Determination of Transport Rates for Arginine and Acetaminophen in Rabbit Intestinal Tissues *in Vitro*

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The *in vitro* Ussing technique was employed to examine transport rates for acetaminophen and arginine across rabbit intestinal tissues. Mannitol and transepithelial conductance were used to monitor the integrity of rabbit intestinal tissues and the basal and stimulated short-circuit current were measured to assess functional viability. Transepithelial transport of acetaminophen, arginine, and mannitol was determined in rabbit jejunum, ileum, and distal colon. Transepithelial transport of arginine in the ileum and jejunum was composed of both passive (nonsaturable) (P_m = 0.06) and saturable components (K_T = 0.6-0.7 m*M*; J_{max} = 0.3-0.4 μmol/hr · cm²). The saturable component of arginine fluxes was abolished by pretreatment of the tissue with serosal ouabain (0.1 m*M*). In the distal colon, both unidirectional arginine fluxes were nonsaturable. In the segments examined, both unidirectional fluxes of acetaminophen were nonsaturable over the concentration range from 0.1 to 30 m*M*. These results provide values for maximal permeabilities attained by molecules traversing both the cellular and the paracellular pathways and, by comparison to their *in vivo* bioavailabilities, provide selection criteria for evaluating drug candidates for oral activity.

KEY WORDS: acetaminophen; arginine; intestine; absorption; mannitol; diffusion; passive transport; carrier-mediated transport; active transport; permeability.

INTRODUCTION

The Ussing technique has been extensively employed for intestinal transport studies, demonstrating the ease and rapidity with which intestinal absorption and metabolism by the gut wall can be evaluated. These studies have elucidated site-specific absorption and effects of xenobiotics on "basal" physiological characteristics of the intestine (1–9).

Assessment of integrity and biological viability is important for *in vitro* studies that aim to define absorption rates and mechanisms. Functional viability of preparations in the Ussing chamber system has been commonly assessed by measurement of transepithelial potential difference (PD) or short-circuit current (I_{sc}) and the change in these parameters elicited by absorptive or secretory stimuli (10–12). The implicit assumption in using electrical characteristics to assess viability is that generation of the PD and thus I_{sc} results from cellular metabolism to produce adenosine triphosphate (ATP). This assumption has been examined with metabolic inhibitors such as 2,4-dinitrophenol (13,14) and/or the Na^+/K^+-ATPase inhibitor, ouabain (13,14). Intestinal integrity can be assessed by monitoring transepithelial conductance (G_t). However, in "leaky" epithelia with G_t values greater than 10 mS/cm², changes in junctional permeability may not be reflected in significant changes in G_t. Therefore, in the present study, mannitol was also employed as a marker for passive permeability and thus as a measure of intestinal integrity (2,15,16).

The emphasis of this study was to distinguish between different intestinal transport mechanisms and to compare rates of transport of actively and passively transported molecules *in vitro*. To do this, transport of the well-absorbed, actively transported amino acid, arginine, was compared to the transport of acetaminophen and mannitol in different intestinal segments from rabbit. These results allow calculation of the permeability for molecules traversing the transcellular and/or paracellular pathways at different sites in the intestine and provide selection criteria for selecting orally active drug candidates.

MATERIALS AND METHODS

Materials. Acetaminophen (4-acetamidophenol), arginine, mannitol, ouabain (G-strophantin), and prostaglandin E_1 (PGE_1) were obtained from Sigma Chemical Co. (St. Louis, MO); 1-octanol was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI); all other chemicals were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Radiolabeled compounds were purchased from New England Nuclear (Boston, MA). Liquid Carbonic (Chicago, IL) was the supplier for 95% O_2–5% CO_2.

