THE PRECIPITATION REACTION

EXPERIMENTS ON MULTIPLE ZONES

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Introduction

Goldsworthy (1928) reported the occurrence of multiple zones of particulation in titrations of horse serum and homologous antisera performed by the optimal proportions method of Dean and Webb (1926). The following observations suggest that the different zones were due to the presence of more than one antigen-antibody system. Taylor (1931) noted two zones in a titration of horse serum and a mixture of anti-horse sera which had individually particulated optimally in widely differing times. He suggested that the antibodies in the component antisera were different, and that the appearance of two zones in the titration of an unmixed antiserum might be due to its containing more than one antibody. Wells (1931) found two zones when titrating human serum against a mixture of antisera, and thought that one component might contain antiglobulin and the other antialbumin. Goldsworthy and Rudd (1935) have examined bizonal anti-horse sera and demonstrated clearly that one zone was due to an albumin-antialbumin system, the second zone to a globulin-antiglobulin system. They cite the occurrence of multiple zones in other immunity reactions.

The experiments to be described in this paper show that by titrating an antiserum containing antibodies for two quite separate substances, crystalline egg albumin and crystalline horse serum albumin, against mixtures of these antigens, it is possible to produce a single zone or two zones of particulation by varying the relative proportions of the two components in the antigenic mixtures. In addition to confirming that multiple zones are, in some cases at least, produced by the existence of more than one antigen-antibody system, the work makes it clear that a single zone is not necessarily indicative of the presence of only one antigen-antibody system.

Secondary zones which appeared many hours after the main or primary zones were seen in titrations of crystalline egg albumin and homologous antisera by Taylor, Adair and Adair (1932) who thought that two antibodies might be present and that egg albumin might not be a single antigen. Similar late zones have been observed in the experiments recorded below; they were situated in the region of antigen excess, and their position in the series was far removed from the main zones with which they did not apparently interfere.

Methods

The preparation of the antisera used in the present work was described by Dean, Taylor and Adair (1935). Titrations have been performed by the method of optimal proportions, in which a wide range of antigen dilutions, each in a volume of 1 c.c., is arranged, so that the amount of antigen is halved in successive tubes. To each tube is added 1 c.c. of an appropriate dilution of antiserum. Such an experiment indicates approximately the proportions necessary for the quickest particulation, and is known as a rough test. A fine test is then set up in which the amount of antigen differs but little from tube to tube, and the proportions are more accurately determined. The result is expressed as the antigen-antibody ratio, *e.g.* a ratio of 1 to 20 means that 1 part by volume of antigen is in optimal proportions with 20 parts of antiserum. All reactions took place at room temperature; 0·85 per cent. saline was used as diluent.

Experimental

Table I outlines experiments in which falling amounts of antigen were titrated against a constant amount of antiserum, 2375 C, diluted 1 in 20. In set A the antigen was egg albumin; in B, serum albumin; and in C, equal parts of these two substances so mixed that in the several tubes the dilution of each antigen was that recorded in column 2. Zones of particulation, similarly placed in all the series, were evident after 25 min. The progress of events in set C, in which the two systems were acting simultaneously, was rather more rapid than in either A or B.

Zones of particulation, secondary to the main or primary zones described above, occurred in all the sets, but only after many hours. The region of the main zone in each set was investigated by fine tests in which the amounts of antigen present differed little from tube to tube. The results, expressed as optimal ratios in terms of 1 per cent. albumin to antiserum, are given in brackets in the tables. The ratio, 1 to 12, in set C refers to each individual antigen.

Table I. *Antiserum titrated against the antigens, individually and in mixture*

Tube	Antigen dilution	Set A Egg alb. 1 %	Set B H.S. alb. 1 %	Set C Egg alb. 1 % H.S. alb. 1 %
1	1 in 8		Zone 7 hours	Zone 7 hours
2	1 in 16			
3	1 in 32	Zone 24 hours		
4	1 in 64			
5	1 in 128			
6	1 in 256	1* } Zone 25 min.	1 } Zone 25 min.	1 } Zone 25 min.
7	1 in 512	2* } (1 to 13)	2 } (1 to 12)	2 } (1 to 12)
8	1 in 1024			
9	1 in 2048			

* Signify the order of particulation.

