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A theoretical approach to precipitin reactions

INSIGHT FROM COMPUTER SIMULATION

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Summary. The theoretical consequences of different hypotheses of the mechanism of precipitin reactions have been evaluated by means of computer simulation. It has been found that the formation of compositionally different complexes in different antigen/antibody mixtures provides a valid explanation of the zoning phenomenon, but this concept fails to explain the absence of free antigen and of antigen in soluble complexes at the point of maximum percipitation.

It is found that the following hypothesis provides an improved qualitative and quantitative explanation of precipitin reactions. In the first stage of the total reaction a series of compositionally different complexes is formed. As the second stage of the total reaction two kinds of processes are proposed. Inherently insoluble complexes precipitate causing the remaining soluble complexes to participate in mutual rearrangements to re-establish a new state of equilibrium in the supernatant. The inherently insoluble complexes, moreover, create a hydrophobic phase, distinct from the supernatant and cause the remaining otherwise soluble complexes to distribute themselves between the two phases according to a partition coefficient. A mathematical apparatus to study the consequences of this hypothesis is presented, and it is demonstrated that the features of precipitin curves can be explained nearly completely this way.

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INTRODUCTION

Precipitin reactions have proven difficult to explain in thermodynamic terms (Talmage & Cann, 1961; Day, 1972). We have adopted the classical concept that precipitin reactions comprise two stages (Marrack, 1938; Topley & Wilson, 1936). Initially, multivalent antigens and bivalent antibodies form a series of compositionally different complexes (Goldberg, 1952; Palmiter & Aladjem, 1966, 1968; Steensgaard, Johansen & Møller, 1975; Steensgaard, Liu, Cline & Møller, 1977). Secondarily, some of these complexes develop a hydrophobic character. They expel water, cohere and form their own phase, the precipitate.

The present work was undertaken to develop and to refine theoretical means for analysis of the consequences of different concepts of the mechanism of precipitin reactions. By use of computer simulation three aspects of precipitin reactions have been

studied, namely: (1) the extent to which the distinguishing features of precipitin curves can be explained solely as a consequence of formation of compositionally different complexes; (2) the possible requirement of an element of non-equilibrium thermodynamics to explain the nature of precipitin reactions; and (3) the implications of an assumption that the inherently insoluble complexes create a hydrophobic phase, distinct from the supernatant, and that the remaining otherwise soluble complexes will be distributed between the two phases according to a partitition coefficient.

THEORETICAL CONSIDERATIONS

In an equilibrium mixture consisting of an f-valent antigen (Ag) and a bivalent antibody (Ab) the equilibrium concentration of the complex $[Ag_iAb_j]$ is given by:

$$(1) \qquad [Ag_i\,Ab_j] = \kappa_{(ij)}^{i+j-1}\,[Ag]^i\,[Ab]^j$$

Assuming that $\kappa_{(ij)}$ functionally can be replaced by the apparent association constant (κ) as obtainable by a Sips analysis mass balance expressions can be formulated as follows:

$$(2) \qquad (Ag) = [Ag] + \sum_{j=1}^{\infty} \sum_{i=g}^{j+1} \kappa^{i+j-1}\, i[Ag]^i\,[Ab]^j$$

$$(3) \qquad (Ab) = [Ab] + \sum_{j=1}^{\infty} \sum_{i=g}^{j+1} \kappa^{i+j-1}\, j[Ag]^i\,[Ab]^j$$

where $g = \text{int}\ (1+(j-1)/f)$ and ( ) denotes total concentrations.

Equations (2) and (3) can be solved simultaneously for known values of (Ag) and (Ab) to yield [Ag] and [Ab] by use of a Newton-Raphson technique (Steensgaard *et al.*, 1977). The program used here is a thoroughly revised and improved version of the original program. Major changes in the new program are:

(1) Initial guesses required to start the Newton-Raphson iterations are simply (Ag) and (Ab), if these are less than 1·0, else 1·0.

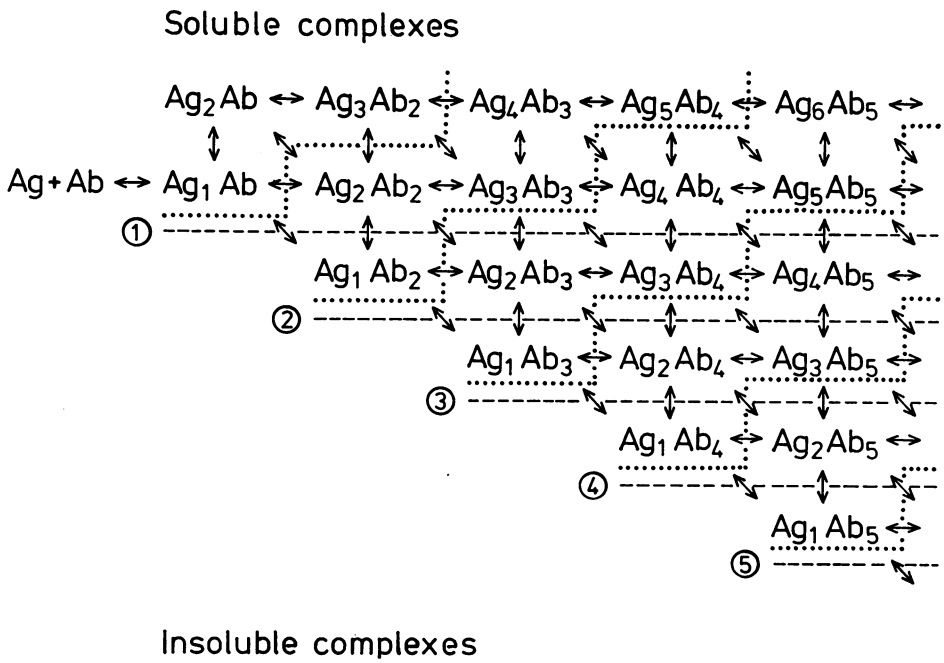

Figure 1. Reaction scheme for the theoretically possible interactions between an antigen (Ag) and a bivalent antibody (Ab). Dotted and dashed lines show the modes of distinctions between supposed soluble and supposed insoluble complexes. Dotted distinction modes are called staircase modes, and dashed distinction modes are called horizontal modes. Numbers at the beginning (to the left) are the mode numbers used in the calculations. (Modified from Steensgaard & Funding, 1974.)

