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Allowance for kinetics of solute partitioning in the determination of rate constants by affinity chromatography

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ABSTRACT

An improved method is described for evaluation of the dissociation rate constant for the solute–matrix interaction from affinity chromatographic elution profiles in which the flow-rate dependence of the variance reflects the combined effects of chemical and partition kinetics. Results of a previous investigation of the interaction between saccharides and concanavalin A immobilized on CPG 3000 are reconsidered in terms of the new procedure, which takes advantage of an analytical expression to separate the two contributions. Although the revised analysis confirms the adequacy of the earlier empirical approach to this problem, it also renders redundant the necessity for the empiricism.

INTRODUCTION

Although affinity chromatography has been proposed as a means of measuring rate constants for over a decade [1–3], reasonable progress towards achievement of that goal is a relatively recent development [4,5]. Major contributing factors to this greatly improved outlook for the determination of rate constants by affinity chromatography have been (i) replacement of zonal by frontal affinity chromatography to avoid the necessity of creating two infinitely sharp boundaries an infinitesimal distance apart, and (ii) use of the flow-rate dependence of the boundary variance to evaluate the kinetic contribution to boundary spreading [6].

In the numerical simulations of affinity chromatographic migration designed to test the feasibility of the intended procedure [4,5] the problem of solute partition was avoided by considering the matrix sites to be immobilized on the surface of an impenetrable matrix. Experimentally, however, it transpires that the required flow-rates dictate the use of porous glass beads for an acceptable concentration of matrix sites to be achieved. Consequently, the contribution of partition kinetics between the mobile and stationary phases needs to be taken into account if the flow-rate dependence of boundary spreading is to be used to evaluate the rate constant for the chemical kinetics of solute dissociation from its complex with affinity-matrix sites.

In our previous attempt to determine the rate

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constant for the dissociation of *p*-nitrophenylmannoside from concanavalin A immobilized on CPG 3000 [5], simple additivity of the partition- and chemical-kinetics contributions was assumed in order to take into account the small but significant dependence of variance upon flow-rate observed in chromatography of the saccharide on an otherwise identical column of underivatized CPG 3000 beads. However, we now show that this empirical assumption may be avoided by using an analytical expression [7] which describes the simultaneous operation of partition and chemical kinetics. In this communication we consider in greater detail the question of partition kinetics by additional numerical simulations of affinity chromatographic migration and by subjecting the earlier results [5] to the improved analytical procedure.

EXPERIMENTAL

Summary of previous protocol

Because the goal of the present investigation is to remove the element of empiricism from an earlier analysis, the affinity chromatography experiments and results to be considered have already been published [5]. However, it seems appropriate to reiterate the essential features of that experimental protocol as a prelude to description of the procedures used to effect the improved analysis.

Briefly, the interaction of *p*-nitrophenylmannoside with immobilized concanavalin A was quantified by subjecting solutions of the sugar (10–50 μM) to frontal chromatography on a standard HPLC column (25 × 0.46 cm) of concanavalin A–CPG 3000, equilibrated with phosphate–chloride buffer (pH 5.5; ionic strength, I 0.5) and operated at flow-rates in the range 2–10 ml/min. The absorbance at 305 nm was recorded continuously by transfer of the ISCO V^4 monitor response to a data acquisition system. Standard procedures were then used to obtain the first moment (mean elution volume, $\bar{V}_A$) and second moment (variance in elution volume, σ_A^2) of the boundaries.

For the corresponding characterization of the competing interaction between methylmannoside and immobilized lectin, the same procedure was followed except that the buffer and applied solutions were supplemented with competing saccharide (100 μM). In a third series of experiments the kinetics of partitioning of *p*-nitrophenylmannoside was examined by subjecting solutions of the saccharide to frontal chromatography on an identical HPLC column of underivatized CPG 3000, equilibrated with the same phosphate–chloride buffer and operated at a similar range of flow-rates. Equivalence of the two columns was gauged by identity of the elution volumes obtained on the underivatized column and on the affinity column in the presence of a saturating concentration of competing saccharide.

