SOME OBSERVATIONS ON THE REACTION BETWEEN HORSE SERUM AND A POOL OF RABBIT ANTIHORSE SERUM

I. THE RELATIVE INHIBITORY EFFECT OF EXCESS ANTIGEN AND EXCESS ANTISERUM

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(With 2 Figures in the Text)

INTRODUCTION

Dean & Webb (1926) showed that, in a series of mixtures containing various quantities of horse serum and the same quantity of a rabbit antihorse serum, one mixture developed discrete particles of precipitate before the others. They found that the process of particulation, or the appearance of discrete particles of precipitate, spread rapidly into the zone of relative antiserum excess, and more slowly into the zone of relative antigen excess. The inhibitory effect of excess antigen was much more marked than that of excess antibody. As used in this context, the term 'inhibition of precipitation' is used to mean a delay in the formation of visible particles of precipitate, and not a decrease in the total weight of precipitate eventually formed. Cruickshank (1946) has shown, in tabular form, the times taken for the formation of visible particles of precipitate by mixtures of various dilutions of horse serum and a rabbit antihorse serum. He has expressed dilutions of antigen in the top row of the table and dilutions of antibody in the first column. The highest concentration of antigen recorded in the table is 1/50. The times taken for the development of visible particles of precipitate, when various dilutions of horse serum and antihorse serum were mixed, have been recorded in the appropriate squares of the table. The optima determined by the α procedure (Dean & Webb optima or constant antibody optima) fall on a straight line, illustrating that the optimal ratio is independent of the antibody concentration. The optima determined by the β procedure (Ramon optima or constant antigen optima) also fall on a straight line, parallel with the first, but situated in the region of antibody excess. On either side of the α procedure optima of this table the times increase, but the inhibition of precipitation is no more marked in the region of antigen excess than in the region of antibody excess. Visible particles of precipitate were formed within 1 hr. in mixtures which contained antigen twelve times stronger than that in the optimal tube.

Boyd (1941) has investigated various precipitating systems, and has shown with extreme clarity the more marked inhibiting effect of excess antigen. Using highly purified antigens, haemocyanin, ovalbumin and diphtheria toxin, Boyd determined the time required for the first appearance of discrete particles of precipitate in mixtures of all practicable dilutions of antigen and antiserum. He recorded these results in tables similar to that of Cruickshank. Boyd points out that mathematically the series of data expressed in such tables as these represent a surface, where time is the third dimension and antigen dilution and antiserum dilution are the two independent variables. Such a surface is not easy to show in two dimensions, but Boyd shows the essential characteristics of the surface by drawing lines through points of equal time, at appropriate intervals, similar to the contour lines used in topographic maps. Boyd refers to these 'contour lines' as 'isochrones', and points out that the position and distance apart of these contour lines serve to characterize the surface. Where changes of time are sudden the isochrones are close together, and where changes of time are more gradual the isochrones are spaced farther apart.

In all examples given by Boyd the inhibition of precipitation is much more marked in the region of antigen excess than in the region of antibody excess. This is well shown by the isochrones. In the region of antigen excess the isochrones are crowded together, whereas in the region of antibody excess they are spaced farther apart.

On the basis of the shape of these isochrones, Boyd divided antisera into two types, which he designated R and H type antisera. The R type antisera gave well-marked α procedure optima, but poorly defined β procedure optima, as the times taken for the development of particles of precipitate in mixtures containing the same dilution of antigen, but varying dilutions of antiserum, were approximately equal in the zone of antibody excess. Moreover, the β procedure optima did not fall on a straight line in the table, showing that the β procedure optimal ratio varied with the antigen

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concentration. These facts were well shown by the shape of the isochrones. In contrast, H type antisera gave well-marked optima by both the α and β procedure, the α and β procedure optimal ratios both being independent of the absolute concentration of the reagents. Antisera prepared in rabbits were usually of the R type, and those prepared in horses of the H type. Duncan (1932) and Taylor (1933), however, found well-marked optima by the β procedure in antisera prepared in rabbits.

