THE PRECIPITATION REACTION.

OPTIMAL PROPORTIONS, NEUTRALITY AND MAXIMAL PRECIPITATION IN MIXTURES OF ALBUMIN AND ANTISERUM.

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(With 1 Figure in the Text.)

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Introduction.

Culbertson (1932) has recently described a method for estimating the antibody content of antisera against crystalline egg-albumin. He determined by tests on supernatant fluids the point at which antigen and antibody neutralised each other. At this neutralisation point he found that antigen and antibody combined in a ratio of 1 to 13, and that the amount of precipitate nitrogen was maximal. The ratio 1 to 13, he stated, held for all the precipitates in the post-zone where antibody was in excess.

The optimal proportions method of titrating either antigen or antibody in the precipitation reaction, described for horse serum and its antisera by Dean and Webb (1926), was believed to indicate the proportions of the reagents which neutralised each other. In contrast to Culbertson's finding Dean and Webb reported that maximal precipitation occurred not at their optimal or neutral point, but where excess of antigen was present.

The present communication records studies made to establish the relationships of the points of neutrality, constant-antibody (Dean and Webb) optimal particulation, and maximal precipitation. The point of maximal precipitation for antisera to crystalline egg-albumin has been determined with precautions calculated to increase the accuracy of the measurements. Additional experiments have been carried out with antisera to crystalline horse-serum albumin. In the experiments of Culbertson and of Dean and Webb the amount of an antiserum was held constant and that of the antigen varied. In order to obtain a more rigorous test of Culbertson's conclusion that there is a constant ratio of antigen nitrogen to precipitate nitrogen of 1 to 13 in the zone of

https://doi.org/10.1017/S0022172400034422 Published online by Cambridge University Press

antibody excess, reverse titrations have been made with constant antigen and varying amounts of antiserum. Taylor (1931, 1933) has shown that in such reverse titrations the proportions of antigen and antibody most favourable for rapid particulation are not those found most suitable in the titration with constant antiserum, and that, with crystalline egg-albumin as antigen, a mean value of 1·6 is given for the quotient $\frac{\text{constant-antigen ratio}}{\text{constant-antibody ratio}}$. Similar results in both the agglutination and precipitation reactions have been reported by Duncan (1932) and Miles (1933). In the present work the constant-antigen optimal proportions have also been correlated with neutrality and amount of precipitate.

Taylor (1933) has summarised evidence in support of Dean and Webb's view that their optimal point, the constant-antibody ratio, gives the proportions in which antigen and antibody neutralise each other. Duncan has provided confirmatory evidence. An admirable review of the subject is given by Topley (1933, p. 64 *et seq.*).

METHODS.

Egg-albumin was purified by seven crystallisations; the preparation and the method of producing antisera in rabbits have been previously described by Taylor, Adair and Adair (1932). The crystalline egg-albumin contained 15·6 per cent. of nitrogen, and this figure has been used in calculating, from the dilutions made, the amount of antigen used in our experiments. Horse-serum albumin was recrystallised three times according to the method of Adair and Robinson (1930). For some of the antisera used in the present work, courses of four instead of six injections of antigen were given, since the animals had been established as antibody producers by earlier injections. The number given to an antiserum is that of the rabbit from which it came, and the letter indicates a particular course and bleeding, 1720 E meaning the fifth bleeding of rabbit 1720. The animals were bled after each course of injections. The optimal proportions titrations were all carried out at room temperature, normal 0·85 per cent. saline being used as a diluent. The nitrogen content of the precipitates was determined by the method of Parnas and Wagner (1921).

CONSTANT-ANTIBODY EXPERIMENTS (Table II).

Antisera were titrated by the Dean and Webb method as described by Taylor (1931, 1933), and Taylor, Adair and Adair (1932). The ratio of antigen to antiserum at the optimal particulation point is expressed in terms of a standard 1 per cent. solution of crystalline egg-albumin. The ratio, 1 to 8, of an antiserum means that 8 parts by volume of antiserum were in optimal proportions with 1 part by volume of 1 per cent. egg-albumin solution. Each antiserum was titrated at least twice, and the ratio assigned was the mean of these titrations expressed to the nearest 0·5.

