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DETERMINATION OF EQUILIBRIUM AND RATE CONSTANTS BY AFFINITY CHROMATOGRAPHY

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SUMMARY

A theory of column chromatography is developed that includes the interaction of macromolecules with matrix-bound ligand and free ligand distributed throughout the column. The theory differs from previous formulations in its exact treatment of chemical kinetics and mass transfer kinetics. Characteristics of the elution profile — specifically the mean and variance— are expressed in terms of variables such as mobile phase velocity, bed height, ligand concentrations, mass transfer rate constants and chemical reaction rate constants. Equations are developed for monovalent and bivalent binding, for binding to ligands on porous and on impenetrable bed particles and for heterogeneous macromolecules. Previous results for the profile peak appearing in the literature are shown to be special cases of the equations presented here, holding only when the rate constants satisfy certain constraints. Our results therefore broaden the range of applicability of chromatography for the study of macromolecular interactions and precisely define its limits with respect to the determinations of both thermodynamic and kinetic parameters.

INTRODUCTION

A number of recent theoretical and experimental advances have extended the applicability of affinity chromatography to the quantitative study of macromolecular interactions, especially to the determination of enzyme–substrate affinities (*e.g.*, ref. 1 and references cited therein). The affinities thus determined correspond closely to values obtained by more conventional methods such as dialysis, in which the reactive partners are uniformly dispersed in solution. However, chromatographic methods yield two types of equilibrium constants, one for reaction between the protein (*i.e.*, the eluted macromolecule) and solution phase ligand and another for reaction between the protein and bead-bound ligand. These are approximately the same¹, as theory predicts² when the conformation of the ligand is unaffected by attachment to the bead.

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In many biological systems, quantitative characterization of reactions between a solution phase molecule and a molecule bound to a surface (e.g., a cellular receptor or membrane enzyme) is of considerable interest. Such reactions probably play an important role in the regulation of a number of cellular processes (see, e.g., ref. 3 and references cited therein). Although the equilibrium constant may be, to an excellent approximation, independent of whether a reactive partner is cell-bound or dispersed (barring a conformational dependence upon surface properties), rate constants will not be the same^{2,4}. Chromatography would seem to be a natural method for the determination of rate constants in both cases, simultaneously. Moreover, in many systems the molecules of interest are heterogeneous in their affinities and in their rate constants for ligand, and a complete understanding of biological regulation requires quantitative characterization of the heterogeneity distribution⁵⁻⁷.

Although several different methods of varying degrees of complexity are available for determining rate constants, equilibrium constants and their distributions, it is evident that a widely available, simple, fast and reliable method for obtaining all this information simultaneously would be very useful. In this paper we develop the initial stages of an approach to these problems by generalizing the basic theory of chromatography presented in our two previous papers^{8,9}.

Briefly, we consider a cylindrical column packed to a height h with beads to which some ligand is covalently linked. The ligand is also uniformly distributed throughout the column. The beads may be either permeable or impermeable to the protein. The protein may be either homogeneous or heterogeneous in its affinity for the ligand. The analysis is confined to zonal chromatography in which the total volume of protein is very small compared to the column volume, and the initial concentration of protein is very low compared to the total ligand concentration.

The theory is developed for proteins with one or two combining sites. We show that for penetrable beads, previously derived equations relating the peak of the profile to equilibrium constants¹ are special cases of our results that are valid only when the rate of chemical equilibration is rapid compared to the rate of mass transfer equilibration (movement in and out of beads), and when the elution profile is symmetric. We also derive expressions for the dispersion in the profile and its relation to rate constants. The relations between the profile dispersion and rate constants, in contrast to relations between the profile mean and equilibrium constants, are useful only when ligand-protein equilibration rates are slow compared to mass transfer equilibration rates.

The possible masking of rate constants by movement in and out of beads suggests that the use of impenetrable beads will be advantageous for the determination of rate constants. We therefore present the theory for this case. We indicate that the rate constants so obtained may differ substantially from those obtained in well-stirred solutions, even when the reaction mechanisms are identical.

COMPETITIVE ELUTION WHEN LIGAND BINDS MONOVALENTLY TO SITES ON POROUS BEADS

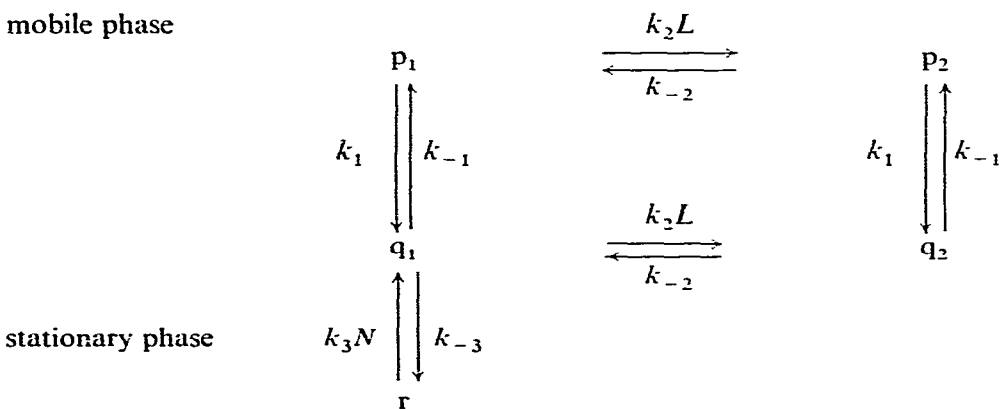
The model

We now formulate an initial boundary value problem that models a small zone affinity chromatography experiment with a single type of molecule in the sample. Some of the notation used here is the same as in ref. 8.

Consider a chromatography column of height h where x measures the distance from the bottom of the bed and u is the velocity in the mobile phase. Let V_0 be the void volume (the volume exterior to the beads) and let V_p be the volume interior to the beads that can be penetrated by the protein molecule under consideration. Assume that the molecule to be studied binds only monovalently to the ligand. The concentration of free ligand in solution is L and the concentration of ligand that is covalently attached to the beads and available for binding protein is N .

Let $p_1(x, t)$ and $p_2(x, t)$ be the probability density functions (per unit column length) at position x at time t that molecules are in the mobile phase and are unbound and bound to free ligand, respectively. Let $q_1(x, t)$, $q_2(x, t)$ and $r(x, t)$ be the probability density functions that molecules are inside the beads and unbound, bound to free ligand and bound to ligand covalently immobilized on the beads, respectively. The rate constants for movement of protein in and out of the beads (*i.e.*, mass transfer) are k_1 and k_{-1} , respectively. For association and dissociation with free ligand they are k_2 and k_{-2} , and for association and dissociation with the bead-bound ligand they are k_3 and k_{-3} . Equilibrium constants are defined by $K_i = k_i/k_{-i}$. The diagram below indicates the transfer rates between the five states:

mobile phase



stationary phase

The size and mass of the ligand are assumed to be small compared to those of the protein so that the protein mass transfer rates are unaffected when it binds solution phase ligand.

