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THE INTESTINAL UNSTIRRED LAYER: ITS SURFACE AREA AND EFFECT ON ACTIVE TRANSPORT KINETICS

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SUMMARY

Uptake into the intestinal mucosa is determined by the rates of penetration of solute molecules across the unstirred water layer and lipid cell membrane of the mucosal cell. The presence of the unstirred water layer results in artifactually low permeability coefficients for passive transport processes and high K_m values for active transport processes. Appropriate correction for this unstirred layer resistance requires knowledge of the effective surface area of the diffusion barrier in the intestine. Using three separate experimental and mathematical approaches this surface area was found to vary from 1.02 cm^2 to 14.24 cm^2 per 100 mg dry weight of rat intestine. These values are very much lower than the 1226 cm^2 and 696 cm^2 per 100 mg area of the microvillus membrane in the jejunum and ileum, respectively, and indicates that the effective surface area of the rate-limiting membrane for such highly permeant solutes as long-chain fatty acids is from 1/100 to 1/200 the actual anatomical surface area. These studies also demonstrate that the presence of the unstirred layer introduces major artifacts into the determination of K_m and J_{max} (maximal transport rate) values for active transport processes.

INTRODUCTION

As a molecule moves from the bulk water phase of the intestinal contents into the intestinal epithelial cell it essentially must pass through two membranes in series, a layer of unstirred water and the lipid membrane of the microvillus surface. While several studies from this laboratory have emphasized the rate-limiting nature of the unstirred layer in the intestine for passive transport processes [1–7] virtually no experimental data are available on the effects of this diffusion barrier on the kinetics of active transport. Yet numerous studies have been published in which conclusions have been drawn concerning the characteristics of carrier-mediated transport processes in the intestinal mucosa based upon comparisons of the apparent transport kinetics for various compounds. If these kinetic parameters are significantly distorted by the presence of the unstirred water layer, then it is apparent that many of the conclusions drawn on the basis of these data must be reevaluated.

It is important, therefore, for the study of both active and passive transport processes in the intestine that appropriate corrections be made for the presence of an

unstirred diffusion barrier. Such corrections, however, require knowledge of the effective surface area of the unstirred water layer in the small bowel. In studies carried out with essentially flat membranes such as frog skin, toad bladder and rabbit gallbladder the surface area of the unstirred layer generally has been assumed to equal that of the membrane through which transport is being measured. Such an assumption cannot be made for a membrane such as intestine where the absorptive surface is composed of a complex series of macro- and micro-folds. Hence, it is of critical importance to determine experimentally the effective surface area of the diffusion barrier in the gut.

The purpose of the present investigation, therefore, is 2-fold. First, studies are reported that provide a means for calculating the effective surface area of the unstirred layer in the rat jejunum and ileum and, second, the effect of this diffusion barrier on the kinetics of active transport of several sugars, amino acids and bile acids is demonstrated.

METHODS

Flux rate determinations

Unidirectional flux rates across the brush border of the rat jejunum and ileum were carried out using techniques developed in this laboratory and described in detail elsewhere [8]. Briefly, the everted mucosa of the intestine was exposed to Krebs bicarbonate buffer containing both a radiolabeled test substance (^{14}C-labeled) and a nonpermeant molecule (^{3}H-labeled) used as a marker of adherent mucosal volume. Incubations were all carried out in Krebs bicarbonate buffer gassed with 5 % CO_2 in O_2 (pH 7.4) at 37 °C in test tubes having an inside diameter of 1.5 cm. At the bottom of each tube was a 5/8 inch round, Teflon-coated stirring bar. In studies designated "stirred" this bar was driven at 1800 rev./min by a magnetic stirring device while the bar was kept stationary in the "unstirred" situation. After an incubation period of 4 min at 37 °C the tissue was washed for 5 s in cold buffer, blotted and transferred to a tared counting vial. After determination of tissue dry weight, the tissue was saponified and counted using an external standardization technique to correct for quenching of the two isotopes [8]. Following correction for mass of test molecules carried over in the adherent mucosal fluid by means of the marker compound, flux rates (J_d) were calculated and have the units nmoles taken up into the mucosa per min per 100 mg tissue dry weight, i.e. nmoles $\cdot$ min^{-1} $\cdot$ 100 mg^{-1}.

Several features of this incubation technique require emphasis. First, we have demonstrated in previous work that (1) the incubation time is sufficient for the unstirred water layers to become uniformly labeled with the nonpermeant marker, (2) the rate of uptake of the various probe molecules is linear with respect to time between 2 and 5 min and extrapolated to zero at 0 time, and (3) the rate of tissue uptake is linear with respect to tissue dry weight [1, 8]. Second, we also have shown that the vigorous stirring employed in some of these studies during the 4-min incubation does not alter either the histology or transport characteristics of the membrane even when the stirring was carried out in the presence of surface active agents such as bile acids or bile acids and phospholipid [1, 8]. Third, we have characterized in detail the transport characteristics of a variety of substances in this system. Uptake of probe molecules that are passively absorbed, e.g. bile acids in the jejunum and fatty acids and

alcohols throughout the gut, manifest uptake rates that are linear with respect to the concentration of the probe molecule in the bulk water phase, show no competition among related compounds, are not altered by metabolic inhibition and generally have low temperature coefficients (except for long-chain saturated and unsaturated fatty acids). In contrast, uptake rates of probe molecules that are actively transported, e.g. bile acids in the ileum and sugars and amino acids throughout the intestine, manifest saturation kinetics and competition among related compounds, are suppressed by metabolic inhibitors and have generally high temperature coefficients [1–4, 7–10] (and unpublished observations). Finally, we have investigated the possibility that significant amounts of the radiolabeled probe molecules might be lost from the tissue as CO_2. In the case of the bile acids and amino acids essentially no $^{14}CO_2$ could be detected after a 4-min incubation. When the intestine was incubated with radiolabeled glucose some oxidation did take place, but the total radioactivity found in CO_2 was never greater than 3 % of the radioactivity present in the tissue sample after a 4-min incubation.

