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ANALYTICAL AFFINITY CHROMATOGRAPHY

I. LOCAL EQUILIBRIUM THEORY AND THE MEASUREMENT OF ASSOCIATION AND INHIBITION CONSTANTS

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SUMMARY

Affinity chromatography can be used as an analytical tool to measure the binding constants for biological interactions. Most chromatography theories are based on the assumption that the equilibrium isotherm is linear, a poor assumption for most biological interactions. This paper presents a local-equilibrium theory for nonlinear zonal and frontal elution chromatography. Results are compared to other commonly-used affinity chromatography elution equations. The effect of isotherm nonlinearity is shown to be relatively small, provided the product of the association constant and feed concentration is less than 1. The major effect of nonlinearity is zone spreading; this can be very important when measuring rate constants. The measurement of binding kinetics by analytical affinity chromatography is discussed in Part II.

INTRODUCTION

In the last ten years affinity chromatography has been gaining popularity as an analytical tool for studying kinetics and thermodynamics of biological interactions[1,2]. In affinity chromatography the partitioning of a solute between the liquid and solid phases, and therefore the retention time in a column, is governed by the strength of binding of the soluble biochemical to immobilized ligands. The rates of diffusion and binding kinetics, on the other hand, govern the spreading of a peak or breakthrough curve. Thus by studying the positions and shapes of effluent concentration profiles one can determine equilibrium binding constants and rate constants for the interaction between the solute and immobilized ligand.

Substances that facilitate or compete with the solute–ligand interaction will influence the solute retention time in an affinity column. Thus affinity experiments can be used to identify inhibitors, immunological cross-reactivities, and in general to

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identify conditions that inhibit or enhance biological binding. The experiments can be performed quantitatively to evaluate binding constants and inhibition constants under a variety of conditions, including temperature, pH, or ionic strength. Dunn[3] has recently published a useful review of the literature on analytical affinity chromatography.

The purpose of this paper is to point out some inconsistencies in and clarify some issues that have arisen from the theoretical treatments of analytical affinity chromatography. A number of elution volume equations have been derived in order to extract equilibrium information from affinity experiments. Most of these equations have been derived under the assumption that the equilibria among all interacting species is reached instantaneously (the local-equilibrium assumption). The resulting equations then relate the elution volume of a solute peak or breakthrough curve to the concentrations of immobilized and soluble ligand, inhibitor, etc. and to the association constants for these interactions. Relaxation of this local equilibrium assumption is discussed in Part II of this paper[4] in connection with the use of analytical chromatography for determining rate constants for biological binding processes.

Another popular assumption has been that of linear equilibrium behavior. In most analytical chromatography the quantity of solute in the sample is very small, and one is nearly always operating in the low-concentration regime where the equilibrium isotherm relating the concentration in the stationary phase to that in the mobile phase is linear. This assumption is rarely justified for affinity chromatography, however. The reason is that the strength of the specific biological interactions is often so great that significant curvature of the isotherm exists over the concentration range of the experiment ($K_L c > 1$). In this paper we will discuss the effect that this equilibrium nonlinearity has on the results of a given experiment and on the correct interpretation of the data.

LOCAL EQUILIBRIUM THEORY

Two fundamentally different procedures for performing analytical affinity experiments are: zonal and frontal elution. In zonal elution experiments the solute is introduced as a (short) pulse, and the elution volume is determined from the first moment of the effluent peak. In frontal elution solute is introduced continuously until the column is completely saturated. The elution volume is then determined by integrating behind the entire effluent concentration profile (the breakthrough curve).

Nichol *et al.*[5] have derived a set of elution volume equations for frontal analysis. They considered several possible schemes for solute binding to free ligand or inhibitor and immobilized ligand. The frontal experiments differ from zonal experiments when the equilibrium relationships are nonlinear, as will be shown below. Therefore equations derived for frontal analysis cannot necessarily be used to interpret zonal data. Although frontal analysis is intrinsically a better technique for determining elution volumes, it suffers from the disadvantage that a larger amount of the solute is needed to saturate the column.

