granulation and can be used as a process parameter to evaluate batch-to-batch as well as formula-to-formula variation. If this procedure is followed, it is worthwhile at one time (only) to correlate the k values to the Q values for a particular sieve fraction of a particular granulation using the equipment (ball mill, rotations per minute, and steel spheres) desired for the testing.

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General Treatment of Competitive Binding of Small Molecules to Macromolecules as Applied to Dynamic Dialysis: Theoretical Analysis

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Abstract □ A mathematical analysis of the dynamic dialysis process is presented, demonstrating how the process can be applied generally to study competitive and noncompetitive binding between small molecules and macromolecules. A law of mass action model for competitive binding with independent sites and classes with equivalent sites (CIE) is considered as a specific case without loss of generality. The escape profiles of two compounds are calculated to illustrate the effect of an increasing degree of binding competition. Noisy data are generated using the CIE model to test the presented method of estimating competitive binding parameters. The parameters estimated by the nonlinear regression technique came close to the true values, considering the degree of noise added to the exact dialysis data. A transformation approach is presented, enabling initial estimates of the binding parameters in the CIE model to be determined by multiple linear regression, thereby eliminating the main problem in the nonlinear estimation. The presented method of analysis is extended to strongly bound compounds, which also bind significantly to the dialysis membrane.

Keyphrases □ Dynamic dialysis—mathematical analysis, competitive binding of small molecules to macromolecules studied □ Models, mathematical—law of mass action for competitive binding with independent sites and classes with equivalent sites □ Binding, competitive—small molecules to macromolecules, mathematical analysis of dynamic dialysis process

Dynamic dialysis has proven valuable for characterizing interactions of small molecules with macromolecules such as drugs and proteins (1–11). Experimentally, it appears to be the simplest and most convenient method available for determining a complete binding profile (3), and its accuracy seems to be as good as equilibrium dialysis and ultrafiltration methods (3). The main disadvantages of dynamic dialysis are the inaccuracy introduced by the classical data treatment (9, 12), which requires differentiation of discrete data (3), and its limitation to molecules that do not bind or adsorb significantly to the dialysis membrane. However, both disadvantages recently were eliminated by a new approach in the data treatment (12).

This paper presents a mathematical analysis that enables the dynamic dialysis process to be extended to the study of competitive binding between small molecules and macromolecules considering any mathematical model for such interaction.

THEORY

To illustrate the general approach, it is appropriate to consider a law of mass action model with competitive binding, independent sites, and equivalence between sites in the binding classes having multiple sites:

$$\bar{\nu}_i = \sum_{j=1}^{N} n_j k_{ij} c_i \left[1 + \sum_{m=1}^{M} k_{mj} c_m\right]^{-1} \qquad \begin{matrix} i = 1, 2, \ldots, M \\ k_{ij} \geq 0 \end{matrix} \qquad \text{(Eq. 1)}$$

This model will be denoted the general CIE model, where $\bar{\nu}_i$ is the number of moles of the ith small molecules ("ligand") bound per mole of macromolecule, n_j is the number of equivalent binding sites in the jth class of sites, k_{ij} is the association constant for the ith compound's binding to the jth binding class, c_i is the free concentration of the ith compound, N is the number of binding classes, and M is the number of compounds competing in their binding to the macromolecule. Most frequently, $M = 2$. If $M = 1$, the CIE model reduces to the general (IE) model, dealing with the binding of one compound (12).

Cases where the compounds compete in their binding to certain, but not all, of the binding classes also are considered in the CIE model by allowing the association constants to take zero values (*i.e.*, $k_{ij} \geq 0$). For example, Compound 1 may bind to two classes of sites, and Compound 2 may bind to two classes. If the two compounds only compete in their binding for one class (*e.g.*, class two for Compound 1 is the same as class one for Compound 2), this situation is described by $k_{11} > 0$, $k_{12} > 0$, $k_{13} = 0$, $k_{21} = 0$, $k_{22} > 0$, and $k_{23} > 0$. Thus, the actual number of classes is $N = 3$.

According to $\bar{\nu}_i = ([c_i] - c_i)/P$, where $[c_i]$ is the total (free plus bound) molar concentration of the ith compound and P is the total molar concentration of macromolecule, Eq. 1 can be written:

$$[c_i] = c_i \left\{1 + P \sum_{j=1}^{N} n_j k_{ij} \left[1 + \sum_{m=1}^{M} k_{mj} c_m\right]^{-1}\right\} \qquad \begin{matrix} i = 1, 2, \ldots, M \\ k_{ij} \geq 0 \end{matrix} \qquad \text{(Eq. 2)}$$

The dynamic process is characterized by the following relationship:

$$d[c_i]/dt = -K_i c_i \qquad \text{(Eq. 3)}$$

where K_i is the dialysis rate constant for the ith compound. Equations 2 and 3 define the kinetics of dialysis. However, the use of these equations in their present form requires differentiation of discrete data, which introduces substantial errors in the determination of the binding parameters (9, 12).

