THE ESTIMATION OF PROTEINS BY THE PRECIPITATION REACTION.

By G. L. TAYLOR,
John Lucas Walker Student,
G. S. ADAIR,
Fellow of King's College, Cambridge,
and MURIEL E. ADAIR,
Beit Memorial Fellow.

(*From the Department of Pathology, the Low Temperature Station and the Biochemical Laboratory, Cambridge.*)

Introduction.

Dean and Webb (1926) showed that when horse serum is mixed with the anti-horse serum prepared by the injection of horse serum into rabbits, the rate of the formation of the particles which results is greatest when the ingredients are in definite proportions. Falling quantities of the antigen, horse serum, are titrated against a constant amount of the antibody-containing antiserum, and the proportions yielding optimal, *i.e.* the quickest particulation, are expressed as the antigen-antibody ratio of the antiserum. An antiserum of which 20 parts by volume react optimally with 1 part of antigen has a ratio of 1 to 20, whilst a weaker antiserum of which 40 parts are needed to react optimally with 1 part of antigen has a ratio of 1 to 40. The method may be used for the estimation of either antigen or antibody.

The experiments on purified protein preparations described in this paper indicate that, in certain cases at least, the precipitation reaction is so specific and so delicate that it can be used to determine the concentrations of pure proteins in mixtures, as, for example, the concentration of crystalline egg-albumin in egg-white. In addition, it seems possible to apply the reaction to more complicated problems such as the concentration of albumin and globulin in serum. The method adopted necessitates the preparation of a pure solution of the antigen of well-defined composition, and the preparation of a suitable antiserum, but, once these have been made, the determinations of the amounts of the antigen, in the unknown mixtures such as serum or egg-white, can be made more rapidly and with less expenditure of material than is necessary for chemical methods.

A description of the manufacture of the antigens and the criteria for the purity of the preparations is given in an appendix. The standard 1 per cent. solutions of protein used in titrations, and for the injection of animals were

made up with 0·85 per cent. saline which was the diluent throughout. All the titrations were performed at room temperature.

OBSERVATIONS ON CRYSTALLINE EGG-ALBUMIN.

An antiserum prepared by the injection of crystalline egg-albumin into a rabbit was titrated against fresh egg-white, and the ratio of egg-white to antiserum optimal for particulation determined. The antiserum was then titrated against a standard 1 per cent. solution of crystalline egg-albumin, and the ratio 1 per cent. albumin to antiserum found. The percentage of crystalline albumin in egg-white was arrived at by dividing the ratio with egg-white by that with 1 per cent. crystalline albumin.

No difficulty was experienced in producing antisera. Of the first batch of ten rabbits, six were given intravenously a first dose of 3 c.c. of a 1 per cent. solution of crystalline egg-albumin; the remaining four received the same dose intraperitoneally. All subsequent injections were intraperitoneal, the same dose being given at intervals of 4 days until six injections in all had been given. The rabbits were bled, about 60 c.c., on the ninth day following the last injection[1]. The first bleeding from these ten animals yielded four antisera satisfactory for "optimal proportions" titrations; three from the rabbits which had received the first injection intravenously, the fourth from those injected entirely by the intraperitoneal route. Ten weeks after bleeding, a second course of injections, entirely intraperitoneal, was begun. Dosage and intervals were the same as before. The second bleedings of those rabbits which had given satisfactory antisera after the first course were good with one exception: in this one case the second bleeding was not so good as the first; however, a rabbit which failed at the first gave a good serum at the second bleeding. When the first group began the second course, five additional rabbits were started on a first course. These five animals received the initial dose intravenously, but only one satisfactory antiserum resulted. Experience with crystalline egg-albumin and horse serum has not shown conclusively that any advantage results from giving initial injections intravenously.