Thus, Dean & Webb, using a horse serum-rabbit antihorse serum system noted that the inhibitory effect of excess antigen was much more marked than that of excess antibody. Boyd, using other systems, has obtained results which are concordant with those of Dean & Webb. The results given by Cruickshank, using the same system as Dean & Webb, do not agree with this finding.

The work reported here is concerned with an investigation into the time required for the formation of visible particles of precipitate in various mixtures of horse serum and a pool of rabbit antihorse serum. The dilutions of the antigen cover a wide range and in the tables, showing the results, the isochrones have been drawn.

MATERIALS AND METHODS

Many extrinsic factors influence the rate of flocculation in the precipitation reaction. The rate of mixing of the reagents, the degree of subsequent agitation and the temperature are three important factors. In order to investigate the time required for the formation of visible particles of precipitate in various mixtures of horse serum and antihorse serum, these extrinsic factors must be carefully controlled. The methods of control are included in the description of the experimental procedure.

Preparation and storage of sera

The same preparation of fresh, unheated horse serum was used throughout the experiments. The antihorse serum (Pool III, Sept. 1946) was obtained from rabbits which had received numerous injections of horse serum by both intravenous and intraperitoneal routes over a number of years, courses of injections alternating with periods of rest. Four rabbits were bled on each of two successive days, and the serum pooled. Using the method of optimal proportions, Taylor (1931) has shown that a pooled antiserum contains the sum of the antibodies present in the individual sera.

All sera were stored frozen at $-20°$C. without preservative. Before use the sera were thawed rapidly by standing the bottle of serum in water at room temperature, and after sufficient serum had been withdrawn for the day's work the sera were immediately returned to the $-20°$C. refrigerator. The serum was stored in small quantities in separate containers, in order to avoid, as far as possible, the cloudiness which usually occurs on repeated freezing and thawing.

Dilution of sera

The dilutions used were serial 1·5 fold dilutions, made by transferring two volumes of a dilution of the horse serum or antihorse serum into one volume of diluent. These dilutions formed a true geometrical series. Sufficient of each dilution of horse serum and antihorse serum for the day's work was made up at the same time. The pipettes used were of the usual serological blow-out variety and the same pipette was used both for measuring out the saline and making up the serial dilutions, thus avoiding some of the errors due to slight variations between pipettes. The serial dilutions of the horse serum covered a range from 1/5 to approximately 1/16,500. Ten serial dilutions of antihorse serum were used, from 1/5 to approximately 1/200. The diluent used throughout was 0·85 % saline.

Method of performing the reaction

In performing the reaction, 0·5 c.c. of an antigen dilution was added to 0·5 c.c. of a dilution of the antiserum in a long narrow tube—internal diameter 0·85 cm. and length 15 cm. The tubes were then placed in a water-bath with one-third of the fluid column immersed. The water-bath was heated by a gas flame and the temperature controlled at 37° C. by means of a thermostat. In this way convection currents were induced within the tubes and produced uniform mixing in all tubes. Moreover, the motion of the fluid made the observation of the first discrete particles of precipitate much easier than in a stationary fluid. The water-bath was fitted with glass windows both in the back and front. A strip light behind the back window gave oblique illumination of the tubes. The contents of the tubes were observed through the front window using a $\times 8$ hand lens. In all mixtures, the essential determination was the time which elapsed between the mixing of the horse serum and the antihorse serum and the first appearance of discrete particles of precipitate. Mixtures that showed visible particles in under 10 min. were observed continuously, no other tubes being watched at the same time. The time taken for development of visible particles was determined with a stop-watch, and recorded to the nearest 6 sec., a fraction of a minute being expressed as a decimal. Mixtures that took longer than 10 min. to develop visible particles were often put up in groups of three or four and observed concurrently. The timing was done to the nearest half minute using an ordinary watch. Mixtures that did not develop visible particles in an hour were abandoned.