The chromatographic behaviour of *p*-nitrophenylmannoside (A) was considered in terms of its interaction with immobilized-lectin sites (X) via the reaction scheme

$$A + X \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AX;\ K_{AX} = k_{+1}/k_{-1} \qquad (1a)$$

k_{+1} and k_{-1} being the respective association and dissociation rate constants, and K_{AX} the association equilibrium constant. Effects of methylmannoside (B) were considered in terms of the analogous scheme

$$B + X \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} BX;\ K_{BX} = k_{+2}/k_{-2} \qquad (1b)$$

for the additional reaction of matrix sites with the competing saccharide.

Evaluation of kinetic parameters for the solute–matrix interaction

For situations in which the elution profile reflects contributions from partition kinetics and the kinetics of the solute–matrix interaction, DeLisi and Hethcote [7] have provided the following analytical expression (their eqn. 222) for the dependence of σ_A^2 upon flow-rate, F.

$$\sigma_A^2 = 2(V_A^* - V_0)\{(1 + K_{AX}[X])^2/k_{-p} + K_{AX}[X]/k_{-1}\}F \qquad (2)$$

Effects of partition kinetics are reflected in eqn. 2 by the appearance of k_{-p}, the rate constant for efflux of partitioning solute from a stationary phase with volume $(V_A^* - V_0)$: V_0 is the volume of

mobile phase (column void volume) and V_A^* the column volume accessible to partitioning solute. Terms in [X], the concentration of uncomplexed matrix sites distributed in the volume $(V_A^* - V_0)$, are eliminated by introducing $\bar{V}_A$, the elution volume obtained from the first moment of the boundary, and taking advantage of the relationship

$$K_{AX}[X] = (\bar{V}_A - V_A^*)/(V_A^* - V_0) \quad (3)$$

which follows directly from eqn. 221 of ref. 7. The flow-rate dependence of the variance then becomes

$$d\sigma_A^2/dF = 2\{(\bar{V}_A - V_0)^2/[k_{-p}(V_A^* - V_0)] + (\bar{V}_A - V_A^*)/k_{-1}\} \quad (4)$$

k_{-p} may be evaluated independently, and hence the rate constant for dissociation of solute–matrix complex (k_{-1}) may be determined as the only parameter of unknown magnitude in eqn. 4. However, because linear kinetics is an inherent assumption in eqns. 2 and 4, the apparent rate constant so determined (k_{-1}^{obs}) must be extrapolated to zero solute concentration in order to meet that condition.

An assumption inherent in the above procedure is the presumed dominance of partition and chemical kinetics contributions to the flow-rate dependence of boundary spreading. Although such neglect of restricted diffusion in the stationary phase is a reasonable approximation for the present system involving partition of small solutes (monosaccharides) within the relatively large pores of CPG 3000, extension of the approach to systems with larger partitioning solutes could well require modification of the quantitative expressions to take into account the effects of the internal diffusional resistance encountered by a macromolecular solute within matrix pores [8]. Allowance for this diffusional phenomenon introduces an element of non-linearity into the variance variation with flow rate by virtue of a dependence on $\sqrt{F}$ [5].

Evaluation of the rate constants for partition kinetics

The rate constant for the kinetics of *p*-nitrophenylmannoside efflux from the stationary phase into the mobile phase (k_{-p}) has been obtained from the corresponding flow-rate dependence of σ_A^2 in experiments on the underivatized CPG 3000 column. Use is made of the relationship

$$k_{-p} = 2(V_A^* - V_0)/(d\sigma_A^2/dF) \quad (5)$$

the expression obtained by setting $\bar{V}_A = V_A^*$ in eqn. 4, which is equivalent to setting [X] = 0 in eqn. 2. On the grounds that the ratio k_{+p}/k_{-p} defines K_p, the equilibrium parameter describing partition of solute between the two phases, the rate constant for influx of solute into the stationary phase has been determined from the expression $k_{+p} = k_{-p}K_p$, where $K_p = (V_A^* - V_0)/V_0$.