Dunn and Chaiken[6], among others, have derived elution volume equations for zonal experiments. Their equations are strictly valid only for *linear* equilibria, that is, for solute concentrations such that $K_L c \ll 1$. This criterion is often practically very difficult to fulfill for biological interactions that are characterized by rather large

association constants: the concentrations required are so small that accurate peak detection can be problematic. The reason that Dunn and Gilbert[7] were successful in comparing their zonal and frontal results was that the interaction was so weak ($K_L \approx 10^2\ M^{-1}$) that they were operating in the linear range where the zonal and frontal theories converge. Some additional important assumptions implicit in these equations and their application to affinity experiments will be discussed below.

The nature of the concentration problem in zonal elution is explained simply as follows: when the equilibrium isotherm is nonlinear, the partition coefficient for the solute in the mobile and stationary phases, and therefore the velocity of the zone, is a function of the local concentration. Ordinary zone spreading that results from diffusional processes, from slow kinetics, or from mixing causes local concentrations to decrease as the zone moves along the column. (It will be shown later that zone spreading can occur even in the limit of local equilibrium.) As a result, the zone velocity is continually decreasing along the column and does not reach a constant value until the local concentrations reach the linear portion of the equilibrium isotherm. Thus the binding constants one would measure from zonal experiments can be expected to depend on the length of the columns used as well as on the concentration in the same pulse. The equilibrium constants would also depend on parameters such as the particle size that increase or decrease zone spreading. Only in the limit of linear partitioning is the zonal elution experiment used to measure equilibrium constants valid, unless, of course, the concentration changes are taken into account by continuously calculating the zone velocities at every column position.[4]

To illustrate this we will start with a widely-used equation[5,6] for the elution volume V_e of a solute P binding to ligand sites L on the particles. To keep the exposition as simple as possible, we will consider here only the binding of P to immobilized ligand with no inhibitor present.

$$V_e = V_0 + \bar{K}_{av} V_s \tag{1}$$

The interaction is described by

$$[P] + [L] \rightleftharpoons [PL] \qquad K_L = \frac{[PL]}{[P][L]} \tag{2}$$

And $\bar{K}_{av}$ is given by

$$\bar{K}_{av} = \left.\frac{[\bar{P}]}{[P]}\right|_{\text{equilibrium}} \tag{3}$$

$[\bar{P}]$, the concentration of P inside the particles, is the sum of the solute bound to affinity sites, which we will call $\rho_p q_P$, and that in the mobile phase within the pores, βc_{iP}. β is the volume fraction of the particle that is available to the diffusing solute, and c_{iP} is the concentration of P in the pore liquid, averaged over the entire particle. [P] is the bulk mobile phase solute concentration, which we will call c_P. V_0 is the column void volume, and V_s is the volume of the beads, equal to the total volume v minus V_0. If ε is the void fraction of the column, then $V_0 = \varepsilon v$ and $V_s = (1 - \varepsilon)v$. Using this nomenclature, eqn. 1 becomes

$$V_e = \left\{\varepsilon + (1 - \varepsilon)\beta + (1 - \varepsilon)\frac{\rho_p q_P}{c_P}\right\}_{\text{equilibrium}} v \tag{4}$$

where we have assumed that at equilibrium $c_{iP} = c_P$. In the absence of adsorption ($q_P = 0$), we retrieve the familiar gel filtration result that says that the elution volume is equal to the fraction of the column available to the solute.

For linear adsorption equilibria ($q_P = Kc_P$), eqn. 4 becomes

$$V_e = \{\varepsilon + (1 - \varepsilon)\beta + (1 - \varepsilon)\rho_p K\} v \tag{5}$$

This is the retention volume obtained from linear theory[8], in the limit of very short pulses.