The investigation of the various mixtures of horse serum and antihorse serum was done in sequence. All the antiserum dilutions were tested against each antigen dilution in succession, beginning with the strongest antigen dilution. The mixing of the dilutions of horse serum and antihorse serum was accomplished as follows: 0·5 c.c. of each antiserum dilution was first transferred from each tube of the bulk antiserum dilutions into the corresponding tube in a row of ten of the long narrow tubes. A separate pipette was used for each antiserum dilution. These pipettes were drawn out of glass tubing, graduated with mercury delivered from a standard mercury pipette, and finally the volume of fluid delivered by them was checked gravimetrically. The rate of delivery of fluid by these pipettes was controlled by using throttled teats. The uniform, slow rate of emptying of the pipettes occasioned by the use of throttled teats avoided variations in the volume of fluid adhering to the inside of the pipette. The antigen dilution was delivered from an all-glass syringe provided with an adjustable stop and automatic spring return. The volume of fluid delivered by the all-glass syringe was checked gravimetrically. By the use of this syringe, the antigen dilution could be added rapidly to the dilution of antiserum in the long narrow tube giving almost instantaneous mixing. Complete mixing was further insured by a brief shake. The stop and spring return on the syringe, made the simultaneous addition of the antigen dilution and the manipulation of the stop-watch an easy procedure. Wilson Smith (1932) has emphasized the importance of mixing the reagents rapidly.

In order to obtain results which were reproducible attention had to be paid to a number of details not outlined above. Many of these have already been stressed by Taylor (1933). Each mixture was handled in an identical way. The tubes and their contained antiserum were placed in the bath to warm before adding the antigen, in order to prevent mist formation. The initial shaking of the tubes, immediately after the addition of the antigen, was limited to a standard number of shakes, and the tubes were wiped clean at this time, and then replaced in the water-bath. Additional shaking, initially or later, was found to hasten the appearance of discrete particles of precipitate. In very hot weather, when the air temperature above the bath approximates that in the bath, convection currents may slow or even stop, and control of the rate of convection currents is necessary. This was accomplished by a trough of iced water placed over the water-bath.

The determination of the moment when discrete particles of precipitate first become definitely visible is a personal matter and for comparable and reproducible results all observations must be made by the same person.

RESULTS

The results of the experiments with horse serum and a pooled rabbit antihorse serum are shown in Fig. 1. Dilutions of horse serum are indicated in the top row of the table, the dilutions being numbered from 1 to 21. The antiserum dilutions are given in the first column and are numbered 1 to 10. These antiserum dilutions correspond with the first ten horse serum dilutions. The time taken for the first appearance of discrete particles of precipitate, when 0·5 c.c. of a dilution of horse serum is mixed with 0·5 c.c. of a dilution of antihorse serum is recorded in the appropriate position in the table. These times are recorded in minutes. The shorter times are expressed to the nearest 6 sec., a fraction of a minute being shown as a decimal; the longer times are expressed to the nearest half minute. The mixtures which are recorded as 'unreadable' developed an extreme turbidity very rapidly. This turbidity split into a number of very large loose floccules of precipitate and it was impossible to say exactly when discrete particles first appeared. The α procedure optima are joined together by a continuous line, and the β procedure optima by a broken line. Isochrones are drawn at 2, 4, 8, 16, 32 and 50 min.

