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Determination of kinetic parameters of a carrier-mediated transport in the perfused intestine by two-dimensional laminar flow model: effects of the unstirred water layer

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The kinetic parameters of a carrier-mediated transport for D-glucose and for taurocholate were determined from rat in situ intestinal single perfusion experiments. The true parameters were obtained by the two-dimensional laminar flow model, in which the solute concentration at the aqueous-intestinal membrane interface can be calculated numerically without assuming the aqueous diffusion layer, discriminating the effects of the unstirred water layer. The true Michaelis constant was 4.5 mM for D-glucose and 1.5 mM for taurocholate. The true maximal transport velocity was 3.4 nmol/s per cm^2 for D-glucose and 0.29 nmol/s per cm^2 for taurocholate. The apparent Michaelis constant was raised by the factor of 6.6 for D-glucose and 3.6 for taurocholate due to the effects of the unstirred water layer. The maximal transport velocity was relatively unaffected by the unstirred water layer in both compounds. The values of the effective (operational) thickness of the unstirred water layer were compatible with those reported previously by employing various experimental methods. The kinetic parameters obtained in vitro everted sacs, for comparison, almost coincided with the true ones in situ. Therefore, the two-dimensional laminar flow model is shown to be valid not only for determining the kinetic parameters of a carrier-mediated transport in situ but also for predicting the absorption rate in situ from the uptake rate in vitro.

Introduction

Is has been proposed that the intestinal absorption rate of highly membrane-permeable substances are restricted by diffusion in the aqueous phase and hence the estimation of the diffusional resistance in the aqueous phase is required in interpreting the experimental data and in predicting the absorption rate under various conditions [1,2]. A most common approach to take into account the aqueous resistance employs a film model in which the aqueous phase is divided into the bulk phase without concentration gradient and the unstirred (stagnant) layer adjacent to the mucosal surface, the aqueous diffusion layer [3]. The experimentally demonstrated [4–7] and theoretically predicted [8–10] biases on the kinetic parameters due to the aqueous resistance, for example the underestimation of the membrane permeability coefficient and the overestimation of the Michaelis constants, are commonly realized as the effects of the unstirred water layer. However, it is troublesome or sometimes impossible to determine experimentally the effective thickness of the un-

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stirred water layer (the aqueous diffusion layer), because it can be decreased but not completely eliminated by shaking [11] or stirring [12,13] in incubation experiments or by a 'segmented flow' [14,15] or increasing flow rate [16,17] in perfusion experiments.

On the other hand, in single perfusion experiments, alternative methods to take into account the aqueous resistance were proposed by Winne [14,16] and by Eliott et al. [18], by assuming that the luminal flow is laminar [19]. In the laminar flow assumption, the solute concentration decreases non-linearly in both axial and radial direction in the intestinal tract, but mathematical allowance for the mass transfer can be made without requiring such an unknown parameter as the effective thickness of the unstirred water layer, as its benefit. In their approach, the approximate solution of the equations derived for the heat transfer in the laminar flow through a circular tube was applied, though only linear transport at the membrane could be considered. We have recently proposed a two-dimensional laminar flow model considering not only passive transport but also Michaelis-Menten-type carrier-mediated transport and water absorption or secretion at the intestinal membrane [20,21], in which the solute concentration profile in the intestinal tract can be numerically calculated. We have previously reported the validity of the model in determination of the membrane permeability coefficients and the reflection coefficients [22].

In this study, we intended to demonstrate that the two-dimensional laminar flow model is applicable to a carrier-mediated transport in situ intestinal single perfusion experiments, using D-glucose and taurocholate as model compounds. The effects of the unstirred water layer on the kinetic parameters, the Michaelis constant and the maximal transport velocity were also investigated by employing a film model and comparing the results with those obtained using in vitro everted sacs.

Theory

Two-dimensional laminar flow model

Basic assumptions are as follows: (1) the intestinal tract is a straight cylindrical tube with a constant radius, neglecting the villous structure; (2) the fluid is incompressible and the axial flow is laminar; (3) the axial diffusion term can be neglected; (4) the fluid flowing through the intestinal membrane is uniform throughout. The non-steady-state equation of the given substance in the intestinal tract in cylindrical co-ordinates is

$$\frac{\partial c}{\partial t} + v\frac{\partial c}{\partial r} + u\frac{\partial c}{\partial x} = D\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial c}{\partial r}\right) \tag{1}$$

and the boundary conditions are

$$\text{at } x = 0\text{: } c = C_{in} \tag{2}$$

$$\text{at } r = 0\text{: } \frac{\partial c}{\partial r} = 0 \tag{3}$$

$$\text{at } r = R\text{: } N = \left\{P_m + 0.5(1-\sigma)v_0 + \frac{V_{max}}{K_m + c}\right\}c \tag{4}$$

where c is the solute concentration; r and x are the radial and axial co-ordinates, respectively,; v and u are the radial and axial components of velocity, respectively; t is the time; D is the diffusion constant, R is the radius of the intestine; C_{in} is the concentration in the inflow solution; v_0 is the radial velocity at the intestinal membrane; σ is the reflection coefficient; P_m, K_m and V_{max} are the true membrane permeability coefficient, the true Michaelis constant and the true maximal transport velocity, respectively; N is the molar flow per unit area. The transport rate across the intestinal membrane, from the mucosal surface to the blood (in which the solute concentration is assumed to be zero), is characterized by the single Michaelis-Menten equation with the phenomenological equation by Kedem and Katchalsky [23] in Eqn. 4. The former represents a carrier-mediated transport and the latter represents a concurrent passive transport with the effect of solvent drag. The parameters P_m, K_m and V_{max} have to be regarded as average values for the overall transport process including not only the entry step into the epithelial cells but also sequential intracellular transport and exit step into the blood. Numerically calculating the time-dependent Eqn. 1 by using a time-stepping procedure, we regarded the asymptotic behavior at a sufficiently long time as the steady-state solution. Details are described in our previous reports [20,21].

