THE DISSIMILARITY OF THE RESULTS OF PRECIPITIN TITRATIONS PERFORMED WITH A CONSTANT AMOUNT OF ANTISERUM AND WITH A CONSTANT AMOUNT OF ANTIGEN.

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Introduction.

Dean and Webb (1926) described an optimal proportions method for the titration of antigen and antibody in the precipitation reaction. They set up falling amounts of horse serum against a constant amount of anti-horse serum and showed that the most rapid particulation took place when the reagents were in definite proportions. These optimal proportions may be expressed as a ratio which serves to indicate the antibody content of an antiserum. An antiserum of which 20 parts by volume react optimally with 1 part of horse serum has a ratio of 1 to 20; a weaker antiserum of which 40 parts are required to react with 1 part of horse serum has a ratio of 1 to 40. They found that the antigenic content of different specimens of normal horse serum was constant.

All their titrations were performed by adding a constant amount of antiserum to each of a series of tubes containing falling amounts of the antigen. Taylor (1931) described experiments in which the reverse procedure was adopted and falling quantities of anti-horse serum were titrated against a constant amount of the antigen. When this reverse method of titration was used it was found that the proportions of antigen and antibody most favourable for rapid particulation were not those which had been found most suitable by titrating a constant amount of antiserum against varying dilutions of horse serum. With a fixed amount of antigen it was seen that particulation occurred in the tube containing the largest amount of antiserum, and a tube in which the antigen and antiserum were optimal, as determined by the usual

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method of titration, merely took its place in the progression of particulation which gradually spread along the series.

Whilst the reverse experiments described in 1931 were sound so far as they went, it is now realised that the series of anti-horse serum dilutions used were not sufficiently extensive. The strength of the antiserum in the first tube of a series was not great enough, whilst the antigen was not used in very high dilution, with the result that optimal particulation occurred in the tube containing the greatest amount of antiserum. Similar tests have been set up in which the anti-horse serum in the first tube has been undiluted, and the earliest particulation has taken place usually in a tube near the end of a series where antibody was greatest, but not actually in the first tube, showing that excess of antiserum beyond a certain point does inhibit the speed of particulation. Nevertheless, the ratios obtained by both methods of titrating horse and anti-horse sera have never been even approximately the same; the reverse ratios have always been at least three times as great as those by the usual method, and anti-horse sera do occur in which the difference is much greater.

In the present work crystalline egg-albumin and its antisera have been examined and the ratios obtained for an antiserum by both methods of titration have never agreed, although in the majority of cases the discrepancy has not been so great as with anti-horse sera.

That the results of titrations by both methods are not identical is of interest since in the Ramon (1923) method of titrating diphtheria toxin and antitoxin the amount of antitoxin is varied and the antigen is constant.

The preparation and characteristics of the crystalline egg-albumin used were described by Taylor, Adair and Adair (1932). All reactions were performed at room temperature, and 0·85 per cent. saline was used as diluent. A few points of practical importance in the performance of precipitation titrations are appended.

Preliminary tests.

In Table 1 is recorded the titration of falling quantities of antiserum 1722 D against a constant amount of the homologous antigen, crystalline egg-albumin. Clean glass test-tubes of uniform calibre were set up in a rack and numbered as in the first column of the table. The test was a rough one in which a series of antiserum dilutions was arranged, each in a volume of 1 c.c., so that the amount of antiserum was halved in successive tubes. The second column gives the dilution of antiserum in the various tubes. 1 c.c. of a 1 in 320 dilution of 1 per cent. crystalline albumin was added to every tube, and the time of the addition noted. The rack was shaken to mix the contents of the tubes, each of which now contained 2 c.c.; the proportion of antigen to antibody being shown in the third column. The progress of the reaction, read at intervals, is outlined in the rest of the table. Tube 1 retained the oily and brownish appearance of the antiserum, as was to be expected since undiluted serum was used. In tube 2 the coloration and oiliness were not nearly

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so marked, whilst in tube 3 they were scarcely obvious; the remaining tubes showed no signs of them. The first six tubes rapidly became opalescent with No. 5 leading. The state of the reaction after 5 min. is shown in the third column. In just under 15 min. very fine particles were seen in tube 5, and shortly afterwards in tube 6. The rest of the series were little altered in the interval between the readings at 5 and 15 min. By the end of an hour there were particles in the first six tubes; tubes 5 and 6 contained flocculi which were beginning to deposit. At 5 hours flocculi were depositing in all tubes from 1 to 6. The deposit was most marked in tubes 4, 5, and 6. Some change had taken place in tube 7, but the remaining tubes were still clear. The signs of the reaction ended abruptly at tube 7, and even in this tube the

Table 1. "*Reverse*" *rough test. Falling amounts of antiserum* 1722 *D titrated against a constant* 1 *in* 320 *dilution of* 1 *per cent. crystalline egg-albumin.*

suggest. = suggestion, opal. = opalescence, mod. = moderate, flocc. = flocculi.