Table I outlines the titration of anti-crystalline egg-albumin serum 1714 B against falling amounts of fresh egg-white. It is similar to Exp. 2 described in Dean and Webb's paper (1926, p. 477). The tabulation is the simpler one adopted by one of us, G. L. T. (1931, p. 57), showing differences of 0·05 c.c. of antigen dilution in some of the tubes. The antiserum was used in a dilution of 1 in 10. The first column describes the tubes in the experiment; E.W.C. and A.S.C. are the egg-white and the antiserum controls respectively. The second column shows the volume of a 1 in 1400 dilution of egg-white 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

[1] *Note.* If such a course begins on a Thursday, the sixth injection falls on a Wednesday. The animals may be bled 9 days later, on a Friday, and the serum dealt with on the Saturday. If the course begins on any other day of the week injections will fall due on Sunday.

appropriate amount of saline. One c.c. of saline was added to tube E.W.C. and 1 c.c. placed in A.S.C. The third column gives the dilution of egg-white which each tube now contained. The fourth column shows the proportion of egg-white to antiserum in each tube after the addition of 1 c.c. of a 1 in 10 dilution of the antiserum to every tube except E.W.C. Each tube now held 2 c.c. The time of the addition of the 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. The controls were unaffected. Tubes $6\frac{1}{2}$ and 6 were equal, and better than the rest. The ratio was taken as 1 to 224.

Table I.

Tube	E.W. 1 in 1400 c.c.	E.W. dilution	E.W. to A.S ratio	Order of degree of particulation after 37 min.
E.W.C.	1·0	1 in 1400	—	—
10	1·0	1 „ 1400	1 to 140	—
9	0·9	1 „ 1555·6	1 „ 155·6	—
8	0·8	1 „ 1750	1 „ 175	—
$7\frac{1}{2}$	0·75	1 „ 1866·7	1 „ 186·7	—
7	0·7	1 „ 2000	1 „ 200	—
$6\frac{1}{2}$	0·65	1 „ 2153·8	1 „ 215·4	1 } Very close
6	0·6	1 „ 2333·3	1 „ 233·3	1 }
$5\frac{1}{2}$	0·55	1 „ 2545·5	1 „ 254·5	—
5	0·5	1 „ 2800	1 „ 280	—
$4\frac{1}{2}$	0·45	1 „ 3111·1	1 „ 311·1	—
4	0·4	1 „ 3500	1 „ 350	—
A.S.C.	—	—	A.S. only	—

Controls unaffected.

Table II gives the results of titrating seven specimens of anti-crystalline egg-albumin serum against fresh egg-white and against a 1 per cent. solution of crystalline egg-albumin. The ratios of the two best tubes in each experiment are given in the table, and the ratio assigned was the whole number nearest

Table II. *Crystallisable albumin content of egg-white.*

Antiserum	Ratio with fresh egg-white	Ratio with 1 % crystalline egg-albumin	% crystallisable albumin in fresh egg-white
1714 B	1 to 215·3–233·3 1 to 224	1 to 30·7 –33·3 1 to 32	$\frac{224}{32} = 7{\cdot}0$
1716 B	1 to 177·7–200 1 to 189	1 to 23·07–25·0 1 to 24	$\frac{189}{24} = 7{\cdot}87$
1720 B	1 to 200 –215·3 1 to 208	1 to 28·5–30·7 1 to 30	$\frac{208}{30} = 6{\cdot}93$
1722 B, mouldy	1 to 333·3–357·1 1 to 345	1 to 46·1 –50 1 to 48	$\frac{345}{48} = 7{\cdot}19$
1716 A	1 to 200 –213·3 1 to 207	1 to 28·5 –30·7 1 to 30	$\frac{208}{30} = 6{\cdot}9$
1722 B, not mouldy	1 to 250 –272·7 1 to 261	1 to 33·3 –36·4 1 to 35	$\frac{261}{35} = 7{\cdot}46$
1717 A	1 to 426·7–457·1 1 to 441	1 to 58·8 –62·5 1 to 61	$\frac{441}{61} = 7{\cdot}23$
1757 A	1 to 514 –554 1 to 534	1 to 66·7 –71·4 1 to 69	$\frac{534}{69} = 7{\cdot}74$