In order to obtain precipitates for nitrogen determinations the following procedure was adopted. A series of tubes was set up containing falling

amounts of antigen, each in a volume of 1 c.c., and to every tube was added 1 c.c. of undiluted antiserum from a 1 c.c. "blow-out" pipette. In Exp. 2 each tube received 1 c.c. of a 1 in 1·5 dilution of the antiserum. Mixing was assured by fairly vigorous shaking. Each series was arranged so as to include a tube in which antigen and antiserum were in constant-antibody optimal proportions (marked with an asterisk in the tables). The tubes were incubated for 2 hours at 37° C. and then left in the refrigerator overnight. Next day the tubes were centrifuged and the supernatant fluids decanted and tested for both antigen and antibody. The precipitates were washed twice by suspension in 0·85 per cent. saline and centrifugalisation, dissolved in glass-distilled water with the addition of a small amount of dilute soda, and analysed for nitrogen.

The possible loss of precipitate by washing in various ways was investigated by the experiment described in Table I. In section A all the tubes contained

Table I. *Exp.* 8. *Antiserum* 1720 F, *ratio* (*constant-antibody*) 1 *to* 14·5.

SECTION A. Eight tubes each containing 1 c.c. of antiserum + 1 c.c. of crystalline egg-albumin 1 %, 1 in 14·5, *i.e.* the optimal amount. Antigen nitrogen 0·1076 mg.

Tube	Washed	Precipitate nitrogen mg.	Ratio Precipitate N / Antigen N
1	Once with saline, then with water	1·164	10·8
1*a*	,, ,,	1·151	10·7
2	Twice with saline	1·135	10·5
2*a*	,,	1·152	10·7
3	Three times with saline	1·126	10·5
3*a*	,,	Broken	—
4	Once with saline	1·169	10·8
4*a*	,,	1·175	10·9

SECTION B. Eight tubes each containing 1 c.c. of antiserum + 1 c.c. of crystalline egg-albumin 1 %, 1 in 7·25, *i.e.* twice the optimal amount. Antigen nitrogen 0·2152 mg.

Tube	Washed	Precipitate nitrogen mg.	Ratio Precipitate N / Antigen N
5	Once with saline, then with water	1·486	6·9
5*a*	,, ,,	1·504	7·0
6	Twice with saline	1·490	6·9
6*a*	,,	1·529	7·1
7	Three times with saline	1·497	7·0
7*a*	,,	1·497	7·0
8	Once with saline	1·529	7·1
8*a*	,,	1·524	7·1

antigen and antibody in constant-antibody optimal proportions; in section B the tubes contained twice the optimal amount of antigen. From the results we concluded that the losses of precipitate due to washing were small enough to be neglected. As a check on the accuracy of the nitrogen estimations occasional tubes in our experiments were duplicated, and in the tables the results obtained from these are bracketed together. Their nitrogen figures are in good agreement except in the case of the last two tubes of Exp. 2, which differed by about 8 per cent. The precipitate nitrogen figures for the last tube of Exp. 7 are very inaccurate, the amount of precipitate being exceedingly small: we have

recorded the figures in order to indicate the order of magnitude of the weight of precipitates resulting from such an excess of antigen.

Ring tests for antigen and antibody in the supernatant fluids were made by methods similar to Culbertson's. To test for antigen about 0·15 c.c. of a good antiserum was pipetted into a small precipitin tube and overlayered by about the same volume of supernatant fluid. The tubes were incubated for an hour at 37° C., read for rings, and their contents thoroughly mixed by stirring with very fine glass rods which were left in the tubes. After a further hour's incubation the experiment was stored overnight in the refrigerator, and next day the tubes were read for precipitates. Antibody was tested for by overlayering about 0·15 c.c. of supernatant with a like amount of crystalline egg-albumin diluted 1 in 20,000: the tubes were treated as were those used in the tests for antigen. As controls every supernatant and the antiserum were overlayered by saline. The results are recorded in the tables; the symbol + indicates that a definite ring was observed, and a + that when the tubes were read next day in conjunction with the controls, there was undoubted evidence of a reaction.

According to Culbertson the points of neutralisation and maximal precipitation are identical. In our experiments, detailed in Table II, ring tests showed the presence of definite antigen excess in the supernatants from tubes yielding the greatest precipitate nitrogen, and that the neutralisation point was identical with, or very close to, the Dean and Webb optimal point. Maximal precipitation occurred not at the optimal point, but where from 1·6 to 2·4 times the optimal amount of antigen was present. Our work with crystalline egg-albumin and its antisera suggested the probability of a constant ratio of antigen nitrogen to precipitate nitrogen at the optimal point, which will be discussed later. In Culbertson's work and in the experiments recorded in Table II, the amounts of precipitate were small in the regions where the antigen content was low, and therefore the observations do not afford a very rigorous test of his conclusion that the ratio of antigen nitrogen to precipitate nitrogen is a constant in the zone of antibody excess. The experiments in Table II (Exp. 2 excepted) indicated that the ratio increased as the content of antigen diminished. More decisive results can be obtained in experiments, described below, in which increasing amounts of an antiserum were added to a constant amount of antigen.