Tube	Antiserum dilution	Crystalline 1 % to A.S. ratio	5 min.	15 min.	1 hour	5 hours	22 hours
1	1 in 1	1 to 320	Oily, brown suggest. of opal.	No change ,,	Very fine particles	Mod. flocc. depositing	Oily, brown fluid, flocc. deposited
2	1 in 2	1 to 160	Very faint opal.	,,	Fine particles	,,	Flocc. deposited
3	1 in 4	1 to 80	,,	,,	Mod. particles	,,	,,
4	1 in 8	1 to 40	Faint opal.	Mod. opal.	Good particles	+Mod. flocc. depositing	,,
5	1 in 16	1 to 20	+Faint opal.	Fine particles	Good flocc. depositing	,,	,,
6	1 in 32	1 to 10	Faint opal.	Very fine particles	Less good flocc. beginning to deposit	,,	,,
7	1 in 64	1 to 5	Suggest. of opal.	Very faint opal.	Faint opal.	Good opal. ? very fine particles	Flocc. depositing
8	1 in 128	1 to 2·5	Clear	Clear	Clear	Clear	Clear
9	1 in 256	1 to 1·25	,,	,,	,,	,,	,,
10	1 in 512	1 to 0·625	,,	,,	,,	,,	,,
11	1 in 1024	1 to 0·3125	,,	,,	,,	,,	,,
12	1 in 2048	1 to 0·15625	,,	,,	,,	,,	,,

evidences were very slight and progress extremely slow. In tube 7 the dilution of antiserum was moderately great (1 in 64), and much greater still, of course, in tubes 8 to 12. It has been definitely established by the work of Welsh and Chapman (1906) that the bulk of the precipitate resulting from the mixture of antigen and antiserum comes from the antiserum. It is not surprising, therefore, that tubes in which the antiserum is greatly diluted should show little or no sign of a reaction; there is so little material to form a precipitate.

In the above experiment, with falling antiserum and constant antigen, the tube containing the greatest amount of antiserum did not particulate first. The point of optimal particulation was in the neighbourhood of tubes 5 and 6, of which the 1 per cent. crystalline albumin to antiserum ratios were 1 to 20 and 1 to 10 respectively.

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The same antiserum, 1722 D, was also titrated by the usual Dean and Webb method and the experiment is described in Table 2.

Again the test was a rough one, but in this case it was the antigen which was halved in successive tubes, and 1 c.c. of a 1 in 10 dilution of antiserum was added to every tube. Readings were taken after the same intervals as in the reverse test, and are recorded in the table. After 12 min. there were particles in tube 7 and shortly afterwards in tube 8, tubes with ratios of

Table 2. *Falling amounts of* 1 *per cent. crystalline egg-albumin titrated against a constant* 1 *in* 10 *dilution of antiserum* 1722 *D.*

Tube	Cryst. alb. 1 % dilution	Crystalline 1 % to A.S. ratio	5 min.	15 min.	1 hour	5 hours	22 hours
1	1 in 2·5	1 to 0·25	Clear (cf. control)	No change	No change	No change	No change
2	1 in 5	1 to 0·5	,,	,,	,,	,,	Faint opal. some gran.* particles
3	1 in 10	1 to 1	,,	,,	,,	,,	Many gran. particles
4	1 in 20	1 to 2	,,	,,	,,	,,	Faint opal.
5	1 in 40	1 to 4	Suggest. of opal.	Suggest of opal.	Mod. opal.	Mod. opal.	Mod. opal. STOP GAP‡
6	1 in 80	1 to 8	Faint opal.	Very good opal.	Mod. flocc. depositing	Flocc. deposited	Flocc. deposited
7	1 in 160	1 to 16	+Faint opal.†	Good particles	Good flocc. deposited	,,	,,
8	1 in 320	1 to 32	Faint opal.	Less good particles	Mod. flocc. depositing	,,	,,
9	1 in 640	1 to 64	Very faint opal.	Faint opal.	Very fine particles	,,	,,
10	1 in 1280	1 to 128	Suggest. of opal.	Suggest. of opal.	Faint opal.	Good particles	Flocculi
11	1 in 2560	1 to 256	Clear	Clear	Suggest. of opal.	Faint opal.	Fine particles
12	1 in 5120	1 to 512	,,	,,	Clear	Suggest. of opal.	Very faint opal.

* gran. = granular. † Particles first seen in tube 7 after 12 min.

‡ STOP GAP is a term used to describe a tube which acts as a buffer between two zones of particulation; such tubes remain turbid and do not clear for a long time, perhaps not for days, and exhibit all the signs of antigen excess, as they contain excess of the antigen responsible for the zone in the tubes below them in the series. Sometimes more than one STOP GAP tube is present between two zones.

1 to 16, and 1 to 32 respectively. These figures and those obtained by the reverse method of titration, namely 1 to 10 and 1 to 20, were sufficiently in agreement to suggest that the point of optimal particulation, as determined by the two methods, might be the same. But when fine tests, in which the amounts of the varied reagent differed but little from tube to tube, were set up by both methods, they gave different values for the proportions of antigen and antibody yielding the quickest particulation.