Film model

A most common approach to take into account the aqueous resistance to employ a film model, in which the luminal concentration profile is approximated by a well-mixed bulk phase concentration with a concentration gradient only in the unstirred water layer (the aqueous diffusion layer) adjacent to the mucosal surface. In this model, assuming that the unstirred water layer area and the membrane area are equal to the same cylindrical surface by neglecting the villous structure, the equation derived by Winne [9] is modified as follows:

$$\text{absorption rate} = \frac{SD}{\delta}\left\{C_b + F - \left(F^2 + qK_mC_b\right)^{1/2}\right\} \qquad (5)$$

where

$$q = \frac{1}{1 + \delta P_m/D}$$

$$F = \tfrac{1}{2}q\left(\frac{K_m}{q} + \frac{\delta V_{max}}{D} - C_b\right)$$

and where the absorption rate is related to the perfused segment; S is the cylindrical surface area of the perfused segment; C_b is the concentration in the bulk phase; δ is the effective thickness of the unstirred water layer. In the present study, this model is used to estimate the values of δ assuming the laminar flow, as a kind of apparent parameter. Its characteristics have already been discussed elsewhere by Elliott et al. [18], and it depends not only on the flow rate and the diffusion constant but also on the membrane permeability and the length of the intestine.

Eliminating the unstirred water layer from the model, absorption rate is represented as follows:

$$\text{absorption rate} = S\left(P_{m,app} + \frac{V_{max,app}}{K_{m,app} + C_b}\right)C_b \qquad (6)$$

where the suffix 'app' means the apparent parameter, which includes the effect of the unstirred water layer.

Methods

Materials. D-[U-^{14}C]Glucose (14.4 mCi/mmol), L-[1-^{14}C]glucose (47.0 mCi/mmol), [G-^{3}H]inulin (430.6 mCi/g), [G-^{3}H]taurocholate (6.6 Ci/mmol), inulin-[^{14}C]carboxylic acid ([*carboxyl*-^{14}C]inulin-carboxyl) (2.4 mCi/g), Protosol (tissue solublizer) and Biofluor (scintillation cocktail) were purchased from New England Nuclear (Boston, MA). D-Glucose (Tokyo Kasei Kogyo Co., Tokyo), L-glucose and taurocholate (Sigma Chemical Co., St. Louis, MO) were commercially obtained. All other reagents were commercially obtained and were of analytical quality.

Animals. Male Wistar rats weighing 300–350 g were fed regular chow and water ad libitum and were not fasted prior to experiments.

Preparations. In situ single perfusion experiments were carried out according to the method described by Winne et al. [15]. The rat was anesthetized with urethane (4.5 ml/kg intraperitoneally, 25% solution). The trachea was cannulated with polyethylene tubing (internal diameter 0.15 cm), and the left carotid artery and the right jugular vein were cannulated with polyethylene tubing (PE 50). Then the abdomen was opened by a midline incision and the inflow and the outflow cannula made of polyethylene tubing (internal diameter 0.3 cm) were attached to a 10 cm segment of the small intestine measured by means of a thread placed on the loop along its curved axis. The segment was internally flushed with saline to remove any residual intestinal contents, and then flushed with air to minimize the amount of residual fluid left in the lumen. The inflow cannula was set at approx. 30 cm distal to the pylorus for the jejunal perfusion of glucose and taurocholate. The outflow cannula was set at approx. 5 cm distal to the ileocecal junction for the ileal perfusion of taurocholate. After injection of heparin (1000 unit/ml, 0.22 ml) and phentolamine (2.5 mg/ml, 0.2 ml) into the jugular vein, the superior mesenteric vein draining the perfused segment was cannulated with polyethylene tubing (internal diameter 0.1 cm) and the blood was drained. Phentolamine was injected to secure the stable and constant blood drainage by preventing the contraction of mesenteric vein. The blood loss was compensated by infusion of fresh heparinized rat blood into the jugular vein, which was taken from other rats immediately before the beginning of the experiment. Blood pressure was monitored continually in the carotid artery. The perfused

segment was put on the flat plate covered with gauze soaked with saline and kept at 37°C with a heat lamp. The intestinal segment was perfused with oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4) containing a probe and inulin as the marker for the intraluminal volume changes at 0.16 ml/min for glucose and 0.84 ml/min for taurocholate by means of a peristaltic pump (Minipuls II, Gilson CO.). After the steady-state condition was achieved, 20 min for glucose and 10 min for taurocholate, the outflow solution and the blood from the mesenteric vein were collected at 5-min intervals for 20 min. At the end of the experiment the outer circumference was measured by a thread leading around the loop.

