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Evaluation of Equilibrium Constants by Affinity Chromatography

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Theoretical expressions are derived for affinity chromatography of systems comprising an acceptor A with one binding site for attachment to a functional group X on the column matrix and one site for interaction with a small ligand B that specifically affects its elution. From a general relationship covering all possible interactions between A, B and X simpler expressions are derived for affinity systems in which only two equilibria operate. Methods are suggested whereby these simpler systems may be characterized in terms of the two pertinent equilibrium constants and the concentration of matrix-bound constituent. The means by which the theory may be adapted to affinity chromatography of acceptors with multiple binding sites for ligand is also illustrated. Results of partition experiments on the Sephadex G-100–lysozyme–D-glucose system in acetate–chloride buffer ($I = 0.17$M), pH 5.4, are used to demonstrate the feasibility of evaluating quantitatively affinity-chromatography interactions. Values of $30\,M^{-1}$ and $1.2\times10^{6}\,M^{-1}$ are obtained for the equilibrium constants for the reactions of lysozyme with glucose and Sephadex respectively, there being only an occasional binding site in the polysaccharide matrix (approximately 1 in 10^5 glucose residues). In a second experimental study the phytohaemagglutinin from *Ricinus communis* is subjected to frontal chromatography on Sepharose 4B in the presence of different concentrations of D-galactose, the results illustrating some of the difficulties and limitations that are likely to be encountered in quantitative studies of affinity-chromatographic systems.

The recently reported use of affinity chromatography on Sepharose–α-lactalbumin to determine the equilibrium constant for the interaction between lactose synthetase and glucose introduced an analytical potential of the technique that had not been considered previously (Andrews *et al.*, 1973). However, the theoretical treatment did not consider explicitly heterogeneous equilibria involving the matrix-bound constituent, which must be reflected in observed elution volumes. The present work treats this aspect and extends the earlier work by considering the behaviour of systems in which addition of ligand decreases the constituent elution volume of acceptor as well as of those exhibiting a ligand-retardation effect. Further, in the theoretical formulation inclusion of the interactions with the column matrix has permitted a more complete characterization of the affinity-chromatography system, since matrix interactions, in addition to those between the acceptor and ligand, may now be studied quantitatively. Features of this general theory are illustrated with the Sephadex–lysozyme–glucose and Sepharose–lectin–galactose systems: the results of Andrews *et al.* (1973) are also reconsidered.

Theory

Model and basic equations

The composition of a solution containing an equilibrium mixture of macromolecular acceptor A, ligand B and complex AB may be defined by the total molar concentrations, $[\bar{A}]$ and $[\bar{B}]$, and the equilibrium constant $K_{AB} = [AB]/[A][B]$. This mixture (assumed rapidly equilibrating) is now introduced on to a column pre-equilibrated with solvent. The stationary phase of the column is an insoluble matrix with covalently bound functional groups X capable of interacting with any of the solute species A, B and AB; special cases where some of these interactions are absent will be discussed after the presentation of the more general formulation.

The relevant heterogeneous equilibria are described as follows:

$$A+X \rightleftharpoons AX; \quad [AX] = K_{AX}[A][X] \quad (1a)$$
$$B+X \rightleftharpoons BX; \quad [BX] = K_{BX}[B][X] \quad (1b)$$
$$B+AX \rightleftharpoons BAX; \quad [BAX] = K_1 K_{AX}[A][B][X] \quad (1c)$$
$$A+BX \rightleftharpoons ABX; \quad [ABX] = K_2 K_{BX}[A][B][X] \quad (1d)$$
$$X+AB \rightleftharpoons XAB; \quad [XAB] = K_3 K_{AB}[A][B][X] \quad (1e)$$

where [ ] denotes molar equilibrium concentrations in the mobile phase. It is assumed that these concentrations are the same in the accessible region of the stationary phase, this defining the term accessible region. X is considered to be uniformly distributed through the volume accessible to A, i.e. $V_0+K^*_{av.}V_s$, where V_0 is the void volume, V_s the volume of the stationary phase and $K^*_{av.}$ the fraction of the stationary phase accessible to A. Two further points could be noted in relation to eqn. (1). First, eqns. (1c) and (1d) encompass cases where sites on A or B are dependent ($K_1 \neq K_{AB}, K_2 \neq K_{AB}$) or independent ($K_1 = K_{AB}$, $K_2 = K_{AB}$). Secondly, eqn. (1e) would be redundant if it merely represented an alternative pathway to eqns. (1c) and (1d) in forming the chemically distinct species BAX and ABX respectively: its inclusion therefore implies that XAB is also a distinct species formed by a link between X and a site on AB created as a result of complex-formation between A and B.

Since affinity chromatography is frequently performed by using a gel as the stationary phase, immobilization of A will arise from liquid–liquid partitioning in addition to binding by X (eqn. 1). It follows that the total concentration of A immobilized is given by:

$$[\bar{A}]_t = K_{AX}[A][X] + Y[A][B][X] + [A](1+K_{AB}[B])K^*_{av.} \quad (2a)$$
$$Y = K_1 K_{AX} + K_2 K_{BX} + K_3 K_{AB} \quad (2b)$$

