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

II. AN ANALYSIS OF THE ANTIGENS CONCERNED IN THE PRODUCTION OF MULTIPLE ZONES

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(With 17 Figures in the Text)

INTRODUCTION

The occurrence of multiple zones of optimal or maximal reaction in serological titrations is a well-recognized phenomenon. Goldsworthy (1928) and Goldsworthy & Rudd (1935) have reviewed the relevant observations of previous workers. Goldsworthy (1928) found that multiple zones of rapid flocculation frequently occurred 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 demonstrated that one zone was due to an albumin-antialbumin system and the other to a globulin-antiglobulin system. Horse serum is to be regarded as consisting of a number of antigens (Landsteiner, 1946), and Goldsworthy & Rudd concluded that multiple zones of rapid flocculation are due, in part at least, to the independent activity of multiple antigens and their specific antibodies. 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 reaction, a single zone does not necessarily indicate a single antigen-antibody system.

In a previous paper, Naylor (1948) reports an investigation into the reaction between horse serum and a pool of rabbit antihorse serum. The occurrence of four zones (four α procedure optima) indicated that at least four antigen-antibody systems were involved in the reaction. The work reported here is an extension of this observation and is directed towards an analysis of the antigens responsible for these multiple zones, together with an investigation into the occurrence of these antigens in preparations of horse serum albumin and globulin.

MATERIALS AND METHODS

The method of preparation of the pool of rabbit antihorse serum (pool III, September 1946) and the experimental procedure adopted throughout this work have been fully described in the previous paper (Naylor, 1948). The horse serum globulin was prepared by the method of Taylor, Adair & Adair (1932) and reprecipitated twice. The horse serum albumin was crystallized four times by the method of Adair & Robinson (1930). The crystalbumin was prepared by the method of Hewitt (1938) and recrystallized five times. The results of the electrophoretic analyses of these protein samples are shown in Figs. 15–17 and described later.

The nomenclature of derivatives of horse serum is confusing, as the terms albumin and globulin are used with differing connotations by different workers. In this paper the term albumin refers to a protein constituent of horse serum which, though not necessarily homogeneous, probably consists of very similar molecules. The preparation of albumin, derived from horse serum and used in this work, is believed to consist largely of albumin, but is also thought to contain other proteins which are vastly different from the albumin and which are present in small amounts as impurities. The term albumin does not refer to that fraction of horse serum not precipitated by half saturation with ammonium sulphate.

The term crystalbumin refers to a protein constituent of horse serum also consisting of similar, but not necessarily, identical molecules. The preparation of crystalbumin used in this work is thought to contain other entirely different proteins present in small amounts as impurities. Crystalbumin and albumin are regarded as similar proteins, but do not necessarily embrace an identical range of molecules, as the technique used in the preparation of crystalbumin excludes less soluble proteins which may be present in albumin preparations and, from crystalline horse serum albumin, Sørensen (1925, 1930) has

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prepared several fractions with similar crystal form but with different solubilities in ammonium sulphate solutions.

The term globulin is used for that fraction of horse serum precipitated by half saturation with ammonium sulphate. Globulin is regarded as an extremely complex mixture of proteins and, though globulin is defined as a fraction of horse serum, small amounts of albumin present in the globulin fraction would, nevertheless, be regarded as impurity. Some proteins, though partially precipitated by half saturation with ammonium sulphate, may not be entirely separated in the globulin fraction, and may consequently occur as impurities in preparations of albumin or crystalbumin where they may conveniently be termed 'globulin impurity'.

It is realized that the constitution of the derivatives of horse serum may differ with variation in the details of the method of preparation and, moreover, they may contain unstable compounds which alter during preparation or storage. In view of these difficulties, and also because proteins regarded as impurities may be present in certain derivatives as noted above, the proteins used in this work are usually referred to as the albumin preparation, the globulin preparation and the crystalbumin preparation; if the meaning is perfectly clear the word 'preparation' is sometimes omitted.

Method of absorption

Absorption of precipitin was conducted by mixing antigen and antiserum in optimal proportions. This method has been used by Duncan (1932) and Goldsworthy & Rudd (1935). The absorptions performed in these experiments were rather unusual as the derivatives of horse serum used for absorbing showed multiple α procedure optima. In the majority of cases the absorption was conducted at a single α procedure optimum, a procedure which is only practicable at the optimum involving the lowest concentration of (the mixed) antigen. This point will be clarified in the account of the experiments. The mixture of antigen and antiserum in optimal proportions was allowed to stand at room temperature for about 1 hr., with occasional shaking, and then stored at $+2°$ C. overnight. Next morning the precipitate was packed by centrifugation and the supernatant absorbed serum separated.

EXPERIMENTAL

The experimental work consists of an investigation into a number of precipitation reactions, each involving an antigen (either horse serum or a purified derivative of horse serum) and an antiserum (either pool III antihorse serum or the same antiserum after absorption in various ways). The investigation of each reaction has been conducted in the same way, and has involved a determination of the times taken for the development of discrete particles of precipitate in various mixtures of the antigen and the antiserum.

The results of each reaction have been recorded in an identical manner in tabular form. Dilutions of the antigen are indicated in the top row of the table. These dilutions are serial 1·5-fold dilutions, beginning at 1/5, and are numbered from 1 to 21. Antiserum dilutions are given in the first column, and are numbered from 1 to 10. These are again serial 1·5-fold dilutions beginning at 1/5 and correspond with the first ten antigen dilutions. In some reactions less than ten antiserum dilutions have been used, but the same numbering of the dilutions has been used throughout. The time taken for the first appearance of discrete particles of precipitate when equal volumes of a dilution of the antigen and a dilution of the antiserum are mixed 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. In general, mixtures which failed to develop discrete particles of precipitate in 1 hr. were discarded, but occasionally mixtures were observed for longer than this period. The α procedure optima are joined together by a continuous line, and the β procedure optima by a broken line. Isochrones (Boyd, 1941) are drawn at appropriate intervals. Each isochrone may be identified by observing the times through which it passes.

In order to enable reference to be made to the positions of the α procedure optima, each figure has letters across it, from A to W, excluding I and O, at the level of antiserum dilution numbered 6. The position of each series of α procedure optima is then referred to as the lettered position through which the series passes. For example, in Fig. 1, the four series of α procedure optima are in positions D, H, M and Q. (The third series of α procedure optima from the left would, if produced, pass through position M.) The letter denoting the position of each series of α procedure optima is also used to indicate the zone in which the series lies. In the course of the description of the figures showing the results, a certain amount of discussion is included under the heading of 'recapitulation' in order to draw comparisons between the several results, and also to explain the purposes underlying the subsequent experiments. Under the description of the figures showing the results, observations incidental to the topic under discussion are noted in small type. A list of the reactions which have been investigated, together with the figure number showing the results, is given below:

Fig. 1. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III.

Fig. 2. Antigen: horse serum albumin, 2·9 %. Antiserum: rabbit antihorse serum, pool III.

Fig. 3. Antigen: horse serum globulin (batch I), 4·26 %. Antiserum: rabbit antihorse serum, pool III.

Fig. 4. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III.

Fig. 5. Antigen: horse serum albumin, 2·9 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4.

Fig. 6. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4.

Fig. 7. Antigen: horse serum globulin (batch II), 4%. Antiserum: rabbit antihorse serum, pool III, absorbed with 4 % horse serum globulin in the proportions of 1:38·4.

Fig. 8. Antigen: horse serum albumin, 2·9 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 4 % horse serum globulin in the proportions of 1:38·4.

Fig. 9. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4, and with 4 % horse serum globulin in the proportions of 1:38·4.

Fig. 10. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 4 % horse serum globulin in the proportions of 1:38·4.

Fig. 11. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4.

Fig. 12. Antigen: horse serum crystalbumin, 2·9 %. Antiserum: rabbit antihorse serum, pool III.

Fig. 13. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 and 1:1.

Fig. 14. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 and 1:1.

Figure 1

Antigen: horse serum.
Antiserum: rabbit antihorse serum, pool III.
Result: four series of α procedure optima at positions D, H, M and Q.

In this reaction 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. It seems probable that these four different zones may correspond with four completely separate and distinct antigen-antibody reactions occurring when horse serum reacts with the pool of antihorse serum.

The series of α procedure optima at position M is only apparent for the three lowest dilutions of the antiserum. In the higher dilutions of the antiserum, although it is no longer apparent, the isochrones still show abrupt changes in direction in this region. It seems that the optima of this zone are succeeded by the antigen excess region of zone Q without any intervening mixtures taking longer to show visible particles of precipitate.

In the case of zone Q, where it is possible to examine the antibody excess region completely, the isochrones correspond with those of Boyd's R type antiserum.

Figure 2

Antigen: horse serum albumin 2·9 %.
Antiserum: rabbit antihorse serum, pool III.
Result: two series of α procedure optima at positions F and Q.

There appear to be two antigens present in this preparation of horse serum albumin. These may be antigenically distinct albumins, or one may be albumin and the other an impurity, possibly globulin. This problem will be discussed later.

The dilutions of the antigen refer to dilutions of a 2·9 % solution of the horse serum-albumin preparation. This concentration of albumin is approximately equal to that of albumin in horse serum, consequently it is logical to compare the positions of the α procedure optima in Fig. 2 with those in Fig. 1, in order to ascertain which zones of Fig. 1 are due to an albumin-antialbumin system. It is at once apparent that both Figs. 1 and 2 show a series of α procedure optima in position Q and, moreover, the particulation times in zone Q of Figs. 1 and 2 correspond very closely in the higher concentrations of the reagents.

