Journal of Chromatography, 597 (1992) 67–82
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 846

Review

Recent developments in quantitative affinity chromatography

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ABSTRACT

This review surveys developments during the past decade in the use of quantitative affinity chromatography as a means of evaluating equilibrium constants for solute–ligand and solute–matrix interactions. Topics include allowance for multivalency of the partitioning solute, removal of the myth that highly substituted affinity matrices are unsuitable for zonal quantitative affinity chromatography, adaptation of the technique to allow characterization of high-affinity interactions and the application of quantitative affinity chromatography theory to the characterization of biospecific adsorption phenomena in cellular systems.

CONTENTS

0021-9673/92/$05.00

1. INTRODUCTION

Quantitative affinity chromatography [1–4] is arguably becoming the most versatile of the methods available for the evaluation of ligand-binding constants [5], this being an assertion that stems from the fact that the experimental parameter measured is the distribution of partitioning solute (acceptor) between liquid and matrix-bound states. Consequently, the only requirement for its application is an ability to measure the position of this equilibrium, irrespective of the ligand concentration required to effect the particular equilibrium distribution of solute [6–8]. Envisaged initially as a technique for characterizing interactions that are too weak for study by conventional means [9], quantitative affinity chromatography has, in the past 5 years, been adapted for the characterization of interactions at the other end of the energy spectrum, namely high-affinity interactions that are too strong for study by conventional means [6–8].

Because the theoretical and experimental aspects of quantitative affinity chromatography have been subjects of detailed consideration in relatively recent reviews [10–12], comprehensive coverage of the technique is discarded here in favour of a presentation that highlights some developments of particular significance and/or interest in the past decade. Topics to be covered include allowance for the effects of multivalency of the partitioning solute [9,13,14], the demise [6,15] of the myth that highly substituted affinity matrices are unsuitable for characterization of ligand binding by zonal affinity chromatography, the study of interactions for which the concentration of free ligand must be established by prior equilibrium dialysis or a gel chromatographic counterpart [6,7,15] and the application of quantitative affinity chromatography theory to the characterization of biological phenomena such as the metabolite-dependent adsorption of glycolytic enzymes to muscle myofibrils [16–18]. This review concludes by drawing attention to an exciting development in quantitative affinity chromatography, namely the recent realization [8,19] that the problem of describing affinity chromatographic behaviour in terms of total ligand concentration is also amenable to analytical solution.

2. EXPRESSIONS FOR ANALYSIS OF AFFINITY CHROMATOGRAPHIC DATA

In previous reviews [5,10–12,20], quantitative expressions have been derived specifically for each type of affinity chromatography scheme: ligand-facilitated elution reflecting competition between ligand and matrix sites for partitioning solute [2,4,9], or between solute and ligand for matrix sites [3,13]; and ligand-retarded desorption reflecting interaction of solute–ligand complexes with matrix sites [3,13,21], or of solute with matrix–ligand complexes [3,13]. However, because these expressions assume a daunting form in situations where the partitioning solute is multivalent in its interaction with matrix sites [9,13], a more general approach is adopted here. Irrespective of the interplay of equilibria that gives rise to solute adsorption by matrix sites, the concentration of partitioning solute associated with the matrix is used to define an effective binding constant for the interaction of the solute constituent with matrix sites. This constitutive equilibrium constant is then rationalized in terms of equilibrium constants for the particular combination of interactions responsible for the affinity chromatographic behaviour. The logical starting point is the interaction of partitioning solute with matrix in the absence of ligand, this being the situation in which the effective binding constant is also the equilibrium constant for the solute–matrix interaction.

2.1. The problem of solute multivalency

In the initial affinity chromatographic studies [1–4] the partitioning solute, A, was considered to be univalent in its interaction with matrix sites, X. However, affinity chromatography of an enzyme such as lactate dehydrogenase on Blue Sepharose [14,22], 10-carboxydecylamino-Sepharose [23,24] or trinitrophenyl-Sepharose [6] entails a situation in which the nucleotide-binding site on each of the four subunits is a candidate for interaction with the matrix. Consequently, evaluation of the intrinsic affinity constant for the solute–matrix interaction, k_{AX}, relies upon account being taken of the enzyme tetravalency. For the interaction of f-valent partitioning solute, A, with univalent matrix sites, X, the binding function, r_f is defined [25] as

$$r_f = ([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})/[\bar{\bar{X}}] \qquad (1)$$

where $[\bar{A}]$ and $[\bar{\bar{A}}]$ denote the liquid-phase and total solute concentrations, respectively, of partitioning solute for a system with an effective total concentration $[\bar{\bar{X}}]$ of matrix sites; in the absence of ligand $(\bar{A})$ is also the free concentration of solute. Adaptation of eqn. 1 to frontal column chromatographic data in an experiment with applied solute concentration $[\bar{A}]$ is effected by noting [14] that the ratio of the accessible volume (V_A^*) to the measured elution volume $(\bar{V}_A)$ defines the proportion of solute in the liquid phase. On making this substitution of $V_A^*/\bar{V}_A$ for $[\bar{A}]/[\bar{\bar{A}}]$, eqn. 1 may be rewritten as

$$r_f[\bar{\bar{X}}] = [\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f} = [\bar{A}]^{1/f}\{(\bar{V}_A/V_A^*)^{1/f} - 1\} \quad (2)$$

The decision to combine the denominator of eqn. 1 with r_f reflects the fact that the effective total concentration of matrix sites is not a parameter of known magnitude in either column chromatographic experiments or partition equilibrium studies.

Provided that a single intrinsic association constant, k_{AX}, governs all solute–matrix interactions, the general counterpart of the Scatchard [26] analysis becomes [25]

$$r_f[\bar{\bar{X}}]/[\bar{A}]^{1/f} = k_{AX}[\bar{\bar{X}}] - fk_{AX}r_f[\bar{\bar{X}}][\bar{A}]^{(f-1)/f} \quad (3)$$

which enables k_{AX} and $[\bar{\bar{X}}]$, the *effective* total concentration of matrix sites, to be calculated from a linear plot of $r_f[\bar{\bar{X}}]/[\bar{A}]^{1/f}$ *versus* $r_f[\bar{\bar{X}}][\bar{A}]^{(f-1)/f}$. In frontal affinity chromatography $[\bar{\bar{A}}]$ is replaced by $(\bar{V}_A/V_A^*)[\bar{A}]$, and $r_f[\bar{\bar{X}}]$ by $\{(\bar{V}_A/V_A^*)^{1/f} - 1\}[\bar{A}]^{1/f}$ (eqn. 2), whereupon eqn. 3 becomes

$$(\bar{V}_A)/V_A^*)^{1/f} - 1 = k_{AX}[\bar{\bar{X}}] - fk_{AX}(\bar{V}_A/V_A^*)^{(f-1)/f} \cdot [\bar{A}]\{(\bar{V}_A/V_A^*)^{1/f} - 1\} \quad (4)$$

Fig. 1a presents such an analysis of frontal chromatographic data for the interaction of *p*-nitrophenylmannoside with immobilized concanavalin A, a system for which f may be assigned a value of unity on the grounds of the small size of the partitioning carbohydrate. Values of 2.4 $(\pm 0.5) \cdot 10^4$ l/mol and 13.3 μM are obtained for the intrinsic binding constant (k_{AX}) and effective matrix-site concentration $([\bar{\bar{X}}])$, respectively. In a corresponding study [27] of the interaction of concanavalin A with the carbohydrate matrix of Sephadex G-50 (Fig. 1b), the partitioning protein has been considered bivalent because of its two equivalent and independent sites for carbohydrates [28]; an intrinsic binding constant of 1.3 $(\pm 0.3) \cdot 10^4$ l/mol and an effective matrix-site concentration of 46.3 μM emanate from this analysis. Fig. 1 serves to emphasize that an obvious prerequisite for application of eqns. 3 and 4 to affinity chromatographic data is the assignment of a magnitude to the solute valence, f, and in that regard the resort to a conventional Scatchard plot ($f = 1$ in

