METHOTREXATE TRANSPORT IN THE HUMAN INTESTINE

EVIDENCE FOR HETEROGENEITY*

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Abstract—The transport of methotrexate (MTX) was investigated in organ-cultured endoscopic biopsy specimens of intestinal mucosa from normal subjects. In biopsy specimens from the proximal small intestine incubated with [^{3}H]MTX (0.1 μM) for 2 hr at pH 5.5, [^{3}H]MTX accumulated in the intracellular fluid to a concentration 3.5-fold higher than that of the medium, but at pH 6.5 and 7.5, the concentration was the same as that of the medium. In biopsy specimens from the cecum incubated under similar conditions, no accumulation against a concentration gradient was found. However, the accumulation of MTX was significantly higher at pH 5.5 than at 7.5. At [MTX] = 0.1 μM, the initial rate of MTX transport in the small intestine was significantly affected by medium pH and was optimal at pH 5.5. The relationship between the initial rate of uptake and medium [H$^+$] was hyperbolic, suggestive of saturability with respect to [H$^+$] with a K_m of 132.2 nm [H$^+$], corresponding to a medium pH of 6.88. At medium [MTX] = 10 μM, this effect was abolished. At pH 5.5, the relationship between the initial rate of uptake and medium [MTX] was sigmoidal, suggestive of a positive cooperativity, with n_{app} of 1.8. [MTX] at 0.5 V_{max} was 20.37 μM. Folic acid inhibited 37% of MTX flux. At pH 7.5, the relationship between the initial rate of uptake of MTX and medium [MTX] was linear. These data indicate the presence of a proton-dependent active transport of MTX in the human proximal small intestine, which is partially shared with folic acid.

Methotrexate (MTX†, amethopterin), a synthetic analog of folic acid, is a potent inhibitor of dihydrofolate reductase, the enzyme that reduces dihydrofolic acid to the metabolically active form, tetrahydrofolate [1]. This drug, initially employed in the treatment of choriocarcinoma and leukemia [1, 2] has been introduced as a therapeutic agent in a wide range of non-malignant diseases such as psoriasis [2], rheumatoid arthritis [3], bronchial asthma [4], primary biliary cirrhosis [5] and inflammatory bowel diseases [6]. Because of the widespread use of this agent in cancer chemotherapy, its transport has been subjected to extensive studies [7–21]. Distinct membrane carriers that mediate influx and efflux of this drug have been described in various cell systems [7]. Altered transport of MTX across the membrane of leukemic cells may result in resistance to this drug, a common problem in cancer chemotherapy [8]. MTX has been widely used as a substrate in studies of intestinal transport of folates. Competitive inhibition of the uptake of folic acid [9–12, 22] and 5-methyl tetrahydrofolate [23] by MTX has been demonstrated in various preparations of intestinal tissue. These observations have suggested that all folates share a common carrier in the intestinal luminal membrane [9, 10]. However, in isolated lymphocytes [13], reticulocytes [14], erythrocytes [15], hepatocytes [16] or leukemic cells [17–21], marked differences in the affinity of transport for various folate substrates were found. These differences may indicate the presence of more than a single carrier for folate in these cells. No study of the transport of MTX in the human intestine has ever been published and, therefore, the information concerning the absorption of this drug in the human intestine is limited. We have recently demonstrated the presence of several carriers for folic acid in the mucosa of the human intestine: active transport across the duodenojejunal mucosa is mediated via two proton-dependent carriers while facilitated diffusion through a low-affinity carrier accounts for uptake of folic acid in the mucosa of the colon and also in the proximal small intestine at neutral or alkaline pH conditions [12]. MTX inhibited competitively both the active transport of folic acid as well as its facilitated diffusion. The purpose of the present study was to further characterize the mechanisms of transport of folate in the human intestine, using MTX as a substrate.

† Abbreviations: MTX, methotrexate; ICF, intracellular fluid; ECF, extracellular fluid; v, initial rate of transport.