Up until this point there is no problem with using these results for both zonal and frontal elution because the elution volumes are independent of the mobile phase concentration. However, this is no longer the case when the equilibrium relation is nonlinear. For example, let us look at the Langmuir isotherm, which one obtains from interactions that are described by eqn. 2. In eqn. 2 [PL] is the concentration of bound P, which we have called $\rho_p q_P$. [L] is the concentration of uncomplexed, active immobilized ligand, which is the difference between the maximum number of accessible binding sites and those that are occupied, $\rho_p(Q_{max} - q_P)$. From eqn. 2, at equilibrium we obtain the familiar Langmuir equilibrium relation

$$q_P = \frac{Q_{max} K_L c_P}{1 + K_L c_P} \tag{6}$$

where K_L is the Langmuir constant, or association constant, of the binding interaction. Substituting this into eqn. 4 gives

$$V_e = \left\{\varepsilon + (1 - \varepsilon)\beta + \frac{(1 - \varepsilon)\rho_p Q_{max} K_L}{1 + K_L c_P}\right\} v \tag{7}$$

We see from eqn. 7 that for nonlinear equilibria the elution volume depends on some mobile phase solute concentration. In frontal elution experiments, this equilibrium concentration is known: it is the concentration of P in the feed. This is the value of c_P everywhere in the column at equilibrium. In zonal experiments, however, the value is always less than the concentration in the feed pulse and it is constantly changing as the pulse moves along the column. It is clear from eqn. 7 that eqn. 1 is strictly valid for zonal elution when $K_L c_P \ll 1$, that is, when one operates in the linear portion of the equilibrium isotherm. Since the binding constants for most biological interactions are large, the pulse concentrations must be extremely small in order to use the zonal equations. The frontal elution equations, on the other hand, are valid for all concentrations. The frontal and zonal equations become equivalent in the low concentration limit.

At this point we will digress slightly in order to consider the equations for elution volume that are commonly used to interpret data from analytical affinity

experiments. Dunn gives the following equation for the elution volume of a solute in the absence of competing ligand (eqn. 9 of ref. 3):

$$V_e = V' + (V' - V_0)\frac{[\text{I–L}]}{K_{IL}} \tag{8}$$

where V' is the unretarded elution volume for the solute [$\{\varepsilon + (1 - \varepsilon)\beta\}v$ in our nomenclature], and V_0 is the void volume ($= \varepsilon v$). K_{IL} is the dissociation constant for the interaction. If we recast eqn. 8 into the nomenclature of this paper, we see that several discrepancies exist between the result, eqn. 9, and eqn. 7.

$$V_e = \left\{\varepsilon + (1 - \varepsilon)\beta + (1 - \varepsilon)\beta\frac{[\text{I–L}]}{K_{IL}}\right\}v \tag{9}$$

First of all, the assumption implicit in using eqn. 8 is that one is operating in the linear regime ($K_L c_P \ll 1$). [I–L], the concentration of immobilized ligand in eqn. 8, is equivalent to $\rho_p Q_{max}$, the total concentration of accessible sites. $\rho_p Q_{max}$ must be determined from the plateau of the Langmuir isotherm or, better, from the intercept of its double-reciprocal plot[9]. Obtaining $\rho_p Q_{max}$ by hydrolysis of the gel[7] will lead to serious errors in the determination of the equilibrium constants since the number of accessible sites is often but a fraction of the total immobilized ligand. Nor should $\rho_p Q_{max}$ be equated to the binding capacity of the gel under the conditions of the zonal experiment: since the solute concentrations are low, the capacity thus measured would be just a fraction of the binding capacity at infinite concentration [$q = Q_{max}K_L c/(1 + K_L c) \ll Q_{max}$ when $K_L c \ll 1$].