(2) A limitation is put on the size of the increment values in the Newton-Raphson procedure. If newly calculated increment values will double the absolute test function values (i.e. $|f_1|$ and $|f_2|$ in Steensgaard *et al.*, 1977) the increment values are halved, and in case (Ag)/(Ab) and/or (Ab)/(Ag) exceed 10·0 the increment values are recursively divided with these ratios instead of 2·0.

(3) In the original program the size of j was limited to 16 or 24, depending on the numerical accuracy desired. In the present program a variable j_{max} value is used, and the size of j_{max} is automatically increased until it exhausts a defined computational accuracy. Choosing that all calculations are to be performed with 10 significant digits, a j_{max} of 16 is sufficient in antigen excess examples, whereas up to 126 may be required in the extreme antibody excess examples.

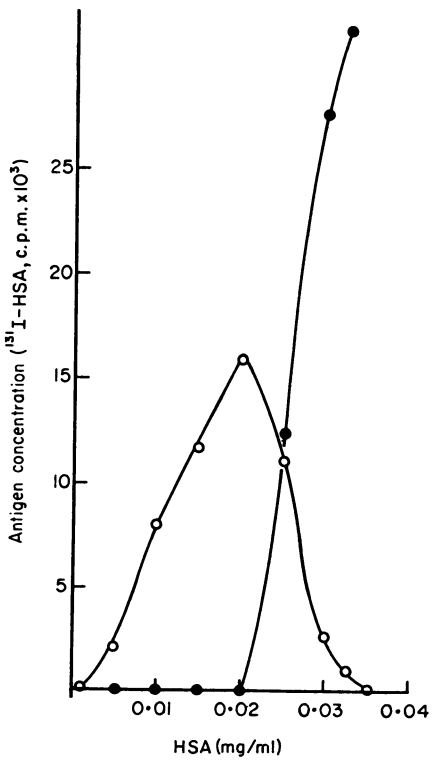

Figure 2. Experimental precipitin curve. The antigen (human serum albumin) was labelled with ^{131}I, and the ordinate shows measured radioactivity in the precipitate (open circles) and in the supernatant (filled circles). Rabbit anti-human serum albumin IgG (Dakopatts, Copenhagen, code 10-001) was used as antibody. The experimental procedure is described in Steensgaard *et al.* (1975).

A series of distinctions between soluble and insoluble complexes will be studied. The distinction modes are shown in Fig. 1. The distinction modes are chosen to represent compromises between: (1) knowledge that soluble complexes can be detected in moderate antigen excess; and (2) the assumption that thermodynamically most likely complexes (f.i. $AgAb_f$) in antibody excess are inherently insoluble (Steensgaard *et al.*, 1975). Staircase distinction mode 1 corresponds to the distinction introduced by Palmiter & Aladjem (1966).

In the basic procedure the amount of precipitated antigen (Ag_{prc}) is calculated from the following expressions to simulate the precipitin curve in Fig. 2:

(a) for horizontal distinction mode n

$$(4)\qquad Ag_{prc}=\sum_{j=n+1}^{\infty}\sum_{i=g}^{j-n}\kappa^{i+j-1}\,i[Ag]^i\,[Ab]^j$$

(b) for staircase distinction mode n

$$(5)\qquad Ag_{prc}=\sum_{j=n+1}^{\infty}\sum_{i=g}^{h}\kappa^{i+j-1}\,i[Ag]^i\,[Ab]^j$$

where $h=\min(2(j-n), j+1)$.

In the re-equilibration studies 'new' total and active concentrations of (Ag) and (Ab) are calculated as follows in each re-equilibration cycle for horizontal distinction mode n:

$$(6)\quad (Ag)=[Ag]+\left(\sum_{j=1}^{\infty}\sum_{i=m}^{j+1}\kappa^{i+j-1}\,i[Ag]^i\,[Ab]^j\right)+\alpha\sum_{j=n+1}^{\infty}\sum_{i=g}^{j-n}\kappa^{i+j-1}\,i[Ag]^i\,[Ab]^j$$

$$(7)\quad (Ab)=[Ab]+\left(\sum_{j=1}^{\infty}\sum_{i=m}^{j+1}\kappa^{i+j-1}\,j[Ag]^i\,[Ab]^j\right)+\alpha\sum_{j=n+1}^{\infty}\sum_{i=g}^{j-n}\kappa^{i+j-1}\,j[Ag]^i\,[Ab]^j$$

$m=\max(g, j-n+1)$

where α is the activity coefficient of insoluble complexes. For staircase distinction modes the summation limits must be changed accordingly.

The phase separation problemacy is treated as follows. Some inherently insoluble complexes as defined by one of the distinction modes are assumed to make up their own hydrophobic phase in contrast to the hydrophilic supernatant. The remaining

complexes (C_s) will then be distributed between the aqueous supernatant and the hydrophobic precipitate as determined by a partition coefficient (κ_p) so that

$$\kappa_p = \frac{[C_{prc}]}{[C_{sup}]} \quad (8)$$

where C_{prc} denote otherwise soluble complexes from the supernatant, but now distributed in the precipitate (PRC), and C_{sup} are complexes in the supernatant. Replacement of concentrations with amounts/volumes leads to

$$\kappa_p = \frac{(C_{prc})\,(V - PRC/\rho)}{PRC/\rho\,(C_{sup})} \quad (9)$$

where V is the total reaction volume, PRC the amount of inherently insoluble complexes, and ρ the density of these complexes.