CONSTANT-ANTIGEN EXPERIMENTS (Table III).

Falling amounts of antiserum were placed in a series of tubes and a constant amount of antigen solution of known strength added to each. The volume of fluid in every tube of a series was made equal by the addition of saline where necessary, and each series contained a mixture in which the reagents were in constant-antibody optimal proportions. Incubation, tests on supernatant fluids, and nitrogen determinations on precipitates were carried out as in the experiments of Table II.

Table II.

SECTION A.

Antiserum	Antigen nitrogen mg.	Precipitate nitrogen mg.	Ratio Precipitate N / Antigen N	Tests on supernatants: For antigen	Tests on supernatants: For antibody
		Exp. 1.			
A.S. 1722E. Ratio (constant-antibody) 1 to 23·5. 1 c.c. A.S. in each tube	0·1328	1·099	8·3	+	—
	0·1062	1·072	10·1	+	—
	0·0797	0·948	11·9	—	—
	*0·0664	0·886	13·3	—	—
	*0·0664	0·880	13·3	—	—
	0·0531	0·786	14·8	—	+
	†0·0398	0·698	17·5	—	+
	0·0332	0·608	18·3	—	+
		Exp. 2.			
A.S. 1720E. Ratio (constant-antibody) 1 to 11·5. 1 c.c. A.S. 1 in 1·5 in each tube	0·2894	Trace	—	+	—
	0·2170	0·644	3·0	+	—
	0·1809	0·925	5·1	+	—
	0·1447	1·133	7·8	+	—
	0·1085	1·004	9·3	+	—
	*0·0904	0·942	10·4	? Trace	—
	0·0723	0·783	10·8	—	+
	†0·0543	0·655	12·1	—	+
	0·0362	0·416	11·5	—	+
	0·0362	0·449	12·4	—	+
	0·0181	0·185	10·2	—	+
	0·0181	0·199	11·0	—	+
		Exp. 3.			
A.S. 1754C. Ratio (constant-antibody) 1 to 21. 1 c.c. A.S. in each tube	0·2377	0·575	2·4	+	—
	0·2080	0·879	4·2	+	—
	0·1783	1·149	6·4	+	—
	0·1486	1·144	7·7	+	—
	0·1337	1·128	8·4	+	—
	0·1189	1·111	9·3	+	—
	0·1040	1·055	10·1	+	—
	0·0891	0·974	10·9	+	—
	*0·0743	0·914	12·3	—	—
	0·0594	0·813	13·7	—	+
	†0·0446	0·677	15·2	—	+
	0·0297	0·500	16·8	—	+
	0·0297	0·512	17·2	—	+
	0·0149	0·253	17·0	—	+
	0·0149	0·246	16·5	—	+
		Exp. 4.			
A.S. 1757 C. Ratio (constant-antibody) 1 to 17. 1 c.c. A.S. in each tube	0·2936	0·413	1·4	+	—
	0·2202	0·824	3·7	+	—
	0·1835	1·088	5·9	+	—
	0·1652	1·145	6·9	+	—
	0·1468	1·140	7·8	+	—
	0·1285	1·120	8·7	+	—
	0·1101	1·066	9·7	+	—
	*0·0918	0·976	10·6	—	—
	0·0734	0·910	12·4	—	+
	†0·0551	0·715	13·0	—	+
	0·0367	0·557	15·2	—	+
	0·0184	0·287	15·6	—	+
	0·0184	0·283	15·4	—	+

* See explanation in text. † See explanation in text.

https://doi.org/10.1017/S0022172400034422 Published online by Cambridge University Press

Table II (*continued*).

SECTION B.

