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Kinetic Constants for Intestinal Transport of Four Monosaccharides Determined under Conditions of Variable Effective Resistance of the Unstirred Water Layer

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Summary. Theoretical considerations have suggested that variations in the resistance of the unstirred water layer (UWL) have a profound effect on the kinetic constants of intestinal transport. In this study, a previously validated *in vitro* technique was employed to determine the unidirectional flux rate of glucose, galactose, 3-O-methyl glucose and fructose into the rabbit jejunum under carefully-defined conditions of stirring of the bulk phase known to yield different values for the effective resistance of the UWL. For each monosaccharide, uptake is much greater when the resistance of the UWL is low than when high. The maximal transport rate, J_d^m, of glucose was half as large as the J_d^m of galactose and 3-O-methyl glucose (3-O-MG), and was twice as great as the J_d^m of fructose. The apparent affinity constant, K_m^*, of glucose is less than that of fructose, which was lower than the K_m^* of galactose and 3-O-MG. The use of the Lineweaver-Burk double reciprocal plot is associated with an overestimation of both J_d^m and K_m^*. This discrepancy between the true and apparent values of the kinetic constants is much greater for lower than for higher values of J_d^m and K_m^*; variations in the resistance of the unstirred layer influences the magnitude and direction of the discrepancy. The apparent passive permeability coefficient is similar for each sugar, but because of the different values of J_d^m, passive permeation contributes relatively more to the uptake of glucose and fructose than of galactose or 3-O-MG. Under conditions of high unstirred layer resistance, differences in uptake rates of the sugars are due to differences in their J_d^m rather than their K_m^*. Kinetic analysis is compatible with the suggestion that the glucose carriers are predominantly near the tip of the villus, whereas those for galactose and 3-O-MG are located along the entire villus and the K_m^* of their carriers at the tip is lower than their K_m^* towards the base of the villus. It is proposed that there are multiple or heterogeneous intestinal carriers for glucose, galactose and 3-O-methyl glucose in the jejunum of the rabbit.

Abbreviations Used in this Paper

C_1 Concentration of the probe molecule in the bulk phase
C_2 Concentration of the probe molecule at the aqueous-membrane interface
d Effective thickness of the intestinal unstirred water layer
D Free diffusion coefficient of the probe molecule

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J_d Unidirectional flux rate
J_d^m Maximal transport rate
J_d^{m*} Apparent maximal transport rate
K_m Michaelis affinity constant
K_m^* Apparent affinity constant
P Passive permeability coefficient
P^* Apparent passive permeability coefficient
rpm Revolutions per minute
Sw Effective surface area of the intestinal unstirred water layer

Overlying the intestinal brush border membrane there is a layer of relatively stagnant water, known as the unstirred water layer [6, 7, 28, 29, 31]. Previous theoretical and experimental findings have suggested that failure to account for the effect of the unstirred water layer may lead to gross errors in the estimation of intestinal transport kinetics, with underestimation of the magnitude of the passive permeability coefficient, and overestimation of the affinity constant [8, 14, 19–21, 27, 30, 33, 34]. Glucose, galactose and 3-O-methyl glucose are thought to share a common intestinal carrier-mediated sugar transport mechanism [1, 13, 32], but there are conflicting results as to their relative and absolute apparent affinity constants, K_m^*, and their maximal transport rates, J_d^m. Some workers have postulated that there may be multiple carriers for these hexoses [3, 4, 10–12, 16–18]. In addition, fructose is absorbed by a membrane carrier distinct from that of glucose and galactose; in contrast to the "active" nature of the transport of glucose and galactose, the uptake of fructose is by facilitated transport [9, 24]. Although there is a multitude of data on intestinal monosaccharide absorption, there are conflicting results on the kinetics, and there is a paucity of data comparing the K_m^* and J_d^m of these four nutritionally important monosaccharides, using comparable animals and techniques. The present study was accordingly undertaken, first to determine the effect of variations in the resistance of the unstirred water layer on the unidirectional flux of glucose, galactose, 3-O-methyl glucose, and fructose into the jejunum of rabbits; second, to estimate their maximal transport rate, apparent affinity constant, and apparent passive permeability coefficients; third, to determine whether there are quantitative limitations in the use of the "linear" transformations of the Michaelis-Menten equation; and finally, to apply these linear transformations of the Michaelis-Menten equation to assess the possibility of the qualitative differences in the intestinal transport process of these sugars.

Materials and Methods

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 Corp., Boston, Mass., and was used as supplied by the manufacturer. Unlabeled and 1-^{14}C-labeled, respectively, monosaccharides were supplied by Sigma Chemical Corporation, St. Louis, Mo., Fisher Scientific Co., Ltd., and New England Nuclear Corp.

Tissue Preparation

Albino New Zealand rabbits were killed by decapitation. As described in detail elsewhere [15, 28] a 10-cm length of proximal jejunum was rapidly removed, rinsed with 50 ml of cold saline, 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, 2 cm in diameter, were cut from the segment using a sharpened steel punch, 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. The incubation chambers were identical to those previously described [15]. To the serosal compartment was added 1.2 ml of Krebs-bicarbonate buffer, and each chamber at 4 °C was constantly oxygenated by a stream of 5% CO_2 in oxygen until it was used in the various experiments. The chambers were first transferred to identical beakers containing oxygenated Krebs-bicarbonate buffer at 37 °C for a preincubation of 30 min. Then they were 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 rpm at which the stirring bar was driven. Stirring rates were altered in a systematic and reproducible manner to yield the previously reported values of the effective thickness and surface area of the unstirred water layer [25].

Determination of Unidirectional Flux Rates

After preincubation, the chambers were transferred to other beakers containing ^{3}H-dextran and various ^{14}C-probe molecules in oxygenated Krebs-bicarbonate buffer at 37 °C. After incubation for 6 min, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for approximately 5 sec. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, divided into two pieces, and blotted on filter paper, after which each half was placed in a tared counting vial. The tissue was dried in an oven overnight 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 [22]. 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 mucosa per min per 100 mg dry wt of tissue.

