent (antigen) (Freundlich, *Colloid and Capillary Chemistry*, p. 176, lines 5 and 6, English translation). Such conditions obtain in an experiment where varying quantities of antigen are titrated against a constant amount of antiserum, and so a constant amount of antibody—the usual titration performed by Dean and Webb (cf. Exp. 1, Part I). That the amount of antibody reacting with antigen has not increased logarithmically with increase in antigen is proved by the fact that excess of antibody is demonstrable in the supernatant fluid in equivalent amount. In the "reverse" experiments a constant amount of antigen has reacted always with the same quantity of antibody, despite increasing concentration of antibody.

(2) If antibody were adsorbent, and antigen were adsorbable material, we should expect a constant amount of antibody to adsorb increasing amounts of antigen with increasing antigen concentration. This increase should be logarithmic. Further the resulting antigen-antibody complexes would not be alike; the proportion of antigen to antibody in the complexes would increase with the increase in antigen concentration. Excess of antibody being traceable in the supernatant fluid in equivalent amount proves that a constant amount of antibody has not reacted with varying amounts of antigen, and that the antigen-antibody complex always contains antigen and antibody in the same relative proportions.

That antigen in excess is not traceable in the supernatant fluid does not contradict the preceding reasonings. Excess of antigen seems to be adsorbable by the antigen-antibody complex, and to exercise a protective action on the micellae of the complex, decreasing the sensitiveness of the complex towards the adsorbable matter present in the mixtures, and so preventing the reaction going to completion. An analogy of the adsorption of excess of one of the constituents in a chemical reaction by the resulting compound is furnished by the reaction between barium nitrate and sulphuric acid. In this case acid in excess is adsorbable by the resulting compound, and is not traceable in the supernatant fluid in equivalent amount.

Heidelberger and Kendall (1929), working with a non-protein antigen from pneumococcus and homologous antisera, have suggested that, in this case, the reaction between antigen and antibody is following a modification of the laws of mass action. They state that more than one type of complex is formed: one part of antibody being capable of reacting with one part of antigen to yield one type of complex, whilst one part of antibody may react with two parts of antigen to form a second type of complex, and so on. The finding of excess of antibody, in equivalent amount, in the supernatant fluid shows that the above conception does not explain the reaction between horse serum and anti-horse serum.

THE SECOND STAGE.

The adsorption by the antigen-antibody complex.

In Exp. 1, Table I, the speed of particulation must depend on some variation in the antiserum content of the tubes, other than their antibody content. If we regard the antigen-antibody complex as adsorbent, and some of the constituents of the horse and anti-horse sera, other than antigen and antibody, as adsorbable materials, we find that the results of Exp. 1 are in accordance with the laws governing the velocity of adsorption in solution, viz. the initial velocity of adsorption is proportional to the concentration of adsorbable matter in the solution (cf. Freundlich, *Colloid and Capillary Chemistry*, p. 170, English translation). In Exp. 1, Tubes 5 to 10 inclusive contain a constant amount of the adsorbent antigen-antibody complex, and increasing amounts of adsorbable material in the form of antiserum, as distinct from antibody. The greater the concentration of antiserum, the greater has been the speed of particulation. "Reverse" experiments with eight different antisera yielded similar results. That the speed of particulation is proportional to the concentration of the adsorbable matter present in a mixture, is strikingly shown by the rest of the experiments to be described in this paper.

There is, therefore, this great difference in the behaviour of antigen and antibody. Antigen in excess is not left behind in the supernatant fluid in equivalent amount; it delays the speed of particulation, and prevents the reaction going to completion. Antibody in excess is found in the supernatant fluid, in equivalent amount, and excess does not prevent completion of precipitation. Antigen is of such a nature that, in excess, it exercises a protective action on the micellae of the complex, and decreases the sensitiveness of the complex towards the adsorbable matter present. No such protective action takes place when antibody is in excess.

Antigen and antibody react in equivalent proportions to produce definite amounts of the antigen-antibody complex. If the amount of the complex is constant in an experiment the speed of particulation is proportional to the amount of adsorbable matter present (Exp. 1, Table I).

