THE DEMONSTRATION OF THE INCREASE OF GLOBULIN IN DIPHTHERIA ANTITOXINS BY THE PRECIPITATION REACTION

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Introduction

In titrations of anti-horse sera against parallel dilutions of (*a*) normal horse serum, (*b*) horse serum globulin and (*c*) horse serum albumin, Taylor, Adair and Adair (1932) found that with some antisera a primary or main zone of optimal particulation (Dean and Webb, 1926) occurred in the (*a*) horse serum and (*b*) globulin series but was absent from the (*c*) albumin series: the precipitin was regarded as antiglobulin. With other anti-horse sera reactions were seen in all three series: presumably these antisera contained both antiglobulin and antialbumin. Since this publication two anti-horse sera have been examined which contained antialbumin but no antiglobulin in the main zone. Recently similar findings have been reported by Goldsworthy and Rudd (1935).

By titrating anti-horse sera, containing only antiglobulin, against a 1 per cent. solution of globulin and against normal horse serum it was found possible to estimate the globulin content of the horse serum. The results of these titrations were expressed as antigen-antibody ratios, *e.g.* 1 part by volume of antiserum, 1193 K, reacted optimally with 177 parts of horse serum, the ratio horse serum to antiserum was 1 to 177; with 1 per cent. globulin the ratio was 1 to 40; the greater the ratio the greater the antigen content. Division of 177 by 40 yielded the percentage, 4·42, of globulin in the horse serum.

It was difficult to reconcile this finding with a statement made by Dean (1931, p. 436). He wrote: "In a series of unpublished experiments R. A. Webb has titrated specimens of normal horse-serum and of diphtheria antitoxin with antisera prepared by the injection of rabbits with normal horse-serum. In other experiments antitoxin and normal horse-serum have been titrated with antiserum prepared by the injection of antitoxin into rabbits. All specimens of both normal and antitoxic horse-serum have reacted in identical proportions with either antisera prepared by the injection of the normal serum or antisera prepared by injection of the antitoxin. The process of immunization with diphtheria toxin appears to have no effect on the antigenic content of horse-serum."

The results of many different workers (Hiss and Atkinson, 1900; Ledingham, 1907; Gibson and Banzhaf, 1910; and others), who have used chemical methods, have shown that the production of diphtheria antitoxin is accompanied by a considerable rise in the globulin content of the serum of the injected horse.

In the experiments described below some anti-horse sera failed to disclose any increase in the antigen content of diphtheria antitoxin, in accordance with Webb's observation. Other anti-horse sera, however, which reacted with globulin only, showed a marked increase, in agreement with the conclusions arrived at by other methods.

METHODS

Titrations were performed by the method of Dean and Webb (1926) as described by Taylor, Adair and Adair (1932). The antigen-antibody ratios assigned to the experiments were the whole numbers nearest the mean of the ratios of the first two tubes to particulate; when the rates of particulation in the tubes on either side of the optimal one were the same, the ratio of the optimal tube was recorded, as in the horse serum series in Table I.

Total globulin, referred to in this paper as globulin, and serum albumin were prepared from horse serum as described by Adair and Robinson (1930). The albumin was recrystallised three times.

All titrations were carried out at room temperature; 0·85 per cent. saline was used as diluent.

Table I. *Titration of anti-horse serum*, 1618 B, *diluted* 1 *in* 20, *against normal horse serum, and against diphtheria antitoxin from horse* 329

Tube	Antigen 1 in 800 c.c.	Antigen dilution	Ratio	Normal horse serum	Antitoxin 329
10	1·0	1 in 800	1 to 40		
9	0·9	1 in 888·8	1 to 44·4		3
8	0·8	1 in 1000	1 to 50		1 } 40 min.
7½	0·75	1 in 1066·6	1 to 53·3	2*	2
7	0·7	1 in 1142·8	1 to 57·1	1* } 25 min.	
6½	0·65	1 in 1230	1 to 61·5	2*	
6	0·6	1 in 1333·3	1 to 66·6		
5½	0·55	1 in 1454·5	1 to 72·7		
5	0·5	1 in 1600	1 to 80		
4½	0·45	1 in 1777·7	1 to 88·8		

* Signifies the order of particulation.

EXPERIMENTAL

Through the kindness of Dr R. G. White of the Belmont Laboratories two samples of fresh diphtheria antitoxin were obtained. These were heated to render them sterile, but received no other treatment. On the day after bleeding the antitoxin from horse 329 which contained 2100 antitoxic units per c.c., as determined by a flocculation test performed at Belmont, was titrated against anti-horse serum 1618 B. The latter had been shown to contain antibodies for

both globulin and albumin in the main zone. At the same time 1618 B was titrated against normal horse serum, the results being set out in Table I.