Tissue Preparation. Distal colon, ileum, and jejunum obtained from New Zealand White rabbits (2–4 kg) which had been fed a standard chow and water *ad libitum* were opened along the mesenteric border and rinsed with an ice-cold bicarbonate-Ringer solution containing (m*M*): 141 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 122 Cl^-, 25 HCO_3^-, 1.6 HPO_4^{2-}, and 0.4 $H_2PO_4^-$. At 37°C this solution has a pH of 7.4 when gassed with 95% O_2–5% CO_2. All tissues were kept at ice-bath temperature and gassed with 95% O_2–5% CO_2 prior to use. Mucosa was stripped of underlying muscle (17–19), mounted in Ussing chambers (3-cm² exposed surface area), and bathed on both tissue surfaces with 12.5 mL of bicarbonate-Ringer solution containing 8 m*M* glucose and 2 m*M* mannitol in the serosal bath and 10 m*M* mannitol in the mucosal bath. Solutions were circulated by gas lift with 95% O_2–5% CO_2 and maintained at 37°C by water-jacketed reservoirs. Tissues were equilibrated for 30–45 min while monitoring PD and I_{sc}. The G_t was calculated from the ratio of I_{sc} to open-circuit PD. In tissues with low values of I_{sc} (<50 μA/cm²), a brief pulse (<2 sec) of direct current was passed across the tissue and the resulting PD change was used to calculate G_t.

Transepithelial Transport Studies. After the 45-min equilibration period, acetaminophen (1 m*M*) or arginine (5 m*M*) was added to the mucosal and serosal bathing solutions; isotope (5 μCi ^{14}C-labeled mannitol and 1–2 μCi ^{3}H-labeled acetaminophen or arginine) was added to the mu-

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cosal [for mucosal (m)-to-serosal (s) fluxes] or the serosal (for s-to-m fluxes) bathing solution, respectively. Samples (1 mL) were taken from mucosal (for s-to-m fluxes) or serosal (for m-to-s fluxes) bathing solutions at 15-min intervals for up to 180 min. To maintain a constant volume, 1 mL of bicarbonate-Ringer solution containing all ingredients of the original bathing solution was added after each sample. Effect of dilution was taken into account for the calculation of fluxes. Samples (100 μL) were taken from the initially labeled bathing solutions before and after time course experiments. Both ^{14}C and ^{3}H were analyzed on a Packard Tri-Carb liquid scintillation spectrometer (Model 4640). Counts per minute were converted to disintegrations per minute using the external standard channel's ratio.

Concentration dependence of unidirectional fluxes of acetaminophen and arginine was determined in six tissues incubated with different concentrations of acetaminophen (0.1, 0.3, 1, 3, 10, and 30 m*M*) and arginine (0.1, 0.3, 1, 3, 5, and 10 m*M*). After an equilibration period of 45 min, nonlabeled and labeled compounds were added. Steady-state fluxes were calculated from two 1-mL samples taken at 30 and 60 min after the addition of isotope. Immediately after measurement of these fluxes 0.1 m*M* ouabain was added to the serosal bathing solution. After a 30- to 45-min reequilibration period, a second 30-min flux was determined.

Data Analysis. Statistical analysis was performed using SYSTAT (SYSTAT Inc., Evanston, IL, 1989). Results are presented as means ± SD. Lines and slopes were calculated using multiple linear regression. Statistical significance between calculated lines was estimated by "indicator" or "dummy variable" regression analysis using the multivariate general linear hypothesis module in SYSTAT. A value of $P < 0.05$ was considered statistically significant.

Carrier-mediated transport parameters of arginine were calculated by fitting the obtained data to the general expression for active transport of solutes:

$$J = \frac{J_{max} * C}{K_T + C} + P_m * C \qquad (1)$$

where J represents the total arginine flux, J_{max} the maximum carrier flux, K_T the concentration required for half-maximal transport, P_m the passive membrane permeability, and C the arginine concentration. Transport parameters were estimated by nonlinear regression analysis using the NONLIN module in SYSTAT.