Although there is a series of α procedure optima at position F in the reaction between the horse serum-albumin preparation and antihorse serum (Fig. 2) there is no such series at a corresponding position in the reaction between horse serum and the antihorse serum (Fig. 1). There are two features of zone F (Fig. 2) worthy of mention. In the first place,

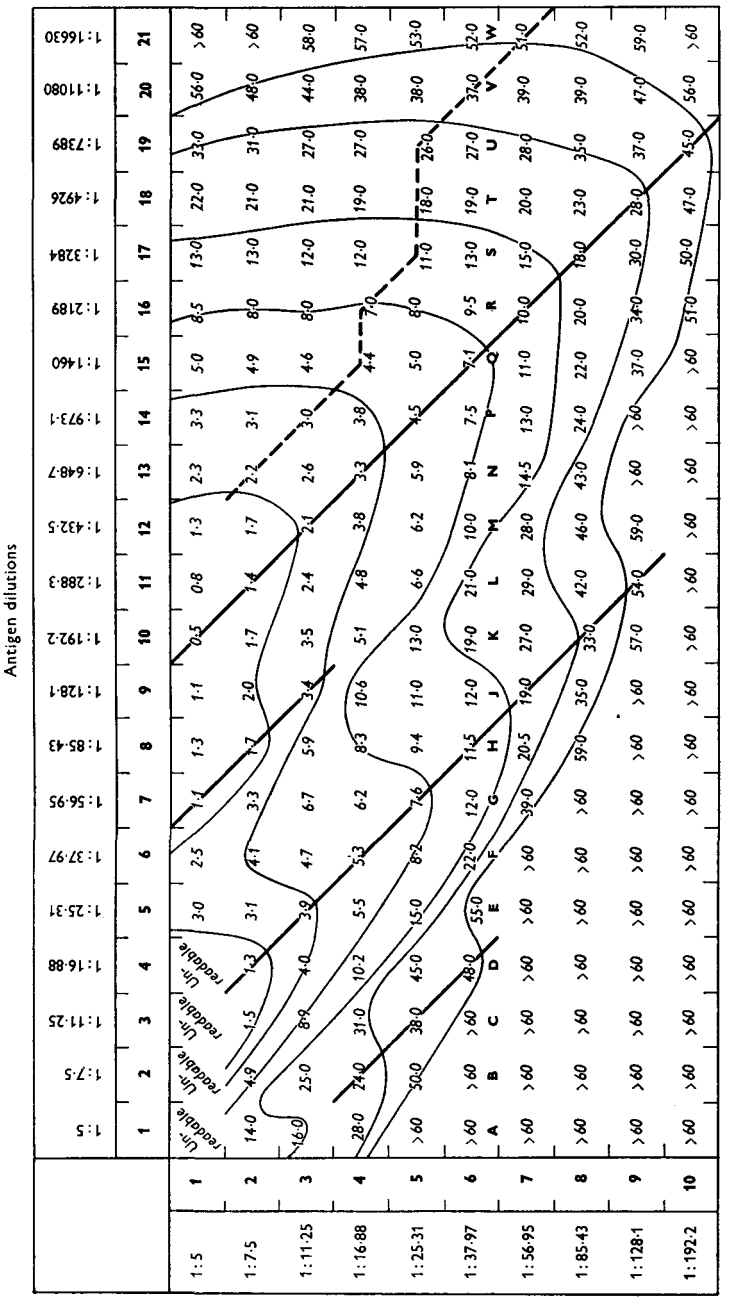

Fig. 1. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III. Isochrones drawn at 2, 4, 8, 16, 32 and 50 min. In this and subsequent figures the method of recording the results is identical. The times taken for the development of discrete particles of precipitate by mixtures of various dilutions of antigen and antiserum are shown in the appropriate positions in the figure. Those times are recorded in *minutes*. —, *isochrones, passing through points of equal time, are drawn at appropriate intervals and each* isochrone may be identified by observing the times through which it passes; —, lines joining α procedure optima; - - -, line joining β procedure optima. Dilutions are numbered in bold type. Capital letters across the figure at the level of antiserum dilution numbered 6 enable reference to be made to the positions of the α procedure optima. 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 determine exactly when discrete particles first appeared.

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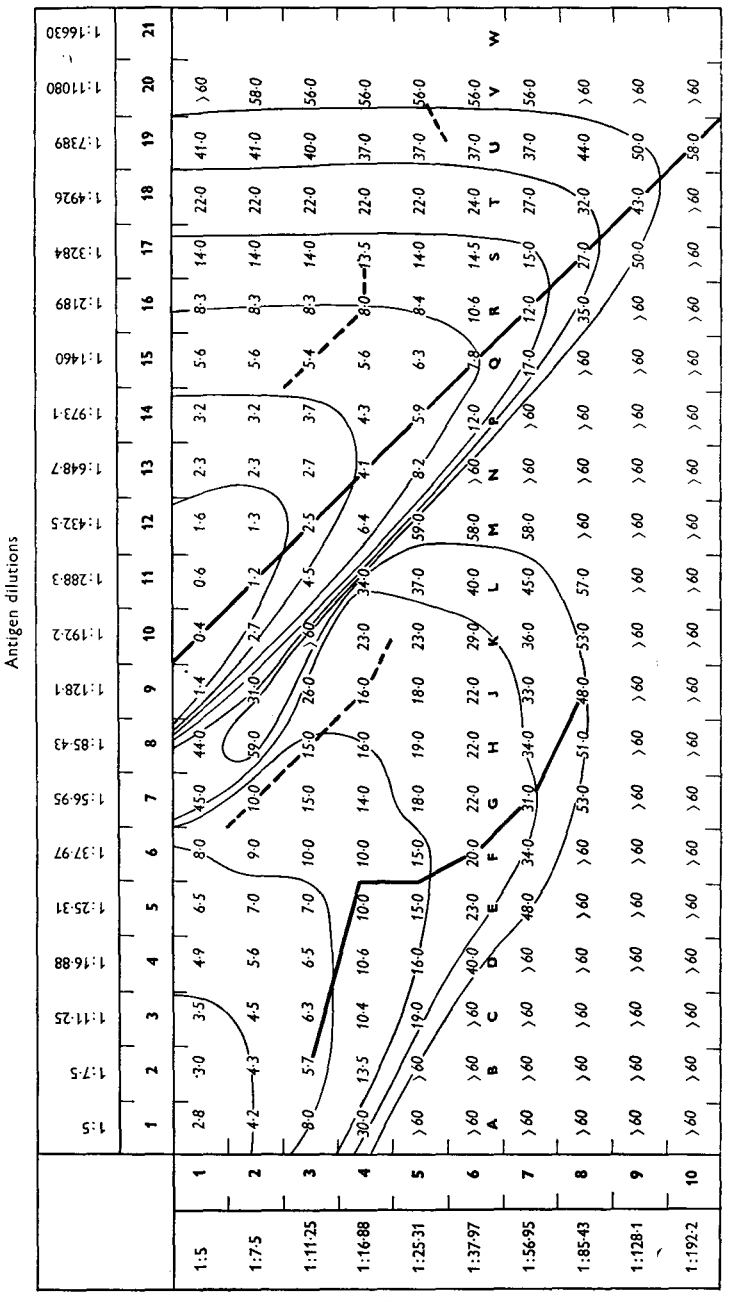

Fig. 2. Antigen: horse serum albumin, 2·9 %. Antiserum: rabbit antihorse serum, pool III. Isochrones drawn at 2, 4, 8, 16, 32 and 50 min.

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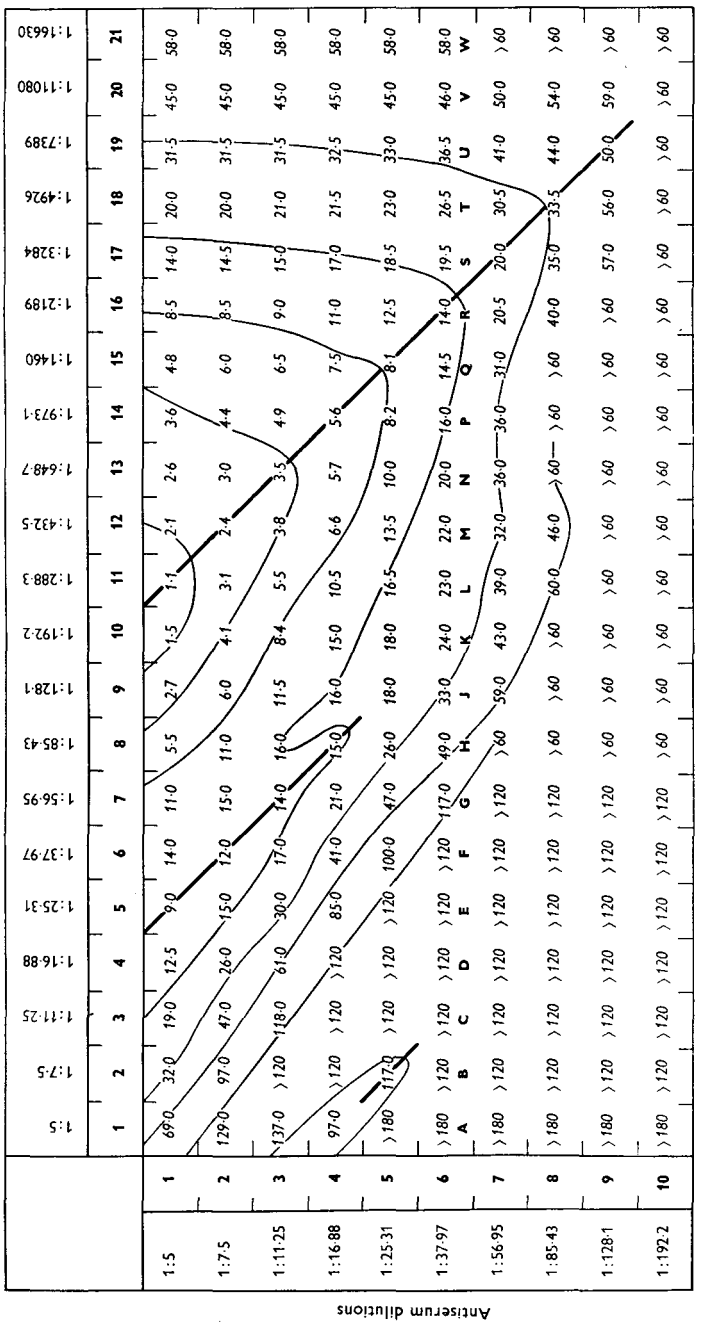

Fig. 3. Antigen: horse serum globulin (batch I), 4·26%. Antiserum: rabbit antihorse serum, pool III. Isochrones drawn at 2, 4, 8, 16, 32, 60 and 120 min. In the investigation of this reaction a number of mixtures were observed for longer than 1 hr.

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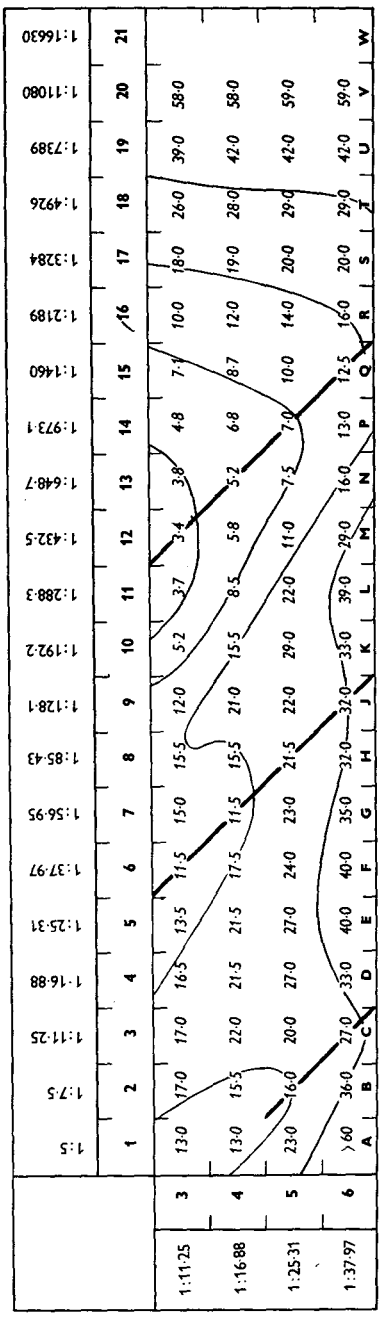

Fig. 4. Antigen: horse serum globulin (batch II), 4%. Antiserum: rabbit antihorse serum, pool III. Isochrones drawn at 4, 8, 16 and 32 min. In this reaction, four antiserum dilutions (nos. 3–6) only have been used, but the method of recording the results is identical with that of the previous figures.