Using a conservation of mass approach as described in ref. 8, we have derived the following system of reaction-convection partial differential equations for the probabilities:

$$\frac{\partial p_1}{\partial t} = u \cdot \frac{\partial p_1}{\partial x} - k_1 p_1 + k_{-1} q_1 - k_2 L p_1 + k_{-2} p_2 \tag{1}$$

$$\frac{\partial p_2}{\partial t} = u \cdot \frac{\partial p_2}{\partial x} - k_1 p_2 + k_{-1} q_2 + k_2 L p_1 - k_{-2} p_2 \tag{2}$$

$$\frac{\partial q_1}{\partial t} = k_1 p_1 - k_{-1} q_1 - k_2 L q_1 + k_{-2} q_2 - k_3 N q_1 + k_{-3} r \tag{3}$$

$$\frac{\partial q_2}{\partial t} = k_1 p_2 - k_{-1} q_2 + k_2 L q_1 - k_{-2} q_2 \quad (4)$$

$$\frac{\partial r}{\partial t} = k_3 N q_1 - k_{-3} r \quad (5)$$

These equations do not have diffusion-related terms since we have assumed that the flow-rate, $F = uV_0/h$, is large enough so that the effects of diffusion can be neglected⁸. We showed⁸ that the sorption-desorption (or mass transfer) equilibrium constant, K_1 , satisfies $K_1 = V_p/V_0$.

The initial layer containing sample molecules at the top of the bed is assumed to be sufficiently small so that it can be considered an instantaneous source (small zone) given by a Dirac delta function. Thus the initial conditions are:

$$p_1(x, 0) = \delta(x - h) \quad (6)$$

$$p_2(x, 0) = q_1(x, 0) = q_2(x, 0) = r(x, 0) = 0 \quad x \neq h$$

Since there are no molecules at the top of the bed after the initial layer leaves,

$$p_1(h, t) = p_2(h, t) = q_1(h, t) = q_2(h, t) = r(h, t) = 0 \quad (7)$$

for $t > 0$. The flow at the bottom of the bed where the elution profile $u \cdot p(0, t)$, is measured, is assumed to be the same as if the bed extended below $x = 0$. Thus we solve the initial boundary value problem on the semi-infinite interval $(-\infty, h)$.

The mean of the passage time

As in ref. 8, we use the model given by eqns. 1-7 to obtain an ordinary differential equation for the mean and variance of the passage time. The mean passage time, $T_1(x)$, is defined as the mean time for molecules starting in the initial layer to move past a position x . The mean elution time, $M_e = T_1(0)$, is the mean time for molecules starting at $x = h$ to leave the bottom of the bed. These definitions and those of higher order moments can be expressed formally as^{8,10,11}

$$T_j(x) = \int_0^{\infty} t^j u [p_1(x, t) + p_2(x, t)] dt \quad (8)$$

where $T_j(x)$ is the j th moment of the passage time to position x . Although explicit solutions of the chromatography model in eqns. 1-7 are not available to use in eqn. 8, we can still obtain expressions for the moments. In particular we show in Appendix I that the mean time satisfies

$$\frac{dT_1(x)}{dx} = -(1 + K_1 + \frac{K_1 K_3 N}{1 + K_2 L})/u - \frac{K_1 K_3 N (k_{-1}/k_{-2}) K_2 L}{u (k_{-1}/k_{-2} + 1 + K_2 L) (1 + K_2 L)} \exp[-B(h - x)/u] \quad (9)$$

where

$$B \equiv (k_{-2} + k_2L)[1 + k_1/(k_{-1} + k_{-2} + k_2L)]$$

Integration of eqn. 9 from x to h gives:

$$T_1(x) = \left(1 + K_1 + \frac{K_1K_3N}{1 + K_2L}\right)\left(\frac{h-x}{u}\right) + \frac{K_1K_3N(k_{-1}/k_{-2})K_2L}{u(k_1 + k_{-1} + k_{-2} + k_2L)(1 + K_2L)^2} \left[1 - \exp\left(-B \cdot \frac{h-x}{u}\right)\right] \quad (10)$$

Thus the mean elution time is:

$$M_e \equiv T_1(0) = \left(1 + K_1 + \frac{K_1K_3N}{1 + K_2L}\right)\left(\frac{h}{u}\right) + \frac{K_1K_3N(k_{-1}/k_{-2})K_2L}{u(k_1 + k_{-1} + k_{-2} + k_2L)(1 + K_2L)^2} [1 - \exp(-Bh/u)] \quad (11)$$

Desorption rate constants k_{-1} seem to vary from approximately 0.01 sec^{-1} for larger beads to approximately 100 sec^{-1} for small beads. The reverse rate constants such as k_{-3} for chemical reactions may range from 10^{-5} to 10^3 sec^{-1} (ref. 12). Since chemical reaction kinetics may be much faster or much slower than sorption-desorption kinetics, we now consider these two limiting cases separately.

Fast chemical reactions

If chemical reaction kinetics are fast compared to the movement of molecules in and out of the beads, then a reasonable approximation is obtained if we assume that chemical equilibrium is attained in the mobile phase and in the stationary phase. In this case

$$p \equiv p_1 + p_2, p_1 = p/(1 + K_2L), p_2 = pK_2L/(1 + K_2L) \quad (12)$$

$$S \equiv q_1 + q_2 + r, q_1 = \frac{S}{1 + K_2L + K_3N}, q_2 = \frac{SK_2L}{1 + K_2L + K_3N}, r = \frac{SK_3N}{1 + K_2L + K_3N} \quad (13)$$

so that the five partial differential eqns. 1-5 reduce to the two following equations:

$$\frac{\partial p}{\partial t} = u \cdot \frac{\partial p}{\partial x} - k_1p + \frac{k_{-1}(1 + K_2L)}{1 + K_2L + K_3N} \cdot S \quad (14)$$

$$\frac{\partial S}{\partial t} = k_1p - \frac{k_{-1}(1 + K_2L)}{1 + K_2L + K_3N} \cdot S \quad (15)$$

Since this system of equations is analogous to that analyzed in ref. 8, we use the results there to find that the mean, M_c , and variance, S_c , are:

$$M_c = \left(1 + K_1 + \frac{K_1 K_3 N}{1 + K_2 L}\right) \left(\frac{h}{u}\right) \quad (16)$$

$$S_c = 2K_1 \left(1 + \frac{K_3 N}{1 + K_2 L}\right)^2 \frac{h}{k_{-1} u} \quad (17)$$