To avoid such errors, it is necessary to eliminate the variables, c_i, that cannot be measured directly by using a technique similar to that pre-

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sented previously (12). To do so, it is useful to introduce a set of variables, s_i, defined by:

$$s_i = -d[c_i]/dt = K_i c_i \qquad \text{(Eq. 4)}$$

which, when substituted into Eq. 2, yields:

$$[c_i] = f_i = \frac{s_i}{K_i}\left\{1 + P\sum_{j=1}^{N} n_j k_{ij}\left[1 + \sum_{m=1}^{M}\frac{k_{mj}}{K_m}s_m\right]^{-1}\right\}$$
$$i = 1, 2, \ldots, M \qquad \text{(Eq. 5)}$$

For simplicity, and to demonstrate the generality of the following approach, it is convenient to switch to a matrix–vector notation. The system of equations given by Eq. 2 can be represented by:

$$[\mathbf{c}] = \mathbf{f}(\boldsymbol{\theta}, \mathbf{c}) \qquad \text{(Eq. 6)}$$

where $\boldsymbol{\theta}$ is a vector containing the parameters in the model, $[\mathbf{c}] = ([c_1], [c_2], \ldots, [c_M])^T$, $\mathbf{c} = (c_1, c_2, \ldots, c_M)^T$, and $\mathbf{f} = (f_1, f_2, \ldots, f_M)^T$ is the vector of functions describing the relationship between $[c_i]$ and $\mathbf{c}$. The variable $\mathbf{c}$ can, similarly to c_i, be eliminated according to Eq. 4 so that Eq. 6 becomes:

$$[\mathbf{c}] = \mathbf{f}(\boldsymbol{\theta}, \mathbf{D}\mathbf{s}) \qquad \text{(Eq. 7)}$$

where $\mathbf{D} = \text{diag}\,(1/K_1, 1/K_2, \ldots, 1/K_M)$ is the diagonal matrix containing the reciprocal of the dialysis rate constants and $\mathbf{s} = (s_1, s_2, \ldots, s_M)^T$. By taking the total differential of the ith row of Eq. 7, the following relationship is obtained:

$$\frac{d[c_i]}{dt} = \sum_{j=1}^{M}\left(\frac{\partial f_i}{\partial s_j}\right)\frac{ds_j}{dt} = -s_i \qquad \text{(Eq. 8)}$$

which, for $i = 1, 2, \ldots, M$, yields a set of equations:

$$\left(\frac{\partial f_1}{\partial s_1}\right)\frac{ds_1}{dt} + \left(\frac{\partial f_1}{\partial s_2}\right)\frac{ds_2}{dt} + \ldots + \left(\frac{\partial f_1}{\partial s_M}\right)\frac{ds_M}{dt} = -s_1 \qquad \text{(Eq. 9a)}$$

$$\left(\frac{\partial f_2}{\partial s_1}\right)\frac{ds_1}{dt} + \left(\frac{\partial f_2}{\partial s_2}\right)\frac{ds_2}{dt} + \ldots + \left(\frac{\partial f_2}{\partial s_M}\right)\frac{ds_M}{dt} = -s_2 \qquad \text{(Eq. 9b)}$$

$$\vdots \qquad \vdots \qquad \vdots \qquad \vdots$$

$$\left(\frac{\partial f_M}{\partial s_1}\right)\frac{ds_1}{dt} + \left(\frac{\partial f_M}{\partial s_2}\right)\frac{ds_2}{dt} + \ldots + \left(\frac{\partial f_M}{\partial s_M}\right)\frac{ds_M}{dt} = -s_M \qquad \text{(Eq. 9c)}$$

These equations are recognized as a linear matrix–vector system:

$$\mathbf{J}\left(\frac{\mathbf{f}}{\mathbf{s}}\right)\frac{d}{dt}\mathbf{s} = -\mathbf{s} \qquad \text{(Eq. 10)}$$

where $\mathbf{J}$ is the Jacobian matrix of $\mathbf{f}$ with respect to $\mathbf{s}$. Thus, the ijth element of the coefficient matrix is:

$$J_{ij} = \frac{\partial f_i}{\partial s_j} \qquad \text{(Eq. 11)}$$

Equation 10 can be rearranged to give:

$$\frac{d}{dt}\mathbf{s} = -\mathbf{J}^{-1}\left(\frac{\mathbf{f}}{\mathbf{s}}\right)\mathbf{s} \qquad \text{(Eq. 12)}$$

which, subject to:

$$\mathbf{s} = \mathbf{s}_0 \qquad \text{at } t = 0 \qquad \text{(Eq. 13)}$$

constitutes an initial value problem that readily can be solved numerically using a well-established technique (13).

RESULTS AND DISCUSSION

The functional relationship between $[\mathbf{c}]$ and t is defined by Eqs. 7, 12, and 13 in parametric form, with $\mathbf{s}$ as the parametric variable. To get $[\mathbf{c}]$ at t, Eq. 12 is integrated from $t = 0$ to time t and the obtained $\mathbf{s}$ is inserted into Eq. 7 to give $[c]$. The $\mathbf{s}_0 = \mathbf{s}_{t=0}$ required in the integration is obtained from Eq. 7 by solving for $\mathbf{s}_0$ corresponding to $[\mathbf{c}]_{t=0}$. Thus, the complete escape profiles ($[c_i]$ *versus* t) can be calculated when the binding parameters, $\boldsymbol{\theta}$, and the initial total concentrations, $[\mathbf{c}]_{t=0}$, are given.

When $\boldsymbol{\theta}$ is to be determined by the nonlinear regression technique from dynamic dialysis data ($[\mathbf{c}]$ *versus* t), either $[\mathbf{c}]_{t=0}$ or $\mathbf{s}_0$ must be treated as additional unknown parameters to be determined simultaneously with $\boldsymbol{\theta}$. The latter choice ($\mathbf{s}_0$) is computationally most convenient, particularly if $\mathbf{s}$ cannot be expressed explicitly in terms of $[\mathbf{c}]$ and $\boldsymbol{\theta}$ in Eq. 7. The initial estimates of the elements of $\mathbf{s}_0$ required in the curve-fitting procedure are simply obtained according to the definition of $\mathbf{s}$ (Eq. 4) as the absolute values of the initial slopes in the $[c_i]$ *versus* t plots of the data.