Results

When the bulk phase was stirred to minimize the effective resistance of the intestinal unstirred water layer, there was a curvilinear relationship between the concentration of monosaccharide in the bulk phase and the unidirectional flux rate (Fig. 1 *A–D*). The uptake of each hexose was much less when the bulk phase was stirred at 200 rpm, or was

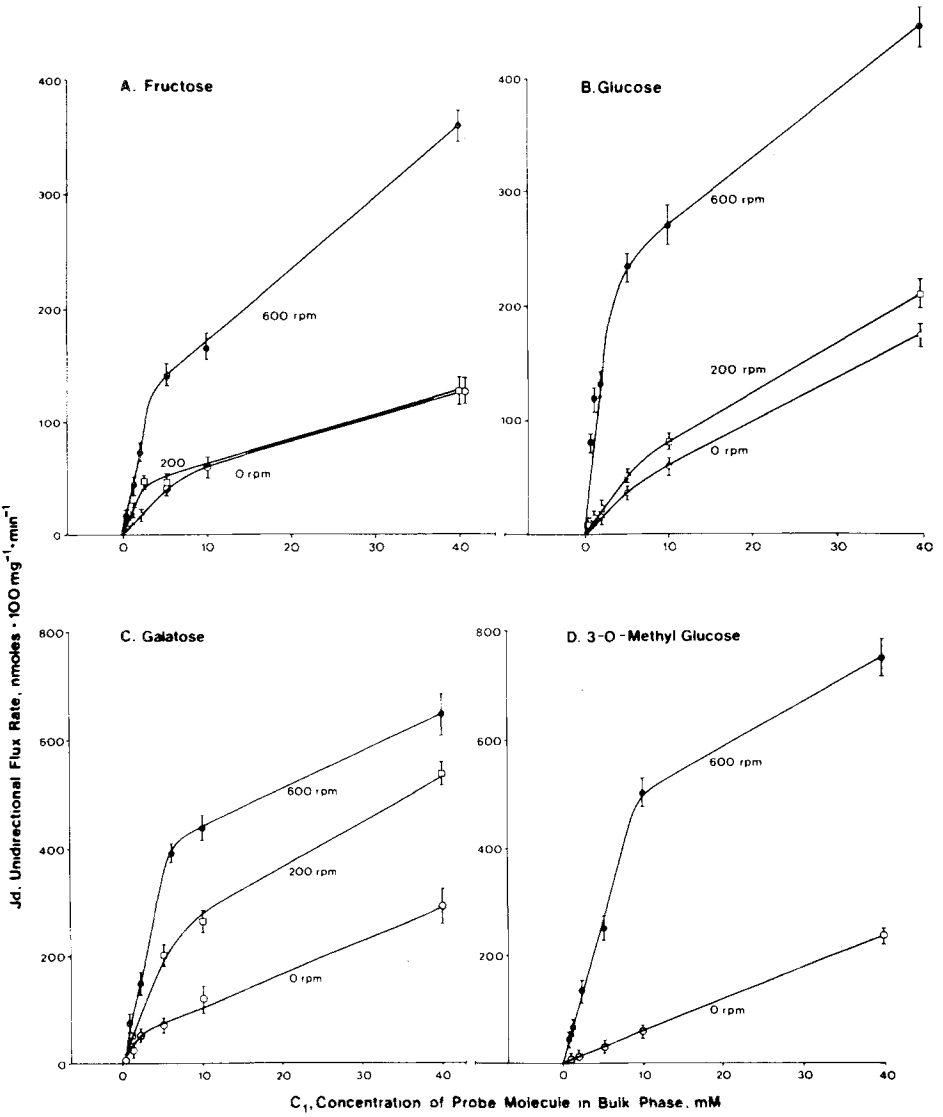

Fig. 1. Unidirectional flux rates of four monosaccharides into rabbit jejunum under conditions selected to yield low, intermediate, and high effective resistance of the unstirred water layer

Table 1. Kinetic constants for the *in vitro* unidirectional flux rate of glucose, galactose, 3-O-methyl glucose and fructose into rabbit jejunum

Monosaccharide probe	Apparent passive permeability coefficient, P^* (nmol·100 mg^{-1}·min^{-1}·mM^{-1})		Maximal transport rate, J_d^m (nmol·100 mg^{-1}·min^{-1})	Apparent Michaelis affinity constant K_m^* (M) (mM)	
	600 rpm	0 rpm		600 rpm	0 rpm
Glucose	5.9±0.5	3.7±0.3	206±20	0.8±0.1	>40
Galactose	6.6±0.4	5.7±0.5	388±39	3.0±0.2	>40
3-O-methyl-glucose	7.0±0.6	5.6±0.5	417±40	4.3±0.3	>40
Fructose	6.7±0.5	2.5±0.3	107±11	1.7±0.2	>40

The mean ±SEM of the kinetic constants for the *in vitro* unidirectional flux rate of the four monosaccharides, glucose, galactose, 3-O-methyl glucose, and fructose, into rabbit jejunum. The bulk phase containing the monosaccharide probe molecules was stirred at 600, 200 or 0 rpm; the magnitude of the apparent passive permeability coefficient P^*, maximal transport rate, J_d^m, and apparent Michaelis affinity constant K_m for the 200 rpm group are not shown in this table, but their values are intermediate between those obtained at 600 and 0 rpm. The experimental data used to obtain these results is shown in Fig. 2. The values for the maximal transport rate were obtained at 600 rpm, and were assumed to represent an acceptable approximation of the true maximal transport rate of the appropriate hexose carrier [27].

unstirred. For most substrate concentrations, the uptake of 3-O-methyl glucose and galactose was much higher than for glucose, which in turn was higher than for fructose.

The magnitude of the apparent passive permeability coefficient of each sugar was assessed from the slope of the linear portion of the concentration curve between 10 and 40 mM; preliminary data demonstrated a linear relationship between uptake and concentration of 10, 20, 40 and 80 mM. These values are shown in Table 1. In addition, the apparent passive permeability coefficient of glucose was determined from the rate of uptake of L-glucose and of D-glucose at 4 °C, and with stirring of the bulk phase at 600 rpm. These values were 5.7±0.5 and 6.0±0.4 nmol·100 mg^{-1}·min^{-1}·mM^{-1}, and they correspond closely to the value of 5.9 nmol·100 mg^{-1}·min^{-1}·mM^{-1} given in Table 1. When the bulk phase was stirred at 600 rpm, the magnitude of the apparent passive permeability coefficient P^* of glucose was similar to that for galactose, 3-O-methyl glucose, and fructose (Table 1). When the bulk phase was unstirred (0 rpm), the magnitude of P^* for each hexose was

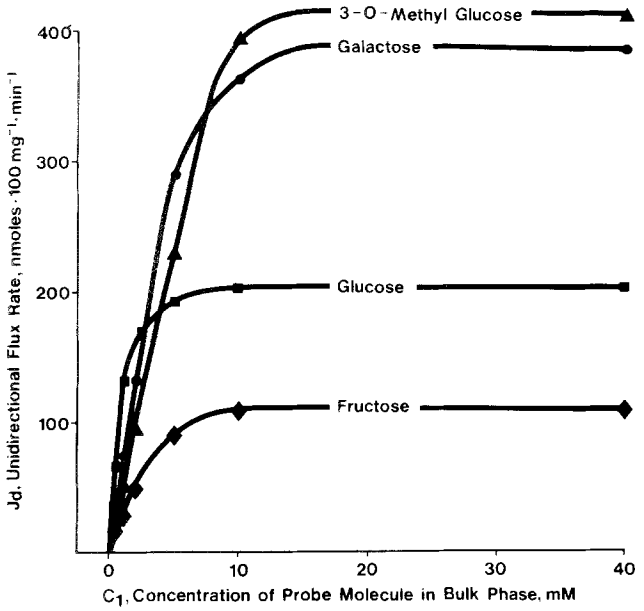

Fig. 2. Comparison of unidirectional flux rates of three monosaccharides into rabbit jejunum under conditions selected to yield low effective resistance of the unstirred water layer with correction of the experimental data for the contribution of the passive component. Each point represents the mean of at least 8 animals

lower. However, the apparent passive permeability coefficient of fructose in the unstirred condition was significantly less than for glucose, which in turn was significantly less than for galactose or 3-O-methyl glucose (Table 1).