Slow chemical reactions

If chemical reaction kinetics are slow compared to mass transfer kinetics, then an approximation is obtained by assuming that mass transfer equilibrium is attained for the unbound molecules and for the molecules bound to free ligand. In this case

$$f \equiv F_1 + q_1 \quad p_1 = \frac{f}{1 + K_1} \quad q_1 = \frac{fK_1}{1 + K_1} \quad (18)$$

$$g \equiv P_2 + q_2 \quad p_2 = \frac{g}{1 + K_1} \quad q_2 = \frac{gK_1}{1 + K_1} \quad (19)$$

so that the five partial differential eqns. 1-5 reduce to the three following equations:

$$\frac{\partial f}{\partial t} = \frac{u}{1 + K_1} \cdot \frac{\partial f}{\partial x} - k_2 L f + k_{-2} g - \frac{k_3 N K_1}{1 + K_1} \cdot f + k_{-3} r \quad (20)$$

$$\frac{\partial g}{\partial t} = \frac{u}{1 + K_1} \cdot \frac{\partial g}{\partial x} + k_2 L f - k_{-2} g \quad (21)$$

$$\frac{\partial r}{\partial t} = \frac{k_3 N K_1}{1 + K_1} \cdot f - k_{-3} r \quad (22)$$

The mean of the passage time can be obtained for these equations in a manner analogous to the procedure used to obtain eqn. 9 from eqns. 1-5. Here we omit most details and give only a few intermediate results and the final results. For notational simplicity we omit the limits on the integrals (0, ∞) and the integration variable indicator dt . Integrating eqn. 22 implies that:

$$\int r = \frac{K_1 K_3 N}{1 + K_1} \int f \quad (23)$$

Integrating the sum of eqns. 20-22 yields:

$$\int \frac{u}{1 + K_1} \cdot (f + g) = 1 \quad (24)$$

The moments of the passage time (eqn. 8) are now:

$$T_j(x) = \int_0^x t^j u(f + g)/(1 + K_1) dt \quad (25)$$

Integrating t times the sum of eqns. 20–22 leads to

$$\begin{aligned} \frac{dT_1(x)}{dx} &= - \int (f + g) - \int r \\ &= \frac{-(1 + K_1)}{u} - \frac{K_1 K_3 N}{u(1 + K_2 L)} \cdot [1 - K_2 L e^{-B(h-x)/u}] \end{aligned} \quad (26)$$

where $B = (k_{-2} + k_2 L)(1 + K_1)$. Integrating eqn. 26 from 0 to h yields:

$$\begin{aligned} M_e = T_1(0) &= \left(1 + K_1 + \frac{K_1 K_3 N}{1 + K_2 L}\right) \left(\frac{h}{u}\right) + \\ &+ \frac{K_1 K_2 L K_3 N}{(1 + K_1) k_{-2} (1 + K_2 L)^2} \cdot (1 - e^{-Bh/u}) \end{aligned} \quad (27)$$

The second moment can be found by a procedure similar to that used in ref. 8. Integrating t^2 times the sum of eqns. 20–22 yields:

$$\frac{dT_2(x)}{dx} = - \frac{2(1 + K_1)T_1(x)}{u} - \frac{2K_1 K_3 N}{1 + K_1} \int t f - \frac{2K_1 K_3 N}{(1 + K_1) k_{-3}} \int f \quad (28)$$

The variance $S(x) = T_2(x) - T_1^2(x)$ satisfies:

$$\frac{dS}{dx} = - \frac{2K_1 K_3 N}{(1 + K_1) k_{-3}} - \frac{2K_1 K_3 N}{1 + K_1} \int t f + 2K_1 T_1(x) \int f \quad (29)$$

Solving simultaneous differential equations similar to A6 and A7 leads to:

$$\begin{aligned} \int t f &= \frac{(1 + K_1)^2 \left[1 + \frac{K_1 K_3 N}{(1 + K_1)(1 + K_2 L)} \right] (h - x)}{u^2 (1 + K_2 L)} + \\ &+ \frac{2K_1 K_3 N K_2 L}{u(1 + K_2 L)^3 k_{-2}} \cdot [1 - e^{-B(h-x)/u}] + \\ &+ \frac{K_2 L [(1 + K_1)(1 + K_2 L) + K_1 K_3 N K_2 L] (1 + K_1)}{u^2 (1 + K_2 L)^2} \cdot (h - x) e^{-B(h-x)/u} \end{aligned} \quad (30)$$

Thus eqn. 29 becomes:

$$\frac{dS}{dx} = - \frac{2K_1K_3N}{(1+K_1)u} \left\{ \frac{K_1K_3NK_2L}{(1+K_2L)^3k_{-2}} + \frac{(1+K_1)K_2L}{k_{-1}(1+K_2L)} + \left[\frac{K_2L(1+K_1)}{k_3(1+K_2L)} - \frac{K_1K_3NK_2L}{(1+K_2L)^2k_{-2}} \right] \cdot e^{-B(h-x)/u} + \frac{K_1K_3N(K_2L)^2}{(1+K_2L)^3k_{-2}} \cdot e^{-2B(h-x)/u} \right\} \quad (31)$$

Integrating eqn. 31 from 0 to h , we find

$$S_e = S(0) = \frac{2K_1K_3N}{(1+K_2L)} \left[\frac{1}{k_{-3}} + \frac{K_1K_2L K_3N}{(1+K_1)k_{-2}(1+K_2L)^2} \right] \left(\frac{h}{u} \right) + \frac{K_2L}{k_{-2}(1+K_2L)^2} \left[\frac{1}{k_{-3}} - \frac{K_1K_3N}{(1+K_1)k_{-2}(1+K_2L)^2} \right] (1 - e^{-Bh/u}) + \frac{K_1(K_2L)^2K_3N}{2(1+K_1)k_{-2}(1+K_2L)^2k_{-3}} \cdot (1 - e^{-2Bh/u}) \quad (32)$$

where $B = (k_{-2} + k_2L)(1 + K_1)$.