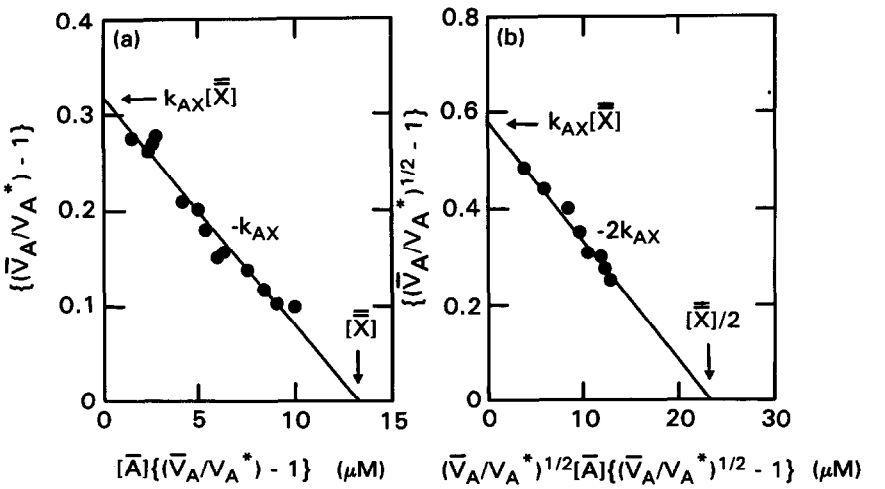

Fig. 1. Evaluation of the intrinsic association constant for the solute matrix interaction, k_{AX}, in frontal affinity chromatographic studies of the interactions of (a) *p*-nitrophenylmannoside with concanavalin A immobilized on glyceryl-CPG 170 and (b) concanavalin A with Sephadex G-50 [27]. Results are plotted according to eqn. 4 with the valences (f) of *p*-nitrophenylmannoside and concanavalin A being taken as 1 and 2, respectively.

eqns. 3 and 4) merely means that unity has been selected as the most appropriate valence.

Although the problem of allowance for solute multivalency has been solved in the sense that eqns. 3 and 4 provide the general counterpart of the Scatchard analysis, such a procedure is open to criticism that the use of a linearly transformed version of the binding equation distorts the consequences of experimental uncertainty. The obvious answer to such criticism is to employ the untransformed binding equation, but for multivalent solutes it transpires that eqn. 3 has been derived without resort to the expression of which it is the linear transform; the latter therefore needs to be identified. By relatively simple rearrangement and multiplication throughout by $f[\bar{\bar{A}}]^{(f-1)/f}$, eqn. 3 may be written as

$$fr_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f} = \frac{fk_{AX}[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}}{1 + fk_{AX}[\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}} \quad (5)$$

from which the dependence of $fr_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f}$ on $f[\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}$ is seen to be a rectangular hyperbolic relationship. Standard non-linear regression analysis of results in such terms may thus be used to yield k_{AX} and the total concentration of matrix sites, $[\bar{\bar{X}}]$.

2.2. Effects of a soluble ligand on solute partition

In affinity chromatography the usual effect of added soluble ligand is either facilitated desorption of solute because of interactions that are competitive with solute partitioning, or retarded desorption through the formation of complexes required for its interaction with the matrix. Consider, first, the commonly encountered affinity chromatographic situation in which the matrix site, X, is essentially an immobilized form of the soluble ligand, S, that is to be added. Clearly, the partition behaviour of the solute requires description in terms of two phenomena, a solute–matrix interaction governed by the intrinsic association constant, k_{AX}, and a solute–ligand interaction governed by a corresponding binding constant, k_{AS}. Nevertheless, despite the fact that there are now two interactions contributing to the overall extent of partitioning, an effective binding function may still be determined from eqn. 1. In this instance $[\bar{\bar{A}}]$ is the solute concentration in the biphasic mixture in which the liquid-phase concentrations of solute (free and complexed) and free ligand are $[\bar{A}]$ and [S], respectively. By conducting a series of frontal chromatographic experiments with a fixed concentration of free ligand and a range of solute concentrations ($[\bar{A}]$), eqn. 3 may be employed to describe the partitioning of solute in terms of an effective association constant, $\bar{k}_{AX}$, where the overbar is used to denote a constituent quantity. There now remains the problem of rationalizing this constitutive equilibrium constant in terms of the equilibria that govern its magnitude.

The value of $\bar{k}_{AX}$ obtained by application of eqn. 3 to results obtained in the presence of ligand is being calculated on the basis that $[\bar{A}]$ is the equilibrium (free) concentration of partitioning solute in the liquid phase. Clearly, the actual concentration of free solute, [A], is smaller than $[\bar{A}]$ because of solute–ligand complex formation. These two concentrations are related by the expression [29]

$$[A] = [\bar{A}]/(1 + k_{AS}[S])^f \quad (6)$$

where k_{AS} is the intrinsic binding constant for the interaction of univalent ligand, S, with f equivalent and independent sites on the partitioning solute, A. As $[\bar{A}]^{1/f}$ and $[A]^{1/f}$ are required for the respective determinations of $\bar{k}_{AX}$ and k_{AX}, it follows that the two solute–matrix affinity constants are related by the expression

$$k_{AX} = \bar{k}_{AX}(1 + k_{AS}[S]) \quad (7)$$

A linear dependence of $k_{AX}/\bar{k}_{AX}$ (or $1/\bar{k}_{AX}$) on [S] is thus predicted for this competitive case.

Table 1 summarizes the dependence of $\bar{k}_{AX}$ on [S] for the four situations that give rise to either a binary or a ternary complex between solute and matrix, the

TABLE 1

EFFECT OF FREE LIGAND CONCENTRATION, [S], ON THE CONSTITUTIVE SOLUTE–MATRIX ASSOCIATION CONSTANT, $\bar{k}_{AX}$, IN QUANTITATIVE AFFINITY CHROMATOGRAPHY

Case	Operative equilibrium constants	Quantitative description
1	k_{AS}, k_{AX}	$k_{AX}/\bar{k}_{AX} = 1 + k_{AS}[S]$
2	k_{XS}, k_{AX}	$k_{AX}/\bar{k}_{AX} = 1 + k_{XS}[S]$
3	k_{AS}, k_{ASX}	$k_{ASX}/\bar{k}_{AX} = 1 + 1/(k_{AS}[S])$
4	k_{XS}, k_{XSA}	$k_{XSA}/\bar{k}_{AX} = 1 + 1/(k_{XS}[S])$

first line (case 1) being the system already considered. From the second entry (case 2) it is clear that the expression describing competition between solute and ligand for matrix sites is formally identical with eqn. 7 (case 1). Such identity of form between expressions also occurs with the two situations involving solute retardation through the formation of a ternary complex between solute, matrix and ligand, either by interaction of the complex AS with a matrix site (case 3 in Table 1) or by interaction of A with a matrix–ligand complex (case 4). In these instances $1/\bar{k}_{AX}$ depends inversely on [S].

