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Experimental Demonstration of the Effect of the Unstirred Water Layer on the Kinetic Constants of the Membrane Transport of D-Glucose in Rabbit Jejunum

Alan B.R. Thomson* and John M. Dietschy

Gastrointestinal-Liver Unit, Department of Internal Medicine, University of Texas Health Science Center, Dallas, Texas 75234, and Division of Gastroenterology, Department of Medicine, University of Alberta, Edmonton, Canada

Summary. The rate of active transport of a probe molecule into the intestinal mucosal cells is determined by the rate of movement of the solute molecule across two barriers, the unstirred water layer and the microvillus membrane of the epithelial cell. Previously a theoretical equation has been derived which described J_d, the velocity of unidirectional flux, as a function of the characteristics of the transport carrier in the membrane and of the resistance of the overlying unstirred water layer (UWL). The predictions of these equations have been tested experimentally by studying the effect of the rate of stirring of the bulk phase on the *in vitro* uptake of D-glucose by rabbit jejunum. These studies demonstrated that, first, alterations in the UWL have a profound effect on the magnitude of the apparent affinity constant, K_m^*, of the active transport process. Second, at bulk phase concentrations in excess of the K_m the passive component of the experimentally determined flux rate becomes of such magnitude as to introduce significant error into the estimate of both the maximal transport rate, J_d^m, and the true K_m. Third, as a result of the UWL, the use of double-reciprocal plots to determine J_d^m and K_m leads to the overestimation of these constants. Finally, failure to account for the UWL leads to important quantitative errors describing a number of the characteristics of the transport process: these include an underestimation of the Q_{10} and the effect of sodium ion on the active transport of glucose in the jejunum. The results confirm that the kinetic characteristics of the uptake of an actively transported molecule are a complex function of the resistance of both the UWL and the mucosal cell membrane, and this transport process can be adequately described by a newly-derived equation. It is apparent that there are serious limitations in the interpretation of much of the previously published data dealing with active transport processes in the intestine, since these studies failed to account for the effect of the UWL.

Unidirectional reflux of solutes into the intestinal mucosal cell is determined by the rate of passage through at least two separate barriers in series, the protein-lipid membrane of the microvillus surface, and the overlying unstirred water layer [5, 6, 10, 13, 14, 18–21]. A mathematical expression has previously been derived which predicts the effect of both of these resistances on active transport processes in the intestine [17]. The present studies were undertaken to experimentally test these predictions. The effective thickness and surface area of this diffusion barrier was varied in a systematic and reproducible manner, and the unidirectional uptake rate of D-glucose into rabbit jejunum was studied. Most previous work dealing with transport in the gastrointestinal tract has assumed that the experimentally determined kinetic parameters describing the active uptake of a particular solute reflect the properties of the membrane carrier itself. However, the results of this study clearly demonstrate that the characteristics of the uptake of a molecule are a complex function of the resistance of both the unstirred water layer and the mucosal cell membrane and that there are serious limitations in the interpretation of much of the previously published data dealing with active transport processes in the intestine.

Methods and Materials

Probe and Marker Compounds

The compound used to measure the adherent mucosal fluid volume, (G-^{3}H) dextran (mol wt approximately 15,000 to 17,000), was obtained from New England Nuclear Corporation, Boston, Mass., and was used as supplied by the manufacturer. Unlabeled and (1-^{14}C) labeled, respectively, D- and L-glucose were supplied by Sigma Chemical Corporation, St. Louis, Mo., Fisher Scientific Company Ltd., Pittsburgh, Pa., and New England Nuclear Corp.

* *Current address:* Department of Medicine, Division of Gastroenterology, 8–104 Clinical Sciences Building, University of Alberta, Edmonton, Alberta, Canada, T6G 2G3.

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Tissue Preparation

Albino New Zealand female rabbits were killed by decapitation. As described in detail elsewhere [10, 18], a 10 to 20 cm length of proximal jejunum was rapidly removed, rinsed with 80 ml of cold saline, and opened along its mesenteric border, and the mucosal surface was carefully washed with a stream of cold saline from a syringe to remove visible mucus and detritus. Circular pieces of intestine were cut from the segment using a sharpened steel punch. These were mounted as a flat sheet in incubation chambers, and clamped between two plastic plates so that the serosal and mucosal surfaces were exposed to separate incubation solutions through apertures in the plates exactly 1 cm in diameter. To the serosal compartment was added 1.2 ml of Krebs-bicarbonate buffer, and each chamber was then placed in a beaker containing KRB buffer at 4 °C and constantly oxygenated by a stream of 5% CO_2 in oxygen until used in the various experiments. The chambers were first transferred to identical beakers containing oxygenated KRB buffer at 37 °C for a preincubation of either 4 min to allow the tissue to equilibrate at this temperature, or for 30 min to allow closure of the intervillus spaces [18]. They were then transferred to other beakers for specific experiments. The preincubation and incubation solutions were mixed at identical stirring rates with circular magnetic bars, and the stirring rates were precisely adjusted by means of a strobe light; stirring rates are reported as the revolutions per minute, rpm, at which the stirring bar was driven. Stirring rates were altered in a systematic and reproducible manner to yield the previously reported values for the effective thickness and surface area of the unstirred water layer [18].

