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EFFECTS OF UNSTIRRED LAYERS ON THE KINETICS OF CARRIER-MEDIATED SOLUTE TRANSPORT BY TWO SYSTEMS

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Equations describing the effect of unstirred layers on the simultaneous transport of a solute via two mediated systems have been derived. It has been previously established that unstirred layers cause convex curvature of Eadie-Hofstee transformations of kinetic data for single mediated transport systems. On the other hand, multiple transport systems produce concave curvature in this plot. A numerical example is presented which illustrates the fact that under some circumstances the opposing effects can nearly cancel out, producing an erroneous apparently linear relation. Furthermore, statistical analysis of these curves suggests that the normal error encountered in physiological transport measurements can readily obscure evidence of unstirred layers. It is suggested that kinetic analysis of transport, particularly in epithelial tissues, should whenever possible, include independent measurements of unstirred layers, employ a broad range of substrate concentrations, and vary the stirring rate of the medium. Evaluation of the 'true' kinetic constants can then be made with the relations derived here. In addition, several limiting cases have been discussed which reduce to simpler kinetic descriptions published earlier.

Introduction

The presence of unstirred layers at absorptive surfaces has been shown to distort the apparent kinetics of carrier mediated transport systems. This problem has been treated both theoretically and experimentally for suspensions of cells [1–3] and epithelial sheets [4–13]. Winne [4] derived the relation between the thickness of unstirred layer, solute diffusion constant, and apparent influx of solute for simple carrier systems and for carrier systems with an added diffusional component [5]. Thomson and Dietschy [7] described the effect of variable unstirred layers which may occur in tissues with villous structures such as intestine. It was demonstrated by Preston et al. [12] and later discussed more extensively by Thomson [6] that the Eadie-Hofstee transformation of kinetic data was sensitive to the effects of unstirred layers. It was shown some time ago that this transformation is generally preferred over the traditional Lineweaver-Burk transformation for kinetic plots [14]. This is because the Eadie-Hofstee plot obscures experimental error less than the Lineweaver-Burk plot by more balanced weighting data points. For similar reasons the Eadie-Hofstee plot is more sensitive to multiple transport systems than the Lineweaver-Burk transformation. Preston et al. [12] and Thomson [6] established that with simple carrier systems and planar absorptive surfaces significant unstirred layers produce convex curvature. On the other hand, two transport systems produce concave curvature. If both conditions are present simultaneously this might lead to an apparent linear relation because of the opposing effects. In this paper, the formal relation between unstirred layers, multiple transport systems and influx rate is derived for a planar membrane. It is illustrated with a numerical model and the implications for transport studies discussed.

Theory

The diffusion rate of a substance across an unstirred layer at the surface of a membrane is given by Eqn. 1:

$$J=\frac{D}{\delta}(C_0-C) \tag{1}$$

where J = flux rate per unit area (e.g., $\mathrm{mol \cdot h^{-1} \cdot cm^{-2}}$); D = diffusion constant of the substance in the aqueous phase ($\mathrm{cm^2 \cdot h^{-1}}$), δ = the 'effective' thickness of the unstirred layer (cm), C_0 = concentration of the solute in the well mixed bulk phase ($\mathrm{mol \cdot l^{-1}}$) and C = concentration of the solute at the surface of the membrane ($\mathrm{mol \cdot l^{-1}}$). Simple carrier mediated transport for two systems is characterized by Eqn. 2:

$$J=\frac{J_1^{\mathrm{m}}(C)}{K_{\mathrm{t}_1}+(C)}+\frac{J_2^{\mathrm{m}}(C)}{K_{\mathrm{t}_2}+(C)} \tag{2}$$

where J = the total unidirectional influx ($\mathrm{mol \cdot h^{-1} \cdot cm^{-2}}$), C = concentration at the surface of the membrane ($\mathrm{mol \cdot l^{-1}}$), K_{t_1} and K_{t_2} = the apparent Michaelis constants for systems one and two, respectively ($\mathrm{mol \cdot l^{-1}}$), and J_1^{m}, and J_2^{m} = apparent maximum transport rate for systems one and two, respectively ($\mathrm{mol \cdot h^{-1} \cdot cm^{-2}}$). At steady state, the flux rate (J) across the unstirred layer and the membrane are equal. Under this condition the concentration at the membrane (C) expressed in terms of bulk phase concentration (C_0) which is:

$$C=C_0-(J\delta/D) \tag{3}$$

Substituting Eqn. 3 in Eqn. 2 and expressing the result in J produces the following rather complicated cubic equation:

$$\begin{aligned}0={}&J^3+J^2\left(-\frac{D}{\delta}(K_{\mathrm{t}_1}+K_{\mathrm{t}_2}+2\,C_0)-J_1^{\mathrm{m}}-J_2^{\mathrm{m}}\right)\\&+J\left(\left(\frac{D}{\delta}\right)^2(K_{\mathrm{t}_1}+C_0)(K_{\mathrm{t}_2}+C_0)\right.\\&\left.+\frac{D}{\delta}\left(2\,C_0(J_1^{\mathrm{m}}+J_2^{\mathrm{m}})+K_{\mathrm{t}_1}J_2^{\mathrm{m}}+K_{\mathrm{t}_2}J_1^{\mathrm{m}}\right)\right)\\&-\left(\frac{D}{\delta}\right)^2C_0\left(J_1^{\mathrm{m}}(K_{\mathrm{t}_2}+C_0)+J_2^{\mathrm{m}}(K_{\mathrm{t}_1}+C_0)\right)\end{aligned} \tag{4}$$

The roots of this equation can be obtained by approximation methods or explicitly using resolvent equations (e.g., for explicit solutions, Refs. 15 and 16). An exact expression for J could be written here but would require an inordinate amount of space. Alternatively, the resolvent equations are presented below, let:

$$p=\left(-\frac{D}{\delta}(K_{\mathrm{t}_1}+K_{\mathrm{t}_2}+2\,C_0)-J_1^{\mathrm{m}}-J_2^{\mathrm{m}}\right) \tag{5}$$

$$\begin{aligned}q={}&\left(\frac{D}{\delta}\right)^2(K_{\mathrm{t}_1}+C_0)(K_{\mathrm{t}_2}+C_0)\\&+\frac{D}{\delta}\left(2\,C_0(J_1^{\mathrm{m}}+J_2^{\mathrm{m}})+K_{\mathrm{t}_1}J_2^{\mathrm{m}}+K_{\mathrm{t}_2}J_1^{\mathrm{m}}\right)\end{aligned} \tag{6}$$

$$r=-\left(\frac{D}{\delta}\right)^2C_0\left(J_1^{\mathrm{m}}(K_{\mathrm{t}_2}+C_0)+J_2^{\mathrm{m}}(K_{\mathrm{t}_1}+C_0)\right) \tag{7}$$

$$\text{then } 0=J^3+pJ^2+qJ+r \tag{8}$$

$$\text{by letting } J=x-\frac{p}{3} \tag{9}$$

$$\text{then } 0=x^3+ax+b \tag{10}$$

$$\text{where } a=\tfrac{1}{3}(3\,q-p^2) \tag{11}$$

$$\text{and } b=\tfrac{1}{27}(2\,p^3-9\,pq+27r) \tag{12}$$

It was found for the numerical values for the kinetic constants employed in this paper, the single positive and real root for J was given by:

$$J=\left(2\left(-\frac{a}{3}\right)^{1/2}\left(\cos\frac{\phi}{3}\right)\right)-\frac{p}{3} \tag{13}$$

$$\text{where } \phi=\arccos\left(-\frac{b}{2\left(-\frac{a^3}{27}\right)^{1/2}}\right) \tag{14}$$

The other roots were negative or imaginary. Several limiting cases for the effect of unstirred layers on two systems can be described. For reference the substituted Eqn. 2 is given below:

$$J=\frac{J_1^{\mathrm{m}}(C_0-(J\delta/D))}{K_{\mathrm{t}_1}+(C_0-(J\delta/D))}+\frac{J_2^{\mathrm{m}}(C_0-(J\delta/D))}{K_{\mathrm{t}_2}+(C_0-(J\delta/D))} \tag{15}$$