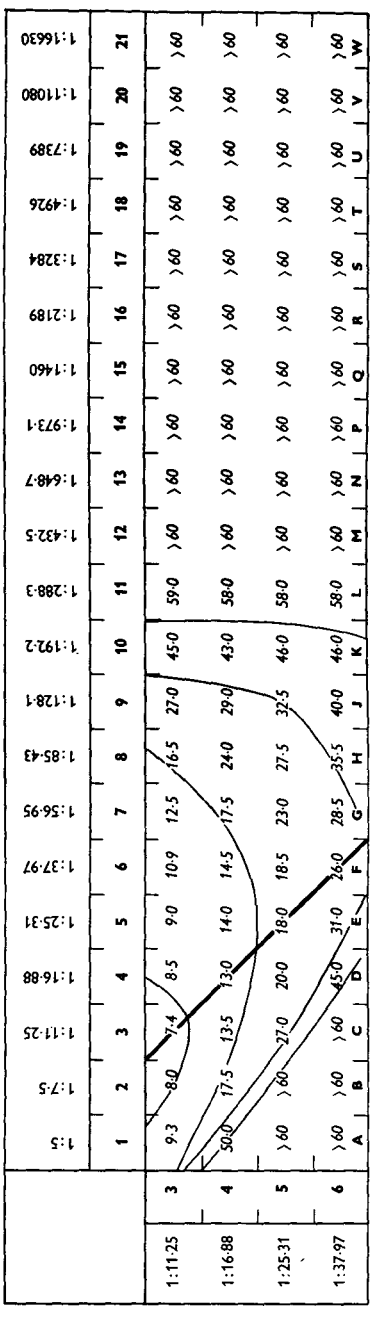

Fig. 5. Antigen: horse serum albumin, 2·9%. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9% horse serum albumin in the proportions of 1 : 38·4. Isochrones drawn at 8, 16, 32 and 50 min.

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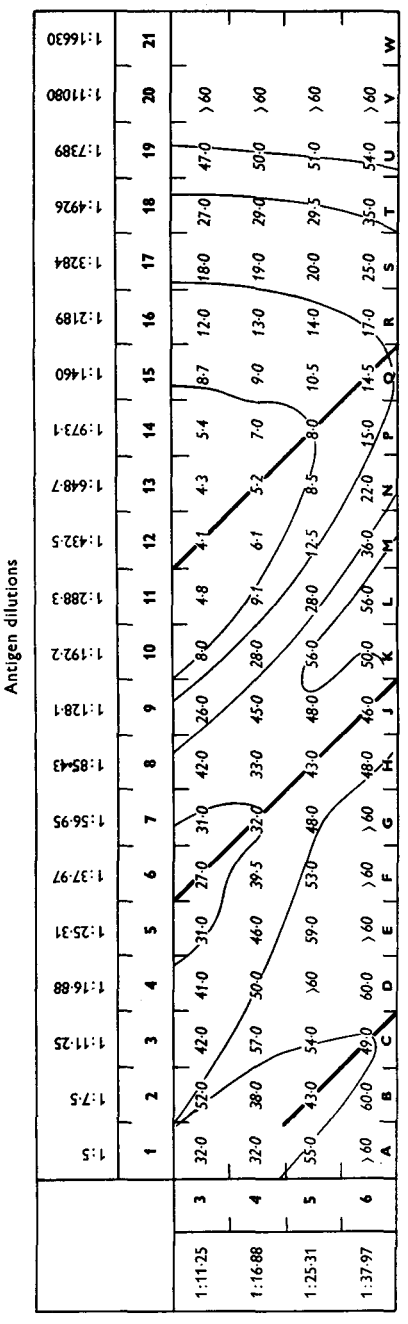

Fig. 6. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1 : 38·4. Isochrones drawn at 8, 16, 32 and 50 min.

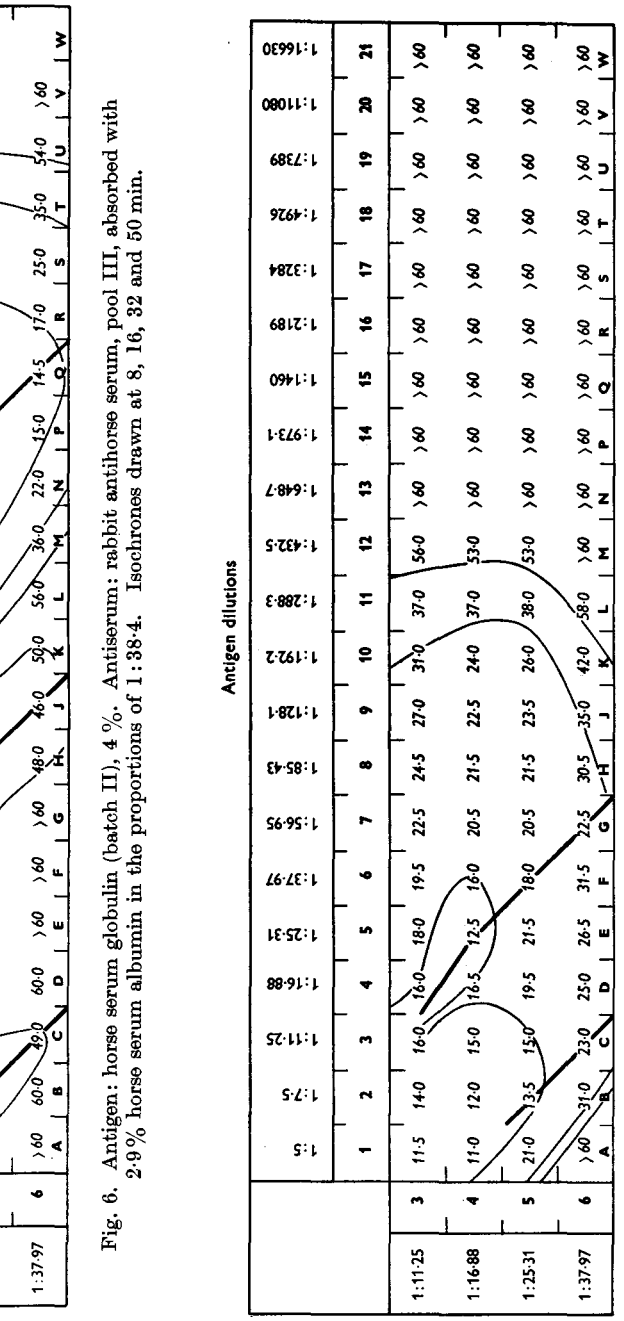

Fig. 7. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 4 % horse serum globulin in the proportions of 1 : 38·4. Isochrones drawn at 16, 32 and 50 min.

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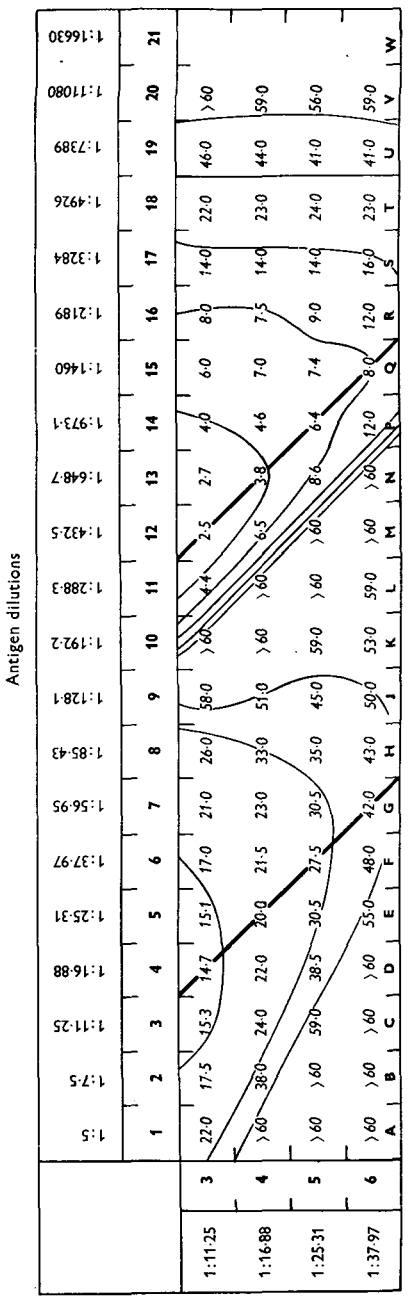

Fig. 8. Antigen: horse serum albumin, 2·9%. Antiserum: rabbit antihorse serum, pool III, absorbed with 4% horse serum globulin in the proportions of 1:38·4. Isochrones drawn at 4, 8, 16, 32 and 50 min.

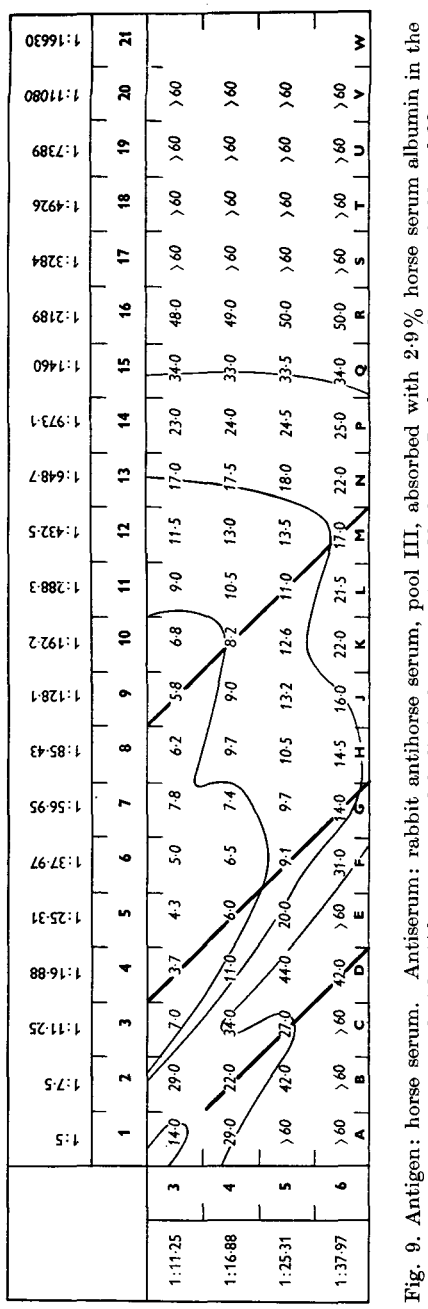

Fig. 9. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9% horse serum albumin in the proportions of 1:38·4 and with 4% horse serum globulin in the proportion of 1:38·4. Isochrones drawn at 8, 16 and 32 min.