Lastly, the dissociation constant K_{IL} in eqn. 8 is equal to β/K_L, and *not* to $1/K_L$. This is true because K_{IL} was defined in the original derivation by Dunn and Chaiken[6] as the dissociation constant of the interaction between the immobilized ligand and solute that has partitioned into the liquid inside the bead. The solute concentration in the liquid inside the pores, based on the particle volume, is βc_P at equilibrium, not c_P. The dissociation constant defined by Dunn and Chaiken is then the product of β and the intrinsic dissociation constant. As a result of this, dissociation constants measured using eqn. 8 will depend on the porosities of the particles used to immobilize the ligand. In fact, if the experiment is performed on solid beads ($V' = V_0$; $\beta = 0$), then eqn. 8 predicts that no retardation occurs, which is not the case. Values of K_{IL} obtained from eqn. 8 will be artificially small and will not be directly comparable to solution values, even when the intrinsic dissociation constants are equal. This problem does not arise with eqn. 7.

One may wonder whether the average solute concentration in the pore liquid does equal the bulk solute concentration at equilibrium, as assumed in this argument. It is possible that the *local* concentration is smaller, as a result of exclusion effects at the pore entrances. If the pores are large compared to the diffusing solute, then little effect on the equilibrium concentration would be expected. However, if the pores are very small, on the order of the size of the solute, then the local concentration of solute around the immobilized ligand may be appreciably smaller than in the bulk solution. There also may be some local concentration effects at the pore surface itself

that result from hydrodynamic or electrostatic interactions with the pore walls and the immobilized ligands. Thus one can argue that equilibrium constants measured by affinity chromatography will differ from those measured in solution, even if the intrinsic association constants are equal and β is known.

The preceding discussion pertains to K_{IL} in all of the zonal elution equations listed in the Dunn review[3].

INHIBITION EXPERIMENTS

Once K_L and $\rho_p Q_{max}$ have been determined by, for example, frontal elution experiments over a range of solute concentrations c_0, the effects of inhibitors or competing soluble ligands can be quantitatively assessed. Once again, the elution volume equations that have been derived for competition experiments often assume that the isotherm is linear. Consider the following binding scheme

$$\begin{aligned} P + L &\rightleftharpoons PL \qquad K_L = \frac{[PL]}{[P][L]} \\ P + I &\rightleftharpoons PI \qquad K_I = \frac{[PI]}{[P][I]} \end{aligned} \tag{10}$$

where P and L are, as before, the solute and immobilized ligand, and I is a soluble inhibitor or competing ligand, present at constant concentration c_I. The elution volume equation for this case is given by Dunn (eqn. 4 of ref. 3) for linear equilibria and $c_I \gg c_0$. The more general equation for both linear and nonlinear equilibria is

$$V_e = \left\{\varepsilon + (1 - \varepsilon)\beta + \frac{(1 - \varepsilon)\rho_p Q_{max} K_L}{1 + K_I c_I + K_L c_0}\right\} v \tag{11}$$

where $c_I \gg c_0$. This can be rearranged in the usual way:

$$\frac{v}{V_e - \{\varepsilon + (1-\varepsilon)\beta\}v} = \frac{1 + K_L c_0}{(1-\varepsilon)\rho_p Q_{max} K_L} + \frac{K_I c_I}{(1-\varepsilon)\rho_p Q_{max} K_L} \tag{12}$$

A plot of the left-hand side *versus* c_I for frontal analysis should give a straight line with intercept $(1 + K_L c_0)/(1 - \varepsilon)\rho_p Q_{max} K_L$ and slope $K_I/(1 - \varepsilon)\rho_p Q_{max} K_L$. c_0 is of course kept constant during these experiments. The quantity $(1 - \varepsilon)\rho_p Q_{max} K_L$ should be determined independently in the absence of inhibitor by a double reciprocal plot of elution volumes at various c_0, as described earlier.