The first two terms of eqn. (2a) follow directly from eqn. (1), and the last term expresses the contribution due to liquid–liquid partitioning. The use of a single value of $K^*_{av.}$ for A and AB, implicit in eqn. (2a), is reasonable when B is a small ligand. The constituent concentration of A in the mobile phase is that in the original solution (Nichol *et al.*, 1967) and is given by:

$$[\bar{A}] = [A](1+K_{AB}[B]) \quad (3)$$

Thus the ratio $\bar{K}_{av.} = [\bar{A}]_t/[\bar{A}]$ may be written as:

$$\bar{K}_{av.} = K_{av.} + \frac{[X](K_{AX}+Y[B])}{1+K_{AB}[B]} \quad (4)$$

$\bar{K}_{av.}$ and $K^*_{av.}$ may be converted into elution volumes by using the relationships (Laurent & Killander, 1964):

$$V^*_A = V_0 + K^*_{av.}V_s \quad (5a)$$
$$\bar{V}_A = V_0 + \bar{K}_{av.}V_s \quad (5b)$$

where $\bar{V}_A$ is the constituent elution volume of A and V^*_A the elution volume of A in the absence of any interaction with X; the latter is obtainable in cases where such interactions do occur only by procedures to be described. Combination of eqns. (4) and (5) yields:

$$(\bar{V}_A - V^*_A) = V_s[X](K_{AX}+Y[B])/(1+K_{AB}[B]) \quad (6)$$

In addition, [X] may be written in terms of the constituent concentration of X by using eqn. (1):

$$[X] = [\bar{X}]/(1+K_{AX}[A]+K_{BX}[B]+Y[A][B]) \quad (7)$$

Combination of eqns. (3), (6) and (7) gives on rearrangement:

$$(\bar{V}_A - V^*_A) = \frac{V_s[\bar{X}](K_{AX}+Y[B])}{(1+K_{AB}[B])(1+K_{BX}[B])+[\bar{A}](K_{AX}+Y[B])} \quad (8)$$

Special cases

The general eqn. (8) will now be discussed in terms of the simplest systems leading to ligand-dependent elution of A. These involve cases where two of the six equilibrium constants, namely K_{AB} and those defined in eqn. (1), are non-zero, the possible combinations being summarized in Table 1 together with the appropriate simplifications of eqn. (8) obtained by setting the remaining equilibrium constants equal to zero. Several points in relation to Table 1 merit comment.

First, it is evident from column 3 that all relationships involve [Ā], and it has been suggested in relation to eqn. (3) that this is the total concentration of A in the applied solution and therefore is of known magnitude. This identity is valid only if the column experiment is conducted to ensure a plateau of solution of initial composition in the elution profile (Winzor & Scheraga, 1963; Nichol & Winzor, 1964). Although concentration dependence of elution volume may be observed in zonal affinity-chromatography experiments (Andrews *et al.*, 1973), this procedure is not recommended for accurate quantitative analysis, because the continuous dilution of the zone in its passage down the column ensures a decreasing value of [Ā] and hence an ever-changing $\bar{V}_A$ (Winzor, 1966). In contrast, in a frontal experiment involving a plateau, not only is [Ā] defined, but also $\bar{V}_A$ may be determined from the median bisector of the A constituent gradient in the elution profile (Gilbert & Kellett, 1971).

Secondly, it is evident from the relationships for cases 1–3 that $1/(\bar{V}_A - V^*_A)$ decreases with increasing [B] (or [$\bar{B}$]) and thus that these cases are characterized by a ligand-induced retardation effect in that $\bar{V}_A$ increases with increasing [B]. On the other hand, for cases 4 and 5 the opposite effect pertains in that $\bar{V}_A$ decreases with increasing [B]. This observation, considered together with the effects specified in the

Table 1. *Systems for which the constituent elution volume of a macromolecular acceptor A in affinity chromatography is affected by introduction of ligand B*

Case	Operative equilibrium constants†	$\bar{V}_A-[B]$ relationship (eqn. 8)	Concentration-dependence‡ V_A	Concentration-dependence‡ V_B
1	K_{AB}, K_3	$\frac{1}{\bar{V}_A-V_A^*}=\frac{1}{V_s[\bar{X}]K_3K_{AB}[B]}+\frac{1+K_3[\bar{A}]}{V_s[\bar{X}]K_3}$	−	−
2	K_{BX}, K_2	$\frac{1}{\bar{V}_A-V_A^*}=\frac{1}{V_s[\bar{X}]K_2K_{BX}[B]}+\frac{1+K_2[\bar{A}]}{V_s[\bar{X}]K_2}$	−	+
3	K_{AX}, K_1	$\frac{1}{\bar{V}_A-V_A^*}=\frac{1}{V_s[\bar{X}]K_{AX}(1+K_1[B])}+\frac{[\bar{A}]}{V_s[\bar{X}]}$	+	−
4	K_{AB}, K_{AX}	$\frac{1}{\bar{V}_A-V_A^*}=\frac{K_{AB}[B]}{V_s[\bar{X}]K_{AX}}+\frac{1+K_{AX}[\bar{A}]}{V_s[\bar{X}]K_{AX}}$	+	−
5	K_{AX}, K_{BX}	$\frac{1}{\bar{V}_A-V_A^*}=\frac{K_{BX}[B]}{V_s[\bar{X}]K_{AX}}+\frac{1+K_{AX}[\bar{A}]}{V_s[\bar{X}]K_{AX}}$	+	+

† The remaining set of two non-zero equilibrium constants is (K_{AB}, K_{BX}), for which eqn. (8) reduces to $\bar{V}_A=V_A^*$. Thus $\bar{V}_A$ is not ligand-dependent in this case.

‡ Concentration-dependence of elution volume observed when reactant A or B is studied separately on the affinity column.

last two columns of Table 1, permits positive identification of the particular case pertinent to an experimental system.