Mean 7·29 %.

https://doi.org/10.1017/S0022172400018064 Published online by Cambridge University Press

the mean of these figures. The last column shows the division of the ratio with egg-white by that with 1 per cent. albumin solution, yielding the percentage of crystalline egg-albumin in egg-white. Two different samples of 1722 B were tested; one had grown a mould, and, as is not unusual, become weaker. Eight estimations were made giving a mean result of 7·29 per cent. The egg-white was freshly prepared from new-laid eggs by shaking with beads and straining through muslin.

OBSERVATIONS ON TOTAL GLOBULIN.

In Table III are set out the results of experiments to determine the amount of total globulin present in normal horse serum. In this case the antisera used were made against a complex antigen, whereas in the egg-albumin series the antigen was a supposedly pure one. The six antisera of Table III react only with globulin in the main zone, and not with serum albumin also. The ratio

Table III. *Total globulin content of horse serum.*

Anti-horse serum	Ratio with horse serum	Ratio with 1 % total globulin	% total globulin in horse serum
1193 K	1 to 166·7–188·2 1 to 177	1 to 37·5–42·8 1 to 40	$\frac{177}{40} = 4{\cdot}42$
1614 B	1 to 45·45–50 1 to 48	1 to 10 –10·7 1 to 10	$\frac{48}{10} = 4{\cdot}8$
1595 C	1 to 76·9 –83·3 1 to 80	1 to 17·1–20 1 to 19	$\frac{80}{19} = 4{\cdot}21$
1568 E	1 to 23·0 –25 1 to 24	1 to 5·7–6·15 1 to 5·9	$\frac{24}{5{\cdot}9} = 4{\cdot}07$
1105 P	1 to 76·9 –83·3 1 to 80	1 to 16 –17·1 1 to 17	$\frac{80}{17} = 4{\cdot}71$
1354 I	1st tube 1 to 27·27 (Tubes on either side equal) 1 to 27	1 to 5·7–6·15 1 to 5·9	$\frac{27}{5{\cdot}9} = 4{\cdot}57$

Mean 4·46 %.

assigned is again the whole number nearest the mean of the ratios of the two best tubes. In the case of ratios less than 10 the figure is given to the first decimal place. When antiserum 1354 I was titrated against horse serum the best tube had the ratio 1 to 27·27: the tubes on either side were equal, so the ratio was taken as 1 to 27.

When anti-horse sera are titrated against parallel dilutions of normal horse serum, total globulin, and serum albumin, particulation usually occurs in the horse serum and globulin series, and is absent in the corresponding part of the serum albumin series.

Table IV outlines such an experiment. Parallel series of dilutions of the three antigens were set up as shown in the second column. Each tube contained 1 c.c. of antigen dilution. To every tube 1 c.c. of a 1 in 10 dilution of anti-horse serum 1354 I was added. The resulting particulation is recorded under the respective antigens. The ratio horse serum to antiserum is about 1 to 30: the ratio total globulin to antiserum is about 1 to 8. There is no corresponding reaction in the albumin series, showing that the antibody of the main zone of

antiserum is against globulin. Some antisera show a reaction in all three series, indicating that antibodies against both globulin and albumin are present. Secondary zones of particulation, which may occur later, do not affect the point under consideration.

When horse serum is titrated against an antiserum containing antibodies against both globulin and albumin it is impossible to be certain which antigen-antibody reaction is represented by the ratio obtained. Such antisera are unsuitable for antigen estimations. Only anti-horse sera with antibody against one fraction are suitable. The best plan is to prepare antisera against single antigens as was done with crystalline egg-albumin. Antisera against the pseudoglobulin, euglobulin, and serum albumin fractions of horse serum are in the course of preparation. The antisera used in the titrations described in Table III although prepared against whole horse serum contained only anti-globulin antibody in the main zone.