Numerical simulation of elution profiles

The advancing elution profiles of saccharide A in the absence and presence of 100 μM competing saccharide B were simulated for a porous matrix by an adaptation of the theoretical plate model of chromatography [9] with the transfer volume set at 1.7% of the mobile phase volume in a plate (segment). In the absence of competing saccharide the chromatographic column initially contained only free matrix sites at the constituent concentration $\bar{C}_X$. In the presence of competing saccharide, the initial equilibrium concentration of B in the mobile phase (volume V_0) of the column, C_B, was assigned the value of 100 μM, whereupon the concentration of uncomplexed matrix sites follows from the conservation equation

$$\bar{C}_X = [X] + K_p K_{BX}[X]C_B \quad (6)$$

The concentrations of B and BX in the accessible volume within the stationary phase $(V_A^* - V_0)$ also follow from the appropriate mass action expressions. The elution profiles were then generated according to the procedure described previously [5], except that the set of differential equations was extended to include the kinetics of partitioning of solute(s) between the mobile phase and the accessible volume within the stationary phase. After each transfer of a volume increment, δV, which corresponds to a time interval (δt) via flow-rate, F, a subroutine for partition and chemical kinetics was called to

evaluate the new distribution of solute(s) between complexed and free states in each column segment. This kinetics subroutine entailed numerical solution of the set of differential equations by the 4th-order Runge–Kutta method.

RESULTS AND DISCUSSION

In the previous attempt to evaluate a dissociation rate constant for the solute–matrix interaction in affinity chromatography [5], we used an empirical approach to the separation of contributions of partitioning kinetics and chemical kinetics to the flow-rate dependence of boundary spreading in frontal chromatography. Subsequently we have become aware that DeLisi and Hethcote [7] had already provided an analytical solution (eqn. 2) to the problem. It is therefore of interest to test the adequacy of the earlier approach. However, a factor that requires consideration is the compatibility of results inferred from eqn. 2 with the earlier thermodynamic and kinetic characterization, which was based on the concept of matrix sites distributed throughout the volume accessible to partitioning solute (V_A^*). Because eqn. 2 is based on a reaction volume confined to the pores of the stationary phase, $(V_A^* - V_0)$, the validity of identifying parameters in eqn. 2 with those in eqn. 1 clearly needs to be checked.

The question is addressed by considering the expressions for mass conservation of partitioning solute that apply to the two thermodynamic descriptions of the system. If the matrix sites are considered to be confined to the volume of stationary phase accessible to partitioning solute, $(V_A^* - V_0)$, the expression for mass conservation of solute (A) within that volume is

$$(V_A^* - V_0)[\bar{A}] = (\bar{V}_A - V_0)[A] \quad (7)$$

where [A] and $[\bar{A}]$ denote the free and total concentrations of solute. By writing the consequent equation for $[\bar{A}]$, it readily follows that

$$([\bar{A}] - [A])/[A] = (\bar{V}_A - V_A^*)/(V_A^* - V_0) = K_3[X] \quad (8)$$

which is, in fact, eqn. 3 because the ratio on the left-hand side defines the product of the free concentration of matrix sites and the association constant for the solute–matrix interaction, designated here as K_3 [7] to distinguish it from the corresponding parameter (K_{AX}) in eqn. 1. Adoption of the convention wherein matrix sites are considered to be distributed throughout the same volume as partitioning solute (V_A^*) leads to the analogous relationship [4]

$$(\bar{C}_A - [A])/[A] = (\bar{V}_A - V_A^*)/V_A^* = K_{AX}C_X \quad (9)$$

where different terminology for the total solute concentration ($\bar{C}_A$) and the concentration of uncomplexed matrix sites (C_X) is used to signify differences in magnitude from their counterparts in eqn. 8. In order to compare the magnitudes of the two equilibrium constants defined by eqns. 8 and 9, we note that the relationship

$$[X](V_A^* - V_0) = C_X V_A^* \quad (10)$$

expresses the condition that the two conventions merely differ in the volume occupied by the same number of matrix sites. By substituting the consequent expression for [X] in eqn. 8 we obtain

$$K_3C_X = (\bar{V}_A - V_A^*)/V_A^* \quad (11)$$

which essentially duplicates eqn. 9 and thereby establishes the identity of equilibrium constants emanating from either convention. We therefore proceed with the envisaged analysis of the earlier experimental results [5].