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In Table II are described experiments in which falling amounts of mixtures of the antigens were titrated against a 1 in 20 dilution of the antiserum. These mixtures differed from that used in set C (Table I) in that they were composed of unequal amounts of the two antigens. In set D each tube contained twice as much horse serum albumin as egg albumin; in set E these proportions were reversed. In sets F and G the proportion of one antigen to the other was 4 to 1; and in H and J 16 to 1.

Table II. *Titrations of the antiserum against mixtures of the two antigens in varying concentrations*

Tube	Antigen dilution: Egg alb. 1 %	Antigen dilution: H.S. alb. 1 %	Result
	Set D		
1	1 in 16	1 in 8	Zone 7 hours
2	1 in 32	1 in 16	
3	1 in 64	1 in 32	
4	1 in 128	1 in 64	
5	1 in 256	1 in 128	
6	1 in 512	1 in 256	1 } Zone 25 min.
7	1 in 1024	1 in 512	2 } (1 to 12)
8	1 in 2048	1 in 1024	
9	1 in 4096	1 in 2048	
	Set F		
1	1 in 32	1 in 8	Zone 7 hours
2	1 in 64	1 in 16	
3	1 in 128	1 in 32	
4	1 in 256	1 in 64	} Zone 25 min.
5	1 in 512	1 in 128	} (1 to 14)
6	1 in 1024	1 in 256	1 } Zone 25 min.
7	1 in 2048	1 in 512	2 } (1 to 13)
8	1 in 4096	1 in 1024	
9	1 in 8192	1 in 2048	
	Set H		
1	1 in 32	1 in 2	Zone 7 hours
2	1 in 64	1 in 4	
3	1 in 128	1 in 8	
4	1 in 256	1 in 16	2 } Zone 30 min.
5	1 in 512	1 in 32	1 } (1 to 19)
6	1 in 1024	1 in 64	
7	1 in 2048	1 in 128	
8	1 in 4096	1 in 256	1 } Zone 30 min.
9	1 in 8192	1 in 512	2 } (1 to 14)
10	1 in 16384	1 in 1024	

Tube	Antigen dilution: Egg alb. 1 %	Antigen dilution: H.S. alb. 1 %	Result
	Set E		
1	1 in 8	1 in 16	Zone 7 hours
2	1 in 16	1 in 32	
3	1 in 32	1 in 64	
4	1 in 64	1 in 128	
5	1 in 128	1 in 256	
6	1 in 256	1 in 512	1 } Zone* 25 min.
7	1 in 512	1 in 1024	2 }
8	1 in 1024	1 in 2048	
9	1 in 2048	1 in 4096	
	Set G		
1	1 in 8	1 in 32	Zone 7 hours
2	1 in 16	1 in 64	
3	1 in 32	1 in 128	
4	1 in 64	1 in 256	} Zone 25 min.
5	1 in 128	1 in 512	} (1 to 12)
6	1 in 256	1 in 1024	1 } Zone 25 min.
7	1 in 512	1 in 2048	2 } (1 to 12)
8	1 in 1024	1 in 4096	
9	1 in 2048	1 in 8192	
	Set J		
1	1 in 2	1 in 32	Zone 7 hours
2	1 in 4	1 in 64	
3	1 in 8	1 in 128	
4	1 in 16	1 in 256	1 } Zone 30 min.
5	1 in 32	1 in 512	2 } (1 to 14)
6	1 in 64	1 in 1024	
7	1 in 128	1 in 2048	
8	1 in 256	1 in 4096	2 } Zone 30 min
9	1 in 512	1 in 8192	1 } (1 to 15)
10	1 in 1024	1 in 16384	

* Ratios { Egg. alb. 1 % to antiserum 1 to 14. H.S. alb. 1 % to antiserum 1 to 13.

Disregarding the secondary zones which became evident after many hours, both D and E showed only one main zone of particulation, although two different antigen-antibody systems were reacting, as was the case in set C. A fine test (Table III) of the region of the main zone in E disclosed two quite definite zones of practically simultaneous particulation, whereas a similar test applied to D revealed only one zone.