Thirdly, it is noted that the relationships are written in terms of V_A^*, but only in cases 1 and 2 does $V_A^*=V_A$, the experimentally observed elution volume of A obtained in the absence of B. However, in cases 3–5, where $K_{AX}\neq 0$ and hence $V_A^*\neq V_A$, it is possible to rewrite the relationships in terms of $(\bar{V}_A-V_A)$. This is achieved by setting $[B]=0$ and $\bar{V}_A=V_A$ in eqn. (8) to obtain the expression:

$$(V_A-V_A^*)=V_s[\bar{X}]K_{AX}/(1+K_{AX}[\bar{A}]) \qquad (9)$$

which may be subtracted from the relevant reciprocated equation in column 3 of Table 1. The resulting expressions for cases 3, 4 and 5 are respectively:

$$\frac{1}{\bar{V}_A-V_A}=\frac{(1+K_{AX}[\bar{A}])^2}{V_sK_1K_{AX}[\bar{X}][B]}+\frac{(1+K_{AX}[\bar{A}])[\bar{A}]}{V_s[\bar{X}]} \qquad (10a)$$

$$\frac{1}{\bar{V}_A-V_A}=-\left\{\frac{(1+K_{AX}[\bar{A}])^2}{V_sK_{AX}K_{AB}[\bar{X}][B]}+\frac{(1+K_{AX}[\bar{A}])}{V_sK_{AX}[\bar{X}]}\right\} \qquad (10b)$$

$$\frac{1}{\bar{V}_A-V_A}=-\left\{\frac{(1+K_{AX}[\bar{A}])^2}{V_sK_{AX}K_{BX}[\bar{X}][B]}+\frac{(1+K_{AX}[\bar{A}])}{V_sK_{AX}[\bar{X}]}\right\} \qquad (10c)$$

where V_A is the elution volume of A in an experiment conducted with A alone at the same concentration $[\bar{A}]$ to which $\bar{V}_A$ refers. It may also be noted that eqn. (10) and those in Table 1 are all written in terms of [B], the equilibrium concentration of unbound B in the mobile phase, which in a frontal experiment is that in the original mixture. If the solution is made by mixing known concentrations of A and B (rather than by dialysis of A against B; see below), the known total concentration of B used to prepare the mixture may be related to [B] by:

$$[B]=[\bar{B}]/(1+K_{AB}[A]) \qquad (11)$$

Clearly, when $K_{AB}=0$ (cases 2, 3 and 5), $[B]=[\bar{B}]$ and hence relationships for these cases are now available in appropriate form. For cases 1 and 4 substitution of eqn. (11) into the first expression of Table 1 and eqn. (10b), respectively, yields:

$$\frac{1}{\bar{V}_A-V_A}=\frac{1+K_{AB}[A]}{V_s[\bar{X}]K_3K_{AB}[\bar{B}]}+\frac{1+K_3[\bar{A}]}{V_s[\bar{X}]K_3} \qquad (12a)$$

$$\frac{1}{\bar{V}_A-V_A}=-\left\{\frac{(1+K_{AX}[\bar{A}])^2(1+K_{AB}[A])}{V_sK_{AX}[\bar{X}]K_{AB}[\bar{B}]}+\frac{1+K_{AX}[\bar{A}]}{V_sK_{AX}[\bar{X}]}\right\} \qquad (12b)$$

It is now possible to discuss the experimental evaluation of $[\bar{X}]$ and the two equilibrium constants appropriate to each case. In all cases a plot of $1/(\bar{V}_A-V_A)$ versus $1/[\bar{B}]$ is suggested. For cases 2, 3 and 5 this double-reciprocal plot is linear, the expressions for the slopes and intercepts being summarized in Table 2. For cases 1 and 4, the double-reciprocal plot is curvilinear and the slopes and intercepts reported in Table 2 for these cases refer to those of the limiting tangent as $1/[\bar{B}]\rightarrow 0$ ($[A]\rightarrow 0$). It is evident from Table 2 that for each case a set of experiments conducted at a fixed value of $[\bar{A}]$ leads to two expressions in the relevant three unknowns and therefore that a third simultaneous equation is required to obtain the complete solution. This may be obtained in various ways. First, it is possible to perform a second set of experiments at a different value of $[\bar{A}]$ and with a range of values of $[\bar{B}]$ (including zero to establish the

Table 2. *Expressions for the slopes and intercepts of plots of* $1/(\bar{V}_A - V_A)$ *versus* $1/[\bar{B}]$ *obtained in affinity chromatography of interacting mixtures of acceptor A and ligand B*

Case	Relevant equation	Slope†	Intercept†
1	Eqn. (12a)	$1/V_s[\bar{X}]K_3K_{AB}$	$(1+K_3[\bar{A}])/V_s[\bar{X}]K_3$
2	Row 2 of Table 1 with $V_A^* = V_A$ and $[B] = [\bar{B}]$	$1/V_s[\bar{X}]K_2K_{BX}$	$(1+K_2[\bar{A}])/V_s[\bar{X}]K_2$
3	Eqn. (10a) with $[B] = [\bar{B}]$	$(1+K_{AX}[\bar{A}])^2/V_sK_1K_{AX}[\bar{X}]$	$(1+K_{AX}[\bar{A}])[\bar{A}]/V_s[\bar{X}]$
4	Eqn. (12b)	$-(1+K_{AX}[\bar{A}])^2/V_sK_{AB}K_{AX}[\bar{X}]$	$-(1+K_{AX}[\bar{A}])/V_sK_{AX}[\bar{X}]$
5	Eqn. (10c) with $[B] = [\bar{B}]$	$-(1+K_{AX}[\bar{A}])^2/V_sK_{AX}K_{BX}[\bar{X}]$	$-(1+K_{AX}[\bar{A}])/V_sK_{AX}[\bar{X}]$