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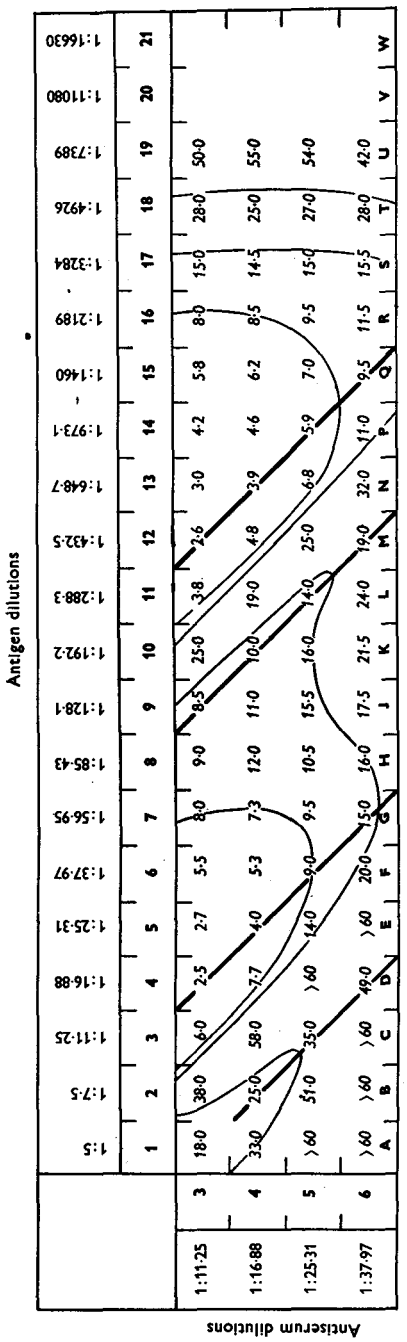

Fig. 10. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 4% horse serum globulin in the proportions of 1:38·4. Isochrones drawn at 8, 16 and 32 min.

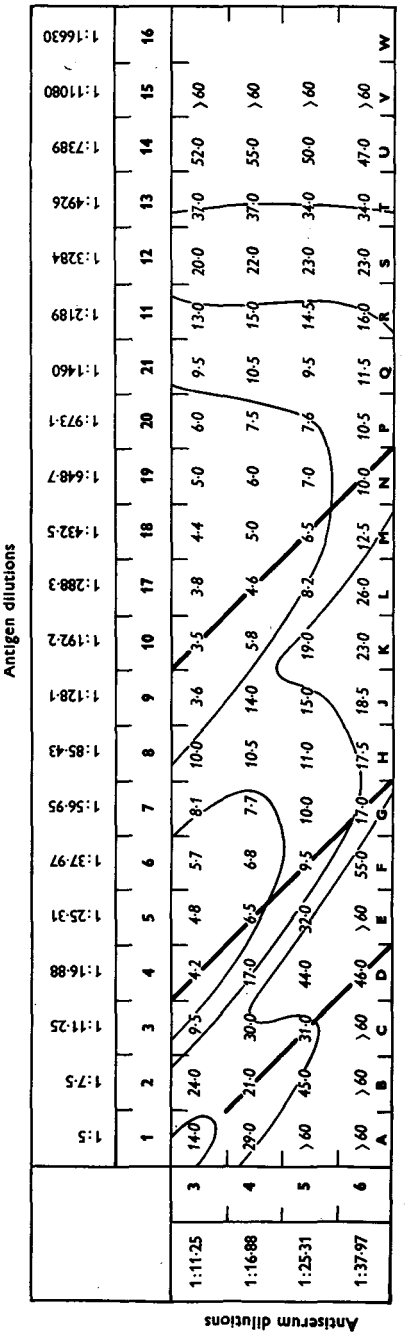

Fig. 11. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9% horse serum albumin in the proportions of 1:38·4. Isochrones drawn at 8, 16 and 32 min.

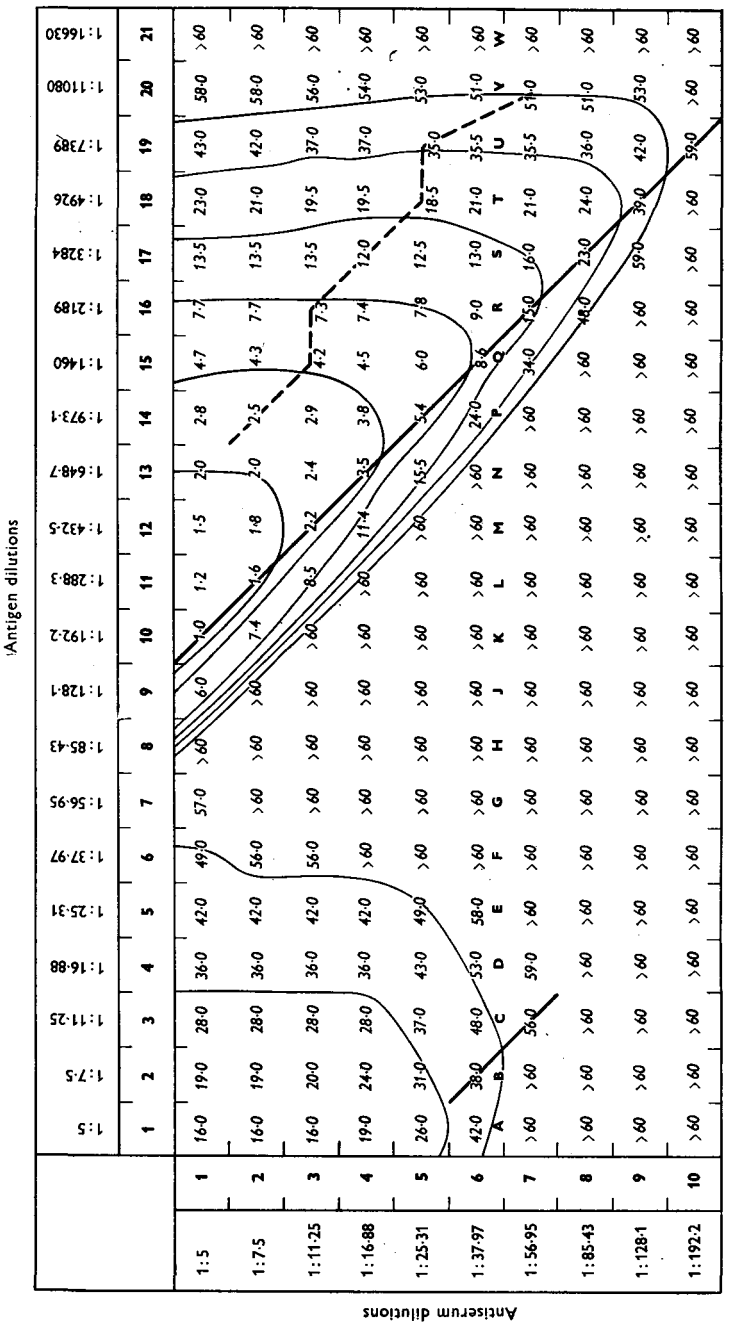

Fig. 12. Antigen: horse serum crystalbumin 2·9 %. Antiserum: rabbit antihorse serum, pool III. Isochrones drawn at 2, 4, 8, 16, 32 and 50 min.

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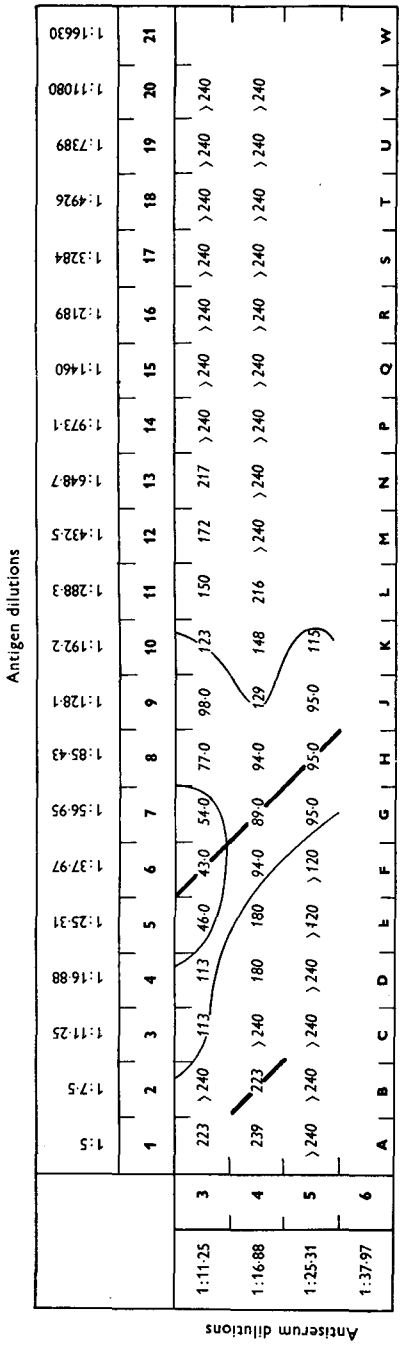

Fig. 13. Antigen: horse serum globulin (batch II), 4 %. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1 : 38·4 and 1 : 1. I-ochrones drawn at 60 and 120 min.

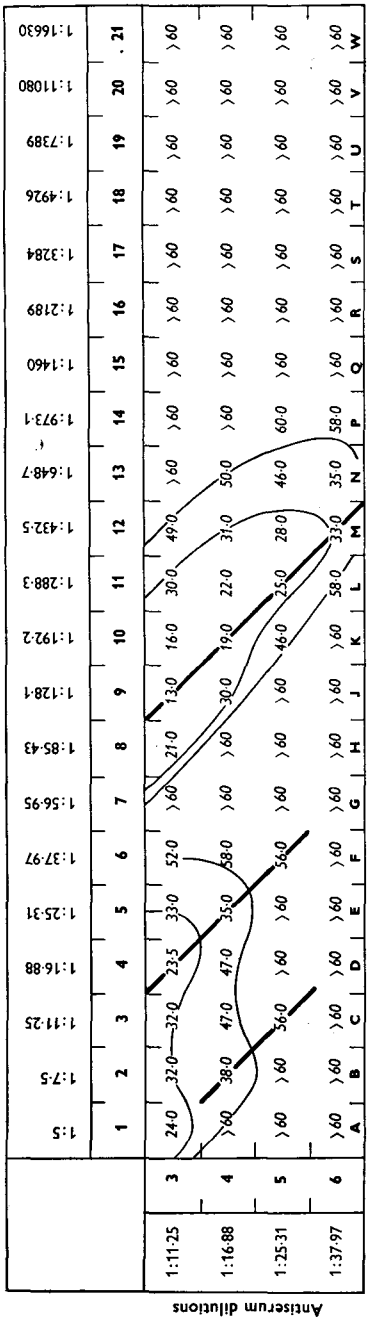

Fig. 14. Antigen: horse serum. Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1 : 38·4 and 1 : 1. Isochrones drawn at 32 and 50 min.