The experimental values of the unidirectional flux rates were corrected for the contribution of the passive component by multiplying each of the six substrate concentrations (0.5–40 mM) by the appropriate apparent passive permeability coefficient shown in Table 1, and subtracting each of these products from the appropriate experimental values shown in Fig. 1 *A–D*. The relationship between monosaccharide uptake and substrate concentration now achieved a plateau (Fig. 2); this plateau represents the magnitude of the maximal transport rate J_d^m, and these values of J_d^m are shown in Table 1. The value of the maximal transport rates of galactose and 3-O-methyl glucose were similar (388 ± 39 and 417 ± 40 nmol·100 mg^{-1}·min^{-1}, respectively), and was twice as high as the J_d^m of glucose (206 ± 20 nmol·100 mg^{-1}·min^{-1}), which in turn was twice as high as the J_d^m of fructose (107 ± 11 nmol·100 mg^{-1}·min^{-1}).

With knowledge of the maximal transport rate of each hexose, it was next possible to estimate, from visual inspection of Fig. 2, the concentration of substrate yielding an uptake rate equal to half of the maximal transport rate ($J_d^m/2$). This value is known as the apparent Michaelis affinity constant, K_m^*, and is shown in Table 1. The apparent affinity constant K_m^* of glucose, 0.8 ± 0.1 mM, was much less than the K_m^* of galactose and 3-O-methyl glucose (3.0 ± 0.2 and 4.3 ± 0.3 mM, respectively). The K_m^* of fructose, 1.7 ± 0.2 mM, was intermediate between that of glucose and the two other monosaccharides. The differences in the values of the K_m^*'s of the four monosaccharides were statistically significant ($P < 0.05$). When the bulk phase was unstirred (0 rpm), the apparent affinity constant of each sugar could not be accurately estimated, but exceeded 40 mM; the fact that the K_m^* in the unstirred situation exceeded 40 mM simply meant that this concentration was too low to saturate the carriers when the effective resistance of the unstirred layers was so high. When the bulk phase was stirred at 200 rpm, the values of the apparent affinity constants for the hexoses were intermediate in magnitude between those obtained when the bulk phase was stirred at 600 rpm, and unstirred, 0 rpm. In addition, at 200 rpm, the differences between the values of the K_m's of the four monosaccharides seen at 600 rpm were obscured: the K_m^* of glucose was now greater than that of galactose, 14 and 5 mM, respectively. Thus variations in the resistance of the unstirred water layer lead to qualitative as well as quantitative differences of the value of the apparent Michaelis constant of the intestinal transport process of the four sugars.

An equation has previously been derived which described the effects of the unstirred water layers on the kinetic parameters of active transport processes in the intestine [27]. Since the estimation of the maximal transport rate is essentially unaffected by the unstirred water layer [19, 25–27, 30, 33], the values of the J_d^m* obtained from Fig. 2 and shown in Table 1 were assumed to represent the true maximal transport rates of the appropriate carriers, and these values were substituted into this previously-derived equation. The previously-reported values of the effective thickness and surface area of the unstirred water layer in rabbit jejunum stirred at 600 rpm are 137 μM and 11.7 $cm^2 \cdot 100\ mg^{-1}$, respectively [28]; these values were then substituted into this equation, which was solved for the values of the true affinity constants, K_m, of the monosaccharides. The values of the K_m's were calculated to be 0.5 mM for glucose, 2.5 mM for galactose, 3.7 mM for 3-O-methyl glucose, and 1.6 mM for fructose. Note that the magnitude of these calculated true affinity constants was

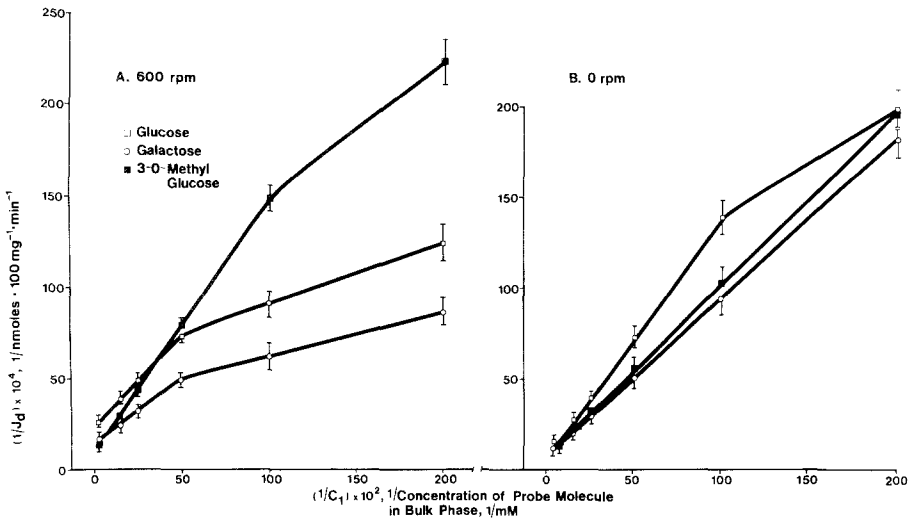

Fig. 3. Estimation of kinetic constants of intestinal transport of three monosaccharides using the Lineweaver-Burk double-reciprocal plot, under conditions selected to yield variable effective resistance of the unstirred water layer. The experimental data shown in Fig. 1 is redrawn according to the plot of $1/J_d$ *vs.* $1/C_1$, where J_d is the unidirectional flux rate, and C_1 is the concentration of the monosaccharide probe molecule in the bulk phase. The bulk phase was stirred at 600 or 0 rpm. Note the different magnitude of the values of $1/J_d$, shown on the *y*-axis of panels *A* and *B*

slightly lower than the value of the apparent affinity constants obtained experimentally when the bulk phase was stirred at 600 rpm and the unstirred layer resistance was low (Table 1).