Because $V \gg PRC/\rho$ and ρ can be regarded constant, equation (9) can be changed to

$$\kappa_p' = \kappa_p \cdot \rho/V = \frac{(C_{prc})}{PRC \cdot (C_{sup})} \quad (10)$$

$$\text{where } (C_{sup}) = (C_s) - (C_{prc}) \quad (11)$$

Equation (10) therefore can be solved to yield (C_{prc}):

$$(C_{prc}) = (C_s)\frac{PRC\,\kappa_p'}{1 + PRC\,\kappa_p} \quad (12)$$

C_s is for horizontal distinction mode n simply obtained as

$$C_s = \sum_{j=1}^{\infty} \sum_{i=m}^{j+1} \kappa^{i+j-1} [Ag]^i [Ab]^j \quad (13)$$

In case of staircase distinction mode n, the summation limits must be changed accordingly.

Assuming that the hydrophobic effect is mediated through the antibody molecules in the complexes in question, PRC is given by equation (14) as exemplified for horizontal distinction mode n:

$$PRC = \sum_{j=n+1}^{\infty} \sum_{i=g}^{j-n} \kappa^{i+j-1}\, j[Ag]^i [Ab]^j \quad (14)$$

Let δ define the degree to which any of these complexes are taken up by the precipitate

$$\delta = \frac{(C_{prc})}{(C_s)} \quad (15)$$

The amount of otherwise soluble antigen (Ag_p) which is taken up by the precipitate is then given by

$$Ag_p = \sum_{j=1}^{\infty} \sum_{i=m}^{j+1} \delta\, \kappa^{i+j-1}\, i[Ag]^i [Ab]^j \quad (16)$$

in case of horizontal distinction mode n. The total amount of antigen in the precipitate is thereafter the sum of Ag_{prc} and Ag_p.

In the theoretical precipitin curves shown in Figs 4, 6, 7, 8 and 9 each curve is based on 12 calculated points (as indicated by crosses). The smooth curve that combines the calculated points is the result of a third degree Lagrangian interpolation between the calculated points. In the calculations presented here κ was taken as $3{\cdot}8\ 10^7$ (litres/mole) as found by Arend & Mannik (1974). The antibody concentration used was $0{\cdot}9\ 10^{-6}$ (moles/litre), and f was taken as 8 in all calculations. Concentrations of antigen and other parameters are given in the text when needed.

The program for these calculations was written in FORTRAN IV. The calculations were performed on a CDC Cyber 173 computer at the Computing Centre (RECAU) at the University of Aarhus. A complete theoretical precipitin curve including 55 re-equilibration cycles and corresponding phase separation calculations needs up to 500 sec of central processing time.

RESULTS AND DISCUSSION

An experimental precipitin curve

A typical precipitin curve for human serum albumin and rabbit anti-human serum albumin 7S antibodies is shown in Fig. 2. It appears from this figure that two features may be regarded as distinctive. The first is a clearly expressed zoning phenomenon so that the precipitin curve reaches the baseline in antigen excess. The second distinguishing feature is that little if any free antigen and/or antigen contained in soluble complexes is present in antibody excess and in the antibody excess part of the equivalence zone. For the system in question free specific antibodies also are absent in the equivalence zone. The ratio of (Ag)/(Ab) in this system is near 0·5 at the point of maximum precipitation.

Theoretical precipitin curves as a consequence of the formation of different complexes

The core of a theoretical explanation of the zoning phenomenon has previously been found in the

