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PROTEIN-PROTEIN INTERACTIONS STUDIED BY COUNTER-CURRENT DISTRIBUTION

I. THEORETICAL COMPUTATIONS

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SUMMARY

Many biological macromolecules are known to interact either with themselves, with other macromolecules or with small compounds. A simple equilibrium method for detecting and quantifying these interactions is to study the mutual influence of the molecules on their respective counter-current distribution in liquid-liquid biphasic systems.

The theoretical counter-current distribution patterns for the components in an interacting system, $A + B \rightleftharpoons AB$, have been calculated for two models in order to establish the boundary conditions and to optimize the experimental procedure. The patterns have been calculated for a range of association constants, partition coefficients and initial concentrations of the two reactants.

INTRODUCTION

It is well established that biological macromolecules in solution may interact with themselves (self-association), with other macromolecules or with small compounds. Many of these interactions are known to be essential for the operation and regulation of metabolic processes. Examples of interacting systems that have been studied extensively are protein-small ligand¹⁻⁶, DNA-small ligand⁷, protein-DNA⁸⁻¹⁰, RNA-RNA¹¹⁻¹³, protein-RNA^{11,14,15} and protein-protein¹⁶⁻²⁵. In some of these instances the biological consequences also have been established.

Of particular interest are protein-protein interactions, especially those between enzymes that catalyse consecutive metabolic steps. Such interactions would probably offer many advantages for the cell, *viz.*, channelling effect, shielding effect. These complexes should be of a dynamic nature, probably involving relatively weak interactions between the proteins. A simple equilibrium method for detecting and quantifying interactions between molecules is to study the mutual influence of the molecules on their respective partition in liquid-liquid biphasic systems^{26,27}. The counter-current distribution (CCD) technique²⁸ provides an accurate method for the determination

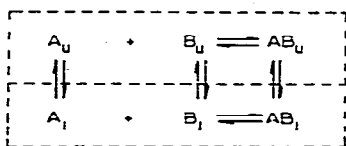
of partition coefficients and therefore should be very useful for detecting even small changes in them. Thus, CCD in aqueous–aqueous biphasic systems²⁹ has been shown to be an efficient method for detecting protein–protein interactions³⁰. This method should be very suitable for the study of interactions involving biological macromolecules, as both of the phases are rich in water (75–95%) and seem to have a stabilizing effect on structure and biological activities. However, it is necessary to make a thorough theoretical study of the CCD of interacting systems in order to establish the boundary conditions of the method as well as to be able to make a physico-chemical analysis of the experimental results.

For this purpose, it was necessary to investigate how the CCD patterns are dependent on the association constant, the partition coefficients and the initial concentrations of the components. The theoretical calculations have been designed for adaptation to future experiments involving macromolecules. We have therefore chosen to start initially only with the reactants and to calculate the distribution patterns in terms of total concentration per tube of each of the reactants, A and B.

The earlier approach used by Bethune and Kegeles^{31–33} for the study of the CCD of 1:1 interacting systems is not useful for our purpose, because they calculated the distribution patterns in terms of the total mass of each of the components in every tube following the dissociation of the complex initially present in unit concentration in the zeroth tube. Their procedure also requires the simplifying assumption that the volumes of the two phases are identical. In this paper, which is the first in a series on protein–protein interactions, only 1:1 interactions are discussed for two sets of experimental conditions.

CALCULATIONS

Consider a biphasic system in which the two phases are immiscible, no volume change occurs upon mixing and equilibration, all solutions are thermodynamically ideal and there is equilibrium within and between the phases before a transfer. If the reaction $A + B \rightleftharpoons AB$ may occur in such a system, then the following equilibrium condition must hold:



where the subscripts u and l refer to the upper and the lower phases, respectively.

The formation of AB from A and B may be described by the association constant, K , defined as

$$K = \frac{[AB]}{[A][B]} \quad (1)$$

where the square brackets denote concentrations in moles per litre. Then the asso-

ciation constants in the upper and the lower phase are given by eqns. 2 and 3, respectively:

$$K_u = \frac{[AB]_u}{[A]_u [B]_u} \quad (2)$$

$$K_l = \frac{[AB]_l}{[A]_l [B]_l} \quad (3)$$

The partition of a substance, Q, in a biphasic system may be described by its partition coefficient, K_Q , defined as

$$K_Q = \frac{[Q]_u}{[Q]_l} \quad (4)$$

Eqns. 5, 6 and 7 then give the partition coefficients K_A , K_B and K_{AB} , respectively:

$$K_A = \frac{[A]_u}{[A]_l} \quad (5)$$

$$K_B = \frac{[B]_u}{[B]_l} \quad (6)$$

$$K_{AB} = \frac{[AB]_u}{[AB]_l} \quad (7)$$

If the right-hand side of eqn. 2 is substituted by eqns. 3, 5, 6 and 7, the relationship between the association constant in the upper phase, K_u , and the association constant in the lower phase, K_l , is given by

$$K_u = K_l \cdot \frac{K_{AB}}{K_A K_B} \quad (8)$$

The subsequent equations are developed for repeated partitions, namely CCD. In this procedure, the lower phase is held stationary and the upper phase is transferred after equilibrium to the next tube in sequence.

The total concentrations of A and B in the i th tube after n transfers are given by eqns. 9 and 10, respectively:

$$[A]_{\text{tot}}^{in} = ([A]_u^{in} + [AB]_u^{in}) \frac{V_u}{V_u + V_l} + ([A]_l^{in} + [AB]_l^{in}) \frac{V_l}{V_u + V_l} \quad (9)$$

$$[B]_{\text{tot}}^{in} = ([B]_u^{in} + [AB]_u^{in}) \frac{V_u}{V_u + V_l} + ([B]_l^{in} + [AB]_l^{in}) \frac{V_l}{V_u + V_l} \quad (10)$$

where V_u and V_l are the volumes of the upper and the lower phase, respectively.

Model 1

In this model, A and B are initially introduced only to tube zero, and consequently eqns. 11 and 12 give the total concentrations of A and B before the first transfer:

$$[A]_{\text{tot}}^{00} = ([A]_u^{00} + [AB]_u^{00}) p + ([A]_l^{00} + [AB]_l^{00}) q \quad (11)$$

$$[B]_{\text{tot}}^{00} = ([B]_u^{00} + [AB]_u^{00}) p + ([B]_l^{00} + [AB]_l^{00}) q \quad (12)$$

where $p = \frac{V_u}{V_u + V_1}$ and $q = \frac{V_1}{V_u + V_1}$. When eqns. 3, 5, 6 and 7 are inserted, eqns. 11 and 12 can be rewritten as

$$[A]_{\text{tot}}^{00} = [A]_i^{00} (\alpha + \gamma K_1 [B]_i^{00}) \quad (13)$$

$$[B]_{\text{tot}}^{00} = [B]_i^{00} (\beta + \gamma K_1 [A]_i^{00}) \quad (14)$$

where $\alpha = pK_A + q$, $\beta = pK_B + q$ and $\gamma = pK_{AB} + q$.

The concentrations of A and B in the i th tube after n transfers can correspondingly be written as

$$[A]_{\text{tot}}^{in} = [A]_i^{in} (\alpha + \gamma K_1 [B]_i^{in}) \quad (15)$$

$$[B]_{\text{tot}}^{in} = [B]_i^{in} (\beta + \gamma K_1 [A]_i^{in}) \quad (16)$$

Given the initial values of A and B, *i.e.*, $[A]_{\text{tot}}^{00}$ and $[B]_{\text{tot}}^{00}$, and assigning values to K_1 and partition coefficients, $[A]_i^{00}$ and $[B]_i^{00}$ can be determined, using eqns. 13 and 14. Once $[A]_i^{00}$ and $[B]_i^{00}$ have been obtained, the equilibrium concentrations of AB, A and B in each phase can be calculated using eqns. 3, 5, 6 and 7. The upper phase is then moved to tube one and the lower phase remains in tube zero. This constitutes the first transfer, immediately after which

$$[A]_{\text{tot}}^{01} = q ([A]_i^{00} + [AB]_i^{00}) \quad (17)$$

$$[B]_{\text{tot}}^{01} = q ([B]_i^{00} + [AB]_i^{00}) \quad (18)$$

$$[A]_{\text{tot}}^{11} = p ([A]_u^{00} + [AB]_u^{00}) \quad (19)$$

$$[B]_{\text{tot}}^{11} = p ([B]_u^{00} + [AB]_u^{00}) \quad (20)$$

Eqns. 15 and 16 are then solved for tubes zero and one using the new values of A and B, yielding the new equilibrium concentrations of A, B and AB in these tubes. This constitutes the equilibrium before the second transfer, which when completed gives