The kinetic constants of the active transport of glucose, galactose, and 3-O-methyl glucose were estimated using the Lineweaver-Burk plot of the experimental values shown in Fig. 1: the reciprocal of the unidirectional flux rate, J_d, was plotted against the reciprocal of the concentration of the probe molecule in the bulk phase, C_1. Note that these values contain both an active and a passive component. When the bulk phase was stirred at 600 rpm, the relationship between $1/J_d$ *vs.* $1/C_1$ was initially linear at lower values of $1/C_1$ (Fig. 3*A*) but the lines deviated from this linear relationship at higher values of $1/C_1$. When the bulk phase was unstirred, the slopes of the curves were different, but the lines converged at high values of $1/C_1$ (Fig. 3*B*). When the deviations of the linearity at higher values of $1/C_1$ were arbitrarily ignored and only the initial linear portions of the relationships were examined, estimates could be made of the apparent maximal transport rate (J_d^m) from the *y*-axis intercept ($1/J_d$), and of the apparent Michaelis affinity constant

Table 2. Kinetic constants for the *in vitro* unidirectional flux rate of glucose, galactose, and 3-O-methyl glucose obtained from the application of "linear" transformations of the Michaelis-Menten Equation to the experimental values

Monosaccharide probe molecule	Maximal transport rate, J_d^m (nmol·100 mg^{-1}·min^{-1})				Apparent Michaelis constant, K_m^*	
	$1/J_d$ *vs.* $1/C_1$		C_1/J_d *vs.* C_1		$1/J_d$ *vs.* $1/C_1$	
	600 rpm	0 rpm	600 rpm	0 rpm	600 rpm	0 rpm
Glucose	400±35	1,225±100	210±20	645±60	5.9±0.4	25±3
Galactose	700±60	1,300±120	450±42	1,250±100	6.3±0.4	20±2
3-O-methyl glucose	700±50	1,250±105	680±60	—	16±2	15±2

The mean ±SEM of the kinetic constants for the *in vitro* unidirectional flux rate of glucose, galactose and 3-O-methyl glucose into rabbit jejunum. The bulk phase containing the monosaccharide probe molecules was stirred at 600 and 0 rpm. The symbols represent unidirectional flux rate, J_d, and concentration of the probe molecule in the bulk phase, C_1. These values of the maximal transport rate and apparent Michaelis constant were obtained by replotting the experimental data shown in Fig. 1, and thus these kinetic constants were derived from data uncorrected for the contribution of passive permeation.

(K_m^*) from the negative x-axis intercept ($1/C_1$). When the resistance of the unstirred layer was lowered by stirring the bulk phase (600 rpm, Fig. 3*A*), the apparent maximal transport rates of galactose and 3-O-methyl glucose estimated from the Lineweaver-Burk plot were similar (700 and 710 nmol·100 mg^{-1}·min^{-1}, respectively), and higher than the J_d^{m*} of glucose (400 nmol·100 mg^{-1}·min^{-1} – Table 2). In contrast, when the bulk phase was unstirred (0 rpm, Fig. 3*B*), the double reciprocal plot demonstrated a common y-axis intercept, suggesting a similar apparent maximal transport rate of approximately 1250 nmol. 100 mg^{-1}·min^{-1} for each hexose (Table 2). Thus, when the bulk phase was unstirred and resistance was high, the estimated J_d^{m*} of each hexose was higher, and the difference between the maximal transport rate of the sugars obtained from Fig. 2 was obscured (Table 1). The relative differences between the apparent affinity constants of the hexoses, estimated using the Lineweaver-Burk plot, were also influenced by the rate of stirring of the bulk phase (Table 2). The apparent affinity constant, K_m^*, of 3-O-methyl glucose estimated from the Lineweaver-Burk plot was much greater than glucose or galactose when the bulk phase was stirred (16, 5.9 and 6.3 mM, respectively), but in contrast the K_m^* of

3-O-methyl glucose was less than that of glucose or galactose when the bulk phase was unstirred (15, 25, and 20 mM, respectively). Note that these values for J_d^m* and K_m^* estimated with the Lineweaver-Burk plot (Table 2) are much higher than those obtained from direct inspection of the relationship between uptake rate and concentration (Table 1).

The values of the maximal transport rate J_d^m and apparent affinity constant K_m^* estimated using the Lineweaver-Burk plot were much closer to the values shown in Table 1 when the bulk phase was stirred to reduce the resistance of the unstirred layer, and when appropriate corrections were made for the contribution of passive permeation. For example, when the unidirectional flux rates of glucose, shown in Figure 2, were replotted according to the Lineweaver-Burk plot, the K_m^* was estimated to be 1.0 mM and the J_d^m was 250 nmol·100 mg^{-1}·min^{-1}; these values correspond closely to those shown in Table 1. Furthermore, when the experimental values (Fig. 1) were corrected for passive permeation (Fig. 2), and the values of the J_d were calculated by substituting appropriate values for K_m, J_d^m, D, d and Sw into a previously derived equation [27], then the relationship between $1/J_d$ *vs.* $1/C_1$ was linear. However, this pertained only under the special condition where unstirred layer resistance was zero.

The magnitude of the maximal transport rates was also estimated from the plot of C_1/J_d *vs.* C_1, where C_1 is the concentration of the hexose in the bulk phase, and J_d is the unidirectional flux rate determined experimentally and shown in Fig. 1. When the bulk phase was stirred at 600 rpm, there was a linear relationship between C_1/J_d *vs.* C_1 at values of C_1 between 5 to 40 mM (Fig. 4); the magnitude of the apparent maximal transport rate J_d^m* was estimated from the reciprocal of the slope of this linear portion of the curve. The values for the J_d^m* of glucose, galactose, and 3-O-methyl glucose were 210, 450 and 680 nmol·100 mg^{-1}·min^{-1}, respectively. These values for the maximal transport rates of glucose and galactose agree approximately with those given in Table 1 (206, 388, and 417 nmol·100 mg^{-1}·min^{-1}, respectively). When the bulk phase was unstirred, the estimated maximal transport rates of glucose and galactose were higher than in the stirred situation, and once again the J_d^m* of galactose was twice as high as that of glucose (1,250 and 645 nmol·100 mg^{-1}·min^{-1}, respectively). In the unstirred condition, there was an essentially zero slope between the plot of C_1/J_d *vs.* C_1 for galactose and 3-O-methyl glucose at concentrations between 5–40 mM, although the relationship was curvilinear at lower values of C_1 (Fig. 4). Thus it was not possible to determine the y-axis intercept