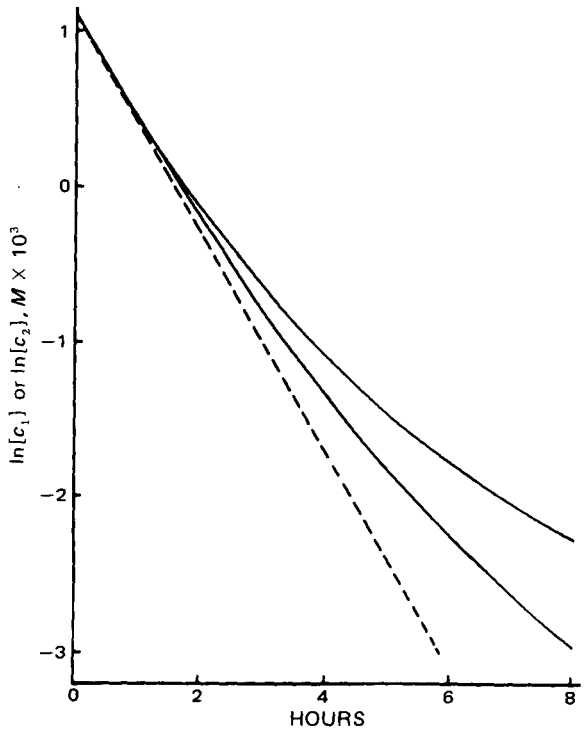

Figure 1—*Theoretical escape curves describing the dynamic dialysis of two compounds competing in their binding to a macromolecular compound according to the CIE model (Eq. 2, M = 2 and N = 1) with one class of sites. The association constant for the two compounds binding to the common binding sites are* $k_1 (= k_{11}) = 5\ mM^{-1}$ *(middle curve) and* $k_2 (= k_{21}) = 10\ mM^{-1}$ *(upper curve). The broken straight line describes the first-order dialysis in the absence of the macromolecular compound. Figures 1–3 show the effect of changing* k_2 *while all other parameters are kept constant:* P = 0.145 mM, n (= n_1) = 2, $K_1 = K_2 = 0.7\ hr^{-1}$, *and* $[c_1]_{t=0} = [c_2]_{t=0} = 3$ mM.

These slopes would normally be determined by extrapolation. To avoid the inconvenience and errors of such extrapolations, it is convenient to define $t = 0$ at the first sampling time. Thus, there is no need to take samples at the very beginning of the experiment, which is very convenient. The accuracy by which the initial slopes are determined does not affect the accuracy of the binding parameters since the slope values are only used as initial guesses for $\mathbf{s}_0$ in the iterative nonlinear estimation.

This treatment (Eqs. 6–13) is completely general and applies to any mathematical model for competitive or noncompetitive binding involving any number of interacting compounds. If the partial derivatives used for the Jacobian matrix (Eq. 11) are not available in an analytical form, they can be evaluated numerically; or if $[\mathbf{c}]$ is not expressed explicitly in the binding model as in Eq. 6, this can also be done numerically. Thus, the method is not restricted by the complexity of the mathematical model for the binding kinetics.

To illustrate this general procedure, consider again the general CIE model (Eq. 2). For this model, the Jacobian matrix is assembled according to Eq. 11 using the following partial derivatives obtained from Eq. 5:

$$\frac{\partial f_i}{\partial s_v} = -\frac{s_i P}{K_v K_i}\sum_{j=1}^{N} n_j k_{ij} k_{vj}\left[1 + \sum_{m=1}^{M}\frac{k_{mj}}{K_m}s_m\right]^{-2}$$
$$i \neq v = 1, 2, \ldots, M \qquad \text{(Eq. 14)}$$

$$\frac{\partial f_i}{\partial s_i} = \frac{1}{K_i} + \frac{P}{K_i}\sum_{j=1}^{N} n_j k_{ij}\left\{1 + \sum_{\substack{m=1 \\ \neq i}}^{M}\frac{k_{mj}}{K_m}s_m\right\}\left[1 + \sum_{m=1}^{M}\frac{k_{mj}}{K_m}s_m\right]^{-2}$$
$$i = 1, 2, \ldots, M \qquad \text{(Eq. 15)}$$

For simplicity, consider the most common case, $M = 2$, with only two compounds competing in their binding to the macromolecule. The inverse of the Jacobian matrix is given by:

$$\mathbf{J}^{-1}\left(\frac{f_1, f_2}{s_1, s_2}\right) = \begin{bmatrix} \frac{\partial f_2}{\partial s_2} & -\frac{\partial f_1}{\partial s_2} \\ -\frac{\partial f_2}{\partial s_1} & \frac{\partial f_1}{\partial s_1} \end{bmatrix} \Big/ \begin{bmatrix} \frac{\partial f_1}{\partial s_1} & \frac{\partial f_1}{\partial s_2} \\ \frac{\partial f_2}{\partial s_1} & \frac{\partial f_2}{\partial s_2} \end{bmatrix} \qquad \text{(Eq. 16)}$$