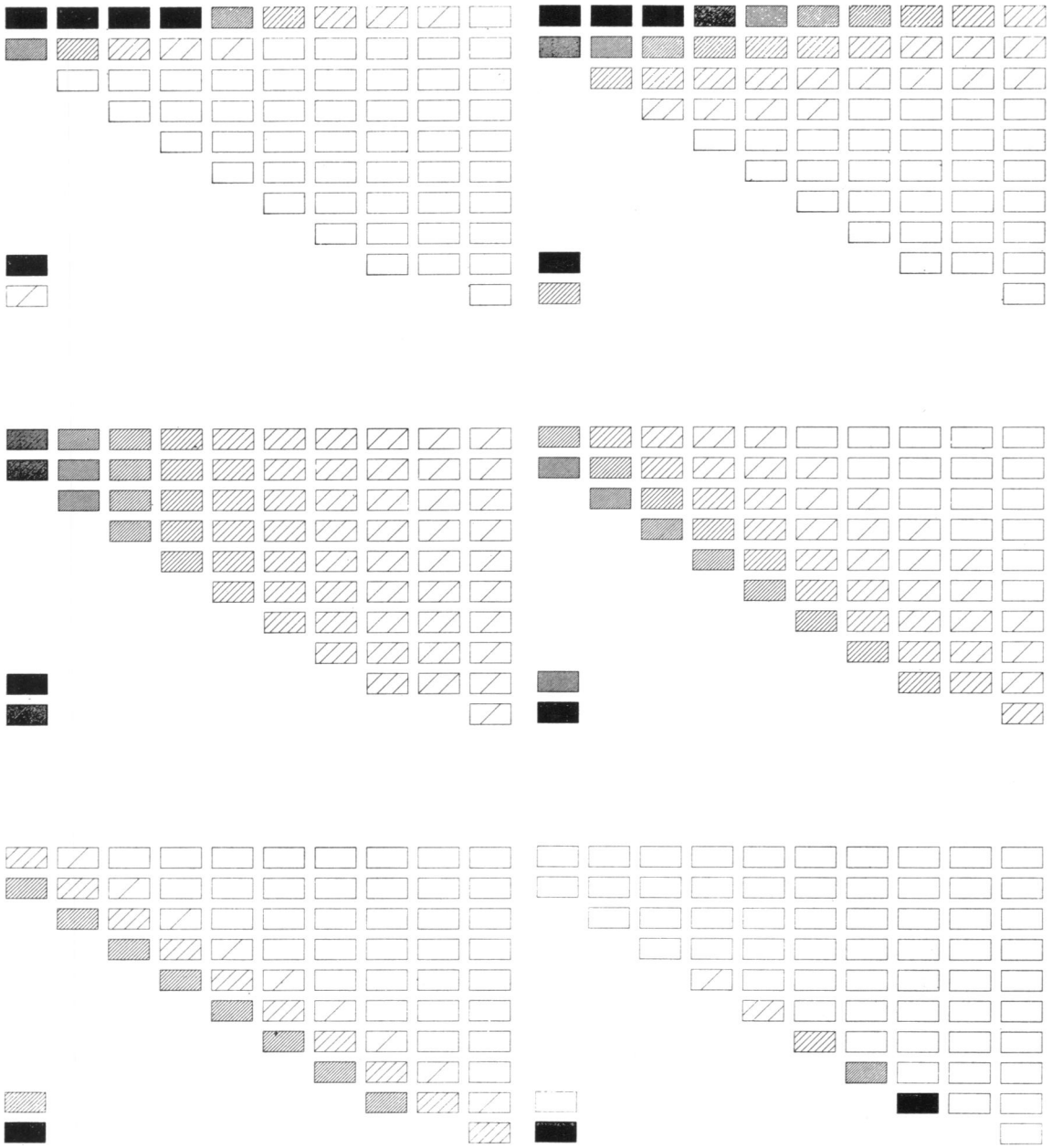

Figure 3. Graphical representation of six different complex distributions at six different (Ag)/(Ab) ratios. Each box represents a single immune complex, and the boxes are arranged in the same way as in the reaction scheme in Fig. 1. Hence, boxes in the top row are from left to right Ag_2Ab, Ag_3Ab_2, Ag_4Ab_3 and so forth. Concentrations are given by the number of 45° lines in each box, and the number of lines (and hence the darkness) in a box is directly proportional to the calculated complex concentrations. The two boxes in the lower left corner of each individual complex distribution pattern show free antigen (upper box) and free antibody (lower box). From the upper left distribution to the bottom right distribution pattern the total (Ag)/(Ab) ratios were: 2·25, 1·27, 0·71, 0·40, 0·23 and 0·13, respectively.

formation of compositionally different complexes at different Ag/Ab ratios (Goldberg, 1952; Palmiter & Aladjem, 1966, 1968; Kubo, 1976), or as a function of the use of different absolute concentrations of antigen and antibody (Steensgaard *et al.*, 1975, 1977). Complex distributions at six selected points of a precipitin curve are shown graphically in Fig. 3. However, the question of the extent to which the formation of compositionally different complexes in a quantitative sense can explain the features of precipitin curves was not settled. Aiming at achieving this a series of theoretical precipitin curves have been calculated employing ten different distinction modes between soluble and insoluble antigen–antibody complexes. For this particular system the horizontal distinction mode 1 and 3, and the staircase distinction modes 2 and 3 (see Fig. 1) are appealing, because it is known from experimental studies that some small antigen rich complexes are soluble in the moderate antigen excess region (Singer, 1957; Steensgaard & Funding, 1974).

The calculated precipitin curves are shown in Fig. 4. It appears directly from this figure that the zoning phenomenon arises irrespective of the distinction mode. The amount of precipitate, however, depends very much on the distinction mode. The horizontal distinction modes yield relatively low precipitin curves with maximum precipitation at (Ag)/(Ab) ratios below 1. The staircase distinction modes in contrast give rise to very high precipitin curves with maximum precipitation at (Ag)/(Ab) ratios above 1. The horizontal distinction modes are therefore regarded most likely, and they will be preferred in the following calculations.

To evaluate the extent to which simple discrimination between soluble and insoluble complexes provide a quantitative explanation of precipitin reactions the amount of unprecipitated antigen has

