THE PRECIPITATION REACTION

EXPERIMENTS WITH AN ANTISERUM CONTAINING TWO ANTIBODIES

By H. R. DEAN, G. L. TAYLOR, *John Lucas Walker Student*, and MURIEL E. ADAIR

From the Department of Pathology and the Biochemical Laboratory, Cambridge

Introduction

Of the chemical structure of antibodies little or nothing is known. It is generally held that the specific properties of an antiserum are closely associated with the globulin fraction. An antiserum may be distinguished from a normal serum by the precipitation of some of its globulins in the presence of the homologous antigen. The formation of a precipitate in a mixture of antigen and antiserum may be explained in either of two ways: (1) The reaction which occurs when antigen is added to antiserum may produce such physical changes in the mixture that normal globulin is precipitated. The reaction takes place between two specific substances, the antigen and the antibody, and is rendered visible by the presence of a non-specific globulin which plays the part of an indicator. (2) Or we may suppose that in the process of immunisation the antigen produces a specific alteration in the structure of the globulin. That is to say, the antibody is a specifically altered globulin, which has undergone some change in its molecular structure, in virtue of which it can react and combine with the homologous antigen.

The experiments which are described below were planned to throw light on the relation of antibody to the globulin of the serum and more particularly to test the second of the above hypotheses.

It was decided to attempt to work to the following plan, namely:

(1) To prepare and purify two separate antigens, crystalline egg albumin and crystalline horse serum albumin.

(2) To immunise rabbits by the injection of both these antigens in the hope of obtaining an antiserum which would yield a satisfactory precipitate when mixed with either antigen.

(3) To determine the optimal proportions for precipitation in mixtures of either antigen with the antiserum.

(4) To determine the amount of precipitate formed when

(*a*) 1 c.c. of the antiserum was added to the optimal amount of egg albumin;

(*b*) 1 c.c. of the antiserum was added to the optimal amount of horse albumin;

https://doi.org/10.1017/S0022172400018982 Published online by Cambridge University Press

(*c*) 1 c.c. of the antiserum was added to a mixture containing the optimal amount of egg albumin and the optimal amount of horse albumin.

(5) To mix the antiserum with egg albumin in optimal proportions and after the reaction was completed to separate the precipitate from the supernatant fluid and

(*a*) to determine the amount of the precipitate;

(*b*) to add to the supernatant fluid its optimal amount of the other antigen horse albumin and to determine the amount of the precipitate.

(6) To mix the antiserum with horse albumin in optimal proportions and after the reaction was completed to separate the precipitate from the supernatant fluid and

(*a*) to determine the amount of the precipitate;

(*b*) to add to the supernatant fluid its optimal amount of egg albumin and to determine the amount of the precipitate.

The results of these experiments may be expressed briefly as follows:

In the first experiment (paragraph (4) above) 1 c.c. of antiserum mixed with the optimal amount of egg albumin produced x amount of precipitate, and 1 c.c. of antiserum mixed with the optimal amount of horse albumin produced y amount of precipitate. 1 c.c. of antiserum added to a mixture containing the optimal amounts of egg albumin and horse albumin yielded $x+y$ amount of precipitate.

In the second experiment (paragraph (5) above) a mixture of 1 c.c. of the antiserum with the optimal amount of egg albumin yielded x amount of precipitate. The supernatant fluid which was equivalent to $\frac{2}{3}$ c.c. of antiserum was mixed with its optimal amount of horse albumin and yielded $\frac{2}{3}y$ precipitate. The mixture of 1 c.c. of antiserum with the optimal amount of horse albumin (paragraph (6) above) yielded y amount of precipitate. The supernatant fluid which was equivalent to $\frac{2}{3}$ c.c. of antiserum yielded $\frac{2}{3}x$ amount of precipitate when mixed with its optimal amount of egg albumin.

As it was convenient to use larger quantities than those given above, and it was thought desirable to test the behaviour of mixtures of antigen and antibody in proportions other than the optimal ones, our actual experiments were rather more complicated than those we have outlined. Since Taylor, Adair and Adair (1934) showed that the weights of such precipitates could be accurately determined from estimations of their nitrogen content, the precipitates have been expressed in terms of their nitrogen content.

Methods

The antigens used were egg albumin, crystallised seven times as described by Taylor, Adair and Adair (1932), and horse serum albumin four times crystallised according to the method of Adair and Robinson (1930). Both antigens contained 15·6 per cent. of nitrogen.