Note. Solutions of crystalline egg-albumin, even quite weak ones, have a great tendency to become stringy or ropy after a very little agitation, owing to denaturation of some of the protein. A stock solution of 2 or 3 per cent. has to be dealt with carefully when a portion is removed and any use of a pipette, other than the most gentle, may cause the formation of one or two strings in the bulk of the supply. The upper four or five tubes of an experiment like the one in Table 2 are apt to be more or less ropy even in the early stages, since the albumin is bound to be subjected to a certain amount of agitation in the

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making of the dilutions, but as the dilutions become greater the tendency to ropiness disappears. To gauge the effect of this stringy formation a parallel set of antigen dilutions was made and, instead of antiserum, 1 c.c. of saline was added to each tube. For the experiment in Table 2 controls for the first eight tubes were set up; a very little stringiness, diminishing with each dilution, was evident immediately in the first six controls, but after 22 hours no obvious change had occurred. Of course, a similar stringiness existed for many hours in the corresponding tubes of the experiment proper, but its effect on the subsequent happenings could be judged by comparison with the controls. By careful handling of experiments much interference from this source can be avoided. In fine tests the antigen dilutions used were so high that difficulties due to stringiness did not arise.

In a rough test with crystalline egg-albumin and a homologous antiserum the limited extent of the zone showing much evidence of a reaction is very striking. In the early stages and for a considerable time, often many hours, good opalescence is confined to four or five tubes, whilst the rest of the tubes on either side of this zone are only very faintly opalescent at the most, and usually quite clear. In several tests like the one in Table 2, the series of antigen dilutions was carried on to the seventeenth or eighteenth tube; nothing happened in these higher dilutions, even after 24 hours, and the narrow reaction zone was flanked on both sides by tubes which were quite clear. This narrow range is in marked contrast to the widespread opalescence which results, usually within a few minutes, when horse serum in varying amounts is titrated against a constant dilution of a good precipitating anti-horse serum as a rough test.

Fine tests.

In Table 3 are set out the details of the fine test performed on antiserum 1722 D by the direct Dean and Webb method, using a constant antiserum dilution. The first column describes the tubes in the experiment, and the second shows the volume of a 1 in 200 dilution of 1 per cent. crystalline egg-albumin delivered into the respective tubes. The volume in each tube was

Table 3. *Falling amounts of* 1 *per cent. crystalline egg-albumin titrated against a constant* 1 *in* 20 *dilution of antiserum* 1722 *D* (*Dean and Webb method*).

Tube	Crystalline egg-albumin 1 % 1 in 200 (c.c.)	Crystalline 1 % dilution	Crystalline 1 % to antiserum ratio	Order of degree of particulation after 22 min.
10	1·0	1 in 200	1 to 10	—
9	0·9	1 in 222·2	1 to 11·1	—
8	0·8	1 in 250	1 to 12·5	—
7½	0·75	1 in 266·7	1 to 13·3	—
7	0·7	1 in 285·7	1 to 14·3	2
6½	0·65	1 in 307·7	1 to 15·4	1
6	0·6	1 in 333·3	1 to 16·7	2
5½	0·55	1 in 363·6	1 to 18·2	—
5	0·5	1 in 400	1 to 20	—
4½	0·45	1 in 444·4	1 to 22·2	—
4	0·4	1 in 500	1 to 25	—
3½	0·35	1 in 571·4	1 to 28·5	—

made up to 1 c.c., where necessary, by the addition of saline. The third column gives the dilution of albumin which each tube now contained. The fourth column shows the proportion of 1 per cent. albumin to antiserum after the addition of 1 c.c. of a 1 in 10 dilution of the antiserum to every tube. The

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fifth column registers the order of the degree of particulation and the time after which it was possible to determine this. Tube $6\frac{1}{2}$ was first with 7 and 6 equal for second place. The ration was assigned as 1 to 15.

The method of assigning a ratio from the results of fine tests has been the same throughout the work to be described. In such an experiment as the one in Table 3, the order of the leading tubes might have varied as follows:

$$\text{A.}\quad \begin{matrix}2\\1\\3\end{matrix}\left\{\begin{matrix}1 \text{ to } 14{\cdot}3\\1 \text{ to } 15{\cdot}4\\1 \text{ to } 16{\cdot}7\end{matrix}\right.$$

, in which case the ratio assigned would have been 1 to 15, the whole number nearest the mean, 14·85, of the ratios of the two leading tubes, 1 to 15·4 and 1 to 14·3.

$$\text{B.}\quad \begin{matrix}3\\1\\2\end{matrix}\left\{\begin{matrix}1 \text{ to } 14{\cdot}3\\1 \text{ to } 15{\cdot}4\\1 \text{ to } 16{\cdot}7\end{matrix}\right.$$

, when the ratio would have been 1 to 16, the whole number nearest the mean, 16·05, of the ratios of the two leading tubes, 1 to 15·4 and 1 to 16·7.

$$\text{C.}\quad \begin{matrix}2\\1\\2\end{matrix}\left\{\begin{matrix}1 \text{ to } 14{\cdot}3\\1 \text{ to } 15{\cdot}4\\1 \text{ to } 16{\cdot}7\end{matrix}\right.$$

, yielding the ratio 1 to 15, the whole number nearest the ratio, 1 to 15·4, of the leading tube.

$$\text{D. Equal}\quad \begin{matrix}1\\1\end{matrix}\left\{\begin{matrix}1 \text{ to } 15{\cdot}4\\1 \text{ to } 16{\cdot}7\end{matrix}\right.$$

, giving the ratio 1 to 16, the whole number nearest the mean of the ratios of the two equal tubes, 1 to 15·4 and 1 to 16·7. In a few reverse tests the mean of the two ratios has fallen exactly midway between two whole numbers, and in these cases the ratio has been assigned as 1 to $x{\cdot}5$.