Antiserum	Antigen nitrogen mg.	Precipitate: Nitrogen mg.	Precipitate: Protein mg.	Precipitate: Weights, mg. 1st	Precipitate: Weights, mg. 2nd	Ratio Precipitate N / Antigen N	Tests on supernatants
			Exp. 9.				
A.S. 1758 D. Ratio (constant-antibody) 1 to 32. 2 c.c. A.S. in each tube	0·2730	0·949	6·27	6·0	6·1	3·5	Not done
	0·2340	1·220	8·06	7·8	7·9	5·2	,,
	0·1755	1·437	9·50	9·6	9·5	8·2	,,
	0·1365	1·268	8·38	8·7	8·7	9·3	,,
	*0·0975	0·999	6·60	6·9	7·2	10·2	,,
	0·0780	0·935	6·18	6·3	6·3	12·0	,,
	0·0585	0·794	5·25	5·0	5·2	13·6	,,

The results of the experiments in which the amount of antigen was constant and that of antiserum varied are given in Table III; they indicate a definite, if only gradual, rise in the ratio of precipitate nitrogen to antigen nitrogen with increasing antibody excess, but although this ratio was practically constant at the optimal point, 1 to 11, its rate of increase with increasing antibody was by no means uniform with different antisera, *e.g.* in Exps. 12 and 13 the ratios were 19·4 and 16·0 respectively in the tubes where eight times the optimal amount of antibody was present.

Both the constant-antibody and the constant-antigen ratios were obtained for the antisera used in Exps. 7, 10, 11 and 12; in each case the quotient $\frac{\text{constant-antigen ratio}}{\text{constant-antibody ratio}}$ agreed well with the value 1·6 previously reported by Taylor (1933). Exps. 7, 10 and 12 included tubes in which the proportions of antigen and antibody corresponded closely to the constant-antigen ratio. In each of these tubes, marked † in the table, tests on supernatant fluids showed the presence of definite antibody excess; in the experiments of Table II the same result was demonstrated by certain tubes, also marked †, in which there was approximately 1·6 times the constant-antibody requirement of antibody.

To provide the large amounts of antiserum needed for Exps. 13 and 14, antisera were pooled. The two used for Exp. 13 both had similar constant-antibody optimal ratios; equal parts were mixed and the resulting mixed antiserum had the same ratio as the components. The two antisera of Exp. 14 had different ratios and different amounts of each were mixed together; the ratio of the mixed antiserum agreed with the ratio calculated from those of the components (see Taylor, 1931).

WEIGHTS OF PRECIPITATES.

In three experiments, 9, 10 and 12, the precipitates were determined gravimetrically. Monax glass tubes which had been dried to constant weight over phosphorus pentoxide were used for the precipitations. The precipitates were centrifuged and then washed once with saline and once with distilled water. The volume of the precipitate was so small that a single washing with water was sufficient to eliminate the sodium chloride. The tubes were dried in

Table III.

Section A.

Antiserum	A.S. c.c.	Saline c.c.	Antigen nitrogen mg.	Precipitate nitrogen mg.	Ratio Precipitate N / Antigen N	Tests on supernatants: For antibody	Tests on supernatants: For antigen
			Exp. 7.				
A.S. 1758 C. Ratio (constant-antibody) 1 to 14, (constant-antigen) 1 to 22. Quotient 22/14 = 1·57	1·8	—	0·1114	1·500	13·5	+	—
	†1·6	0·2	,,	1·469	13·2	+	—
	1·4	0·4	,,	1·417	12·7	+	—
	1·2	0·6	,,	1·334	12·0	? +	—
	*1·0	0·8	,,	1·244	11·2	—	—
	0·75	1·05	,,	1·123	10·1	—	? +
	0·5	1·3	,,	0·792	7·1	—	+
	0·5	1·3	,,	0·789	7·1	—	+
	0·25	1·55	,,	0·0084	0·075	—	+
	0·25	1·55	,,	0·0028	0·025	—	+
			Exp. 11.				
A.S. 1756 D. Ratio (constant-antibody) 1 to 38, (constant-antigen) 1 to 57·5. Quotient 57·5/38 = 1·51	8·0	—	0·0411	0·512	12·5	+	—
	6·0	2·0	,,	0·498	12·1	+	—
	4·0	4·0	,,	0·518	12·6	+	—
	2·0	6·0	,,	0·484	11·8	+	—
	*1·0	7·0	,,	Broken	—	—	? +
	0·5	7·5	,,	0·145	3·5	—	+
			Exp. 13.				
A.S. 1758 E and 1754 E equal parts. Ratio of mixed A.S. (constant-antibody) 1 to 15·5. Ratio of components 1 to 15·5	12·0	4·0	0·1006	1·701	16·9	+	—
	8·0	8·0	,,	1·612	16·0	+	—
	6·0	10·0	,,	1·463	14·5	+	—
	4·0	12·0	,,	1·559	15·5	+	—
	2·0	14·0	,,	1·364	13·6	+	—
	*1·0	15·0	,,	1·119	11·1	—	—
	*1·0	15·0	,,	1·120	11·1	—	—
	0·5	15·5	,,	0·575	5·7	—	+
			Exp. 14.				
Mixed A.S. 1720 G (20 c.c.). Ratio (constant-antibody) 1 to 9. 1756 E 30 c.c.). Ratio (constant-antibody) 1 to 36. Ratio of mixed A.S.: titration 1 to 16·5, calculated 1 to 16·36	15·0	—	0·0945	1·184	12·5	+	—
	10·0	5·0	,,	1·142	12·1	+	—
	8·0	7·0	,,	1·127	11·9	+	—
	6·0	9·0	,,	1·085	11·5	+	—
	4·0	11·0	,,	1·086	11·5	+	—
	2·0	13·0	,,	1·015	10·7	+	—
	*1·0	14·0	,,	0·924	9·8	—	—
	*1·0	14·0	,,	0·930	9·8	—	—