Application of the present approach to the characterization of a solute–ligand interaction is illustrated in Fig. 2, which presents the results of a frontal affinity chromatographic study of the effect of NADH on the elution of lactate dehydrogenase from trinitrophenyl-Sepharose [6]. From Fig. 2a, which plots the results in accordance with eqn. 4, NADH is clearly inhibiting the binding of this tetravalent enzyme to the affinity matrix. The series of experiments conducted in the absence of NADH (○) yields values of 1.5×10^4 l/mol for k_{AX} and 28 μM for the effective total concentration of matrix sites ($[\bar{\bar{X}}]$). Advantage is then taken of the fact that the same concentration of matrix sites must also apply to the other series of experiments with (▲) 5 μM and (●) 20 μM NADH. As predicted by eqn. 7, the values of $\bar{k}_{AX}$ so deduced exhibit a linear dependence on free coenzyme concentration (Fig. 2b), the slope of which signifies a magnitude of $1.3 \cdot 10^5$ l/mol for k_{AS}.

2.3. *Zonal affinity chromatography*

In view of the labour-intensive nature of the above characterization of a solute–ligand interaction, it is not surprising that quicker methods have been sought. To that end, zonal affinity chromatography of solute on a column pre-equilibrated with a range of ligand concentrations has been the most popular technique [1,2,4,30–42], but there are also a few examples where frontal affinity chromatography at a single solute concentration has been used to generate the same type of information [21,43–45]. These methods all rely upon truncation of eqn. 3 as [6,15]

$$r_f[\bar{\bar{X}}]/[\bar{A}]^{1/f} = \bar{k}_{AX}[\bar{\bar{X}}] \tag{8}$$

or, in its column chromatographic context,

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = \bar{k}_{AX}[\bar{\bar{X}}] \tag{9}$$

On substitution of eqn. 7 for $\bar{k}_{AX}$ in the situation where ligand and matrix sites compete for solute (case 1), eqn. 9 becomes

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = k_{AX}[\bar{\bar{X}}]/(1 + k_{AS}[S]) \tag{10}$$

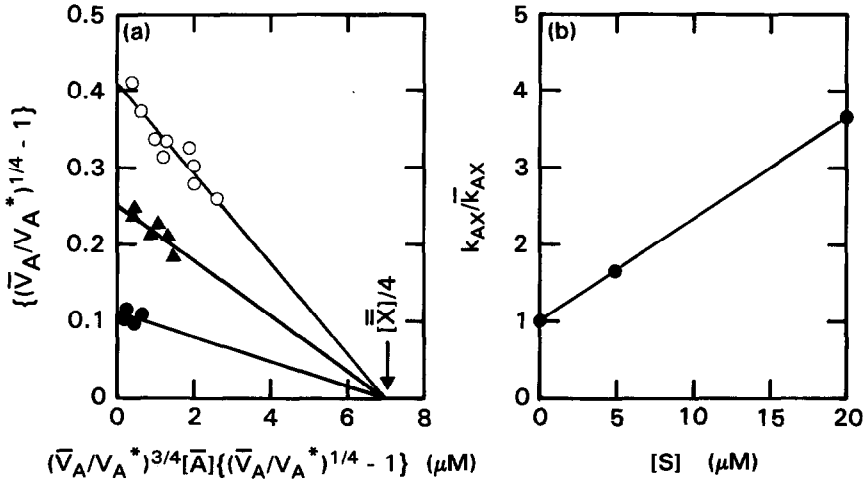

Fig. 2. Characterization of the interaction between NADH and rabbit muscle lactate dehydrogenase by frontal chromatography of the enzyme on trinitrophenyl-Sepharose [6]. (a) Multivalent Scatchard plots for determination, via eqn. 4, of the intrinsic association constant (k_{AX}) in the absence of coenzyme (○) and its constitutive counterpart ($\bar{k}_{AX}$) in the presence of free concentration, [S], of (▲) 5 μM and (●) 20 μM NADH. (b) Plot of the resultant $\bar{k}_{AX}$ values in accordance with eqn. 7 to obtain k_{AS} for the enzyme–NADH interaction.

An obvious advantage of this analysis is the absence of any terms in solute concentration. Consequently, the inability to ascribe a unique value to the ever-changing solute concentration in a migrating zone poses no impediment to the application of eqn. 10 to elution volumes ($\bar{V}_A$). However, consideration of such results in terms of eqns. 4 and 7 rather than eqn. 10 shows that the quantity defined as the solute–ligand binding constant by the truncated expression (eqn. 10) is actually $k_{AS}/(1 + k_{AX}[\bar{A}])$ [3]. Although this approach does provide a direct measure of the ligand-binding constant if $k_{AX}[\bar{A}] \ll 1$, its obvious drawback is uncertainty about the magnitude of k_{AX}, which may differ substantially from that of k_{AS} even when the immobilized reactant and ligand are essentially identical. For example, k_{AX} for the interaction of aldolase with cellulose phosphate is 50 000 l/mol, whereas $k_{AS} = 400$ l/mol [9].

A second limitation of any analysis based on eqn. 10 is its inability to provide a value for the effective total concentration of matrix sites ($[\bar{\bar{X}}]$), which means that there is no internal means of assessing the validity of the inherent assumption that $f[\bar{A}] \ll [\bar{\bar{X}}]$, which stems from the fact that $[\bar{\bar{X}}]$ is being used as an approximate concentration of free sites [6,15]. An obvious way to guarantee validity of this approximation that $[X] \approx [\bar{\bar{X}}]$ is to employ an affinity matrix with a very large concentration of immobilized reactant sites. Such affinity matrices are recommended for use in solute purification studies and, indeed, the only factor against their use for quantitative affinity chromatography seems to be the assertion [46] that preparative affinity matrices are unsuitable for the quantitative characterization of solute–ligand binding constants. This assertion, which clearly contradicts the present conclusion that a highly substituted matrix should be the preferred choice for zonal affinity chromatographic studies, has been traced [6] to the manner in which the dependence of $\bar{V}_A$ on ligand concentration was being analysed.

At that time eqn. 10 was being written as the following linear transform:

$$1/\{(\bar{V}_A/V_A^*)^{1/f} - 1\} = 1/(k_{AX}[\bar{\bar{X}}]) + k_{AS}[S]/(k_{AX}[\bar{\bar{X}}]) \quad (11)$$

which requires k_{AS} to be evaluated as the ratio of the slope to the ordinate intercept of the linear dependence of $1/\{(\bar{V}_A/V_A^*)^{1/f} - 1\}$ on free ligand concentration, [S]. Because a large value of $[\bar{\bar{X}}]$ results in the ordinate intercept being indistinguishable from zero, it was recommended [46] that a lower concentration of immobilized reactant be used to allow better delineation of the ordinate intercept.

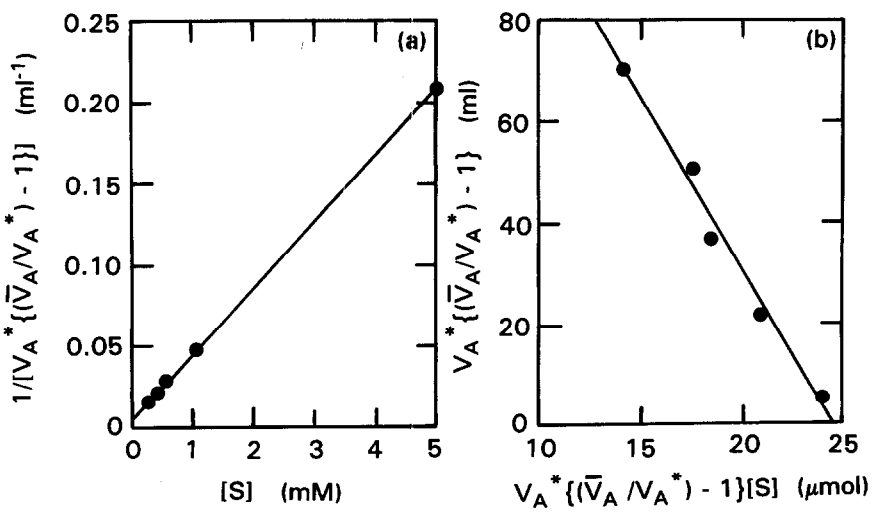

Fig. 3. Quantitative evaluation of the interaction between *p*-aminobenzamidine and trypsin by zonal affinity chromatography on *p*-aminobenzamidine-Sepharose. (a) Results in the form originally presented [46]; (b) their reassessment [6] in terms of eqn. 12. Adapted with permission from ref. 20.