$$[A]_{\text{tot}}^{02} = q ([A]_i^{01} + [AB]_i^{01})$$

$$[B]_{\text{tot}}^{02} = q ([B]_i^{01} + [AB]_i^{01})$$

$$[A]_{\text{tot}}^{12} = p ([A]_u^{01} + [AB]_u^{01}) + q ([A]_i^{11} + [AB]_i^{11})$$

$$[B]_{\text{tot}}^{12} = p ([B]_u^{01} + [AB]_u^{01}) + q ([B]_i^{11} + [AB]_i^{11})$$

$$[A]_{\text{tot}}^{22} = p ([A]_u^{11} + [AB]_u^{11})$$

$$[B]_{\text{tot}}^{22} = p ([B]_u^{11} + [AB]_u^{11})$$

By repetition of this process, the final CCD curves are obtained for n transfers, where n is the total number of transfers.

Model 2

The only difference between models 1 and 2 is in the mode of the initial introduction of A and B. In model 1, both A and B are introduced only to tube zero, but in model 2, only A is introduced initially to tube zero whereas B is introduced in the same concentration to all the tubes.

This does not affect the equilibrium concentrations before the first transfer in relation to model 1. However, immediately after the first transfer, eqns. 18 and 20 become

$$[A]_{\text{tot}}^{01} = q ([A]_i^{00} + [AB]_i^{00}) + p [B]_u^{00} \quad (21)$$

$$[B]_{\text{tot}}^{11} = p ([B]_u^{00} + [AB]_u^{00}) + q [B]_i^{00} \quad (22)$$

In the same manner as before, eqns. 15 and 16, containing the new values of A and B, are solved for the new equilibrium concentrations of A, B and AB in tubes zero and one. The second transfer is then carried out, and after it has been completed we have

$$[A]_{\text{tot}}^{02} = q ([A]_i^{01} + [AB]_i^{01})$$

$$[B]_{\text{tot}}^{02} = q ([B]_i^{01} + [AB]_i^{01}) + p [B]_u^{01}$$

$$[A]_{\text{tot}}^{12} = p ([A]_u^{01} + [AB]_u^{01}) + q ([A]_i^{11} + [AB]_i^{11})$$

$$[B]_{\text{tot}}^{12} = p ([B]_u^{01} + [AB]_u^{01}) + q ([B]_i^{11} + [AB]_i^{11})$$

$$[A]_{\text{tot}}^{22} = p ([A]_u^{11} + [AB]_u^{11})$$

$$[B]_{\text{tot}}^{22} = p ([B]_u^{11} + [AB]_u^{11}) + q [B]_i^{21}$$

As before, the CCD curves are obtained for n transfers by repetition of this process.

It is necessary to use a high-speed computer, as this process involves the solution of a large number of equation systems to investigate a range of arbitrarily chosen values of the variable parameters, *i.e.*, the association constants, the partition coefficients and the initial concentrations of A and B. The program, written in Fortran for the CDC CYBER-172, was kindly supplied by Dr. Gunnar Eriksson, Department of Inorganic Chemistry, University of Umeå³⁴.

The results of the calculations were checked in three ways:

(1) the total amount of A and B in all tubes after every transfer should equal the amount initially introduced;

(2) if $K_u = K_t = 0$, for any values of partition coefficients and initial concentrations of A and B, the resulting curves should be indistinguishable from the calculated binomial curve for a single substance with the same partition coefficient;

(3) if $K_A = K_B = K_{AB}$, for any values of association constants, the resulting curves should fit perfectly a calculated binomial curve with the same partition coefficient.

In all calculations the total amount of material in all tubes never differed from that initially introduced by more than 5 parts in 10^7 . The second and third check were both satisfied to 1 part in 10^6 .

RESULTS

The association constant and the volume of the upper phase were identical with those of the lower phase in all calculations. This reduces eqn. 8 to

$$K_A K_B = K_{AB} \quad (23)$$

The calculations were carried out for the number of transfers, n , set at 59. To investigate whether or not an obtained curve has changed shape or position, it should be compared with a calculated binomial curve. However, this comparison can be done equally well with a curve obtained for association constants of $1 M^{-1}$ and initial concentrations of $1 mM$, because on the scale used in the graphs this curve will coincide with a binomial curve. Further, a binomial curve will change only its magnitude as the initial concentration is varied.

The above relationship between the partition coefficient of the complex, K_{AB} , and those of the reactants, K_A and K_B , divides the results naturally into three sets for each model. In the first set, the partition coefficient of the complex lies between those of the reactants. According to eqn. 23, this condition is met only when the partition coefficient of one of the reactants is less than 1.0 and the other is greater than 1.0.