Section B.

Antiserum	A.S. c.c.	Saline c.c.	Antigen nitrogen mg.	Precipitate: Nitrogen mg.	Precipitate: Protein mg.	Precipitate: Weights (mg.) 1st	Precipitate: Weights (mg.) 2nd	Ratio Precipitate N / Antigen N	Tests on supernatants: For antibody	Tests on supernatants: For antigen
				Exp. 10.						
A.S. 1754 D. Ratio (constant-antibody) 1 to 14, (constant-antigen) 1 to 22. Quotient 22/14 = 1·57	4·2	0·8	0·1950	2·875	19·02	18·9	19·0	14·7	+	—
	3·85	1·15	,,	2·861	18·91	18·7	18·7	14·7	+	—
	3·5	1·5	,,	2·796	18·48	18·4	18·5	14·3	+	—
	3·15	1·85	,,	2·781	18·38	17·9	18·1	14·3	+	—
	†2·8	2·2	,,	2·656	17·55	17·5	17·5	13·6	+	—
	2·45	2·55	,,	2·516	16·63	16·5	16·6	12·9	+	—
	2·1	2·9	,,	Broken	—	15·2	15·2	—	+	—
	*1·75	3·25	,,	2·202	14·55	14·5	14·6	11·3	—	—
	1·4	3·6	,,	2·031	13·42	13·0	13·2	10·4	—	+
	1·05	3·95	,,	1·693	11·18	11·2	11·3	8·7	—	+
	0·7	4·3	,,	1·137	7·51	7·5	7·5	5·8	—	+
				Exp. 12.						
A.S. 1722 G. Ratio (constant-antibody) 1 to 14·5, (constant-antigen) 1 to 22. Quotient 22/14·5 = 1·52	8·0	—	0·1076	2·088	13·80	14·0	13·8	19·4	+	—
	6·0	2·0	,,	1·991	13·16	13·0	12·9	18·5	+	—
	4·0	4·0	,,	1·942	12·84	12·9	12·9	18·0	+	—
	2·0	6·0	,,	1·588	10·49	10·7	10·7	14·8	+	—
	†1·5	6·5	,,	1·438	9·51	9·9	9·9	13·4	+	—
	*1·0	7·0	,,	1·222	8·08	8·2	8·2	11·4	—	—
	*1·0	7·0	,,	1·208	7·98	8·3	8·2	11·2	—	—
	0·5	7·5	,,	0·641	4·24	4·6	4·6	6·0	—	+

https://doi.org/10.1017/S0022172400034422 Published online by Cambridge University Press

a high vacuum over phosphorus pentoxide. The first weighing was made after drying overnight, the second a day or two later after further drying. The results recorded in sections B of Tables II and III show that there was good agreement between the two weighings. The nitrogen contents of the precipitates were then determined. It was found that the percentage of nitrogen in the precipitates was approximately equal to the value 15·13 per cent., given for globulin (Adair and Robinson, 1930). Ten determinations in Exp. 10 showed a range of variation from 15·0 to 15·4. In the other experiments the masses of the precipitates were smaller and the range of variation was somewhat greater. The precipitates contained an antigen, egg-albumin, with a nitrogen content of 15·6 per cent., but in the zone of antibody excess the ratio of the precipitate nitrogen to the total antigen added was usually greater than 10, and in view of the generally accepted conclusion that antibodies are found in the globulin fraction of the antiserum, it is not surprising that the percentage of nitrogen in the precipitates agreed with the value obtained for globulin. In a mixture composed of 10 g. of globulin per gramme of albumin the nitrogen percentage is 15·17. The difference between this figure and the value recorded for pure globulin is less than the experimental error. The figure 15·13 has been used in the calculations of protein content from nitrogen determinations, which have been recorded in the tables for comparison with the gravimetric determinations. The agreement between the two results affords evidence that the precipitates are composed of protein, for if they contained appreciable amounts of adsorbed inorganic salts or compounds poor in nitrogen, the gravimetric results should exceed the values calculated from nitrogen determinations.