There are four quite separate and distinct series of α procedure optima; that is, for each dilution of antiserum there are four dilutions of horse serum which, on mixing with the antiserum, give discrete particles of precipitate faster than mixtures on either side of them. The α procedure optima of each series lie on a straight line showing that the antigen-antibody ratios at the individual optima of any one series are equal and independent of the dilution of the antiserum, as stated by Dean & Webb (1926). Each series of α procedure optima appears to be quite distinct and to lie in the centre of a separate zone, the isochrones of one zone, changing direction quite abruptly as they run into those of a neighbouring zone. For convenience, these four zones may be referred to as zones A, B, C and D, from left to right. It seems possible that these four different zones may correspond with four completely separate and distinct antigen-antibody reactions. There is no evidence that these reactions form mixed precipitates, at least where the antigen excess of one system reacts in the presence of the antibody excess of another system, as the isochrones of one zone are not distorted by the presence of another zone but simply show abrupt changes in direction. Also in mixtures such as the one containing antigen dilution numbered 6 and antibody dilution numbered 2, this mixture lying in the antibody excess region of zone B and antigen excess region of zone C, particles of precipitate appeared in a turbid supernatant fluid. The particles of precipitate may correspond with the antibody excess of zone B, and the turbidity with

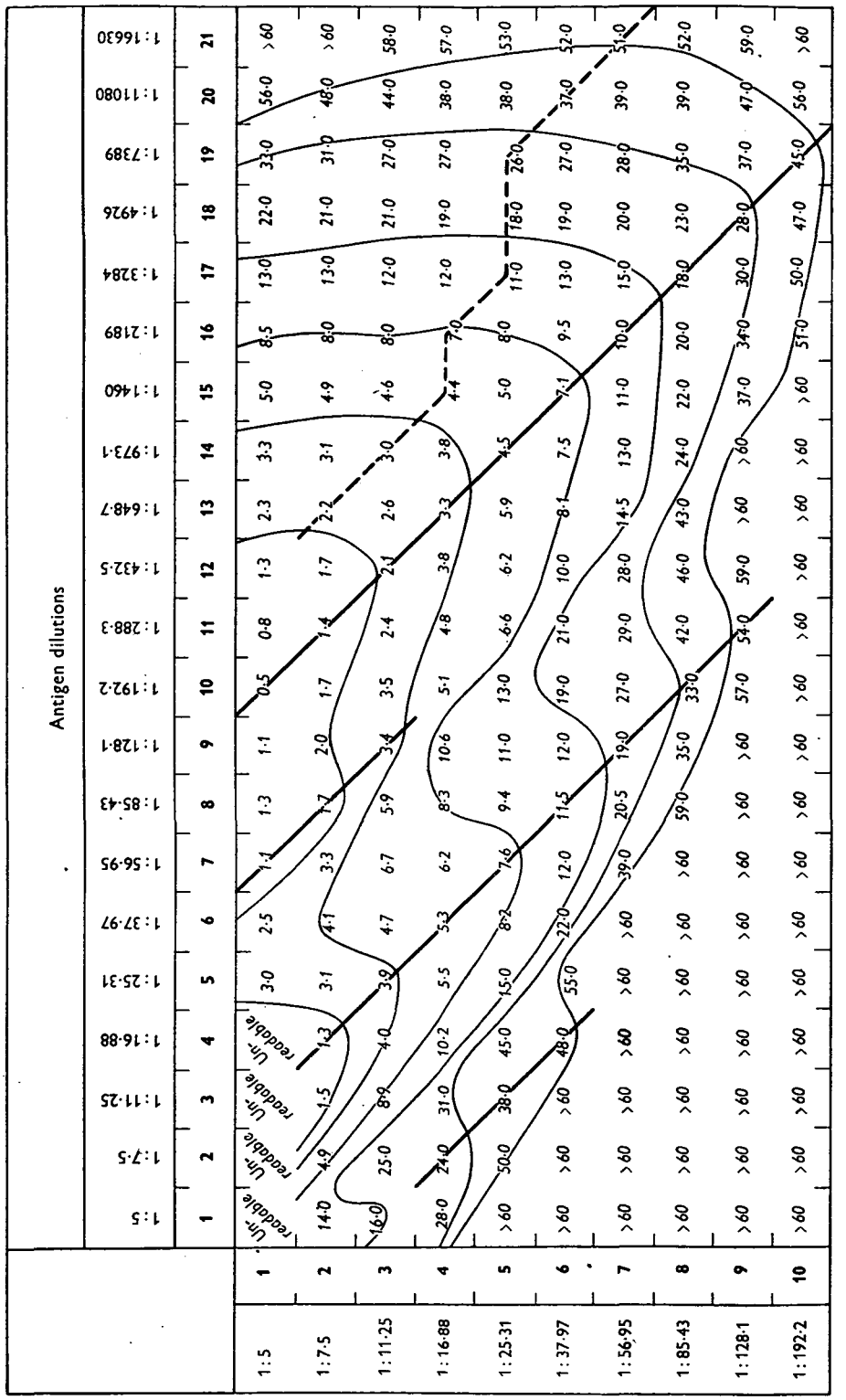

Fig. 1. The times taken for the development of discrete particles of precipitate by mixtures of various dilutions of horse serum and antihorse serum are shown in the appropriate positions in the figure. These times are recorded in minutes. — Isochrones, passing through points of equal time, drawn at 2, 4, 8, 16, 32 and 50 min.; — lines joining α procedure optima; - - - line joining β procedure optima. For ease of reference the dilutions are numbered in bold type.

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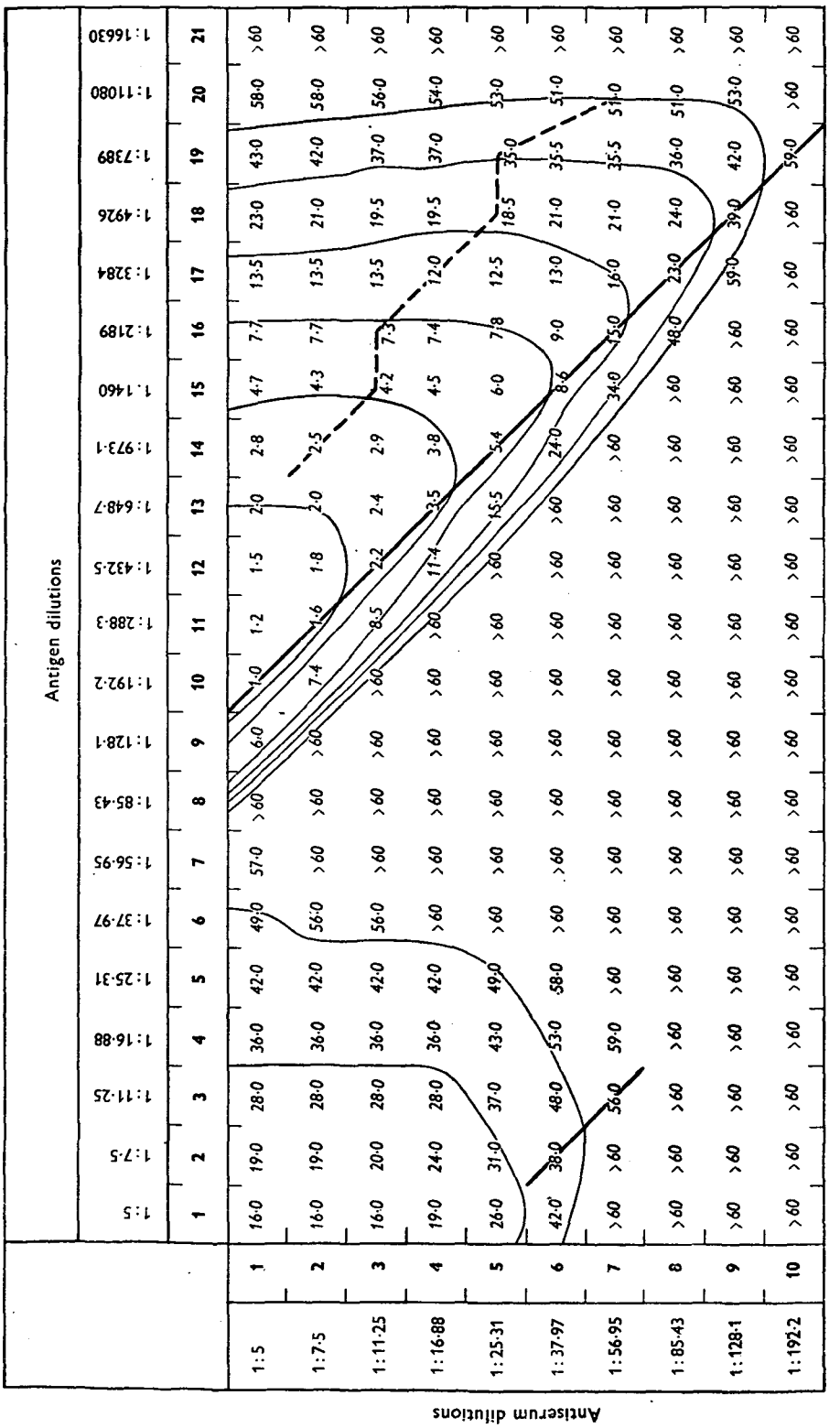