The effect of column flow-rate on the variance of the advancing elution profile in frontal chromatography of *p*-nitrophenylmannoside (40 μM) is shown (●) in Fig. 1a, together with the corresponding dependence (■) obtained on an identical column of underivatized CPG 3000. The latter yields a value of 0.011 ml min for $d\sigma_A^2/dF$, which needs to be combined with V_A^* and V_0 in eqn. 5 to obtain k_{-p}, a prerequisite for determining k_{-1}, the rate constant for dissociation of saccharide–matrix complex, via eqn. 4.

A value of 3.58 ml for V_A^* has been determined experimentally, but direct measurement of the void volume (V_0) was precluded by unavailability of a sufficiently large solute to guarantee its complete exclusion from the 300 nm pores of the

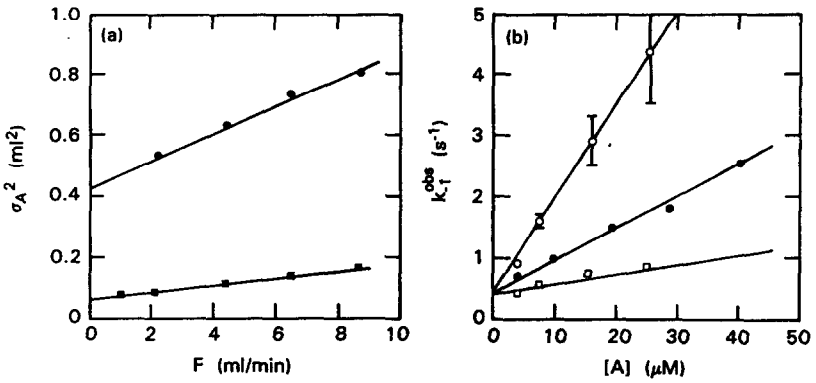

Fig. 1. Chromatographic evaluation of the rate constant for desorption of *p*-nitrophenylmannoside from concanavalin A immobilized on CPG 3000. (a) Published [5] effect of flow-rate on the variance of elution profiles of the saccharide (40 μM) on the affinity column (●) and identical CPG 3000 column devoid of matrix sites (■). (b) Effect of *p*-nitrophenylmannoside concentration on the apparent rate constant for saccharide desorption obtained (eqn. 4) from the flow-rate dependence of the variance of the advancing (○) and trailing (□) elution profiles: ● = corresponding dependence derived from the advancing elution profile in experiments where the applied solution and equilibrating buffer were supplemented with 100 μM methylmannoside.

glass matrix. For columns of CPG beads with similar mesh but smaller pore size, direct measurement of V_0 has yielded estimates of 0.485 [10] and 0.476 [11] for V_0/V_t, the ratio of the void and total column volumes. Indeed, a similar value, 0.480, emanates from the manufacturer's specification of pore volume (1.07 ml/g) for the present beads, the density of glass (2.6 g/ml) and consideration of the volume of glass to be $(V_t - V_A^*)$. For the present column with a total volume of 4.15 ml we shall therefore take V_0 as 2.00 ml, which corresponds to a value of 0.482 for V_0/V_t. On the basis of the above magnitudes for V_0, V_A^* and $d\sigma_A^2/dF$, the partition rate constant for efflux of *p*-nitrophenylmannoside from the stationary phase of CPG 3000 (k_{-p}) is 4.79 s^{-1}.

Consideration of the results for *p*-nitrophenylmannoside obtained from advancing (○) and trailing (□) elution profiles on the affinity column in terms of eqn. 4 leads to essentially linear dependences of k_{-1}^{obs} upon [A] (Fig. 1b), the ordinate intercepts of which signify a rate constant (k_{-1}) of 0.40 (±0.05) s^{-1} for dissociation of the saccharide from its complex with immobilized concanavalin A. Furthermore, the corresponding analysis of results obtained from the advancing profile in the presence of 100 μM methylmannoside (●, Fig. 1b) also extrapolates to the same intercept. Comparison of these findings with those from the previous empirical analysis (Fig. 7 of ref. 5) reveals general agreement. First, essentially the same dissociation rate constant (0.40 *cf.* 0.42 s^{-1}) is obtained by use of the analytical expression encompassing the combined effects of chemical and partition kinetics, thereby establishing the adequacy of the simple subtractive procedure adopted previously [5]. Secondly, an essentially identical ordinate intercept from results obtained in the presence of competing saccharide contrasts with the previous experimental finding, but confirms the inference drawn from simulations of affinity chromatographic migration (Fig. 4 of ref. 5) that the two ordinate intercepts should coincide. Thirdly, in keeping with previous findings, both experimental and simulated, the concentration dependence of k_{-1}^{obs} in the presence of competing saccharide is smaller. It is therefore of interest to ascertain whether the ratio of these two slopes can be used to deduce the magnitude of k_{-2} by comparison with the corresponding ratios from results of simulations of chromatographic behaviour for a range of k_{-2} values.