RESULTS

Viability of Intestinal Preparations. At the end of experiments (~255–260 min) under control conditions, 10 m*M* glucose was added to the mucosal bathing solution of ileum and jejunum or 10 μ*M* PGE_1 was added to the serosal bathing solution of the distal colon. Addition of glucose or PGE_1 elicited a rapid increase in I_{sc} which has been previously shown to be due to active Na^+ absorption or Cl^- secretion, respectively (10,11), providing a qualitative assessment of tissue viability, since ATP is required for these active transport processes.

Time Courses of Acetaminophen and Arginine Transport. Cumulative time courses for unidirectional fluxes of acetaminophen and arginine are depicted in Figs. 1 and 2. The m-to-s fluxes of acetaminophen are equivalent in ileum and distal colon (Fig. 1). In addition, m-to-s and s-to-m fluxes of acetaminophen in distal colon are equivalent (1.0 and 1.2%/hr · cm^2, respectively). In ileum, however, there is a small but significant difference between the unidirectional fluxes of acetaminophen (m-to-s, 1.2%/hr · cm^2; s-to-m, 0.8%/hr · cm^2). The explanation for these differences in unidirectional fluxes of acetaminophen cannot be determined from the present studies. However, as will be seen from the concentration dependence of acetaminophen transport, there does not appear to be a carrier-mediated component.

Arginine fluxes in jejunum, ileum, and distal colon are presented in Fig. 2. In jejunum and ileum, the m-to-s fluxes are significantly greater than the corresponding s-to-m fluxes, while in the distal colon, both the m-to-s and the s-to-m fluxes are identical. Ileal m-to-s transport is 1.1%/hr · cm^2, whereas the s-to-m flux is 0.4%/hr · cm^2. The m-to-s flux in jejunum is 0.9%/hr · cm^2, while the s-to-m flux is 0.2%/hr · cm^2. For the distal colon the m-to-s and s-to-m fluxes are identical (0.2%/hr · cm^2). In separate studies, the effect on I_{sc} of sequential additions of arginine (5 m*M*), leucine (5 m*M*), and glucose (8 m*M*) to the luminal bathing solution of ileum was examined. The maximal changes in I_{sc} occurring within 10 min after the addition of arginine, leucine, and glucose were 2.0 ± 0.2, 3.3 ± 0.3 and 3.9 ± 0.3 μEq/hr · cm^2, respectively (data not shown). These results are consistent with stimulation of Na^+-coupled cotransport by arginine as has been reported for neutral amino acids (e.g., leucine) and for glucose (9).

Transport of mannitol in the three intestinal segments was measured concomitantly with the fluxes of arginine or acetaminophen. No difference in m-to-s or s-to-m fluxes were seen in any of the intestinal segments examined. Mannitol transport rates in distal colon, ileum, and jejunum were 0.03, 0.06, and 0.09%/hr · cm^2, respectively (16).

Concentration Dependence for Acetaminophen or Arginine Transport. From Figs. 1 and 2 it can be seen that

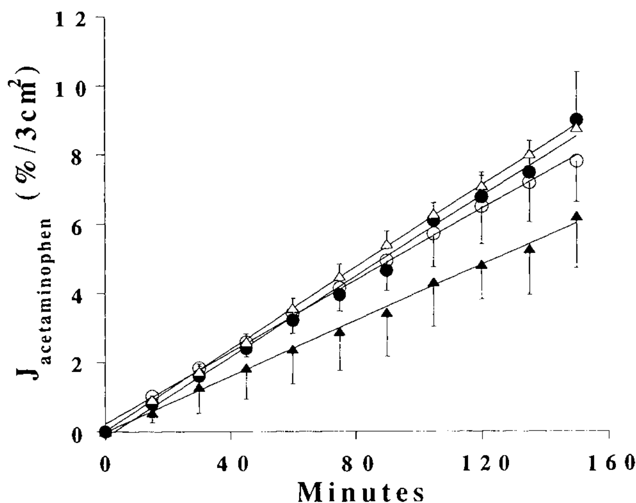

Fig. 1. Time course for [^{3}H]acetaminophen transport in rabbit ileum (m-to-s, open triangles, n = 3; s-to-m, filled triangles, n = 4) and distal colon (m-to-s, open squares, n = 4; s-to-m, filled squares, n = 2).