Determination of mucosal surface area

In order to relate our transport data to surface area, we determined the area of the jejunum and ileum present in 100 mg dry weight of each of these tissues. Three sacs 2 cm in length were prepared from the proximal and distal small intestine. One sac was placed in a counting vial, dried overnight and the tissue dry weight determined in the usual manner. The other two sacs were fixed in Bouins solution and imbedded in paraffin. One of the two blocks was used for cutting transverse sections and the other for cutting longitudinal sections. The mucosal surface area was then calculated according to the method of Fisher and Parsons [11].

Determination of unstirred layer thickness

The thickness of the unstirred layer in the intestine was measured by the method of Diamond [12]. This technique involves measurement of the half-time ($t_{\frac{1}{2}}$) for evolution of an electrokinetic or diffusion potential across a biologic membrane. Knowing the diffusion constant (D) for the molecule used to induce the potential difference, one can calculate the thickness of the unstirred water layer (d) by means of the formula

$$d = \left(\frac{D \cdot t_{\frac{1}{2}}}{0.38}\right)^{\frac{1}{2}}$$

To measure these half-times an everted segment of intestine similar in length to those used in the incubation experiments was tied over an open glass cannula at one end and fixed to a closed cannula at the other. The serosal compartment was filled with buffer through the glass cannula, and the potential difference across the intestinal wall was measured using salt bridges placed in the outer, mucosal and inner, serosal solutions. All electrical transients were generated by emersion of the gut segments in buffer solutions containing sucrose at varying concentrations up to 100 mM. In this range we have demonstrated that the steady state potential difference essentially is linear with respect to the concentration of added sucrose, i.e. the value of $\Delta PD/\Delta m$ osM $\cdot$ kg^{-1} is constant.

Diffusion coefficients

Except for fatty acids, diffusion coefficients, D, were normalized to the value

of sucrose at 37 °C, $0.69 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1} [3] and were calculated from the relationship $DM^{\frac{1}{2}} = 12.7 \cdot 10^{-5}$ where $M^{\frac{1}{2}}$ equals the square root of the molecular weight of each test solute. Published values were utilized for the fatty acids 4:0, 9:0 and 12:0 [3, 13].

RESULTS

Effect of varying the thickness of the unstirred layer on the kinetics of active transport

Initial experiments were designed to evaluate the effect of varying the thickness of the unstirred water layer on the kinetics of active transport. Previous studies have demonstrated that sugars and amino acids are actively transported at all levels of the small intestine while bile acids are actively transported in the ileum [1, 9, 10, 14, 15] (and unpublished observations). In these studies, therefore, the kinetics of the uptake step were examined for the sugars D-glucose and D-galactose, the amino acid L-serine and the bile acids taurocholate and taurodeoxycholate during absorption into the ileal mucosal cell. The thickness of the diffusion barrier was varied by altering the rate of stirring of the bulk incubation solution.

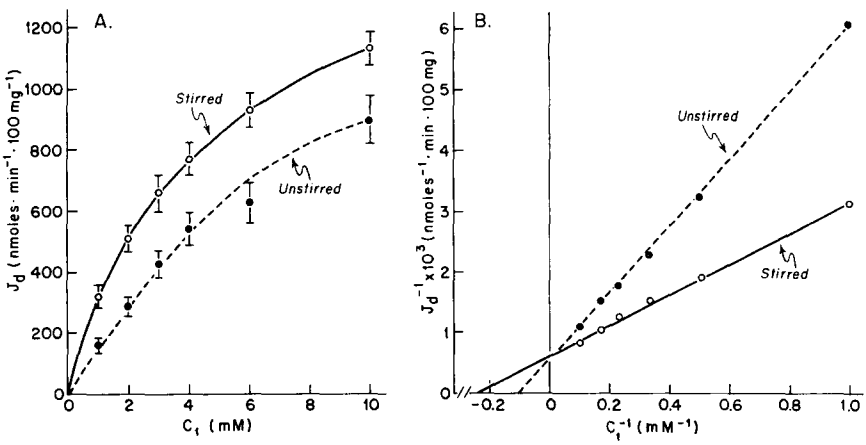

Fig. 1. Active transport rates of glucose as a function of concentration in the bulk buffer phase. Rates of glucose uptake (J_d) into the ileum were determined at various concentrations of the sugar in the bulk buffer phase (C_1) under both stirred and unstirred conditions and are shown in Panel A. These same data are replotted as reciprocals in Panel B: the two regression curves were fitted to the data by the method of least squares. Each point represents the mean ± S.E. for values determined in 12 animals.

In Fig. 1A is illustrated the relationship between the rate of ileal uptake of D-glucose, J_d, and the concentration of the probe molecule in the bulk buffer phase, C_1, under stirred and unstirred conditions. As is apparent stirring results in a leftward shift of the kinetic curve. When the mean values of J_d shown in Fig. 1A are plotted in the conventional double reciprocal fashion, as shown in Fig. 1B, it is evident that the most dramatic effect of stirring is to rotate the best fit straight lines about a near-common intercept on the Y-axis. Thus, stirring appears to have relatively little effect on the apparent maximal achievable transport rate, $^*J_{max}$, but does significantly lower the apparent Michaelis constant, $^*K_m$, for the transport carrier.