MATERIALS AND METHODS

The protocol of this study has been reviewed and approved by the Human Research Review Committee of the Hadassah University Hospital. Mucosal biopsy specimens were procured, after informed consent, from patients undergoing routine upper gastrointestinal endoscopy or colonoscopy for

diagnostic purposes. None of the patients had diarrhea or any clinical evidence of malabsorption. Indications for upper gastrointestinal endoscopy included evaluation of upper gastrointestinal symptoms or follow up of previously diagnosed peptic disease. Colonoscopy was indicated either for the evaluation of gastrointestinal bleeding, overt or occult, or for follow-up after previous polypectomy. In all patients, the mucosa was found to be normal.

Upper gastrointestinal endoscopy was performed after an overnight fast and sedation with diazepam, 5 mg i.v. Endoscopic biopsy specimens were obtained as distally as possible, usually about 10 cm distal to the papilla. Colonoscopy was preceded by bowel preparation using lavage with isotonic solution as described by Davis *et al.* [24]. Premedications were pethidine hydrochloride (50 mg) plus diazepam (5 mg), i.v. Colonoscopic biopsy specimens were obtained from the cecum, transverse and sigmoid colon. From each subject, 4–6 biopsy specimens were procured, and these biopsies were used for a single experiment.

After excision, biopsy specimens were kept in normal saline and within 5 min were gently mounted, mucosal surface up, on a stainless steel screen which was placed in the central well of a plastic culture dish (Falcon, Cockeysville, MD, U.S.A.). Culture medium (0.8 mL) was then added and the tissue was cultured at 37° in atmosphere of 5% CO_2, 95% room air. The culture medium was RPMI 1640 (Bio Lab, Israel), supplemented with glutamine and 10% fetal calf serum. The medium did not contain folate polyglutamates. The pH was adjusted with phosphate buffer, 0.15 M. The final medium osmolarity was 280 mOsm/kg. After 20 min, the medium was removed and the tissue was incubated in the same medium containing [³H]MTX (~1.0 µCi/nmol, 0.1 µmol/L) and inulin [¹⁴C]carboxylic acid (~0.05 µCi/mL) as a marker of the extracellular volume [25]. After exposure to the radioactive medium, the tissue was blotted, weighed and homogenized in 1.0 mL of water. Aliquots of the tissue homogenate were dissolved in scintillation fluid and counted for 5 min in a liquid scintillation spectrometer programmed to count ³H and ¹⁴C radioactivities simultaneously. The total water content of the tissue was determined for the various experimental conditions as the difference between the wet weight of the tissue and its dry weight (= weight after drying at 100° for 20 hr). The intracellular fluid (ICF) volume was calculated as the difference between total tissue water and extracellular fluid (ECF) volume (=inulin space) [25]. Uptake of MTX into the ICF compartment was calculated from the ³H counts in the tissue homogenate, the [¹⁴C]inulin space and the tissue weight. Results were expressed either in pmol/µl ICF or as the ratio of the ³H counts in the ICF to those in the medium.

The nature of ³H counts in the tissue homogenates was evaluated by paper chromatography. Biopsy specimens were organ-cultured for 2 hr in a medium containing [³H]MTX (0.1 µM) at pH 5.5 or 7.5. The tissue was then washed three times in NaCl (0.154 M) and homogenized in 0.5% sodium carbonate. Aliquots (10 µL) of the tissue homogenates were subjected to paper chromatography using 0.5%
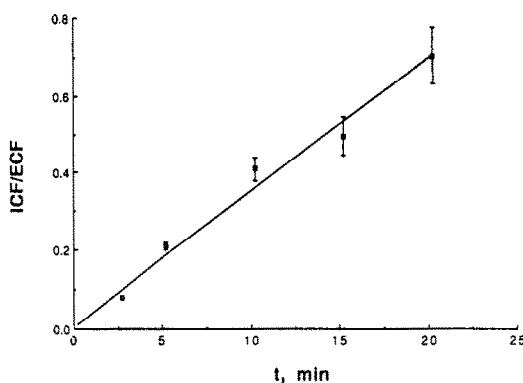