In each of the sets F and G, where the disparity between the concentrations of the two antigens was greater, two well-marked main zones appeared. Reference to the antigen dilutions in the optimal tubes of sets A and B (Table I) shows that in F the optimal point at tube 4 was due to the egg albumin, that

in tube 6 to horse serum albumin, whilst in G the zone at tube 4 was due to horse serum albumin and that in tube 6 to egg albumin. A similar state of affairs resulted in sets H and J, but the greater difference in the concentration of the two antigens caused a still wider separation of the main zones. In H and J the zones due to egg albumin were moved towards that end of the series where antibody was in excess. In both, the optimal tubes of the zones due to egg albumin contained 1 per cent. egg albumin diluted 1 in 512, whereas in all previous sets the optimal tube of such zones contained a dilution of 1 in 256. Fine tests confirmed this change.

These results show that by varying the relative concentrations of the antigens used two zones of particulation may be produced, one due mainly to egg albumin and the other mainly to horse serum albumin. Moreover, it is possible to arrange the extent of the separation of the two zones, or to cause

Table III. *Fine tests of the main zones of precipitation in sets* D *and* E (*Table II*)

Tube	Antigen dilution: Egg alb. 1 %	Antigen dilution: H.S. alb. 1 %	Reading 31 min.	Antigen dilution: Egg alb. 1 %	Antigen dilution: H.S. alb. 1 %	Reading 25 min.
	Set E			Set D		
10	1 in 80	1 in 160		1 in 160	1 in 80	
9	1 in 88·8	1 in 177·7		1 in 177·7	1 in 88·8	
8	1 in 100	1 in 200		1 in 200	1 in 100	
7	1 in 114·3	1 in 228·6	1	1 in 228·6	1 in 114·3	
6	1 in 133·3	1 in 266·6	2 } Zone (1 to 13)	1 in 266·6	1 in 133·3	
5	1 in 160	1 in 320		1 in 320	1 in 160	
4	1 in 200	1 in 400		1 in 400	1 in 200	
3½	1 in 228·5	1 in 457·1		1 in 457·1	1 in 228·5	1
3	1 in 266·6	1 in 533·3	1	1 in 533·3	1 in 266·6	2 } Zone (1 to 12)
2½	1 in 320	1 in 640	2 } Zone (1 to 14)	1 in 640	1 in 320	
2	1 in 400	1 in 800		1 in 800	1 in 400	

the optimal points of the two reactions to approximate so that only one zone is detectable. Similar results were found with two other specimens of antiserum.

Table III records a fine test on the region of the main zone of set E (Table II). A series of falling amounts of the mixture of antigens was arranged to contain the concentrations of the respective antigens given in the second and third columns. Each tube contained 1 c.c. of antigen mixture, and received 1 c.c. of a 1 in 20 dilution of the antiserum, 2375 C. A reading taken after 31 min. showed two easily differentiated zones of optimal particulation, that in tubes 7 and 6 due to horse serum albumin, the one in tubes 3 and 2½ to egg albumin. The continued progress of particulation in the tubes between the zones finally obliterated the differentiation. The ratio of 1 per cent. horse serum albumin to antiserum in tube 7 was 1 to 11·4; in tube 3 the ratio 1 per cent. egg albumin to antiserum was 1 to 13·3. Still finer tests yielded the values 1 to 13·3 and 1 to 13·9. A similar study of the main zone of set D revealed only one zone, as stated previously. Since in some cases only one main zone may result from the reactions between two different antigen-antibody systems, it is clear that a single zone does not necessarily signify the presence of a single antigen.

The results of the fine tests show that in the majority of the experiments the presence of the other antigen-antibody system has not caused profound changes of the proportions of an antigen and antiserum necessary for optimal particulation. Marked alterations of ratio were found only in the zones due to egg albumin in sets H and J; in these sets the two antigens were mixed in proportions of 16 to 1.

SUMMARY

1. An antiserum containing antibodies for two unrelated antigens, crystalline egg albumin and crystalline horse serum albumin, has been prepared.

2. In titrations of the antiserum against mixtures of these antigens it has been possible to produce a single zone or two zones of optimal particulation by varying the relative concentrations of the components in the antigenic mixtures. It is evident, therefore, that whilst (*a*) a single zone does not necessarily indicate a single antigen-antibody system, (*b*) multiple zones suggest the presence of more than one antigen-antibody system.

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