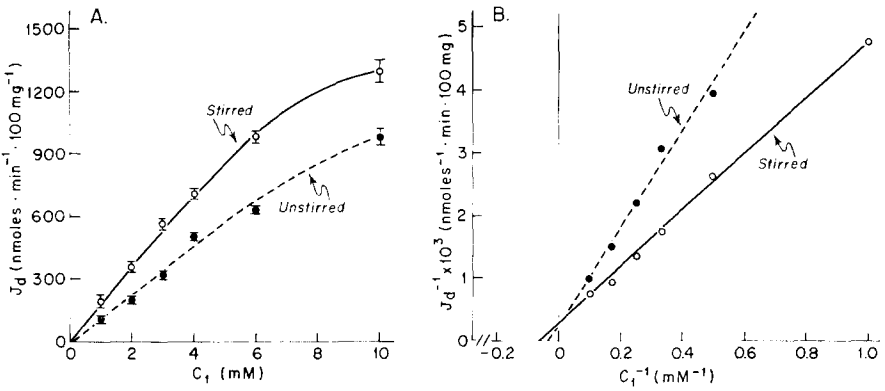

Fig. 2. Active transport rates of L-serine as a function of concentration in the bulk buffer phase. The conditions of this experiment are identical to those described in Fig. 1.

Two other examples of the effect of stirring on the kinetics of active transport are shown in Figs 2 and 3 for L-serine and taurocholate, respectively. Again, in both cases stirring shifts the curve to the left and, in the double reciprocal plots of the data, causes only minor changes in the value of $^*J_{max}$ but does significantly lower $^*K_m$.

Using these double reciprocal plots the best fit values for $^*K_m$ and $^*J_{max}$ were determined for these three compounds as well as for D-galactose and taurodeoxycholate, and these are shown in the first and second columns, respectively, in Table I. The range of concentrations used in these studies was deliberately selected to span the $^*K_m$ region. In addition, however, J_d also was measured for each of these compounds at much higher values of C_2, usually at 15 and 25 mM. In each instance the uptake rates found at these two higher concentrations were essentially equal suggesting that the maximal transport rate had been achieved. These values, therefore, were combined and are shown in the third column of Table I: they are considered to reflect the true value of the maximal transport rate, J_{max}.

Three points warrant emphasis concerning these data. First, stirring had a

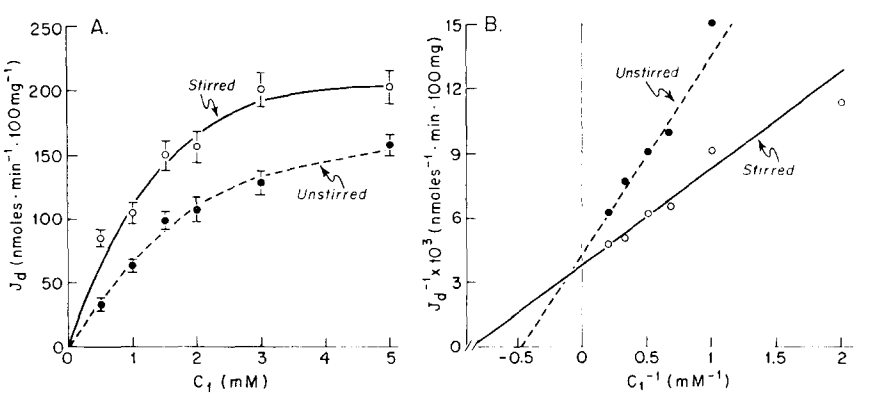

Fig. 3. Active transport rates of taurocholate as a function of the concentration of the probe molecule in the bulk buffer phase. The conditions of this experiment are identical to those described in Fig. 1.

TABLE I

APPARENT TRANSPORT PARAMETERS FOR THE ACTIVE UPTAKE OF SEVERAL REPRESENTATIVE COMPOUNDS ACROSS THE BRUSH BORDER OF THE RAT ILEUM

Rates of uptake, J_d, were determined at 6 different concentrations of each compound in ileums from 12 different rats under both stirred (1800 rpm) and unstirred (0 rpm) conditions. These concentrations were carefully selected to span the region of the apparent K_m for each compound. The best fit linear regression curve was then determined by the method of least squares after plotting the data as reciprocal values. By this means the apparent values for the maximal transport rate, $*J_{max}$, and the bulk phase concentration necessary to give half-maximal velocity, $*K_m$, were determined for each compound and are shown in the first two columns. In addition, J_d was measured at two much higher concentrations, usually at 15 and 25 mM; the observed values of J_d found at these two higher bulk phase concentrations were essentially the same and were considered to represent a direct measure of the maximal transport rate. These values, designated J_{max}, are given in the third column.