The delay in particulation in the tubes containing excess of antibody in an experiment where a constant amount of antiserum is titrated against falling quantities of horse serum (the usual titration performed by Dean and Webb (cf. Exp. 1, Part I)), is not due to excess of antibody, but to the deficiency of antigen. There is not enough antigen to react with all the antibody present, and as the amount of antigen diminishes, so does the amount of the resulting antigen-antibody complex decrease. The concentration of antiserum, and so of adsorbable material, as distinct from antibody, is the same in every tube. The tube containing the largest amount of antigen-antibody complex, and no excess of antigen, is the first to particulate. This is the "Optimal Tube," in which antigen and antibody are present in the proportions necessary to react with each other completely, without excess of either. Thus we have this further

fact: the speed of particulation is dependent on the amount of the adsorbent antigen-antibody complex present in the mixture. The greater the amount of complex, the greater the speed of particulation.

EXPERIMENTS TO SHOW THE PRESENCE OF EXCESS OF ANTIBODY IN SUPERNATANT FLUIDS.

It is often found that two different anti-horse sera, having the same ratio and behaving normally in mixtures, take varying times to particulate, when they are mixed with horse serum in optimal proportions, and in the same concentrations. If a mixture of the two antisera is titrated against horse serum it can be shown that they both contain the same amount of the same antibody (Part I). Differences in the constituents of the antisera, other than their antibody content, appear to be responsible for the variation in the speed in particulation. These differences also affect the weights of the resulting precipitates. In Table VIII on p. 488 of Dean and Webb's paper (1926) are shown the weights of the resulting precipitates when two antisera of the same ratio were mixed in the same proportions with the same amount of horse serum. Both antisera had ratios of 1 to 50, yet the weight of precipitate in one case was about $1\frac{1}{2}$ times the weight in the other. The antiserum containing the more adsorbable matter particulates the more rapidly. The relation between the speed of particulation and the amount of adsorbable matter present is most strikingly shown in experiments performed to demonstrate the presence of antibody in a supernatant fluid. If a quantity of horse serum is mixed with, say, twice the equivalent amount of antiserum, and after the completion of the reaction the whole is centrifuged and the supernatant fluid is decanted from the precipitate, it is possible to show the presence of the excess of antibody in the supernatant fluid. On mixing such a supernatant fluid with a further amount of antigen (horse serum), particulation may not take place for many hours, perhaps not overnight. Hartley (1925), with extracted sera, showed that when most of the serum elements, other than antigen and antibody, were removed, precipitation no longer occurred.

When falling quantities of horse serum are titrated against a supernatant fluid of this sort, the particulation may be so slow and the size of the particles so small, that it may be impossible to detect in which tube of a series the optimal particulation has occurred. The first reaction in the mixture has so depleted it of its content of adsorbable matter that, when the supernatant fluid is allowed to react with a further amount of antigen (horse serum), there is relatively so little adsorbable material present as to make the reaction very slow and difficult to read. However, if adsorbable matter is included in the mixture, the reaction is hastened and the size of the particles increased, so that the optimal tube is more easily picked out. Adsorbable matter may be added in varying ways. Normal rabbit serum may be added, although the content of adsorbable material varies greatly in different normal rabbit sera. The supernatant fluid may be mixed with a further amount of the antiserum, and

titrated against horse serum. These procedures provide adsorbable elements which hasten particulation and facilitate the reading of the result.

H. R. Dean (1912, 1916) showed that the addition of guinea-pig serum would increase the amount of precipitate, and Hartley (1925) used normal rabbit serum to increase the amount of precipitate.

Exp. 2 (Table II). Titration of a supernatant fluid, with the addition of adsorbable material in the form of more antiserum.

10 c.c. of a 1 in 370 dilution of horse serum, and 10 c.c. of a 3 in 10 dilution of antiserum 1105 I (ratio 1 to 37) were mixed, and centrifuged after 16 hours. The supernatant fluid was decanted from the precipitate. 5 c.c. of the supernatant fluid were mixed with 5 c.c. of a 1 in 10 dilution of antiserum 1105 I, and titrated against falling quantities of horse serum.

Table II.

Tube	H.S. 1 in 300 (c.c.)	H.S. dilution	Order of degree of particulation
10	1·0	1 in 300	After 1 hour 30 min.
9	0·9	1 in 333·3	—
8	0·8	1 in 375	1 } Very close
7	0·7	1 in 428	1 } Very close
6	0·6	1 in 500	—
5	0·5	1 in 600	—
4	0·4	1 in 750	—
3	0·3	1 in 1000	—
2	0·2	1 in 1500	—
1	0·1	1 in 3000	—

The supernatant fluid was opalescent. Controls of horse serum and saline, and of the antiserum mixture and saline were set up. The antiserum control was faintly opalescent, but did not change even after many hours.