† For cases 1 and 4 the expressions refer to those of the limiting tangents drawn as $1/[\bar{B}] \to 0$.

new V_A). This procedure is applicable to all cases and is the only method available for treating case 1 where K_{AX} and K_{BX} are both zero. Secondly, in cases 2–5 inclusive, where one or both reactants interact with X, use may be made of chromatography experiments conducted with one reactant alone studied at a series of concentrations. The method may be illustrated with the A reactant, whereupon eqn. (9) is appropriate. The simultaneous solution of eqn. (9) written for three values of V_A, termed V_j, and the corresponding $[\bar{A}]_j$ ($j = 1,2,3$) leads to:

$$K_{AX} = \frac{(V_3 - V_1)([\bar{A}]_2 - [\bar{A}]_1) - (V_1 - V_2)([\bar{A}]_1 - [\bar{A}]_3)}{[\bar{A}]_2(V_1 - V_2)([\bar{A}]_1 - [\bar{A}]_3) - [\bar{A}]_3(V_3 - V_1)([\bar{A}]_2 - [\bar{A}]_1)} \quad (13a)$$

$$[\bar{X}] = \frac{(V_1 - V_2)(1 + K_{AX}[\bar{A}]_1)(1 + K_{AX}[\bar{A}]_2)}{V_sK_{AX}^2([\bar{A}]_2 - [\bar{A}]_1)} \quad (13b)$$

which are the parameters required together with the expressions in Table 2 to complete the appropriate solutions; V_A^* is also available from eqns. (9) and (13). The third method of approaching the problem applies to cases 4 and 5. Comparison of the expressions for the intercepts (Table 2) with eqn. (9) shows that these intercepts are $-1/(V_A - V_A^*)$ in both cases. Since V_A has been determined from an experiment conducted with A alone at the same $[\bar{A}]$ used to determine the intercept, the value of the constant V_A^* is now available. Thus, in relation to experiments conducted with A alone, eqn. (9) may be plotted as $1/(V_A - V_A^*)$ versus $[\bar{A}]$ to yield a line of slope $1/V_s[\bar{X}]$ and intercept $1/V_s[\bar{X}]K_{AX}$.

Alternative experimental procedures

Although the preceding sections have referred explicitly to frontal column experiments, the theory is readily adapted to describe equilibrium partition experiments. Thus combination of eqns. (3), (4) and (7) yields:

$$(\bar{K}_{av.} - K_{av.}^*) = \frac{[\bar{X}](K_{AX} + Y[B])}{(1 + K_{AB}[B])(1 + K_{BX}[B]) + [\bar{A}](K_{AX} + Y[B])} \quad (14)$$

which is directly analogous to eqn. (8), noting that the parameter V_s is absent. It follows that the expressions in Table 2 continue to apply with omission of V_s. Accordingly, partition experiments may also be used to evaluate $[\bar{X}]$ and the pertinent equilibrium constants (Table 1) by the procedures already described.

Reference is now made to the technique of pre-equilibrating a column with a solution of ligand of known concentration before application of the equilibrium mixture. Provided that $\bar{V}_A < V_B$, passage of the acceptor through the column would be associated with adjustment of the equilibrium concentrations of AB and A such that the concentration of unbound B is the pre-equilibrating concentration. However, a zonal procedure would not suffice because $[\bar{A}]$ would no longer be defined, and it would thus be necessary to apply a volume of mixture that ensured the existence of a small plateau of acceptor terminating before V_B. There are evident dangers in this design of experiment when a ligand-retardation effect operates, but if the conditions can be achieved, it follows that the quantity written as [B] in Table 1 and eqn. (10) is now known (the pre-equilibrating concentration). These expressions show that plots of $1/(\bar{V}_A - V_A)$ versus $1/[B]$ are linear for all cases with slopes and intercepts given in Table 2. The advantage of this procedure is that for cases 1 and 4 the need to construct limiting tangents is obviated. Since this advantage is a direct consequence of knowing [B], it will apply also to experiments in which the reaction mixture has been dialysed exhaustively against a known concentration of ligand before its application to a column pre-equilibrated with either diffusate or solvent (frontal analysis).

Multiple binding sites

So far consideration has only been given to situations where A possesses a single binding site per

molecule for B. Although a similar set of cases as described in previous sections may be considered in relation to multiple binding, it sufficies to illustrate the approach with a simple system, likely to be encountered in practice, where the same p sites per molecule of A are capable of binding X or B, ensuring a competitive situation in affinity chromatography analogous to case 4. The constituent concentration of A applied to the column is given by:

$$[\bar{A}] = [A](1+k_{AB}[B])^p \quad (15)$$

where k_{AB} is the intrinsic association constant (Klotz, 1946), related to the successive equilibrium constants describing the formation of AB_j ($j = 1, 2, \ldots p$) by:

$$K_j = (p-j+1)k_{AB}/j \quad (16)$$

It will further be assumed that, because of steric requirements, A attaches to X at only one site with a site-binding constant k_{AX} related to the stoicheiometric binding constant K_{AX} by:

$$K_{AX} = pk_{AX} \quad (17)$$

Provided that the binding of A to X leaves the remaining $(p-1)$ sites unaltered with respect to their affinity for B, the total concentration of A immobilized by binding to X and liquid–liquid partition is:

$$[\bar{A}]_t = pk_{AX}[A][X](1+k_{AB}[B])^{p-1} + [A](1+k_{AB}[B])^p K^*_{av.} \quad (18)$$