Compound	Degree of stirring	$*K_m$ (mM)	$*J_{max}$ (nmoles · min^{-1} · 100 mg^{-1})	J_{max} (nmoles · min^{-1} · 100 mg^{-1})
D-Glucose	unstirred	10.3± 3.1	2050±350	1460±250
	stirred	3.7± 1.2	1666±210	1523±123
D-Galactose	unstirred	38.6±10.1	2340±435	1847±350
	stirred	11.2± 2.5	1975±125	1700±290
L-Serine	unstirred	40.0±13.2	4700±417	1535±176
	stirred	12.7± 3.9	3640±320	1702±381
Taurocholate	unstirred	2.4± 0.4	250± 25	210± 15
	stirred	1.2± 0.3	235± 37	235± 25
Taurodeoxy-cholate	unstirred	0.8± 0.2	92± 10	85± 11
	stirred	0.5± 0.1	85± 6	80± 16

marked effect upon $*K_m$ but relatively little effect on $*J_{max}$. Second, the maximal transport rates determined directly, J_{max}, were consistently lower than those calculated indirectly, $*J_{max}$, from the double reciprocal plots. Third, the relative reduction of $*K_m$ brought about by stirring was roughly proportional to the value of $*J_{max}$ for a particular probe molecule; thus, when the various test molecules are arranged in order of decreasing values of $*J_{max}$ stirring decreases $*K_m$ by a factor of 3.2 (L-serine), 3.4 (D-galactose), 2.8 (D-glucose), 2.0 (taurocholate) and 1.6 (taurodeoxycholate).

Effect of varying the thickness of the unstirred layer on passive permeability coefficients

Passive permeability coefficients were determined for the saturated fatty acids 4:0, 9:0 and 12:0 in the ileum and for taurodeoxycholate, deoxycholate and taurocholate in the jejunum. In practice, J_d values were measured under stirred and unstirred conditions at concentrations of the probe molecules in the bulk buffer phase that varied from 0.1 to 1.0 mM. All data, however, are normalized to a bulk phase concentration of 1 mM so that the apparent passive permeability coefficients, $*P$, have the units nmoles · min^{-1} · 100 mg^{-1} · mM^{-1}.

Under both stirred and unstirred conditions, $*P$ increased as progressively less polar probe molecules were tested. When the bulk buffer phase was unstirred, apparent permeability coefficients equaled 14.2±0.6, 18.3±1.4, 20.2±2.4, 35.4±4.6,

61.5±4.6 and 110.0±15.2 nmoles · min^{-1} · 100 mg^{-1} · mM^{-1}, respectively, for taurocholate, taurodeoxycholate, 4:0 fatty acid, 9:0 fatty acid, deoxycholate and 12:0 fatty acid. With stirring *P for these respective compounds increased to 16.6±1.2, 26.5±3.5, 30.9±5.0, 52.0±7.3, 156.7±8.3 and 330.0±25.4 nmoles · min^{-1} · 100 mg^{-1} · mM^{-1}. These data represent mean values ±1 S.E. for determinations in intestinal segments from 6 to 10 animals. It is evident that the relative effect of stirring varies directly with the apparent permeability coefficient. Thus, stirring increased *P by a factor of 1.2 (taurocholate), 1.4 (taurodeoxycholate), 1.5 (4:0 fatty acid), 1.5 (9:0 fatty acid), 2.5 (deoxycholate), and 3.0 (12:0 fatty acid).

Mean thickness of the unstirred layer under stirred and unstirred conditions

Under unstirred conditions the average thickness of the unstirred water layer equaled 198±5 μm and 217±5 μm in the jejunum and ileum, respectively, from 8 different animals. The mean thickness of this diffusion barrier was reduced by vigorous stirring of the bulk phase to 141±4 μM and 159±5 μm, respectively.

Surface area of the rat small bowel mucosa

For comparative purposes, it was necessary to determine the anatomical surface area of the small intestinal preparations utilized in these transport studies. Particular portions of the surface area are shown schematically in Fig. 4 while experimentally determined or calculated values are given in Table II. In each case a particular parameter of surface area is expressed as cm^2 per 100 mg dry wt of tissue. Thus, such data allow comparisons between uptake rates of the various probe molecules and anatomical surface area.

As shown in Table II the minimum cylindrical serosal surface area present in 100 mg dry weight of jejunum and ileum equaled 7.7±0.3 and 7.2±0.5 cm^2, respectively (line E, Fig. 4 and Table II). The minimum cylindrical surface area at the villus tips (line C, Fig. 4 and Table II) equaled 9.5±0.3 and 8.2±0.6 cm^2 · 100

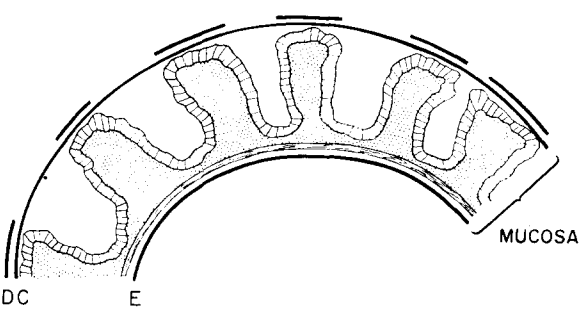

Fig. 4. Schematic representation of various parameters of surface area of the small intestine of the rat. As described in Methods, everted intestinal segments were fixed, and longitudinal and transverse sections were prepared for determination of surface area relationships. This figure represents a line drawing of the photomicrograph prepared from one such transverse section. The letters E, C and D show three different surface areas that were measured, and they correspond to data presented in lines E, C and D, respectively, of Table II. It should be noted that the rat has leaf-shaped villi so that in transverse section they appear as broad widely spaced structures, as shown in this diagram, while in corresponding longitudinal sections they appear as narrow tightly packed structures.

TABLE II

DETERMINATION OF VARIOUS PARAMETERS OF SURFACE AREA OF JEJUNUM AND ILEUM OF THE RAT

Values B, C and E were determined experimentally as described in the text. Value A was calculated by multiplying the values in B times 24. The value of D was determined by multiplying value C by the percent of the cylindrical surface area estimated to overlie the villus tips. Values C, D and E correspond to the dimensions illustrated diagrammatically in Fig. 5.