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the α procedure optima are not clear cut. For each dilution of antiserum there are a number of antigen dilutions each of which gives visible particles of precipitate in approximately the same time. Therefore, it seems possible that there are really two series of α procedure optima in this region, close together.

The second interesting feature of zone F is the marked inhibition of precipitation in the region of relative antiserum excess; this is Boyd's H type antiserum. This delay in the formation of visible particles of precipitate in the antibody excess region of zone F may be an inherent property of the antibody concerned in the production of this zone, or it may be due to an interference with this antigen-antibody reaction by the antigen-antibody reaction responsible for zone Q. On the other hand, the inhibition in the antiserum excess region of zone F may be apparent rather than real, as the mixtures in the antigen excess region of zone Q developed a marked turbidity very rapidly. This marked turbidity, which persisted a long time before discrete particles of precipitate became apparent, could easily obscure the fine particles of precipitate formed in the antibody excess region of zone F.

Figure 3

Antigen: horse serum globulin (batch I), 4·26 %.
Antiserum: rabbit antihorse serum, pool III.
Result: three series of α procedure optima at positions C, K and R.

There are three separate series of α procedure optima probably representing three distinct antigens present in the preparation of horse serum globulin.

The concentration of globulin used, 4·26 %, corresponds approximately with the concentration of total globulin in horse serum, and again the positions of the α procedure optima may be compared with those occurring in the reaction between horse serum and antihorse serum (Fig. 1). Both Figs. 1 and 3 have a series of α procedure optima at positions C or D and again at positions Q or R. Variations in position of α procedure optima involving one tube only cannot be regarded as significant, as slight variations in concentration, and other factors, may cause the optimum to fall in either of two adjacent tubes if the true optimum lies somewhere between the two. There is no optimum in Fig. 1 corresponding in position to optimum K of Fig. 3.

The particulation times of zone R in this figure 3 correspond fairly closely with those of zone Q in the reaction between horse serum and antihorse serum (Fig. 1).

Figure 4

Antigen: horse serum globulin (batch II), 4 %.
Antiserum: rabbit antihorse serum, pool III.
Result: three series of α procedure optima at positions C, J and Q.

In this reaction, four antiserum dilutions (nos. 3–6) only have been used, but the method of recording the results is identical with that of the previous figures.

This reaction is the same as the previous one except that a different batch of horse serum globulin was used. This batch of globulin also shows three series of α procedure optima and their positions correspond very closely with those shown by batch I (see Fig. 3).

The two batches of horse serum globulin were prepared in an identical manner. The α procedure optima obtained with the two batches correspond very closely in position (see Figs. 3 and 4), but a striking difference is the difference in the particulation times of the zones C. This difference affects the zones C only, and may be due to some difference in the physical state of the antigen responsible for this zone in the two batches of globulin.

Recapitulation

[Discussion of results shown in Figs. 1–4 and introduction to description of Figs. 5 and 6.]

The reactions of the antihorse serum with horse serum, the preparation of horse serum albumin and the preparation of horse serum globulin all show multiple α procedure optima. As each α procedure optimum probably represents an individual antigen-antibody reaction, it seems that the reactions so far investigated all involve a number of antigen-antibody systems. The position of each series of α procedure optima, as shown in the figures, is comparable with the optimal proportions of the antigen-antibody reaction responsible for that series of optima, as each position represents a definite ratio of the two reagents in the mixtures. If, for example, in the reaction between horse serum and antihorse serum, two antigen-antibody systems should have the same optimal proportions, then the series of α procedure optima produced by each system will be superimposed in the diagram of the results and only one zone of reaction will be apparent. It is only when the optimal proportions of two reactions differ that their optima show as two separate series. Hence each series of optima shown in the figures may represent two or more antigen-antibody reactions which have the same optimal proportions.

Apart from other considerations, it seems probable that, of the antigens involved in the reaction between the horse serum albumin preparation and the antiserum (Fig. 2), the antigen responsible for optimum Q (i.e. the optimum occurring with the lowest concentration of the albumin preparation) is the major constituent of the albumin preparation, as the difference in optimal proportions of the optima F and Q is probably more of a reflexion of the difference in concentrations of the two (or more) antigens present in the albumin preparation than a difference in the molecular weight of these antigens or in the concentrations of their homologous antibodies in the antiserum. Similar considerations may be applied to the reaction between the globulin preparation and the antiserum.

It seems logical to suppose that an antibody contained in the antiserum will react in the same proportions with its homologous antigen, whether that antigen be contained in horse serum or a purified derivative of horse serum (i.e. a preparation of horse serum albumin or horse serum globulin). Consequently, if the antiserum is titrated against horse serum and also against a purified derivative of horse serum, whose protein content equals the concentration of that purified derivative in horse serum, it seems reasonable to deduce that each individual antigen of the purified derivative of horse serum will produce a series of α procedure optima corresponding in position with the series of α procedure optima produced by that same antigen contained in horse serum. Hence by titrating antihorse serum against horse serum and equivalent concentrations of horse serum albumin and globulin preparations, it would seem possible to ascertain which optima of the horse serum reaction are due to albumin and which to globulin by comparing the positions of the series of α procedure optima produced in each reaction.

An obvious source of error in this procedure lies in the fact that preparations of albumin and globulin each contain a number of distinct antigens which may be present in different relative proportions from their proportions in horse serum, because certain antigens may be neither completely excluded from, nor completely included in, a particular derivative. This source of error will be discussed later, but before doing this the positions of the series of α procedure optima in the reactions so far described are compared.

On comparing the positions of the series of α procedure optima in the reactions of the antiserum with horse serum and with equivalent concentrations of the preparation of horse serum albumin and the preparation of horse serum globulin, it is apparent that each reaction shows a series of optima at position Q. The occurrence of an α procedure optimum with horse serum, and with equivalent concentrations of albumin and globulin all at the same position (that is, with the same proportions) has been observed by Taylor *et al.* (1932) (see also Taylor, 1935). The most likely interpretation of this phenomenon is that the zone Q of the reaction of horse serum with antihorse serum is composed of two separate antigen-antibody reactions which have approximately the same optimal ratio and consequently appear as only one zone. In order to confirm this, and also to exclude the possibility that the phenomenon is due to an antibody to 'horse serum protein' which reacts with equivalent concentrations of both albumin and 'globulin', the antiserum was absorbed with the horse serum-albumin preparation in the proportions of optimum Q, that is 1:38·4, in order to remove the antibody responsible for the zone Q of the reaction with the albumin preparation.

Absorption of precipitin by mixing antigen and antiserum in optimal proportions is a well-recognized procedure, but the absorption of an antiserum with an antigen exhibiting multiple zones, that is an antigen which really consists of several separate antigens, introduces a number of difficulties. Absorption at the optimum involving the lowest concentration of the (mixed) antigen only completely removes the antibodies responsible for that zone, and so enables deductions to be made about the nature of these antibodies from the subsequent reactions of the absorbed serum. When absorptions are conducted in this manner at the optimum involving the lowest concentration of the mixed antigen, any other reactions taking place do so under the conditions of relative antibody excess and a certain amount of the antibodies responsible for other optima are removed as well. The amount of other antibodies removed probably depends on the degree of difference between the optimal ratios of the various reactions. If absorption is conducted in the proportions of some optimum other than that involving the lowest concentration of the (mixed) antigen, the antibodies responsible for this optimum are removed in the precipitate and others are combined with under the conditions of antigen excess, thus producing an absorbed serum in which two or more antibodies have either been removed or prevented from combining visibly with antigen. The reactions of such an absorbed serum could not possibly provide conclusive evidence of the nature of the antibodies involved in the production of the various optima. Moreover, the absorbed serum would contain soluble antigen-antibody complexes formed in antigen excess which might interfere with the subsequent reactions of the absorbed serum. The only antibodies which can be completely absorbed alone are those responsible for the optimum involving the lowest concentration of the (mixed) antigen and the majority of the absorptions to be described have been conducted at such an optimum. When absorption of several antibodies is necessary, the antiserum is absorbed with the (mixed) antigen at several optima in turn, first mixing the reagents in the proportions of the optimum involving the lowest concentration of the antigen and then in the proportions of the optimum involving the next lowest concentration of the antigen (determined by titration after the first absorption) and so on, removing the precipitate after each stage of the absorption, thus avoiding the production of an absorbed serum containing soluble antigen-antibody complexes formed in antigen excess.

These considerations may be clarified by taking as an example the absorption of the antiserum with the albumin preparation. If the antiserum and the sample of albumin were mixed in the proportions of optimum F a precipitate would form and the anti-

body responsible for zone F would probably be completely removed, but the supernatant fluid would contain a soluble antigen-antibody complex of the reactants responsible for zone Q, formed in antigen excess. This is undesirable as at least one antibody has been removed in the precipitate, and at least one other antibody has been combined with under conditions of antigen excess producing a soluble complex. The reactions of this absorbed serum could not provide good evidence of the nature of the antibody responsible for zone Q because at least two antibodies have been removed or prevented from combining visibly with antigen. Moreover, the soluble antigen-antibody complexes might interfere with subsequent reactions of the absorbed serum. When the antiserum and the preparation of albumin are mixed in the proportions of optimum Q the antibodies responsible for this zone are completely removed, but a certain amount of the antigen responsible for zone F is also unavoidably added as well, and probably causes the removal of a small amount of the antibody responsible for zone F. The reactions of this absorbed antiserum are capable of providing evidence on the nature of the reactants responsible for zone Q.

The reactions of this absorbed antiserum (absorbed with the preparation of albumin in the proportions of optimum Q (i.e. 1:38·4)) with the preparations of horse serum albumin and horse serum globulin were then investigated and the results are shown in Figs. 5 and 6.

Figure 5

Antigen: horse serum albumin, 2·9 %.
Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 (optimum Q).
Result: one series of α procedure optima at position F.

Absorption of the antiserum with the horse serum-albumin preparation at the proportions of optimum Q has completely removed the antibody responsible for this optimum, and the absorbed antiserum shows a single zone with the horse serum albumin at position F (cf. Fig. 2).

Although the mixtures containing antiserum dilutions numbered 1 and 2 were not investigated, the zone F shown in this figure does not exhibit marked inhibition in the region of antiserum excess. Therefore it appears that the marked inhibition in the region of antiserum excess of zone F in the reaction of the horse serum albumin with the unabsorbed antiserum (Fig. 2) is not due to an inherent property of the antibody responsible for this zone, but is due either to interference with the antigen-antibody reaction responsible for zone F by the reaction responsible for zone Q, or to a masking of the fine particles formed in the antiserum excess region of zone F by the marked turbidity occurring in the antigen excess region of zone Q (see description of Fig. 2). Hence it seems clear that the presence of multiple zones can lead to erroneous conclusions regarding the occurrence of marked inhibition of precipitation in the region of antiserum excess of a particular zone.

Figure 6

Antigen: horse serum globulin (batch II) 4 %.
Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 (optimum Q).
Result: three series of α procedure optima at positions C, J and Q.