Table 4 outlines the fine test performed on 1722 D by the reverse method. A 1 in 6 dilution of the antiserum was made and of it the amounts shown in the second column were placed in the respective tubes. The volume of each tube was made up to 1 c.c. with saline to yield the dilutions given in column three. 1 c.c. of a 1 in 360 dilution of 1 per cent. crystalline egg-albumin was added to every tube, for which the antigen-antibody ratios are in the fourth column. Tube $4\frac{1}{2}$ was the first to particulate. Tubes 5 and 4 were difficult to differentiate from each other and were taken as being equal. The ratio of 1 to 27 was assigned. Tube $2\frac{1}{2}$ with a ratio of 1 to 15, and containing antigen and antibody in the proportions found to be optimal by the usual method of titration, particulated in due course and took its place in the order of particulation. In the reverse fine test on antiserum 1722 D tube $2\frac{1}{2}$ did not contain the antiserum in a dilution of 1 in 20, the dilution of the antiserum used in all the tubes of the fine test by the usual method (Table 3). But, with some of the other antisera, fine tests by both methods were arranged so that the reverse test included a tube in which the reagents were in optimal proportions according to the ordinary method of titration, and, in addition, the absolute

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antiserum content of this tube was the same as that of the tubes in the usual fine test. Even so, such a tube, a replica of the optimal one in the usual form

Table 4. *Falling amounts of antiserum* 1722 *D titrated against a constant* 1 *in* 360 *dilution of* 1 *per cent. crystalline egg-albumin. A reverse fine test.*

Tube	A.S. 1722 D 1 in 6 (c.c.)	Antiserum dilution	Crystalline 1 % to antiserum ratio	Order of degree of particulation after 25 min.
8	0·8	1 in 7·5	1 to 48	—
7	0·7	1 in 8·57	1 to 42	—
6½	0·65	1 in 9·23	1 to 39	—
6	0·6	1 in 10	1 to 36	—
5½	0·55	1 in 10·90	1 to 33	—
5	0·5	1 in 12	1 to 30	2
4½	0·45	1 in 13·3	1 to 27	1
4	0·4	1 in 15	1 to 24	2
3½	0·35	1 in 17·14	1 to 21	—
3	0·3	1 in 20	1 to 18	—
2½	0·25	1 in 24	1 to 15	—
2	0·2	1 in 30	1 to 12	—

of test, took its place in the order of particulation and was invariably beaten by some of the other tubes which contained more antiserum.

On the whole it was more difficult to read reverse fine tests than those performed by the usual method, in that absolutely undoubted readings could usually be obtained with the latter in a less advanced stage of particulation than was the case with the former. There was no doubt about the readings in reverse titrations, but in the early stages of particulation the differences between adjacent tubes were not so marked as in titrations by the usual method, and consequently it was necessary to wait until particulation was more advanced before making an undoubted reading. This delay in reading and the fact that in the usual form of test in Table 3, the amount of antigen in the optimal tube was slightly greater than that used in the corresponding reverse test in Table 4, explain why the time of particulation in the reverse one is recorded as being a little longer than in the direct test.

Twelve antisera made against crystalline egg-albumin have been examined by both methods of titration. Ten have behaved as did 1722 D in giving by the two methods ratios which were not very different; the experiments are detailed in Table 5. The behaviour of the other two antisera, bleedings of the same rabbit, will be described later. Seven rabbits are represented in the table; three animals each contribute two bleedings. The figures obtained by dividing the reverse ratio by the direct ratio are given in the fourth column. The highest value is 1·8 (two cases), and the lowest 1·33. The mean of ten values is 1·6015. When the method of assigning a ratio is remembered, and consideration is given to the effect of small differences in either the dividend or the divisor on the quotient of say 28·5 by 18, the figures in Table 5 do show fairly good agreement. Although the number of antisera reported is only small, and no definite conclusions can be drawn, it does appear likely that the figure 1·6 may be a constant.

As mentioned above, two antisera, 1716 C and 1716 D, behaved differently. In a rough test on 1716 C by the direct method the two best tubes had ratios of 1 to 32 and 1 to 16. A fine test gave the ratio as 1 to 19. The rough test

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Table 5. *Ten anti-crystalline egg-albumin sera examined by the usual Dean and Webb method of titration and by the reverse method.*