	Jejunum ($cm^2 \cdot 100\ mg^{-1}$)	Ileum ($cm^2 \cdot 100\ mg^{-1}$)
A. mucosal surface area including microvillus surface	1226.0	696.0
B. mucosal surface area excluding microvillus surface	51.1±4.0	29.0±3.1
C. cylindrical surface area at tips of villi	9.5±0.3	8.2±0.6
D. minimum cylindrical surface area overlying tips of villi	4.3	3.2
E. cylindrical surface area at serosal surface	7.7±0.3	7.2±0.5

mg^{-1} in these same two respective areas of the small intestine. The total mucosal surface area, excluding microvilli, in the jejunum equaled 51.1±4.0 $cm^2 \cdot 100\ mg^{-1}$ while in the ileum equaled 29.0±3.1 $cm^2 \cdot 100\ mg^{-1}$.

Two other important values are calculated in Table II. First, based upon the published value that microvilli increased the mucosal surface area in the rat by a factor of 24 [16], the mucosal surface area, including microvilli, equals 1226 $cm^2 \cdot 100\ mg^{-1}$ and 696 $cm^2 \cdot 100\ mg^{-1}$, respectively, in the jejunum and ileum. Second, the percent of the minimum cylindrical surface area at the villus tips (line C, Table II) estimated to actually overlie villi, as shown diagrammatically by line D in Fig. 4, was estimated on the histologic sections to equal 45 and 39 %, respectively, in the everted jejunal and ileal preparations used in these studies. From these two values the minimum surface area of the exposed villus tips was estimated to equal 4.3 $cm^2 \cdot 100\ mg^{-1}$ in the jejunum and 3.2 $cm^2 \cdot 100\ mg^{-1}$ in the ileum (line D, Table II).

DISCUSSION

In the presence of a significant unstirred layer the concentration of the probe molecule at the aqueous–lipid interface, C_2, is reduced below the concentration of the molecule in the bulk perfusion solution, C_1. The magnitude of this reduction is given by the expression

$$C_2 = C_1 - J\left(\frac{d}{D}\right) \tag{1}$$

where J is the flux rate of the probe molecule across the unstirred layer, d equals the thickness of the diffusion barrier and D is the free diffusion coefficient for the molecule. Consequently, as recently derived by Winne [17], the rate of active transport of a particular solute is given by the expression

$$J = 0.5\frac{D}{d}\left[K_m + C_1 + \frac{J_{max}\,d}{D} \pm \sqrt{\left(K_m + C_1 + J_{max}\frac{d}{D}\right)^2 - 4\left(\frac{d}{D}\right)(J_{max}C_1)}\right] \qquad (2)$$

where K_m and J_{max} represent the true Michaelis constant and maximal transport velocity, respectively, for the membrane carrier.

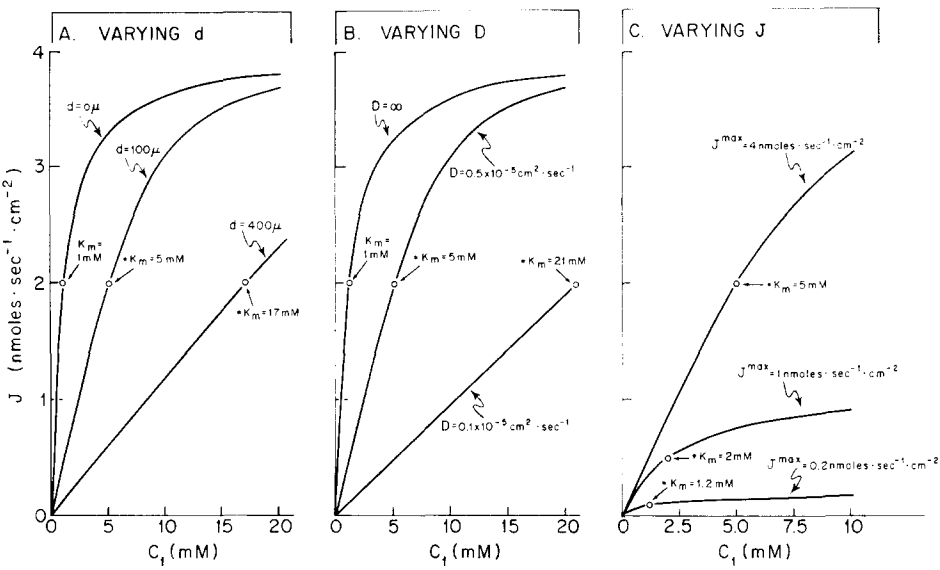

Fig. 5. Theoretical relationship between the kinetics of active transport and variations in unstirred layer thickness, diffusivity of the probe molecule and uptake rates. This diagram illustrates the manner in which active transport kinetics are altered by changes in the thickness (d) of the unstirred water layer (Panel A), by changes in the free diffusion coefficient (D) of the probe molecule (Panel B) and by changes in the absolute flux rate (J) across the unstirred layer (Panel C). J_{max} and K_m represent the true maximal transport rate and Michaelis constant, respectively, for the membrane carrier while $*K_m$ represents the apparent Michaelis constant. In Panel A, d is varied, J_{max} equals 4 nmoles $\cdot$ s^{-1} $\cdot$ cm^{-1}, K_m equals 1 mM and D equals $0.5 \cdot 10^{-5}$ cm^2 $\cdot$ s^{-1}. In Panel B, D is varied, J_{max} equals 4 nmoles $\cdot$ s^{-1} $\cdot$ cm^{-2}, K_m equals 1 mM and d equals $1 \cdot 10^{-2}$ cm. In Panel C, J_{max}, and therefore J, is varied, K_m equals 1 mM, d equals $1 \cdot 10^{-2}$ cm and D equals $0.5 \cdot 10^{-5}$ cm^2 $\cdot$ s^{-1}. In these examples the hypothetical membrane is considered to be flat and of known surface area so that the flux rates, J, are given in the units nmoles $\cdot$ s^{-1} $\cdot$ cm^{-2}; hence, the term S_w which is used to correct the experimentally determined flux rates, J_d, to J does not enter into these calculations.