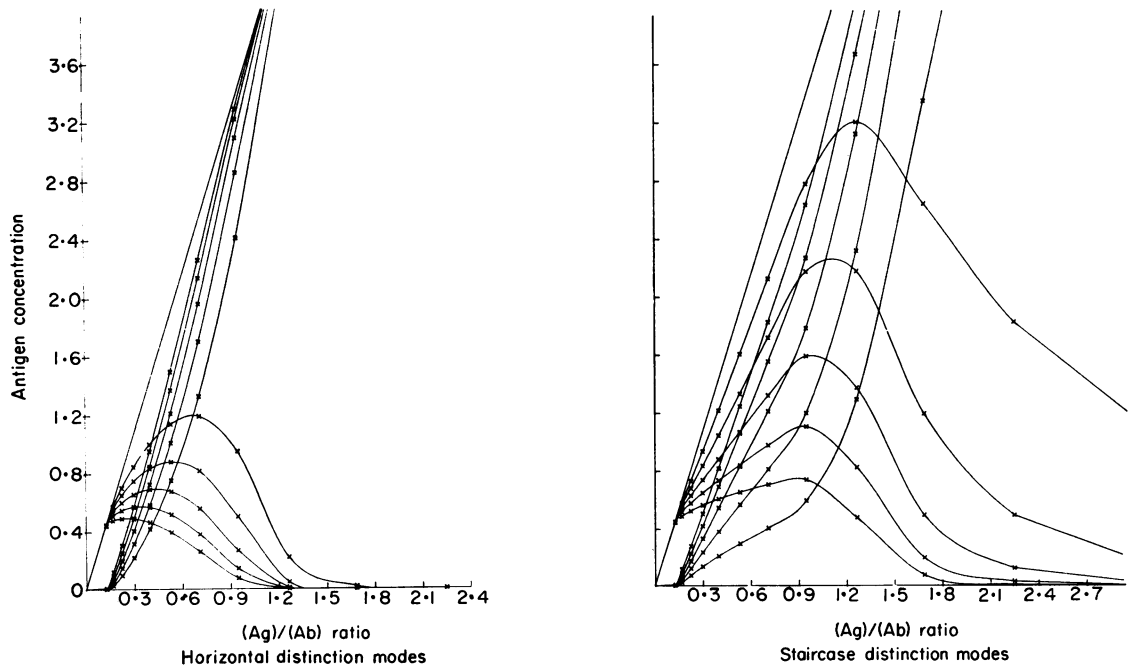

Figure 4. Calculated precipitin curves using different distinction modes. The antibody concentration was kept constant ($=0{\cdot}9 \times 10^{-6}$M), and the antigen concentration varied. Antigen concentration is expressed as total (Ag)/(Ab). The lower curves, showing a zoning phenomenon are the calculated precipitin curves using different distinction modes. The straight line shows the total amount of antigen in the system. The curved 'parabolic' curves show the combined concentration of free and complexed antigen in the theoretical supernatant. In the left part of the figure horizontal distinction modes were tested, and in the right part of the figure staircase distinction modes were tested. Mode number 1 is in both cases the highest precipitin curve, and increasing mode number gives lower and lower precipitin curves. The curves of free and complexed antigen can be identified accordingly. The antigen concentration is based on arbitrary units as they would appear if the antigen was radioactively labelled (f.i. in 1000 c.p.m.).

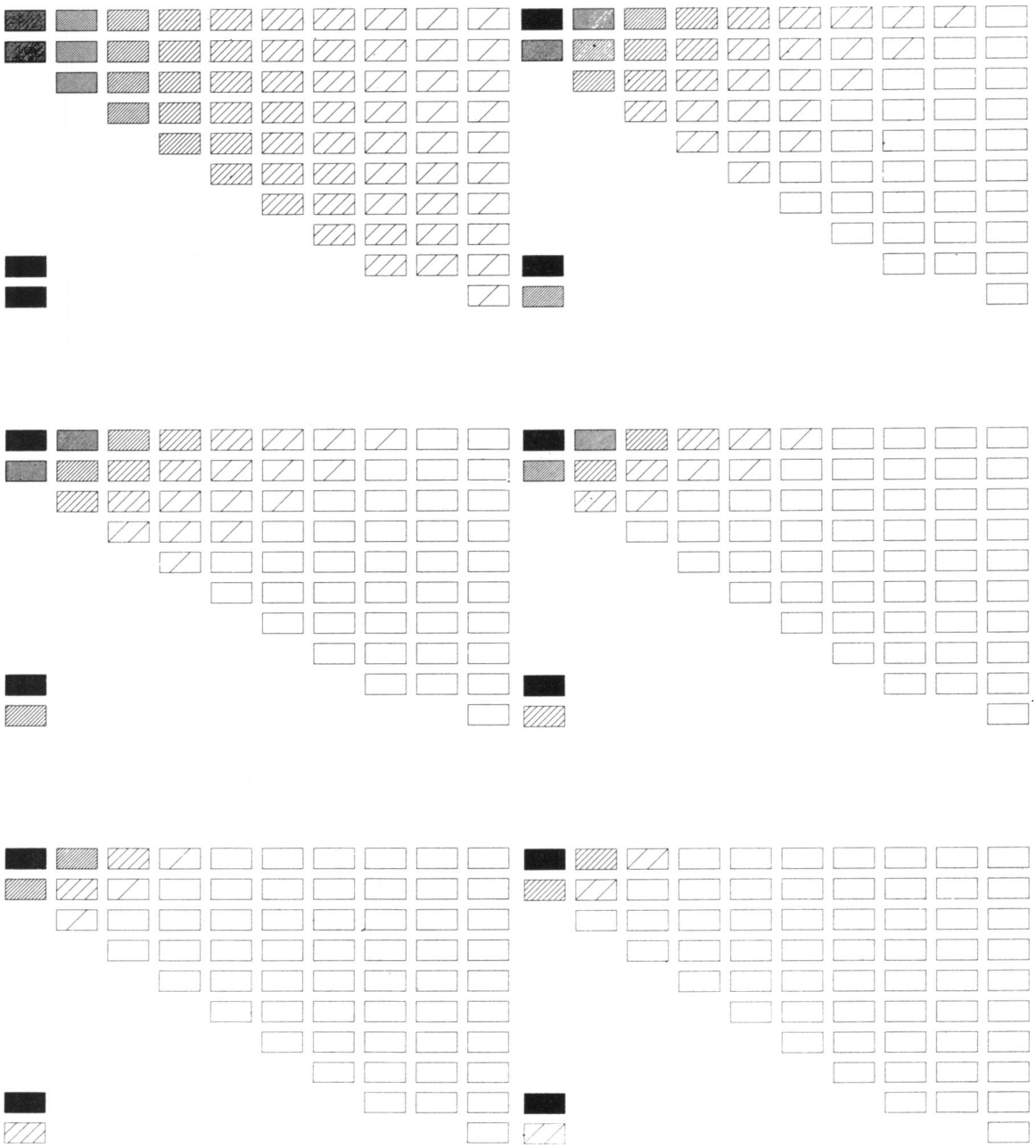

Figure 5. Calculated changes in complex distributions following precipitation of inherently insoluble complexes. The example is taken at the point of maximum precipitation by use of horizontal distinction mode 1. From the upper left complex distribution to the bottom right complex distribution the re-equilibration cycle numbers are 1, 2, 3, 8, 21 and 55.