Table IV.

Tube	Antigen dilution	Ratio	Normal horse serum	Total globulin 1 %	Serum albumin 1 %
1	1 in 10	1 to 1	—	—	Very faint opalescence in tubes 1, 2 and 3 after 30 min.
2	1 „ 20	1 „ 2	—	—	
3	1 „ 40	1 „ 4	—	2	
4	1 „ 80	1 „ 8	—	1, after 25 min.	No reaction
5	1 „ 160	1 „ 16	3	3	„ „
6	1 „ 320	1 „ 32	1, after 20 min.	—	„ „
7	1 „ 640	1 „ 64	2	—	„ „
8	1 „ 1280	1 „ 128	—	—	„ „
9	1 „ 2560	1 „ 256	—	—	„ „
10	1 „ 5120	1 „ 512	—	—	„ „

Discussion.

Four anti-horse and five anti-crystalline egg-albumin sera had been tested before any enquiry was made to find out what figures had been obtained for these estimations by other methods. The comparison of our results with those obtained by other methods is given in Table V.

Table V.

Crystalline albumin in egg-white (%)	
6·0	Hopkins (1900)
8·5	Wu and Ling (1927)
7·29	(Optimal proportions)
Total globulin in horse serum (%)	
4·57	Hammarsten (1878)
4·07	Gibson and Banzhaf (1910)
4·46	(Optimal proportions)

It will be observed that we estimated 1·3 per cent. more albumin than Hopkins. He states that 60 grm. of crude crystalline albumin should be obtained from a litre of fresh egg-white; which will be less than the total amount originally present. The figure 8·5 per cent. is calculated from the data of Wu and Ling, who assumed that all the crystalline egg-albumin is coagulated by

shaking, and that conalbumin escapes coagulation. In the case of globulin, the results obtained by different workers show a certain amount of variation. Our value is in good agreement with that given by Hammarsten.

Our titrations were all carried out by the Dean and Webb method in common use in the Department of Pathology at Cambridge. There was no particular attempt to get especially accurate ratios, either by making the differences between the tubes of a series unusually small, or by using slightly differing series of antigen dilutions, each containing tubes with ratios about the optimal point. The employment of such refinements might lead to still more certain results. The method is very simple, if antisera are available, and may be useful when the estimation of protein is required.

APPENDIX.

The preparation of pure egg-albumin and its characterisation.

The purification of egg-albumin is a matter of importance, because egg-white contains a number of substances which may act as antigens in addition to the crystallisable egg-albumin. Since the protein is not very stable, the preparation must be made under favourable conditions, or protein derivatives may be formed. In view of these possible complications, it is desirable to test the purity of the preparations. Hopkins (1900) showed that the optical rotation of egg-albumin remains constant after repeated crystallisations. The work of Sørensen (1917) shows that measurements of solubilities and osmotic pressures serve as tests to characterise pure egg-albumin.

These tests require comparatively large amounts of material. In our experiments the bulk of the two preparations we made was required for injections into rabbits, but even if the amount of material available is small, it is possible to make refractometric and osmometric tests of the type illustrated in Table VI.

In our preparations the first crystallisation of egg-albumin was made by the method of Hopkins (1900). The crystals were centrifuged, washed, redissolved, and recrystallised as described in a previous paper by Adair and Robinson (1930). The egg-albumin preparations differed from serum-albumin, in that the crystals formed readily after the addition of ammonium sulphate, and in many crystallisations it was not necessary to add the solution of ammonium sulphate and acetic acid used in the work on serum-albumin. The elimination of conalbumin, mucoid and other impurities by repeated crystallisations has been discussed in detail by Sørensen (1917), who finds that no appreciable amount of mucoid and conalbumin remains after three crystallisations and the usual washings. In our experiments, the egg-albumin was crystallised seven times, and on each occasion the crystals were separated and washed by centrifugalisation. The possibility that a globulin is present as an impurity must be considered, since Svedberg and Nichols (1926) suggested

that crystallised egg-albumin is mixed with a globulin which can be precipitated by electrodialysis.