As shown in Fig. 5 this equation can be used to generate several theoretical curves that illustrate how changes in each of the three variables, i.e. d, D and J, alter the apparent kinetics of active transport. In Panel A, the thickness of the unstirred layer was varied from 0 to 400 μm while values for D, J_{max} and K_m were arbitrarily assigned as noted in the legend. In this example the true Michaelis constant. K_m, for the transport carrier was set equal to 1 mM; the presence of an unstirred layer only 25 μm thick results in an apparent Michaelis constant, $*K_m$, of 2 mM while diffusion barriers 100 μm and 400 μm thick give $*K_m$ values of 5 and 17 mM, respectively. Thus, unstirred layers with dimensions commonly encountered in biological membranes may be expected to profoundly distort K_m values for active transport processes.

Similarly, increasing the molecular weight of the probe molecule and thereby lowering its diffusivity (lowering D) or increasing J_{max} and thereby increasing flux across the unstirred layer (increasing J) causes a marked artifactual increase in the Michaelis constant as shown in Panels B and C, respectively.

In the studies reported here an attempt was made to vary the thickness of the unstirred layer by varying the degree of stirring of the bulk buffer phase. However, it is conceivable that the enhanced uptake seen with stirring was not the result of thinning of the unstirred layer but rather was due to opening of new sites on the villus surface for both active and passive transport, Two observations, however, argue strongly against this latter possibility, First, there was no significant effect of stirring on the directly determined maximal transport rates, J_{max}, of the various probe molecules (third column, Table I) and second, stirring enhanced passive uptake in direct proportion to the apparent passive permeability coefficients for the various solutes. Both of these observations would be expected if the primary effect of stirring was to reduce the thickness of an unstirred layer and neither would be anticipated if stirring simply opened new sites for passive and active transport.

Accepting, then, that stirring reduces the value of d, it is apparent that the experimental data on active transport reported in this paper are entirely consistent with the theoretical predictions illustrated in Fig. 5. First, if these considerations apply to the physiological situation, then altering d should not change J_{max}; as shown in Table I this was consistently the case. Second, $*K_m$ values should vary in the same direction as d; again, this was demonstrated experimentally. Third, a given incremental change in d should alter the $*K_m$ values for a series of solutes in direct proportion to their J_{max} values (assuming that the value of D for the members of the series did not vary greatly). As noted in Results this also was found to be the case: stirring reduced $*K_m$ by a factor of only 0.62 for taurodeoxycholate which has a low mean J_{max} of approximately 82 nmoles $\cdot$ min^{-1} $\cdot$ 100 mg^{-1}, whereas the reduction of $*K_m$ was relatively much greater for the compounds with higher maximal transport rates.

As recently predicted by Winne [17], there is yet another source of error in defining the kinetics of active transport. Eqn 2 does not take the form of a rectangular hyperbole; therefore, the reciprocal of J should not relate in a linear fashion to the reciprocal of C_1. When such data are analyzed by the conventional double reciprocal method, the derived value for the maximal transport rate should be artifactually high: furthermore, the magnitude of this artifact would be expected to vary directly with the thickness of the unstirred layer. This effect is seen in the present studies, as shown in Table I, where the maximal transport rates derived from analysis of the double reciprocal plots, $*J_{max}$, were always higher than those determined directly, J_{max}. Furthermore, for a given probe molecule, $*J_{max}$ was consistently higher in the unstirred situation. Thus, the presence of an unstirred layer leads not only to an overestimation of the Michaelis constant, but, in addition, the value of the maximal transport rate may be seriously in error if data analysis by double reciprocal plots has been carried out.

It is apparent from these considerations that accurate values of K_m for active transport processes and of permeability coefficients for molecules passively transported cannot be obtained unless one takes into account unstirred layer effects. However, equations 1 and 2 use a flux term, J, that must have the units of mass crossing the diffusion barrier per unit time per cm^2 of unstirred layer. The experimentally deter-

mined flux rates in the present study, J_d, were normalized to 100 mg dry tissue weight. If, for purposes of calculation, S_w is defined as the effective surface area of the unstirred layer in an amount of rat small intestine equivalent to 100 mg tissue dry weight, then

$$J = \frac{J_d}{S_w} \tag{3}$$

where S_w has the units $cm^2 \cdot 100\ mg^{-1}$. Thus, in equations 1 and 2 the term $\frac{J_d}{S_w}$ may be substituted for J.

In order to utilize these equations, however, it is first necessary to determine a value for S_w under the conditions of these studies. Using flux data reported in this paper as well as other data from this laboratory it is possible to calculate values of S_w using three different experimental and mathematical approaches; these involve an analysis of (1) the effect of stirring on the kinetics of active transport, (2) the effect of stirring on passive permeation and (3) maximum rates of passive uptake of compounds whose rates of absorption essentially are limited by the unstirred water layer.