The antigen-antibody ratio with normal horse serum was 1 to 57, and with the antitoxin 1 to 52. They are not identical, but the variation is small, and no great difference in antigen content between the normal serum and the antitoxin is indicated by this experiment.

An exactly similar experiment was then set up using anti-horse serum 1568 E, which did not react with serum albumin in the main zone, but with globulin only. Table II describes these titrations; the ratio with horse serum was 1 to 22, but when the antitoxin was set up in an exactly similar series of dilutions to that shown for horse serum in the first half of the table the earliest particulation took place in the last tube, No. 3½, the optimal point not being included in the series. The antitoxin dilutions in the second half of the table were then arranged and the ratio 1 to 55 was obtained. Suitable experiments

Table II. *Anti-horse serum*, 1568 E, *in a constant dilution of* 1 *in* 20 *titrated against normal horse serum, and against diphtheria antitoxin from horse* 329

Horse serum					Antitoxin 329				
Tube	Horse serum 1 in 300 c.c.	Horse serum dilution	Ratio	Order of particulation	Tube	Antitoxin 329 1 in 800 c.c.	Antitoxin dilution	Ratio	Order of particulation
10	1·0	1 in 300	1 to 15		10	1·0	1 in 800	1 to 40	
9	0·9	1 in 333·3	1 to 16·6		9½	0·95	1 in 842·1	1 to 42·1	
8	0·8	1 in 375	1 to 18·75		9	0·9	1 in 888·8	1 to 44·4	
7	0·7	1 in 428·6	1 to 21·4	2	8½	0·85	1 in 941·2	1 to 47·1	
6½	0·65	1 in 461·5	1 to 23	1 } 50 min.	8	0·8	1 in 1000	1 to 50	3
6	0·6	1 in 500	1 to 25	3	7½	0·75	1 in 1066·6	1 to 53·3	1 } 1 hour
5½	0·55	1 in 545·4	1 to 27·3		7	0·7	1 in 1142·9	1 to 57·1	2 } 15 min.
5	0·5	1 in 600	1 to 30		6½	0·65	1 in 1230	1 to 61·5	
4½	0·45	1 in 666·6	1 to 33·3		6	0·6	1 in 1333·3	1 to 66·6	
4	0·4	1 in 750	1 to 37·5		5½	0·55	1 in 1454·5	1 to 72·7	
3½	0·35	1 in 1000	1 to 50		5	0·5	1 in 1600	1 to 80	

showed that in the reaction between 1568 E and normal horse serum there was no point of optimal particulation corresponding to that obtained with the antitoxin (1 to 55); whilst, as stated above, no optimal particulation occurred between this antiserum and the antitoxin in the dilution found optimal for horse serum. Ratios of 1 to 22 with normal horse serum and of 1 to 55 with the antitoxin indicate that the content of the antigen responsible for these reactions was much greater in the antitoxin than in the normal horse serum. The reaction would appear to be between globulin and antiglobulin.

To make certain that there was no optimal particulation between 1618 B and the antitoxin in a dilution corresponding to the increased antigen content demonstrated by 1568 E, an extensive series of antitoxin dilutions was made against 1618 B, but only the one optimal point (1 to 52), recorded in Table I, was revealed. This result suggests that the reactions shown by this antiserum and the antitoxin were due to albumin and antialbumin.

Increase in the globulin content of antitoxin from another horse, 328, was shown by a titration against 1568 E, in which the second antitoxin particulated in 1 hour 5 min. and the ratio was 1 to 64.

The ampoule of 1568 E in use was tested against total globulin and the ratio 1 per cent. total globulin to antiserum was 1 to 5·7. If this figure be divided into 22, the ratio obtained with 1568 E and horse serum, the percentage of total globulin in the horse serum is 3·86. Similarly the percentage in antitoxin 329 is 55 divided by 5·7, *i.e.* 9·65, and in antitoxin 328, 64 divided by 5·7, *i.e.* 11·23. The figures for 328 are higher than those for 329, although 329 contained many more antitoxic units, 2100, than did 328 with 1300 units. It is generally agreed that the increase in globulin content is not always parallel to the increase in antitoxin.