When there is no free ligand ($L = 0$), the mean and variance simplify significantly to:

$$M_e = (1 + K_1 + K_1K_3N)h/u \quad (33)$$

$$S_e = \frac{2K_1K_3N}{k_{-3}} \cdot \frac{h}{u} \quad (34)$$

COMPETITIVE ELUTION WHEN MOLECULES BIND BIVALENTLY TO SITES ON POROUS BEADS

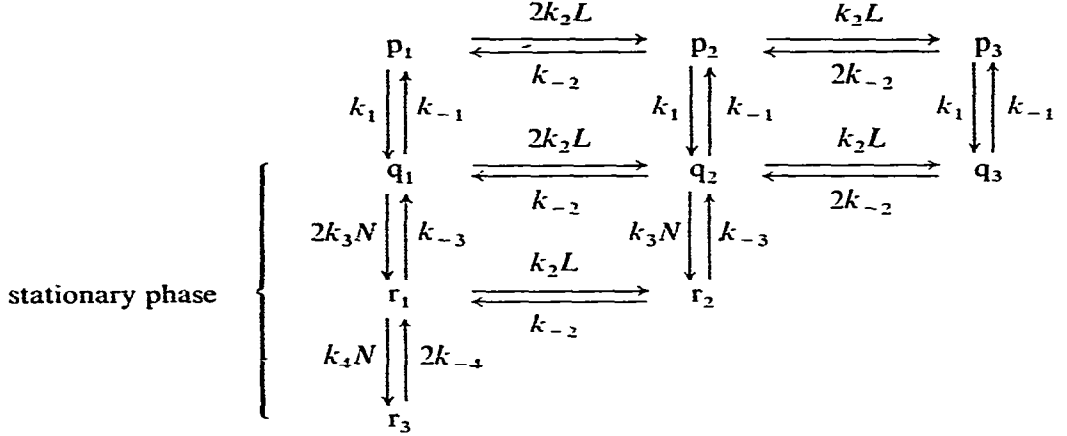
The model

In this section a model is formulated for a small zone affinity chromatography experiment with a homogeneous population of molecules with two identical binding sites. IgG and IgA myeloma proteins fall into this category. The molecules can first bind monovalently and then bivalently to the ligands. As before the concentration of free ligand in the solution is L and the concentration of ligand that is covalently attached to the beads and available for binding protein molecules is N .

Let $p_1(x, t)$, $p_2(x, t)$ and $p_3(x, t)$ be the probability density functions at point x at time t that a molecule in the mobile phase has both sites empty, one site bound to a free ligand and both sites bound to a free ligand, respectively. Let $q_1(x, t)$, $q_2(x, t)$ and $q_3(x, t)$ be the corresponding probability density functions for molecules interior to a bead. Let $r_1(x, t)$, $r_2(x, t)$ and $r_3(x, t)$ be the probability density functions that a

molecule inside a bead has, respectively, one site empty and one bound to an attached ligand, one site bound to a free ligand and one bound to an attached ligand and both sites bound to attached ligands. The transfer rates between the nine states are indicated in the diagram below:

mobile phase



The mass balance equations for this model are:

$$\frac{\hat{c}p_1}{\hat{c}t} = u \cdot \frac{\hat{c}p_1}{\hat{c}x} - k_1 p_1 + k_{-1} q_1 - 2k_2 L p_1 + k_{-2} p_2 \quad (35)$$

$$\frac{\hat{c}p_2}{\hat{c}t} = u \cdot \frac{\hat{c}p_2}{\hat{c}x} - k_1 p_2 + k_{-1} q_2 + 2k_2 L p_1 - k_{-2} p_2 - k_2 L p_2 + 2k_{-2} p_3 \quad (36)$$

$$\frac{\hat{c}p_3}{\hat{c}t} = u \cdot \frac{\hat{c}p_3}{\hat{c}x} - k_1 p_3 + k_{-1} q_3 + k_2 L p_2 - 2k_{-2} p_3 \quad (37)$$

$$\frac{\hat{c}q_1}{\hat{c}t} = k_1 p_1 - k_{-1} q_1 - 2k_2 L q_1 + k_{-2} q_2 - 2k_3 N q_1 + k_{-3} r_1 \quad (38)$$

$$\frac{\hat{c}q_2}{\hat{c}t} = k_1 p_2 - k_{-1} q_2 + 2k_2 L q_1 - k_{-2} q_2 - k_2 L q_2 + 2k_{-2} q_3 - k_3 N q_2 + k_{-3} r_2 \quad (39)$$

$$\frac{\hat{c}q_3}{\hat{c}t} = k_1 p_3 - k_{-1} q_3 + k_2 L q_2 - 2k_{-2} q_3 \quad (40)$$

$$\frac{\hat{c}r_1}{\hat{c}t} = 2k_3 N q_1 - k_{-3} r_1 - k_2 L r_1 + k_{-2} r_2 - k_4 N r_1 + 2k_{-4} r_3 \quad (41)$$

$$\frac{\hat{c}r_2}{\hat{c}t} = k_3 N q_2 - k_{-3} r_2 + k_2 L r_1 - k_{-2} r_2 \quad (42)$$

$$\frac{\partial r_3}{\partial t} = k_4 N r_1 - 2k_{-4} r_3 \quad (43)$$

The initial conditions, boundary conditions, definitions of moments and other assumptions are similar to those in the previous section.

Fast chemical reactions

If the chemical reactions are fast compared to the movement of molecules in and out of beads, then a reasonable approximation is obtained if chemical equilibrium is assumed. In this case

$$p \equiv p_1 + p_2 + p_3, \quad p_1 = \frac{p}{(1 + K_2 L)^2}, \quad p_2 = \frac{p 2K_2 L}{(1 + K_2 L)^2},$$

$$p_3 = \frac{p(K_2 L)^2}{(1 + K_2 L)^2} \quad (44)$$

$$s \equiv q_1 + q_2 + q_3 + r_1 + r_2 + r_3,$$

$$q_1 = \frac{s}{(1 + K_2 L)^2 + 2K_3 N(1 + K_2 L) + K_3 N K_4 N} \quad (45)$$

$$q_2 = 2K_2 L q_1, \quad q_3 = (K_2 L)^2 q_1, \quad r_1 = 2K_3 N q_1, \quad r_2 = K_2 L r_1, \quad r_3 = K_4 N r_1 / 2 \quad (46)$$

so that the nine partial differential equations reduce to the following two equations:

$$\frac{\partial p}{\partial t} = u \cdot \frac{\partial p}{\partial x} - k_1 p + k_{-1} \cdot \frac{(1 + K_2 L)^2}{(1 + K_2 L)^2 + 2K_3 N(1 + K_2 L) + K_3 N K_4 N} \cdot s \quad (47)$$

$$\frac{\partial s}{\partial t} = k_1 p - k_{-1} \cdot \frac{(1 + K_2 L)^2}{(1 + K_2 L)^2 + 2K_3 N(1 + K_2 L) + K_3 N K_4 N} \cdot s \quad (48)$$

Since these differential equations are similar to those analyzed in ref. 8, we can use the results there to obtain:

$$M_c = \left[1 + K_1 + \frac{2K_1 K_3 N}{1 + K_2 L} + \frac{K_1 K_3 N K_4 N}{(1 + K_2 L)^2} \right] \left(\frac{h}{u} \right) \quad (49)$$

$$S_c = \frac{2K_1}{k_{-1}} \left[1 + \frac{2K_3 N}{1 + K_2 L} + \frac{K_3 N K_4 N}{(1 + K_2 L)^2} \right] \left(\frac{h}{u} \right) \quad (50)$$