On comparing the results of the reaction of the horse serum-globulin preparation and the unabsorbed antiserum (Fig. 4) with those of the reaction of the horse serum-globulin preparation and the antiserum absorbed with albumin (this figure), the α procedure optima are seen to be unchanged in position. Hence removal of the antibody responsible for zone Q in the reaction between the albumin preparation and the antiserum does not effect the antibody responsible for zone Q in the reaction between the globulin preparation and the antiserum.

The only noteworthy difference between the results shown in Figs. 4 and 6 is that the absorbed antiserum shows longer particulation times for zones C and J.

Recapitulation

[Discussion of results shown in Figs. 5 and 6 and introduction to description of Figs 7 and 8.]

The results demonstrate that the reactions of the antiserum with horse serum and equivalent concentrations of the sample of albumin and the sample of globulin all show a series of α procedure optima at position Q (Figs. 1, 2 and 4). On removing the antibody responsible for zone Q of the albumin reaction from the antiserum, the optimum Q of the globulin reaction is left unchanged (Figs. 5 and 6). This serves to show that the antigen responsible for optimum Q in the reaction of the albumin preparation is quite distinct from the antigen responsible for optimum Q in the reaction of the globulin preparation, and that the optimum Q in the reaction between horse serum and the antihorse serum is probably composed of two separate and distinct optima, one involving the major antigenic constituent of the albumin preparation and the other involving the chief constituent of the globulin preparation. In order to confirm this result the antiserum was absorbed with horse serum globulin at the proportions of optimum Q and the reaction of the absorbed antiserum with horse serum globulin and horse serum albumin investigated. These results are shown in Figs. 7 and 8.

Figure 7

Antigen: horse serum globulin (batch II), 4%.
Antiserum: rabbit antihorse serum, pool III, absorbed with 4% horse serum globulin in the proportions of 1:38·4 (optimum Q).
Result: two series of α procedure at positions C and G.

After absorption of the antiserum with globulin at the proportions of optimum Q, the zone Q has completely disappeared; that is to say, the antibody responsible for this zone has been completely removed. On comparing the results of this reaction (Fig. 7) with the results of the reaction between the globulin and the unabsorbed antiserum (Fig. 4) a most interesting apparent shift in optima has taken place. The unabsorbed serum gives optima at positions C, J and Q and the absorbed serum gives optima at positions C and G. It appears as though the optimum at position J had moved to position G, i.e. a shift to the left as viewed in these figures. If absorption at optimum Q had also removed a fraction of the antibody responsible for zone J in Fig. 4, then it would be expected that the absorbed antiserum would show an optimum further to the right (i.e. with higher dilutions of the antigen), as an antiserum which contains less antibody than another, shows an optimum with a higher dilution of the antigen.

One hypothesis which might explain these facts is to suppose that in the reaction between the preparation of horse globulin and the antiserum (Fig. 4) there are in reality four α procedure optima, the three apparent ones at positions C, J and Q, and another not apparent due to its proximity to others at position F or G. On absorption of the antiserum with globulin at the proportions of optimum Q, part of the antibody responsible for zone J is also removed, its particulation time becomes longer, and on investigating the reaction of the absorbed antiserum with globulin, the optimum G is apparent, while the optimum at J is now obscured in the antibody excess region of zone G. An examination of particulation times in the neighbourhood of these zones lends support to this hypothesis, as the differences in particulation times of mixtures containing the same dilution of antiserum and different dilutions of antigen are small. If this hypothesis is correct the preparation of horse serum globulin probably consists of at least four distinct antigens.

Figure 8

Antigen: horse serum albumin, 2·9%.
Antiserum: rabbit antihorse serum, pool III, absorbed with 4% horse serum globulin in the proportions of 1:38·4 (optimum Q).
Result: two series of α procedure optima at positions G and Q.

Absorption of the antibody responsible for optimum Q of the reaction of the antiserum with the globulin does not remove the antibody responsible for optimum Q of the reaction between the albumin preparation and the antiserum.

Recapitulation

[Summary of results shown in Figs. 5–8 and introduction to description of Figs. 9–11.]

The investigations so far described have shown that zone Q of the reaction between horse serum and antihorse serum (Fig. 1) is probably composed of two superimposed zones: one involving the chief antigenic constituent of the albumin preparation, and the other involving an antigen present in the globulin preparation.

A possible source of error in this conclusion lies in the fact that the preparation of purified albumin or globulin (both samples contain several distinct antigens) may involve the partial, but not the complete, loss of one or more antigens, so that the relative proportions of the antigens present in these purified derivatives of horse serum differ from their relative proportions in horse serum. If such a difference occurs, then it is not possible to identify the antigens responsible for the α procedure optima of the reaction between horse serum and the antihorse serum by a simple comparison of the positions of the α procedure optima occurring in the reactions of the antiserum with horse serum, and with equivalent concentrations of horse serum-albumin and horse serum-globulin preparations. This consideration is far more likely to influence the minor antigenic components than the major ones, as the minor antigenic components, which give α procedure optima with high concentrations of horse serum or derivatives of horse serum, are probably present in small amounts only and their concentration can differ greatly in various preparations without appreciably affecting the total protein concentration. However, this source of error raises the question of whether the antigen responsible for zone Q of the reaction between the antiserum and the globulin preparation and the antigen responsible for zone Q of the reaction between the antiserum and the albumin preparation have the same concentrations in horse serum as they have in the globulin and albumin preparations respectively; in other words, it raises the question of whether in fact there are two superimposed optima at position Q of the reaction between horse serum and the antihorse serum. The separate antigens responsible for the zones Q in the reactions of the albumin and globulin preparations with the antiserum are probably the major constituents of their respective preparations, and it is unlikely that the source of error under discussion will effect the major constituents of the preparations. Nevertheless, in order to investigate this question evidence of two superimposed optima at position Q of the

reaction between horse serum and antihorse serum was sought by testing the antiserum absorbed with both the globulin and albumin preparations, with the albumin preparation alone and with the globulin preparation alone (all in the proportions of optimum Q) using horse serum as antigen. These results are shown in Figs. 9–11.

Figure 9

Antigen: horse serum.
Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 (optimum Q) and with 4 % horse serum globulin in the proportions of 1:38·4 (optimum Q).
Result: three series of α procedure optima at positions D, G and M.

Absorption of the antiserum with both the albumin and globulin preparations in the proportions of 1:38·4 (optimum Q) removes the antibodies responsible for zone Q of the reaction between horse serum and the antihorse serum, and leaves the other three optima of this reaction almost unchanged in position (cf. Fig. 1).

Figure 10

Antigen: horse serum.
Antiserum: rabbit antihorse serum, pool III, absorbed with 4 % horse serum globulin in the proportions of 1:38·4 (optimum Q).
Result: four series of α procedure optima at positions D, G, M and Q.

These optima correspond in position (almost exactly) with those present in the reaction between horse serum and the unabsorbed antiserum (Fig. 1). The complete removal of the antibody responsible for zone Q in the globulin-antihorse serum reaction (Fig. 4) apparently produces no marked change in the results of the reaction of the antiserum with horse serum. Hence the antigen-antibody reaction responsible for zone Q in the globulin-antihorse serum reaction (Fig. 4) is not apparent as a separate α procedure optimum in the reaction between horse serum and the antihorse serum.

Figure 11

Antigen: horse serum.
Antiserum: rabbit antihorse serum, pool III, absorbed with horse serum albumin in the proportions of 1:38·4 (optimum Q).
Result: three series of α procedure optima at positions D, G and N.

The results shown in this figure, with the apparent lack of a series of α procedure optima at position Q, appear to show that absorption of the antiserum with the albumin preparation alone removes the antibody responsible for optimum Q of the reaction between horse serum and the antihorse serum. However, on comparing these results (Fig. 11) with those of the reaction between horse serum and the antiserum absorbed with both the albumin and globulin preparations (Fig. 9) it is seen that zone M of Fig. 9 and zone N of Fig. 11 differ considerably. Zone N of Fig. 11 shows visible particles of precipitate under 1 hr. with antigen dilution numbered 19, whereas zone M of Fig. 9 does not show visible particles of precipitate under an hour with any antigen dilution above that numbered 16. Hence it seems highly probable that zone Q is still present in the reaction between horse serum and the antiserum absorbed with albumin alone (Fig. 11), but the optimum is obscured in the antibody excess region of zone N.

Recapitulation

[Summary of the results shown in Figs. 9–11 and introduction to description of Figs. 12–14.]

The results of the investigation of the reactions between horse serum and the antiserum absorbed with both the albumin and globulin preparations, with the globulin preparation alone and with the albumin preparation alone (Figs. 9–11), also support the view that the optimum Q of the reaction between horse serum and the antihorse serum (Fig. 1) consists of two superimposed optima, one involving the chief antigenic constituent of the albumin preparation, and the other involving an antigen contained in the globulin preparation. But the results are not conclusive owing to the probable obscuring of an optimum at position Q of the reaction between horse serum and the antiserum absorbed with the albumin preparation (Fig. 11).

The reactions about to be described were undertaken in order to investigate the nature of the zone F in the reaction between the horse serum albumin preparation and the antiserum (Fig. 2). This zone might represent an antigenically distinct albumin or it might represent globulin or other impurity in the albumin preparation. This problem was investigated by two methods. In the first place the reaction between a horse serum crystalbumin preparation and the antihorse serum was investigated and the results are shown in Fig. 12. Secondly, the pool of antihorse serum was absorbed with the albumin preparation in the proportions of both optimum Q and optimum F. This absorption was conducted in two stages. The antiserum was first absorbed in the proportions of optimum Q and after the precipitate had been removed by centrifugation more of the albumin preparation was added so that the reagents were then mixed in the proportions of 1:1, i.e. the proportions of optimum F after absorption of the antiserum with the albumin preparation in the proportions of optimum Q (see Fig. 5). A second precipitate formed and was removed. The absorbed serum now failed

to react with the horse serum-albumin preparation. In conducting the second stage of the absorption considerable excess of the antigen responsible for zone Q had been added and remained in the absorbed serum. The reactions between the absorbed serum and horse serum globulin and horse serum were then investigated and the results are shown in Figs. 13 and 14.

Figure 12

Antigen: horse serum crystalbumin, 2·9 %.
Antiserum: rabbit antihorse serum, pool III.
Result: two series of α procedure optima at positions B and Q.

On comparing these results (Fig. 12) with the results of the reaction between the albumin preparation and the antiserum (Fig. 2), it is seen that both show a similar zone with an optimum at position Q. It appears that the antigen responsible for this zone is the chief constituent of both the albumin and the crystalbumin preparations and might be termed the 'true' albumin. The reaction of the albumin preparation with the antiserum shows a second optimum at position F (Fig. 2), whereas the reaction of the crystalbumin preparation shows a second optimum at position B (Fig. 12). This variation in the position of the second optimum would seem to indicate that optimum Q of both the albumin and crystalbumin reactions represents the only true albumin, and that zone F of the albumin reaction and zone B of the crystalbumin reaction represent impurities, the proportion of impurity being much less in the crystalbumin than in the albumin preparation as shown by the position of the second optimum in the two reactions. [A decrease in concentration of a particular antigen in a mixture is manifest in these diagrams by a relative shift to the left of the optimum concerned.] These results indicate that the crystalbumin preparation has far less impurity than the albumin preparation.