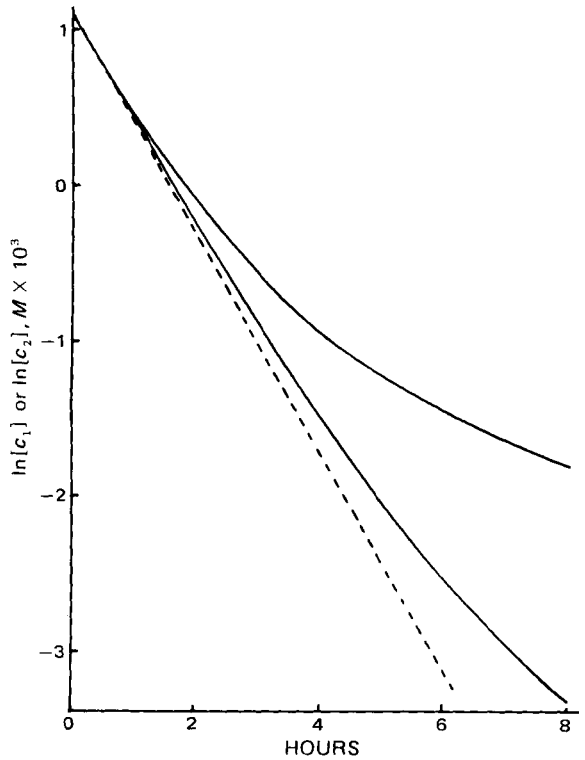

Figure 2—*Theoretical escape curves describing the dynamic dialysis of two compounds competing in their binding to a macromolecular compound according to the CIE model (Eq. 2, M = 2 and N = 1) with one class of sites. The association constant for the two compounds binding to the common binding sites are* k_1 (= k_{11}) = 5 mM^{-1} *(middle curve) and* k_2 (= k_{21}) = 25 mM^{-1} *(upper curve). The broken straight line describes the first-order dialysis in the absence of the macromolecular compound.*

which, when substituted into Eq. 12, yields the following differential equations:

$$\frac{ds_1}{dt} = \frac{-\left(\frac{\partial f_2}{\partial s_2}\right) s_1 + \left(\frac{\partial f_1}{\partial s_2}\right) s_2}{\left(\frac{\partial f_1}{\partial s_1}\right)\left(\frac{\partial f_2}{\partial s_2}\right) - \left(\frac{\partial f_2}{\partial s_1}\right)\left(\frac{\partial f_1}{\partial s_2}\right)} \qquad \text{(Eq. 17)}$$

$$\frac{ds_2}{dt} = \frac{-\left(\frac{\partial f_1}{\partial s_1}\right) s_2 + \left(\frac{\partial f_2}{\partial s_1}\right) s_1}{\left(\frac{\partial f_1}{\partial s_1}\right)\left(\frac{\partial f_2}{\partial s_2}\right) - \left(\frac{\partial f_2}{\partial s_1}\right)\left(\frac{\partial f_1}{\partial s_2}\right)} \qquad \text{(Eq. 18)}$$

where the partial derivatives are given by Eqs. 14 and 15.

The functional relationship among the experimentally available variables $[c_1]$, $[c_2]$, and t is now given in parametric form (with s_1 and s_2 as parameter variables) by Eqs. 5 and 14–18. The procedure by which this functional relationship is established is as follows. Equations 17 and 18 are integrated numerically from $t = 0$ to time t, using an appropriate, well-established technique such as a Runge–Kutta or a multistep method (13).

The obtained s_1 and s_2 values then are substituted into Eq. 5, $i = 1, 2$, to give the $[c_1]$ and $[c_2]$ at that particular time t. This two-step process, when repeated for various t values, defines the $[c_1]$, $[c_2]$ *versus* t functional relationship.

Having defined this relationship, a nonlinear regression technique can be used to estimate the binding parameters by simultaneous curve fitting to experimental $[c_1]$ *versus* t and $[c_2]$ *versus* t dialysis data. The dialysis rate constant, K_i, can be determined from ln (c_i) *versus* t plots in a separate experiment in the absence of the macromolecular compound or it can be determined directly by the nonlinear regression technique by considering K_i as an unknown parameter (12). The initial estimates of $[s_1]_{t=0}$ and $[s_2]_{t=0}$ are obtained graphically as the absolute values of the initial slopes from plots of $[c_1]$ *versus* t and $[c_2]$ *versus* t. As explained, the accuracy of this estimation will not affect the accuracy of the estimated binding parameters.

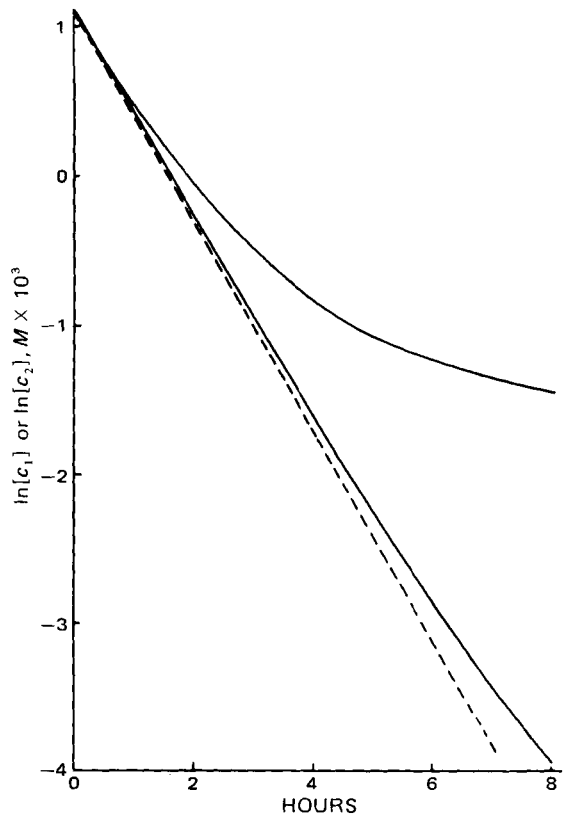

Figure 3—*Theoretical escape curves describing the dynamic dialysis of two compounds competing in their binding to a macromolecular compound according to the CIE model (Eq. 2, M = 2 and N = 1) with one class of sites. The association constant for the two compounds binding to the common binding sites are* k_1 (= k_{11}) = 5 mM^{-1} *(middle curve) and* k_2 (= k_{21}) = 100 mM^{-1} *(upper curve). The broken straight line describes the first-order dialysis in the absence of the macromolecular compound.*