Determination of Unidirectional Flux Rates.

After preincubation, the chambers were transferred to other beakers containing (G-^{3}H) dextran and various (^{14}C) probe molecules dissolved in oxygenated KRB buffer at 37 °C and pH 7.4. After incubation for various periods of time, the experiment was terminated by removing the chamber and quickly rinsing the tissue in iced saline for approximately 5 sec. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, divided into two equal pieces, blotted on filter paper, and placed in a tared counting vial. The tissue was drived in an oven overnigth and the dry weight determined. The sample was then saponified with NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes [14]. The rate of uptake, J_d, was calculated after correcting the total tissue ^{14}C radioactivity for the mass of the probe molecule present in the adherent mucosal fluid. These rates were expressed as the nanomoles of the probe molecule taken up into the tissue per 100 mg dry wt of tissue per min (nmol/100 mg/min).

Abbreviations and Units used in This Paper.

C_1, concentration of the probe molecule in the bulk aqueous phase, mM; C_2, concentration of the probe molecule at the aqueous-microvillus interface, mM; D, free diffusion coefficient of the probe molecule, cm^2/min; d, effective thickness of the unstirred water layer, cm; Sw, effective surface area of the unstirred water layer, m^2/100 mg; J_d, experimentally determined unidirectional rate of uptake of glucose normalized to tissue dry wt, nmol/100 mg/min; K_m, true affinity constant of the intestinal transport process, mM; P, true permeability coefficient of the microvillus membrane, nmol/100 mg/min/mM; P^*, apparent permeability coefficient, nmol/100 mg/min/mM; Q_{10}, ratio of transport measured at two temperatures 10° apart; Q_{10}^*, apparent Q_{10}; rpm, the revolutions per minute at which the stirring bar was driven to mix the bulk phase; KRB, Krebs-Ringer-Bicarbonate buffer; UWL, the unstirred water layer.

Results

Initial experiments were designed to evaluate the conditions which must be satisfied in order for the technique to be valid to demonstrate unidirectional flux. First, the rate of tissue uptake must be linear with respect to time, and, further, this function must extrapolate to zero uptake at zero time. As shown in Fig. 1 *A*, the uptake of D-glucose was linear with respect to time between 4 and 16 min, with specimens preincubated for both 4 and 30 min. It is also apparent that glucose uptake extrapolated to zero at zero time. Second, the marker compound used to estimate the adherent mucosal fluid volume must become uniformly labeled within the designated experimental incubation period. As demonstrated in Fig. 1 *B*, [G-^{3}H] dextran equilibrated with the adherent mucosal fluid only after about 4 min in specimens preincubated for both 4 and 30 min, and stirred at 600 rpm. In addition, while not shown in this figure, 6 min incubation was required for equilibration of the dextran marker when the bulk phase was unstirred. With incubation periods between 12 and 16 min there was an apparent increase in the volume of the adherent mucosal fluid. As a result of the failure of the marker to equlibrate before 4 min, the uptake of glucose at 1 and 2 min was overestimated by at least 100% (Fig. 1 *A*). Thus, short incubation periods could not be employed to measure the rate of unidirectional flux since these brief periods did not allow complete and uniform labeling of the extracellular fluid layers immediately adjacent to the microvillus surface. Third, none of the probe molecule should be lost into the serosal compartment. Less than 1% of the ^{14}C-activity in the tissue appeared in the serosal fluid in incubation times less than 12 min (Fig. 1 *C*); thereafter the percentage of counts rose sharply so that over 10% of the activity in the tissue appeared in the serosal compartment after 16 min. Since each of these criteria was fulfilled by incubations of between 6 and 12 min in both the unstirred and highly stirred experimental conditions, subsequent kinetic studies of the effect of stirring on the unidirectional flux of D-glucose were performed using 8-min incubation periods.

As demonstrated in Fig. 2 *A*, there was a curvilinear relationship noted between the concentration of D-glucose in the bulk phase and the unidirectional flux. In samples of intestine preincubation for 30 min, increasing the rate of stirring of the bulk phase from 0 to 600 rpm was associated with a marked increase in J_d at concentrations below 10 mM glucose. In con-