Figure 13

Antigen: horse serum globulin.
Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 (optimum Q) and 1:1 (optimum F).
Result: two series of α procedure optima at positions D and J.

On comparing the results of this reaction with those of the reaction between globulin and the unabsorbed serum (Fig. 4), and also with those of the reaction between globulin and the serum absorbed with the albumin preparation in the proportions of optimum Q (Fig. 6), it is clear that removal of the antibodies responsible for zone F of the reaction between the albumin preparation and the antiserum removes the antibodies for zone Q of the reaction between the globulin preparation and the antiserum. In other words, the horse serum-albumin preparation contains an antigen which is also present in the horse serum-globulin preparation and which is responsible for zone Q of the reaction between globulin and the antiserum. But absorption of the antiserum with the globulin preparation in the proportions of optimum Q does not remove the antibodies responsible for zone F of the albumin reaction as shown by the presence of a second zone G in the reaction between the horse serum-albumin preparation and the antiserum absorbed with globulin in the proportions of optimum Q (Fig. 8). This may be explained on the view that zone F of the reaction between the albumin preparation and the antiserum is really composed of two superimposed zones, one due to an antigen present at a much higher concentration in the globulin preparation and a second antigen not apparently present in the globulin preparation.

Figure 14

Antigen: horse serum.
Antiserum: rabbit antihorse serum, pool III, absorbed with 2·9 % horse serum albumin in the proportions of 1:38·4 (optimum Q) and 1:1 (optimum F).
Result: three series of α procedure optima at positions D, G and M.

The results of the previous reaction establish the fact that zone F of the reaction between the albumin preparation and the antiserum is probably the result of two antigen-antibody reactions. The antigen of one is an antigen present at a much higher concentration in the globulin preparation, but there is no evidence of the presence of the other antigen in globulin. In order to try and correlate this second antigen with an antigen of horse serum the reaction between horse serum and the antiserum absorbed with the albumin preparation in the proportions of both optimum Q and optimum F was investigated. The results of this reaction (Fig. 14) fail to correlate the second antigen responsible for zone F in the albumin reaction with any zone in the reaction of the antiserum with horse serum, as this figure shows three optima in the same positions as the results of the reaction between horse serum and the antiserum absorbed with the albumin and globulin preparations in the proportions of optimum Q (Fig. 9). The absence of a series of α procedure optima at position Q in the reaction between horse serum and the antiserum completely absorbed with albumin (Fig. 14) confirms the previous results. Optimum Q of the reaction between horse serum and the antiserum is the result of two antigen-antibody reactions; both antigens are present in the albumin preparation, one is the true albumin and the other is globulin impurity.

The isochrones of zone M (Fig. 14) correspond with those of Boyd's H type antiserum. In the reaction between horse serum and the antihorse serum absorbed with the albumin and globulin preparations both in the proportions of optimum Q (Fig. 9), where the isochrones of zone M can also be seen in the antiserum excess region without interference from neighbouring zones, the isochrones are of the R type. The possibility that the marked inhibition in the region of antiserum excess may be due to the excess albumin contained in the antiserum used for the investigation of this reaction (Fig. 14) is interesting in view of the work by Matthews (1948) on the influence of protein concentration on the R and H types of antisera.

Recapitulation

[Summary of the results shown in Figs. 12–14 and a discussion of uncorrelated optima.]

The results serve to show that the horse serum-albumin preparation contains three antigens. One of these antigens is regarded as the substance referred to as albumin as it is the chief antigenic constituent of the albumin preparation and there is a similar, if not identical, antigen present in the crystalbumin preparation at approximately the same concentration. The crystalbumin preparation contains less of both the other antigens than the albumin preparation and these are consequently regarded as impurities. The possibility that one of the impurities is absent from crystalbumin was not investigated. Of the two impurities one is present at a much higher concentration in the horse serum-globulin preparation than the albumin preparation and is regarded as a globulin, whereas the other has not been correlated with another antigen, either in the globulin fraction or in horse serum. The optimum due to this antigen may be obscured in the reaction between horse serum and the antiserum. Goldsworthy & Rudd (1935) concluded that their preparation of horse serum albumin contained globulin, and Hewitt (1936, 1937, 1938) has described the occurrence of three distinct proteins in the fraction of horse serum which is not precipitated by half saturation with ammonium sulphate.

In the course of all these experiments the optima D, H and M of the reaction between horse serum and the antiserum (Fig. 1) have not been correlated with optima in the reaction between the albumin or globulin preparations and the antiserum. Likewise several optima of the reaction between globulin and the antiserum (C, J (Fig. 4), and possibly another optimum G (Fig. 7)) have not been correlated with other optima. It is possible that these two groups of optima represent the same three antigen-antibody systems, one or more of the antigens being only partially separated in the globulin preparation. But no definite conclusions on this point can be drawn from the experimental results, as absorption tests involving these antigen-antibody reactions have not been performed.

The experimental results show many remarkable variations in the absolute particulation times of the same antigen-antibody reaction under different conditions. No attempt has been made to describe, collate or interpret these variations as so many factors, both known and probably unknown, are simultaneously involved. These factors possibly include the effect of heterologous antigen, variations in protein concentration, partial absorption of antibodies due to the use of mixtures of antigens for this purpose and the mutual influence of mixed precipitating systems.

CONCLUSIONS REGARDING THE ANTIGENIC CONSTITUTION OF HORSE SERUM, THE HORSE SERUM-ALBUMIN PREPARATION AND THE HORSE SERUM-GLOBULIN PREPARATION

In the foregoing account of the experimental results, much attention has been paid to the correlation of α procedure optima in order to ascertain which antigens of horse serum are present in a horse serum-albumin preparation and which in a horse serum-globulin preparation. A number of conclusions have been reached regarding the antigens concerned in the production of the various α procedure optima studied, and these have been collected below.

The reaction between horse serum and antihorse serum

This reaction involves at least five antigen-antibody systems. The antigens responsible for the α procedure optima of the reaction may be enumerated as follows:

Optima D, H and M. Possibly due to antigens present in the horse serum-globulin preparation. No evidence was found suggesting that the antigens are present in the albumin preparation.

Optimum Q. This optimum really represents two optima as it is the result of two antigen-antibody reactions which have approximately the same optimal proportions.

(1) One of these antigens is present in both the horse serum-albumin and globulin preparations, though it is present at a much higher concentration in the globulin preparation and has been regarded as a globulin which is present as an impurity in the albumin preparation. This antigen is responsible for optimum Q of the reaction between the globulin preparation and the antiserum and partially responsible for optimum F of the reaction between the albumin preparation and the antiserum.

(2) The other antigen is present in the albumin preparation where it is responsible for optimum Q of the reaction between the albumin preparation and the antiserum. No evidence suggested the presence of this antigen in the globulin preparation.

The reaction between the horse serum-albumin preparation and antihorse serum

This reaction involves three antigen-antibody reactions. The antigens responsible for the α procedure optima of the reaction may be enumerated as follows:

Optimum F. This optimum is the result of two antigen-antibody reactions which have approximately the same optimal proportions.

(1) One of these antigens is a globulin impurity. It is present at a much higher concentration in the horse serum-globulin preparation where it is responsible for optimum Q of the reaction between the globulin preparation and the antiserum. It is partially responsible for optimum Q of the reaction between horse serum and the antiserum.

(2) The second antigen was not correlated with another antigen, either in horse serum or the horse serum-globulin preparation.

Both of these antigens responsible for optimum F are regarded as impurities as their concentration in the crystalbumin preparation is much less than that in the albumin preparation. The possibility that one is completely absent from the crystalbumin preparation was not investigated.

Optimum Q. Due to an antigen which is probably the substance usually referred to as albumin. This antigen is partially responsible for optimum Q of the reaction between horse serum and the antiserum, and no evidence was found of the presence of the antigen in the globulin preparation.

The reaction between the horse serum-globulin preparation and antihorse serum

This reaction involves at least three antigen-antibody systems and possibly a fourth.

The antigens responsible for the α procedure optima of the reaction may be enumerated as follows:

Optima C (Fig. 4), G (Fig. 7) and J (Fig. 4). The antigens responsible for these optima have not been studied in detail. No evidence suggested the presence of these antigens in the horse serum-albumin preparation.

Optimum Q. Due to an antigen which is partially responsible for optimum Q of the reaction between horse serum and the antiserum. This antigen also occurs as an impurity in the horse serum-albumin preparation, where it is partially responsible for optimum F of the reaction between the albumin preparation and the antiserum.

RESULTS OF ELECTROPHORETIC ANALYSES OF THE HORSE SERUM DERIVATIVES USED AS ANTIGENS

An identical technique was used in the electrophoretic analysis of the albumin, crystalbumin and globulin preparations. In each case a sample of the preparation was dialysed at 0° C. against a buffer M/30-Na_2HPO_4 + M/30-KH_2PO_4 (pH 6·91 at 0° C.). Dialysis was continued for 72 hr. with frequent changes of buffer. Electrophoresis was conducted at 3·8° C. with the voltage adjusted to 200 V. Photographs taken after 2 hr. electrophoresis of the albumin and crystalbumin preparations and after 3 hr. electrophoresis of the globulin preparation are shown in Figs. 15, 16 and 17 respectively.

The analysis shows that the albumin preparation contains two components. The crystalbumin preparation likewise contains two components, but the subsidiary peak is far smaller than the subsidiary peak of the albumin preparation. In other words, by electrophoretic criteria, the crystalbumin preparation approaches homogeneity more nearly than the albumin preparation. The globulin preparation contains four components, none of which migrate at a rate approaching that of albumin. These results of the electrophoretic analyses are in complete agreement with the serological results, except that the serological results demonstrated the presence of three antigens in the albumin preparation, whereas electrophoresis revealed two components only.