Antiserum	Dean and Webb titration. Ratios of best tubes	Reverse titration. Ratios of best tubes	Reverse ratio / Normal ratio quotient
1722 C	2 1 to 17·14 1 1 to 18·45 2 1 to 20 Ratio 1 to 18	2 1 to 30 1 1 to 27 3 1 to 24 Ratio 1 to 28·5	$\frac{28·5}{18}=1·58$
1720 C	2 1 to 25 1 1 to 26·7 2 1 to 28·6 Ratio 1 to 27	2 1 to 49·5 1 1 to 45 3 1 to 40·5 Ratio 1 to 47	$\frac{47}{27}=1·74$
1757 B	2 1 to 33·3 1 1 to 36·4 3 1 to 40 Ratio 1 to 35	2 1 to 58·5 1 1 to 54 2 1 to 49·5 Ratio 1 to 54	$\frac{54}{35}=1·54$
1714 C	2 1 to 16 1 1 to 20 2 1 to 24 Ratio 1 to 20	3 1 to 40 1 1 to 37·3 2 1 to 34·6 Ratio 1 to 36	$\frac{36}{20}=1·8$
1722 D	2 1 to 14·3 1 1 to 15·4 2 1 to 16·7 Ratio 1 to 15	2 1 to 30 1 1 to 27 2 1 to 24 Ratio 1 to 27	$\frac{27}{15}=1·8$
1720 D	2 1 to 16 1 1 to 17·7 2 1 to 20 Ratio 1 to 18	3 1 to 30 1 1 to 27 2 1 to 24 Ratio 1 to 25·5	$\frac{25·5}{18}=1·42$
1757 C	2 1 to 13·3 1 1 to 14·54 2 1 to 16 Ratio 1 to 15	3 1 to 30 1 1 to 27 2 1 to 24 Ratio 1 to 25·5	$\frac{25·5}{15}=1·7$
1754 B	3 1 to 28·6 1 1 to 30·8 2 1 to 33·3 Ratio 1 to 32	2 1 to 54 1 1 to 49·5 3 1 to 45 Ratio 1 to 52	$\frac{52}{32}=1·625$
1758 B	2 1 to 33·3 1 1 to 36·4 3 1 to 40 Ratio 1 to 35	3 1 to 58·5 1 1 to 54 2 1 to 49·5 Ratio 1 to 52	$\frac{52}{35}=1·48$
1756 B	3 1 to 23 1 1 to 25 2 1 to 27·3 Ratio 1 to 26	2 1 to 36 1 1 to 33 3 1 to 30 Ratio 1 to 34·5	$\frac{34·5}{26}=1·33$

Mean for 10 antisera: 1·6015.

Table 6. *Falling amounts of antiserum* 1716 *C titrated against a constant* 1 *in* 320 *dilution of* 1 *per cent. crystalline egg-albumin.*

Tube	Antiserum dilution	Crystalline 1% to A.S. ratio	5 min.	30 min.	40 min.	6 hours
1	1 in 1	1 to 320	Oily, brown; faint opal.*	Fine particles	2	Flocculi depositing
2	1 in 2	1 to 160	+Faint opal.*	Mod. particles 1	1	,,
3	1 in 4	1 to 80	Faint opal.*	Fine particles	3	,,
4	1 in 8	1 to 40	Very faint opal.	Very fine particles	Fine particles	,,
5	1 in 16	1 to 20	Suggest. of opal.	Faint opal.	Very fine particles	,,
6	1 in 32	1 to 10	Clear	Very faint opal.	Faint opal.	Very fine particles
7	1 in 64	1 to 5	,,	,,	? Suggest. of opal.	Faint opal.
8	1 in 128	1 to 2·5	,,	,,	Clear	Clear
9	1 in 256	1 to 1·25	,,	,,	,,	,,
10	1 in 512	1 to 0·625	,,	,,	,,	,,

* Very, very fine particles in tube 2 at 12 min. and in tubes 1 and 3 at 20 min.

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byt he reverse method is described in Table 6. Tube 2, containing a great deal of antiserum, particulated first; its ratio was 1 to 160. Tube 1, ratio 1 to 320, particulated second. An attempt was made to get the reverse ratio more closely, and the experiment of Table 7 was set up. It was impossible to decide in which tube particulation occurred first, but there was no doubt that tubes 1, 2 and 3 were the leading ones, and it seems probable that the ratio was 1 to 200 at least, and perhaps 1 to something more than 200; a result in keeping with the rough test in Table 6. Owing to shortage of antiserum it was not possible to pursue the search of this ratio further: experiments in which antiserum is used undiluted, or in dilutions of 1 in 2, and so on, are very costly in material.

In addition to the determination of the reverse ratio of 1716 C the experiment in Table 7 had another object. Since the antisera in Table 5 had ratios

Table 7. *Falling amounts of antiserum* 1716 *C titrated against a constant* 1 *in* 640 *dilution of* 1 *per cent. crystalline egg-albumin.*

Tube	Antiserum dilution	Crystalline 1 % to A.S. ratio	Order of degree of particulation after 2 hours
1	1 in 2	1 to 320	1?
2	1 in 2·5	1 to 256	1?
3	1 in 3	1 to 213·3	1?
4	1 in 4	1 to 160	—
5	1 in 5	1 to 128	—
6	1 in 6	1 to 106·7	—
7	1 in 8	1 to 80	—
8	1 in 10	1 to 64	—
9	1 in 12	1 to 53·3	—
10	1 in 16	1 to 40	—
11	1 in 20	1 to 32	—
12	1 in 24	1 to 26·7	—
13	1 in 28	1 to 22·9	—
14	1 in 32	1 to 20	—
15	1 in 36	1 to 17·7	—
16	1 in 40	1 to 16	—

No sign of second zone even after 24 hours.