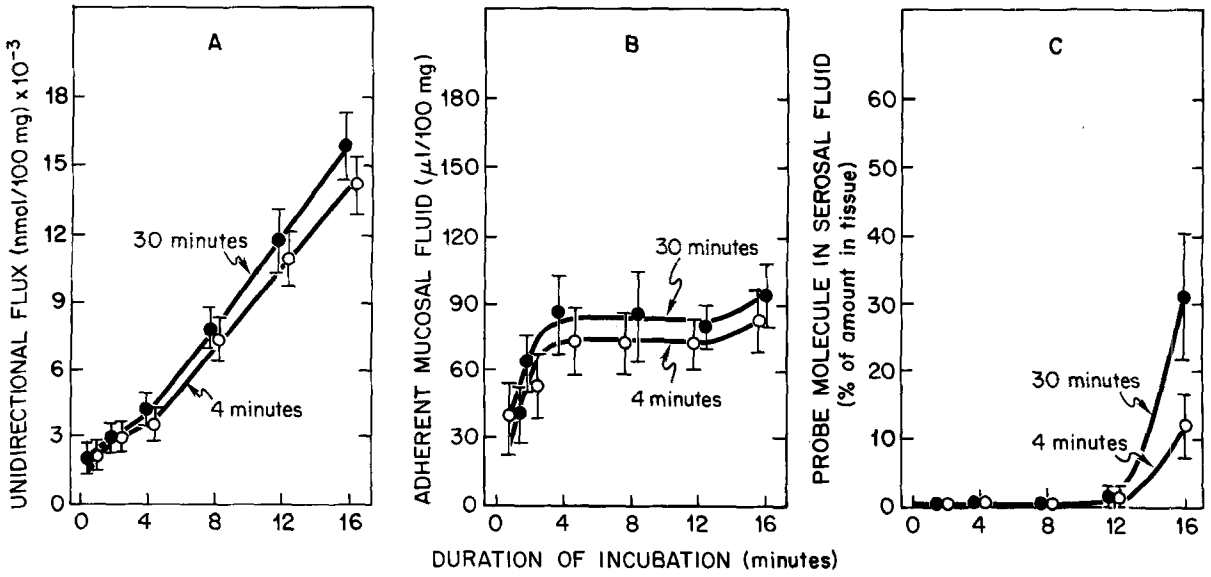

Fig. 1. Validation of the technique employed to demonstrate unidirectional reflux. Discs of rabbit jejunum were stirred *in vitro* at 600 rpm and were preincubated for 4 or 30 min before transferring to beakers containing 120 mM D-glucose for varying periods of 1, 2, 4, 8, 12 or 16 min. Each point represents the mean ± SEM of 15 to 18 measurements. *(A)*: The time course for mucosal uptake of D-glucose at periods of varying duration of incubation. *(B)*: The time course for the estimation of the adherent mucosal fluid volume. *(C)*: Appearance of the [^{14}C] D-glucose in the serosal fluid, expressed as a percentage of the C^{14} counts in the jejunal tissue

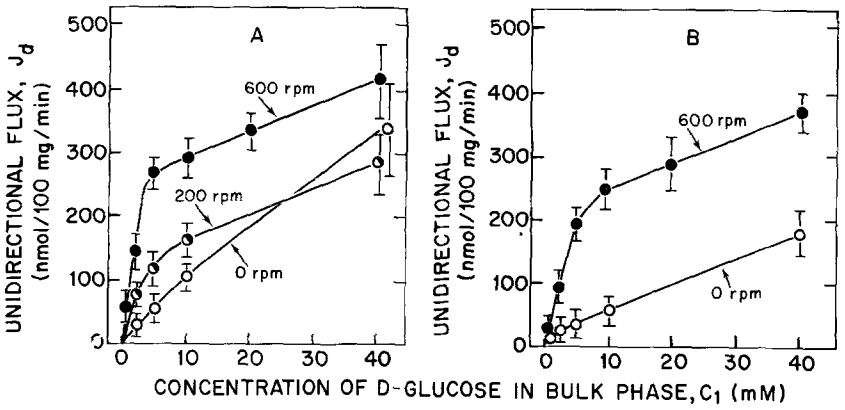

Fig. 2. Effect of stirring of the bulk phase on the unidirectional flux of D-glucose. Jejunal discs were preincubated for either 30 min *(A)* or 4 min *(B)*, while being stirred at 600, 200 or 0 rpm. The samples were then incubated for 8 min in ^{14}C-labeled solutions of concentrations of D-glucose varying from 1 to 40 mM. During this time, the same rate of stirring was maintained for each specimen

trast, stirring had much less effect on solute uptake at higher concentrations; indeed, at concentrations of 80 and 120 mM, the uptake rates were equal at all these stirring rates. As shown in Fig. 2*B*, in tissue specimens preincubated for only 4 min, stirring had a marked effect on J_d, even at the higher substrate concentrations. As previously demonstrated under these conditions the intervillus spaces are open and the effective thickness of the unstirred water layer is considerably increased [18].

To accurately evaluate an active intestinal transport process, one must correct for the experimentally determined unidirectional flux for the passive component which proceeds concurrently [15]. It was necessary, therefore, to estimate the apparent passive permeability coefficient, P^*, of the rabbit jejunum for glucose at these same stirring rates. Three techniques were employed. At substrate concentrations several times the true affinity constant of the transport process, the maximal transport velocity of the active component is approached, the active component of the total uptake process becomes independent of substrate concentration, and any further experimentally-determined increase in uptake is due to the continued uptake by the passive process(es). Accordingly, the apparent passive permeability coefficient of the rabbit jejunum for D-glucose was determined from the slope of the kinetic curve between 10 and 120 mM glucose. Secondly, L-glucose has the same mol wt and polarity as D-glucose and is either passively absorbed or has

Table 1. Estimation of the apparent passive permeability coefficient (P^*) of the rabbit jejunum for D-glucose under different conditions of preincubation and stirring