Fig. 2. The times taken for the development of discrete particles of precipitate by mixtures of various dilutions of horse serum crystalbumin and antihorse serum are shown in the appropriate positions in the figure. These times are recorded in minutes. — Isochrones, passing through points of equal time, drawn at 2, 4, 8, 16, 32 and 50 min.; — lines joining α procedure optima; - - - line joining β procedure optima. For ease of reference the dilutions are numbered in bold type.

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the antigen excess of zone C. In each of the zones A, B and C, there is a crowding of the isochrones in the antigen excess region of that zone and a spacing out of the isochrones in the antibody excess region. These facts show quite clearly that in these three zones the inhibition of precipitation is much more marked in the region of antigen excess than in the region of antibody excess. This is not true for zone D. The α procedure optimum of zone C is only apparent for the three lowest dilutions of the antiserum. In the higher dilutions of antiserum, although it is no longer apparent, the isochrones still show abrupt changes in direction in the region of this zone. It seems that the optimum of this zone is succeeded by the antigen excess of zone D without any intervening mixtures taking longer to show visible particles. It appears as though the slow increase in the time required for the first appearance of discrete particles in the antigen excess region of zone D, illustrated by the wide spacing of the isochrones in this region, is due to the presence of zone C.

In the case of zone D, where it is possible to examine the antibody excess region completely, the isochrones correspond with Boyd's R type. The times taken for the formation of visible particles of precipitate in a series of mixtures containing the same dilution of horse serum and various dilutions of antiserum are almost the same in the region of antibody excess; consequently, the β procedure optima are not well marked. The ratio of antigen to antibody at each β procedure optimum varies with the antigen concentration.

By using a chemically purified fraction of horse serum it is possible to examine an antigen-antibody system completely, without masking of the antigen excess and antibody excess regions by neighbouring zones. In Fig. 2 are shown the results of experiments using horse serum crystalbumin and the same pooled antihorse serum. The crystalbumin was prepared by the method of Hewitt (1938). The experimental procedure and the method of recording the results are identical with those for horse serum. The dilutions of crystalbumin shown are dilutions of a 2·9% solution. In the case of crystalbumin there are two zones. These are well separated and consequently the right-hand zone, as seen in the table, can be examined completely. This zone shows very well the marked inhibition of precipitation in antigen excess, and the consequent crowding together of the isochrones in this region.

DISCUSSION

Goldsworthy (1928) described the occurrence of multiple zones of rapid particulation in the titration of rabbit antihorse sera by the optimal proportions method of Dean & Webb (1926). Goldsworthy & Rudd (1935) investigated antihorse sera showing two zones when titrated against horse serum, and showed that one zone was due to an albumin-antialbumin system, and the other to a globulin-antiglobulin system. Taylor & Adair (1935) titrated an antiserum containing antibodies to crystalline egg albumin and crystalline horse serum albumin against mixtures of the two antigens, and found that one or two zones of rapid precipitation appeared depending on the relative proportions of the two antigens in the mixture. They pointed out that whereas multiple zones suggest the presence of more than one antigen-antibody system, a single zone does not necessarily indicate a single antigen-antibody system.