In vitro uptake experiments were carried out by using everted sacs prepared by a modification of the procedure described by Wilson and Wiseman [24]. After the rat had been killed by decapitation, an approx. 10-cm segment of small intestine corresponding to the in situ single perfusion experiments was cut out and internally rinsed with cold saline. Then the segment was everted with the aid of a glass rod. Four 2.5-cm length sacs containing 0.4 ml of probe free oxygenated Krebs-Ringer-bicarbonate buffer as a serosal fluid were made out of the segment, and stored in the ice-cold probe-free buffer until incubation. A sac had one long thread for carrying. After preincubation for 4 min, a sac was incubated at 37°C and 100 cycles/min in a 50 ml flask containing 20 ml of oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4) with a probe and inulin as the marker for the adherent mucosal fluid. At the end of the incubation period the sac was quickly removed, rinsed in ice-cold buffer and blotted on the filter paper. Half of the sac was placed in a tared counting vial and the wet tissue weight was determined.

Solutions. Oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4) containing a known concentration of unlabeled probe with tracer amount of labeled probe and labeled inulin as the marker for the intraluminal volume change or for the adherent mucosal fluid was used as the perfusion solution or as the incubation medium. ^{3}H-labeled inulin was used with ^{14}C-labeled glucose and ^{14}C-labeled inulin was used with ^{3}H-labeled taurocholate.

The osmolality of the perfusion solution was maintained to be constant by changing NaCl content in order to prevent the intraluminal volume change [22]; 425 mosmol/kg for glucose and 390 mosmol/kg for taurocholate, as taurocholate caused some water secretion.

The incubation medium in vitro was maintained isoosmotic (290 mosmol/kg) by changing the NaCl content.

Analyses. 10 ml scintillation cocktail (Biofluor) was added to the 100-μl aliquots of the inflow solution, the outflow solution and the incubation medium, and the radioactivity was determined by liquid scintillation counting.

The 50-μl aliquots of blood were solubilized in 0.5 ml of a 2:1 mixture of ethanol and protosol (60°C, 1h) and decolorized with 0.3 ml 30% hydrogen peroxide (60°C, 0.5 h); 10 ml scintillation cocktail and 0.5 ml 0.5 M HCl were added, and the radioactivity was determined.

Tissues were solubilized in 2 ml of protosol (60°C, 5h) and decolorized with 0.2 ml of 30% hydrogen peroxide (60°C, 1h); 10 ml scintillation cocktail were added, and the radioactivity was determined.

Representation and treatment of data. The absorption rate (nmol/min per 10 cm) in situ as the average of four sampling periods was in principle calculated from disappearance of the probe from the lumen after correcting for the intraluminal volume change and monitored by appearance in the mesenteric venous blood. Only in the jejunal perfusion of taurocholate was the appearance rate in the mesenteric venous blood used as the absorption rate, because the concentration difference between the inflow and the outflow solution was too small to give reliable values for the disappearance rate from the lumen. The uptake rate (nmol/min per 100 mg) in vitro was calculated from the amount of the probe in the tissue after correcting for the amount in the adherent mucosal fluid, and related to the 100 mg wet tissue weight.

The inner circumference was determined by multiplying the measured outer circumference by 0.83, the ratio of the inner to the outer circumference [15]. The radius of the small intestine, R (cm) was calculated by R = (inner circumference)/2π. Neglecting the villous structure, the cylindrical surface area, S (cm^2) was determined by $S = 2\pi RL$, where the length of the

perfused segment, L was 10 cm. The diffusion constant, D (cm^2/s) at 37°C was calculated from that of D-glucose at 25°C [25], considering that it is inversely proportional to the square root of the molecular weight and is proportional to the absolute temperature. For analysis by the two-dimensional laminar flow model, the maximum velocity at the inlet, u_0 (cm/s) was calculated by $u_0 = 2Q/\pi/R^2/60$, where Q (ml/min) is the perfusion rate.

In all data analyses the membrane permeability coefficient for D-glucose and for taurocholate were determined beforehand from the jejunal transport rate of L-glucose and taurocholate, respectively. Then the Michaelis constant and the maximal transport velocity for jejunal transport of D-glucose and for ileal transport of taurocholate were determined.

In the analysis by the two-dimensional laminar flow model, terms related to the radial component of velocity (v and v_0) were omitted in Eqns. 1 and 4 because there was no significant intraluminal volume change in average. The P_m (cm/s) was determined by a graphic method, using absorption rate versus P_m curve calculated by omitting the Michaelis-Menten equation in Eqn. 4 [20,22]. At the lower concentration, $c \ll K_m$, the absorption rate related linearly to c and Eqn. 4 simplifies to:

$$N = \left(P_m + \frac{V_{max}}{K_m} \right) c \tag{7}$$

and $P_m + V_{max}/K_m$ (cm/s) could also be determined by a graphic method. The V_{max}/K_m (cm/s) was determined by subtracting P_m from $P_m + V_{max}/K_m$. Fixing the value of V_{max}/K_m, K_m and V_{max} were determined so as to fit best the experimental data, using residual sum of squares as an index.

In the analysis by the film model, the logarithmic mean concentration was applied to the concentration in the bulk phase by $C_b = (C_{in} - C_{out})/\ln(C_{in}/C_{out})$, where C_{out} is the concentration in the outflow solution [26]. The δ (cm) was determined by fitting Eqn. 5 to the experimental data by a non-linear regression method, using P_m, K_m and V_{max} determined by the two-dimensional laminar flow model as known parameters. Therefore, δ is only an operational quantity which represents the diffusional resistance in the aqueous phase in the assumption of the laminar flow. The apparent parameters were determined by fitting Eqn. 6 to the experimental data by a non-linear regression method.