Results obtained with the phosphocellulose–aldolase–fructose diphosphate system (Masters *et al.*, 1969) provide support for this assumption. By division of eqn. (18) by eqn. (15) it follows that:

$$(\bar{K}_{av.} - K^*_{av.}) = pk_{AX}[X]/(1+k_{AB}[B]) \quad (19)$$

Moreover:

$$[\bar{X}] = [X]\{1+pk_{AX}[A](1+k_{AB}[B])^{p-1}\} \quad (20)$$

Combination of eqns. (15), (17), (19) and (20) gives:

$$(\bar{K}_{av.} - K^*_{av.}) = K_{AX}[\bar{X}]/(1+k_{AB}[B]+K_{AX}[\bar{A}]) \quad (21)$$

Eqn. (21) is identical with eqn. (14) in appropriate form for case 4 ($K_{BX} = 0$, $Y = 0$), with k_{AB} recognized as the intrinsic binding constant. This means that if the system were studied by any of the suggested methods, K_{AX} and $[\bar{X}]$ would be obtainable from studies with A alone at different $[\bar{A}]$ and hence k_{AB} from studies with A in the presence of B. It is noted that affinity chromatography has not distinguished in this competitive situation between single and multiple binding of B to A. Therefore without independent evaluation of p it is not possible to evaluate the stoicheiometric constants K_j (eqn. 16) unless $p = 1$ when $k_{AB} = K_{AB}$.

Experimental

Materials

Thrice-crystallized lysozyme was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and Sephadex G-100, Sepharose 4B and Blue Dextran 2000 were supplied by Pharmacia, Uppsala, Sweden. Before use the Sephadex was washed thoroughly with water, dehydrated with ethanol and dried at 55°C.

Phytohaemagglutinin was isolated from the seeds of *Ricinus communis* by the method of Nicolson & Blaustein (1972), which involves salt extraction of the seed meal followed by $(NH_4)_2SO_4$ fractionation and affinity chromatography on agarose (Sepharose 4B) with 0.1 M-lactose as the specific eluent. The resulting agglutinin preparation differed from that of Nicolson & Blaustein (1972) in that no heterogeneity could be detected by velocity sedimentation or by chromatography on Sephadex G-100. The material corresponds to the RCA_I fraction of Nicolson *et al.* (1974).

Methods

Partition experiments. Equilibrium partition experiments of similar design to those described by Ackers (1964) and Ogston & Silpananta (1970) were performed at room temperature (13–16°C) in test tubes fitted with Quickfit stoppers. To each tube containing a known amount (400±10mg) of Sephadex G-100 were added weighed amounts of acetate–chloride buffer, I 0.17M, pH5.4 (0.15M-NaCl, 0.02M-sodium acetate, pH adjusted with acetic acid), and acetate–chloride buffer with the same ionic composition and pH but containing 1M-glucose: in each case the total volume of buffer added was 10ml. After addition of 2ml (V_a) of lysozyme solution of predetermined concentration c_a, the tubes were reweighed to give a more precise estimate of V_a and then left to equilibrate for 20h. A sample of the supernatant from each tube was then obtained by filtration. Its concentration (c_0) and also c_a were determined spectrophotometrically at 280nm by using an extinction coefficient ($E^{1\%}_{1cm}$) of 26.35 (Sophianopoulos *et al.*, 1962). Experiments were also performed in which 2ml of buffer or of Blue Dextran (0.5mg/ml) were substituted for the addition of lysozyme, a wavelength of 625nm being used for measuring concentrations of the coloured polysaccharide.

Results were analysed in terms of the partition coefficient $\bar{K}_{av.}$ (Laurent & Killander, 1964), the ratio of the concentrations of lysozyme (free and complexed) in the stationary and mobile phases. In the present design of experiment $\bar{K}_{av.}$ is obtained from the expression:

$$\bar{K}_{av.} = (w_s/c_0 V_0)(V_0/V_s) = w_s/c_0 V_s \quad (22)$$

where $c_0 V_0$ denotes the product of the concentration (mg/ml) and volume of lysozyme in the outer phase, V_s is the volume of stationary phase and w_s is the weight of lysozyme in any form in that phase; this last quantity is simply the difference between the amount of lysozyme added and that present in the outer phase at equilibrium ($w_s = c_a V_a - c_0 V_0$). The method of obtaining V_0 and V_s, values of which are required for application of eqn. (22), was as follows. Combination of the weights of buffer, glucose buffer and lysozyme solution with the appropriate densities (1.007, 1.076 and 1.007 respectively) yielded the volume of liquid added to each tube, the total volume of slurry (V_b) being obtained by addition of a term for the volume of the gel matrix: a value of 0.60 for the partial specific volume of Sephadex (Edmond *et al.*, 1968) was used to calculate this volume from the weight of Sephadex in each tube. Stationary-phase volumes (V_s) were obtained by multiplying the weight of Sephadex in each tube by 11.1, the volume of stationary phase per g of Sephadex G-100, this multiplication factor (±0.2) having been obtained from twelve experiments with Blue Dextran. V_0 was then taken as the difference between V_b and V_s.

Values of $\bar{K}_{av.}$ so obtained were related to glucose concentration [$\bar{B}$] on the basis that the glucose was uniformly distributed in the liquid added to each tube, and to lysozyme concentration [$\bar{A}$] by the relationship $[\bar{A}] = c_0/M_A$, with M_A, the molecular weight of lysozyme, taken as 14400 (Sophianopoulos *et al.*, 1962).