Without competing free ligand

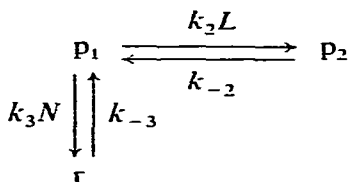
If there is no competing free ligand ($L = 0$), then the nine differential eqns. 35–43 reduce to four differential equations for p_1 , q_1 , r_1 and r_3 . For those equations, the usual procedure leads to:

$$M_e = (1 + K_1 + 2K_1K_3N + K_1K_3NK_4N)\left(\frac{h}{u}\right) \quad (51)$$

$$S_e = 2K_1 \left[\frac{(1 + 2K_3N + K_3NK_4N)^2}{k_{-1}} + \frac{2K_3N(1 + K_4N/2)^2}{k_{-3}} + \frac{K_3NK_4N}{2k_{-4}} \right] \cdot \left(\frac{h}{u}\right) \quad (52)$$

MONOVALENT BINDING TO LIGAND ATTACHED TO IMPENETRABLE BEADS

The model here is similar to the first model described except that the beads are impenetrable and the ligands are covalently attached to their surfaces. Consider a univalent protein and let $p_1(x, t)$, $p_2(x, t)$ and $r(x, t)$ be the probability density functions that it is unbound, bound to a free ligand and has its site bound to a bead ligand, respectively. The transfer diagram is:



The mass balance equations are:

$$\frac{\partial p_1}{\partial t} = u \cdot \frac{\partial p_1}{\partial x} - k_2Lp_1 + k_{-2}p_2 - k_3Np_1 + k_{-3}r \quad (53)$$

$$\frac{\partial p_2}{\partial t} = u \cdot \frac{\partial p_2}{\partial x} + k_2Lp_1 - k_{-2}p_2 \quad (54)$$

$$\frac{\partial r}{\partial t} = k_3Np_1 - k_{-3}r \quad (55)$$

The eqns. 53–55 are similar to eqns. 20–22 so that we can convert the results there to obtain

$$M_e = \left(1 + \frac{K_3N}{1 + K_2L}\right)\left(\frac{h}{u}\right) + \frac{K_2LK_3N}{k_{-2}(1 + K_2L)^2} \cdot (1 - e^{-Bh/u}) \quad (56)$$

$$S_e = \frac{2K_3N}{1 + K_2L} \left[\frac{1}{k_{-3}} + \frac{K_2LK_3N}{k_{-2}(1 + K_2L)^2} \right] \left(\frac{h}{u} \right) + \\ + \frac{K_2L}{k_{-2}(1 + K_2L)^2} \left[\frac{1}{k_{-3}} - \frac{K_3N}{k_{-2}(1 + K_2L)} \right] (1 - e^{-Bh/u}) + \\ + \frac{(K_2L)^2K_3N}{2k_{-2}(1 + K_2L)^2k_{-3}} \cdot (1 - e^{-2Bh/u}) \quad (57)$$

where $B = (k_{-2} + k_2L)$.

When there is no free competing ligand ($L = 0$), these expressions simplify to:

$$M_e = (1 + K_3N)h/u \quad (58)$$

$$S_e = \frac{2K_3N}{k_{-3}} \cdot \frac{h}{u} \quad (59)$$

Denizot and Delaage¹³ used the random walk approach of Giddings and Eyring¹⁴ to develop a theory of affinity chromatography for monovalent binding of molecules to ligands. Their expression for the mean of the elution profile is

$$\text{mean} = E(t') = E(t_0)(1 + k/k') \quad (60)$$

where $E(t_0)$ is the expected elution time for a particle that does not bind, and k/k' is the equilibrium constant of binding (K_3N in our notation). For porous beads $E(t_0) = (1 + K_1)h/u$ so that eqn. 60 in our notation becomes:

$$M_e = (1 + K_1)(1 + K_3N)h/u \quad (61)$$

This is clearly not the same as eqn. 11, 16 or 27 with $L = 0$ so that their theory does not seem to be applicable to porous beads. This is consistent with Chaiken's¹ finding of unrealistic rate constants when the Denizot and Delaage theory was applied to the elution of ribonuclease through a bed of porous beads. The difficulty, as they indicate, is that their formulas assume that mass transfer in and out of the stationary phase is insignificant. In fact, for impenetrable beads $E(t_0) = h/u$ so that eqn. 60 is the same as our result, eqn. 58. The expression of Denizot and Delaage for the variance is

$$\sigma^2 = \frac{2k}{k'^2} \cdot E(t_0) + \left(1 + \frac{k}{k'} \right)^2 \sigma_0^2 \quad (62)$$

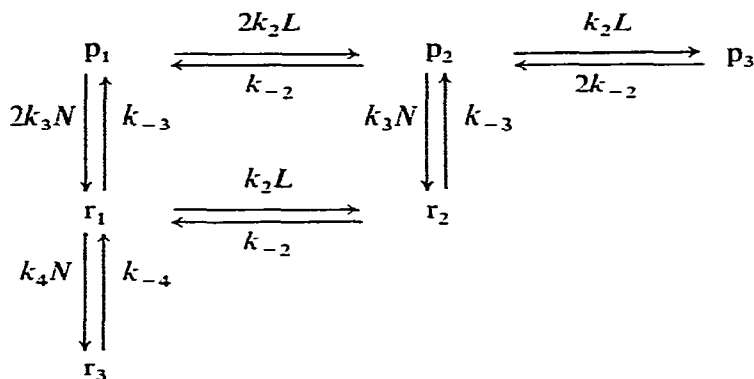
where σ_0^2 is the variance of t_0 . For impenetrable beads $E(t_0) = h/u$ and $\sigma_0^2 = 0$ when the effects of diffusion are negligible so that eqn. 62 in our notation becomes eqn. 59. Thus their results do apply to chromatography with impenetrable beads.

It is important to recognize, however, that the forward and reverse rate constants obtained when one of the reactants is attached to a large particle, such as a bead or a cell, may differ by several orders of magnitude from the values obtained when both reactants are dispersed in solution^{2,4}. The differences are present when the reactions are diffusion limited and are consequences of the Brownian movement of ligand in the presence of a large surface (e.g., a bead)¹⁵. An additional complication is

that in a column chromatography experiment, the reactions may be mediated by transport rather than diffusion. If this is the case, rate constants obtained by chromatography experiments would have to be interpreted entirely differently from rate constants obtained in well stirred solutions, and quantitative agreement between the two would be fortuitous and surprising. Nevertheless, rate constants obtained by chromatography, in which flow is a dominant process, may be more relevant to physiological situations where flow is also dominant, than well stirred solution data.