The kinetic parameters in vitro were determined by fitting following Eqn. 8 to the experimental data by a non-linear regression method.

$$\text{uptake rate} = \left(P_m^* + \frac{V_{max}^*}{K_m^* + C_m} \right) C_m \tag{8}$$

The P_m^*, K_m^* and V_{max}^* are the apparent permeability coefficient (nmol/100 mg per mM), the apparent Michaelis constant (mM) and the apparent maximal transport velocity (nmol/100 mg), respectively. C_m is the concentration in the medium. These parameters are expected to represent only the entry step into the epithelial cells, but may possibly be distorted slightly by the effects of the unstirred water layer, if eliminated incompletely. In order to compare with parameters obtained in situ, P_m^* and V_{max}^* were converted by multiplying the correcting factors as follows:

$$P_m^* \times \frac{W/100}{2\pi R/0.83} \times \frac{1}{1000} (\text{cm/s}) \tag{9}$$

$$V_{max}^* \times \frac{W/100}{2\pi R} (\text{nmol/s per cm}^2) \tag{10}$$

where W (mg/cm) is the wet tissue weight in vitro and R is the radius in situ. As the passive transport is proportional to the surface area, P_m^* was normalized by the outer surface area of the intestinal cylinder which corresponded to the mucosal surface area of the everted sacs.

Results are represented as the mean ± S.D.

Results

The experimental conditions and the values used for data analyses are listed in Table I. We performed two series of experiments, a glucose series and a taurocholate series. It is generally accepted that the intestinal carrier for D-glucose has no affinity for L-glucose and that L-glucose is transported only passively in the intestine. It is also generally accepted that the carrier for taurocholate exists only in the lower ileum but not in the jejunum and that taurocholate is transported only passively in the jejunum. Therefore, the membrane

permeability coefficient determined for the jejunal transport of L-glucose was adopted as that for D-glucose in the glucose series, considering that physicochemical properties concerning the membrane permeability by passive transport are not different between D- and L-glucose. The membrane permeability coefficient determined for the jejunal transport of taurocholate was adopted as that for taurocholate in the taurocholate series, considering that properties concerning the membrane permeability by the passive transport are not different between the ileal and the jejunal membrane. Using the membrane permeability coefficient thus obtained as a known parameter, the Michaelis constant and the maximal transport velocity of a carrier-mediated transport were determined for the jejunal transport of D-glucose in the glucose series and for the ileal transport of taurocholate in the taurocholate series.

Absorption kinetics in situ perfused intestine

As is generally accepted, the jejunal absorption of D-glucose and the ileal absorption of taurocholate showed concentration-dependent saturation kinetics, indicating the presence of carrier-mediated transport (Fig. 1). On the other hand, the jejunal absorption of L-glucose and the jejunal absorption of taurocholate showed much lower concentration-independent linear kinetics, indicating the lack of carrier-mediated transport (Table II).

The true parameters obtained by the two-dimensional laminar flow model (Eqns. 1–4) and the apparent parameters obtained by the application of the simple Michaelis-Menten equation with a passive component (Eqn. 6) are listed in Table V, and the curves corresponding to the true parameters are diagrammed in Fig. 1. In our analysis by the two-dimensional laminar flow model, the independent variable is the concentration in the inflow solution, C_{in}, which is strictly controlled, and the dependent variable is the absorption rate, which is calculated from C_{in}, the observed concentration in the outflow solution, C_{out} and another strictly controlled value, the perfusion rate, Q. The length, L and the radius, R, of the intestine used, the axial velocity of the fluid flow, u, and the diffusion constant, D, are needed for calculation by the model in addition to the parameters to be determined P_m, K_m and V_{max}. The L value is experimentally set to be 10 cm, R has little variance (Table I) and u is calculated from Q and R by the assumption of the laminar flow. The diffusion constant reported for the diffusion of the probe in the aqueous solution was used as the most plausible value for D. The error in the estimation of P_m can be neglected because the passive transport components are relatively minor ones. The S.D. of

TABLE I

EXPERIMENTAL CONDITIONS AND VALUES USED FOR DATA ANALYSES

	Glucose series	Taurocholate series
Perfusion rate (ml/min)	0.16	0.84
Maximum velocity at the inlet[a] (cm/s)	0.030	0.134
Length in situ (cm)	10	10
Radius in situ (cm)	0.23 ± 0.02 (21)[b]	0.26 ± 0.02 (18)[b]
Diffusion constant[c] ($\times 10^{-6}$ cm^2/s)	7.04	4.16
Wet tissue weight in vitro (mg/cm)	139 ± 22 (15)[b]	138 ± 30 (15)[b]

[a] Maximum velocity at the inlet, u_0, was calculated from the perfusion rate, Q, and radius, R, by $u_0 = 2Q/\pi/R^2/60$.

[b] Values represent the mean ± S.D. (n), where n is the number of experiments.

[c] Diffusion constant at 37°C was calculated from that of D-glucose at 25°C [25], considering that it is inversely proportional to the square root of the molecular weight and is proportional to the absolute temperature.

TABLE II

JEJUNAL ABSORPTION RATE OF L-GLUCOSE AND TAUROCHOLATE IN SITU PERFUSION EXPERIMENTS

The absorption rate was normalized by the concentration in the inflow solution, C_{in}. Experimental conditions are listed in Table I. Values represent the mean ± S.D. of three experiments.