Fig. 1. Accumulation of [³H]MTX in human small bowel biopsy specimens as a function of time. Biopsy specimens were preincubated at pH 5.5 for 20 min and were subsequently exposed to a medium of the same pH containing [³H]MTX (0.1 µmol/L) and inulin[¹⁴C]-carboxylic acid for the indicated time periods. The data are mean ratios of intracellular to extracellular ³H counts of 4 experiments. Error bars represent SEM. Where absent, SEM merge into the data points. For 20 min, the uptake of [³H]MTX is linear with time and fits the relation $y = 0.0349x$ ($r^2 = 0.97$).

sodium carbonate as a solvent. After 14 hr of chromatography, the areas containing MTX were identified and extracted. The percentage of ³H counts associated with MTX was determined as the ratio of ³H counts in the extract to the ³H counts in an aliquot of the tissue homogenate.

In preliminary experiments, the time course of [³H]MTX uptake in the tissue was studied. In biopsy specimens from the proximal small intestine and from the cecum, uptake of MTX was linear with time during at least 20 min of exposure, both at pH 5.5 as well as at 7.5. Data for the proximal small intestine at pH 5.5 are shown in Fig. 1. Therefore, in subsequent experiments, a 10-min exposure to [³H]MTX was employed for determination of initial rate of transport.

Statistical analysis. Each of the experiments was implemented on biopsy specimens from at least four different subjects. Data reported are means ± SEM. In the setting of more than two experimental conditions, the results were first evaluated by one-way analysis of variance. If the *F* ratio indicated that the results of the various experimental conditions differed significantly, the Scheffé's multiple contrasts procedure was employed for the pairwise comparison of the various experimental conditions. In the case of two experimental conditions, *t*-tests were performed. Linear regression analysis was performed using the least squares method. Significance of the regression was evaluated by the *t*-statistic. All statistical tests were two-tailed with alpha of less than 5%.

Kinetic rate constants and their standard errors were derived by fitting the Michaelis–Menten or the Hill equations to the experimental data. This was performed using the SPSSX-NLR statistical package [26] implemented on the Vax computer (Digital

Table 1. Percentage of ^{3}H counts associated with methotrexate in homogenates of intestinal mucosa

Region	pH	N	Counts associated with methotrexate (%)*
Proximal small intestine	5.5	9	92.9 ± 6.9
	7.5	6	98.8 ± 13.7
Cecum	5.5	7	100.4 ± 10.2
	7.5	6	88.5 ± 18.3

* The percentage of ^{3}H counts in tissue homogenates associated with methotrexate was determined by paper chromatography, as described in Materials and Methods. Values are means ± SEM.

Equipment Corporation, Marlboro, MA, U.S.A.) of the Hebrew University-Hadassah Medical School. The goodness of fit of these models to the data was assessed by the F statistic. This test uses the residual sum of squares as the total variation. The smaller the F, the better the fit [27].

Materials. [^{3}H]MTX (20 Ci/mM) and inulin[^{14}C]-carboxylic acid (9.0 mCi/mmol) were purchased from Amersham Radiochemicals (Amersham Laboratories, Buckinghamshire, U.K.). Prior to use, the radioactive MTX was purified by chromatography on Whatman No. 1 chromatography paper (Whatman International Ltd, Maidstone, U.K.), using 0.5% sodium carbonate as a solvent. The purity of inulin[^{14}C]carboxylic acid was 99% by paper chromatography. All other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and were of the highest grade available.

RESULTS

The water content of the organ-cultured tissue (mean ± SE) was 83.0 ± 0.6, 82.9 ± 0.6, 83.0 ± 0.9 and 83.4 ± 0.7% of wet tissue weight for duodenum, cecum, transverse and sigmoid colon and did not change appreciably during the incubation periods employed in these studies. The [^{14}C]inulin space was 14.6 ± 0.8% of wet tissue weight after incubation for 20 min and increased to 35.9 ± 3.8% after 2 hr. The [^{14}C]inulin space was unaffected by medium pH.