First case

If $J_1^{\mathrm{m}} \gg J_2^{\mathrm{m}}$ and K_{t_1} and K_{t_2} are not very different or $J_2^{\mathrm{m}} \rightarrow 0$ the flux J is essentially given by the relation for a single system described by Winne [4] and Wilson and Dietschy [11].

$$J=0.5\frac{D}{\delta}\left(\left(K_{\mathrm{t}_1}+C_0+J_1^{\mathrm{m}}\frac{\delta}{D}\right)^2 - \left(4C_0J_1^{\mathrm{m}}\frac{\delta}{D}\right)\right)^{1/2} \tag{16}$$

Second case

If $K_{\mathrm{t}_2} \gg K_{\mathrm{t}_1}$ and J_1^{m} and J_2^{m} are not greatly different, then system two functions as a diffusion component when C_0 is in the range of K_{t_1}. Since $K_{\mathrm{t}_2} \gg (C_0-(J\delta/D))$:

$$J_2=\frac{J_2^{\mathrm{m}}(C_0-(J\delta/D))}{K_{\mathrm{t}_2}}=K_{\mathrm{D}}(C_0-(J\delta/D)) \tag{17}$$

$$\text{where } K_{\mathrm{D}}=\frac{J_2^{\mathrm{m}}}{K_{\mathrm{t}_2}} \tag{18}$$

$$\text{then } J=\frac{J_1^{\mathrm{m}}(C_0-(J\delta/D))}{K_{\mathrm{t}_1}+(C_0-(J\delta/D))} + K_{\mathrm{D}}(C_0-(J\delta/D)) \tag{19}$$

K_{D} equals an apparent diffusion coefficient for membrane flux. The value for J is then given by Eqn. 20. A similar equation was also derived by Winne [5]

$$J=\frac{0.5b}{1-(K_{\mathrm{D}}\delta/D)}-\left(0.25\left(\frac{b}{1\text{-}(K_{\mathrm{D}}\delta/D)}\right)^2 - \frac{D}{\delta}C_0\frac{J_1^{\mathrm{m}}+K_{\mathrm{D}}+K_{\mathrm{t}_1}+K_{\mathrm{D}}C_0}{1-(K_{\mathrm{D}}\delta/D)}\right)^{1/2} \tag{20}$$

$$\text{where } b=K_{\mathrm{t}_1}(D/\delta)+C_0(D/\delta) + J_1^{\mathrm{m}}+2K_{\mathrm{D}}C_0+K_{\mathrm{D}}K_{\mathrm{t}_1} \tag{21}$$

Third case

When $K_{\mathrm{t}_1}=K_{\mathrm{t}_2}$ and $J_1^{\mathrm{m}}=J_2^{\mathrm{m}}$ then the mediated component acts essentially like a single system but the apparent maximum velocity is twice the maximum velocity for each system. Therefore, the effect of unstirred layers is given by Eqn. 16 with $J_{\mathrm{app}}^{\mathrm{m}}=2J_1^{\mathrm{m}}=2J_2^{\mathrm{m}}$.

Fourth case

If $C \ll K_{\mathrm{t}_1}$ and K_{t_2} then J is related in a nearly linear fashion to the concentration at the membrane surface. The flux is then given by:

$$J=\frac{(K_{\mathrm{t}_1}J_2^{\mathrm{m}}+K_{\mathrm{t}_2}J_1^{\mathrm{m}})C_0}{K_{\mathrm{t}_1}K_{\mathrm{t}_2}+K_{\mathrm{t}_1}J_2^{\mathrm{m}}(\delta/D)+K_{\mathrm{t}_2}J_1^{\mathrm{m}}(\delta/D)} \tag{22}$$

The apparent transport rate is therefore directly proportional to the bulk phase concentration. In the absence of unstirred layers, the above equation reduces to:

$$J=\frac{(K_{\mathrm{t}_1}J_2^{\mathrm{m}}+K_{\mathrm{t}_2}J_1^{\mathrm{m}})C_0}{K_{\mathrm{t}_1}K_{\mathrm{t}_2}} \tag{23}$$

Hence, the transport rate is decreased in the presence of unstirred layers by factors dependent on δ/D.