Substance	C_{in} (mM)	Absorption rate/C_{in} (nmol/min per 10 cm per mM)
L-Glucose	20	5.9 ± 2.3
	50	4.8 ± 1.1
	100	6.1 ± 1.5
Taurocholate	0.1	2.0 ± 1.4
	1	2.1 ± 0.8
	2	2.0 ± 0.6
	4	2.2 ± 1.4

the passive transport component for D-glucose is about 30% of itself but only about 5% of the total absorption rate, even at the saturated portion. With regard to taurocholate, the passive transport component is only about 5% of the total absorption rate, even at the highest C_{in}. The K_m and V_{max} are the essential parameters to be determined. In general, the simulation at the saturated portion is sensitive to the value of V_{max}. The K_m is concerned with the deviation from the linearity. Then simulations were carried out by changing the values of K_m and V_{max}, using the true values shown in Table V as the standard, namely by 50% for K_m and by 10% for V_{max} of D-glucose and by 20% for both parameters of taurocholate. Most experimental data are within the rage of these simulations as shown in Fig. 1. Therefore, it is expected that the values of K_m and V_{max} are in these ranges. The linear portion is mainly restricted by the radial diffusion of the probe in the aqueous phase in these high V_{max}/K_m compounds, the unstirred water layer effect. So it is relatively insensitive to the values of K_m and V_{max}.

Comparing the apparent parameters with the true ones, although the membrane permeability coefficients were little different to each other and the difference in the maximal transport velocities was relatively insignificant in both compounds, the apparent Michaelis constants were greatly overestimated; from 4.5 to 29.7 mM for D-glucose (factor of 6.6) and from 1.5 to 5.4 mM for taurocholate (factor of 3.6). This overestimation of the Michaelis constants indicates the considerable effects of the unstirred water layer. The values of the effective thickness of the unstirred water layer obtained by Eqn. 5 were 644 ± 56 μm in the glucose series and 395–33 μm in the taurocholate series.

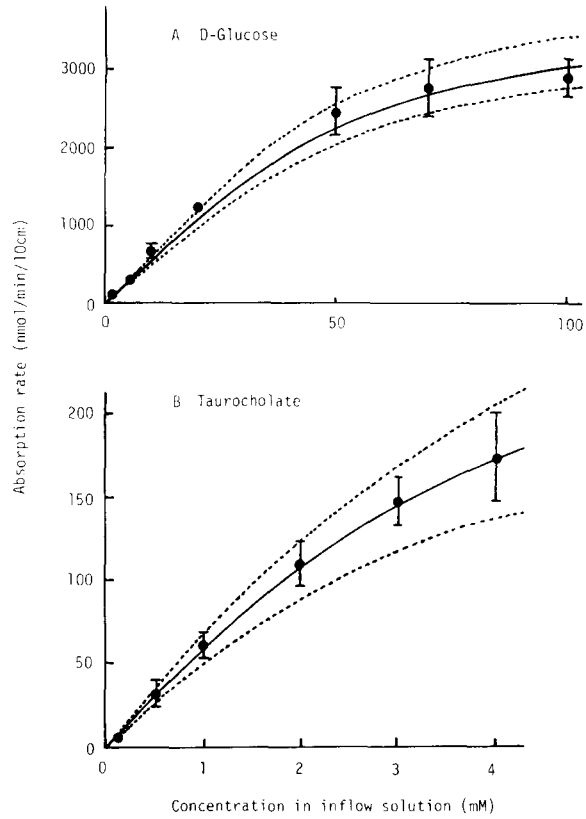

Fig. 1. Jejunal absorption rate of D-glucose (A) and ileal absorption rate of taurocholate (B) versus concentration in the inflow solution in in situ perfusion experiments. Experimental conditions are listed in Table I. Simulations were carried out by using the two-dimensional laminar flow model (Eqns. 1–4). the solid curves correspond to the combination of the true values of K_m and V_{max} shown in Table V. The values of K_m and V_{max} were then changed, using the true values as the standard: by 50% for K_m and by 10% for V_{max} in panel A (D-glucose) and by 20% for both parameters in panel B (taurocholate). The upper limits which correspond to the combination of the lowest K_m and the highest V_{max} and the lower limits which correspond to the combination of the highest K_m and the lowest V_{max} are shown by the dotted lines. Each point and vertical bar represents the mean $\pm$S.D. of three experiments.

Time-courses of uptake in vitro everted sacs

The time-courses of the uptake in vitro everted sacs are shown in Fig. 2. In all cases there was no detectable transport to the serosal fluid. Analyzing the data of the jejunal uptake of L-glucose and the jejunal uptake of taurocholate by a linear regression analysis with the least-squares method, the initial binding to the mucosal membrane obtained from the intercept of the ordinate was negligible for L-glucose (binding/$C_m = 0.33 \pm 1.16$ nmol/100 mg per mM) but significant for taurocholate (binding/$C_m = 2.24 \pm 0.80$ nmol/100 mg per mM). So we obtained the jejunal uptake rate of L-glucose from the initial 5-min uptake without correcting for the initial binding (Table III). The jejunal uptake rate of taurocholate was obtained from the slope of the linear relation between uptake and time (Table IV). The initial binding of taurocholate to the jejunal membrane was considered to be nonspecific because it showed no saturability,