BIVALENT BINDING TO LIGAND ATTACHED TO IMPENETRABLE BEADS

The model here is similar to the second model described except that the beads are impenetrable. Using the same notation, the transfer diagram is:



The mass balance equations for this model are:

$$\frac{\partial p_1}{\partial t} = u \cdot \frac{\partial p_1}{\partial x} - 2k_2 L p_1 + k_{-2} p_2 - 2k_3 N p_1 + k_{-3} r_1 \tag{63}$$

$$\frac{\partial p_2}{\partial t} = u \cdot \frac{\partial p_2}{\partial x} + 2k_2 L p_1 - k_{-2} p_2 - k_2 L p_2 + 2k_{-2} p_3 - k_3 N p_2 + k_{-3} r_2 \tag{64}$$

$$\frac{\partial p_3}{\partial t} = u \cdot \frac{\partial p_3}{\partial x} + k_2 L p_2 - 2k_{-2} p_3 \tag{65}$$

$$\frac{\partial r_1}{\partial t} = 2k_3 N p_1 - k_{-3} r_1 - k_2 L r_1 + k_{-2} r_2 - k_4 N r_1 + 2k_{-4} r_3 \tag{66}$$

$$\frac{\partial r_2}{\partial t} = k_2 L r_1 - k_{-2} r_2 + k_3 N p_2 - k_{-3} r_2 \tag{67}$$

$$\frac{\partial r_3}{\partial t} = k_4 N r_1 - 2k_{-4} r_3 \tag{68}$$

When there is no free ligand ($L = 0$), these six equations reduce to three differential equations. Following the preceding procedure, we find:

$$M_e = (1 + 2K_3N + K_3NK_4N)h/u \quad (69)$$

$$S_e = \frac{4K_3N}{k_{-3}} \left[1 + K_4N + \frac{(K_4N)^2}{4} \right] \left(\frac{h}{u} \right) + \frac{K_3NK_4N}{k_{-4}} \cdot \frac{h}{u} \quad (70)$$

PEAKS AND MEANS OF THE ELUTION PROFILE

Our theory of elution chromatography^{8,9} leads, when diffusion is negligible, to a mean elution time $M_e = (1 + K_1)h/u$, where the equilibrium constant $K_1 = V_p/V_0$. Since the volume eluted up to time t is the flow-rate, $F = V_0u/h$, multiplied by the time t , our formula for the profile mean becomes $V_e = FM_e = V_0 + V_p$. This formula is formally the same as a standard formula for the peak of the elution profile. The conditions under which this formula is applicable and comparisons of the peaks and the means are given in refs. 8 and 9.

Using the theory for monovalent binding to ligand immobilized in porous beads which assumes local equilibration of both mass transfer and chemical kinetics, Dunn and Chaiken¹⁶ obtained the following important relation

$$\frac{1}{V - V_0} = \frac{1 + [L]/K_L}{(V_0 - V_m)[LM]/K_{LM}}$$

where V is the peak of the elution profile, V_0 is the volume at which the protein elutes in the absence of interaction ($V_0 + V_p$ in our notation), V_m is the void volume (V_0 in our notation), $[LM]$ is the concentration of immobilized ligand (N in our notation), K_{LM} is the dissociation constant for interaction of protein with immobilized ligand ($1/K_3$ in our notation), $[L]$ is the concentration of free ligand (L in our notation) and K_L is the dissociation constant for interaction of protein with free ligand ($1/K_2$ in our notation). Thus in our notation, their formula becomes:

$$V = V_0 + V_p + V_p \cdot \frac{K_3N}{1 + K_2L} \quad (71)$$

Using eqn. 11 which is valid when diffusion is negligible, we find that the profile mean as a function of eluted volume is

$$V_e = FM_e = V_0u/h M_e = V_0 + V_p + \frac{V_p K_3 N}{1 + K_2 L} + \frac{u}{h} \cdot V_p \cdot \frac{K_3 N (k_{-1}/k_{-2}) K_2 L}{(k_1 + k_{-1} + k_{-2} + k_2 L)(1 + K_2 L)^2} \cdot [1 - \exp(-Bh/u)] \quad (72)$$

where $B = (k_{-2} + k_2 L)[1 + k_1/(k_{-1} + k_{-2} + k_2 L)]$. For fast chemical reactions the expression for the mean V_e found from eqn. 16 is eqn. 72 without the last term.

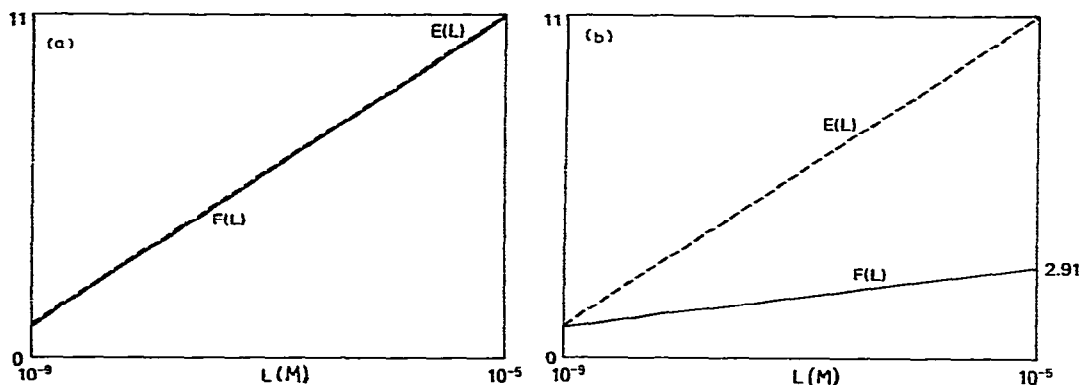


Fig. 1. $F(L)$ given by eqn. 74 is compared with the linear function $E(L)$ given by eqn. 73 for $k_{-2} = 10^{-2}$ and 10^{-4} sec^{-1} in a and b, respectively.

The contribution of the last term in eqn. 72 is not easy to determine since it contains many parameters. However, we can study the significance of this term for some typical parameter values. For fast chemical reactions the eqn. 72 without the last term can be manipulated to obtain the following linear function of L :

$$E(L) = \frac{V_p}{V_c - (V_0 + V_p)} = \frac{1}{K_3 N} + \frac{K_2}{K_3 N} \cdot L \quad (73)$$

The analogous equation obtained from eqn. 72 is:

$$F(L) = \left[\frac{K_3 N}{1 + K_2 L} + \frac{u}{h} \cdot \frac{K_3 N (k_{-1}/k_{-2}) K_2 L}{(k_1 + k_{-1} + k_{-2} + k_2 L)(1 + K_2 L)^2} \cdot (1 - e^{-Bh/u}) \right]^{-1} \quad (74)$$

Let $h/u = 10^3 \text{ sec}$, $k_1 = 0.02 \text{ sec}^{-1}$, $k_{-1} = 0.01 \text{ sec}^{-1}$, $K_2 = 10^6 \text{ M}^{-1}$, $K_3 = 10^6 \text{ M}^{-1}$, $N = 10^{-6} \text{ M}$ and let k_{-2} take on the values 10^{-2} and 10^{-4} sec^{-1} . Plots of $E(L)$ and $F(L)$ for these parameter values are given in Fig. 1. Notice that $E(L)$ and $F(L)$ both look like straight lines so that the apparent linearity of an observed curve does not imply that the approximation is valid. Although the intercepts of $E(L)$ and $F(L)$ are the same, the slopes are often different so that the approximation 73 could give good estimates for $K_3 N$, but poor estimates of K_2 . Since $E(L)$ and $F(L)$ almost coincide for $k_{-2} = 0.01 \text{ sec}^{-1}$, the chemical reaction rates would be called fast for the above parameter values if $k_{-2} \geq 0.01 \text{ sec}^{-1}$.