been calculated also and the results hereof are inserted in Fig. 4. These curves show that considerable amounts of antigen either free or contained in soluble complexes are predicted by the basic theory at the point of maximum precipitation, and this prediction contrasts with the experimental finding, as shown in Fig. 2. We therefore conclude that the formation of compositionally different complexes in different antigen–antibody mixtures still can be regarded as the core of the explanation of precipitin reactions, but a second stage is required to provide a complete explanation. Two possible treatments of a second stage of precipitin reactions will be discussed in the following two sections.

Introduction of an element of non-equilibrium thermodynamics

Consider the following two stage model of precipitin reactions. In the first stage complexes are formed according to the references mentioned previously. In a second stage some selected complexes gradually loose their water solubility, leading to formation of a precipitate. Loss of water solubility in this sense means that less of these complexes are available for immune specific interactions with other complexes. In thermodynamic terms this can be dealt with by assigning an activity coefficient lower than 1·0 to insoluble complexes. Now, if some complexes gradually loose their water solubility, the composition

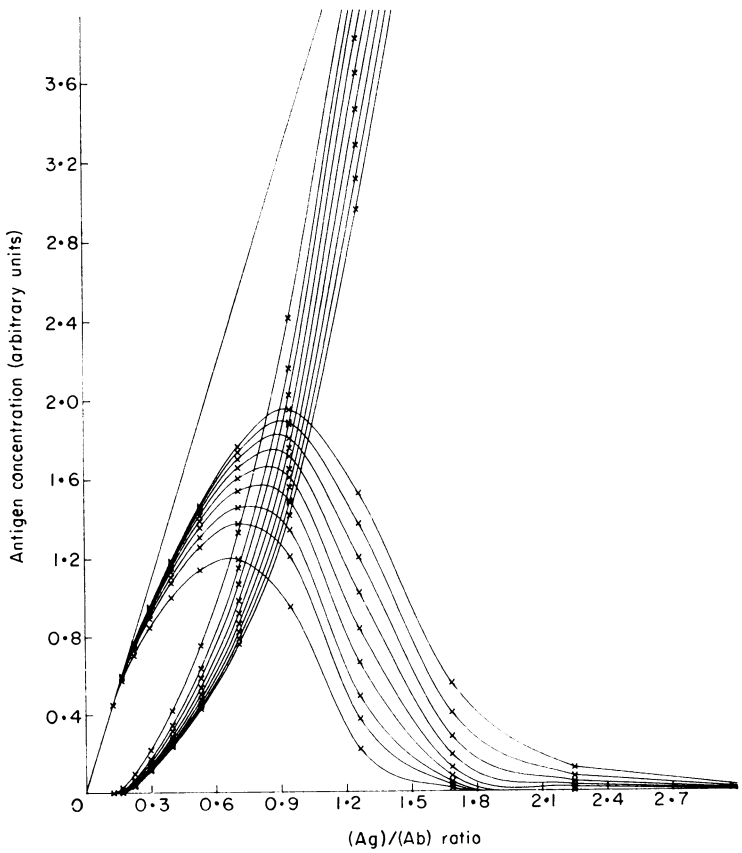

Figure 6. Development of calculated precipitin curves after introduction of re-equilibration cycles. The figure is principally composed as Fig. 4. The lowest precipitin curve is the calculated precipitin curve using horizontal distinction mode 1 to determine the amount of precipitate formed in the first cycle. The precipitin curves above the lowest show the increment in calculated amount of precipitate after re-equilibration cycles number 2, 3, 5, 8, 13, 21, 34 and 55. $\alpha = 0{\cdot}0$ and $f = 8$.

of soluble complexes must change aiming at re-establishing a new state of equilibrium. A simple example could be that in antibody excess the complex $AgAb_f$ is prevailing. This complex is likely to belong to the class of insoluble complexes. As it precipitates, the equilibrium is disturbed and more of the complex will be formed by mutual re-arrangements of other complexes in the soluble phase. Whether or not a new state of equilibrium will be reached, taking into consideration re-equilibrium of soluble complexes as well as an equilibrium between soluble and insoluble complexes will depend on the practical circumstances when experimental precipitin curves are made.

The implications of this hypothesis can be evaluated by computer simulation as follows. Initially concentrations of all possible complexes are calculated as in the previous section. Then a certain amount of the complexes which by the distinction mode in question are regarded insoluble, are taken out of the calculations. Following that new equilibrium complex concentrations are calculated, and again a given amount of complexes is mathematically removed. These calculations are repeated as many times as desired for the purpose in question. The resulting gradual shift in the composition of the supernatant is shown in Fig. 5, which gives six different complex distributions after re-equilibration cycle numbers 1, 2, 3, 8, 21, and 55. To demonstrate the effects of re-equilibration most clearly the activity coefficient of insoluble complexes was set to zero.