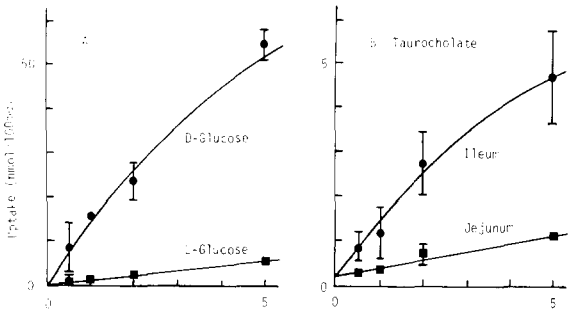

Fig. 2. Time-courses of the jejunal uptake of D- and L-glucose (A) and the ileal and the jejunal uptake of taurocholate (B) into the tissue in vitro everted sacs. Concentration in the medium was 1 mM for glucose and 0.1 mM for taurocholate. A time-course was obtained by using four everted sacs from a rat. Data are the mean ±S.D. of three time-courses. Data of the jejunal uptake of L-glucose and taurocholate were analyzed by a linear regression analysis with the least-square method. Curves for the jejunal uptake of D-glucose and the ileal uptake of taurocholate were drawn by inspection.

namely, no reduction of 'binding/C_m', when the concentration in the medium, C_m, was raised. The binding coefficient of 2.13 nmol/100 mg per mM, the mean of 'binding/C_m' at $C_m = 0.1$ and 2 mM, was used in the following correction of the ileal uptake rate of taurocholate. The jejunal uptake rate of D-glucose and the ileal uptake rate of taurocholate were obtained from the initial 0.5-min uptake because they appeared to be reduced, perhaps owing to the back flux of the probes from the tissue to the incubation medium as the incubation was prolonged (Fig. 3). The characteristics of the initial binding of D-glucose to the jejunal membrane and those of taurocholate to the ileal membrane were assumed to be the same as those of L-glucose to the jejunal membrane and of taurocholate to the jejunal membrane, respectively; namely, correction for the initial binding was made for taurocholate by using the binding coefficient obtained from the jejunal uptake, but was not made for D-glucose.

TABLE III

JEJUNAL UPTAKE RATE OF L-GLUCOSE IN VITR EVERTED SACS

The uptake rate was obtained from the initial 5-min uptake into the tissue without correcting for the initial binding to the mucosal membrane and was normalized by the concentration in the medium, C_m. Values (nmol/min per 100 mg per mM) represent the mean ±S.D. of three experiments.

	C_m (mM)		
	1	5	20
Uptake rate/C_m	0.83±0.15	0.85±0.16	0.80±0.05

Uptake kinetics in vitro everted sacs

The experiments in this section were carried out to examine the validity of the two-dimensional laminar flow model by comparing the parameters obtained in situ with those obtained in vitro.

The jejunal uptake of D-glucose and the ileal uptake of taurocholate showed saturation kinetics (Fig. 3) and the jejunal uptake of L-glucose showed much lower linear kinetics (Table III), in the same manner as the absorption in situ perfused intestine. Although the jejunal uptake rate of taurocholate showed saturability, namely the reduction of 'uptake rate/C_m' when C_m was raised (Table IV), it was considered that the contribution of a saturable component, if it existed, could be practically neglected, because the jejunal uptake rate was much lower than the ileal uptake rate and because there was no sign of a saturable component in the in situ perfused jejunum. The 'uptake rate/C_m' at higher concentration, 0.95 nmol/min/100 mg per mM, was adopted as P_m^* in Eqn. 8 to minimize the effect of a possible saturable component.

TABLE IV

JEJUNAL UPTAKE RATE AND INITIAL BIDING TO JEJUNAL MUCOSAL MEMBRANE OF TAUROCHOLATE IN VITRO EVERTED SACS

A time-course of the uptake into the jejunal tissue was obtained by using four everted sacs from a rat, and was analyzed by a linear regression analysis with the least-square method. The uptake rate was determined from the slope and the initial binding to the mucosal membrane was determined from the intercept of the ordinate. The uptake rate and the initial binding were normalized by the concentration in the medium, C_m. Values represent the mean ±S.D. of three experiments.

	C_m (mM)	
	0.1	2
Uptake rate/C_m (nmol/min per 100 mg per mM)	1.80±0.03	0.95±0.17
Binding/C_m (nmol/100 mg per mM)	2.24±0.80	2.02±0.61

TABLE V

KINETIC PARAMETERS OBTAINED IN THE MODEL ANALYSES

Parameters are mean ±S.D., except for the true Michaelis constants and the true maximal transport velocities in situ.

Substance	Method	Parameters		
		Michaelis constant (mM)	Maximal transport velocity (nmol/s per cm^2)	Membrane permeability coefficient (10^{-6} cm per s)
D-Glucose	in situ[a]	4.5	3.4	7.0 ± 2.1
	in situ, app[b]	29.7 ± 6.8	3.9 ± 0.3	6.5 ± 1.8
	in vitro[c]	7.8 ± 3.0	3.4 ± 0.6	10.8 ± 1.5
Taurocholate	in situ[a]	1.5	0.29	2.2 ± 1.0
	in situ, app[b]	5.4 ± 1.9	0.41 ± 0.09	2.2 ± 1.0
	in vitro[c]	1.4 ± 0.5	0.38 ± 0.07	11.2 ± 2.1

[a] True parameters were obtained by applying the two-dimensional laminar flow model (Eqns. 1–4) to the experimental data in situ (Fig. 1 and Table II).