Fifth case

When C at the membrane surface is much greater than either K_{t_1} or K_{t_2}, then the carrier systems are saturated. Therefore, $(K_{\mathrm{t}_{1,2}}+(C_0-(J\delta/D))) \approx (C_0-(J\delta/D))$ and for each system $J_{1,2}=J_{1,2}^{\mathrm{m}}$. Consequently,

$$J=J_1^m+J_2^m \tag{24}$$

This simply confirms that at high enough concentrations enough substrate penetrates to the surface of the membrane to drive both transport systems at maximum velocity.

It should be noted that parallel arguments can be developed to solve for the concentration (C) at the membrane surface in the presence of unstirred layers. These equations may be useful in some circumstances but have been deleted to conserve space. In any event the value for C can be simply calculated by substituting J in Eqn. 3.

Sixth case

The expression of the kinetic effects described previously is, of course, dependent on the particular values for the diffusion constant D, and the unstirred layer thickness δ. It is apparent from Eqn. 3, $C = C_0 - (J\delta/D)$, that as δ becomes large the concentration at the membrane becomes small such that at very large δ, $C \to 0$. The unstirred layer becomes rate limiting for transport. Therefore, even in the presence of mediated systems, the rate of solute flux will resemble diffusion kinetically. On the other hand, if δ is very small $C \to C_0$ and the rate of influx shows simple saturation kinetics.

Numerical model and discussion

In order to illustrate the conclusions presented here, a computer model was used. Values for the kinetic constants were chosen and the unstirred layer and bulk phase concentration were varied. For convenience the following values were selected $J_1^m = J_2^m = 5$ mM, $K_{t_1} = 1$ mM and $K_{t_2} = 10$ mM. The unstirred layer thickness and diffusion constant were combined as the ratio D/δ for these calculations. This is useful because the apparent resistance of the unstirred layer depends in part on the chemical nature of the substance transported which determines its diffusion constant. D/δ is essentially the permeability constant for the unstirred water layer. As pointed out by Wilson and Dietschy [11] transported substances with large diffusion constants are less influenced by unstirred layers than substances with low diffusion constants.

Fig. 1 presents an Eadie-Hofstee plot of the calculated example. When D/δ is large the curve approximates that which would be observed in the absence of unstirred layers. As D/δ becomes progressively smaller, the unstirred layer imparts progressively increasing convexity to the curves. In the midpoint range (about $D/\delta = 3.2$), the curves are almost linear. Finally as the unstirred layer becomes large the diffusion across the unstirred layer becomes rate limiting. The curve then approaches a vertical line characteristic of simple diffusion. If the bulk phase concentration is very large, then even in the presence of large unstirred layers the flux rate, J, approaches the maximal velocity of the two systems ($J^m = J_1^m + J_2^m = 10$ mM) as indicated previously (fifth case). It should be pointed out that for any particular set of values for the kinetic constants a family of curves (such as presented in Fig. 1), can be generated. The degree of concave curvature and opposing effect of unstirred layers will be more or less accentuated depending on the disparity of values in systems one and two.