Complete precipitin curves were computed also, and an example is shown in Fig. 6. In these calculations horizontal distinction mode 1 was used and the activity coefficient was again assumed to be zero for all insoluble complexes. Fifty-five iterative cycles

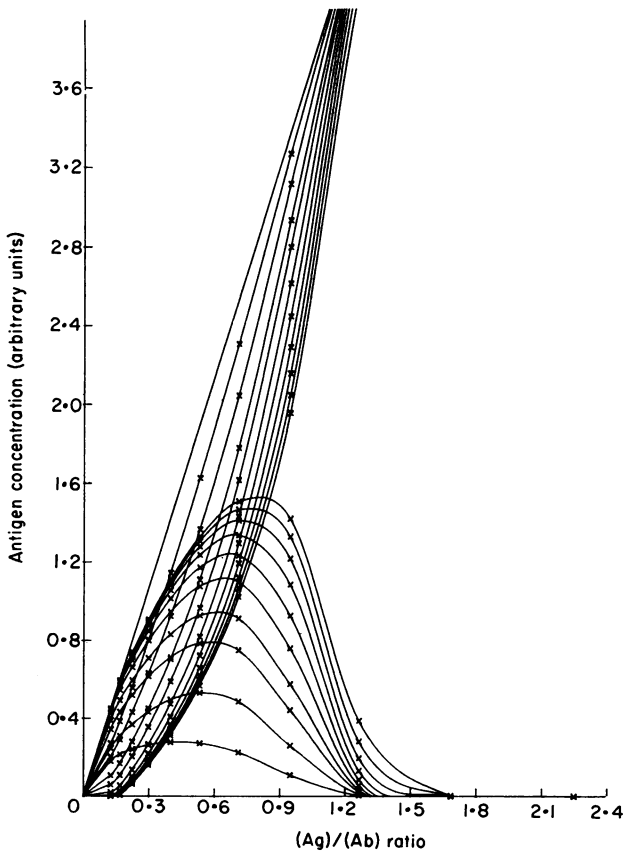

Figure 7. Development of calculated precipitin curves after introduction of re-equilibration cycles. The figure is calculated as in Fig. 6, but employs horizontal distinction mode 3 and $\alpha = 0{\cdot}6$, $f = 8$.

were run, and to illustrate the development against a new state of equilibrium intermediate results from re-equilibration iterations number 1, 2, 3, 5, 8, 13, 21, 34 and 55 (a Fibonacci row) are shown in the figure. The figure demonstrates that the introduction of re-equilibration steps does improve the theory with respect to formation of precipitate on the antibody excess side of the equivalence point. Moreover, less precipitate is formed in each new cycle, suggesting that a state of equilibrium eventually can be reached.

It can be seen in Fig. 6 that the point of maximum precipitation moves in the direction of the higher total (Ag)/(Ab) ratios with increasing numbers of re-equilibration steps. Maximum precipitation at lower total (Ag)/(Ab) ratios can be obtained by use of other distinction modes. Fig. 7 gives the calculated results using horizontal distinction mode 3 and an activity coefficient of 0·6.

It should be noted that re-equilibration of the soluble complexes as discussed here comprises processes that continuously move against a changing state of equilibrium, and the theoretical treatment is therefore taken as non-equilibrium thermodynamics. Experimental precipitin curves are usually made so that the antigen–antibody mixtures are incubated at 37° initially for a short period of time and followed by a very much longer incubation at 4°. The incubation at low temperature is likely to favour cohesion of those complexes which eventually ends up in the precipitate, whereas the low temperature is likely to decelerate rearrangements of soluble complexes. It is therefore not certain that a final state of equili-

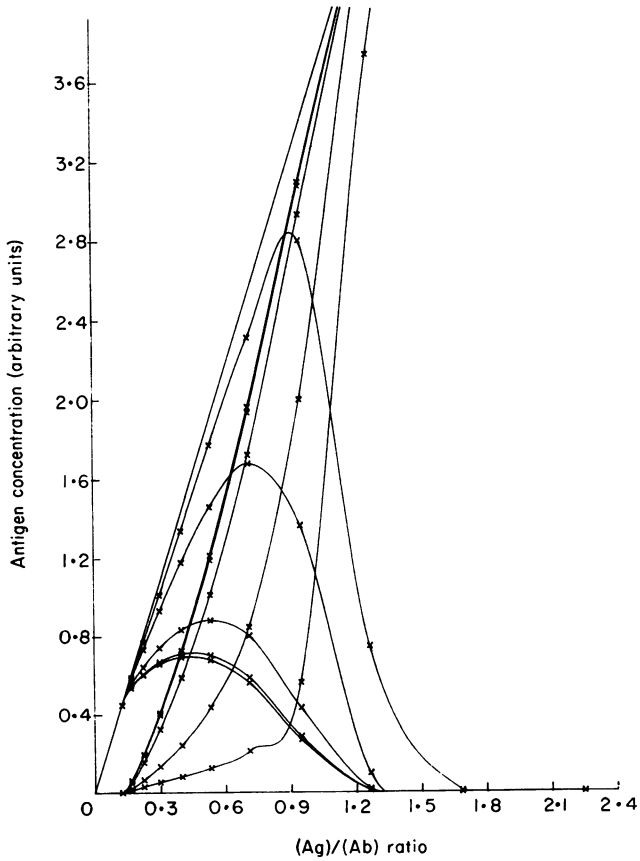

Figure 8. Calculated precipitin curves after introduction of a phase separation process. Horizontal distinction mode 3 is used as example and appears directly as the lowest precipitin curve. The curves above the lowest precipitin curve show the implications of the following κ'_p-values: 0κ, $10^{-3}\kappa$, $10^{-2}\kappa$, $10^{-1}\kappa$ and κ in increasing order.

brium is reached, and we have accordingly preferred to show the development against the final state of equilibrium here.