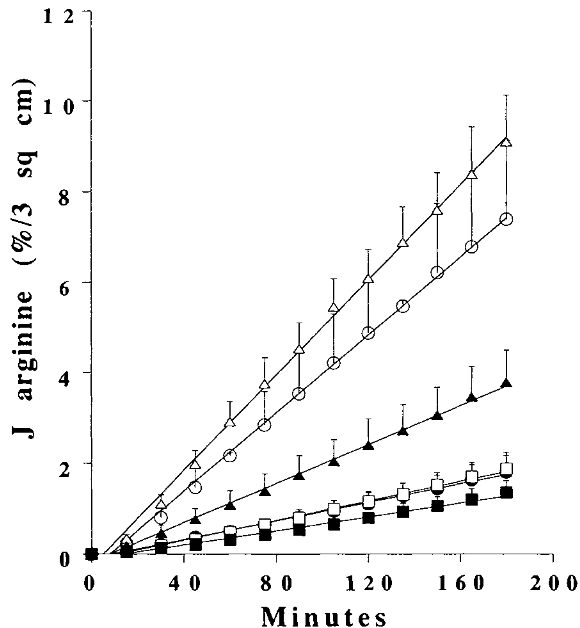

Fig. 2. Time course for [^{3}H]arginine transport in rabbit jejunum (m-to-s, open circles, n = 4; s-to-m, filled circles, n = 3), ileum (m-to-s, open triangles, n = 4; s-to-m, filled triangles, n = 4), and distal colon (m-to-s, open squares, n = 5; s-to-m, filled squares, n = 3).

steady-state fluxes were achieved within 30 min. Acetaminophen fluxes in both ileum and distal colon are linear functions of acetaminophen concentration up to 30 m*M* (data not shown) and were not altered by pretreatment of tissues with serosal ouabain (0.1 m*M;* data not shown). Indicator regression analysis showed no difference between ileal and colonic acetaminophen transport rates, in the presence or absence of ouabain.

Unidirectional fluxes of [^{3}H]arginine as a function of arginine concentration are presented in Figs. 3–5. From Figs. 3–5 it is evident that the m-to-s fluxes of arginine in the jejunum and ileum are composed of saturable and linear components, while the m-to-s flux in distal colon and the s-to-m fluxes in all segments are linear functions of concentration. Addition of ouabain (0.1 m*M*) to the serosal bathing solution of jejunum and ileum resulted in elimination of the saturable components of the m-to-s arginine fluxes without altering the s-to-m fluxes. In distal colon, addition of ouabain (0.1 m*M*) to the serosal bathing solution had no effect on either unidirectional flux. In all tissue segments, ouabain abolished I_{sc} (indicating that active Na^+ transport was eliminated), consistent with inhibition of Na^+/K^+-ATPase activity (data not shown; 13,14).

Data from m-to-s arginine studies in jejunum and ileum were fit to the expression for carrier-mediated transport processes [Eq. (1)]. From the obtained transport parameters, theoretical curves were calculated for the carrier-mediated transport component (K_T) and the passive component. In

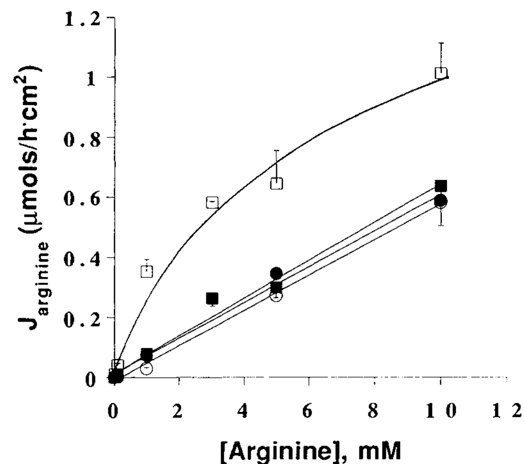

Fig. 4. Concentration dependence of [^{3}H]arginine fluxes in rabbit ileum (n = 2–4) in the absence or presence of serosal ouabain (0.1 m*M*). Fluxes are depicted by open squares (m-to-s) and open circles (s-to-m) in control tissues and filled squares (m-to-s) and filled circles (s-to-m) in the presence of ouabain.