Although such action certainly increases the precision with which the magnitude of the ordinate intercept may be determined, it also increases the likelihood that eqn. 11 is not a valid quantitative expression because of its reliance on a truncated form (eqn. 8) of the binding equation (eqn. 3). As noted previously [6,15,20], the solution to this dilemma is to analyse results in terms of another linear transform of eqn. 10, namely

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = k_{AX}[\bar{\bar{X}}] - k_{AS}[S]\{(\bar{V}_A/V_A^*)^{1/f} - 1\} \quad (12)$$

whereupon the solute–ligand binding constant is evaluated from the slope of the linear plot of $\{[\bar{V}/V_A^*)^{1/f} - 1\}$ as a function of $\{(\bar{V}_A/V_A^*)^{1/f} - 1\}$ [S]. Verification of this inference is provided by Fig. 3, which presents not only the "uninterpretable" results for the *p*-aminobenzamidine-facilitated elution of trypsin from *p*-aminobenzamidine-Sepharose [46], but also their replot in accordance with eqn. 12: a value of 6800 (±1300) l/mol is obtained for k_{AS} from the slope. By rendering possible the quantitative analysis of results obtained under conditions where the enforced assumption that [X] ≈ $[\bar{\bar{X}}]$ is most likely to be valid, eqn. 12 obviously achieves the breakthrough required for unequivocal characterization of ligand binding by zonal affinity chromatography.

3. EXPERIMENTAL DEFINITION OF THE FREE LIGAND CONCENTRATION

Because the equations for the quantitative description of affinity chromatographic behaviour are expressed in terms of free ligand concentration (Table 1), the ability to ascribe a value to [S] is clearly a prerequisite for the application of those expressions. For some applications this requirement has posed no problems, but for others special procedures have had to be devised.

3.1. Low-affinity solute–ligand interactions

In the initial quantitative affinity chromatographic studies the emphasis centred on the characterization of relatively weak interactions (k_{AS} = 10^2–10^4 l/mol). Consequently, the concentrations of ligand required to effect changes in solute distribution between liquid and matrix-bound states was sufficiently high compared with [$\bar{A}$] to justify the approximation that [S] ≈ [$\bar{S}$]. Examples of partition equilibrium and frontal chromatographic investigations in which this approximation was made include the evaluation of k_{AS} for the binding of monosaccharides to lysozyme [3] and lectins [15,27], of various competitive inhibitors to trypsin [44] and of phosphate to aldolase [9,16]. No such approximation is inherent in zonal affinity chromatographic studies, which entail the application of a small zone of solute to a column pre-equilibrated with a known concentration of ligand. This affinity chromatographic equivalent of the Hummel–Dreyer gel chromatographic procedure [47] generates a solute zone migrating in the presence of a concentration of free ligand equal to that used for column pre-equilibration, the only proviso being that solute and solute–ligand complexes migrate at the same rate in the liquid phase [48].

3.2. High-affinity solute–ligand interactions

In considerations of the quantitative characterization of solute–ligand interactions, attention is usually drawn to the limitations of equilibrium dialysis that are imposed by the necessity to employ a sufficiently high concentration of solute for the difference between total and free ligand concentrations to be measurable, a factor that led to the development of quantitative affinity chromatography. Another, although less well publicized, limitation of many conventional binding methods is their inapplicability to interactions for which dissociation of the complex occurs in a concentration range where the measurement of total ligand concentration, let alone a concentration difference, is beyond the scope of available assay procedures. By 1985 it was recognized [5] that quantitative affinity chromatography had potential in this area also, provided that exhaustive dialysis against multiple changes of ligand solution was used to bring a solute solution into dialysis equilibrium with a series of ligand solutions whose concentrations had been defined through their preparation by weight-dilution of a stock solution with an experimentally measurable concentration. Affinity chromatographic studies of the dialysed solute solutions then allows $\bar{V}_A$ to be obtained as a function of [S], and hence k_{AS} to be determined by application of eqn. 7 to those results. Initial studies exploited the interaction of NADH

with lactate dehydrogenase to illustrate the feasibility of the approach [6,14] with a system for which k_{AS} could be obtained readily by other methods. The example chosen for illustrative purposes in this review is the use [7] of methotrexate-Sepharose to characterize the binding of methotrexate to the binary complex formed between dihydrofolate reductase and NADPH, an interaction for which k_{AS} is in the vicinity of 10^9 l/mol [49].

Calculations based on a binding constant of that magnitude signified the feasibility of quantifying the interaction of methotrexate (S) with enzyme–NADPH complex (A) provided that the concentrations of both reactants were nanomolar or lower. These restrictive conditions clearly placed stringent demands on the design of the experimental affinity chromatographic study on methotrexate-Sepharose [7]. The first precaution taken was the inclusion of polyethylene glycol (2 mg/ml) in all buffers to stabilize enzyme activity, presumably by minimization of adsorptive losses. Second, to ensure effective saturation of the coenzyme-binding sites on the dihydrofolate reductase (and hence justify consideration of the binary complex as A), the buffer was also supplemented with 20 μM NADPH, a concentration at least 25-fold (possibly 200-fold) greater than the dissociation constant for the enzyme–NADPH interaction. Third, since overnight dialysis led to unacceptably high losses of enzymic activity, this step was replaced by zonal gel chromatography on Sephadex G-25 in order to define the concentrations of free methotrexate in the enzyme solutions to be subjected to frontal affinity chromatography. Finally, the use of 0.1–0.2 nM dihydrofolate reductase in the affinity chromatography necessitated resort to a continuous enzyme activity assay for monitoring the column effluent.

Fig. 4a presents two typical elution profiles, which clearly do not allow the evaluation of $\bar{V}_A$, because a plateau corresponding to the applied concentration is not being attained. Further, the appearance of enzyme activity in the column effluent coincides with the accessible volume (V_A^*). Whereas the predicted behaviour (eqns. 4 and 7) is based on the presumption that chemical equilibrium is maintained throughout chromatographic migration, the form of the elution profiles indicates a rate constant for dissociation of the enzyme–NADPH–methotrexate complex that is insignificant in relation to the rate of species separation effected by the affinity chromatographic procedure. The elution profile thus reflects the frontal affinity chromatographic behaviour of the ternary complex in the mixture, and the discrepancy between the enzyme activities of the applied mixture and the observed plateau corresponds to the concentration of free enzyme–