by the reverse method which were about 1·6 times as great as those found by the direct method, it was thought worth while to find out if 1716 C would show, by a suitably arranged test, optimal particulation at a corresponding point. The very extensive range of antiserum dilutions in Table 7 was set up, but only one zone of particulation was seen. In tube 11 antigen and antibody were present in about those proportions which would have been optimal for the reverse titration if the two ratios of 1716 C had borne to each other the same sort of relationship as had been found with the rest of the antisera. For $19 \times 1{\cdot}6$ gives 30·4. Tube 11, however, took its place in the order of particulation, as did tube 14, which, with a ratio of 1 to 20, contained the reagents in almost the optimal proportions, 1 to 19, found by the direct titration. Ratios of 1 to 19 with constant antiserum, and of 1 to 200, or thereabouts, by the reverse method, are widely different and would seem to indicate that the greater the amount of antiserum added to constant antigen the greater was the speed of particulation. This appears to be true for this antiserum

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1716 C up to a point where something like ten times the optimal amount of antiserum, as determined by the usual type of titration, has been added. For 1716 D, the next bleeding, the reverse ratio was 1 to 78, and the direct ratio 1 to 16. An extensive reverse fine test was performed and again a tube with a ratio about 1·6 times the optimal ratio, determined by the usual method, took its place in the order of particulation. With 1716 D, therefore, the reverse ratio was about five times as great as the direct ratio; a difference not so big as with 1716 C which had one ratio about five times as great as the other.

Constancy of optimal proportions in reverse titrations.

In their experiments with horse and anti-horse sera Dean and Webb (1926) showed that when a constant amount of an antiserum was titrated against falling quantities of the antigen, the proportions of the two reagents giving the quickest particulation remained the same, although the absolute quantities of the ingredients might be altered. That is to say, if a particular antiserum were used in a constant dilution of 1 in 5 in one test, and of 1 in 10, 1 in 20, 1 in 40 and so on in others, the antigen-antibody ratio of the optimal tube in the first series was the same as the ratio of the best tubes in all the other sets. To discover if the optimal proportions of antigen and antibody were similarly constant in reverse titrations, suitable experiments were arranged using crystalline egg-albumin and its antisera.

Parallel series of falling amounts of antiserum 1754 B were titrated against (*a*) a constant 1 in 320 dilution of 1 per cent. crystalline albumin, (*b*) a constant 1 in 640 dilution, and (*c*) a constant 1 in 1280 dilution. In all three sets optimal particulation took place in tubes in which the ratio of crystalline albumin 1 per cent. to antiserum was 1 to 40; the ratios of the second tubes were all 1 to 80. Similar experiments on two other antisera, 1714 C and 1722 C, gave corresponding results. With all three antisera second zones of particulation were observed. The second zones appeared later than the first or main zones and were situated on that side of the first one where antibody was in excess. Taylor, Adair and Adair (1932) reported the occurrence of late zones in titrations of crystalline egg-albumin and its antisera by the direct Dean and Webb method, and pointed out that possibly they were due to a second antigen and its antibody. The finding of second zones in titrations by the reverse method is of additional interest.

The results of reverse fine tests on several antisera indicate more conclusively that the proportions of antigen and an antiserum favourable for the quickest particulation are the same although the absolute amounts of the ingredients may differ. In Table 8 are grouped four antisera upon each of which fine tests by the reverse method were performed using first a constant antigen dilution of 1 in 360 of 1 per cent. crystalline albumin and secondly a constant dilution of 1 in 180. When the latter strength of antigen was used the absolute quantities of antigen and antiserum at the optimal point were double those at the corresponding point in the test with the 1 in 360 dilution.

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The ratios and order of the three leading tubes are given for most of the experiments; in two cases tubes next each other in a series were equal for first place.

The two antisera, 1716 C and D, which had not fallen into line with the rest of the antisera investigated by both methods of titration, were also shown by rough tests to react optimally in definite proportions when the amounts of the reagents were varied.

Table 8. *Reverse fine tests using different strengths of constant antigen.*

Antiserum	Ratios of best tubes using crystalline 1 % 1 in 360 constant	Ratios of best tubes using crystalline 1 % 1 in 180 constant
1722 C	2 1 to 30 1 1 to 27 3 1 to 24	Equal { 1 to 30 1 to 27
1720 C	Equal { 1 to 45 1 to 42	2 1 to 49·5 1 1 to 45 3 1 to 40·5
1757 C	2 1 to 30 1 1 to 27 2 1 to 24	3 1 to 30 1 1 to 27 2 1 to 24
1756 B	2 1 to 36 1 1 to 31·5 3 1 to 27	2 1 to 36 1 1 to 33 3 1 to 30

The experiments described to demonstrate the constancy of optimal proportions in reverse titrations are not very numerous or exhaustive, because each test requires a large amount of the necessarily limited supply of antiserum. Taken as a whole, however, they furnish definite evidence that over a considerable range the optimal proportions are constant.

Experiments were also performed to show that the optimal proportions of crystalline egg-albumin and a homologous antiserum were constant in titrations by the usual Dean and Webb method.

Discussion.