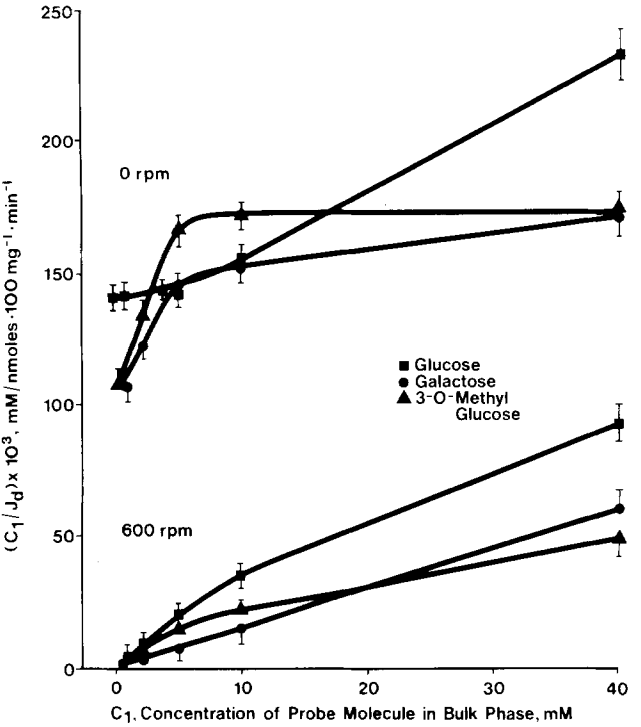

Fig. 4. Estimation of kinetic constants of intestinal transport of three monosaccharides, using the Eadie-Hofstee plot, under conditions selected to yield variable effective resistance of the unstirred water layer. The experimental data shown in Fig. 1 is redrawn according to the plot of C_1/J_d *vs.* C_1, where C_1, is the concentration of the monosaccharide probe molecule in the bulk phase, and J_d is the unidirectional flux rate

by extrapolation, and accordingly the K_m^* could not be estimated using this plot.

The third "linear" transformation of the Michaelis-Menten equation is the plot of J_d *vs.* J_d/C_1. When the experimental values shown in Fig. 1 were replotted, a complex nonlinear relationship was obtained which precluded estimation of the magnitude of either K_m^* or J_d^m (Fig. 5*A*). However, when the experimental values obtained under conditions of low unstirred layer resistance were first corrected for passive permeation (Fig. 2), the relationship between J_d *vs.* J_d/C_1 became curvilinear (Fig. 5*B*). The apparent affinity constants were estimated for the slope of the linear portion of the curve, and these values of the K_m's were 1.3 mM for glucose, and 3.8 mM for galactose. These values approximated the values of the K_m^* of 0.8 mM for glucose and 3.0 mM for galactose shown in Table 1. The magnitude of the *y*-axis intercept (J_d) gives the

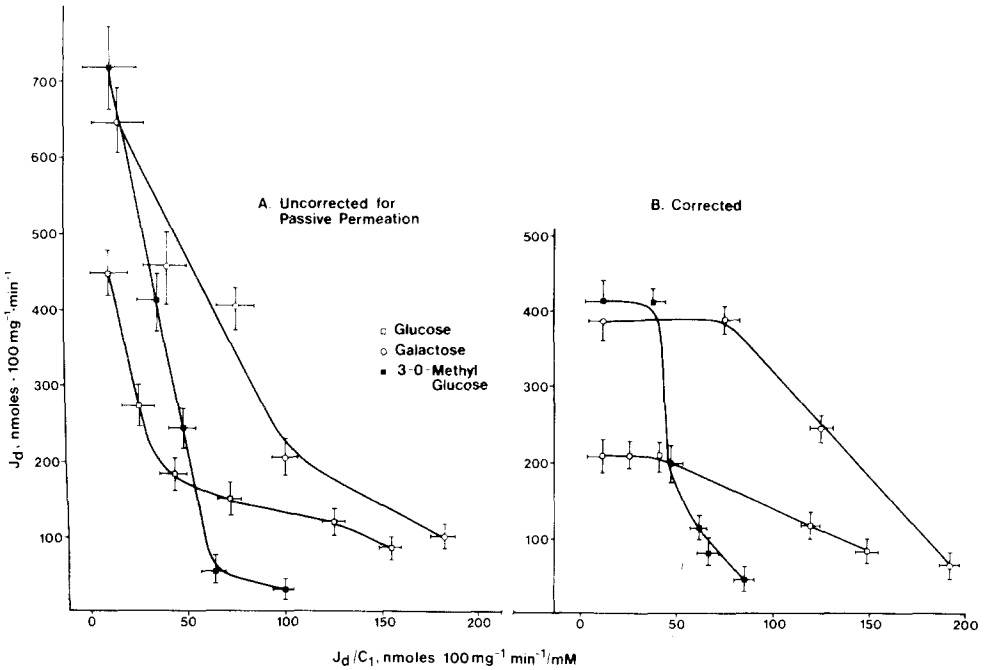

Fig. 5. Estimation of kinetic constants of intestinal transport of three monosaccharides using the plot of J_d *vs.* J_d/C_1, both corrected and uncorrected for passive permeation, under conditions selected to yield low effective resistance of the unstirred water layer. The unidirectional flux rate is represented by J_d, and C_1 is the concentration of the probe molecule in the bulk phase. The experimental data shown in Fig. 1 is redrawn according to the plot of J_d *vs.* J_d/C_1

value of the apparent maximal transport rate, J_d^m*. Because of the curvilinear nature of the relationship between J_d *vs.* J_d/C_1, the value of J_d^m could not be estimated with any accuracy, but clearly the intercept would be higher for galactose and 3-O-methyl glucose than for glucose, and would therefore indicate a larger value of J_d^m* for galactose and 3-O-methyl glucose than for glucose.

Discussion

When the bulk phase is stirred to minimize the effective resistance of the intestinal unstirred water layer, there is a marked difference between the maximal transport rate J_d^m for glucose, galactose, 3-O-methyl glucose, and for fructose (Table 1). This relative difference was demonstrated using each of the "linear" transformations of the Michaelis-Menten equation: $1/J_d$ *vs.* $1/C_1$, (Fig. 3*A*), C_1/J_d *vs.* C_1 (Fig. 4*A*), and

J_d *vs.* J_d/C_1 (Fig. 5*B*). However, the absolute value of the maximal transport rates is influenced by the method of estimation. Direct inspection of the concentration curves corrected for the contribution of passive permeation (Fig. 2) yielded values of J_d^m (Table 1) which were considerably lower than those obtained from the Lineweaver-Burk plot (Table 2).