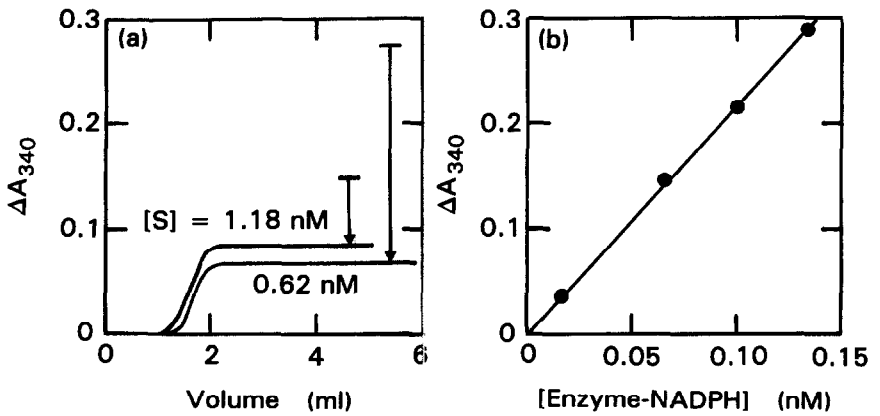

Fig. 4. Evaluation of the binding constant for the interaction between methotrexate and dihydrofolate reductase–NADPH complex by frontal affinity chromatography of the enzyme–NADPH complex on methotrexate-Sepharose. (a) Elution profile obtained for mixtures (0.1 and 0.2 nM enzyme) in which the indicated free concentrations of methotrexate were established by zonal gel chromatography on Sephadex G-25: the vertical arrows denote the difference between the assay responses of the eluted plateau and the applied solution. (b) Calibration plot for interpretation of this difference in assay response in terms of the molar concentration of free enzyme–NADPH complex. Adapted with permission from ref. 7.

NADPH complex (A) that has been removed from the mixture by adsorption to the methotrexate-Sepharose. Conversion of these absorbance differences at 340 nm to a corresponding free concentration of A (via Fig. 4b) allowed the evaluation of k_{AS} as $([\bar{A}] - [A])/([A][S])$: a value of $2 \cdot 10^9$ l/mol is obtained for the interaction of methotrexate with coenzyme-saturated dihydrofolate reductase [7].

Although the study of high-affinity interactions poses problems in relation to establishing the free ligand concentration, determination of the mixture composition is seen to be a relatively simple matter because of the ability to remove one reactant essentially without effect on the equilibrium position. Indeed, advantage is taken of this procedural simplification in the application of affinity chromatography theory to the characterization of antigen–antibody interactions by competitive radioimmunoassay [50–52] and ELISA [53,54] techniques.

3.3. Interactions involving macromolecular ligands

In situations where the solute and ligand are both macromolecular, the use of eqns. 4 and 7 to characterize the solute–ligand interaction is precluded by inability to use dialysis for establishing the magnitude of free ligand concentration, [S]. However, such systems may be characterized by using eqn. 12 in conjunction with elution volumes, $\bar{V}_A$, obtained by zonal chromatography of A on an affinity column pre-equilibrated with defined concentrations ([S]) of ligand. In that regard it is important to choose an affinity matrix for which V_A^* describes not only the accessible volume for A but also for all solute–ligand complexes [48], a requirement that can obviously be met by choosing an affinity matrix that excludes the partitioning solute and hence AS complex(es). The feasibility of this approach has been illustrated [15] by quantitative characterization of the interaction between concanavalin A and ovalbumin by zonal affinity chromatography of the lectin on a column of Sephadex G-50 pre-equilibrated with the glycoprotein.

Results of those zonal affinity chromatographic experiments, which entailed the application of a small zone of concanavalin A (50 μl, 94 μM) to an 8-ml column (12.5 × 0.9 cm I.D.) pre-equilibrated with buffer supplemented with ovalbumin (1.2–14.0 μM), are analysed in Fig. 5 on the basis of eqn. 12 and bivalency of concanavalin A [28]. Although the

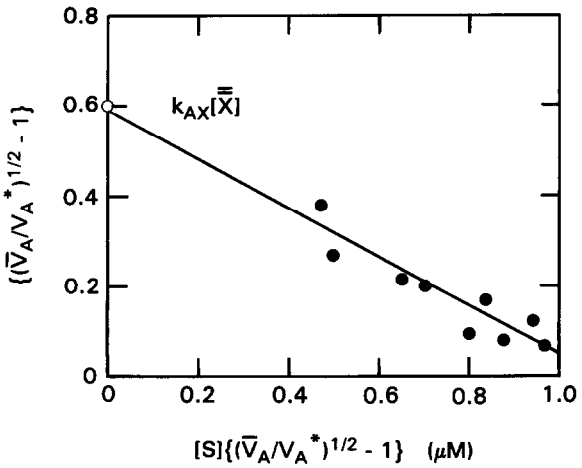

Fig. 5. Characterization of the interaction between concanavalin A and ovalbumin by zonal affinity chromatography of the lectin on a Sephadex G-50 column pre-equilibrated with various concentrations of ovalbumin [27]. (●) Results plotted in accordance with eqn. 12; (○) independent estimate of the ordinate intercept ($k_{AX}[\bar{\bar{X}}]$) obtained by frontal chromatography of concanavalin A in the absence of ovalbumin.

results (●) clearly conform with the linear dependence predicted by eqn. 12, the fact that the interaction of concanavalin A with the Sephadex G-50 matrix was confined to the bead surface made it necessary to justify experimentally the use of an analytical expression containing an inherent assumption that $f[\bar{A}] \ll [\bar{\bar{X}}]$. An independent estimate (○) of the ordinate intercept ($k_{AX}[\bar{\bar{X}}]$) was therefore obtained [14] by frontal affinity chromatography of concanavalin A on the same column. Use of the truncated quantitative expression for analysis of the zonal data is clearly justified, whereupon the slope of Fig. 5 may be used to evaluate k_{AS}. An intrinsic association constant of 5.3 (± 0.8)$\cdot 10^5$ l/mol is obtained for the interaction of ovalbumin with concanavalin A under the conditions of the experiment (pH 5.5, $I = 0.5$).

4. INTERACTION OF ALDOLASE WITH MUSCLE MYOFIBRILS

The biological advantages of metabolite-dependent myofibrillar adsorption of enzymes as a means of regulating glycolytic flux were being extolled long before the availability of acceptable evidence to indicate physiological significance of the phenom-

enon. That experimental support for the concept of metabolite-dependent binding of glycolytic enzymes to the myofibrillar matrix has come from a combination of partition equilibrium studies and quantitative affinity chromatography theory [16–18,55].

4.1. Evidence of active-site involvement

Partition equilibrium studies of the interaction between aldolase and muscle myofibrils have provided quantitative evidence for the existence of a significant extent of aldolase adsorption under conditions physiological with respect to pH and ionic strength [16,17,55]. Further, the concept of metabolite dependence of the equilibrium between soluble and adsorbed enzyme states is supported by studies of the effect of phosphate on the adsorption phenomenon [16]. Fig. 6a, which presents a plot of those results in accordance with eqn. 3, establishes that phosphate is certainly inhibiting the binding of aldolase to the myofibrillar matrix. In addition, Fig. 6b indicates that the dependence of $\bar{k}_{AX}$ on [S] conforms with eqn. 7 and hence the concept of phosphate as a competitive inhibitor of aldolase adsorption by myofibrils. That the equilibrium constant deduced from the slope of Fig. 6b refers to k_{AS} (case 1 in Table 1) rather than k_{XS} (case 2) is borne out by the essential identity of its magnitude (400 l/mol) with the corresponding constant (350 l/mol) obtained from enzyme kinetic studies of aldolase inhibition by phosphate [9]. Additional evidence in favour of active-site involvement in the aldolase–myofibril interaction has been provided by the demonstration that the myofibrillar matrix inhibits competitively the aldolase-catalysed cleavage of fructose 1,6-bisphosphate [56].