(1) Analysis using the effect of stirring on the kinetics of active transport into the intestinal mucosa

As shown in Figs 1–3 the effect of stirring is to shift the kinetic curve for active transport to the left so that $^*K_m$ values are lowered. Using Eqn 1 and substituting the appropriate expression for J (from Eqn 3) the value of C_2 at any point on the kinetic curve can be calculated in both the stirred and unstirred situation as follows:

$$C_2^s = C_1^s - \left(\frac{J_d^s}{S_w}\right)\left(\frac{d^s}{D}\right) \tag{4}$$

$$C_2^u = C_1^u - \left(\frac{J_d^u}{S_w}\right)\left(\frac{d^u}{D}\right) \tag{5}$$

where the superscripts s and u refer to the values of C_1, C_2, J_d and d in the stirred and unstirred situation, respectively. Since the true K_m for the transport carrier presumably is unaltered by stirring, it follows that for any value of J_d the concentration of the test molecule at the membrane interface must be the same in the stirred and unstirred situation, i.e. $C_2{}^s$ must equal $C_2{}^u$ at a given value of J_d; thus, in this particular circumstance Eqns 4 and 5 may be combined to yield

$$C_1^s - \left(\frac{J_d}{S_w}\right)\left(\frac{d^s}{D}\right) = C_1^u - \left(\frac{J_d}{S_w}\right)\left(\frac{d^u}{D}\right) \tag{6}$$

By rearranging terms and solving for S_w the following expression is obtained

$$S_w = \frac{(J_d)(d^u - d^s)}{(D)(C_1^u - C_1^s)} \tag{7}$$

Hence, this equation will yield a value for S_w given the experimentally determined values of C_1 in the stirred and unstirred situation necessary to produce a given rate of active absorption, J_d. Inherent in this derivation, it should be emphasized, is the assumption that S_w is the same in the stirred and unstirred situations for the actively transported compounds. In practice, in making these calculations the experimentally

determined values of J_{max} in the stirred and unstirred condition given in Table I were averaged and 0.5 J_{max} was determined; the values of C_1^s and C_1^u necessary to achieve this particular value of J_d were then determined graphically from plots of the transport data like those shown in Figs 1A–3A. Using values for J_d, C_1^s and C_1^u so derived from the experimental data in Table I and the determined mean values for d^s and d_u, the effective surface area of the unstirred layer varied from 2.75 to 5.18 $cm^2 \cdot 100\ mg^{-1}$ as shown in coumn 1, line A of Table III.

TABLE III

THE EFFECTIVE SURFACE AREA OF THE UNSTIRRED WATER LAYER IN THE JEJUNUM AND ILEUM OF THE RAT

The surface area of the unstirred layer per 100 mg dry weight of jejunum and ileum was calculated using the three mathematical procedures outlined in the Discussion. In column 1, S_w was calculated using the measured mean values of d which equal 141 ± 4 μm (stirred, 1800 rev./min and) 198 ± 5 μm (unstirred) in the jejunum and 159 ± 5 μm (stirred, 1800 rev./min) and 217 ± 5 μm (unstirred) in the ileum. In column 2 the value of d^s was assumed to equal 50 μm in both the jejunum and ileum.

Method	Compound	Tissue	Effective surface area of the unstirred layer, S_w ($cm^2 \cdot 100\ mg^{-1}$)	
			Using mean values of d	using minimum value of d^s
A. Analysis of active transport kinetics	D-glucose	ileum	5.18	14.91
	D-galactose	ileum	2.75	7.91
	L-serine	ileum	4.28	12.33
	taurocholate	ileum	3.85	11.10
	taurodeoxycholate	ileum	4.61	13.28
Mean values from ileal studies			4.13	11.91
B. Stirring effect on passive permeation	4:0 fatty acid	ileum	0.47	1.34
	9:0 fatty acid	ileum	1.19	3.45
	12:0 fatty acid	ileum	1.39	5.34
	taurodeoxycholate	jejunum	0.97	2.52
	deoxycholate	jejunum	1.53	3.92
	taurocholate	jejunum	1.66	4.32
Mean values from ilela studies			1.02	3.38
Mean values from jejunal studies			1.39	3.59
C. Passive uptake of compounds limited by unstirred layer	12:0 fatty acid	jejunum	12.22	4.33
	10:0 alcohol	jejunum	16.25	5.76
Mean values from jejunal studies			14.24	5.05

(2) *Analysis using the effect of stirring on the kinetics of passive permeation into the intestinal mucosa*

As also shown in the present studies, stirring increases the value of apparent passive permeability coefficients. Since the rate of passive permeation of the mucosal cell membrane is a linear function of C_2 it follows that the change in C_2 brought about by stirring can be calculated from the ratio of the passive flux rates measured under stirred and unstirred conditions, i.e.

$$X = \frac{J_d^s}{J_d^u} \tag{8}$$

where X denotes the relative change in concentration of a probe molecule at C_2 brought about by stirring.