The magnitude of the apparent maximal transport rate, J_d^m*, estimated from the Lineweaver-Burk plot, was influenced by the resistance of the unstirred water layer. When the resistance of the unstirred layer was high, the J_d^m* of glucose, galactose and 3-O-MG was much higher than when the unstirred layer resistance was low (Fig. 3 and Table 2). If the linear portion of the relationship at lower values of $1/C_1$ and high unstirred layer resistance (Fig. 3*B*) is extrapolated to the *y*-axis, approximately identical values are found for the J_d^m* of the hexoses (Table 2). Therefore a true difference between the values of the maximal transport rates may be obscured by the use of the Lineweaver-Burk plot to estimate this kinetic constant under conditions where unstirred layer resistance is high. Thus, it is likely that the failure of previous studies to demonstrate such a clear difference between the maximal transport rates of fructose, glucose, galactose and 3-O-methyl glucose is due to the use of Lineweaver-Burk plots and to the use of experimental data obtained under conditions of high unstirred layer resistance. Indeed the magnitude of this error may be further accentuated depending upon which points in the nonlinear Lineweaver-Burk plot are arbitrarily omitted.

Winne has previously demonstrated that the use of this double-reciprocal plot is associated with overestimation of the magnitude of J_d^m* [30]. This error is due to failure to account for the effective resistance of the unstirred water layer, and of failure to correct for the contribution of passive diffusion that proceeds concurrently with carrier-mediated transport [2, 23]. Indeed, when the bulk phase is stirred to minimize the effective resistance of the unstirred layer, and when the experimental values are corrected for passive permeation, the J_d^m* of glucose, galactose and 3-O-methyl glucose obtained with the Lineweaver-Burk plot are much closer to those values shown in Table 1. Furthermore, when a recently derived equation is used to calculate the value of the true affinity constant [27], and when previously measured values for the effective thickness and surface area of the unstirred layer [28] are also substituted into this equation, it becomes possible to calculate theoretical values of J_d^m under the special condition of zero unstirred layer resistance; when the values of $1/J_d$ *vs.* $1/C_1$ are plotted, a linear relationship is

obtained, yielding estimates of the kinetic constants close to those shown in Table 1. Thus, the use of the Lineweaver-Burk plot to estimate the magnitude of kinetic constants may be associated with both quantitative and qualitative errors, but these errors may be abolished by correcting for both the contribution of passive permeation as well as by correcting for the effective resistance of the unstirred water layer.

The plot of C_1/J_d *vs.* C_1 may be useful to estimate the magnitude of the apparent maximal transport rate J_d^m*. Theoretical considerations have shown that the value of the maximal transport rate J_d^m* estimated from this plot deviates less than 20% from the magnitude of the true maximal transport rates [25]. If the experimental values shown in Fig. 1 are analyzed using this plot, then the values of the J_d^m* of glucose and galactose but not 3-O-methyl glucose approach those values of the maximal transport rate, shown in Table 1: the J_d^m* of galactose is still over twice as high as that of glucose. Estimation of the apparent maximal transport rate J_d^m*, from the plot of J_d *vs.* J_d/C_1 (Fig. 5) requires extrapolation of the *y*-axis. It was not possible to accurately assess the value of J_d^m* since this relationship is curvilinear, both with (Fig. 5*B*) and without correction for passive permeation (Fig. 5*A*). However, although the value of J_d^m could not be accurately assessed, it was clear that the *y*-axis extrapolation, and therefore the J_d^m*, for galactose and 3-O-methyl glucose was considerably higher than for glucose. Theoretical considerations have shown that the deviation from linearity of the plot of C_1/J_d *vs.* C_1, and J_d/C_1 is due in part to the presence of a passive component and an unstirred water layer [26, 27]. Indeed, when the appropriate corrections are made for passive permeation and the effective resistance of the unstirred layer, then these relationships become linear and the values of the kinetic constants are close to those shown in Table 1. The limitations imposed upon the use of these transformation plots to estimate the correct value of the maximal transport rates of glucose, galactose, and 3-O-methyl glucose are due, therefore, to two factors: failure to correct for the effective resistance of the unstirred layer, and failure to correct for the contribution of passive permeation. Only once appropriate corrections have been made is it valid to estimate the maximal transport rate using these transformation plots. Failure to make these corrections may lead to both qualitative and quantitative errors in the estimation of this important kinetic constant: qualitative differences between the maximal transport rates of the different monosaccharides may be obscured, and the absolute values of the J_d^m's may be greatly overestimated.

What influence do variations in the magnitude of the affinity constant or maximal transport rate have on the discrepancy between the true and apparent values of these kinetic constants, as estimated with the Lineweaver-Burk plot? On the assumption that the values of the affinity constants of the hexose transport process(es) shown in Table 1 are close approximations to the true values, then the discrepancy between these values and those estimated using the Lineweaver-Burk plot (Table 2) are indeed inversely proportional to the value of K_m itself. For example, when the bulk phase was stirred, the affinity constants of glucose were 0.8 mM (Table 1) and 5.9 mM (Table 2), a discrepancy of 738%. In contrast, when the affinity constant was higher, as for 3-O-methyl glucose, the discrepancy was 372% (4.3 mM, Table 1, and 16.0 mM, Table 2). When the resistance of the unstirred layer is high, the difference between the true and apparent values of the affinity constant should be even greater when K_m is small than when large. The difference between the affinity constants shown in Tables 1 and 2 was at least 50-fold for glucose (0.8 and 40 mM), but was only ninefold for 3-O-methyl glucose (4.3 and 40 mM). Thus, the Lineweaver-Burk plot is associated with overestimation of apparent affinity constants, even when unstirred layer resistance is low, and the magnitude of the discrepancy between true and apparent values is influenced by the value of the kinetic constants themselves.

Is the discrepancy between the values of the maximal transport rate J_d^m shown in Table 1 and those estimated using the Lineweaver-Burk plot (Table 2) also influenced by the magnitude of J_d^m? On the assumption that the values of the maximal transport rates shown in Table 1 are close approximations of the true values, then the discrepancy between these values and those estimated using the Lineweaver-Burk plot (Table 2) are also inversely proportional to the value of the J_d^m itself. The maximal transport rate for glucose was 206 and 1225 nmol $\cdot$ 100 mg^{-1} $\cdot$ min^{-1} ($1/J_d$ *vs.* $1/C_1$, 0 rpm), using the different methods shown in Tables 1 and 2, respectively. In contrast to this sixfold difference when J_d^m was relatively low, the discrepancy was only threefold when J_d^m was relatively higher, as for galactose (388 nmol$\cdot$100 mg^{-1}, Table 1, compared with 1250 nmol$\cdot$100 mg$^{-1}\cdot$min^{-1} for J_d^m of galactose estimated from the Lineweaver-Burk plot, Table 2, at 0 rpm).