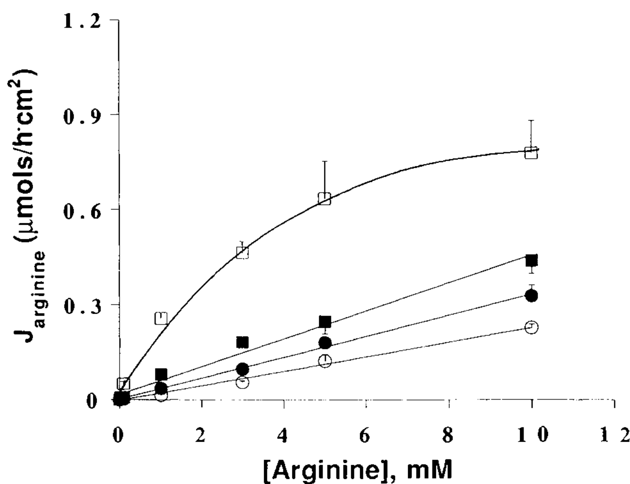

Fig. 3. Concentration dependence of [^{3}H]arginine fluxes in rabbit jejunum (n = 3–4) in the absence or presence of serosal ouabain (0.1 m*M*). Fluxes are depicted by open squares (m-to-s) and open circles (s-to-m) in control tissues and filled squares (m-to-s) and filled circles (s-to-m) in the presence of ouabain.

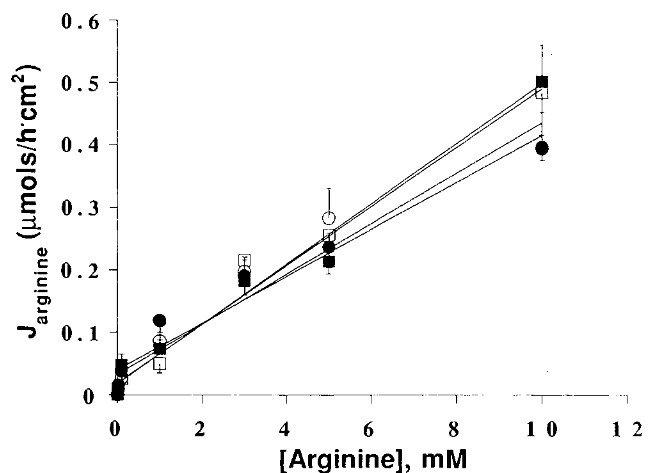

Fig. 5. Concentration dependence of [^{3}H]arginine fluxes in rabbit distal colon (n = 3–4) in the absence or presence of serosal ouabain (0.1 m*M*). Fluxes are depicted by open squares (m-to-s) and open circles (s-to-m) in control tissues and filled squares (m-to-s) and filled circles (s-to-m) in the presence of ouabain.

jejunum, the maximum transport rate is 0.3 μmol/hr · cm² and the concentration for half-maximal transport is 0.7 m*M*, while these values in ileum are 0.4 μmol/hr · cm² and 0.6 m*M*, respectively. In both ileum and jejunum, the passive membrane permeability is calculated to be 0.06 cm/hr. This value is in good agreement with the values of 0.04 and 0.06 cm/hr obtained from the s-to-m fluxes in jejunum or ileum, respectively, and the slopes of the m-to-s fluxes in jejunum and ileum in the presence of ouabain of 0.04 and 0.06 cm/hr, respectively.

In the distal colon, both unidirectional fluxes of arginine are linear functions of arginine concentration over the range of 1 to 10 m*M*. The slopes and intercept of the m-to-s flux are 0.05 cm/hr and 0.02 nmol/hr · cm², respectively, and for the s-to-m flux are 0.03 cm/hr and 0.04 nmol/hr · cm², respectively.