Example of CIE Model with $M = 2$ and $N = 1$—To show the practicality of this approach and to illustrate the dynamic dialysis behavior in a situation with competitive binding, the CIE model in its simplest form ($M = 2$ and $N = 1$) is now considered. The following equations are obtained from Eqs. 5, 14, and 15 with $M = 2$ and $N = 1$:

$$[c_1] = f_1 = \frac{s_1}{k_1} + \frac{nPk_1K_2s_1}{K_1K_2 + k_1K_2s_1 + k_2K_1s_2} \qquad \text{(Eq. 19)}$$

$$[c_2] = f_2 = \frac{s_2}{K_2} + \frac{nPk_2K_1s_2}{K_1K_2 + k_1K_2s_1 + k_2K_1s_2} \qquad \text{(Eq. 20)}$$

$$\frac{\partial f_1}{\partial s_1} = \frac{1}{K_2} + \frac{nPk_1K_2(K_1K_2 + k_2K_1s_2)}{(K_1K_2 + k_1K_2s_1 + k_2K_1s_2)^2} \qquad \text{(Eq. 21)}$$

$$\frac{\partial f_2}{\partial s_2} = \frac{1}{K_2} + \frac{nPk_2K_1(K_1K_2 + k_1K_2s_1)}{(K_1K_2 + k_1K_2s_1 + k_2K_1s_2)^2} \qquad \text{(Eq. 22)}$$

$$\frac{\partial f_1}{\partial s_2} = -\frac{nPk_1K_2k_2K_1s_1}{(K_1K_2 + k_1K_2s_1 + k_2K_1s_2)^2} \qquad \text{(Eq. 23)}$$

$$\frac{\partial f_2}{\partial s_1} = -\frac{nPk_1K_2k_2K_1s_2}{(K_1K_2 + k_1K_2s_1 + k_2K_1s_2)^2} \qquad \text{(Eq. 24)}$$

The dynamic dialysis behavior can now be calculated as discussed using Eqs. 17–24. Three escape profiles were calculated[1] (Figs. 1–3) with the following common parameters: $P = 0.145$ mM; $n = 2$; $K_1 = K_2 = 0.7$ hr^{-1}; $[c_1]_{t=0} = [c_2]_{t=0} = 3$ mM; $k_1 = 5$ mM^{-1}; and $k_2 = 10$, 25, and 100 mM^{-1}. The association constant, k_2, for the competing second compound (top curves in Figs. 1–3) is the only parameter that differs in the three cases.

[1] Calculations were done using an IBM 370 digital computer. The integration of the differential equations was done using a Runge–Kutta algorithm (14). The accuracy of the integration was checked by step reduction. The solution of Eqs. 19 and 20 for s_0 for given values of $[c_1]_{t=0} = [c_2]_{t=0}$ was done using the Newton algorithm.

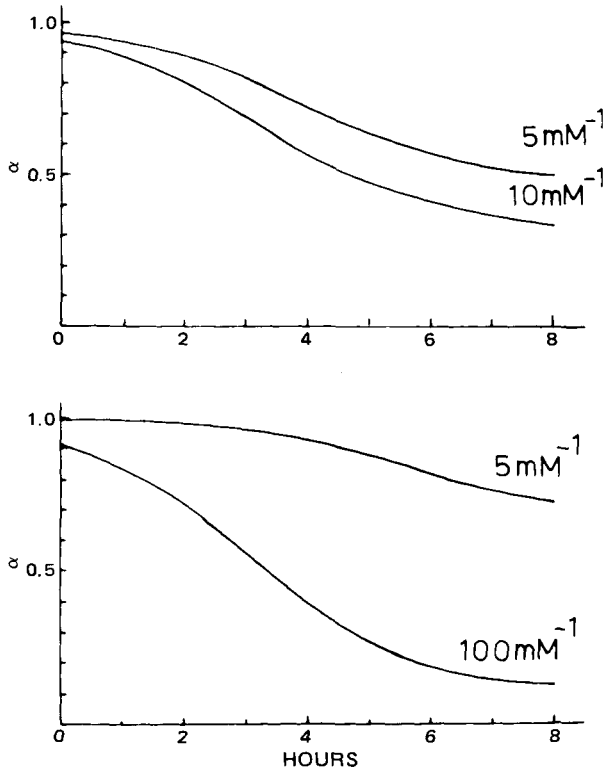

Figure 4—*Calculated variation of the fraction of unbound form* (α = c/[c]) *of two compounds in a dynamic dialysis cell where the compounds compete in their binding to a macromolecular compound according to the CIE binding model (Eq. 2,* M = 2 *and* N = 1*) with one class of sites. The association constant for the binding of the two compounds to the common binding sites are indicated on the curves. The other parameter values are the same for the upper and lower graphs and identical to the parameters in Figs. 1 and 3, respectively.*

Semilogarithmic plots are used to illustrate the effect of binding on the escape of the two compounds from the compartment containing the macromolecular compound. If there is no binding, the escape would be first order and follow the broken line (|slope| = K). The increasing deviation in the slopes of the two dialysis curves observed at decreasing concentrations is expected according to Eq. 4 because of the increased degree of binding at lower concentrations. This deviation becomes more pronounced for the second compound as its association constant increases. The association constant for the first compound is kept constant (k_1 = 5 mM^{-1}) in all three cases. However, the deviation of its escape curve (the middle curves in Figs. 1–3) from that of the broken line becomes less significant as the association constant of the second compound increases. This behavior agrees with the corresponding increased degree of displacement of the first compound as the binding of the second compound to the common binding sites becomes stronger. This pattern in the displacement is clearly seen when the fractions of unbound compounds, α, are plotted *versus* time (the upper and lower graphs in Fig. 4 correspond to Figs. 1 and 3, respectively).