Why the proportions of the two reagents yielding optimal particulation in titrations by Dean and Webb's method are not the proportions which yield optimal particulation in reverse tests, I cannot say. That they are different raises the question as to which of these proportions, if either, represents the point at which antigen and antibody neutralise each other. The evidence of the titrations of the supernatant fluids resulting from mixtures of horse and anti-horse sera in various proportions, reported by Dean and Webb (1926) and confirmed for mixtures containing excess of antibody by Taylor (1931), seems to indicate that the point of neutrality coincides with the point of optimal particulation determined by Dean and Webb's method. Dean and Webb found that when the two reagents were mixed in optimal proportions the antigen and antibody disappeared completely or almost completely from the supernatant fluid. In mixtures containing excess of antibody the whole

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of the antigen disappeared, whilst the equivalent amount of antibody was traceable in the supernatant. When antigen was in excess, the titration of the supernatants was difficult and unreliable because they remained turbid. A few supernatants from mixtures of crystalline egg-albumin and excess antiserum have been examined by experiments similar to those described by Taylor (1931), and results pointing to the presence of the equivalent amount of antibody have been obtained.

Many workers using different methods have reported contradictory results from experiments on supernatant fluids. Eisenberg (1902) stated that from every mixture there remained some antigen and antibody in the supernatant. In no case did v. Dungern (1903), working with antisera prepared against the plasma of the octopus and various crustacea and molluscs, find that both antigen and antibody remained together in the supernatant. He mixed different strengths of antigen with a constant amount of antiserum, and in his series he always found a middle zone in which the reagents combined quantitatively and went out of solution as a precipitate. With horse serum and egg-white, and their antisera, Weil (1916) found that the supernatants contained both reagents, but in experiments with crystalline egg-albumin and its antiserum, the supernatants contained either antigen or antibody, but never both. Bayne-Jones (1917), using edestin and crystalline egg-albumin thrice recrystallised, could not confirm Weil's results; the supernatants always contained both antigen and antibody. Opie (1923) regards crystalline egg-albumin, six times recrystallised, as an almost, but not quite, pure antigen, and thinks the simultaneous presence of antigen and antibody in supernatant fluids is best explained by the assumption of a multiplicity of antigens. Marrack and Smith (1931) state that Taylor's (1931) work "raises the suspicion that the most rapid flocculation may be the result of several factors and have no special significance; nevertheless, we have found that the supernatants from the mixture in optimum proportions always became cloudy when either more antigen or more antibody was added, while the supernatants from mixtures in other proportions gave precipitates with either antigen or antibody, but not with both." They were working with azo- and iodo-proteins prepared with horse serum pseudoglobulin and crystalline egg-albumin. Wilson Smith (1932) reports the qualitative demonstration in supernatant fluids of excess antigen and excess antibody on the appropriate sides of the optimal flocculating point of series of mixtures of the soluble specific substance of pneumococcus Type I and homologous antisera.

The evidence on the whole suggests that by using purified antigens and their antisera it will be possible to demonstrate that the optimal point of particulation, as determined by Dean and Webb's method of titration, does represent the point of neutrality of antigen and antibody.

The results of Taylor's (1931) experiments led to the conclusion that antibody *per se* did not inhibit the reaction, but from the later work reported in the present paper, it is obvious that excess of antiserum does inhibit the speed

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of particulation; although the inhibitory effect of excess antibody is not nearly so marked as that of antigen. These later findings necessitate some modification of the views expressed in 1931 with regard to the part played by adsorption of non-specific elements in determining the speed of particulation.

Apart from theoretical considerations as to the nature of the precipitation reaction, the results of reverse tests have another interest. The Ramon (1923) method of titrating diphtheria toxin antitoxin is performed by adding varied amounts of antibody to a constant amount of antigen. The results of such tests, although in most cases bearing definite relationship to the results obtained by animal tests, are usually not identical with them (see Glenny and Okell, 1924). Whether the titration of diphtheria toxin antitoxin by flocculation experiments in which the amount of antitoxin was constant and that of toxin varied would yield values the same as those found by titrating constant antigen against varied antibody, is of interest. Values determined by tests in which the amount of antibody was constant might be found to be more nearly coincident with the values obtained by animal tests than are those yielded by the Ramon test as performed at present.

In a personal communication Prof. G. Dreyer told me that he has titrated diphtheria toxin and antitoxin by both methods; he found that the results were not in agreement and in some cases there were great discrepancies.

Some points of practical importance in the performance of optimal proportions titrations.

Details of the performance of titrations by the method of optimal proportions were given by Dean and Webb (1926). One or two points, the outcome of experience with the reaction, seem worthy of record. My earliest work was done with ordinary test-tubes which varied very considerably in calibre, and in the thickness and colour of the glass from which they were made. The selection of ten or a dozen tubes to form a uniform series took an appreciable time, and was often very troublesome. It is of the utmost importance that the internal diameter of the tubes in a series used for the detection of the earliest particulation should be the same. The inclusion in a series of a tube markedly different from its neighbours has been found to cause incorrect readings. All the work to be described has been performed with "cordite" tubes. Although these tubes are not absolutely uniform, they are a great improvement on the earlier ones, and it is comparatively easy to select a uniform series. They are made of thin glass and are of a fairly narrow bore and their use greatly facilitates the reading of titrations.