Exp. 2, Table II, is an example of an experiment, where adsorbable matter, in the form of further antiserum, is added to the supernatant fluid. A 3 in 10 dilution of anti-horse serum 1105 I was mixed with an equal amount of a 1 in 370 dilution of horse serum. The ratio of this antiserum, previously determined, was 1 to 37, so the mixture contained three times the antibody necessary to react with the antigen present. In the 20 c.c. of the primary mixture the dilution of the antiserum became 3 in 20. If only one of the three parts of antibody reacted with the antigen, the other two parts should remain in the supernatant fluid, in amount corresponding to an antiserum dilution of 2 in 20, or 1 in 10. On mixing equal parts of the supernatant fluid and a 1 in 10 dilution of the antiserum, there should result the equivalent of a 1 in 10 dilution of antiserum. A large part of the adsorbable matter had been removed during the first stage of the experiment, but that the supernatant still contained antibody in amount equal to a 1 in 10 dilution of the antiserum, *i.e.* the equivalent amount, was shown by the titration. It was impossible to decide between Tubes 8 and 7. A tube containing a 1 in 370 dilution of horse serum would react optimally with a 1 in 10 dilution of antiserum, and the experimental result of the titration agrees well with such figures. This experiment demonstrates that the excess of antibody remains in the supernatant fluid in equivalent amounts. Had the

supernatant fluid contained no antibody, then the dilution of the antiserum used in the titration would have been 1 in 20, and Tube 4 would have been expected to be the optimal one; if less than the equivalent amount had been present, a tube between 4 and 8 would have been optimal. The slight difference between the experimental and the calculated result, in that it was impossible to decide between Tubes 8 and 7, comes well within the limits of experimental error. It is open to objection that the volume of the supernatant fluid is not quite 20 c.c., but the error arising therefrom is so small as not to be of any importance.

Exp. 3 (Table III). Titration of supernatant fluid without addition of adsorbable matter.

The supernatant fluid used in Exp. 2, Table II, was, without addition, titrated against falling amounts of horse serum.

Table III.

Tube	H.S. 1 in 300 (c.c.)	H.S. dilution	Order of degree of particulation after 7 hours	
10	1·0	1 in 300	—	
9	0·9	1 in 333·3	1	Very close
8	0·8	1 in 375	1	
7	0·7	1 in 428	—	
6	0·6	1 in 500	—	
5	0·5	1 in 600	—	
4	0·4	1 in 750	—	
3	0·3	1 in 1000	—	
2	0·2	1 in 1500	—	
1	0·1	1 in 3000	—	

Controls were set up, and remained unaffected.

In this experiment the supernatant fluid used in Exp. 2, having an antibody content equal to that of a 1 in 10 dilution of antiserum, was used without any addition. It was not possible to read the result until 7 hours had passed from the time the experiment was set up. Tubes 9 and 8 were the best ones, again indicating that the supernatant fluid contained the excess of antibody in equivalent amount. This experiment, although not readable until after 7 hours, was very much quicker than most of those in which supernatant fluid, without addition, was used.

Exp. 4 (Table IV). Titration of supernatant fluid with the addition of more antiserum.

10 c.c. of a 1 in 370 dilution of horse serum, and 10 c.c. of a $1\frac{1}{2}$ in 10 dilution of antiserum 1105 I (ratio 1 to 37) were mixed. After $3\frac{1}{2}$ hours the mixture was centrifuged. To 9 c.c. of the supernatant fluid was added 1 c.c. of antiserum 1105 I, and a titration against falling quantities of horse serum performed.

The antibody content of the supernatant fluid in this case should be equal to that of a 1 in 40 dilution of the antiserum. When 1 c.c. of antiserum is added to 9 c.c. of the supernatant fluid the resulting 10 c.c. contain the equivalent of the antibody from 1·225 c.c. of antiserum, corresponding to a dilution of 1 in

Table IV.

Tube	H.S. 1 in 270 (c.c.)	H.S. dilution	Order of degree of particulation after 1 hour 20 min.
10	1·0	1 in 270	—
9	0·95	1 in 284·2	2
8	0·9	1 in 300	1
7	0·85	1 in 318	3
6	0·8	1 in 337·5	—
5	0·75	1 in 360	—
4	0·7	1 in 385	—
3	0·6	1 in 450	—
2	0·5	1 in 540	—

Horse serum and antiserum controls unaffected.