Each of a series of rabbits was injected intraperitoneally with 4 c.c. of a 1 per cent. solution of one antigen and immediately afterwards with a similar

amount of the second antigen. Six such injections were given at four-day intervals. At each dose the order of injecting the two antigens was reversed; egg albumin being given first on one occasion, serum albumin first on the next. Nine days after the last dose the animals were bled. As the antisera produced by the first course were not strong enough for our purpose, a second course of injections exactly similar to the first was given. For our work we used the second batch of antisera.

Antisera were titrated against each antigen by the optimal proportions method of Dean and Webb (1926) 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 antigen. Antiserum 2373 B had a ratio of 1 to 45 with 1 per cent. egg albumin, which means that 45 parts by volume of antiserum were in optimal proportions with one part by volume of 1 per cent. egg albumin solution. The ratio of this antiserum with serum albumin was 1 to 17·5; 17·5 parts of antiserum were in optimal proportions with one part of 1 per cent. serum albumin solution.

Antiserum was exposed to the action of each antigen in turn by mixing a portion with one antigen and adding the second antigen to the supernatant fluid separated from the resulting precipitate. Equal portions of antiserum were placed in suitable tubes, to one was added egg albumin and to the other serum albumin. The mixtures were incubated for 2 hours at 37° C. and stored overnight in the refrigerator. Next day, after centrifugalisation, the supernatant fluids were decanted and to a portion of the one originally treated with egg albumin was added serum albumin, so that the proportions of antigen and antiserum equalled those in the mixture containing antiserum and serum albumin. Similarly, a portion of the supernatant fluid from the mixture originally treated with serum albumin was mixed with egg albumin.

After washing twice by suspension in 0·85 per cent. saline and centrifugalisation, the precipitates were dissolved in glass-distilled water with the addition of a small amount of dilute soda, and analysed for nitrogen by the method of Parnas and Wagner (1921). Taylor, Adair and Adair (1934) showed that the loss of precipitate due to such washing is small enough to be negligible.

RESULTS

Three antisera were investigated; details of the experiments and results appear in Table I. In the tubes labelled S supernatant fluids from mixtures of antiserum and one antigen were added to the second antigen; e.g. tube 4S in Exp. 1 contained the supernatant from tube 4. In the tubes of Exps. 1 and 2 the volume of the mixture was always 6 c.c., either 3 c.c. of antiserum and 3 c.c. of antigen dilution, or 4 c.c. of supernatant fluid and 2 c.c. of a corresponding dilution of the second antigen. It will be appreciated that 4 c.c. of supernatant fluid correspond to 2 c.c. of antiserum. In tube 2 of Exp. 1, 3 c.c. of antiserum were mixed with 3 c.c. of egg albumin dilution in optimal proportions; 4 c.c. of the resulting supernatant fluid and its optimal amount of serum albumin in a

volume of 2 c.c. were placed in tube 2S. Tubes 4 and 4S were similar save that the order of adding the antigens was reversed. Tube 4S, containing two-thirds of the supernatant fluid resulting from the interaction of 3 c.c. of antiserum and its optimal amount of serum albumin, received two-thirds the amount of egg albumin in tube 2, and the precipitate in tube 4S was about two-thirds of that in tube 2. Similarly, with serum albumin as antigen, the precipitate in tube 2S was almost exactly two-thirds of that in tube 4.