The time elapsing between the setting up of a fine test and the making of a definite reading is difficult to record absolutely. Some observers can read reactions at an earlier stage than others; this is probably largely a matter of experience, but acuteness of vision may play some part. Dean and Webb (1926, p. 479) describe the difficulties of reading fine experiments in which the antigen dilutions differ little from tube to tube. They state that in many cases it is possible to detect the tube in which particles first appear and there may be a short interval of time in which particles are present in one tube and in one tube only. I have discussed this point with Dr A. Q. Wells and in our joint experience such cases are very rare. Usually in a fine test very tiny particles can be detected in a good many tubes long before a reading can be made. These particles are very fine and can most

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easily be seen where the reading-lamp light, shining through the fluid, is brightest; the rest of the fluid being apparently turbid. At a later stage the even turbidity in one tube will suddenly be lost and a cracked or mosaic appearance become evident, which is often for a time confined to one tube. It is at this point that I try to make my readings, and it is almost certainly the moment, referred to by Dean and Webb, at which discrete particles make their appearance. Often the mottled appearance occurs almost simultaneously in three or four tubes, but within a short time particles are bigger in one tube than in the rest. In spite of these difficulties Wells and I have placed the first three tubes in the same order in the majority of experiments we have read together. We have sometimes differed as to which of two adjacent tubes was actually first, but as the ratio assigned was the mean of the ratios of the two tubes our results were the same. With some antisera the optimal tube can be picked out without any difficulty whilst with others the selection needs great care and attention. Occasionally the reading of reactions performed with a particular antiserum has been so difficult that its use has been discontinued.

Some observers remove two or three tubes from the rack containing a series, and examine them separately. I advise strongly against this practice, as agitation may hasten particulation and lead to faulty readings. If it is thought desirable to wipe the outsides of tubes during the progress of particulation, they should all be removed, one after the other, and be subjected to an equal amount of disturbance.

If the supply of an antiserum is stored in a single container, the removal of samples at intervals is very liable to result in infection of the serum and the appearance of moulds, and in many cases loss of antibody content evidenced by an increase in the ratio. To avoid infection antisera have been stored in separate ampoules, which, to preserve the antibody content, are made of brown glass and are, when filled, stored in a dark cool place. Some ampoules hold about 2·5 c.c., others about 5 c.c. or 10 c.c. according to the purpose for which it is intended to use the serum. In the great majority of cases these precautions are successful, but they have one drawback. Very occasionally the contents of one ampoule will be found to yield a different ratio from other ampoules of the same antiserum tested at the same time. Inferior quality of the glass may possibly be responsible. Although the frequency of such happenings is not high, and during the first month or two after bleeding they have not been noticed, a worker must bear the possibility constantly in mind. The likelihood of error due to this source can be eliminated by performing related titrations from the same supply of antiserum dilution, made up, if necessary, from the pooled contents of different ampoules. Whenever another ampoule is opened it should be re-tested against an antigen, the ratio between which and the contents of earlier ampoules is known. After a time the ratio of all the supply of an antiserum may change; the ratio always becomes greater, indicating loss of antibody content. Only by re-testing the antiserum against a known antigen every time it is used can the possibility of error be avoided. The ratio of an antiserum, once determined, cannot with safety be assumed to be the same days afterwards. If antisera are used within a month or two of bleeding changes in ratio will be seldom met with.

Conversation with several workers has shown that the production of good precipitating antisera against crystalline egg-albumin and other antigens is regarded as difficult. Details of injections of crystalline egg-albumin into rabbits are given by Taylor, Adair and Adair (1932). The use of several rabbits, five at least, and the giving of more than one course of injections is to be recommended. Failure to produce a satisfactory antiserum after one course of injections, given to one or two rabbits, is not surprising, and the production of a good serum in these circumstances would be most fortunate.

The points here mentioned may seem trivial, but experience has established their importance.

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SUMMARY.

1. Antisera, made against crystalline egg-albumin, have been examined by titrating a constant amount of antiserum against falling amounts of the antigen (the usual Dean and Webb method), and also by the reverse procedure in which the amount of antigen was constant and that of antiserum varied. The ratios by the two methods were never found to agree; nevertheless, in the majority of cases the relationship which the ratio by one method bore to the ratio by the other method was of the same order, and it is possible that this relationship is constant for crystalline egg-albumin and its antisera.

2. The conclusions reached in my previous work (1931) require modification, because in those experiments insufficiently extensive ranges were used and as a result it was found that when varying amounts of anti-horse serum were titrated against a fixed amount of antigen, particulation was quickest in the tube containing most antiserum.

3. Titrations by the reverse method over a fairly considerable range have provided evidence that the proportions of crystalline egg-albumin and a given homologous antiserum favourable for the most rapid particulation are constant despite absolute quantitative changes. The proportions in which these reagents particulate most rapidly are similarly constant in titrations by the usual Dean and Webb method.

4. Why the two methods of titration give different results I am unable to explain, but the point of optimal particulation found by the Dean and Webb method probably coincides with the point of neutrality of antigen and antibody. Since in the Ramon (1923) method of titrating diphtheria toxin and antitoxin the reverse method, with constant antigen and varying antiserum, is used, the work reported here suggests that the determination of the relationship to each other and to animal values of the results of titrations of toxin and antitoxin by both methods might yield helpful information.

5. Some points of practical importance in the performance of optimal proportions titrations are appended.

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