Experimental conditions used to estimate P^* of the rabbit jejunum for D-glucose	Apparent permeability coefficient, P^*, nmol/100 mg/min/mM				
	Preincubation 30 min			Preincubation 4 min	
	0 rpm	200 rpm	600 rpm	0 rpm	600 rpm
1. Slope of D-glucose kinetic curve, high substrate concentrations	3.4±0.3	4.2±0.3	5.7±0.5	3.6±0.3	4.0±0.4
2. Slope of L-glucose kinetic curve	3.3±0.5	–	5.5±0.5	3.0±0.5	3.7±0.3
3. Slope of D-glucose kinetic curve at 4 °C, corrected to 37 °C	4.1±0.5	–	5.8±0.6	–	–
4. Mean value	3.6±0.4	4.2±0.3	5.7±0.5	3.3±0.4	3.9±0.3

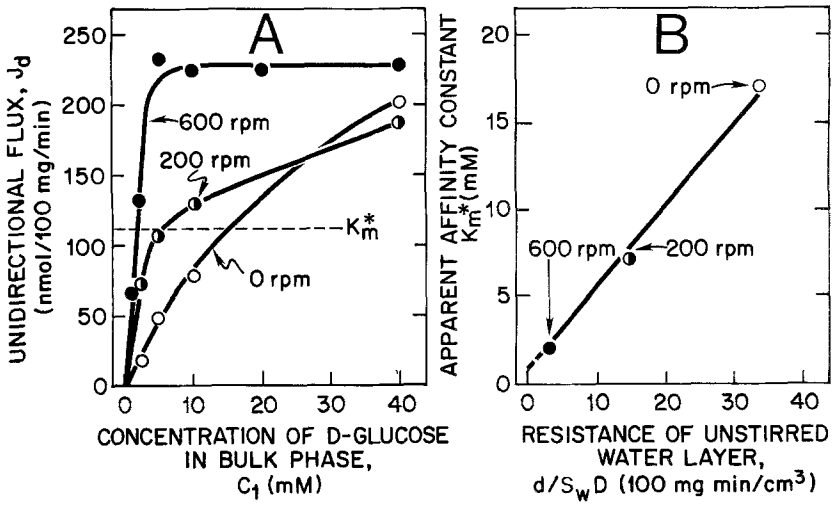

Fig. 3. Effect of stirring the bulk phase on the kinetic constants of the active transport for D-glucose in jejunal tissue preincubated 30 min. The experimentally determined unidirectional flux (Fig. 2) was corrected for passive permeation (Table 1) in order to obtain an accurate assessment of the kinetic constants of the active transport process. *(A)*: Increasing the rate of stirring of the bulk phase from 0 to 200 to 600 rpm was associated with a marked decline in the magnitude of the apparent affinity constant. K_m^*, from 17.7 to 6.8 to 1.9 mM, respectively. *(B)*: Relationship between the effective resistance of the unstirred water layer, $d/Sw \cdot D$, and the apparent affinity constant, K_m^*. The magnitude of the effective thickness, d, and effective surface area, Sw, of the unstirred water layer, as well as the free diffusion coefficient of D-glucose, have been reported previously [18]. When these values at each stirring rate were substituted into the formula $d/Sw \cdot D$, a measure of the effective resistance of the unstirred water layer was obtained [17]. Note that when $d/Sw \cdot D = 0$, $K_m^* = K_m$. Thus, from extrapolation, the true affinity constant K_m is predicted to be approximately 0.8 mM

an extremely low affinity for the glucose carrier [1]. Thus the slope of the L-glucose kinetic curve was used as an approximation of the P^* of D-glucose. Thirdly, the activity of active transport processes is markedly inhibited in the cold. The slope of the kinetic curve of D-glucose transport at 4 °C was assumed to represent the passive component of D-glucose uptake occurring at that temperature. Using the Q_{10}^* value of 1.2 obtained in a subsequent experiment (Fig. 4), the apparent permeability coefficient for D-glucose was calculated at 37 °C. As shown in Table 1, there was close agreement between the apparent passive permeability coefficient of D-glucose obtained by the three techniques. Note that P^* was affected by stirring the bulk phase: the mean calculated P^* rose from 3.6 to 5.7 nmol/100 mg/min/mM when the rate of stirring of the bulk phase was increased from 0 to 600 rpm (Table 1).

With knowledge of these apparent passive permeability coefficients for D-glucose, it was next possible to correct the experimentally determined values of unidirectional flux for the contribution of the passive component, and thereby obtain a more accurate evaluation of the active membrane transport process for this sugar. As shown in Fig. 3, in jejunal tissue preincubated 30 min and stirred at 600 rpm, there was a rapid increase in the active unidirectional flux of D-glucose as the concentration of glucose in the