Some months later Dr White sent two further specimens of diphtheria antitoxin, one specimen came from horse 329, which had provided one of the first antitoxins tested, the second was a pooled antitoxin to which phenol had been added; both contained over 1000 antitoxic units per c.c. By this time antisera

Table III. *Diphtheria antitoxin titrated against antisera with different types of antibody content*

Antiserum	Normal horse serum	Antitoxin 329 (second sample)	Pooled antitoxin
1826 B (made against serum albumin)	3 (1 to 17·14), 1 (1 to 18·46), 2 (1 to 20) } 45 min.	Equal 1 (1 to 18·46), 1 (1 to 20) } 40 min.	2 (1 to 17·14), 1 (1 to 18·46), 2 (1 to 20) } 45 min.
	Ratio assigned 1 to 19 Albumin percentage 2·57	Ratio assigned 1 to 19 Albumin percentage 2·57	Ratio assigned 1 to 18 Albumin percentage 2·43
	Ratio: 1 % serum albumin to antiserum 1 to 7·4		
1308 M (made against whole horse serum, main zone against both globulin and albumin)	2 (1 to 30·7), 1 (1 to 33·3), 3 (1 to 36·4) } 20 min.	2 (1 to 28·6), 1 (1 to 30·7), 3 (1 to 33·3) } 17 min.	2 (1 to 28·6), 1 (1 to 30·7), 3 (1 to 33·3) } 23 min.
	Ratio assigned 1 to 32 Albumin percentage 2·8	Ratio assigned 1 to 30 Albumin percentage 2·63	Ratio assigned 1 to 31 Albumin percentage 2·72
	Ratio: 1 % serum albumin to antiserum 1 to 11·4		
1193 R (made against whole horse serum, main zone against globulin only)	2 (1 to 28·6), 1 (1 to 30·7), 2 (1 to 33·3) } 55 min.	3 (1 to 57·1), 1 (1 to 61·5), 2 (1 to 66·7) } 50 min.	2 (1 to 57·1), 1 (1 to 61·5), 2 (1 to 66·7) } 60 min.
	Ratio assigned 1 to 31 Total globulin percentage 4·8	Ratio assigned 1 to 64 Total globulin percentage 10	Ratio assigned 1 to 59 Total globulin percentage 9·22
	Ratio: 1 % total globulin to antiserum 1 to 6·4		

had been prepared against the serum albumin fraction of horse serum, and the later antitoxins were tested against (*a*) antiserum 1826 B made against serum albumin, (*b*) anti-horse serum 1308 M which reacted in the main zone with both globulin and albumin, and (*c*) anti-horse serum 1193 R, in which the main zone reacted with globulin, but not with albumin. Each antiserum was also tested against normal horse serum, and the ratios of the optimal tubes, the assigned ratios, the speeds of particulation, the ratios obtained using 1 per cent. solutions of the fractions, and the protein contents of the antigen are all contained in Table III.

Antiserum 1193 R was also titrated against ranges of dilutions of both antitoxins which covered the ratio 1 to 31, obtained with this antiserum and normal horse serum. No point of optimal particulation was observed in this part of the series, nor was any optimal point found with normal horse serum and 1193 R in the region of 1 to 60, about which point particulation was quickest

with both the antitoxins, proving, as did the exactly similar experiments performed with the earlier antitoxins and antiserum 1568 E, that the content of the antigen was much greater in the antitoxins than in normal horse serum. Suitably arranged tests were performed with both specimens of antitoxin and antiserum 1308 M which demonstrated that there was no optimal particulation with this antiserum at a point corresponding to the increased globulin content of the antitoxins, and so proved that the ratios obtained with the antitoxins and 1308 M were definitely the result of reactions between albumin and anti-albumin.

A consideration of the results of the experiments performed with the diphtheria antitoxins shows that the demonstration of their increased globulin content is possible by the method of optimal proportions if suitable antisera are used. It seems likely that, in the experiments mentioned by Dean (1931), Webb used antisera which reacted with both fractions, and was actually recording the reactions due to albumin. Quickly acting antisera appear to be those which do contain both antibodies and it would be natural to choose such good precipitating sera. It is just possible, but not likely, that he used antisera with albumin antibodies only in the main zone, but these sera appear to be very rare. In the work reported here antisera reacting in the main zone with both fractions have yielded results similar to those found by Webb.

Summary

1. By titration against horse serum and its protein fractions anti-horse sera can be divided into three groups, according to the antibodies responsible for the main or earliest particulating zone, namely the sera containing antibodies against (*a*) globulin only, (*b*) albumin only, and (*c*) both globulin and albumin.

2. For the quantitative estimation of a protein fraction in a horse serum it is necessary to use an antiserum containing a main zone antibody acting with that protein only.

This is illustrated in experiments, designed to show the increased globulin content of diphtheria antitoxin, in which antisera containing antibodies against both globulin and albumin failed to register an increase in the globulin content.

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