Malanikova and Turkova[10], in a widely referenced paper on analytical affinity chromatography, compared results obtained from frontal and zonal elution inhibition experiments. The elution volume equations they used to interpret their data assume linear equilibria, although they were clearly operating in the nonlinear regime. In order to evaluate $(1 - \varepsilon)\rho_p Q_{max}$, the concentration of accessible binding sites, a pulse of concentrated protein was applied to the column. They then eluted at low pH and used the amino acid content of the eluted protein to determine the amount that had been bound to the affinity beads. In this way they measured the concentra-

tion of binding sites to be $1.2 \cdot 10^{-5}$ *M*. This procedure is incorrect for several reasons. In the first place, the amount of solute bound to a column in such an experiment depends on the concentration of solute in the pulse and how that pulse spreads out in the column. The *maximum* number of binding sites is filled only in the limit $c_0 \rightarrow \infty$. What is more important, in order to find the equilibrium uptake, one must be sure to saturate the column. This means that the solute must be fed continuously in a frontal analysis mode until the outlet concentration equals the feed concentration. The capacity at that particular c_0 can then be found from the area behind the breakthrough curve (in the absence of inhibitor). For example, Malanikova and Turkova[10] show a breakthrough profile for trypsin adsorbing onto *p*-aminobenzamidine-NH_2-Spheron in their Fig. 4. The area behind the curve for $c_1 = 0$ is roughly 41 ml. The volume accessible to a tracer is 8.5 ml, and therefore the protein in 32.5 ml of feed was adsorbed onto their column. At a feed concentration of 0.15 mg/ml = $6.25 \cdot 10^{-6}$ *M* the total uptake was 4.88 mg ($2.03 \cdot 10^{-7}$ moles). As the total column volume was 9.55 ml, $(1 - \varepsilon)\rho_p q_P = 2.1 \cdot 10^{-5}$ *M*. In other words, the column capacity under these conditions was 75% higher than the "working capacity" that these authors measured.

The equilibrium capacity measured from the breakthrough profile is still not equal to $(1 - \varepsilon)\rho_p Q_{max}$. However, we can estimate $(1 - \varepsilon)\rho_p Q_{max}$ from the equation from the Langmuir isotherm (eqn. 6) and K_L. If we use the association constant for the complex formed between trypsin and free *p*-aminobenzamidine ($5.3 \cdot 10^4$ M^{-1}), we find

$$(1-\varepsilon)\rho_p Q_{max} = (1-\varepsilon)\rho_p q_P \left(\frac{1 + K_L c_0}{K_L c_0}\right) = 8.5 \cdot 10^{-5}\ M$$

This error in $(1 - \varepsilon)\rho_p Q_{max}$ accounts for the high K_L observed and attributed by Malanikova and Turkova[10] to nonspecific adsorption. The value of $5.3 \cdot 10^4$ M^{-1} for K_L is consistent with the data if they are interpreted using eqn. 12.

The same problem appears in the zonal experiments. Since the retardation of the solute peak is proportional to the product of K_L and the number of accessible binding sites, a value of Q_{max} that is too low would lead to a measured association constant that is too high by the same factor. The zonal data of Malanikova and Turkova[10] show significant nonlinear equilibrium effects: asymmetries in the exiting peaks and tailing that makes calculation of the peak moments difficult. These authors erroneously equated the volume at the peak maximum to the elution volume, a procedure that underestimates the true elution volume. Frontal analysis is clearly preferable in this situation.

EXACT SOLUTION FOR NONLINEAR LOCAL-EQUILIBRIUM ZONAL ELUTION

Eqn. 1 is not a valid starting point for nonlinear zonal chromatography (for $K_L c_P \geq 1$). Even if the diffusion and binding kinetics are very fast and local equilibrium can be assumed, the square pulse shape is not conserved as the pulse moves down the column. In systems with nonlinear isotherms, the pulse instead develops a front that moves at a certain concentration-dependent velocity. It has a slower-moving trailing edge that serves to dilute the pulse as it progresses. This is clear from

looking at the solution to the differential equations for single-solute nonlinear chromatography[11]. Instead of starting from eqn. 1, we will look at the governing partial differential equations to find the solution for the exiting concentration profile for a single solute pulse and Langmuir equilibrium. The governing equations are

$$u_0 \frac{\partial c_P}{\partial z} + \varepsilon \frac{\partial c_P}{\partial t} + (1 - \varepsilon) \frac{\partial s}{\partial t} = 0 \tag{13}$$

$$s = \rho_p q_P + \beta c_P = \frac{\rho_p Q_{max} K_L c_P}{1 + K_L c_P} + \beta c_P \tag{6a}$$