In numerical simulations of the affinity chromatography in the absence and presence of competing saccharide B (100 μM) k_{-1} has been taken as 0.42 s^{-1} (Fig. 1b), k_{-p} as 4.79 s^{-1} and k_{+p} as 3.49 s^{-1}; k_{-2} ranged between 0.2 and 2.0 s^{-1}. Parameters carried over from the previous

investigations included $K_{AX} = 24\,000\ M^{-1}$ [5,12] and $K_{AB} = 9\,000\ M^{-1}$ [5]. Whereas previously the volume increment could be set at 10% of the mobile-phase volume in a segment, a much smaller transfer volume had to be used to accommodate the 20-fold larger magnitudes of the partition rate constants without violating the validity of the substitution of $\delta c/\delta t$ for $\partial c/\partial t$ in the numerical integration of continuity equations [13–16].

As in the previous simulations [5] the dependences of k_{-1}^{obs} upon concentration of *p*-nitrophenylmannoside in the presence and absence of inhibitor exhibited a common ordinate intercept (as in Fig. 1b), and a ratio of slopes dependent upon the magnitude assigned to k_{-2}, the rate constant for dissociation of the methylmannoside–matrix complex (Fig. 2). On the basis of this calibration plot and the slope ratio of 0.36 (±0.03) inferred from the experimental results (Fig. 1b), the rate constant for the dissociation of methylmannoside from immobilized concanavalin A is 0.8 (±0.2) s^{-1}, which substantiates the value of 1 s^{-1} reported previously [5].

By confirming the findings of its predecessor [5], this revised analysis has served two useful roles. First, it certainly verifies the adequacy of the previous empirical approach to allowance for the contribution of partition kinetics to the flow-rate dependence of the variance of the eluted boundary of partitioning solute. More importantly, however, it draws attention to the availability of an analytical expression for making such allowance, and thereby eliminates the need for the earlier empiricism. Finally, this study has highlighted the existence of two different conventions for description of the solute–matrix interaction. From the thermodynamic viewpoint it is immaterial whether the solute–matrix interaction is considered to occur throughout the volume accessible to the partitioning solute or to be restricted to the accessible volume of the stationary phase. Consequently, awareness of the convention pertaining to a particular quantitative expression is important in any attempt to assign physical meaning to an experimental parameter derived therefrom.

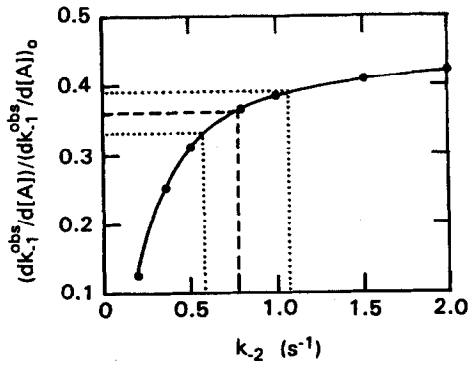

Fig. 2. Revised calibration plot (*cf.* Fig. 5 of ref. 5), based on results of numerical simulations, for evaluating k_{-2}, the rate constant for dissociation of methylmannoside from immobilized concanavalin A, from its effect on the affinity chromatographic behaviour of *p*-nitrophenylmannoside. Broken lines indicate the magnitude of the experimentally determined ratio of slopes (Fig. 2b) and the consequent estimate of k_{-2}: dotted lines define the error envelope associated with the extent of experimental uncertainty inherent in the experimentally evaluated slope.

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