In a later paper, Sjögren and Svedberg (1930) showed that material recrystallised twice and electrodialysed was free from the impurity referred to above. Our preparations were not electrodialysed, but the risk of contamination with any globulin is considerably less than in twice recrystallised preparations, as our protein was recrystallised and washed six times.

The results of experiments made to characterise our preparations are summarised in Table VI.

Table VI.

	Prep. 1	Prep. 2
Nitrogen in grm. per 100 grm. dry protein	15·61	15·59
Temperature of refraction measurement	16·7° C.	15·8° C.
Specific refraction increment	0·001809	0·001818
Osmotic pressure ratio P/C	3·7	3·6

C = grm. albumin per 100 c.c. solution.

P = pressure in mm. of mercury at 0° C. at pH 4·64 (range of pressures from 10 to 20 mm.).

In the first place it will be observed that the nitrogen content of the two preparations, determined by the method of Kjeldahl, are practically identical and they agree with the figure 15·64 obtained by Sørensen (1917), who used similar methods.

A previous determination of the specific refraction increment of albumin has been made by Haas (1918), who gives figures ranging from 0·00171 to 0·00184. His mean value for albumin in water is about 0·00179.

A few measurements of the osmotic pressures of the preparations were made under conditions where the egg-albumin was equilibrated at 0° C. with an acetate buffer mixture containing 0·10 mol. of acetic acid plus 0·10 mol. of sodium acetate per litre. As a first approximation, it may be concluded that in both preparations the osmotic pressure ratios are equal. Most of Sørensen's measurements were made on more concentrated solutions, but his tables include a few observations on solutions containing from 2 to 4 per cent. of protein dissolved in solutions containing ammonium sulphate, in which the ratio P/C ranges from 3·7 to 4·2, and it may be inferred that the osmotic pressure and molecular weight of the protein in our preparations and in Sørensen's must have been equal within the limits of experimental error.

Marrack and Hewitt (1929) have measured the osmotic pressure of albumin twice recrystallised and the value of the ratio P/C calculated from their data is from about 3·79 to 3·93 in dilute solutions, in approximate agreement with the results obtained in this work for material six times recrystallised.

It has been stated that in the case of albumin solutions free from salts, the molecular weight is 34,000 (Sørensen, 1917; Svedberg and Nichols, 1926). In a later paper, Nichols (1930), using the centrifugal method, has obtained a value of about 34,000 for egg-albumin in the presence of salts. The theoretical osmotic pressure ratio P/C of a substance of this molecular weight is 5·0, whereas the observed ratios range from about 3·6 to 3·9. Since these values have been

obtained on material twice and six times recrystallised, it is unlikely that they can be attributed to accidental impurities such as the globulin referred to by Svedberg and Nichols. In view of the difference between the results obtained by the osmotic and by the centrifugal methods, the molecular weight of egg-albumin must be discussed later when more data are available.

An active antiserum made against hen serum was set up against the second of our crystalline albumin preparations, and against egg-white. No reaction occurred with the crystalline albumin and only a slight one with egg-white, so, according to Hektoen and Cole (1928), this preparation can be regarded as free from conalbumin. Unfortunately, the first preparation was not tested in this way, but, as it was made in the same manner as the second, it is reasonable to suppose it was free from conalbumin also. In any case the second preparation was the antigen in all the titrations described in this paper.

Although the purity of our preparations would appear to be established by the tests to which they were subjected, there is one other point which seems worthy of record. When some of the antisera were titrated against a fairly wide range of crystalline egg-albumin dilutions, two zones of optimal particulation were observed. In these cases the second zone appeared many hours after the first, and was situated well up in the region of antigen excess. What the significance of this second zone is we do not know; that it is due to the reaction between a second antigen and its antibody, is, of course, a possibility. The hypothesis that egg-albumin is not a simple substance is suggested by the recent work of Sørensen (1930), who has shown that the solubility of albumin is not entirely independent of the total mass of protein. The observation of two zones in one of our first four antisera, and in three of the five of our second batch, interested us greatly.