In addition to these quantitative differences in the magnitude of J_d^m and K_m^* obtained using the Lineweaver-Burk plot, qualitative errors are introduced: the K_m^* of 3-O-methyl glucose was higher than that of the other sugars when the bulk phase was stirred, but was lower when the

bulk phase was unstirred (Table 2). Also, when the bulk phase was stirred, the maximal transport rate of the hexoses were different when the bulk phase was stirred, but similar when the bulk phase was unstirred (Table 2). Since the Lineweaver-Burk plot introduced both qualitative and quantitative errors into the estimation of the kinetic constants, it is suggested that this plot should be used with discretion to estimate the kinetic constants of intestinal transport processes unless the effective resistance of the unstirred layer is low or of known magnitude and unless the contribution of passive permeation has been taken into account.

To assess the magnitude of the kinetic constants of carrier-mediated transport, it is is necessary to first assess the magnitude of passive permeation. The apparent passive permeability coefficient of glucose was estimated using three techniques: the slope of the glucose concentration curve at high values of C_1, the rate of uptake J_d of L-glucose, and the J_d of D-glucose at 4 °C. These values closely agreed, and were assumed to represent the passive permeation of glucose into the rabbit jejunum. However, these values are likely to be less than the true permeability coefficient of the intestine to glucose, since passive permeation is also influenced by the effective resistance of the unstirred layer. This arises from the difference in the concentration of the probe molecule in the bulk phase in the intestinal lumen (C_1), and the concentration at the aqueous-membrane interface (C_2). The value of C_2 can be estimated from Eq. (4):

$$C_2 = C_1 - \frac{J_d \cdot d}{D \cdot Sw} \tag{1}$$

where J_d is the experimentally determined unidirectional flux rate, D is the free diffusion coefficient of the probe molecule, d is the effective thickness of the unstirred water layer, and Sw is its effective surface area. Substituting the appropriate values of C_1, J_d, D and the published values of d and Sw for rabbit jejunum stirred at 600 rpm [28], then the value of C_2 for glucose may be shown to be 0.9 mM, when C_1 is 1 mM. Thus the true value of the passive permeability coefficient of glucose is greater than the apparent value of 5.9 nmol·100 $mg^{-1} \cdot mM^{-1}$ (P^*/C_1), and is estimated to be 6.6 nmol·100 $mg^{-1} \cdot min^{-1}$ (P^*/C_2).

The apparent passive permeability coefficients of galactose, 3-O-methyl glucose and fructose were estimated from the slope of the linear portion of the concentration curve at high values of substrate concentration (Fig. 1*B–D*). The rationale of and validity for this approach is

as follows: the equation for active transport is

$$J = \frac{J^m \cdot C_2}{K_m + C_2} \tag{2}$$

where J is the absorption rate, J^m is the maximal transport rate, K_m is the affinity constant, and C_2 is the concentration of the probe molecule at the aqueous-membrane interface. The equation for passive permeation is [28]:

$$J = PC_2 \tag{3}$$

where P is the passive permeability coefficient. Thus the total rate of absorption of a probe molecule which is transported by both active and passive processes is:

$$J = \frac{J^m \cdot C_2}{K_m + C_2} + PC_2. \tag{4}$$

When $C_2 \gg K_m$, J becomes approximately proportional to J^m [5]. For values of C_2 several times greater than K_m, the ratio J/J^m approaches 1, i.e., further increases in C_2 produce further increases in J by a factor PC_2, the contribution of passive permeation. The estimated values of the apparent affinity constants of glucose, galactose, and 3-O-methyl glucose were 0.8, 3.0 and 4.3 mM, respectively (Table 1). Although these K_m^*'s were estimated under conditions selected to minimze the effect of the unstirred layer, the resistance of the unstirred layer was still of sufficient magnitude to allow for a small overestimation of the true affinity constants. Thus the true affinity constants of these hexoses is less than the values given in Table 1, and at the bulk phase concentrations of 10 to 40 mM, the value of C_2 would indeed be much greater than the value of K_m ($C_2 \gg K_m$), further uptake would be approximately proportional to J_d^m, and the slope of the concentration curves between 10–40 mM would therefore represent a valid estimate of passive permeation (PC_2).

An assessment of the adequacy of the correction for passive permeation can be made from inspection of the slope of the relationship between J_d *vs.* J_d/C_1 obtained under conditions of low unstirred layer resistance (Fig. 5*A*). Theoretical considerations [26] have shown that this complex curvilinear relationship is achieved only when the experimental values contain a passive component. After correction for this component the shape of the relationship changes (Fig. 5*B*). This new form is compatible with adequate correction for passive permeation. Although small varia-

tions are present between the mean values of the apparent passive permeability coefficients of the four hexoses obtained when the bulk phase was stirred at 600 rpm (Table 1), these differences are small and statistically insignificant ($P < 0.05$), and it is likely that these values represent a reasonable approximation of the true passive permeability coefficient and therefore of the contribution of passive permeation under conditions of low unstirred layer resistance.

The apparent passive permeability coefficients of the hexoses were lower when the bulk phase was unstirred (Table 1). The estimated K_m^* of each monosaccharide obtained when unstirred layer resistance was high exceeded 40 mM, and thus the slope of the line between 10 and 40 mM at 0 rpm does not represent passive permeation alone but a combination of passive and carrier-mediated transport. Thus these apparent passive permeability coefficients for the four monosaccharides obtained at 0 rpm do not reflect either the quantitative or qualitative aspects of passive membrane permeability towards the sugars. What is the explanation for the lower values for the apparent permeability coefficients of glucose and fructose than of galactose and 3-O-methyl glucose, under conditions of high unstirred layer resistance? When the bulk phase is unstirred, the value of the apparent affinity constant of each hexose is greatly increased (Table 1), and uptake becomes more dependent upon the value of J_d^m and P [Eq. (4)]. Since the value of the apparent permeability coefficient of the hexoses is similar when unstirred layer resistance is low (Table 1), the most likely explanation for the higher rates of uptake of galactose and 3-O-methyl glucose than of glucose and fructose under conditions of high unstirred layer resistance is the much larger values of their maximal transport rates (388 and 417 nmol/$100\ mg^{-1} \cdot min^{-1}$ *vs.* 206 and 107 $nmol \cdot 100\ mg^{-1} \cdot min^{-1}$, respectively, Table 1). Thus, the values of the apparent passive permeability coefficients estimated under conditions of high unstirred layer resistance reflect the effective resistance of the unstirred layer, the maximal transport rate, as well as the true passive permeability coefficient. Accordingly, the apparent passive permeability coefficients of hexoses cannot be accurately estimated in the presence of an unstirred water layer of significant dimensions.