The second set includes cases in which the partition coefficient of the complex is either greater or less than those of both reactants. This condition is valid only when the partition coefficients of both reactants are greater or less than 1.0.

In the third set, both reactants have the same partition coefficients, which differ from that of the complex, *i.e.*, $K_A = K_B \neq 1$.

Model 1

Set 1. $K_A < K_{AB} < K_B$. As A and B are only labels, this also includes $K_B < K_{AB} < K_A$. The initial concentration of A was set to $1 mM$ and the values of K_A and K_B were assigned as 0.5 and 2.0, respectively, in all cases of this set.

In Fig. 1a, the calculated curves for $K_u = K_l = 1 M^{-1}$ and $[B]_{tot}^{00} = [A]_{tot}^{00}$ are shown. When K_u and K_l are increased to $10^4 M^{-1}$, the adjacent sides of the curves approach each other and the heights of the curves are lowered, but their positions are not changed much, as can be seen in Fig. 1b in comparison with Fig. 1a.

When the association constants are increased further, the overlapping of the curves becomes greater and the positions of the curves approach each other. Finally, at infinite association constants, the curves overlap each other completely and are indistinguishable from a binomial curve calculated for a single substance with $K_s = 1.0$. If the initial concentration of one of the reactants is increased, the change in shape and position of the other curve becomes more pronounced as the association constants are increased. This is shown in Fig. 1c and d, where $K_u = K_l = 100 M^{-1}$ and $10^4 M^{-1}$, respectively, and $[B]_{tot}^{00} = 100 mM$. With increasing association constants, A will move more rapidly and, obviously, a corresponding amount of B will move more slowly. Finally, at infinite association constants, the curve of A will fit perfectly a binomial curve calculated for the partition coefficient of the complex. However, the curve of B will not fit a binomial curve because it is made up of both free B and B bound as AB.

For the opposite case, when A is in excess, the resulting curves of A and B will be the mirror images of the curves obtained when B is in excess.

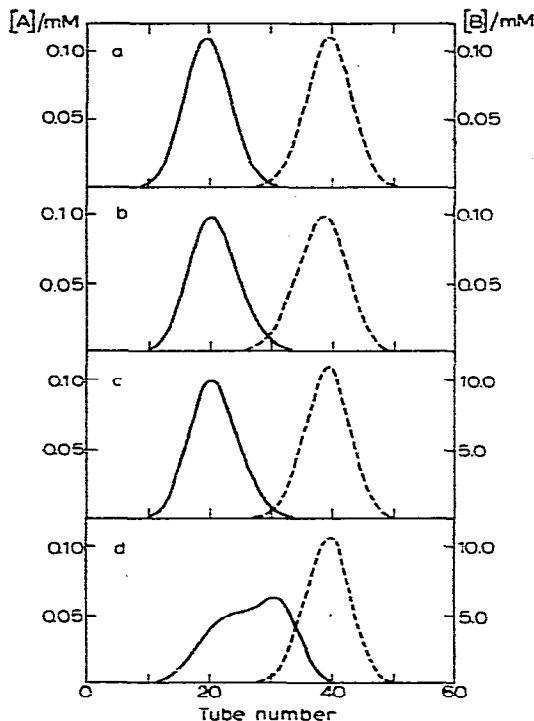


Fig. 1. Theoretical CCD curves according to model 1 of an interacting system, A (—) and B (---), with $K_A = 0.5$ and $K_B = 2.0$. (a) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_1 = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_1 = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$, $[B]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$ and $K_u = K_1 = 100 \text{ M}^{-1}$; (d) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$, $[B]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$ and $K_u = K_1 = 10^4 \text{ M}^{-1}$.

Set 2. $K_{AB} < K_A < K_B < 1$ or $1 < K_B < K_A < K_{AB}$. In this set, $K_A = 0.2$ and $K_B = 0.5$ were assigned for all cases. For partition coefficients greater than unity, *i.e.*, $K_A = 5.0$ and $K_B = 2.0$, the resulting curves will be the mirror images of the curves obtained for $K_A = 0.2$ and $K_B = 0.5$.