Eqn. 13 is the continuity relation for a solute P in a packed bed, and eqn. 6a states that equilibrium is reached between the mobile and stationary phases at every point in the column. The particle concentration s includes both the adsorbed material and the solute in the pores of the affinity particles. The inlet solute pulse has concentration c_0 and is of duration t_0.

$$c(0,t) = \begin{cases} c_0 & 0 \leqslant t \leqslant t_0 \\ 0 & t > t_0 \end{cases} \tag{14}$$

The solution to this problem is straightforward using the method of characteristics[11]. For a column of length L, the elution times for the pulse front (t_f) and the trailing edge (t_e) are

$$t_f = t_0 + \frac{L}{u_0}\left\{\varepsilon + (1-\varepsilon)\beta + \left(\sqrt{(1-\varepsilon)\rho_p Q_{max} K_L} - \sqrt{t_0 u_0 K_L c_0 / L)}\right)^2\right\} \tag{15}$$

$$t_e = t_0 + \frac{L}{u_0}\{\varepsilon + (1-\varepsilon)\beta + (1-\varepsilon)\rho_p Q_{max} K_L\} \tag{16}$$

The concentrations are given by

$$K_L c = \sqrt{\frac{\rho_p (1-\varepsilon) Q_{max} K_L}{u_0 (t - t_0) - \{\varepsilon + (1-\varepsilon)\beta\} L}} - 1 \qquad (t_f \leqslant t \leqslant t_e) \tag{17}$$

If

$$t_0 > \rho_p (1-\varepsilon) Q_{max} K_L \frac{L}{u_0} \frac{K_L c_0}{(1 + K_L c_0)^2}$$

then the concentrations are given by

$$c = \begin{cases} c_0 & (t_f \leqslant t \leqslant t_x) \\ \text{eqn. 17} & (t_x \leqslant t \leqslant t_e) \end{cases} \tag{18}$$

where

$$t_f = \frac{L}{u_0}\left\{\varepsilon + (1-\varepsilon)\beta + \frac{(1-\varepsilon)\rho_p Q_{max} K_L}{(1 + K_L c_0)}\right\}$$

$$t_x = t_0 + \frac{L}{u_0}\left\{\varepsilon + (1-\varepsilon)\beta + \frac{\rho_p(1-\varepsilon)Q_{max}K_L}{(1 + K_L c_0)^2}\right\}$$

The time at which the trailing edge concentration falls back to zero, t_e, is equal to the elution time of the trailing edge in the linear theory (eqn. 5). This is not surprising since at the trailing edge the solute concentration has become so small that $K_L c \ll 1$.

Representative chromatograms are plotted in Fig. 1. Unlike the behavior for linear equilibria, the pulse retention times and shapes depend on the initial concentration c_0. In the absence of dispersive effects caused by departures from equilibrium, a sharp pulse front moves along the column followed by a more dilute tail. The velocity of the trailing edge is less than that of the leading edge, and the pulse is diluted as it moves down the column. Thus for nonlinear equilibria there is band spreading that arises independently of diffusion and mixing. This has important consequences when one wishes to use zone spreading to determine kinetic constants.

In the limit of very large pulses ($t_0 \to \infty$), one obtains the frontal elution result, eqn. 7.