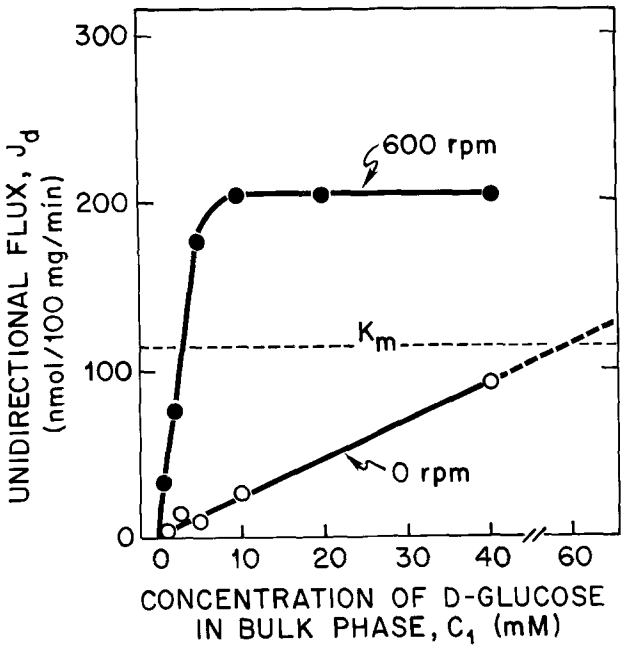

Fig. 4. Effect of stirring the bulk phase on the kinetic constants of the active transport for D-glucose in jejunal tissue preincubated 4 min. The samples were either stirred at 600 rpm or were unstirred, and the active unidirectional flux was calculated as described in the legend of Fig. 3. The values of the kinetic parameters K_m^* and J_d^m are shown in Table 2. Note that increasing the rate of stirring from 0 to 600 rpm was associated with a marked decline in the magnitude of K_m^* from 60 to 3.3 mM

Table 2. Estimation of the kinetic constants K_m^* and J_d^m of the active transport process for D-glucose in the rabbit jejunum under different conditions of preincubation and stirring

Type of plot of experimental data used to estimate the kinetic constants of the Jejunal active transport of D-glucose	Preincubation 30 min		Preincubation 4 min	
	0 rpm	600 rpm	0 rpm	600 rpm
A. Apparent Michaelis constant, K_m^* (mM)				
1. C_1 *vs.* J_d, Figs. 3 and 5	17.7	1.9	60	3.3
2. $1/C_1$ *vs.* $1/J_d$, with correction for passive permeation	33	2.4	14.3	5.0
3. $1/C_1$ *vs.* $1/J_d$, without correction for passive permeation	100	4.3	63	4.5
B. Maximal transport rate, J_d^m (nmoles/100 mg/min)				
1. C_1 *vs.* J_d, Figs. 3 and 5	230	230	210	210
2. $1/C_1$ *vs.* $1/J_d$, with correction for passive permeation	400	280	400	280
3. $1/C_1$ *vs.* $1/J_d$, without correction for passive permeation	1000	300	1000	400

bulk phase was increased from 0.5 to 10 mM; thereafter a plateau was approached and J_d became independent of C_1 and, presumably equalled the maximal transport rate. In this highly stirred condition, J_d^m for D-glucose was approximately 230 nmol/100 mg/min and the K^* was 1.9 mM. With lesser degree of stirring, there was a decline in the magnitude of the unidirectional flux of D-glucose from low, but not from high substrate concentrations. Thus, stirring had no effect on J_d^m, but markedly affected the magnitude of K_m^*. As the resistance of the unstirred water layer increased as the rate of stirring was lowered from 600 to 200 to 0 rpm the K_m^* rose from 1.9, to 6.8, to 17.7 mM, respectively. When the known values of d, Sw and D at the different rates of stirring [18] are substituted into the formula [17] for the unstirred water layer resistance, i.e., $d/Sw \cdot D$, a direct linear relation was noted between $d/Sw \cdot D$ and the apparent Michaelis constant, K_m^*, obtained experimentally under different rates of stirring (Fig. 3*B*). When the resistance of the unstirred water layer is zero, the apparent affinity constant equals the true affinity constant of the membrane carrier (i.e., when $d/Sw \cdot D = 0$, $K_m^* = K_m$). By extrapolating the relationship shown in Fig. 3*B*, it is apparent that the true constant of the carrier for D-glucose in the rabbit jejunum is approximately 0.8 mM. It should be emphasized that this predicted true affinity constant is considerably less than previously published values [4, 12, 16], none of which were corrected for unstirred layer resistance.

When the unidirectional flux of D-glucose in samples preincubated 4 min (Fig. 2), rather than 30 min as shown in Fig. 3, was corrected for passive permeation (Table 1), a curvilinear relationship was noted in stirred samples, with a plateau in J_d at bulk phase concentrations above 10 mM. In contrast, where both stirred and unstirred samples approached J_d^m (Fig. 3*A*), a linear relation was noted between J_d and C_1 in unstirred samples preincubated 4 min, and J_d at 40 mM was still only 40% of J_d^m (Fig. 4). The apparent affinity constant, K_m^*, was also markedly different in the stirred and unstirred condition, equalling 3.3 and 60 mM, respectively (Table 2).

A common practice in studies related to intestinal transport is to obtain kinetic constants from the double-reciprocal, Lineweaver-Burk plot derived from the Michaelis-Menton equation. Reworking the experimental data for the unidirectional flux of D-glucose given in Figs. 2 and 3*A* clearly demonstrates that this mathematical approach introduces a significant error into the estimation of K_m^* and J_d^m. The estimated true affinity constant for the active transport of D-glucose

Table 3. Effect of the addition of polyethylene glycol (PEG) on the unidirectional flux of D-glucose in rabbit jejunum preincubated 30 min and stirred at 600 rpm

Composition of bulk phase	Concentration of D-glucose in bulk phase, C_1	
	0.1 mM	120.0 mM
Krebs-Ringer-bicarbonate (KRB)	8.7±2.1	1076± 84
KRB+10% PEG	4.6±0.7	1071±211