DISCUSSION

In this study, transport rates for arginine and acetaminophen have been compared. These molecules were selected since it is known from the literature that arginine will be efficiently absorbed *in vivo* by a carrier-mediated transport process (20) and that acetaminophen, a relatively lipophilic molecule, is also efficiently absorbed by an apparently passive mechanism (21). Transport rates for these well-absorbed molecules were further compared to the transport rates for mannitol, which is reported to be transported via the paracellular pathway (2). Transport rates for mannitol were also used as a measure of tissue integrity as described previously (16). Results from these studies reveal that the well-absorbed molecules acetaminophen and arginine have transport rates of 1.2 and 1.1%/hr · cm², respectively, in the rabbit small intestine compared to a rate of <0.1%/hr · cm² for mannitol. These values therefore represent upper limits for the flux rate which can be determined in *in vitro* studies in rabbit small intestine. By comparison to published reports for bioavailability of these molecules *in vivo*, these values correlate with 8–40% for mannitol and >90% for arginine and acetaminophen (20–22). In rabbit distal colon, the upper limit of transport is defined by the transport of acetaminophen (1.2%/hr · cm²), which is approximately 40-fold greater than the transport rate for mannitol (0.03%/hr · cm²). Although a variety of factors ultimately contributes to the oral activity of a molecule, in many instances, lack of intestinal permeability is the rate-limiting factor (23). Thus, in studies designed to evaluate the potential oral activity of a molecule, measurement of intestinal permeability and comparison to values obtained for arginine, acetaminophen, and mannitol will provide an initial selection criteria for evaluation *in vivo* and the segmental absorption studies provide information relevant to development of modified release dosage forms.

These studies also provide information regarding the mechanisms involved in transport. Thus, for arginine, as has been reported previously, absorption occurs via a carrier-mediated, sodium-dependent (hence the ouabain sensitivity) mechanism present in the small intestine only. In rabbit distal colon, arginine transport appears to be entirely passive. Of interest is the observation that the passive permeability of arginine is greater than that of mannitol. This may be due to a cellular transport pathway not accessible to mannitol or to a selective junctional permeability which favors positively charged molecules. Further studies will be required to determine the mechanisms responsible for these differences in passive permeability.

For acetaminophen, transport appears to occur by an entirely passive process in both large and small intestine. Metabolism of acetaminophen by brush border peptidases does not appear to account for the transport rates observed in this study since fluxes are similar in both ileum and distal colon (lack of brush border enzymes). The higher passive permeability of the intestine to acetaminophen compared to mannitol may be related to its octanol/Ringer buffer partition coefficient ($\log K_{oct/Ringer} = 0.19 \pm 0.01$). Thus, acetaminophen may traverse the intestine via a predominantly cellular route.

Mannitol is a carbohydrate (MW 182.2) that shows low rates of uptake in most mammalian cells and little absorption through lipid membranes and is thought to be confined to the paracellular pathway for diffusion (2,15,22). In the present studies, tissue integrity was assessed with mannitol fluxes, as described previously (16). Tissues with mannitol transport rates exceeding 0.15, 0.14, and 0.06%/hr · cm² in jejunum, ileum, and distal colon, respectively, were excluded. No significant changes in mannitol permeability were observed in experiments with acetaminophen or arginine or in the presence of the Na^+/K^+-ATPase inhibitor, ouabain.

Transport of arginine in rabbit ileum and jejunum is an active Na^+-dependent carrier-mediated process. Previous studies in rabbit ileum with the Ussing technique have provided information about the transport systems responsible for basic amino acid transport and it has been reported that arginine is a potent inhibitor of lysine uptake through the Ly_2 system (24–26).

These studies demonstrate the utility of evaluating transport *in vitro* with the Ussing technique for predicting regional differences in absorption, potential for oral activity, and mechanisms involved in transepithelial transport.

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