Frontal chromatography. A column (1 cm × 10 cm) of Sepharose 4B was thermostatically maintained at 20°C and pre-equilibrated with phosphate–chloride buffer, pH 7.2 (0.2 M-NaCl, 0.005 M-NaH_2PO_4, pH adjusted with NaOH) at a flow rate of 1.5–2.0 ml/min. A solution of agglutinin (0.4 mg/ml) in the phosphate–chloride buffer and containing an appropriate concentration [$\bar{B}$] of D-galactose (0.05–10 mM) was then applied to the column, a sufficient volume being added to ensure the existence of a plateau of original composition in the elution profile. The column eluate was collected in 1.0–1.5 ml fractions, the precise volume of each fraction being determined by weight, and analysed spectrophotometrically at 280 nm. Between experiments the column was regenerated by washing with 0.1 M-D-lactose, after which the column was re-equilibrated with the phosphate–chloride buffer.

Results and Discussion

We now wish to consider results obtained with three experimental systems, each having been studied by a different technique, in order to illustrate the potential and also limitations of affinity chromatography for the quantitative study of interactions.

Sepharose–α-lactalbumin–lactose synthetase–monosaccharides

Results of zonal studies of the lactose synthetase system have already been presented in the recommended double-reciprocal format (Fig. 2 of Andrews *et al.*, 1973) and interpreted in terms of an interaction between a lactose synthetase–monosaccharide complex and the matrix-bound α-lactalbumin (case 1). Andrews *et al.* (1973) took the ratio intercept/slope as measuring K_{AB}, whereas in fact it measures $K_{AB}(1+K_3[\bar{A}])$ (Table 2). However, for the lactose synthetase–monosaccharide case the resulting error is very small. From the values quoted for the concentration of Sepharose-bound α-lactalbumin and the applied concentration of lactose synthetase, the error entailed by neglecting the matrix interaction term ($K_3[\bar{A}]$) would have been less than 1%. Further quantitative consideration of the data in terms of K_3 and [$\bar{X}$] is not warranted, since no unique value of [$\bar{A}$], the lactose synthetase concentration, is available from zonal chromatography.

Sephadex–lysozyme–glucose

The anomalous gel-chromatographic behaviour of lysozyme on Sephadex is well established, Whitaker (1963), for example, having commented that the enzyme exhibits a larger elution volume than globular proteins of similar size. Preliminary zonal experiments on Sephadex G-100 showed that inclusion of D-glucose (or *N*-acetyl-D-glucosamine) in the solvent decreased the elution volume of lysozyme, and hence it is reasonable to assign the anomalous gel-chromatographic behaviour of the enzyme to reversible adsorption to the polysaccharide matrix of the Sephadex, with glucose a competitor for the binding site on the lysozyme. The normal gel-chromatographic behaviour of glucose precludes its interaction with the matrix. The Sephadex G-100–lysozyme–glucose system thus affords a convenient model for case 4, provided that conditions are selected to eliminate complications arising from reversible dimerization of the enzyme (Sophianopoulos & Van Holde, 1964): lysozyme has been shown to be monomeric in acetate–chloride buffer, *I* 0.17 M, pH 5.4 (Sophianopoulos *et al.*, 1962), the conditions used in the present study.

Results of equilibrium partition experiments on the Sephadex G-100–lysozyme–D-glucose system at pH 5.4 are summarized in Fig. 1. Clearly they conform with the qualitative prediction for case 4 that the partition coefficient $\bar{K}_{av.}$ decreases with increasing glucose concentration, the results shown in Fig. 1(*a*) being from experiments with $[\bar{A}] = 8.3\,\mu M$. Further, the double-reciprocal plot is essentially linear, and thus construction of the limiting tangent as $1/[\bar{B}] \rightarrow 0$ presents no difficulties. Least-squares calculations yield values of 1.33 for $(1+K_{AX}[\bar{A}])^2/K_{AB}K_{AX}[\bar{X}]$ and 4.0 for $(1+K_{AX}[\bar{A}])/K_{AX}[\bar{X}]$, these

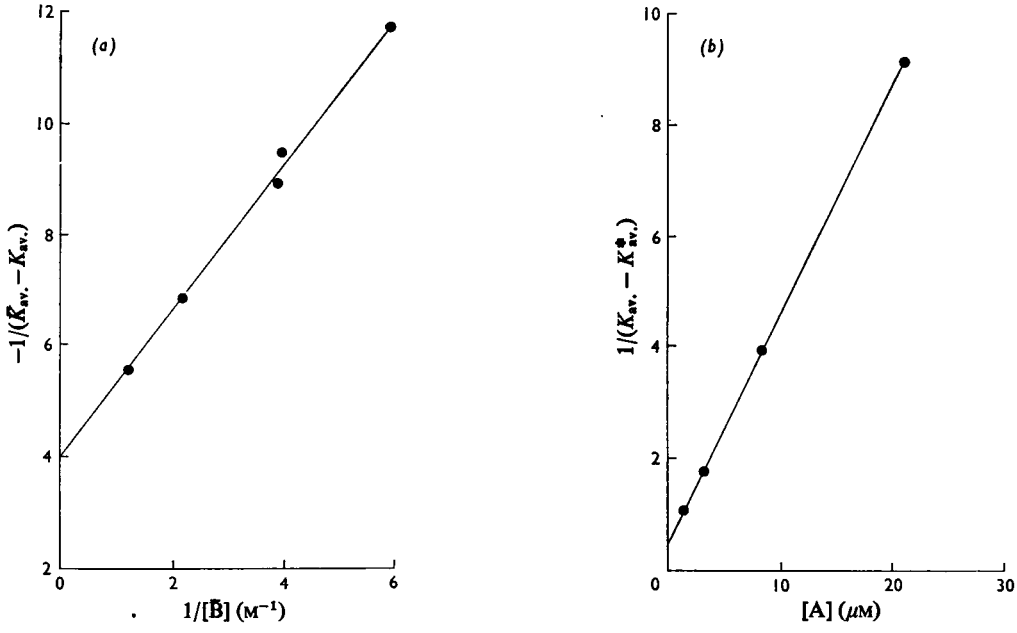

Fig. 1. *Quantitative evaluation of the interactions in the Sephadex–lysozyme–glucose system by partition experiments*