Table I

Antiserum	Tube	A.S. c.c.	Supt. c.c.	Antigen: Egg alb. 1 %	Antigen: Ser. alb. 1 %	Antigen: Nitrogen mg.	Precipitate nitrogen mg.	Ratio Precipitate N / Antigen N
				Exp. 1				
2373 B	2	3	—	3 c.c., 1 in 45	—	0·1040	1·784	17·2 } †
Ratios: with egg	4S	—	4	2 c.c., 1 in 45	—	0·0693	1·313	18·9 }
alb. 1 % 1 to 45;	4	3	—	—	3 c.c., 1 in 17·5	0·2674	2·488	9·3 }
with ser. alb. 1 %	2S	—	4	—	2 c.c., 1 in 17·5	0·1783	1·676	9·4 }
1 to 17·5	1	3	—	3 c.c., 1 in 32	—	0·1463	2·596	17·7 }
	3S	—	4	2 c.c., 1 in 32	—	0·0975	1·588	16·3 }
	3	3	—	—	3 c.c., 1 in 13	0·3600	2·925	8·1 }
	1S	—	4	—	2 c.c., 1 in 13	0·2400	2·006	8·4 }
				Exp. 2				
2374 B	1	3	—	3 c.c., 1 in 54	—	0·0867	0·736	8·5 }
Ratios: with egg	4S	—	4	2 c.c., 1 in 54	—	0·0578	0·458	7·9 }
alb. 1 % 1 to 54;	5S	—	4	2 c.c., 1 in 54	—	0·0578	0·466	8·1 }
with ser. alb. 1 %	6S	—	4	2 c.c., 1 in 54	—	0·0578	0·428	7·4 }
1 to 14·5	2	3	—	3 c.c., 1 in 27	—	0·1734	1·099	6·3
	3	3	—	3 c.c., 1 in 108	—	0·0434	0·440	10·1
	4	3	—	—	3 c.c., 1 in 14·5	0·3228	2·513	7·8 }
	1S	—	4	—	2 c.c., 1 in 14·5	0·2152	1·641	7·6 }
	5	3	—	—	3 c.c., 1 in 7·25	0·6455	2·773	4·3 }
	2S	—	4	—	2 c.c., 1 in 7·25	0·4303	1·875	4·4 }
	6	3	—	—	3 c.c., 1 in 29	0·1614	1·561	9·7 }
	3S	—	4	—	2 c.c., 1 in 29	0·1076	1·000	9·3 }
				Exp. 3*				
2375 B	2	3	—	3 c.c., 1 in 17·5	—	0·2674	2·773	10·4 }
Ratios: with egg	1S	—	6	2 c.c., 1 in 17·5	—	0·1783	1·843	10·3 }
alb. 1 % 1 to 17·5;	1	3	—	—	3 c.c., 1 in 8·75	0·5349	4·539	8·5 }
with ser. alb. 1 %	2S	—	6	—	2 c.c., 1 in 8·75	0·3565	3·094	8·7 }
1 to 8·75	3	3	—	3 c.c., 1 in 17·5	3 c.c., 1 in 8·75	—	7·048	—

* The volume of each tube in Exp. 3 was 9 c.c., saline being added where necessary.

† Tubes containing comparable amounts of antigen are bracketed together.

As both antigens contained 15·6 per cent. of nitrogen, this figure has been used in calculating the amount of antigen nitrogen in our experiments. The weight of precipitate per unit of antigen in a mixture is expressed in terms of the precipitate nitrogen/antigen nitrogen ratio; for tubes 2 and 4S these ratios were very similar, and for tubes 4 and 2S almost identical. In tubes 1, 3S, 3 and 1S of Exp. 1 the mixtures contained antigen in excess of the optimal amount, and here again the precipitate per unit of antigen was almost the same from antiserum and from supernatant fluid.

In Exp. 2 mixtures were set up containing optimal, twice the optimal, and half the optimal amounts of antigen, thus tubes 4S, 5S, and 6S contained supernatant fluid from mixtures of antiserum and different amounts of serum albumin; the addition of the optimal amount of egg albumin to each of these

supernatant fluids produced similar quantities of precipitate, and, per unit of egg albumin, precipitates similar to that in tube 1 in which were 3 c.c. of antiserum and its optimal amount of egg albumin. Tubes 4 and 1S both contained the optimal amount of serum albumin, and their precipitate nitrogen/antigen nitrogen ratios were nearly identical, as were the ratios of tubes 5 and 2S, to which had been added twice the optimal dose of serum albumin, and of tubes 6 and 3S which contained half the optimal serum albumin. That the precipitate nitrogen/antigen nitrogen ratios varied with differing proportions of antigen and antibody was shown by Taylor, Adair and Adair (1934).

In Exp. 3 the total volume of each mixture was 9 c.c., saline being added where necessary. Tubes 2 and 1S received optimal amounts of egg albumin; precipitate nitrogen/antigen nitrogen ratios were almost identical, as they were in tubes 1 and 2S with optimal serum albumin. In tubes 1S and 2S, 6 c.c. of supernatant fluid were included, corresponding to 2 c.c. of antiserum. In tube 3 the antiserum was mixed simultaneously with optimal amounts of both egg albumin and serum albumin, and 7·048 mg. of precipitate nitrogen resulted; antiserum and egg albumin yielded 2·773 mg. of nitrogen (tube 2), antiserum and serum albumin 4·539 mg. of nitrogen (tube 1), totalling 7·312 mg. of nitrogen. Apparently the amount of precipitate resulting from the reaction between one antigen and its antibody was quite unaffected by the reaction, occurring in the same mixture, between the second antigen and its antibody.