THE EFFECTS OF EXCESS OF ANTIGEN AND OF ANTIBODY.

In the constant-antibody experiments (Table II) it has been shown that the points of optimal particulation and maximal precipitation were not identical, but that the greatest amount of precipitate was obtained when from 1·6 to 2·4 times the optimal amount of antigen was present. Greater excess of antigen quickly led to diminution and disappearance of the precipitate. The quantitative relationships are shown in the graphs in Fig. 1, Exps. 4 and 6. The points lie on a smooth curve which rises rapidly to the optimal ratio, and more slowly to a maximum, at which the amount of precipitate is about 20 per cent. greater than that in the optimal tube. On the side of antigen excess the curve falls rapidly. The curves in Fig. 1, Exps. 7 and 10, showing the amounts of precipitate in constant-antigen experiments, are of a very different type. At low concentrations of antiserum very little precipitate is formed, but after about a quarter of the constant-antibody optimal amount of antiserum has been added a curve of hyperbolic type is obtained which rises steeply up to and beyond the optimal tube, and then approximates towards a limiting value. In Exps. 7 and 10 no very considerable excess of antiserum was present, but that the curve does not reach a maximum with the addition of very great excess is shown by the data of Exps. 11, 12, 13

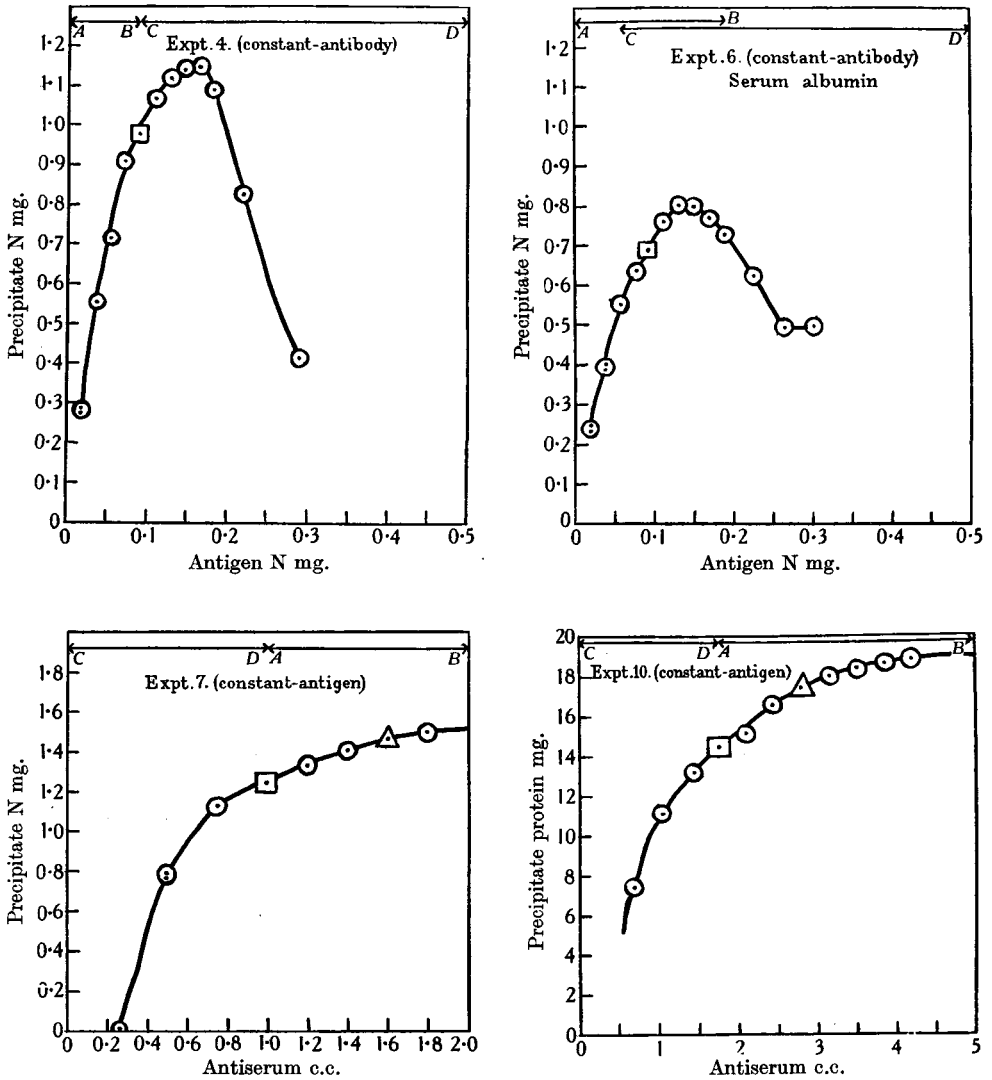

Fig. 1.

Exp. 4. Ordinates: precipitate nitrogen in milligrammes from 1 c.c. of antiserum. Abscissae: antigen (egg-albumin) nitrogen in milligrammes. The Dean and Webb (constant-antibody) optimal point is represented by a square. The line from *A* to *B* indicates the presence of antibody, that from *C* to *D* the presence of antigen in the supernatant fluid.

Exp. 6. Resembles Exp. 4, but the antigen was serum albumin.

Exp. 7. Ordinates: precipitate nitrogen in milligrammes with antigen (egg-albumin) nitrogen constant, 0·1114 mg. Abscissae: c.c. of antiserum. The constant-antigen optimum represented by a triangle.

Exp. 10. Ordinates: precipitate protein in milligrammes determined gravimetrically with antigen (egg-albumin) constant, 1·25 mg. of protein. Abscissae: c.c. of antiserum.

https://doi.org/10.1017/S0022172400034422 Published online by Cambridge University Press