The implication of a phase separation on the distribution of soluble complexes

Formation of a precipitate means that some complexes form their own hydrophobic phase which is distinctly different from the aqueous supernatant phase. The remaining complexes therefore have a choice between a hydrophobic and a hydrophilic phase. Some experimental evidence for a rather high affinity of even small antigen–antibody complexes towards the hydrophobic phase might be found in the observation that little if any free or complexed antigen can be found in the supernatant in antibody excess and at equivalence (cf. Fig. 2), whereas considerable amounts of such complexes are predicted by the basic theory (cf. Fig. 3), and also by the theories of Goldberg (1952) and of Palmiter & Aladjem (1966).

To study this problem a series of calculations were performed employing κ_p'-values ranging from $10^{-3}\kappa$ to 1κ. The calculated results are shown in Fig. 8. Horizontal distinction mode 3 was used as example. It appears from this figure that the concept of a phase separation between some inherently insoluble complexes and the supernatant, with a concomitant distribution of otherwise soluble complexes between these two phases, offers a very appealing explanation of the absence of antigen in the antibody excess region, when κ_p'-values larger than $10^{-1}\kappa$ are used.

General discussion

In the foregoing discussion we have by means of computer simulation of precipitin reactions shown that the zoning phenomenon as such can be regarded as an inherent feature of the formation of compositionally different complexes at different points of a precipitin curve. However, it was found also that complex formation as such was insufficient to explain the absence of antigen and antigen containing complexes in the supernatant at the point of maximum precipitation.

The implication of a second stage in precipitin reactions has been evaluated for two physically likely concepts of the nature of the supposed second stage of these reactions. Re-equilibration of the soluble complexes after partial removal of selected complexes was found to offer a partial explanation. The assumption that some inherently insoluble complexes form their own hydrophobic phase and attract some otherwise soluble complexes is found to offer a very appealing explanation of the absence of soluble antigen in the equivalence zone. These two possible explanations of the nature of precipitin reactions are not mutually exclusive. As a final calculation, the programs were combined, so that computer simulation of precipitin reactions comprised both possible second stage reactions. Horizontal distinction mode 5, $\kappa_p' = 0{\cdot}1\kappa$ and $\alpha = 0{\cdot}6$ was chosen as test example, and the calculated results are given in Fig. 9. It can be seen in this figure that the zoning phenomenon is very clearly expressed and that the majority of antigen in the antibody excess zone is now predicted to be precipitated. Moreover, the point of maximum precipitation approaches the experimentally found value.

The choice of parameters has been a special problem in the calculated examples in this communication. The aim has been a general discussion of the theory of precipitin reactions more than a discussion of the human serum albumin–anti-human serum albumin system. We have, therefore, chosen to vary the parameters of the same basic systems as much as possible. Ten different distinction modes have been used, and those of horizontal distinction modes 3 and 5 yield very realistic results. The activity coefficient of insoluble complexes (α) determines speed in development of the reaction with time. High α-values therefore give a fast theoretical development of the theoretical precipitin reactions. If the calculations are repeated an indefinitely large number of times the final precipitin curves will be very much alike and independent of the size of α. The partition coefficient (κ_p') of the proposed phase separation process seemingly needs to approach the same order of magnitude as the association constant (κ) of complex formation to be efficient. However, because the tendency of choosing the hydrophobic phase of antibody molecules is elicited after specific binding of antigen, the hydrophobicity might very well be a specific property of reacted antibody molecules. In this sense κ_p' values approaching the size of κ values are not unrealistic.

We shall accordingly conclude that a theoretical model comprising a primary stage of complex formation and a secondary stage with phase separation processes and continuous re-equilibration of soluble complexes predicts the outcome of precipitating antigen–antibody interactions to a large extent.

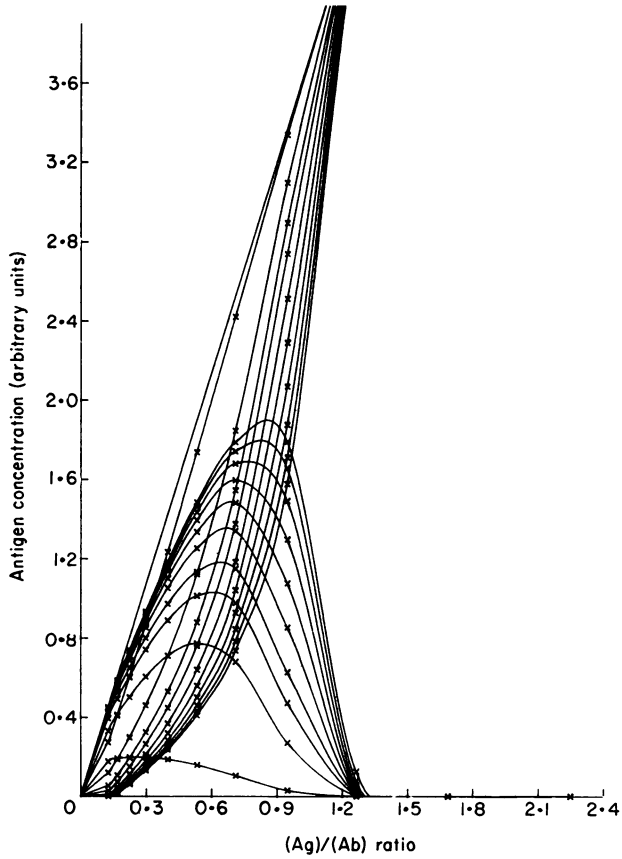

Figure 9. Development of calculated precipitin curves combining re-equilibration cycles and phase separation processes. The composition of the figure is as described in the text to Fig. 4. The theoretical parameters were: horizontal distinction mode 5, $\alpha=0{\cdot}6$, $\kappa_p'=0{\cdot}1\kappa$ and $f=8$.

Hence, the concepts presented here provide a useful qualitative and quantitative explanation of precipitin reactions.

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