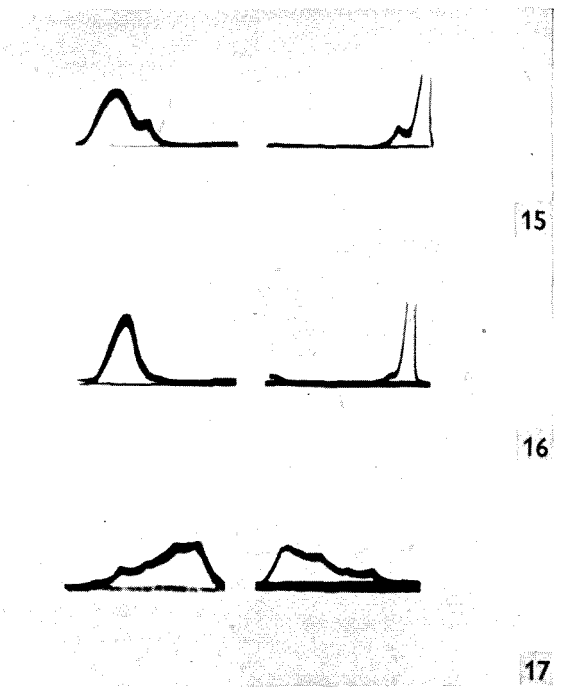
DISCUSSION

The experimental results may be interpreted as showing that the multiple zones of rapid particulation occurring in the titration of a pool of rabbit antihorse serum by the optimal proportions method of Dean & Webb are due to the independent activity of the multiple antigens present in horse serum and their homologous antibodies in the antihorse serum. In this, the results confirm the conclusion reached by Goldsworthy & Rudd (1935). Horse serum was found to consist of at least five antigens, and the results also show that a horse serum-albumin preparation and a horse serum-globulin preparation each consist of a number of separate antigens. The globulin preparation used in these experiments consists of at least three antigens, and evidence was obtained of a fourth. The horse serum-albumin preparation was found to contain three antigens, one of which is probably the true albumin, the other two being impurities; one of these impurities is present at a much higher concentration in the globulin preparation and may be regarded as a globulin impurity in the albumin. Electrophoretic analysis of the horse serum derivatives used in this work gave concordant results.

Throughout this work much attention was paid to the correlation of α procedure optima in order to ascertain which antigens of horse serum are present in the horse serum-albumin preparation and which in the horse serum-globulin preparation. A considerable degree of success was obtained in this respect, many of the antigens involved in the reaction

between horse serum and the antiserum being identified as antigens concerned in the reactions between the antiserum and the albumin or globulin preparations. It is of particular interest to note that in the course of this work the absorption of an antiserum containing antibodies to many antigens, by a mixture of antigens, was found to be a practicable procedure for the removal of antibodies to a single antigen provided the absorption is conducted in the proportions of the α procedure optimum which involves the lowest concentration of the mixed antigen. It was ascertained that a single zone of rapid particulation may be the result of two antigen-molecular weight of these antigens or to differences in the concentrations of their homologous antibodies in the antihorse serum.

The method of antigenic analysis used in the present series of experiments may have a number of applications. The method is based on the identification and correlation of α procedure optima occurring in the parallel titrations of a single antiserum against a mixed antigen and equivalent concentrations of physically or chemically prepared derivatives of it, supplemented by absorption of precipitins at individual and multiple α procedure optima and retitration of the absorbed sera. In the first place



Figs. 15–17. Electrophoretic analyses of the horse serum derivatives used as antigens. Fig. 15: albumin. Fig. 16: crystalbumin. Fig. 17: globulin.

antibody reactions whose optimal proportions are approximately equal, thus confirming the results of Taylor & Adair (1935). However, it seems clear that a single antigen-antibody reaction is manifested by a single zone of rapid particulation, and that the occurrence of two or more zones is due to the use of a mixed system. The results are compatible with the view that differences in the optimal proportions of the multiple α procedure optima shown by the reaction between horse serum (or a derivative of horse serum) and the antihorse serum are largely due to differences in the concentrations of the antigens in horse serum and less to differences in the

this method could be applied to the analysis of naturally occurring protein mixtures such as animal sera or egg white. Fractions of the mixed antigen, prepared by physical or chemical methods, could be subjected to serological analysis, which could guide further fractionation procedures and provide a method of identification and nomenclature of the constituent proteins based on antigenic individuality. Secondly, the method could be used, perhaps more easily, as a means of testing the purity of proteins prepared by physical or chemical methods. It appears that the titration of a single antigen by the optimal proportions method gives rise to the presence

of only one optimum, and hence the criterion of a single optimum indicative of a single antigen could be used as a test of the purity of a protein preparation. The method enables the impurity present in proteins prepared by different methods to be compared quantitatively and absorption tests could be used to identify the impurity qualitatively. The method is extremely sensitive and could, for example, detect and estimate globulin impurity in an albumin preparation at a concentration several thousand times less than that of globulin in horse serum. It seems to be extremely difficult to produce protein preparations which consist of a single antigen only, and the chief advantages of this method are that it does not depend on the preparation of highly purified protein samples containing a single antigen for *in vitro* tests or for animal injection, nor does it rely on the use of antisera containing antibodies to a single antigen.

There are a number of important points involved in the analysis of α procedure optima. The determination of the number and positions of α procedure optima present in a precipitation reaction requires the use of several dilutions of the antiserum. A complete series of dilutions such as are used in the present investigation is probably unnecessary, but optima may be missed if a single dilution of antiserum alone is used. Optima occurring with high concentrations of the antigen are particularly liable to be missed. A wide range of antigen dilutions, particularly at the more concentrated end of the scale, is also essential. In estimating traces of impurity the use of dilute antiserum and concentrated antigen is necessary in order to determine the optimal ratio of the impurity. In correlating the α procedure optima occurring in the parallel titrations of a single antiserum against a mixed antigen and equivalent concentrations of fractions of it, the equivalent concentrations need be only approximate as the dilution steps of the antigen are large.

The method of preparation of the antiserum is of some importance. The antiserum should be prepared against the natural mixture of antigens under investigation, and the aim should be an antiserum containing antibodies to all the antigens of the mixture. A pool of antisera is extremely convenient as a large volume of antiserum enables an extensive series of experiments to be performed and, moreover, a pool of antisera is more likely to contain antibodies for all the component antigens of a mixture, as some animals fail to respond to all the antigens of an injected mixture (Hektoen & Welker, 1925; Goldsworthy & Rudd, 1935; Taylor *et al.*, 1932).

The presence of antibodies for all the antigens in a natural mixture is particularly important in the detection of impurities present in purified proteins prepared from this natural mixture as the search for impurities is limited by the antibody content of the antiserum used. The presence of non-antigenic impurities, and antigenic impurities for which no antibodies exist in the test antiserum cannot be detected by this method. Hooker & Boyd (1936) used absence of a second zone as additional proof of the purity of their preparation of crystalline egg albumin. They used homologous antisera in searching for impurity. Although this indicates that they were not using a mixed system, a more rigorous test for purity of the albumin would be provided by the use of anti-egg white sera shown to contain antibodies for the possible impurities.

The results obtained in the optimal proportions titrations of mixed systems must be interpreted with caution, as there are a number of possible sources of error. While a single antigen-antibody reaction produces a single zone, a single zone may be the result of two or more antigen-antibody reactions which overlap due to the similarity in the optimal proportions of each system at the concentrations of the reagents used. This means that separate reactions do not always show as separate optima, and one zone may obscure another. This difficulty is hardly of importance in testing the purity of proteins, as a moderately pure protein is most unlikely to contain sufficient of an antigenic impurity to give an optimum at the same proportions of the reagents as the protein under preparation. In analysing a mixed antigen, absorptions with chemically prepared derivatives are necessary to differentiate and identify antigens which give superimposed α procedure optima. Absorption of precipitin at individual α procedure optima by a mixture of antigens is limited to the removal of the antibodies involved in the optimum occurring with the lowest concentration of the mixed antigen. This has proved to be successful: it would probably not be so satisfactory if the proportions of the optimum at which the absorption is to be carried out were similar to those of other optima of the reaction. Absorption of antibodies to a number of antigens may be conducted by mixing the reagents in the proportions of several α procedure optima in turn and removing the precipitate and titrating the antiserum after each stage of the absorption, thus avoiding the presence of soluble antigen-antibody complexes, formed in antigen excess, in the absorbed serum. A fraction of a mixed antigen may contain a number of antigens whose relative concentration differs from that in which they were originally present in the mixed antigen. Consequently, the parallel titrations of an antiserum against the mixed antigen and an equivalent concentration of the fraction will not enable direct correlation between α procedure optima to be made. This consideration is unlikely to affect the major antigenic component of the fraction, but the problem must be solved by a series of absorptions.

The occurrence of non-specific cross-reactions may

be a source of error in the suggested method of analysis but, throughout the experiments, no evidence of distinct zones of reaction due to heterologous antigen-antibody reactions was noted. Although it is known that heterologous antigen-antibody reactions exhibit α procedure optima (Duncan, 1932; Adair & Hamilton, 1939), it is not known whether heterologous antigen-antibody reactions can give a second zone in the presence of the homologous reaction. Taylor & Adair (1935) observed that the presence of another antigen-antibody system may cause changes of the proportions of an antigen and antiserum necessary for optimal particulation.

Various physical and chemical methods are available for examining the homogeneity of a protein preparation and for defining and differentiating the constituent proteins of a mixture. These methods include electrophoresis, sedimentation and determinations of solubility, each method depending on a different property of the protein molecules. Of these physico-chemical methods, solubility measurements are generally considered to be capable of providing reliable and sensitive criteria of homogeneity. Immunological methods are valuable as they are extremely sensitive. The exhibition of a single α procedure optimum by a protein preparation serves to show that it consists of a single antigen (after considering the sources of error noted earlier), and the preparation satisfies this criterion of homogeneity. Although such a preparation may fail to satisfy other criteria of homogeneity, all the antigenic substances present in the preparation act together as a single antigen. Whether the reaction between a mixture of two proteins and their homologous antibodies will exhibit one or two α procedure optima, must depend on the degree of similarity of the proteins and probably also on the degree of specificity of the antibodies. What this 'degree of similarity' is must await future work. The presence of a single antigen in a protein preparation is an extremely sensitive criterion of homogeneity, and the technique of defining and differentiating the constituent proteins of a mixture on the basis of antigenic individuality appears to be very useful.

SUMMARY AND CONCLUSIONS

1. The reaction between horse serum and a pool of rabbit antihorse serum exhibiting four α procedure optima has been investigated with a view to analysing the antigens involved in the production of the multiple zones.

2. The following conclusions have been reached regarding the antigenic constitution of horse serum, a horse serum-albumin preparation and a horse serum-globulin preparation:

Horse serum consists of at least five antigens.

The horse serum-albumin preparation contains three antigens, two of which are probably impurities, one of these being a globulin.

The horse serum-globulin preparation consists of three antigens and evidence was obtained of a fourth.

Electrophoretic analysis of these protein preparations gave concordant results.

3. Multiple zones of rapid particulation in the precipitation reaction are due to the independent activity of multiple antigens and their homologous antibodies.

4. The absorption of precipitin from a mixture of antibodies by a mixture of antigens at a single α procedure optimum is both a useful and a practicable procedure.

5. Attention is drawn to the fact that the method of antigenic analysis used in the present series of experiments, involving the correlation of α procedure optima occurring in the parallel titrations of a single antiserum against a mixed antigen and equivalent concentrations of physically or chemically prepared fractions of it supplemented by absorption of precipitins at individual and multiple α procedure optima and retitration of the absorbed sera, could be applied to the identification and nomenclature of proteins occurring in natural mixtures, and also to the quantitative and qualitative appraisal of the purity of proteins prepared by physical or chemical means. The method does not rely on the preparation of highly purified protein samples containing a single antigen.

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