Estimation of Competitive Binding Parameters: A Simple Example—A binding parameter estimation situation was simulated by first calculating 2 × 11 exact dynamic dialysis data points equally spaced over 8 hr, using the CIE model with M = 2, N = 1, P = 0.145 mM, n = 2, k_1 = 5 mM^{-1}, k_2 = 10 mM^{-1}, $[c_1]_{t=0} = [c_2]_{t=0}$ = 3 mM, and $K_1 = K_2$ = 0.7 hr^{-1}. Random deviates from a Gaussian distribution with zero mean and various standard deviations were then added as errors to the exact data[2]. The standard deviations of the Gaussian distribution were chosen so that there was a 95% probability that the relative error would fall within ±5% of the exact value.

The CIE model was then fitted to the noisy data using a general nonlinear regression program, FUNFIT, written for time-sharing use (16). The data were weighted inversely proportional to the observed [c] values according to the theory of least squares (17) since the standard deviations of the errors were chosen proportional to the exact [c] values. It was assumed that the dialysis rate constants K_1 and K_2 were determined in a separate experiment in the absence of the macromolecular compound, so only n, k_1, and k_2 remained to be determined.

[2] The deviates were obtained by the inverse method, using a uniform distribution pseudo-random number generator (15).

Table I—Competitive Binding Parameters Obtained from Simulated Dynamic Dialysis Data Considering the CIE Binding Model (Eq. 2, M = 2 and N = 1)

	n	k_1, mM^{-1}	k_2, mM^{-1}	r
True values[a]	2	5	25	—
Linear regression[b]	3.79	2.76	28.9	—
First nonlinear regression[c]	2.07	5.02	25.3	0.9998
Second nonlinear regression[d]	2	5.15	24.7	0.9997

[a] Values from which exact dynamic dialysis data were calculated and Gaussian noise added to simulate experimental data. [b] Values obtained by the approach described in the *Appendix*. These values were used as initial estimates in the first nonlinear regression. [c] Values obtained by fitting simultaneously by least squares the two regression equations defined by Eqs. 17–24 in dynamic and parametric form to [c_1] *versus* t and [c_2] *versus* t data (Fig. 5). [d] Values were obtained as explained in footnote c, but the value for n was fixed as a constant as the integer value closes to n in the first nonlinear estimation to agree with the binding model.

The difficulty of obtaining good initial parameter estimates is often the main problem in nonlinear parameter estimation. Therefore, it would not simulate a real experimental situation to have *a priori* knowledge about the true parameters in the simulation study. It is shown in the *Appendix* how initial estimates can be obtained for the CIE model by the multiple linear regression technique.

The initial estimates of n_1, k_1, and k_2 were obtained in this way using Eqs. A12–A14, and the estimates of $[s_1]_{t=0}$ and $[s_2]_{t=0}$ were obtained graphically as discussed. Although the linear regression technique itself is not accurate enough to be used as a method for estimating CIE binding parameters, it appears to be valuable to get good initial estimates for the nonlinear estimation (Table I).

The nonlinear regression technique demonstrated very good agreement

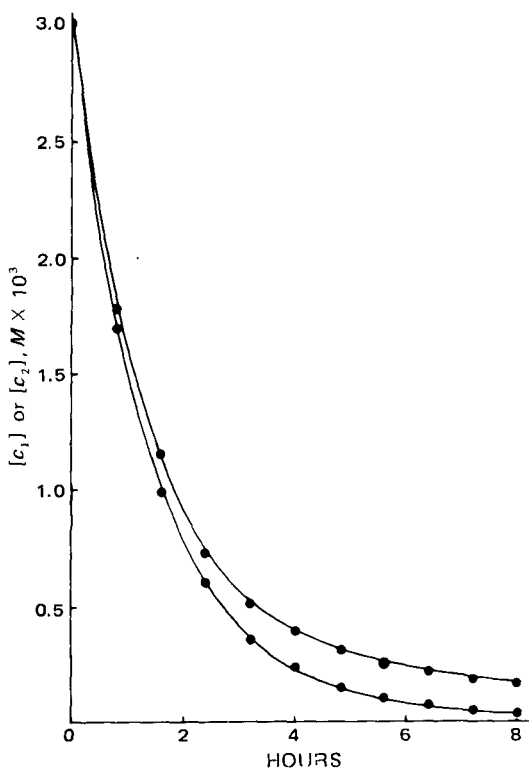

Figure 5—*Simultaneous nonlinear least-squares estimation of competitive binding parameters for two compounds competing in their binding to a macromolecular compound according to the CIE model (Eq. 2,* M = 2 *and* N = 1*). The 2 × 11 dynamic dialysis data points are simulated data with random Gaussian deviates added as noise (for details, see text). The two regression equations fitted simultaneously by least squares to the* [c_1] versus t *and* [c_2] versus t *data are defined in dynamic and parametric form by Eqs. 17–24. The estimated binding parameters are given in Table I.*

between observed and calculated values of the dynamic dialysis data (Fig. 5). The estimated binding parameters came close to the true values, considering the degree of noise added to the true data (Table I).

The dynamic dialysis technique combined with the presented mathematical treatment appears to be a generally applicable and very powerful tool for studying binding interaction between small molecules and macromolecules. However, before the technique can be applied, it is necessary to establish, in the absence of the macromolecular compound, that there is no interaction between the small molecules (*e.g.*, no complexation), in which case Eq. 3 cannot be used. Furthermore, it must be ensured that the experimental conditions are such that the dialysis through the membrane is first order with respect to the free form of the small molecules in the concentration range of interest (3).

If one or more of the small molecules are highly bound, a significant binding to the dialysis membrane may be observed in the preliminary investigations not involving the macromolecular compound and this procedure cannot be applied directly. However, the *Appendix* shows how the method can be extended to include such interactions.