After 2 hr of organ culture of biopsy specimens from the proximal small intestine and from the cecum, most of the ^{3}H counts in the tissue were associated with MTX (Table 1). The effect of medium pH on the accumulation of [^{3}H]MTX in the mucosa of the proximal small intestine and of the cecum is illustrated in Fig. 2. The accumulation of MTX in the tissue is expressed as the ratio of ^{3}H counts in the ICF to those in the medium (ICF/ECF ratio) after incubation for 2 hr. A ratio greater than 1.0 denotes accumulation against a concentration gradient, suggestive of active transport. It is evident that accumulation of [^{3}H]MTX is significantly affected by medium pH both in the proximal small intestine ($F = 39.12$; P = 0.0001) as well as in the

cecum ($t = 2.14$; P = 0.04). In the small intestine at pH 5.5, MTX accumulated in the ICF to a concentration approximately 3.5-fold higher than that of the medium. However, at pH 6.5 and 7.5, the ratio of concentrations of MTX in the ICF to the medium was not significantly different from 1.0. The differences in the ratio of ^{3}H counts in the ICF to the medium between pH 5.5 and 6.5 and between 5.5 and 7.5 were significant. By contrast to the small intestine, MTX did not accumulate against a concentration gradient in the cecum. To gain further insight into the mechanism of the effect of pH on transport of MTX, kinetic experiments were performed. Figure 3 shows the effect of medium pH on the initial rate of [^{3}H]MTX uptake into the mucosa of the proximal small intestine at a concentration of 0.1 μM. The pH conditions in these experiments are within the physiologic range for the human proximal small intestine in the postprandial state [28–30]. The initial rate of MTX uptake (v) in the small intestine was significantly affected by medium pH (P = 0.011) and was optimal at pH 5.5. In the cecum, no significant effect of pH on the initial rate of transport was noted (data not shown). In Fig. 4, the uptake values from Fig. 3 were plotted as a function of medium H$^+$ concentrations. The relationship between v and [H$^+$] was hyperbolic. A Michaelis–Menten equation of the form:

$$v = V^*_{max}[H^+]/(K_m + [H^+])$$

was fitted to the data. In this equation, V_{max} = maximal rate of MTX transport, and K_m = [H$^+$] at $0.5 \, V_{max}$. The fit of this model to the data was good, as indicated by $F = 0.11$. The kinetic parameters obtained were: $V_{max} = 0.0392 ± 0.0040$ pmol/μL ICF/10 min, and $K_m = 132.2$ nM [H$^+$], corresponding to a pH of 6.88. At a concentration of 10 μM in the medium, the effect of pH on the initial rate of transport of MTX was abolished (data not shown). The effect of medium concentrations of MTX on v was investigated using MTX concentrations ranging from 0.1 to 60 μM. The results of these studies are presented in Fig. 5. At pH 5.5, the relationship between v and [MTX] was sigmoidal (Fig. 5A). The Hill equation:

$$v = (V^*_{max}[MTX]^n)/(K' + [MTX]^n)$$

was fitted to the data. In this equation, n is the number of substrate binding sites per carrier, and K' is a constant comprised of various interaction factors and the intrinsic dissociation constant [31]. The F test of goodness of fit of this model to the data yielded a value of 0.21, indicating a good fit [27]. The kinetic parameters obtained were as follows: $V_{max} = 14.23 ± 2.32$ pmol/μL ICF/10 min; $n = 1.80 ± 0.50$ and $K' = 223.6$. From these data, the concentration of MTX at $0.5 \, V_{max}$ was calculated to be 20.32 μM, and the cooperativity index, the ratio between [MTX] at $0.9 \, V_{max}$ and $0.1 \, V_{max}$ was 11.57. When this experiment was repeated in the presence of folic acid (60 μM), the concentration of MTX at $0.5 \, V_{max}$ was not appreciably changed at 21.4 μM (data not shown). At pH 7.5, the relationship between v and [MTX] was linear and conformed to the equation $v = 0.206*$ [MTX] ($r^2 = 0.88$; Fig. 5B).
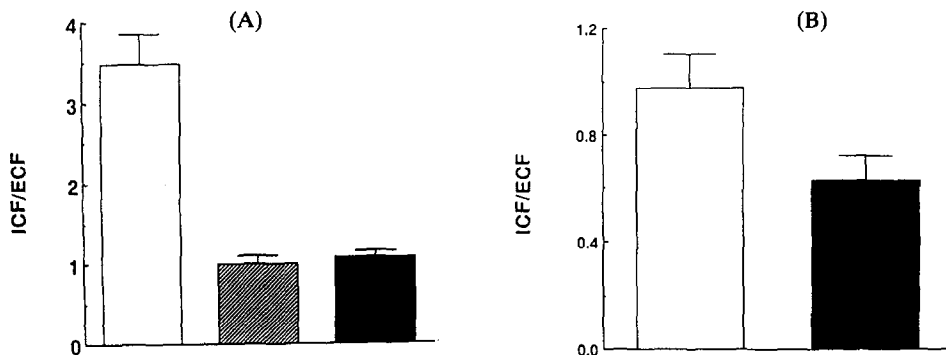
Fig. 2. Effect of pH on accumulation of [³H]MTX in organ cultured mucosa of human small intestine (A) and cecum (B). Biopsy specimens were organ-cultured for 2 hr at the indicated pH conditions. The medium contained [³H]MTX (0.1 μmol/L, and inulin[¹⁴C]carboxylic acid. The accumulation of [³H]-MTX in the tissue is expressed as the mean ratios of ³H counts in the intracellular fluid to the counts in the medium. The error bars indicate SEM. Open bars: pH 5.5; cross hatched bars: pH 6.5; solid bars: pH 7.5. For the small intestine: N = 10; $F = 39.12$; $P = 0.0001$. The differences between pH 5.5 and 6.5 and between 5.5 and 7.5 were significant ($P < 0.05$). For the cecum, N = 11; $P = 0.04$.
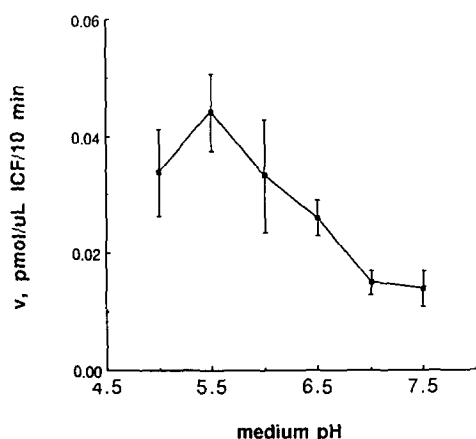

Fig. 3. Effect of medium pH on the initial rate of transport of [³H]MTX in the organ-cultured mucosa of human small intestine. Biopsy specimens were organ cultured at the indicated pH conditions. After 20 min, the preincubation medium was removed and the tissue was cultured for 10 min in a medium of the same pH, containing [³H]MTX (0.1 μmol/L) and inulin[¹⁴C]carboxylic acid. Data are means of 6 experiments. Error bars indicate SEM. The effect of pH was significant ($F = 3.63$; $P = 0.01$).
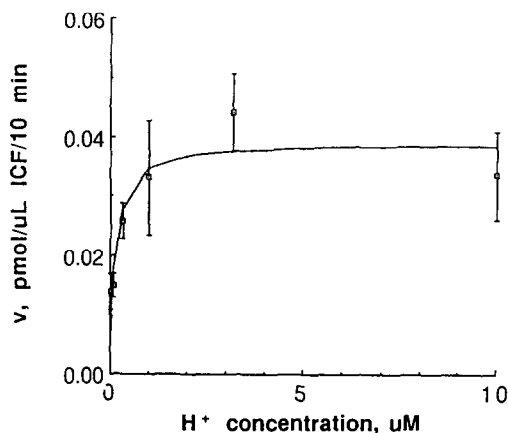
Fig. 4. Effect of medium H⁺ concentration on transport of MTX into the mucosa of the small intestine. Uptake rates from Fig. 3 were plotted as a function of medium [H⁺]. The line is a least-squares fit of the Michaelis–Menten equation to the data.