Sephadex G-100 (0.4g) was allowed to equilibrate with 12ml of acetate–chloride buffer, *I* 0.17M, pH 5.4, containing lysozyme (0.35–3.35mg) and D-glucose ranging in concentration from 0 to 0.835M. (*a*) Effect of glucose concentration on the partition coefficient ($\bar{K}_{av.}$) of lysozyme in experiments with 1.4mg of enzyme; (*b*) concentration-dependence of $K_{av.}$ for lysozyme in the absence of glucose. For further details see the text.

being obtained from the slope and intercept respectively (Table 2). As noted above, since $\bar{K}_{av.} \rightarrow K^{*}_{av.}$ as $1/[\bar{B}] \rightarrow 0$, the intercept also represents $1/(K_{av.}-K^{*}_{av.})$. Combining this value of 0.25 for $(K_{av.}-K^{*}_{av.})$ with the 0.885 obtained for $K_{av.}$ at this particular [Ā], we obtain 0.635 for $K^{*}_{av.}$, the partition coefficient reflecting solely the gel-chromatographic distribution of lysozyme. This value is within the range 0.58–0.67 obtained for ribonuclease (Andrews, 1964; Whitaker, 1963), a protein of comparable molecular weight and exhibiting normal gel-filtration behaviour. Evaluation of $K^{*}_{av.}$ permits the use of eqn. (9) in relation to results obtained with different concentrations of A alone (Fig. 1*b*). From the slope (4.2×10^{5}) and intercept (0.35) of the linear plot, values of 2.4μM and $1.2\times10^{6}M^{-1}$ are obtained for [$\bar{X}$] and K_{AX} respectively. The remaining parameter (K_{AB}) may now be calculated from the slope of the double-reciprocal plot (Fig. 1*a*). The value of $30M^{-1}$ so obtained for K_{AB} is of comparable magnitude with the $50M^{-1}$ for the binding of *N*-acetyl-D-glucosamine to lysozyme at pH 5.4 (Kowalski & Schimmel, 1969).

From the values of [$\bar{X}$] and K_{AX} it is evident that the abnormal gel-chromatographic behaviour of lysozyme is the consequence of fairly strong binding ($K_{AX}=1.2\times10^{6}M^{-1}$) to a relatively small number of binding sites on the Sephadex matrix. Indeed, since the concentration of anhydroglucose residues in the present system (0.4g of Sephadex in about 12ml) is approximately 0.2M, only 1 in 10^{5} matrix residues is acting as a binding site for lysozyme. In view of the strong binding affinity of these few binding sites and the known preference of the enzyme for *N*-acetylated polysaccharides, a possible explanation of the adsorption is that an occasional glucose residue of the Sephadex chain possesses an *N*-acetyl group, the bacterial origin of the polysaccharide matrix providing a feasible source for the nitrogen. Whatever the origin of the lysozyme adsorption may be, it should be recalled that the Sephadex–lysozyme–glucose system was introduced to provide a model for testing the possibility of defining quantitatively an affinity-chromatographic system, and to this end the experiment has proved successful.

Sepharose–lectin–galactose

Ricinus communis agglutinin binds tightly to Sepharose but may be eluted by the inclusion of

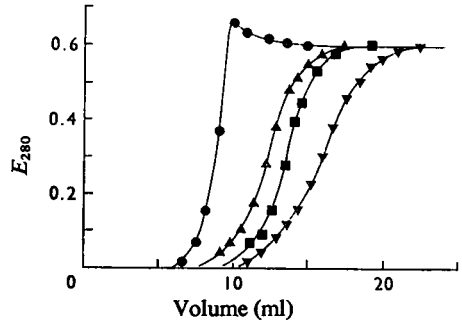

Fig. 2. *Elution profiles (advancing side) in frontal chromatography of Ricinus communis agglutinin on Sepharose in the presence of* D-*galactose*

A column of Sepharose 4B (1cm×10cm) was pre-equilibrated with phosphate–chloride buffer, pH7.2, and then eluted with mixtures of the agglutinin (0.4mg/ml) and D-galactose in the phosphate–chloride medium: ●, 10mM, ▲, 1mM, ■, 0.6mM, ▼, 0.4mM-galactose. In each case the centroid of the boundary (Longsworth, 1943) was taken as $\bar{V}_A$ to allow for differences in the shapes of boundaries. The pronounced overshoot of the plateau in the experiment with highest galactose concentration probably indicates a kinetically controlled rate of migration owing to slow desorption of the lectin (Meggitt *et al.*, 1973).