APPENDIX

Equation 5 can, after rearrangement, be written:

$$\frac{P}{\frac{K_i[c_i]}{s_i} - 1} = \sum_{j=1}^{N}\left[\frac{1}{n_j k_{ij}} + \frac{1}{n_j k_{ij}}\sum_{m=1}^{M}\frac{k_{mj}}{K_m}s_m\right] \qquad \text{(Eq. A1)}$$

which is recognized as a linear expression of the form:

$$y_i = a_{oi} + \sum_{m=1}^{M} a_{im}s_m \qquad i = 1, 2, \ldots, M \qquad \text{(Eq. A2)}$$

where:

$$y_i = \frac{P}{\frac{k_i[c_i]}{s_i} - 1} \qquad \text{(Eq. A3)}$$

$$a_{oi} = \sum_{j=1}^{N}\frac{1}{n_j k_{ij}} \qquad \text{(Eq. A4)}$$

$$a_{im} = \sum_{j=1}^{N}\frac{k_{mj}}{n_j k_{ij} K_m} \qquad \text{(Eq. A5)}$$

Thus, multiple linear regression of y_i ($i = 1, 2, \ldots, M$) on s_m ($m = 1, 2, \ldots, M$) yields $M(M + 1)$ regression coefficients, which are functions of the n's, the k's, and the K's only. To evaluate y_i, the K's must be determined in a separate experiment in the absence of the macromolecular compound so that only the n's and k's remain to be determined from Eqs. A4 and A5. The number of parameters is, therefore, $N + (N)(M) = M(N + 1)$ to be determined from $M(M + 1)$ equations (coefficients).

The solution to this system requires that $M(N + 1) \leq M(M + 1)$, *i.e.*, that $N \leq M$, so the multiple linear regression approach cannot be used to estimate the n's and k's in a CIE model if the number of classes is more than the number of competing compounds.

The solution of Eqs. A4 and A5 when $N = M = 2$ is given by:

$$n_1 = \frac{1}{a_{11}K_1} + \frac{1}{a_{21}K_2} \qquad \text{(Eq. A6)}$$

$$n_2 = \frac{1}{a_{12}K_1} + \frac{1}{a_{22}K_2} \qquad \text{(Eq. A7)}$$

$$k_{11} = \frac{a_{21}K_1K_2(a_{11}a_{22} - a_{12}a_{21})}{(a_{11}K_1 + a_{21}K_2)(a_{22}a_{o1} - a_{12}a_{o2})} \qquad \text{(Eq. A8)}$$

$$k_{22} = \frac{a_{12}K_1K_2(a_{11}a_{22} - a_{12}a_{21})}{(a_{12}k_1 + a_{22}K_2)(a_{11}a_{o2} - a_{21}a_{o1})} \qquad \text{(Eq. A9)}$$

$$k_{12} = \frac{a_{21}K_1K_2(a_{11}a_{22} - a_{12}a_{21})}{(a_{11}K_1 + a_{21}K_2)(a_{22}a_{o1} - a_{12}a_{o2})} \qquad \text{(Eq. A10)}$$

$$k_{21} = \frac{a_{11}K_1K_2(a_{11}a_{22} - a_{12}a_{21})}{(a_{11}K_1 + a_{21}K_2)(a_{22}a_{o1} - a_{12}a_{o2})} \qquad \text{(Eq. A11)}$$

where a_{oi} and a_{im} ($i, m = 1, 2$) are the constant term and the mth regression coefficient, respectively, obtained using the ith regression equation defined by Eq. A2. In the specific example already considered ($M = 2, N = 1$), the initial estimates of the binding parameters were obtained using the following equations:

$$n = \frac{1}{K_1a_1} \qquad \text{(Eq. A12)}$$

$$k_1 = \frac{K_1a_1}{a_o} \qquad \text{(Eq. A13)}$$

$$k_2 = \frac{K_2a_2}{a_o} \qquad \text{(Eq. A14)}$$

where the second subscript of the k's and a's has been dropped for simplicity since there is only one class of sites. The linear regression equation used in this case is $y_1 = a_o + a_1s_1 + a_2s_2$, where $y_1 = p/(K_1[c_1]/s_1 - 1)$, from which a_{o1}, a_1, and a_2 were determined using the noisy dialysis data ($[c_1]$ and $[c_2]$ *versus* t). The s_1 and s_2 values would usually be found by fitting an empirical equation[3] to the dialysis data and differentiating this function to get s_1 and s_2 at the various observation times. However, in this simulation, the s_1 and s_2 values were obtained from the exact s values by adding errors in the same way as was done with the $[c_1]$ and $[c_2]$ values.

If a small molecule binds to the dialysis membrane, it can often be considered as a Langmuir-type adsorption phenomenon (5), which is mathematically analogous to binding to a single class of sites and is described by:

$$\bar{\nu}_i^* = \frac{n_i^*k_i'c_i}{1 + k_i'c_i} \qquad \text{(Eq. A15)}$$

where $\bar{\nu}_i^*$ is the amount of the ith compound bound per amount of available membrane material and k_i' is an "association constant" for membrane binding. The quantity n_i^* does not have the same meaning as n previously defined but is introduced to establish a mathematical analogy. Equation A15 may also be written:

$$(c_b)_i = \frac{n_i^*k_i^*c_i}{1 + k_i^*c_i} \qquad \text{(Eq. A16)}$$

where $(c_b)_i$ is the concentration of the bound form of the ith compound and:

$$k_i^* = \frac{k_i'w}{u_iV} \qquad \text{(Eq. A17)}$$

where w is the amount of membrane material available for binding, u_i is the molecular weight of the ith compound, and V is the volume of the dialysis compartment. Equation A16 can, according to Eq. 4, be written:

$$(c_b)_i = g_i = \frac{n_i^*k_i^*s_i}{K_i + k_i^*s_i} \qquad k_i^* \geq 0 \qquad \text{(Eq. A18)}$$