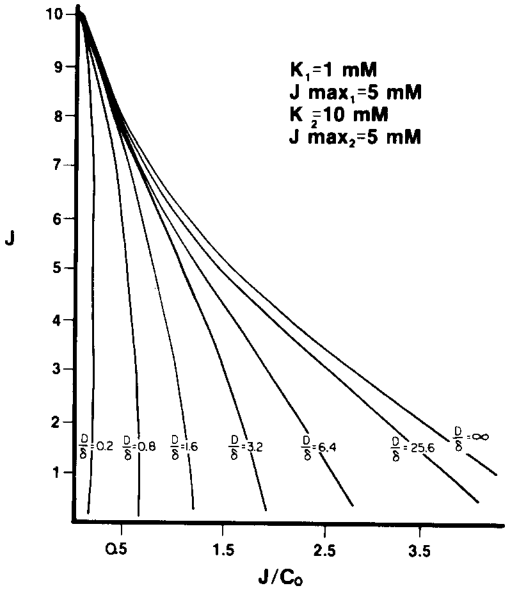

Fig. 1. An Eadie-Hofstee plot of unidirectional solute influx (J) by two transport systems versus J/C_0 in the presence of unstirred layers. C_0 is the bulk phase solute concentration. This is a calculated numerical example with $K_{t_1} = 1$ mM and $J_1^m =$ 5 mM and with $K_{t_2} = 10$ mM and $J_2^m = 5$ mM. The ratio D/δ is the diffusion constant (D) divided by the thickness of the unstirred layer (δ). D/δ was varied from 0.2 to infinity (no unstirred layer present).

As a method of estimating how closely the curves approach linearity for intermediate values of D/δ, the data were treated as linear and fitted with linear regression (Fig. 2). The goodness of fit can be characterized by the standard statistical measures correlation coefficient and the coefficient of variation for the slope and intercepts. Fig. 3

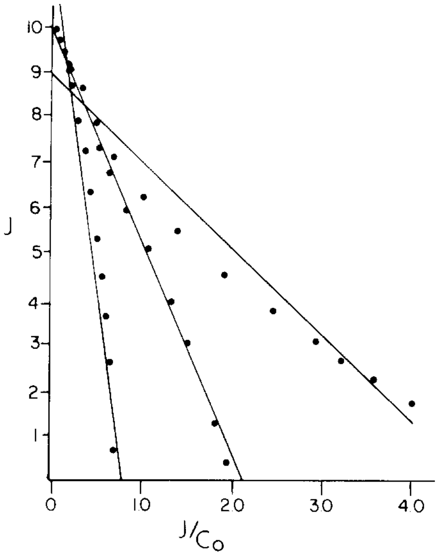

Fig. 2. Linearizing effect of unstirred layers on biphasic transport curves due to two systems. Lines calculated from linear regressions are presented for selected curves from Fig. 1. The points are calculated values of J for various values of D/δ. The solid lines are the corresponding lines calculated by linear regression ($n = 15$, points were selected throughout data range). The correlation coefficients for these curves are $R = 0.972$, 0.999, 0.979 respectively for $D/\delta = 0.8$, 3.2 and infinity.

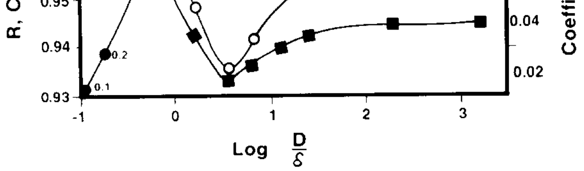

Fig. 3. Variation of correlation coefficient (R) (● ——— ●) and coefficients of variation for the slope (○ ——— ○) and intercepts (■ ——— ■) for linear regressions of the data presented in Fig. 1. The abscissa is $\log(D/\delta)$ and the ordinates either R (left) or coefficients of variation (right scale). The individual values for D/δ are given below each corresponding data point.

presents a plot of these data ($n = 15$). It is apparent that at $D/\delta = 3.2$ the fit to a linear function is very good, $R = 0.999$. The absolute value of R may be higher or lower depending on the particular values selected for the kinetic constants and the population and range of values selected for the regression analysis. However, the relative value of R for a particular set of kinetic constants and using consistent data sampling procedures covering a wide range of values, give an estimate of apparent linearity.