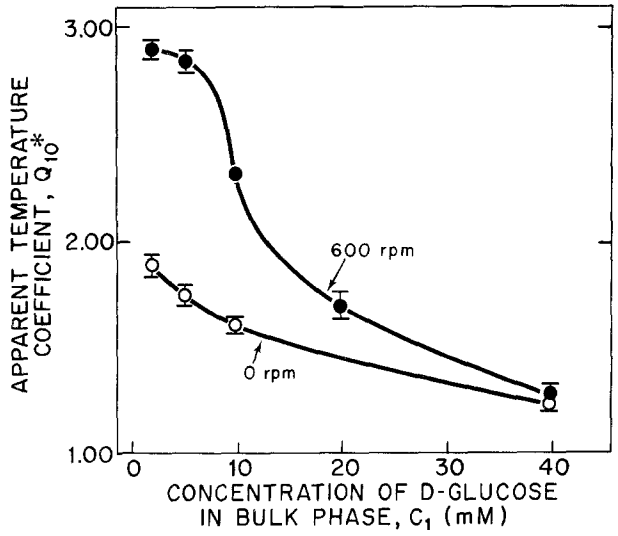

Fig. 5. Effect of stirring of the bulk phase on the estimation of the apparent temperature coefficient, Q_{10}^*, of the intestinal transport process for D-glucose in jejunal tissue preincubated 30 min. The unidirectional flux of varying concentrations of glucose in the bulk phase, C_1, was determined at 37 and 4 °C in tissue preincubated 30 min and stirred at either 600 or 0 rpm. The Q_{10}^*'s at the two different rates of stirring were then plotted as a function of C_1

is less than 1 mM (Fig. 3*B*); in jejunal samples preincubated 30 min and stirred at 600 rpm, a value of 1.9 mM for the K_m^* and 230 nmol/100 mg/min for J_d^m was derived from visual inspection of Fig. 3*A*. When these same data were subjected to Lineweaver-Burk analysis, the K_m^* values equalled 2.4 and 4.3, respectively, using data corrected and uncorrected for passive permeation (Table 2). This error in estimation of K_m^* was even greater in the unstirred condition, when K_m^*'s of over 100 mM were obtained. Furthermore, application of the double-reciprocal plot results in gross overestimation of the maximal transport rate J_d^m (Table 2).

The free diffusion coefficient of the probe molecule D is also a factor in determining the resistance of the unstirred water layer, and it has been predicted that changes in D will reduce unidirectional flux, but only from solute concentrations below the value of the afinity constant [10]. Polyethylene glycol (PEG) was used to increase the viscosity and, therefore, decrease the diffusivity of glucose in the bulk phase. The addition of 10% PEG to 10 mM D-glucose in KRB increased the relative viscosity from 1 to 5.7 (V. Sallee, *personal communication*). As shown in Table 3, this change in viscosity was associated with a 50% reduction in glucose uptake from a bulk phase concentration of 0.1 mM, while there was little effect from a concentration of 120 mM.

An appreciation of the effect of the unstirred water layer is necessary to obtain accurate estimates of the temperature coefficient of the intestinal transport process for D-glucose. The magnitude of the Q_{10}^* was greater at lower than at higher concentrations of glucose in the bulk phase (Fig. 5). This effect was further accentuated by stirring. Because the relationship between C_1 and Q_{10}^* is curvilinear (Fig. 5), it is not possible to estimate accurately the true temperature

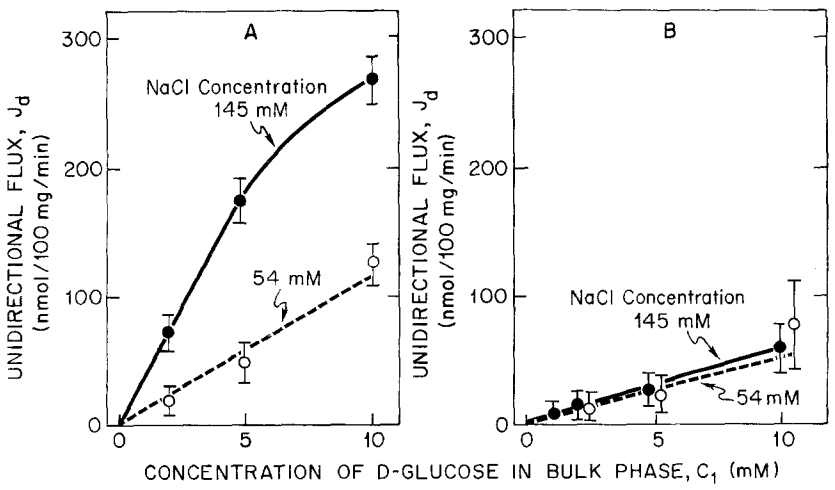

Fig. 6. Effect of stirring the bulk phase on the unidirectional flux of D-glucose in jejunal tissue preincubated 4 min with normal (145 mM) and low (54 mM) concentrations of sodium chloride in the bulk phase. The low sodium concentration was achieved by substituting D-mannitol for an isotonic amount of sodium chloride. bulk phase was stirred at 600 rpm to minimize the effective resistance of the unstirred layer; lowering the concentration of sodium chloride in the bulk phase was associated with a fall in unidirectional flux rate J_d and a rise in the magnitude of the apparent affinity constant, K_m^*. *(B)*: In contrast, when the bulk phase was unstirred and the unstirred layer resistance was high, varying the sodium concentration had no effect on J_d or K_m^*

coefficient; however, an approximation of this value may be made by plotting Q^*_{10} at a bulk phase concentration of 2 mM as a function of varying resistance of the unstirred water layer. By extrapolation, a value of 3.0 was obtained, which represents a minimum value for the true Q_{10} of the active transport mechanism for D-glucose.