and 14. It was expected that excess of antibody beyond a certain point would decrease the precipitate, but even when fifteen times the neutralising amount was added no diminution occurred (Exp. 14), although the rate of particulation progressively decreased with excess of antibody beyond the constant-antigen optimal tube. It is possible that still greater excess would inhibit the precipitate, but in our work very gross excess has failed to do so. It is obvious that the effects of excess antigen are very different from those of excess antibody, and although it has long been taught that excess of either ingredient in the precipitation reaction leads to inhibition, antigen acts far more powerfully than antibody. It may well be that deficiency of antigen rather than excess of antibody is mainly responsible for the diminution of precipitate seen in those tubes of a constant-antibody titration which contain much less than the optimal amount of antigen.

THE RATIO OF ANTIGEN TO PRECIPITATE NITROGEN.

Culbertson stated that, in a neutral mixture of crystalline egg-albumin and homologous antiserum, the ratio between antigen and precipitate nitrogen is constant, 1 to 13. Our ten ratios in Tables II and III with a maximum of 13·3 and a minimum of 9·8 have a mean value of 11·15, and the ratios in section A of Table I are in good agreement. He states further that this ratio is maintained throughout the post-zone where antibody is demonstrable in the supernatant fluids; on this point our evidence is not in agreement. As mentioned above it definitely suggests a steady rise in the ratio with increasing excess of antibody. Where antigen was in excess we found, as did Culbertson, that the ratio diminished. In the case of egg-albumin and serum-albumin it is not possible to determine the antigen nitrogen in the precipitates, and the variations of the ratios referred to above do not prove that the composition of the precipitate is variable, because a part of the total antigen added may remain in the supernatant fluids, even in the zone of antibody excess where tests fail to reveal the presence of free antigen. The precipitates obtained with certain antigens including azo-proteins can be analysed as described by Marrack and Smith (1931), who showed that the composition of the precipitate is variable. It is not unlikely that the same conclusions apply to egg-albumin, but, on the other hand, the conditions for obtaining an antigen of constant composition are more favourable in the case of a homogeneous protein like egg-albumin. The work of Svedberg (1930) and his colleagues has shown that native proteins are easily changed into mixtures of particles of different sizes by chemical treatment.

EXPERIMENTS WITH HORSE-SERUM ALBUMIN AND HOMOLOGOUS ANTISERA (Table IV).