The results described here seem compatible with the view that there are at least four different systems, four different antigen-antibody reactions, taking place when horse serum reacts with a pooled antihorse serum. There may be more than four systems, these others being obscured because they either give α procedure optima at approximately the same horse serum-antihorse serum ratio as the ones noted and take approximately the same time to give visible particles of precipitate, or take much longer to give visible particles in all mixtures than the observed systems.

In each zone of Fig. 1, except zone D, to which reference has already been made, and in Fig. 2, there is a marked crowding of the isochrones in the antigen excess region of each zone and a spacing out of the isochrones in the antibody excess region. These facts show quite clearly that excess antigen has a more marked inhibitory effect than excess antiserum. Fig. 2 (crystalbumin as antigen) shows this particularly well. These results agree with the findings of Dean & Webb and of Boyd. From the results reported here, with any given dilution of antibody the formation of visible particles of precipitate is delayed for at least 1 hr. by an antigen solution approximately four times as strong as that giving the most rapid formation of visible particles of precipitate. It seems probable that Cruickshank's table is misleading in that it suggests that inhibition of precipitation is no more marked in antigen excess than it is in antibody excess.

The experimental results may afford an explanation for the relatively slight delay in formation of visible particles in the region of antigen excess noted by Cruickshank. It is possible that in the region of antigen excess of his table, there are one or more α procedure optima, not apparent as separate optima due to their proximity, just as the α procedure optimum of zone C in the results given is not apparent with the higher antiserum dilutions. Furthermore, it is possible that, had he recorded results for horse serum dilutions stronger than 1/50, other α procedure optima would have been apparent.

In the results given it is seldom possible to examine the shape of the isochrones in the antibody excess region of a zone, due to overlapping of zones, but where this can be done the isochrones conform to Boyd's R type. This is well shown by zone D of the horse serum-antihorse serum reaction, and the horse serum crystalbumin-antihorse serum reaction.

Where the α procedure optima are far apart, as with zones A and B and zones B and C, the absolute distinction of the isochrones and the abrupt changes in their direction support the view that the appearance of visible particles depends solely upon which of the systems gives particles first at the concentration of horse serum and antihorse serum present in the mixture under observation, quite independently of the other reactions going on in the same mixture. In the data, as presented, all that can be seen is the interaction of the antibody excess of one zone and the antigen excess of another. These facts may be cited as evidence in favour of the view that the second stage of the precipitation reaction is specific, and that in mixed precipitating systems visible particles of precipitate are not formed by the indiscriminate and non-specific aggregation of antigen-antibody complexes, at least, when the particles of one reaction are formed in antibody excess and those of the other are formed in antigen excess.

SUMMARY

1. The particulation times of a series of mixtures of horse serum and a pool of rabbit antihorse serum have been determined. The results have been presented in a table and isochrones drawn. Four distinct α procedure optima were found, suggesting that there may be at least four different antigen-antibody reactions concerned when horse serum reacts with a rabbit antihorse serum.

2. The reaction between horse serum crystalbumin and the same pooled rabbit antihorse serum has been investigated, and recorded similarly.

3. Inhibition of precipitation was found to be more marked in the region of antigen excess than in the region of antiserum excess. Evidence is presented in support of the view that the occurrence of multiple zones may obscure this observation.

4. The results are compatible with the view that the second stage of the precipitation reaction is specific.

5. The isochrones correspond with Boyd's R type.

I am extremely grateful to Dr R. I. N. Greaves for constant help and advice, and I wish to express my thanks to Mrs M. E. Adair for providing me with horse serum crystalbumin.

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(*MS. received for publication* 3. II. 48.—Ed.)