Finally, glucose transport has also been shown to be influenced by luminal sodium concentration [2, 3]. Fig. 6*A* shows that the uptake of glucose falls when the concentration of sodium in the bulk phase is reduced, and the K^*_m is increased from 3.3 to greater than 10 mM. However, when unstirred layer resistance is high in the unstirred condition, varying the sodium concentration in the bulk phase had no apparent effect on either glucose uptake or on the value of the K^*_m.

Discussion

We have previously derived the following equation [17] which describes the effect of six variables on the kinetic relationship between solute concentration and active intestinal transport:

$$J^n_d = (0.5)(D)\left(\frac{S^n_w}{d^n}\right)\left[C_1 + K^n_m + \frac{f_n d^n J^m_d}{S^n_w D} \pm \sqrt{\left(C_1 + K^n_m + \frac{f_n d^n J^m_d}{S^n_w D}\right)^2 - 4C_1\left(\frac{f_n d^n J^m_d}{S^n_w D}\right)}\right] + \frac{S^n_w P C_1}{1 + \frac{d^n P}{D}}. \quad (1)$$

The resistance to the passage of the probe molecule through the unstirred water layer overlying the intestinal villi is described by the ratio $d/Sw \cdot D$. The membrane-related parameters affecting solute flux are K_m, the true Michaelis constant, J^m_d, the maximal transport rate, and fn, that proportion of J^m_d which occurs at each of n arbitrarily designated equal segments of the intestinal villus. The purpose of this paper was to test experimentally a number of predictions which derive from this equation [17] concerning the influence of unstirred water layers on the quantitative and qualitative aspects of the gastrointestinal absorption of actively transported solutes.

The first point to emphasize from this equation is that unidirectional uptake of a solute like glucose involves both an active and passive component. In order to obtain accurate kinetic constants for the active component, the flux rates must be corrected for the passive component and the magnitude of this correction, in turn, is influenced by unstirred layer resistance. Thus, in order to obtain reasonable estimates of the kinetic constants from Fig. 2, therefore, accurate approximations of the passive permeation of D-glucose must be obtained. Using three experimental approaches, it was confirmed as predicted that P^* was inversely related to $d/Sw \cdot D$ (Table 1). The experimentally determined unidirectional flux of glucose was thereby adjusted for the contribution of the passive component.

Visual inspection of the kinetic curves of the active transport for D-glucose confirm the prediction that increasing the resistance of the unstirred water layer leads to an error in the estimation of the magnitude of the affinity constant (Figs. 3*A–B* and 4, Table 2). The magnitude of this error is directly proportional to $d/Sw \cdot D$ (Fig. 4*B*), and in the presence of high unstirred layer resistance there may be a hundredfold discrepancy between K_m and K^*_m (Table 2). Therefore, no conclusion may be reached regarding this kinetic property of the intestinal membrane unless appropriate corrections are first made for the effect of the unstirred water layer.

The second prediction that derives from this equation is that, in general, there should be an inverse relationship between the rate of active solute transport and the effective resistance of the unstirred layer. Alterations in the magnitude of d and Sw, and therefore in $d/Sw \cdot D$, may be achieved by systematically varying the rate of stirring of the bulk phase [18]. By this means, it has been confirmed in many different types of experiments that an increase in unstirred water layer resistance is consistently associated with a shift to the right of the kinetic curve relating concentration of probe molecule in the bulk phase, and unidirectional flux (Figs. 2*A–B*, 3*A* and 4), with a consequent overestimation of the magnitude of the K^*_m.

Third, even when the magnitude of d and Sw are taken into account, it was predicted that J_d will be influenced by changes in the free diffusion coefficient [17]. These changes in D would be expected to affect J_d only at concentrations of the solute less than the K_m value. Using PEG to increase the viscosity of the bulk phase and thereby reduce the magnitude of D, no effect was noted on the uptake of glucose from concentrations of 120 mM (Table 3). However, this concentration is many times greater than the K_m, and it is therefore not surprising that no effect was achieved. With a bulk phase concentration below the K_m, (0.1 mM), a 50% inhibition in glucose uptake was noted (Table 3). These experimental findings confirm, therefore, that alterations in each factor describing the resistance of the unstirred water layer, $d/Sw \cdot D$, will give rise to predictable alterations in the unidirectional flux of an actively transported solute.

A fourth prediction may be made from Eq. (1).