Fig. 2a shows the calculated curves obtained for $K_u = K_1 = 1 \text{ M}^{-1}$ and $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$. When the association constants are increased, the shape and position of the curves are affected, as can be seen in Fig. 2b. A is moving slower so that both the position and height of its curve are changed in comparison with Fig. 2a. The same also appears for B, but the change is much more apparent. When the association constants are increased, larger amounts of both A and B are moving with the speed of the complex, *i.e.*, more material is moving slower. For infinite association constants, all A and B will be in the complex form and the completely overlapping curves which result will be indistinguishable from a single binomial curve.

If the initial concentration of one of the reactants is increased to 100 mM , the curve of the other reactant will change most noticeably in position and shape, as can be seen in Fig. 2c and d. If B is in excess its curve will only broaden, whereas the position of the other curve will be retarded when the association constants are increased. The change is more noticeable when A is in excess. Then, both the shape and position of the curve of B are changed with increasing association constants. For infinite association constants, the curve of the reactant present at smaller concen-

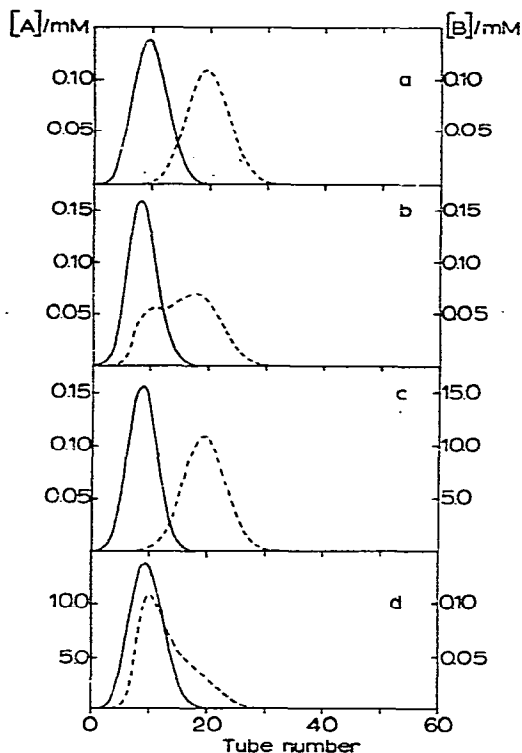


Fig. 2. Theoretical CCD curves according to model 1 of an interacting system, A (—) and B (---), with $K_A = 0.2$ and $K_B = 0.5$. (a) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_l = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_l = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$, $[B]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$ and $K_u = K_l = 10^4 \text{ M}^{-1}$; (d) $[A]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$, $[B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_l = 10^4 \text{ M}^{-1}$.

tration will fit a binomial curve calculated with the same partition coefficient as the complex, whereas the other curve will be slightly broadened.

Set 3. $K_{AB} < K_A = K_B < 1$ or $1 < K_A = K_B < K_{AB}$. For the first alternative of this set, the partition coefficients of A and B were both assigned the value 0.5. For the second alternative (with partition coefficients inverted), the curves will be mirror images of the curves calculated for $K_A = K_B = 0.5$. When the initial concentrations of A and B are equal, their curves will always coincide.

Fig. 3a shows the calculated curves, which are the same as the binomial ones, for association constants of 1 M^{-1} and the same initial concentration of A and B, *i.e.*, 1 mM .

With increasing association constants, both A and B move slower, causing a change in both position and shape of the curves (Fig. 3b). As before, an increased initial concentration of one of the reactants causes its curve to be relatively unaffected compared with the curve of the other reactant when the association constants are increased (Fig. 3c).

This set also corresponds to the dimerization process, $A + A \rightleftharpoons A_2$. The dimerization curves are simply obtained by summing the calculated curves of A and B.

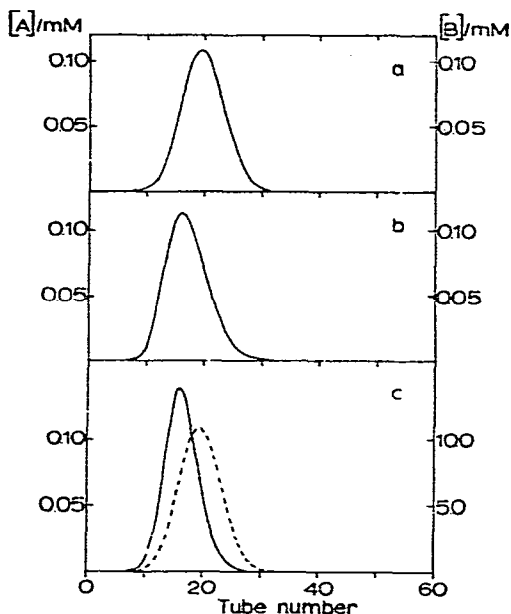


Fig. 3. Theoretical CCD curves according to model 1 of an interacting system, A (—) and B (---), with $K_A = K_B = 0.5$. (a) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_i = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{\text{oo}} = [B]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_i = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$, $[B]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$ and $K_u = K_i = 100 \text{ M}^{-1}$.