Although the above findings signify (a) the existence of aldolase in free and myofibril-bound states and (b) the sensitivity of the equilibrium position to changes in flux of glycolytic metabolites, those predictions relate to interactions in calcium-free media, and hence do not take into account any effects of the increased calcium ion concentration that triggers muscle contraction.

4.2. Effect of calcium ion on enzyme adsorption

The effect of calcium ion concentration on the myofibrillar adsorption of aldolase is summarized in Fig. 7a, from which it is evident that a change in $[Ca^{2+}]$ from (○) 0 to (△) 35 μM decreases substantially the magnitude of $\bar{k}_{AX}$ from its initial value (k_{AX}) of $3.3 \cdot 10^5$ l/mol. This decreased strength of aldolase binding is undoubtedly due to the myofibrillar interaction of calcium ion with troponin, which possesses four sites for this metal ion [57]. However, because two of these sites would have been saturated at the lowest concentration (1 μM) of Ca^{2+} used in Fig. 7a, the observed concentration-dependent decrease in myofibrillar adsorption of aldolase must reflect the binding of calcium ion to the two low-affinity troponin sites that are responsi-

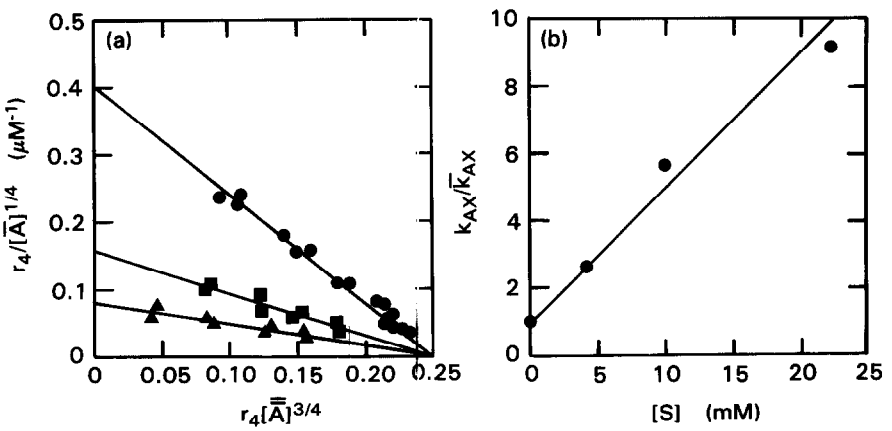

Fig. 6. Characterization of the interaction between phosphate and aldolase (pH 6.8, $I = 0.158$) by means of the effect of the ligand on the binding of enzyme to muscle myofibrils [16]. (a) Multivalent Scatchard plots (eqn. 3) of results obtained in the absence of phosphate (●) and in the presence of (■) 4 and (▲) 10 mM phosphate, [S]. (b) Plot of the resultant $\bar{k}_{AX}$ values in accordance with eqn. 7 to obtain k_{AS} for the enzyme–phosphate interaction. Adapted with permission from refs. 20 and 16, respectively.

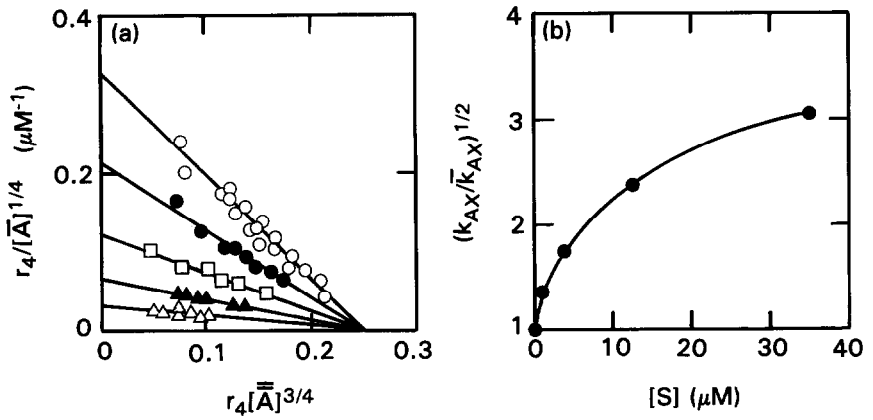

Fig. 7. Effect of calcium ion on the binding of aldolase to muscle myofibrils (pH 6.8, I = 0.158). (a) Multivalent Scatchard analysis (eqn. 3) for evaluation of the intrinsic association constant (k_{AX}) in the absence of metal ion (○) and its constitutive counterpart in the presence of free $CaCl_2$ concentrations, [S], of (●) 1.0, (□) 4.0, (▲) 12.5 and (△) 35 μM. (b) Test of the results for conformity with eqn. 14 for competitive binding of aldolase and Ca^{2+} ion by myofibrils. Adapted with permission from ref. 18.

ble for the regulation of muscle contraction [58,59].

In the binding of an f-valent solute (aldolase, A) to a matrix site (X) that also has the capacity to bind two molecules of a univalent ligand (calcium ion, S), there are potentially three solute–matrix equilibria to be considered: that between A and free matrix sites, governed by intrinsic affinity constant k_{AX}, another between A and a singly liganded matrix site, governed by k_{AXS}, and one between A and a ligand-saturated matrix site, for which the intrinsic affinity constant is k_{AXSS}. This more complicated situation has not been treated in Section 2, but comparable treatment leads to the conclusion [18] that

$$\bar{k}_{AX} = \{k_{AX} + 2k_{AXS}k_{XS}[S] + k_{AXSS}(k_{XS}[S])^2\}/(1 + k_{XS}[S])^2 \quad (13)$$

where k_{XS} is the intrinsic binding constant for the ligand–matrix interaction. If the interactions of ligand and solute with matrix sites are mutually exclusive ($k_{AXS} = k_{AXSS} = 0$), eqn. 13 simplifies to

$$(k_{AX}/\bar{k}_{AX})^{1/2} = 1 + k_{XS}[S] \quad (14)$$

which predicts a linear dependence of $(k_{AX}/\bar{k}_{AX})^{1/2}$ on [S] for this classical competitive inhibition. The curvilinear form of the experimental plot (Fig. 7b) signifies that the inhibition of the aldolase–myofibril interaction by calcium ion is not competitive, a result consistent with earlier inferences that the tropomyosin (not the troponin) component of myofibrils is involved in aldolase adsorption [55,60].

The next step is to use eqn. 13 to evaluate k_{XS}, k_{AXS} and k_{AXSS}, the thermodynamic constants governing the non-competitive inhibition of the aldolase–myofibril interaction by Ca^{2+}. On the grounds that $\bar{k}_{AX}$ approaches k_{AXSS} in the limit of infinite ligand concentration, the dependence of $\bar{k}_{AX}$ on 1/[S] (Fig. 8a) yields a value of $3.3 \cdot 10^4$ l/mol for k_{AXSS} from the ordinate intercept. In order to complete the characterization, k_{XS} and k_{AXS} have been evaluated [18] by assigning a magnitude to the ratio k_{AXS}/k_{AXSS} and assessing its relevance by constancy of the consequent k_{XS} values that emanate from eqn. 13 for the various ligand concentrations, [S]. The results of this procedure for three assigned ratios are illustrated in Fig. 8b, from which the conclusion is reached that $k_{AXS} = k_{AXSS} = 3.3 \cdot 10^4$ l/mol and $k_{XS} = 2.3 \cdot 10^5$ l/mol.