[b] Apparent parameters were obtained by applying Eqn. 6 to the experimental data in situ (Fig. 1 and Table II).

[c] Parameters were obtained by applying Eqn. 8 to the experimental data in vitro (Fig. 3, Tables III and IV) and converted by Eqns. 9 and 10.

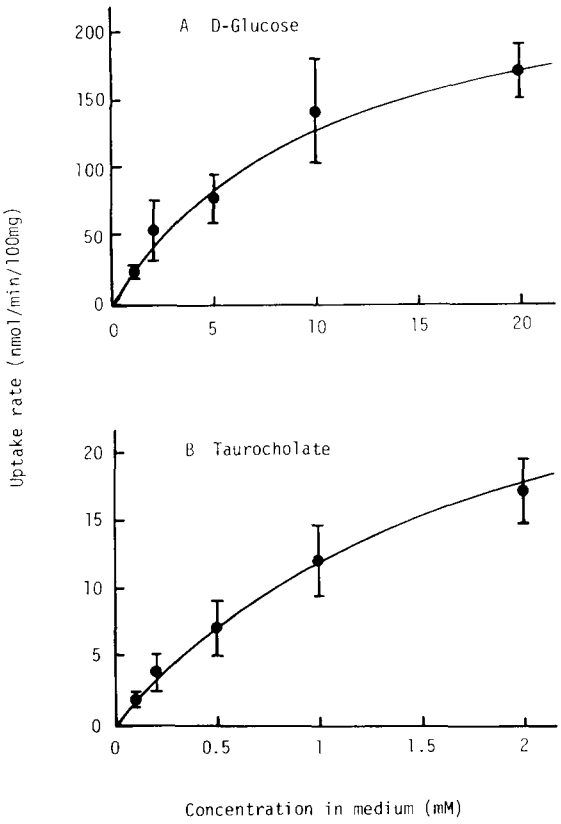

Fig. 3. Jejunal uptake rate of D-glucose (A) and ileal uptake rate of taurocholate (B) versus concentration in medium in vitro everted sacs. The uptake rate was obtained from the initial 0.5-min uptake into the tissue. Correction for the initial binding to the mucosal membrane was made for taurocholate by using the binding coefficient obtained from the jejunal uptake but was not made for D-glucose. Curves were diagrammed by applying Eqn. 8, and the obtained parameters are listed in Table V after conversion by Eqns. 9 and 10 in order to compare with those obtained in situ. Each point and vertical bar represents the mean ±S.D. of three experiments.

Kinetic parameters were obtained by the application of Eqn. 8 and converted by Eqns. 9 and 10 in order to compare with those obtained in situ (Table V). The Michaelis constants obtained in vitro, i.e., 7.8 mM for D-glucose and 1.4 mM for taurocholate, showed much better consistency with the true ones in situ than with the apparent ones in situ, corresponding to the generally accepted idea that the effects of the unstirred water layer in vitro are much less or negligible compared with those in situ. The maximal transport velocities obtained in vitro were also consistent with those obtained in situ. The membrane permeability coefficient in vitro was raised by the factor of 5.1 for taurocholate but was little changed for D-glucose.

Discussion

Details of the effects of the unstirred water layer on the kinetic parameters of the intestinal absorption predicted by the two-dimensional laminar flow model have been described in our

previous simulation studies [20,21]. The most conspicuous features are the reduction of the membrane permeability coefficients and the increase in the Michaelis constants. These effects increase as V_{max}/K_m or P_m increases and as the perfusion rate decreases. Also, they are not inconsistent with those reported experimentally [6,7,14–17] and theoretically by employing the film models [8–10]. In this study, the membrane permeability coefficient is so small as to be little affected in both compounds, namely the membrane limited. On the other hand, the carrier-mediated transport ability indicated by V_{max}/K_m, $7.4 \cdot 10^{-4}$ cm/s for D-glucose and $1.9 \cdot 10^{-4}$ cm/s for taurocholate, is two orders larger than the value of P_m in each compound. Therefore, the transport process across the intestinal membrane is characterized mainly by a carrier-mediated transport in each compound and a considerable effect of the unstirred water layer on the Michaelis constant has been demonstrated, namely in the overestimation in the apparent constant as compared with the true one by the factor of 6.6 for D-glucose and 3.6 for taurocholate. The lesser bias of the Michaelis constant for taurocholate is due to the lower V_{max}/K_m and the higher perfusion rate. The maximal transport velocities are relatively unaffected, as predicted theoretically.

The values of the effective thickness of the unstirred water layer were compatible with those reported in rat perfusion experiments by various methods: 410–430 μm by measuring the development of osmotically induced potential difference [27], 486 ± 40 μm [14] and 460 ± 150 μm [15] at $Q = 0.5$ ml/min by the segmented flow technique, from 212 μm ($Q = 4.213$ ml/min) to 708 μm ($Q = 0.247$ ml/min) by changing the perfusion rate [17], 600–700 μm at $Q = 0.2$ and 0.4 ml/min by comparing with the results in vitro [28]. The effective thickness seems to be reduced remarkably by increasing the perfusion rate, though this operational parameter is theoretically expected to depend on various factors such as the membrane permeability of the probe, the carrier-mediated transport ability and the length of the perfused segment. An enormously large value, 1500 ± 400 μm at $Q = 0.5$ ml/min, was also presented by Winne et al. [15]. It should be remembered that this parameter does not signify the existence of any unstirred stagnant water layer (the aqueous diffusion layer), but is only an index of the diffusional resistance in the aqueous phase [3].