Model 2

The values of the partition coefficients used for the calculations of this model were the same as those for model 1. Further, the initial concentrations of B in each tube used for all calculations were 1 mM.

Set 1. $K_A < K_{AB} < K_B$. As before, when the association constants are set equal to 1 M^{-1} and $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$, neither the curve of A nor the curve of B is affected, as shown in Fig. 4a. With increasing association constants, the curve of A approaches a binomial curve calculated with the same partition coefficient as for the complex. The curve of B, on the other hand, shows a peak and a dip, as can be seen in Fig. 4b.

If the initial concentration of A is increased, the most significant change will be seen on the curve of B, whereas the curve of A is relatively unaffected in comparison with Fig. 4a. This is shown in Fig. 4c, where $K_u = K_i = 100 \text{ M}^{-1}$ and $[A]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$. With increasing association constants, more and more of B will be retarded, as can be seen in Fig. 4d, where $K_u = K_i = 10^4 \text{ M}^{-1}$. To the same extent, more and more of A will move faster, resulting in a broadening and distortion of the curve of A.

Set 2. $K_{AB} < K_A < K_B < 1$ or $1 < K_B < K_A < K_{AB}$. Fig. 5a shows the calculated curves obtained for $K_u = K_i = 1 \text{ M}^{-1}$ and $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$. Neither the curve of A nor the curve of B is affected.

Increasing amounts of complex will be formed with increasing association constants, and therefore A and B will both move slower, producing a change in both the shape and position of their curves (Fig. 5b). If the initial concentration of A is