8·16. The ratio of the antiserum is 1 to 37. The horse serum dilution to react optimally with this amount of antibody should be 1 in 37 × 8·16, *i.e.* 1 in 302. In this experiment it was possible to place the first three tubes in order of particulation. The dilution of horse serum, 1 in 300, in the optimal tube, No. 8, confirms the fact that the equivalent amount of the antibody excess is in the supernatant fluid. Without the addition of some adsorbable material to the supernatant fluid in this experiment it would have been most difficult, perhaps impossible, to determine the amount of antibody present with any degree of certainty. The amount of adsorbable matter left in the supernatant being so small, many hours would have passed before the particulation occurred, and the particles would have been so small as to make it practically impossible to get an accurate reading.

Exp. 5 (Table V). Titration of supernatant fluid, with the addition of adsorbable material, in the form of normal rabbit serum.

10 c.c. of a 1 in 340 dilution of horse serum, and 10 c.c. of a 1 in 5 dilution of anti-horse serum 1193 E (ratio 1 to 34) were mixed, and centrifuged after 15 hours. To 10 c.c. of the supernatant fluid were added 2 c.c. of normal rabbit serum; the resulting 12 c.c. were titrated against falling amounts of horse serum.

Table V.

Tube	H.S. 1 in 600 (c.c.)	H.S. dilution	Order of degree of particulation after 1 hr. 50 min.
10	1·0	1 in 600	—
9	0·95	1 in 631	—
8	0·9	1 in 666·7	—
7	0·85	1 in 706	—
6	0·8	1 in 750	3
5	0·75	1 in 800	1
4	0·7	1 in 857	2
3	0·65	1 in 923	—
2	0·6	1 in 1000	—
1	0·5	1 in 1200	—

Controls were set up: one contained saline, normal rabbit serum, and horse serum; another held saline and horse serum; in the third were saline, supernatant fluid, and normal rabbit serum. They were unaffected.

The supernatant fluid should contain antibody equal in amount to a 1 in 20 dilution of antiserum 1193 E. The 12 c.c. of the mixed supernatant fluid and rabbit serum should contain antibody in amount equal to a 1 in 24 dilution of the antiserum. The ratio of the antiserum is 1 to 34. Therefore, the dilution of

horse serum containing the equivalent amount of antigen should be 1 in 34 × 24, *i.e.* 1 in 816. Tube 5, 1 in 800, was the first to particulate; Tube 4, 1 in 857 was the second, which demonstrates that the supernatant fluid contains antibody in amount equal to a 1 in 20 dilution of the antiserum. Another experiment, in which 5 c.c. of this supernatant fluid were added to 5 c.c. of a 1 in 10 dilution of the antiserum, was performed and the result in this case showed the presence of an equivalent amount of antibody in the supernatant fluid.

In another experiment the supernatant fluid, obtained by mixing less than the equivalent amount of horse serum with antiserum, was subjected to the action of a further amount of antigen, again less than the equivalent amount for the antibody in the fluid. The presence of the proportionate amount of antibody was demonstrable in the ultimate supernatant fluid. It was thought that this fluid would contain relatively so little matter other than antibody, that it might be possible to pass antibody through a collodion membrane. Three attempts were made with such fluids, but the presence of antibody could not be shown in the dialysates.

In 18 experiments, carried out in one or other of the ways described in Exps. 2 to 5, it was possible to demonstrate the presence of an equivalent amount of antibody in the supernatant fluid. Six different anti-horse sera were tested in the course of these experiments. The amount of the antiserum excess varied greatly. It was least in an experiment where one and an eighth times the equivalent quantity was mixed with horse serum, and greatest where three times the appropriate amount of antiserum was included.

A seventh antiserum, 1396 B, behaved differently. When twice the equivalent amount of the antiserum was mixed with horse serum, it was not possible to show the presence of any excess of antibody in the supernatant fluid. Not only was antibody not traceable in equivalent amount, there was apparently none at all. This experiment was repeated with the same result. When three times the proportionate amount of antiserum was used, antibody was found, but not in equivalent amount. Two parts should have been left in the supernatant fluid, the third part uniting with antigen. Only one part was traceable. This was repeated and confirmed. It seemed that, in the case of this particular antiserum, excess of antibody was removable by the precipitate, up to about the point where there was present twice the equivalent quantity; beyond this point excess was left in the supernatant fluid. Antiserum 1396 B was one which acted with unusual rapidity, so much so that it had been set aside from the others, used very sparingly, and generally regarded as a special one. By being so rapid it gave evidence of containing a great amount of adsorbable material. There seemed no reason to believe that, in this one case, antigen and antibody were not reacting in equivalent proportions. It is probable that this antiserum contained adsorbable matter in such unusually large amounts, as to cause the adsorption of excess of antibody up to a point.