Since explicit solutions of the model in eqns. 1-7 are not available, it is not possible to assess the difference between the peak and the mean as we did for elution chromatography in ref. 9. Of course, the peak and the mean are equal when the elution profile is symmetric and their difference increases as the elution profile becomes less symmetric. We conclude that the eqn. 71 is only a reasonable approximation when diffusion is negligible, the chemical reaction kinetics are fast compared to the mass transfer kinetics in and out of the beads and the profile is nearly symmetric.

Eilat *et al.*¹⁷ have used a similar local equilibrium model to derive a formula for bivalent binding to ligand attached to porous beads. In our notation their formula for the peak V becomes:

$$V = V_0 + V_p + V_p \left[\frac{2K_3N}{1 + K_2L} + \frac{(K_3N)^2}{(1 + K_2L)^2} \right] \quad (75)$$

Our formula 49, which assumes that diffusion is negligible and that the chemical kinetics are fast compared to the mass transfer in and out of the beads, becomes

$$V_c = V_0 + V_p + V_p \left[\frac{2K_3N}{1 + K_2L} + \frac{K_3NK_4N}{(1 + K_2L)^2} \right] \quad (76)$$

where V_c is the mean of the elution profile as a function of eluted volume. Thus when the rate constant restrictions delineated above hold, our results reduce to an expression that is formally identical to the one derived by Eilat *et al.*¹⁷. Aside from the distinction between K_3 and K_4 in eqn. 76, the only substantive difference between eqns. 75 and 76 is that the former involves the elution profile peak, whereas the latter involves the mean—a distinction that will be important when profiles are asymmetric.

ESTIMATING EQUILIBRIUM CONSTANTS FROM MEANS OF ELUTION PROFILES

A procedure for determining the mass transfer equilibrium constant, K_1 , from the mean of the elution profile without chemical reactions was outlined in the discussion in ref. 8. We now outline a procedure for obtaining the chemical equilibrium constants K_2 and K_3 for monovalent binding with porous beads.

First, follow the procedure in ref. 8, *i.e.*, determine a flow-rate, F , which is sufficient to guarantee that the effects of diffusion can be neglected and then find the mass transfer equilibrium constant K_1 . Here we assume that the beads are large enough so that the chemical reactions are fast compared to the mass transfer in and out of the beads. In the case of monovalent binding, measure V_c for various values of L and calculate V_p from V_c and V_0 . By fitting a straight line to points plotted according to eqn. 73, values of K_3N and K_2 can be estimated from the intercept and slope. The concentration, N , of ligand attached to the beads which is available for protein binding can be estimated by washing off molecules from saturated beads as in ref. 17 so that K_3 can be determined from the value of K_3N .

In the case of bivalent binding, eqn. 76 can be converted to:

$$\frac{V_c - (V_0 + V_p)}{V_p} = \frac{2K_3N}{1 + K_2L} + \frac{K_3NK_4N}{(1 + K_2L)^2} \quad (77)$$

The left side of eqn. 77 obtained from data can be plotted as a function of L and then with K_2 , K_3N and K_4N as parameters, the best fit of eqn. 77 to the data points can be determined. If N has been determined as above, then K_3 and K_4 can be found from the values of K_3N and K_4N .

ESTIMATING RATE CONSTANTS FROM VARIANCES OF ELUTION PROFILES

The dispersion or variance of the elution profile is due to factors such as diffusion, non-equilibration of mass transfer kinetics, non-equilibration of the chemical reactions and velocity heterogeneity. We assume that the condition given in ref. 8 on the flow-rate, which guarantees that the contribution of mass transfer to the dispersion dominates that of velocity heterogeneity and diffusion is satisfied.

The expressions derived in this paper for the variances of the elution profile involve not only equilibrium constants, but also rate constants so that it may be possible to estimate rate constants from measured variances. The possibility of measuring rate constants by affinity chromatography was considered by Denizot and Delaage¹³; however, as noted here, their results are only applicable to impenetrable beads. If chemical reaction kinetics are fast compared to mass transfer kinetics, then the principal contribution (see eqns. 17 and 50) to the variance involves the mass transfer rate constant, k_{-1} . Since the desorption rate constant k_{-} can be determined by elution chromatography, there does not seem to be any reason to determine k_{-1} by affinity chromatography.

In order to determine chemical reaction rate constants such as k_{-3} from experiments using porous beads, the chemical reactions must be slow compared to mass transfer kinetics. For example, k_{-3} could be determined for monovalent binding from observed variances by using eqn. 34, assuming that K_1 , K_3N , h and u have been measured previously. If the chemical reactions are fast compared to the movement in and out of the beads, then it may be desirable to use impenetrable beads with the ligand covalently attached to the surfaces of the beads. In this case for monovalent binding, k_{-3} could be determined using eqn. 59 if K_3N , h and u have been previously determined.

THE EFFECTS OF HETEROGENEITY

The effects of heterogeneity in the size, shape and weight of the molecules and in the uniformity in packing, size and structure of the beads were discussed in ref. 9. Here we assume that the physical properties of the molecules and the beads are uniform, but the molecules are heterogeneous in their chemical reaction properties such as their affinity for ligand. This heterogeneity is usually due to a distribution of reverse rate constants. Since we have assumed that the concentration of molecules is small so that the chemical kinetics are linear, it follows that molecules with different equilibrium constants behave independently and that an average moment is the sum over the distribution of the separate moments.