4.3. Aldolase adsorption and muscle contraction

On the basis of the above analysis, the effect of calcium ion on the binding of aldolase to muscle myofibrils is a ten-fold decrease in the intrinsic association constant for the enzyme–myofibril interaction on the binding of metal ion to either or both of the matrix sites for calcium. The validity of this interpretation is strenthened further by the fact that the value of $2 \cdot 10^5$ l/mol for k_{XS} inferred from curve-fitting the aldolase adsorption data essentially

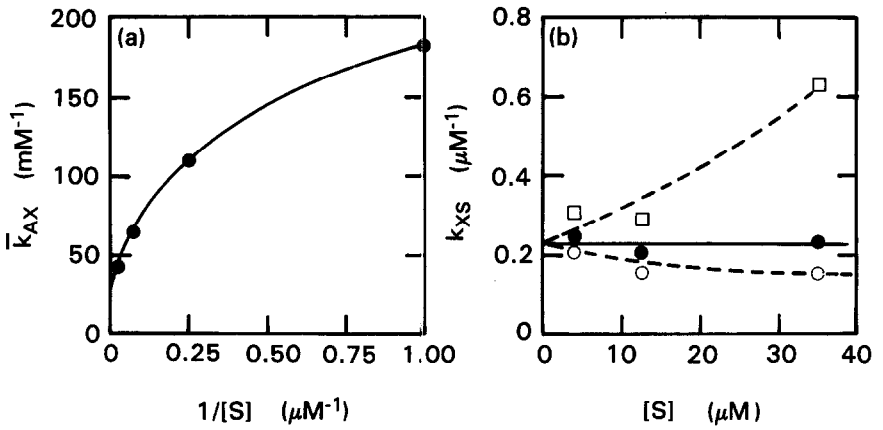

Fig. 8. Determination of thermodynamic parameters for the non-competitive inhibitory effect of calcium ion on the binding of aldolase by muscle myofibrils. (a) Evaluation of the intrinsic association constant, k_{AXSS}, for the interaction of the enzyme with calcium-saturated myofibrils. (b) Dependence on $CaCl_2$ concentration ([S]) of the apparent intrinsic association constant for the calcium–myofibril interaction (k_{XS}) that is calculated from eqn. 13 on the basis of k_{AX}, k_{AXSS} and assigned magnitudes of (○) 0.5, (●) 1.0 and (□) 2.0 for the ratio k_{AXS}/k_{AXSS}.

duplicates the experimentally measured intrinsic affinity constant for the interaction of calcium ion with the troponin C subunit [57], thin filaments [61] and intact muscle [62,63].

The conclusion is therefore reached that the interactions of calcium with troponin that govern the regulation of muscle contraction are also responsible for the diminished affinity for aldolase that is exhibited by myofibrils in the contracting state. Although the binding of metal ion to both regulatory sites was considered initially to be required for the onset of muscle contraction [57], that concept has now been disputed [59]. From the present study the binding of calcium ion to either regulatory site suffices to inhibit aldolase binding, presumably because of the same conformational perturbations that induce the relative movement of thin and thick filaments to achieve muscle contraction. The postulate that myofibril-bound enzyme acts as a facilitator of glycolytic flux certainly loses some appeal as the result of this demonstration of partial disintegration of the aldolase–myofibril complex at the onset of muscle contraction, at the very stage when any need to maximize glycolysis is greatest.

Irrespective of any implications regarding speculation about the physiological role of glycolytic enzyme adsorption to myofibrils, the above study serves to emphasize the potential of affinity chromatography as a quantitative probe of many problems associated with biological function.

5. AFFINITY CHROMATOGRAPHY THEORY REVISITED

An obvious limitation of the above analyses is their reliance on expressions for $\bar{k}_{AX}$ in terms of free ligand concentration, which is a legacy of the fact that binding constants are defined as appropriate ratios of the equilibrium concentrations of product and reactant species. Further, the forms of the affinity chromatography equations written in terms of [S] seemed so complicated at the time of their derivation [9,13] that the concept of an analytical solution in terms of total ligand concentration, [$\bar{S}$], seemed out of the question. However, a return to this problem a decade later [8] has shown that expression of the affinity chromatography equations in terms of [$\bar{S}$] is a relatively simple matter. For the sake of completeness, it seems appropriate to end a review on recent developments in quantitative affinity chromatography with an outline of the latest advance, even though the topic is accorded greater consideration elsewhere in this issue [64].

5.1. Analysis in terms of total ligand concentration

In affinity chromatographic systems where the

competition is between ligand and matrix sites for partitioning solute (case 1 in Table 1), the total ligand concentration in the liquid phase, $[\bar{S}]$, may be introduced into the quantitative analysis by noting that the expression for the binding of a univalent ligand to an f-valent solute [29], namely

$$r = ([\bar{S}] - [S])/[\bar{A}] = fk_{AS}[S]/(1 + k_{AS}[S]) \quad (15)$$

is readily rearranged to provide the relationship between [S] and $[\bar{S}]$. Specifically,

$$[S] = [\bar{S}] - fk_{AS}[\bar{A}][S]/(1 + k_{AS}[S]) \quad (16)$$

Use of eqn. 7 to eliminate the terms in $k_{AS}[S]$ then leads to the expression

$$R = 1 + k_{AS}\{[\bar{S}] - (R - 1)f[\bar{A}]/R\} \quad (17)$$

where $R = k_{AX}/\bar{k}_{AX}$ has been substituted for the experimentally measured ratio of intrinsic affinity constants for the solute–matrix interaction in the absence and presence of ligand. By measuring this ratio (R) at a series of total ligand concentrations, the value of the binding constant for the solute–ligand interaction (k_{AS}) may thus be determined from the slope of the dependence of R on $\{[\bar{S}] - (R - 1)f[\bar{A}]/R\}$.

5.2. Application to the antithrombin–heparin system

The greater versatility of quantitative affinity chromatography as the result of this advance is illustrated with the problem that precipitated the need for the development of analytical expressions in terms of total ligand concentration, namely the affinity chromatographic characterization of the interaction between antithrombin III and high-affinity heparin with heparin-Sepharose as matrix [8]. The fact that the solute (antithrombin) and ligand (heparin) were both macromolecular precluded equilibrium dialysis as a means of establishing free ligand concentrations, and advantage was to be taken of the greater accuracy with which partition equilibrium results may be obtained by resort to a recycling partition equilibrium technique (Fig. 9), in which all mixtures are prepared by successive additions of solute or ligand to the one sample of affinity matrix [8,9,65]. Consequently, the only ligand concentration of known magnitude was the total ligand concentration, $[\bar{S}]$, which could be determined by dividing the amount of ligand added by the accessible volume, V_A^*.

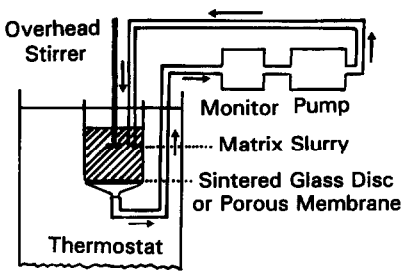

Fig. 9. Schematic representation of the recycling partition equilibrium system. Adapted with permission from ref. 65.