It is clear that unstirred layers in the presence of two mediated systems could easily be confused with a single system without unstirred layers. The use of kinetic criteria alone, then, is not sufficient to assure transport is occurring by a single system. Additional information such as competitive inhibition studies and careful evaluation of unstirred layers is necessary. Of course, the apparent linearity does require a more or less fortuitous combination of particular values for the kinetic constants, the diffusion constant, and the thickness of the unstirred layer. However, given the usual size of error in measurement in physiological data, it appears that it might be rather difficult to detect unstirred layer interaction with two systems. In the example given, correlation coefficients of $R = 0.97$ or larger apply to D/δ values ranging from 0.8 to infinity. The apparent K_t evaluated using linear regression for the curve where $D/\delta = 0.8$ is 15.1 mM and for the curve where $D/\delta = 3.2$, $K_t = 4.68$ mM. Rather sizeable errors in 'apparent K_t' result from unstirred layers even though statistically the curves are fit reasonably well by linear expressions. The greater the difference in the kinetic constants K_{t_1} and K_{t_2}, the more easily the presence of two systems is observed. Therefore, the range of D/δ which could be confused with a linear function is smaller.

The physiological significance of the effects of unstirred layers on transport via two systems should be readily apparent. Dugas et al. [13] and Wilson and Dietschy [11] have confirmed the presence of unstirred layers under experimental conditions in intestinal tissue. Unstirred layers were also shown to influence the absorption of dissolved organic molecules by marine invertebrates [17,18]. It is also known that multiple systems exist for transport of substances such as amino acids. For example, the scheme of Christensen et al. [19–21] for classification of amino acid transport systems suggests that many amino acids are carried simultaneously by two and possibly three systems. In intestine, it has been accepted for some time that transport of the major classes of amino acids occurs primarily via single systems (one each for acid, neutral, basic, amino, and β-amino acids) which have very low or negligible activity for other classes of amino acids [22–24]. However, recently Sepulveda and Smith [25,26] have suggested that two systems might be present for neutral amino acids. Glycine has also been shown to have partial affinity for the imino acid transport system [27]. These data suggest that analysis of transport in epithelia must take cognizance of this possibility. It is clear that analysis of multiple systems in the presence of unstirred layers by using the Eadie-Hofstee plot can lead to serious errors in evaluation of the kinetic constants. This is particularly true if a narrow range of concentrations is used for kinetic experiments.

Thomson and Dietschy [7] have taken account of villous shape in unstirred layer effects on kinetics of single systems. (The equations derived here apply to planar membranes). Thomson [6] observes apparent concave curvature in the Eadie-Hofstee plot as a result of the distribution of transport sites along the villus. The unstirred layer varies in thickness from the top of the villus to the bottom. The 'apparent' transport characteristics due to this variable unstirred layer thickness are functionally similar to multiple transport systems with differing K_t values. For example, assume a single transport system could be classed experimentally as 'superficially' distributed and as 'deeply' distributed [9]. With two different mean depths of unstirred layers, it is functionally equivalent to two transport systems of different K_ts characterized by a single unstirred layer with some average thickness. Even though there are, of course, theoretical differences, kinetically these two cases would appear nearly indistinguishable.

It is clear that the naive application of the Eadie-Hofstee plot to evaluate kinetic parameters of transport systems which have unstirred layers can lead to serious errors. On the other hand, the sensitivity of this plot to unstirred layers makes it useful as a diagnostic tool. The convex curvature of an Eadie-Hofstee plot or a change in apparent kinetic constants with stirring rate should immediately suggest the possible existence of unstirred layers. If two transport systems are present, increased stirring may result in conversion of an apparent linear Eadie-Hofstee plot to a biphasic concave plot. The data presented suggest that some independent method of measurement of unstirred layers should be used whenever possible. These methods include measuring equilibration of extracellular probe molecules [10], electrokinetic effects [8,9,28,29] and estimates based on measuring shape of surface structures. The method of Dugas et al. [13] which measures flux as a function of stirring rate in the medium and then extrapolates to infinite stirring rates could be generally applied for kinetic studies, even if other techniques are not. It should also be emphasized that a broad range of substrate concentrations should be employed. This assists in detection of both multiple systems and unstirred layers. Within a narrow concentration range it is even easier to obtain an erroneous kinetic relationship.

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