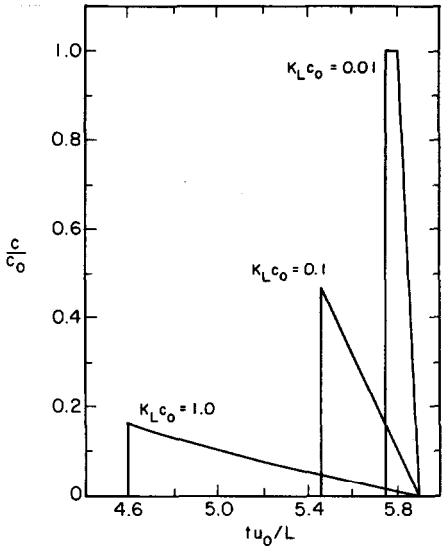

Fig. 1. Chromatograms calculated for nonlinear local-equilibrium theory (eqns. 15–18). $\varepsilon + (1-\varepsilon)\beta = 0.8$; $(1-\varepsilon)\rho_p Q_{max} = 5$; $t_0 u_0/L = 0.1$. The concentration of the pulse has a strong influence on both the shape and position of the exiting peak.

COMPARISON BETWEEN THE LINEAR AND NONLINEAR THEORIES

We would like to know the error to be expected if one nonetheless uses eqn. 5, the linear equation, to determine association constants for nonlinear systems. To assess this we will compare the elution time of the centers of gravity of the exiting pulses for a range of values of $K_L c_0$. This elution time is just the average retention time or first moment of the peak, defined by

$$\mu_1 = \frac{\int_0^\infty c(L,t)t\,dt}{\int_0^\infty c(L,t)\,dt} \tag{19}$$

(Defining the elution volume as the volume at which the peak concentration is a maximum can lead to serious errors when the peaks are asymmetric.) Performing the integration of eqn. 19, we can calculate μ_1 for pulses of various feed concentrations in order to see the effect of the isotherm nonlinearity on pulse retention times. Results are plotted in Fig. 2 for $(1-\varepsilon)\rho_p Q_{max} K_L = 5$ along with the retention times calculated for frontal experiments (eqn. 7) and linear theory.

Up to concentrations such that $K_L c_0 \approx 1$, there is little concentration effect

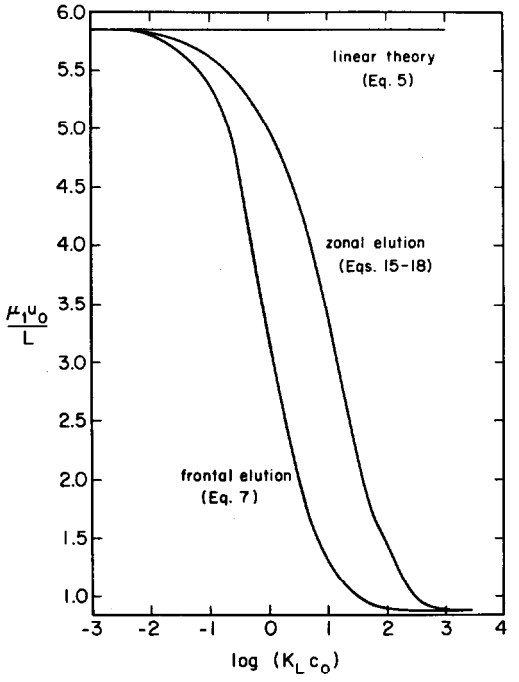

Fig. 2. Average elution times for zonal and frontal elution experiments.

on the mean retention time, although from Fig. 1 we see that the shapes of the peaks calculated from the exact nonlinear local equilibrium theory are quite different from the shape of the peak in the linear limit. The approximately 20% difference between K_L obtained from the comparison between the linear and nonlinear theories at $K_L c_0 = 1$ will in a real experiment be even less, because mixing and diffusional band-spreading will aid the dilution process. The effect on retention time quickly increases, however, for $K_L c_0 > 1$.