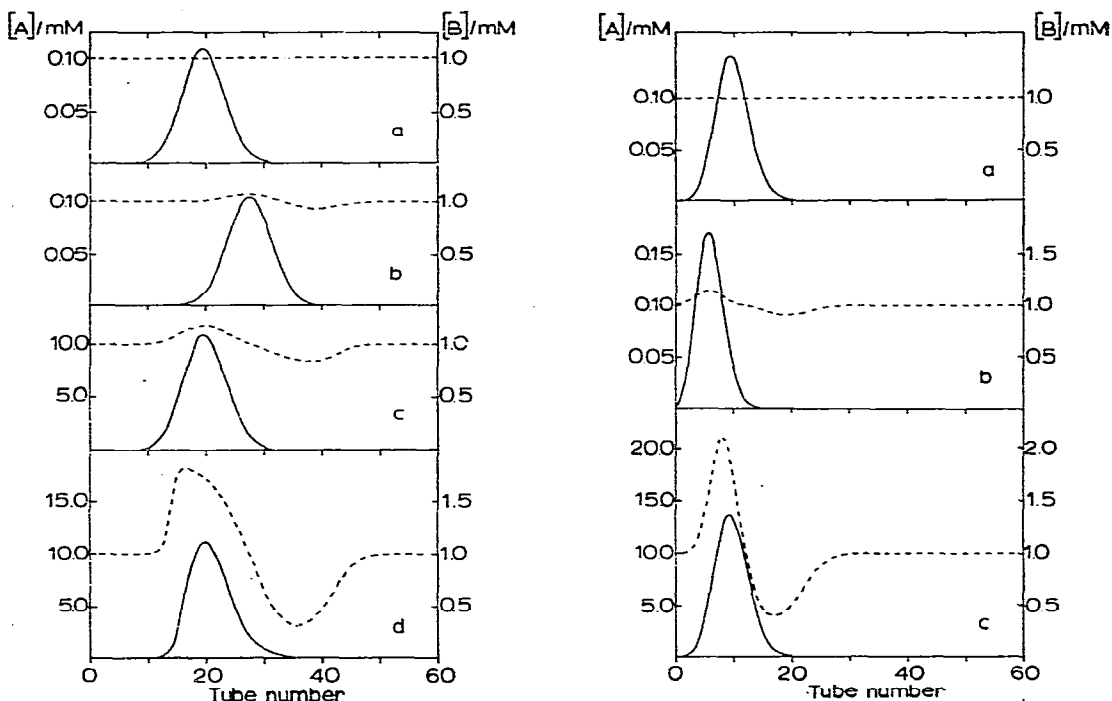


Fig. 4. Theoretical CCD curves according to model 2 of an interacting system, A (—) and B (---), with $K_A = 0.5$ and $K_B = 2.0$. In all instances the initial concentration of B in every tube was 1 mM. (a) $[A]_{\text{tot}}^{00} = 1 \text{ mM}$ and $K_u = K_i = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{00} = 1 \text{ mM}$ and $K_u = K_i = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{00} = 100 \text{ mM}$ and $K_u = K_i = 100 \text{ M}^{-1}$; (d) $[A]_{\text{tot}}^{00} = 100 \text{ mM}$ and $K_u = K_i = 10^4 \text{ M}^{-1}$.

Fig. 5. Theoretical CCD curves according to model 2 of an interacting system, A (—) and B (---), with $K_A = 0.2$ and $K_B = 0.5$. In all instances the initial concentration of B in every tube was 1 mM. (a) $[A]_{\text{tot}}^{00} = 1 \text{ mM}$ and $K_u = K_i = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{00} = 1 \text{ mM}$ and $K_u = K_i = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{00} = 100 \text{ mM}$ and $K_u = K_i = 100 \text{ M}^{-1}$.

increased, the most significant change will be seen on the curve of B, whereas the curve of A is relatively unchanged (Fig. 5c).

Set 3. $K_{AB} < K_A = K_B < 1$ or $1 < K_A = K_B < K_{AB}$. The unaffected curves of A and B, obtained for association constants of 1 M^{-1} and $[A]_{\text{tot}}^{00} = 1 \text{ mM}$, are shown in Fig. 6a. When the association constants are increased, more of both A and B will move slower, causing a change in both position and shape of their curves (Fig. 6b).

An increased initial concentration of A gives rise to a pronounced change in the shape of the curve of B, whereas the curve of A is relatively unaffected, as can be seen in Fig. 6c. The lack of variation in shape and position of the curve of the reactant in excess is dependent on the extent of excess. On the scale we have chosen for the graphs, a relatively unchanged curve will be obtained for the reactant which is in 50–100-fold excess. When the excess is lower, the resulting curves will begin to approach the cases where the initial concentrations are equal.

An important consequence, not shown in the figures, is that no matter how the association constants, partition coefficients and initial concentrations are chosen, the complex will never move in front of or lag behind the free reactants, A and B.