Consideration of the results shows that the injection of two different antigens into a rabbit produced in its serum homologous precipitins which were quite separate and distinct. When the antiserum was mixed with either of the antigens, the antibody for the other antigen took no part in the resulting reaction. The independence was maintained in mixtures containing excess of antigen, excess of antibody, and antigen and antibody in optimal proportions, and would appear to be unaffected by variations of the proportions in which the reagents were mixed. That the sum of the precipitates in tubes 1 and 2 of Exp. 3 equalled that in tube 3 shows that the precipitate from either antigen and its antibody was not affected by the presence of the second reaction in the same mixture.

Taylor, Adair and Adair (1934), by ring tests, found neither antigen nor antibody in the supernatant fluids from mixtures of egg albumin and its antiserum in optimal proportions. The antigen nitrogen/precipitate nitrogen ratios of such mixtures were always about 1 to 11, and further work has confirmed this figure. Culbertson (1932) stated that the proportion at the neutral point was 1 to 13, Heidelberger and Kendall (1934) reported 1 to 12. Only one of the three antisera made against two antigens has given a figure, 1 to 10·4, which approximates to 1 to 11 (Exp. 3). In Exps. 1 and 2 the ratios were 1 to 18 and 1 to 8. At present we have no explanation of these discrepancies: by ring tests the supernatant fluids from the mixtures in question were apparently neutral. Optimal mixtures of horse serum albumin and the three antisera yielded ratios

more nearly alike (1 to 8); they agree with those reported by Taylor, Adair and Adair (1934) for horse serum albumin and its antiserum.

Conclusion

The serum of a rabbit injected with two separate antigens—egg albumin and horse serum albumin—has been shown to contain two distinct precipitable substances, an anti-egg precipitable substance and an anti-horse precipitable substance. Each of these precipitable substances is specific and, while it reacts with its homologous antigen, is indifferent to the presence of the other antigen. Nitrogen estimations by Breinl and Haurowitz (1930), Marrack and Smith (1931), and Taylor, Adair and Adair (1934) suggest that such precipitable substances are of the nature of globulins. That is to say, immunisation is a process in which a fraction of the serum globulin undergoes an alteration whereby it becomes precipitable by the homologous antigen. Our results support the suggestions made by one of us (Dean, 1916 and 1931), that there is an aggregation of the globulin particles around the antigen and a precipitation of the globulin, and are apparently consistent with the views very clearly enunciated by Marrack (1934) that antibodies are globulins.

REFERENCES

Adair, G. S. and Robinson, Muriel E. (1930). The specific refraction increments of serum-albumin and serum-globulin. *Biochem. J.* **24**, 993.

Breinl, F. und Haurowitz, F. (1930). Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-Hämoglobin-Serum und Bemerkungen über die Natur der Antikörper. *Zeitschr. Physiol. Chem.* **192**, 45.

Culbertson, J. T. (1932). A quantitative study of the precipitin reaction with special reference to crystalline egg albumin and its antibody. *J. Immunol.* **23**, 439.

Dean, H. R. (1916). The mechanism of the serum reaction. *Brit. Med. J.* **2**, 749.

—— (1931). *A System of Bacteriology*, **6**, 424. London: H.M. Stationery Office.

Dean, H. R. and Webb, R. A. (1926). The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. *J. Path. and Bact.* **29**, 473.

Heidelberger, M. and Kendall, F. E. (1934). Quantitative studies on the precipitation reaction: the rôle of multiple reactive groups in antigen-antibody union as illustrated by an instance of cross-precipitation. *J. Exp. Med.* **59**, 519.

Marrack, J. R. (1934). *The chemistry of antigens and antibodies.* M.R.C. Report, Chap. II. London: H.M. Stationery Office.

Marrack, J. and Smith, F. C. (1931). Quantitative aspects of immunity reactions: the precipitin reaction. *Brit. J. Exp. Path.* **12**, 30.

Parnas, J. K. und Wagner, R. (1921). Ueber die Ausführung von Bestimmungen kleiner Stickstoffmengen nach Kjeldahl. *Biochem. Zeitschr.* **125**, 253.

Taylor, G. L. (1931). The results of some quantitative experiments on the serum precipitation reaction. *J. Hygiene*, **31**, 56.

—— (1933). The dissimilarity of the results of precipitin titrations performed with a constant amount of antiserum and with a constant amount of antigen. *Ibid.* **33**, 12.

Taylor, G. L., Adair, G. S. and Adair, Muriel E. (1932). The estimation of proteins by the precipitation reaction. *Ibid.* **32**, 340.

—— —— —— (1934). The precipitation reaction: optimal proportions, neutrality and maximal precipitation in mixtures of albumin and antiserum. *Ibid.* **34**, 118.

(*MS. received for publication* 10. XII. 1934.—Ed.)