The true parameters obtained by the two-dimensional laminar flow model are not affected by the factors related to the intraluminal aqueous phase such as the perfusion rate, because it has already been demonstrated that the true membrane permeability coefficient of progesterone, one of the highly membrane permeable and unstirred water-layer-limited probes, did not depend on the perfusion rate, indicating the validity of the model [22]. The validity of the laminar flow assumption has also been presented by Winne [14,16] and Amidon et al. [19]. Winne has reported that the reduction of the effective thickness of the unstirred water layer predicted by the laminar flow assumption for linear transport corresponded to the experimentally obtained values by employing the segmented flow technique or by increasing the perfusion rate. Amidon et al. proposed that the laminar flow in a cylindrical tube is the most appropriate assumption for the in situ single perfusion experiments from the residence time distribution analysis.

The Michaelis constants and the maximal transport velocities obtained in vitro were almost consistent with the true ones in situ, corresponding to the generally accepted idea that the effects of the unstirred water layer in vitro are much less or negligible compared with those in situ. Although the Michaelis constant obtained in vitro for D-glucose was 70% larger than the true one in situ, this difference is negligible compared with one order larger apparent one in situ. Although the membrane permeability coefficient obtained in vitro for taurocholate was approx. 5-fold larger than that obtained in situ, it is still negligible at the lower concentration because it is less than 1/20 of V_{max}/K_m. Therefore, we can predict the absorption rate in situ from the uptake rate in vitro by the two-dimensional laminar flow model.

The true Michaelis constant in situ (4.5 mM) and the Michaelis constant in vitro (7.8 mM) obtained in our study for D-glucose are slightly larger than the Michaelis constants reported for D-glucose by various in vitro methods: 2.2 mM [29] and 1.7 mM [30]. The true Michaelis constant in situ (1.5 mM) and the Michaelis constant in vitro (1.4 mM) obtained in our study for

taurocholate are also larger than the Michaelis constants reported for taurocholate in vitro; 0.2 mM [31] and 0.24 mM [32]. These larger Michaelis constants obtained in our studies may be attributed to the additional resistance in situ, the intracellular transport and the exit step into the blood, and to the incomplete reduction of the effects of the unstirred water layer in vitro. If the unstirred water layer is removed completely, the parameters in vitro can represent only the entry step into the epithelial cells. Additional resistances of the intracellular transport and the exit step can raise the Michaelis constants in situ, because the true parameters in situ include not only the entry step but also the sequential steps into the blood. Incomplete reduction of the unstirred water layer can somewhat raise the Michaelis constants in vitro [4–5,11–13]. The apparent Michaelis constant in situ (29.7 mM) obtained in our study for D-glucose is compatible with those reported previously in rat perfusion experiments; 18 mM [27], 23 mM [33] and 47 mM [34]. The maximal transport velocity in situ and that in vitro obtained in our study for D-glucose were also compatible with those reported previously. Although one order smaller apparent values of the Michaelis constant and the maximal transport velocity for taurocholate were presented by Schiff et al. [35], their values are considered to be greatly distorted because they measured the mucosa-to-serosa transport, including the serosal tissue as another resistance, and because the in vitro perfusion technique employed is insecure in the preservation of the viability and the physiology of the intestine. In our study, the membrane permeability coefficient obtained in situ appears to be reduced to a much greater extent for taurocholate than for D-glucose, compared with that obtained in vitro. This suggests the presence of a larger resistance for taurocholate in the sequential steps into the blood or the presence of a facilitating exit mechanism specific for D-glucose such as Na^+-independent carrier-mediated transport [36]. Further investigations concerning the effects of the unstirred water layer in vitro and the transport mechanism in situ should be required to elucidate these discrepancies of the parameters in detail.

The critical discrepancy between the apparent Michaelis constant obtained in situ and that obtained in vitro, which sometimes amounts to be an order of magnitude, should be overcome. This is the point presented in this study. The logarithmic mean concentration may not be as appropriate as C_b for use in estimating the apparent parameters, δ, $K_{m,app}$ and $V_{max,app}$ when fractional absorption is large or inhomogeneous distribution of absorption capacity along the intestinal tract is possible, as is the case for D-glucose. However, the error in the estimation of C_b would be at most 10 or 20% and not exceed 40%, as the fractional absorption of D-glucose ranged from 0.15 to 0.4. The severalfold overestimation in the apparent Michaelis constant in situ cannot be overcome by this extent of error in C_b. Although the estimation of δ may also be affected by the error in C_b, it is such a hybridized parameter that detailed discussion is too difficult. By using our two-dimensional laminar flow model, this critical point was practically overcome as the effect of the unstirred water layer. Moreover, a method for evaluating quantitatively the effects of the unstirred water layer on the kinetic parameters of the intestinal absorption in situ under the various conditions was offered. Therefore, the two-dimensional laminar flow model is valid not only for determining the kinetic parameters of a carrier-mediated transport in situ but also for predicting the absorption rate in situ from the uptake rate in vitro. However, to our regret, statistical errors are not given for the true Michaelis constants and the true maximal transport velocities because, at present, no non-linear regression computer program can be applied to our calculation method due to the enormous time required even for only a single calculation, which contains many iterations to obtain a steady-state value. Therefore, a further improvement in the calculation method will be needed.

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