Thus, C_2 in the unstirred situation equals

$$C_2^u = C_1^u - \left(\frac{J_d^u}{S_w}\right)\left(\frac{d^u}{D}\right) \tag{9}$$

while in the stirred condition, using the relationship expressed in Eqn 8, the following equation applies

$$XC_2^u = C_1^s - \left(\frac{J_d^s}{S_w}\right)\left(\frac{d^s}{D}\right) \tag{10}$$

Setting both Eqns 9 and 10 equal to $C_2{}^u$ and taking advantage of the fact that passive flux rates are all normalized to the same bulk phase concentration, i.e., C_1 is equal in both situations, the following expression is obtained

$$C_1 - \left(\frac{J_d^u}{S_w}\right)\left(\frac{d^u}{D}\right) = \left[C_1 - \left(\frac{J_d^s}{S_w}\right)\left(\frac{d^s}{D}\right)\right]\left[\frac{1}{X}\right] \tag{11}$$

Solving for S gives the equation

$$S_w = \frac{(X)(J_d^u)(d^u) - (J_d^s)(d^s)}{(D)(XC_1 - C_1)} \tag{12}$$

Hence, this equation will yield a value of S_w based upon the effects of stirring on passive permeation of the intestinal mucosa. It should again be pointed out that this derivation is based on the assumption that S_w is the same for the passive absorption of the various probe molecules in the stirred and unstirred condition. Using the data reported in these studies on the effect of stirring on three fatty acids and three bile acids, the effective surface area of the unstirred water layer varied from 0.47 to 1.66 $cm^2 \cdot 100\ mg^{-1}$ as shown in column 1, line B of Table III.

(3) Analysis using maximal rates of passive uptake of compounds whose rates of absorption essentially are limited by the unstirred water layer

In other work from this laboratory [3] we have shown that for highly permeant molecules such as the 12:0 fatty acid (*P equals 395 nmoles $\cdot$ $min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$) and 10:0 alcohol (*$P$ equals 581 nmoles $\cdot$ $min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$) the major resistance to uptake is the unstirred water layer and not the cell membrane, i.e. $P \gg D/d$. Thus, during the passive absorption of such compounds C_2 essentially equals 0 so that after substituting J_d/S_w for J and rearranging terms Eqn 1 equals

$$S_w = \frac{(J_d)(d)}{(C_1)(D)} \tag{13}$$

Hence, this equation will yield values of S_w in the special circumstance where J_d is measured for compounds where the rate-limiting step to mucosal uptake is the unstirred layer. As shown in column 1 of line *C* this equation gives values of 12.22 $cm^2 \cdot 100\ mg^{-1}$ and 16.25 $cm^2 \cdot 100\ mg^{-1}$ using the 12:0 fatty acid and 10:0 alcohol,

respectively, which are considerably higher than the values calculated by the previous two methods.

However, all values in column 1 of Table III are calculated using the experimentally determined mean values of d^s, i.e. 141 and 159 μm in the jejunum and ileum, respectively. In other work from this laboratory we have shown that such mean values do not accurately reflect the dimensions of the diffusion barrier overlying the sites of absorption on the upper villi. At high stirring rates, for example, the thickness of the unstirred layer may be as small as 50 μm over the tips of the villi. While an analysis of this problem will be published elsewhere, we have recalculated the values of S_w obtained from all three experimental approaches after setting d equal to 50 μm. In this way both a minimum and maximum value for S_w in each experimental situation is obtained: these values are shown in column 2 of Table III.

The complexity of the problem of defining precise values for S_w and d in any given experimental situation is apparent when one considers that these parameters are not just determined by the anatomy of the intestinal mucosa and unstirred layer at a particular stirring rate but they also are, to some extent, influenced by the permeability characteristics of the probe molecule itself. For example, for a highly permeant molecule whose passive uptake is limited by the unstirred layer it is reasonable to assume that nearly all absorption takes place at the villus tips and, therefore, the most appropriate values for d^s and S_w might be 50 μm and 5.05 $cm^2 \cdot 100\ mg^{-1}$, respectively, in the stirred situation in the jejunum (column 2, line C, Table III). On the other hand, for an actively transported probe molecule that may be absorbed through a much greater area of the mucosal surface it is equally reasonable to assume that the mean diffusion distance is greater than 50 μm and that the effective surface area of the diffusion barrier through which movement of the probe molecule occurs is also greater than the minimum value. Hence, as shown in Table III, for actively transported molecules S_w presumably lies somewhere between 4.13 and 11.91 $cm^2 \cdot 100\ mg^{-1}$. Obviously, considerably more work is required to precisely fix these values for each type of transport.

Despite these uncertainties, however, it is apparent that the calculated range of values of S_w allows one major conclusion to be reached, i.e. the effective surface area of the diffusion barrier in the intestine is very much less than the anatomical surface area of the mucosal membrane. This is best appreciated by comparing the value of S_w with the anatomical surface areas shown in Table II. Clearly, for passively absorbed molecules the effective surface area of the unstirred layer is very close to the minimum cylindrical surface area at the tips of the villi and approximately 1/200 of the surface area of the microvillus membrane. In the case of probe molecules that are actively transported, even using the minimum value of d^s, the effective surface area of the unstirred layer is only 1/60 the area of the lipid cell membrane.

Finally, it also should be emphasized that while the unstirred water layer may become absolutely rate limiting for passive absorption into the intestinal mucosa, this barrier can never be rate limiting for active absorption. That is, the unstirred layer will grossly increase the value of $^*K_m$ but will not prevent the system from achieving J_{max} provided a high enough concentration of the solute can be achieved in the bulk solution at C_1. The magnitude of this unstirred layer effect upon $^*K_m$ values can be appreciated from the present data: in the case of glucose, for example, $^*K_m$ equals 10.3 and 3.7 mM, respectively, in the unstirred and stirred situations while the true

K_m for the carrier is lower still, probably in the range of 1 mM. Thus, published K_m values for active transport systems in the intestine may be in error by many fold.

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