Culbertson reported that he was unable to apply his neutralisation method to the titration of rabbit anti-horse sera, because it was impossible to demonstrate a point of neutralisation in antisera to this complex antigen. We have

encountered similar difficulties in the titration of antisera to horse-serum albumin, although the albumin had been purified by three recrystallisations. These experiments are outlined in Table IV. Ring tests disclosed the simultaneous presence of both antigen and antibody in the supernatant fluids from a fairly wide range of tubes, including the optimal one, and therefore it was impossible to determine a neutral point. In the experiments with crystalline egg-albumin no supernatant fluid gave reactions for both antigen and antibody.

Table IV.

Antiserum	Antigen nitrogen mg.	Precipitate nitrogen mg.	Ratio $\frac{\text{Precipitate N}}{\text{Antigen N}}$	Tests on supernatants: For antigen	Tests on supernatants: For antibody
		Exp. 5.			
A.S. 1825D. Ratio 1 % alb. to A.S. (constant-antibody) 1 to 8. 1 c.c. A.S. in each tube	0·6240	1·106	1·8	+	—
	0·5460	1·774	3·2	+	—
	0·4680	1·943	4·2	+	—
	0·3900	2·029	5·2	+	+
	0·3510	2·037	5·8	+	+
	0·3120	2·016	6·5	+	+
	0·2730	1·926	7·1	+	+
	0·2340	1·805	7·7	+	+
	*0·1950	1·660	8·5	+	+
	0·1560	1·505	9·6	+	+
	0·1170	1·361	11·6	+	+
	0·0780	1·040	13·3	+	+
	0·0390	0·599	15·4	+	+
	0·0390	0·603	15·5	+	+
		Exp. 6.			
A.S. 1930C. Ratio 1 % alb. to A.S. (constant-antibody) 1 to 16·5. 1 c.c. A.S. in each tube	0·3025	0·499	1·6	+	—
	0·2647	0·495	1·9	+	—
	0·2269	0·623	2·7	+	—
	0·1891	0·725	3·8	+	+
	0·1701	0·769	4·5	+	+
	0·1513	0·798	5·3	+	+
	0·1324	0·805	6·1	+	+
	0·1135	0·761	6·7	+	+
	*0·0945	0·688	7·3	+	+
	0·0756	0·637	8·4	+	+
	0·0567	0·551	9·7	+	+
	0·0378	0·398	10·5	—	+
	0·0378	0·386	10·2	—	+
	0·0189	0·246	13·0	—	+
	0·0189	0·238	12·6	—	+

One hypothesis, which may account for the findings with serum albumin, is that this protein is a mixture of different antigens. Sørensen (1930) isolated, from crystalline serum albumin, a number of fractions which differed in their solubilities.

Conclusions.

1. When the nitrogen contents of precipitates from mixtures of crystalline egg-albumin and homologous antisera in varying proportions have been determined, and the supernatant fluids from the precipitates have been examined for residual antigen and antibody, it has been found that:

(*a*) There is a point of neutralisation where neither antigen nor antibody is in the supernatant fluid. At this point the mean of our values for the ratio

https://doi.org/10.1017/S0022172400034422 Published online by Cambridge University Press

antigen nitrogen to precipitate nitrogen is 1 to 11·15; this figure may be a constant.

(*b*) The neutral point appears to be identical with, or very close to, the Dean and Webb (1926), the constant-antibody, optimal point.

(*c*) When increasing amounts of antigen are added to a constant amount of antiserum, the greatest precipitate nitrogen is found, not at the neutral point, but where excess of antigen is present. Further excess of antigen leads to diminution and disappearance of the precipitate.

(*d*) When increasing amounts of antiserum are added to a constant amount of antigen, the first particulation takes place not at the neutral point, but where 1·6 times the neutralising amount of antibody is present. At this constant-antigen optimal point antibody is demonstrable in the supernatant fluid. The precipitate nitrogen increases when increasing amounts of antiserum are added, and even fifteen times the neutralising amount of antibody failed to decrease the precipitate. It is possible that still further antibody excess may diminish the precipitate.

(*e*) The inhibitory effects of excess antigen are much more marked than those of excess antibody.

2. All, or nearly all, of the precipitate appears to be protein, since in some cases precipitates have been weighed, and the weights determined corresponded very closely with the weight of protein calculated from estimations of the precipitate nitrogen.

3. Tests on supernatant fluids failed to demonstrate a neutral point when crystalline horse-serum albumin and homologous antisera were examined. The explanation may be that serum albumin contains more than one antigen.

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(*MS. received for publication* 22. XI. 1933.—Ed.)