where the function g_i denotes the concentration membrane bound of the ith compound, which must be added to f_i to get $[c_i]$. Therefore, when one or more compounds are bound (adsorbed) significantly to the dialysis membrane, the following two equations:

$$[\mathbf{c}] = \mathbf{f} + \mathbf{g} \qquad \text{(Eq. A19)}$$

$$\frac{d}{dt}\mathbf{s} = -\mathbf{J}^{-1}\left(\frac{\mathbf{f} + \mathbf{g}}{\mathbf{s}}\right)\mathbf{s} \qquad \text{(Eq. A20)}$$

should replace Eqs. 7 and 12, respectively. For the sake of generality, the function g_i may be any binding or adsorption model describing the membrane binding with or without competitive effects. The most accurate way of accounting for the effect of membrane binding is first to determine the membrane binding parameters (n^* and k^* in this case) in the absence of the macromolecular compound in a separate experiment. These parameters can then be fixed as constants in the "correction term," $\mathbf{g}$, when fitting the corrected model (Eq. A19) to reduce the dimensionality of the nonlinear estimation, thereby getting more reliable estimates of the remaining (variable) parameters.

SYMBOLS

a_{im} = mth regression coefficient in ith linear regression equation (Eq. A2) used to obtain initial estimates of binding parameters n's and k's

a_{oi} = constant term in ith linear regression equation (Eq. A2) used to obtain initial estimates of binding parameters n's and k's

$a_o = a_{o1}$ (see a_{oi})

$a_1 = a_{11}$ (see a_{im})

$a_2 = a_{21}$ (see a_{im})

α = fraction unbound ($\alpha = c_i/[c_i]$)

[3] A spline function seems to be the best choice because of its desirable smoothness and flexibility (18).

c_i = free (unbound) molar concentration of ith compound
$\mathbf{c} = (c_1, c_2, \ldots, c_M)^T$ (see c_i)
$[c_i]$ = total (free + bound) molar concentration of ith compound
$[\mathbf{c}] = ([c_1], [c_2], \ldots, [c_M])^T$ (see $[c_i]$)
$(c_b)_i$ = "membrane bound concentration" of ith compound
D = diag$(1/K_1, 1/K_2, \ldots, 1/K_M)$ = diagonal matrix containing reciprocals of dialysis rate constants
f_i = function describing relationship between $[c_i]$ and $\mathbf{c}$ or $[c_i]$ and $\mathbf{s}$
$\mathbf{f} = (f_1, f_2, \ldots, f_M)^T$ (see f_i)
g_i = function describing relationship between $(c_b)_i$ and s_i
$\mathbf{g} = (g_1, g_2, \ldots, g_M)^T$ (see g_i)
i = subscript ($i = 1, 2, \ldots$)
j = subscript ($j = 1, 2, \ldots$)
$\mathbf{J}\left(\frac{\mathbf{f}}{\mathbf{s}}\right)$ = Jacobian matrix of $\mathbf{f}$ with respect to $\mathbf{s}$
J_{ij} = ijth element of Jacobian matrix
k_{ij} = association constant for ith compound's binding to jth class of binding sites on macromolecule
k_i = k_{i1} (see k_{ij})
k_i^* = membrane binding "association constant" of ith compound
K_m = dialysis rate constant for mth compound
m = subscript ($m = 1, 2, \ldots$)
M = number of compounds
n_j = number of binding sites in jth class of sites
n_i^* = mathematical analog to n_i in Langmuir-type membrane "binding" model
n = n_1 (see n_j)
N = number of binding classes
$\bar{\nu}_i$ = number of moles of ith compound ("ligand") bound per mole of macromolecule
$\bar{\nu}_i^*$ = amount of ith compound bound per amount of available membrane material
P = total molar concentration of macromolecular compound
θ = parameter vector containing elements such as n_j, k_{ij}, and P or other parameters used in the particular mathematical model $\mathbf{f}$ describing binding kinetics
r = correlation coefficient
s_i = absolute value of slope in a $[c_i]$ *versus* t plot at time t. This quantity is used as a parameter variable in the parametric representation of the variables $[c_i]$ and t
$\mathbf{s} = (s_1, s_2, \ldots, s_M)^T$ (see s_i)
$\mathbf{s}_0$ = $\mathbf{s}$ evaluated at $t = 0$
t = time
T = transpose
u_i = molecular weight of ith compound
υ = subscript ($\upsilon = 1, 2, \ldots$)
V = volume of dialysis compartment
w = weight of available membrane material
y_i = ith dependent variable in transformed set of linear regression equations (Eq. A2) used to obtain initial estimates of the binding parameters (n's and k's) by multiple linear regression technique

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Analysis of Steroid Phosphates by High-Pressure Liquid Chromatography: Betamethasone Sodium Phosphate

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Abstract □ A sensitive, automatable high-pressure liquid chromatographic procedure is presented for the determination of steroid phosphates. Quantitation is described for betamethasone sodium phosphate in dosage forms in the presence of polar excipients. The separation of a multicomponent mixture of steroid phosphates also is reported.

Keyphrases □ Betamethasone sodium phosphate—high-pressure liquid chromatographic analysis in dosage forms □ High-pressure liquid chromatography—analysis, betamethasone sodium phosphate in dosage forms □ Glucocorticoids—betamethasone sodium phosphate, high-pressure liquid chromatographic analysis in dosage forms □ Steroid phosphates, various—high-pressure liquid chromatographic analysis in dosage forms

Steroid phosphates are highly effective anti-inflammatory agents produced in ophthalmic, injectable, and solid dosage forms. Reported analyses utilize spectrophotometric procedures preceded by extraction and/or reaction methods (1–3). These procedures are time consuming and relatively difficult. Two chromatographic methods have been reported: TLC of the methyl ester and ion chromatography (4, 5). This laboratory previously used

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