In contrast to the values of the K_m^*, changes in the dimensions of the unstirred water layer should have no effect on the maximal transport rate, J_d^m [17]. The unidirectional flux of D-glucose from a bulk phase concentration of 120 mM was greater in the stirred than in the unstirred condition, 1232 ± 64 and 959 ± 214 nmol/100 mg/min, respectively; however, this difference was due to the effect of stirring on passive permeation, since it was abolished after adjustment for the contribution of the passive component (Table 1). Thus, when this correction was made, J_d^m was similar in jejunal tissue preincubated for 30 min and stirred at different rates (Fig. 3*A*), and in tissue subjected to stirring but preincubated for 4 or 30 min (Table 2). As a result of the very high resistance in the unstirred samples preincubated 4 min, the K_m^* was sufficiently increased that glucose uptake achieved only 40% of J_d^m, even at a bulk phase concentration of 40 mM (Fig. 4). Thus, in the presence of a very high diffusion resistance it may be necessary to use very high solute concentrations to achieve J_d^m.

Fifth, since the relationship between intestinal absorption and substrate concentration is not described by a rectangular hyperbole in the presence of the unstirred water layer, it is theoretically invalid to derive kinetic constants from double-reciprocal plots [22]. Thus, as demonstrated in the present study, the use of the double-reciprocal plot may be associated with a 100-fold error in the estimation of K_m, and a five fold error in J_d^m (Table 2).

A sixth effect of unstirred layer resistance is that it may change the appearance of the transport curves relating substrate concentration and uptake and thereby lead to a misinterpretation of the nature of the transport process. For example, when the unstirred layer resistance is high, the K_m^* is markedly increased (Table 2) and the relationship between J_d and C_1 may appear linear (Fig. 4) even though the membrane carrier in fact has a low affinity constant and a relatively high maximal transport rate. Thus, without first correcting for the effect of the unstirred water layer, it is inappropriate to pass judgment on the active or passive nature of a transport process simply from inspection of the results of kinetic studies.

Seventh, the presence of a significant unstirred layer resistance tends to obscure any effect bringing about a change in the true affinity constant of the membrane carrier. In the present study, under highly stirred conditions, reduction in the bulk phase sodium concentration had a profound effect on glucose transport process (Fig. 6). In contrast, in the unstirred situation, this effect was totally obscured.

Finally, there is another important effect of unstirred layers demonstrated in the present study, having to do with estimation of activation energies for the transport process. In general, the magnitude of the Q_{10}, and therefore the activation energy, will vary inversely with the degree to which the uptake of a particular solute is diffusion-limited or involves a significant passive component. For example, at low concentrations of substrate, passive permeation is relatively unimportant, and unidirectional flux is extremely sensitive to changes in the resistance of the unstirred water layer. Thus, only when $d/Sw \cdot D$ is reduced by stirring and low concentrations of glucose are used is it possible to estimate the Q_{10} of the jejunal transport process for D-glucose (Fig. 5). When unstirred layer resistance is higher, the apparent Q_{10} falls and the correct characteristic of the membrane carrier is underestimated. Furthermore, values for Q_{10} must not be estimated using substrate concentrations far in excess of the K_m: as C_1 exceeds K_m, the unidirectional flux due to active transport becomes increasingly proportional to J_d^m and further increases in uptake become a reflection of the passive component (Eq. (1)). Thus, the lower Q_{10}^* observed at a bulk phase glucose concentration of 40 mM (Fig. 5) reflects a complex effect of the unstirred water layer on both the active and passive components of the total transport process.

From these considerations it is apparent that unstirred water layers may influence the results of studies designed to evaluate active transport processes in the intestine. Most previous studies of intestinal transport have assumed that the experimentally determined kinetic parameters describing active uptake of a particular solute reflect the properties of the membrane carrier. The experiments in this paper clearly demonstrate that there may be serious limitations in the interpretation of much of the previously published data dealing with active transport processes in the intestine. Indeed, a number of recent studies have alleged to show an effect of variations in the resistance of the unstirred layer, but even this work is open to criticism. Using everted rat jejunal sacs, Wilson et al. [21] showed that extremely vigorous stirring of the bulk phase increased uptake of D-glucose. This effect of stirring on uptake was more pronounced at higher than at low concentrations, a result anticipated where the major effect of stirring is on the passive rather than the active component. Indeed, in these studies, the unstirred layer resistance could not have been uniformly reduced around the free-floating sacs, and no correction was made for the contribution of the passive component. Lukie and co-worker [10] showed that stirring of the bulk phase increased the uptake by rabbit jejunal discs of both a low and high dose of glucose and amino acids;

however, complete kinetic studies were not performed. *In vivo* studies by Winne [23] in rats and by Rey et al. [11] and Lewis et al. [9] in humans have shown greater uptake of D-glucose at high rates of intestinal perfusion intended to reduce the thickness of the unstirred layer. Once again, however, the effect was more pronounced at higher than at lower doses, indeed even the fast rates of perfusion were unlikely to have significantly reduced the unstirred layer resistance *in vivo*. While the studies of Dugas et al. [7] showed an influence on glucose uptake of vigorous shaking of guinea pig intestinal rings, the magnitude of the effective thickness and surface area of the unstirred layer were not measured, the contribution of the passive components was not taken into account, and a potentially invalid kinetic analysis was used. Thus it is apparent from these considerations that much work will be required to dissect out the respective contribution of the diffusion barrier and the membrane carriers to the overall kinetic characteristics of the active transport process; in addition, it will be particularly important in the future to examine the manner in which diseases of the gastrointestinal tract alter each of these processes.

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