In frontal analysis the error that would result from using a linear elution volume equation (eqn. 5) instead of the exact ones (eqns. 7 and 12) is of course much greater. When $c_I = 0$ the difference is a factor of $1 + K_L c_0$. In the work of Malanikova and Turkova[10] this factor alone leads to a 33% error.

CONCLUSIONS

Current zonal elution theory is based entirely on the assumption that one is operating in the linear region of the equilibrium isotherm. This assumption may be difficult to justify for biological interactions that have highly nonlinear equilibrium behavior. To investigate nonlinear effects on the equilibrium binding constants one would measure using analytical affinity chromatography, we have used a simple chromatography model: local-equilibrium with a Langmuir isotherm[11]. Upon comparing the results from this model to those from the linear theory, we see that there is relatively little difference in the predicted average retention times, as long as $K_L c_0 < 1$ (Fig. 2). Hethcote and DeLisi[12] have derived an approximate expression for the mean retention time for nonlinear chromatography without the local-equilibrium assumption. They also conclude that the effect of isotherm nonlinearity on retention time is relatively small.

The major effect of isotherm nonlinearity is zone spreading. From the chromatograms plotted in Fig. 1, it is clear that a considerable degree of zone spreading occurs even when diffusion and kinetics are fast. This means that one must be extremely careful to operate in the linear range when using zonal affinity chromatography to measure kinetic constants. An alternative is to include isotherm nonlinearity in a chromatography theory that includes kinetics. To be useful, this theory should be able to distinguish between the effects of finite mass transfer rates and the biological binding kinetics. One such model will be presented in Part II of this paper.[4]

An alternative to the zonal method is frontal elution. Since the concentration c_P at equilibrium is known ($= c_0$) for the frontal case, the exact equations for the elution volume (eqns. 7 and 12) can be used. Therefore frontal elution is not limited to such low concentrations. Frontal methods can also be used to determine kinetic constants.

SYMBOLS

c solute concentration in liquid (M)
c_i average solute concentration in pore liquid (M)
c_{iP} average concentration of P in pore liquid (M)
c_I concentration of inhibitor in liquid (M)
c_0 feed or pulse concentration (M)

c_P concentration of P in liquid (M)
q average sorbate concentration (mmoles g^{-1} particle)
q_P average concentration of sorbed P (mmoles g^{-1} particle)
s average concentration inside particles ($= \rho_p q_P + \beta c_{iP}$)
t_0 pulse length (s)
u_0 liquid superficial velocity (cm s^{-1})
v total column volume (cm^3)
z distance along column (cm)
[I] concentration of inhibitor in liquid ($= c_I$) (M)
[I–L] concentration of immobilized ligand ($= \rho_p Q_{max}$) (mmoles cm^{-3} particle)
K linear equilibrium distribution coefficient (cm^3 g^{-1} particle)
$\bar{K}_{av}$ distribution coefficient defined by eqn. 1 (dimensionless)
K_L Langmuir or association constant (M^{-1})
K_{IL} dissociation constant ($= \beta/K_L$) (M)
L column length (cm)
[L] concentration of uncomplexed ligand [$= \rho_p(Q_{max} - q_P)$] (mmoles cm^{-3} particle)
[P] concentration of solute in liquid ($= c_P$) (M)
[$\bar{P}$] total concentration of P inside particles ($= \rho_p q_P + \beta c_{iP}$) (mmoles cm^{-3} particles)
[PL] concentration of bound solute ($= \rho_p q_P$) (mmoles cm^{-3} particle)
Q_{max} maximum number of available sites (mmoles g^{-1} particle)
V_e elution volume (cm^3)
V_0 column void volume (cm^3)
V_s column solids volume (cm^3)
V' unretarded elution volume [$= \{\varepsilon + (1 - \varepsilon)\beta\}v$] ($cm^3$)
β particle volume fraction available to solute
ε column void fraction
μ_1 average retention time (s)
ρ_p particle density (g cm^{-3} particle)

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