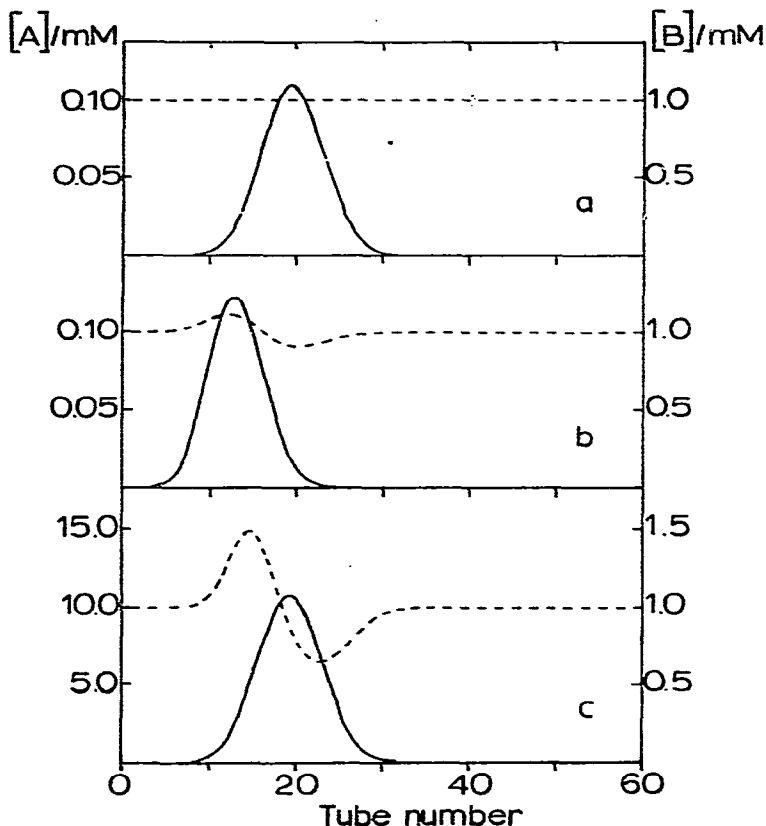


Fig. 6. Theoretical CCD curves according to model 2 of an interacting system, A (—) and B (---), with $K_A = K_B = 0.5$. In all instances the initial concentration of B in every tube was 1 mM. (a) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_l = 1 \text{ M}^{-1}$; (b) $[A]_{\text{tot}}^{\text{oo}} = 1 \text{ mM}$ and $K_u = K_l = 10^4 \text{ M}^{-1}$; (c) $[A]_{\text{tot}}^{\text{oo}} = 100 \text{ mM}$ and $K_u = K_l = 100 \text{ M}^{-1}$.

DISCUSSION

The calculations presented show that CCD of substances in a 1:1 interacting system can be exactly predicted if the assumptions regarding the ideality of the equilibrium and partition steps hold. Under such conditions, the influence of one substance on the CCD or partition of another substance is evidence for an interaction between these two substances.

The shape and position of the distribution curves, *i.e.*, the total concentration of each substance in every tube, are not only determined by the association constants but also by the initial concentrations of the interacting substances. This is an obligatory consequence of the law of mass action (eqn. 1). The higher the initial concentrations of the interacting substances are, the greater is the amount of complex formed and the greater are the changes in the shape and position of the curves. Hence, a slight difference between interacting and non-interacting distribution curves can be enhanced by increasing the initial concentration of one or both of the substances involved.

The effect of the interaction on the theoretical distribution curves also depends on the partition coefficients of both the interacting substances and the complex formed, as is evident from the figures. By adjusting the partition coefficients according to the three sets, it is possible to confirm the existence of an interaction. More information about the interacting system can be obtained by letting both of the substances alternately be in excess.

The CCD technique is also applicable to the quantitative determination of molecular interactions, *i.e.*, the determination of the association constant. When carrying out an experiment both to detect and to quantify interactions, it is necessary not only to distribute the two substances together at different concentrations, but also separately to obtain the partition coefficients of the substances. The partition coefficient of the complex formed is then given by eqn. 23, assuming that the association constants in the upper and lower phase, respectively, are identical, or more generally by eqn. 8.

By calculating distribution curves from these partition coefficients, the assigned values of the association constants and the known initial concentrations of the substances, it should be possible to find a best fitting theoretical pair of curves to match the experimental curves. From these calculated curves, it is then possible to obtain the association constants.

The choice of biphasic system is independent of these two models, but for molecules of biological origin we believe that aqueous–aqueous biphasic systems are the most useful. These systems, developed by Albertsson²⁹, are advantageous in many respects; the advantages include the solubility and stability of macromolecules in these systems, the water content and, particularly, the ease with which the partition coefficient can be adjusted by changing the composition of the biphasic system.

It has recently been shown that poly(ethylene glycol), one of the polymers used in these systems, favours the interaction between malate dehydrogenase and citrate synthase²⁴. Therefore, interactions might, at least between proteins, be more likely to occur in aqueous–aqueous biphasic systems than in buffer solutions. The solubility of proteins and other macromolecules can also easily be increased at least 5-fold by adding zwitterions, *i.e.*, betaine or glycine, to the biphasic system without altering the partition coefficients. This enhanced solubility sets a boundary for the minimal value of the association constants for interacting proteins and macromolecules to 50–100 M^{-1} , below which it is not possible to detect an interaction. Still weaker interactions can be detected between macromolecules and small ligands, because the initial concentration of a small ligand can be made much higher than that of a macromolecule. The choice of model is dependent on both the nature and the amount available of the substances under study.

When working with enzymes, generally the total enzymatic activities and not the total concentration are measured in every tube. Therefore, a change in distribution curves according to the first model need not necessarily mean that the enzymes are interacting. It might, however, be an activation of one of the enzymes without complex formation. With the second model it is possible to distinguish between an interaction and an activation, because a change in distribution curves due to an interaction will always have an appearance similar to those in Figs. 4–6. On the other hand, the second model needs larger amounts of material than does the first.

The CCD technique in aqueous–aqueous biphasic systems according to model

I has, in fact, already been used to show the existence of a specific interaction between malate dehydrogenase and aspartate aminotransferase³⁰ and an interaction between haemoglobin and carbonic anhydrase³⁵. These models are at present being applied to the study of interactions between enzymes of the glycolytic system and of the tricarboxylic acid cycle, and also dimerizations of proteins.

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