Previous studies have shown differences in the apparent affinity constants K_m^* of glucose, galactose and 3-O-methyl glucose: while some workers have shown a lower value of the K_m^* of glucose than of galactose, others have shown the converse [3,4,11,12,16–18]. The results of this study are compatible with the suggestion that this discrepancy is due

to the effect of the unstirred water layer, and the method used to estimate the value of the Michaelis constants. When the bulk phase was stirred, the K_m^* of glucose was less than that of galactose, which in turn was less than 3-O-methyl glucose (Table 1). When the bulk phase was unstirred, the K_m^* of each hexose exceeded 40 mM (Table 1); at an intermediate rate of stirring of the bulk phase (200 rpm), the K_m^* of glucose was greater than galactose, 14 and 5 mM, respectively. Using the Lineweaver-Burk plot of the experimental values obtained when the bulk phase was unstirred demonstrated that the magnitude of the K_m^* of 3-O-methyl glucose was lower than that of glucose or galactose (15, 25 and 20 mM, respectively), but when the bulk phase was stirred at 600 rpm, the K_m^* of 3-O-methyl glucose was now significantly higher than the other two hexoses (Table 2). Thus, the variability in the previously reported relative values of the affinity constants of hexose transport in the intestine is likely to be due at least in part to differences in the effective resistance of the unstirred water layer, and to the use of the Lineweaver-Burk plot.

Despite the serious limitations in the use of the three "linear" transformations of the Michaelis-Menten equation to estimate the quantity of the kinetic constants, they do provide the means to assign certain qualitative interpretations to transport processes. For example, when the resistance of the unstirred water layer is high and adequate corrections have been made for the contribution of passive permeation, the relationship between C_1/J_d *vs.* C_1 is linear over a wide range of values of C_1. At lower values of C_1, however, the relationship deviates either upwards or downwards from an extension of the linear portion of the line obtained at higher values C_1. Upward deviation, with a higher value of the y-axis intercept, is provided by several conditions, including increasing resistance of the unstirred layer, increasing values of K_m, decreasing values of J_d^m, or distribution of most of the transport sites along the upper portion of the villus [25]. For theoretical purposes, the villus is not considered as a homogeneous unit, since the effective resistance of the unstirred layer may vary at different sites along the villus [28], and since the transport characteristics of the mucosal cells at different points along the villus may in fact vary. Downward displacement of the relationship between C_1/J_d *vs.* C_1 can only be achieved when three conditions prevail together: when the transport sites are distributed along the villus, when the effective unstirred layer resistance varies along the villus, and when the affinity constant of the transport sites near the base of the villus is higher than the transport sites at the villus tip [25]. It must

be emphasized that the relationship between C_1/J_d *vs.* C_1 at high values of unstirred layer resistance (0 rpm) is clearly different for glucose on the one hand, and galactose and 3-O-methyl glucose on the other (Fig. 4): there is upward deviation from the extension of the linear portion of the relationship between C_1/J_d *vs.* C_1 for glucose obtained at higher values of C_1, and downward deviation for galactose and 3-O-methyl glucose. Theoretical considerations have suggested that assuming adequate correction for the contribution of passive permeation, this qualitative relationship betwen C_1/J_d *vs.* C_1 is compatible with the suggestion that the distribution of transport sites along the villus is different for glucose than for galactose and 3-O-methyl glucose: glucose transport is likely to occur near the tips of the villus, whereas the transport of galactose and 3-O-methyl glucose is likely to occur along the length of the villus, and the affinity constants are likely to be lower at the tip of the villus than towards the base.

The apparent affinity constants for the unidirectional flux of fructose, glucose, galactose, and 3-O-methyl glucose into jejunum of mature rabbits estimated under conditions of stirring the bulk phase approach the value of the true affinity of the membrane for the hexoses [27]. Although variations in the maximal transport rates are associated with obligatory changes in the estimation of the magnitude of the apparent affinity constants [27], this applies only under conditions of high unstirred layer resistance and would therefore not affect the estimation of the affinity constants when the bulk phase was stirred and effective unstirred layer resistance was low. The experimental values contain a passive component (Table 1); while appropriate corrections are necessary to accurately estimate the affinity constants from the experimental values (Fig. 1), passive permeation comprises less than approximately 3% of the value of the experimental undirectional flux rate of glucose galactose or 3-O-methyl glucose at substrate concentrations equal to their appropriate K_m^*. Although the contribution of passive permeation to the experimental values is greater when the maximal transport rate is low, this becomes an important consideration only for values of C_2 exceeding K_m^* [Eq. (4), Table 1]. Furthermore, since the apparent passive permeability coefficients P^* were similar for each hexose (Table 1), any error in the estimation of the absolute value of the apparent passive permeability coefficient would have a similar effect on the estimation of the apparent affinity constant. It is difficult experimentally to completely eliminate the effective resistance of the unstirred layer, but the effective resistance of the unstirred layer was low when the bulk phase was stirred at 600 rpm

[28] and was similar for each probe molecule. Thus, these experimentally determined results support the suggestion that the relative affinities of the intestinal membrane for the carrier-mediate transport of these sugars is glucose < fructose < galactose < 3-O-methyl glucose. It must be stressed, however, that the value for the apparent affinity constant K_m^* of a probe molecule may reflect a mean effective value for a series of affinity constants of the carrier. If the affinity constant of all the carriers of galactose, for example, are similar at each site along the villus, then the apparent affinity constant would also be the same as the effective K_m^* at each site along the villus. However, if the affinity constant of the galactose carrier varied at different sites along the villus, then the experimentally determined value would simply reflect the average of the relative contribution of the carriers with varying affinities. Thus the K_m^* of galactose of 3 mM could represent the result of the influence of carriers with lower and higher values of K_m^*. Therefore, these values of the affinity constant are only operational terms representing the net effect of the result of possible interaction of a host of carriers with different affinities. This concept is supported by the theoretical and experimental observations that the value of the apparent affinity constant is influenced by variations in the effective resistance of the unstirred water layer, the maximal transport rate, the distribution of the transport sites along the villus, as well as by changes in the value of the true affinity constant itself [27]. Further studies must now be undertaken to determine whether differences in the apparent affinity constant of sugar transport under a variety of conditions, such as different sites along the intestine, in animals of different ages, or in different species, are due to differences in the true affinity constant of the membrane carriers, or due to variations in the many other factors which may influence the experimental estimation of the apparent affinity constant.

It is speculated that there must be either at least two intestinal carriers for glucose, galactose and 3-O-methyl glucose or the carriers for these hexoses are heterogeneous. The finding of a markedly dissimilar J_d^m of glucose from that of galactose or 3-O-methyl glucose supports the suggestion that there are more carriers for the latter two sugars than for glucose. This also implies that there must be two separate carrier systems, one of which is not shared by glucose; otherwise, the maximal transport rate of glucose would be similar to that of galactose and 3-O-methyl glucose. On the other hand, there may be a heterogeneous population of carriers with different affinity and maximal transport capacity for glucose and for galactose or 3-O-methyl glucose. Competition studies

must now be performed to attempt to determine the relative affinities and maximal transport rates of the hexose carriers, and to determine whether these carriers are heterogeneous.

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