For monovalent binding to ligands in porous beads, let $n(K_2, K_3)$ be the probability density function for the equilibrium constants K_2 and K_3 . Define the average of the j th moment of the passage time as:

$$\overline{T_j}(x) = \int_0^x \int_0^x n(K_2, K_3) \int_0^x t^j \text{up}(x, t; K_2, K_3) dt dK_2 dK_3$$

For fast chemical reactions the average mean and variance are:

$$\overline{M_e} = \left[1 + K_1 + K_1 \left(\frac{\overline{K_3 N}}{1 + K_2 L} \right) \right] \left(\frac{h}{u} \right) \quad (78)$$

$$\overline{S_e} = \frac{2K_1}{k_{-1}} \left(1 + \frac{K_3 N}{1 + K_2 L} \right)^2 \left(\frac{h}{u} \right) \quad (79)$$

For $K_2 L \gg 1$ we find:

$$\frac{\overline{M_e} - (1 + K_1)h/u}{K_1 h/u} = \left(\frac{\overline{K_3}}{K_2} \right) \left(\frac{N}{L} \right) \quad (80)$$

Since K_2 and K_3 differ only in their diffusive parts, they are highly correlated so that $(\overline{K_3}/K_2)$ is approximately a constant.

For $K_2 L \ll 1$, we find:

$$\frac{\overline{M_e} - (1 + K_1)h/u}{K_1 h/u} = \overline{K_3} N \quad (81)$$

$$\overline{S_e} = \frac{2K_1 h}{k_{-1} u} \frac{1}{(1 + K_3 N)^2} = \frac{2K_1 h}{k_{-1} u} \cdot \{1 + 2\overline{K_3} N + N^2 [\overline{K_3}^2 + (\overline{K_3}^2 - \overline{K_3}^2)]\} \quad (82)$$

Assuming K_1 , h and u are known, $\overline{K_3}$ could be obtained from measurements of $\overline{M_e}$ as a function of N by using eqn. 81. Assuming K_1 , k_{-1} , h , u and $\overline{K_3}$ are known, the variance $\overline{K_3}^2 - \overline{K_3}^2$ of the distribution of K_3 could be determined from measurements of $\overline{S_e}$ by using eqn. 82. Thus, both the mean and variance of the distribution of K_3 can be obtained.

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APPENDIX I

Differential equation for the mean passage time

Direct integration with respect to t on the interval $(0, \infty)$ of eqn. 5 implies:

$$\int_0^{\infty} r(x, t) dt = K_3 N \int_0^{\infty} q_1(x, t) dt \quad (A1)$$

For notational simplicity we omit in succeeding expressions the limits of integration and the integration variable indicator (dt). The simultaneous equations obtained by integrating eqns. 3 and 4 and then using A1 are:

$$(k_{-1} + k_2 L) \int q_1 - k_{-2} \int q_2 = k_1 \int p_1 \quad (A2)$$

$$-k_2L \int q_1 + (k_{-1} + k_{-2}) \int q_2 = k_1 \int p_2 \quad (\text{A3})$$

The solutions of eqns. A2 and A3 are

$$\int q_1 = K_1[k_{-2} \int (p_1 + p_2) + k_{-1} \int p_1]/A \quad (\text{A4})$$

$$\int q_2 = K_1[k_2L \int (p_1 + p_2) + k_{-1} \int p_2]/A \quad (\text{A5})$$

where $A = k_{-1} + k_{-2} + k_2L$.

The simultaneous ordinary differential equations obtained by integrating eqns. 1 and 2 and then using 6, A4 and A5 are:

$$u \cdot \frac{d}{dx} \int p_1 = [k_1 + k_2L - k_1(k_{-1} + k_{-2})/A] \int p_1 - (k_{-2} + K_1k_{-2}/A) \int p_2 - \delta(x - h) \quad (\text{A6})$$

$$u \cdot \frac{d}{dx} \int p_2 = -(k_2L + k_1k_2L/A) \int p_1 + [k_1 + k_{-2} - k_1(k_{-1} + k_2L)/A] \int p_2 \quad (\text{A7})$$

The solutions of these equations using eqn. 7 are

$$\int p_1 = \frac{1}{u(1 + K_2L)} \{1 + k_2L \cdot \exp[-B(h - x)/u]\} \quad (\text{A8})$$

$$\int p_2 = \frac{1}{u} - \int p_1 \quad (\text{A9})$$

where $B = (k_{-2} + k_2L)(1 + k_1/A)$.

If the differential eqn. 5 is multiplied by t and integrated on the interval $(0, \infty)$, then:

$$k_3N \int t q_1 - k_{-3} \int t r = - \int r \quad (\text{A10})$$

Integration of t times the sum of eqns. 3-5 leads to:

$$k_1 \int t (p_1 + p_2) - k_{-1} \int t (q_1 + q_2) = - \int (q_1 + q_2) - \int r \quad (\text{A11})$$

If t times the sum of eqns. 1-5 is integrated, then:

$$\frac{\hat{c}}{\hat{c}t} \int t u (p_1 + p_2) = - \int (p_1 + p_2) - \int (q_1 + q_2) - \int r \quad (\text{A12})$$

Eqn. 9 is obtained by using eqns. 8, A1, A4, A5, A8, A9 and A12.

REFERENCES

- 1 I. M. Chaiken, *Anal. Biochem.*, 97 (1979) 1.
- 2 C. DeLisi and F. W. Wiegel, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 5569.

- 3 C. DeLisi and R. Blumenthal (Editors), *Physical Chemistry of Cell Surface Events and Cellular Regulation*, Elsevier, New York, 1978.
- 4 C. DeLisi, *Mol. Immunology*, 18 (1981) 507.
- 5 T. P. Werblin and G. W. Siskind, *Immunochemistry*, 9 (1972) 987.
- 6 A. K. Thakur and C. DeLisi, *Biopolymers*, 17 (1978) 1075.
- 7 C. DeLisi, *Biopolymers*, 17 (1978) 1385.
- 8 H. W. Hethcote and C. DeLisi, *J. Chromatogr.*, 240 (1982) 269.
- 9 C. DeLisi, H. W. Hethcote and J. W. Brettler, *J. Chromatogr.*, 240 (1982) 283.
- 10 C. DeLisi and H. Hethcote in T. C. J. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques (Proc. 4th Int. Symp., Veldhoven, The Netherlands, June 22-26, 1981; Analytical Chemistry Symposia Series, Vol. 9)*, Elsevier, Amsterdam, Oxford, New York, 1982, p. 63.
- 11 G. Weiss, in I. Brigogine (Editor), *First Passage Times in Chemical Physics (Advances in Chemical Physics, Vol. 13)*, Wiley, New York, 1967, p. 1.
- 12 I. Pecht and O. Lancet, in I. Pecht and R. Ringler (Editors), *Chemical Relaxation in Molecular Biology*, Springer, New York, 1977.
- 13 F. C. Denizot and M. A. Delaage, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 4840.
- 14 I. C. Giddings and H. Eyring, *J. Amer. Chem. Soc.*, 59 (1956) 416.
- 15 H. C. Berg and E. M. Purcell, *Biophys. J.*, 20 (1977) 193.
- 16 B. Dunn and I. M. Chaiken, *Biochemistry*, 14 (1975) 2343.
- 17 D. Eilat, I. M. Chaiken and W. M. McCormack, *Biochemistry*, 18 (1979) 790.