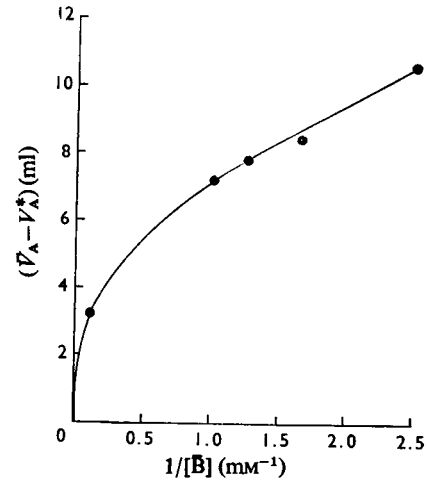

Fig. 3. *Attempts to evaluate quantitatively the interactions involved in affinity chromatography of the agglutinin from Ricinus communis on Sepharose*

Plot of the results from Fig. 2 in the form suggested by eqn. (23). For further details see the text.

either D-lactose or D-galactose in the eluting medium. The present Sepharose–lectin–galactose system has been studied at pH 7.4 by frontal chromatography, and from Fig. 2 the behaviour is qualitatively similar to that observed with Sephadex–lysozyme–glucose in that the extent of adsorption decreased progressively with increasing monosaccharide concentration. However, so strong is the binding of the protein in the absence of galactose (or lactose) that treatment of the results by the procedure recommended for case 4 is not possible because determination of V_A is impracticable. We therefore return to Table 1 for expressions in terms of V_A^*, an approximate estimate of which is available from the size of A (Goldberg & Bloch, 1972). The present lectin has a molecular weight of 140000, for which K_{av}^* should be in the vicinity of 0.55 ($V_A^* = 5.4$ml for the 7.9ml column of Sepharose 4B used). In terms of $[\bar{B}]$, the total galactose concentration, the relevant expression relating the various parameters may be expressed as:

$$\bar{V}_A - V_A^* = \frac{V_s[\bar{X}]K_{AX}(1 + K_{AB}[A])}{(1+K_{AB}[A])(1+K_{AX}[\bar{A}])+K_{AB}[\bar{B}]} \quad (23)$$

As $[\bar{B}] \to \infty$, $(\bar{V}_A - V_A^*) \to V_s[\bar{X}]K_{AX}/K_{AB}[\bar{B}]$, and thus this formulation requires construction of a limiting tangent as $1/[\bar{B}] \to 0$ of a plot of $(\bar{V}_A - V_A^*)$ versus $1/[\bar{B}]$. Such treatment of the present results leads to a decidedly curvilinear plot (Fig. 3), from which the slope of the limiting tangent cannot be obtained with any precision. Indeed, similar treatment of the Sephadex–lysozyme–glucose results also produced a curvilinear plot from which the correct limiting tangent could not be deduced. The reason for this difficulty is that although the $(1+K_{AB}[A])$ terms in eqn. (23) may decrease rapidly to unity as $[\bar{B}]$ increases, much higher ligand concentrations are required, so that the $(1+K_{AX}[\bar{A}])$ term makes a negligible contribution to the magnitude of the denominator, a requisite for the envisaged limiting tangent. We therefore conclude that the results obtained with the lectin system cannot be treated quantitatively, and present them to illustrate difficulties and limitations that may be encountered in application of this quantitative treatment of affinity chromatography.

General Discussion

From a theoretical treatment of interactions involved in affinity chromatography has arisen a series of suggested procedures that in principle allows quantitative evaluation of a simple affinity system involving two equilibria. This potential has been realized with one of the three experimental systems considered, namely Sephadex–lysozyme–glucose, and should be realizable with a second, the Sepharose–α-lactalbumin–lactose synthetase–glucose system, by resort to frontal instead of zonal studies.

However, no such success was achieved with the Sepharose–lectin–galactose system, owing partly to inability to construct experimentally the limiting tangent for which a theoretical expression was available. The experimental limitation stems from the very large magnitudes of K_{AX} and K_{AB}, the two equilibrium constants involved. In this connexion it is noted that obviating the need for limiting tangents by predialysing the lectin against galactose would not necessarily have guaranteed successful quantitative evaluation, which requires additionally the ability to distinguish experimentally between $(1+K_{AX}[\bar{A}])/K_{AX}$ and $[\bar{A}]$. This limitation is not only common to cases 4 and 5, but comparable impasses apply to cases 1 and 2 when $K_3[\bar{A}] \gg 1$ and $K_2[\bar{A}] \gg 1$ respectively. A further problem is encountered in all cases when $[\bar{X}]$ is very large, in that the ordinate intercept may become indistinguishable experimentally from zero. These observations imply that the better an affinity-chromatography system is from the preparative viewpoint, the worse-suited it becomes for quantitative investigation. On the other hand, the method is well-suited to the study of weak interactions, where methods such as equilibrium dialysis have severe limitations.

Finally it is noted that the theoretical treatment has relevance to a field far wider than the problem of characterizing affinity-chromatography systems devised as research aids. Indeed, we hope that it may find application in the study of biological systems comprising two interactions among three reactants, one of which is immobilized on the particulate fractions of cells.

Note Added in Proof (Received 6 September 1974)

After this manuscript was submitted a paper by Dunn & Chaiken (1974) was published on the quantitative interpretation of zonal affinity-chromatography data on systems conforming with the present case 4. However, the method is based on the erroneous assumption that their eqn. (17), which in our terminology becomes:

$$\frac{1}{\bar{V}_A - V_A^*} = \frac{1}{K_{AX}(V_A^* - V_0)[X]} + \frac{K_{AB}[B]}{K_{AX}(V_A^* - V_0)[X]}$$

is linear in [B]. Expression of the variable parameter [X] in terms of $[\bar{X}]$ and the consequent introduction of $[\bar{A}]$ shows that the quantity that Dunn & Chaiken (1974) determined from the slope/intercept was in fact $K_{AB}/(1+K_{AX}[\bar{A}])$, not K_{AB} as claimed. Andrews *et al.* (1973) fell into a similar error, to which we have already drawn attention.

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