Table VII.

Tube	Crystalline egg-albumin 1 %	Zones of particulation
1	1 in 1·25	—
2	1 „ 2·5	—
3	1 „ 5	2
4	1 „ 10	1 } after many hours
5	1 „ 20	—
6	1 „ 40	—
7	1 „ 80	—
8	1 „ 160	3
9	1 „ 320	1 } 1 hr. 10 min.
10	1 „ 640	2
11	1 „ 1280	—
12	1 „ 2560	—

Table VII is a very abbreviated form of protocol recording the titration of anti-crystalline egg-albumin serum 1716 B, in a dilution of 1 in 10, against a series of dilutions of 1 per cent. solution of the antigen. Actually the series was taken to tube 18 which had a dilution of 1 in 163,840; nothing was seen in these greater dilutions, so they are omitted. The exact time of the appearance of the second zone was difficult to determine, the reaction being so slow in that region; after standing overnight, the two separate zones were clear cut.

Goldsworthy (1928) described the appearance of multiple zones in titrations of horse and anti-horse sera.

Total globulin.

The preparation of horse-serum globulin was made as described previously by Adair and Robinson (1930), but the protein was dialysed against a 0·85 per cent. NaCl solution instead of phosphate buffer mixtures. An improvement was made in the process of separating the globulin. After the filtration, which was carried out in a room at 0° C., the filter containing the protein precipitate was pressed between dry filter papers until nearly all of the fluid was removed. The precipitate could then be made to adhere to a spatula pressed upon it, and by rolling the spatula over the filter paper, the protein could be collected without appreciable loss. It could then be dissolved, filtered, and reprecipitated.

Summary.

1. The amount of crystallisable albumin in egg-white and the amount of total globulin in horse serum have been estimated by means of the precipitation reaction. The results are in good agreement with those obtained by other methods.

2. For such estimations it appears advisable to use only antisera prepared against individual proteins.

3. A description of the manufacture and the characteristics of the pure proteins used is appended.

REFERENCES.

Adair, G. S. and Robinson, Muriel E. (1930). *Biochem. J.* **24**, 993.
Dean, H. R. and Webb, R. A. (1926). *J. Path. and Bact.* **29**, 473.
Gibson, R. B. and Banzhaf, E. J. (1910). *J. Exp. Med.* **12**, 411.
Goldsworthy, N. E. (1928). *J. Path. and Bact.* **31**, 525.
Haas, A. R. C. (1918). *J. Biol. Chem.* **35**, 119.
Hammarsten, O. (1878). *Arch. f. d. ges. Physiol.* **17**, 413.
Hektoen, L. and Cole, A. G. (1928). *J. Infect. Dis.* **42**, 1.
Hopkins, F. G. (1900). *J. Physiol.* **25**, 306.
Marrack, J. and Hewitt, L. F. (1929). *Biochem. J.* **23**, 1079.
Nichols, J. B. (1930). *J. Amer. Chem. Soc.* **52**, 5176.
Sjögren, B. and Svedberg, T. (1930). *Ibid.* **52**, 5187.
Sørensen, S. P. L. (1917). *Compt. Rend. lab. Carlsberg*, **12**, 24.
—— (1930). *Ibid.* **18** (No. 5), 45.
Svedberg, T. and Nichols, J. B. (1926). *J. Amer. Chem. Soc.* **48**, 3081.
Taylor, G. L. (1931). *J. Hygiene*, **31**, 56.
Wu, H. and Ling, S. M. (1927). *Chinese J. Physiol.* **1**, 431.

(*MS. received for publication* 22. xii. 1931.—Ed.)