The length of time elapsing, between the mixing of the ingredients and centrifuging, seemed to be of no importance in any of these experiments. The

shortest interval was 1½ hours, the longest 24 hours. No mixture was centrifuged until the reaction had apparently come to an end. The reaction was judged complete when flocculi seemed no longer to be settling on the precipitate. This does not mean that the mixtures were centrifuged immediately the reaction was complete. In many cases they were left for hours after the reaction was complete, quite often overnight.

The work described in this paper has been limited to the serum precipitation reaction. The results are not entirely in agreement with those of workers with other immunity reactions. The presence of an equivalent amount of antibody in the supernatant fluid is not in agreement with the "Danysz Effect" (1902). The findings in the "Danysz Effect" are approximated by those in the experiments with antiserum 1396 B, where excess of antibody was not traceable in equivalent amount. It is likely that the adsorbable matter in this antiserum may also be adsorbent. The antigen-antibody complex may adsorb the adsorbable matter, and the adsorbable matter may in turn adsorb excess of antibody. In the "Reverse" experiments, the results of titrating a constant amount of antigen against varying quantities of antibody do not coincide with the results of the titration of toxin and antitoxin by the Ramon (1923) method. Excess of antibody *per se* has not appeared to inhibit the serum precipitation reaction. Bordet's teaching, that the composition of the antigen-antibody compound varied according to the proportions of the two ingredients present, has not been confirmed. Dean and Webb (1926) found that excess of antigen was removed to a great extent by the precipitate. I do not think that this is proof that the excess of antigen has reacted with antibody. Excess of antigen may have been adsorbed by the antigen-antibody complex.

Whilst the present work was in progress a report by workers in America was noticed (Wu, Sah, and Li, 1929). They used iod-albumin as antigen, and by injecting it into rabbits obtained a homologous antiserum. Quantitative experiments revealed the amount of antigen present in the precipitates resulting from mixtures of antigen and antiserum in various proportions. Their results suggested that the reaction between antigen and antibody was chemical; agreeing with the conclusions arrived at in this paper.

In January 1930 in a private communication to Prof. H. R. Dean, Prof. W. W. C. Topley stated that Mr Tatham of his laboratory, following an examination of the weights of precipitates given in Dean and Webb's paper (1926, Table IX, p. 489) had formed the opinion that the figures were suggestive of some sort of adsorption process being concerned in the reaction. Mr Tatham considered that antigen and antibody combined according to chemical laws, and that subsequently the antigen-antibody compound adsorbed some of the excess of antibody which was present in the supernatant fluid. This conception of a specific adsorption of antibody is different from the non-specific adsorption of other serum elements which I believe to take place. The hypothesis of Mr Tatham has this in common with my own view that both assume a chemical union followed by a process of adsorption and both afford a basis of reconciliation between the theories of Ehrlich and Bordet. The

presence in the supernatant fluid of the equivalent amount of antibody disproves the theory that excess of antibody is adsorbed by the antigen-antibody complex.

CONCLUSIONS.

1. Antigen and antibody react in equivalent proportions, in the serum precipitation reaction, to form an antigen-antibody complex which is adsorbent.

2. The complex adsorbs suitable matter from the surrounding medium.

3. Excess of antigen is adsorbed by and exercises a protective action on the micellae of the complex, and prevents completion of the reaction. Antibody in excess does not affect the reaction in any way, but in the great majority of cases is traceable in the supernatant fluid, in equivalent amount.

4. The speed of particulation depends on the amount of complex and adsorbable matter present.

5. There is some evidence that in certain cases the nature and quantity of the adsorbable matter present may be such as to cause excess of antibody to be removed from the supernatant fluid. In such cases the adsorbable matter may possibly be adsorbent to the excess of antibody.

6. Antisera vary in the amount of antibody content, and in the amount and nature of the adsorbable matter they contain.

7. The addition of adsorbable matter is a useful way of hastening, and facilitating, the reading of reactions.

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