After equilibration of 6 ml of heparin-Sepharose slurry with buffer, an aliquot (10 or 20 μl) of antithrombin stock solution was added to the stirred slurry, sufficient time being allowed for attainment of partition equilibrium so that $[\bar{A}]$ for a mixture with total solute concentration $[\bar{A}]$ could be recognized by constancy of the UV monitor response. Repetition of the procedure with a further four or five antithrombin aliquots generated sufficient ($[\bar{A}]$, $[\bar{A}]$) data for characterizing the antithrombin–matrix interaction via eqn. 3. Successive additions of heparin aliquots then yielded the ($[\bar{A}]$,$[\bar{A}]$) data required for the application of eqn. 3 to obtain $\bar{k}_{AX}$ as a function of $[\bar{S}]$. Fig. 10a presents the plot of results for the first half of the experiment (antithrombin addition) according to eqn. 3 with $f = 1$ [66,67] and $[\bar{X}]$, the total concentration of matrix sites, expressed as $\{(V_A^*)_0/V_A^*\}[\bar{X}]_0$: this takes into account the systematic increase in V_A^* and consequent decrease in $[\bar{X}]$ from their respective initial values, $(V_A^*)_0$ and $[\bar{X}]_0$, that arise from each addition of solute. From these three experiments conducted with the same heparin-Sepharose slurry at (▲) 15, (○) 25 and (●) 35°C, $[\bar{X}]_0 = 2.2$ μM and k_{AX} (defined by the slopes) varies inversely with temperature (Table 2). Although larger than k_{AX}, the values of k_{AS} obtained by graphical analysis of the second half of the experiment on the basis of eqn. 17 (Fig. 10b) show the same trend (Table 2).

5.3. Implications of the development

The expression of quantitative affinity chromatography theory in terms of total concentration of competing ligand is an important breakthrough for studies of high-affinity interactions, because it obviates the need for ascertaining the magnitude of [S]. Although the free ligand concentration may, for

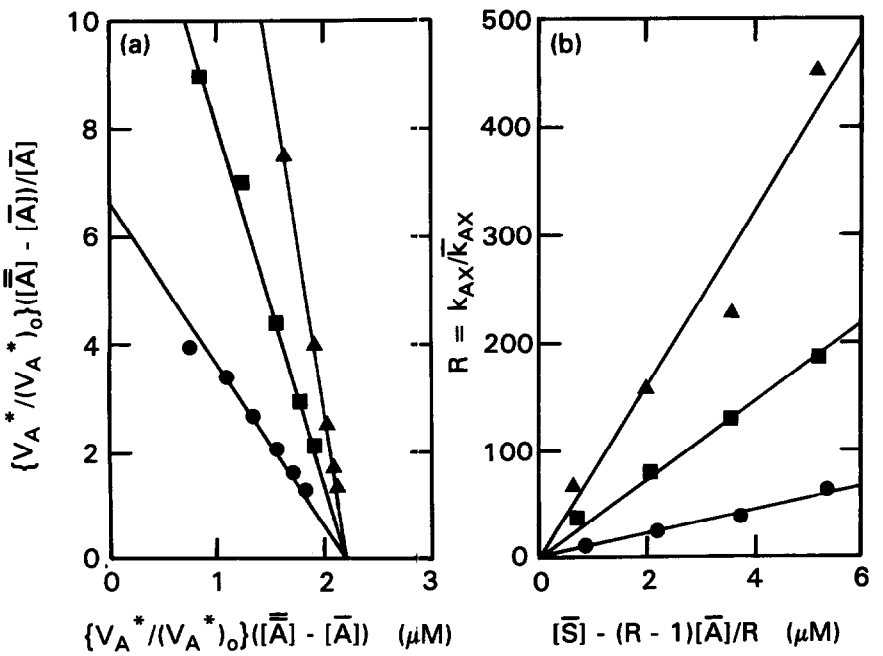

Fig. 10. Evaluation of binding constants for interactions of antithrombin III with high-affinity heparin at (▲) 15, (■) 25 and (●) 35°C. (a) Scatchard plots for evaluation of k_{AX} and $[\bar{X}]_o$, the initial effective concentration of matrix sites in a recycling partition study with a slurry of heprin-Sepharose. (b) Determination of the corresponding binding constants for the heparin–antithrombin interaction (k_{AS}) by addition of soluble heparin to displace matrix-bound protein, the results being plotted in accordance with eqn. 17. Adapted with permission from ref. 8.

small ligands, be established by exhaustive dialysis against copious volumes of ligand solution with known concentration [6], there are certainly technical problems associated with ensuring that dialysis equilibrium has been attained [7]. Indeed, as the strength of the interaction increases, the volume of the dilute ligand solution required for attainment of dialysis equilibrium with a detectable concentration of partitioning solute becomes prohibitively excessive. This difficulty would also pervade evaluation of the binding constant by zonal affinity chromatography of solute on a column pre-equilibrated with ligand, where the above problem of dialysate volume size becomes translated into management of an affinity column with prohibitively excessive length. Theoretical expressions such as eqn. 17 (and its counterparts for the other cases [64]) have thus removed an impediment to the use of quantitative affinity chromatography for the study of tight binding. The stages thus seems to be set for the characterization of many high-affinity interactions, in the blood-clotting system and elsewhere, that have hitherto been unquantifiable because of essentially stoichiometric complex formation at the reactant concentrations required for studies of ligand binding by classical methods.

TABLE 2

TEMPERATURE DEPENDENCE OF BINDING CONSTANTS FROM AN AFFINITY CHROMATOGRAPHIC STUDY OF THE INTERACTION BETWEEN HIGH-AFFINITY HEPARIN AND ANTITHROMBIN III

Results inferred from Fig. 10.

Constant	Temperature (°C)		
	15	25	35
10^{-6} k_{AX} (l/mol)[a]	13.0 ± 1.1	6.8 ± 0.3	3.0 ± 0.3
10^{-7} k_{AS} (l/mol)[a]	8.0 ± 2.2	3.4 ± 0.3	1.0 ± 0.2

[a] Based on a value of 2.2 μM for $[X]_o$, the initial concentration of immobilized heparin. Uncertainty is expressed as 2 S.E.M.

6. CONCLUSIONS

This review has highlighted developments in the field of quantitative affinity chromatography during the past decade, a period that began with a theoretic al advance that solved the problem of allowance for multivalency of the partitioning solute [9,13], and ended with another theoretical development, the realization that the theoretical description of affinity chromatographic behaviour is amenable to analytical solution in terms of total ligand concentration [8]. Highlights of the intervening period included the adaptation of the expressions for zonal affinity chromatography [2,4] to render them suitable for quantitative studies with the highly substituted affinity matrices that are used in preparative work. The era under review also heralded the application of quantitative affinity chromatography theory to the characterization of systems in which the affinity matrix is a biological tissue. Whereas the other developments may be classified as refinements of quantitative affinity chromatographic procedures, the characterization of the metabolite dependence of aldolase adsorption to muscle myofibrils puts an end to the concept of the technique as simply another method for evaluating equilibrium constants. Quantitative affinity chromatography has clearly provided an inroad into the problem of characterizing the interplay of biphasic equilibria that effect the control of physiological, hormonal and immunological responses involving surface receptors.

7. SYMBOLS

- A — f-Valent partitioning solute
- R — Ratio of solute–matrix association constants in the absence and presence of ligand
- S — Univalent ligand
- $\bar{V}_A$ — Measured elution volume of partitioning solute
- V_A^* — Elution volume of (or volume accessible to) A in the absence of interaction with matrix
- X — Univalent matrix site
- f — Valence of partitioning solute
- $[i]$ — Molar concentration of species i (i = A, S or X)
- $[\bar{i}]$ — Total concentration of species i (A or S) in liquid phase
- $[\bar{\bar{i}}]$ — Total concentration of species i (A, S or X) in the system
- k_{ij} — Intrinsic association constant for interaction between species i and j (any two of A, S and X)
- $\bar{k}_{AX}$ — Effective (constitutive) association constant for the solute–matrix interaction in the presence of ligand
- r — Klotz (Scatchard) binding function
- r_f — Counterpart of r for an f-valent species

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