Figure 6 shows the effect of folic acid on the initial rate of transport of MTX in the duodenojejunal mucosa at pH 5.5. In the presence of folic acid at increasing concentrations, the initial rate of uptake of MTX decreased exponentially from 0.041 ± 0.003 pmol/μL ICF/10 min in the absence of folic acid to a plateau of 0.026 ± 0.005. Thus, folic acid inhibited only 36.6% of MTX flux.

DISCUSSION

Our previous studies of transport of folic acid in

biopsy specimens of the mucosa of the human intestine using the same experimental methodology have revealed that transport of folic acid was highly pH-dependent [12]. In biopsy specimens organ-cultured for 2 hr in the presence of [³H]folic acid, the vitamin accumulated in the ICF to concentrations more than two-fold higher than in the medium at pH 5.5 and 6.5, but not at 7.5 [12]. For MTX, a similar trend was noted. However, the spectrum of this pH sensitivity was different since accumulation of MTX against a concentration gradient was demonstrable only at pH 5.5. The accumulation of MTX against a concentration gradient may have reflected its binding to intracellular dihydrofolate reductase rather than its active transport. However, active transport is a more plausible explanation, because if the pH effect on accumulation of MTX
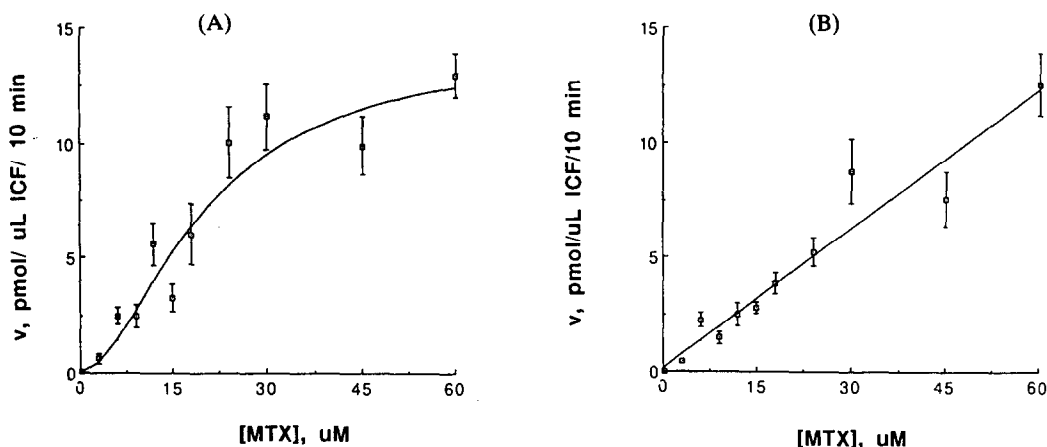
Fig. 5. MTX transport into organ-cultured mucosa of human small intestine as a function of medium MTX concentration. (A) At pH 5.5. (B) At pH 7.5. Biopsy specimens were organ cultured for 20 min at pH 5.5 or 7.5 and were subsequently exposed for 10 min to a medium of the same pH containing [^{3}H]MTX at various concentrations and inulin[^{14}C]carboxylic acid. The data are means of 6 experiments. Error bars indicate SEM; where absent, the error bars merge into the data points. The lines are least-squares fits of the Hill equation (A) or of a linear model (B) to the data.
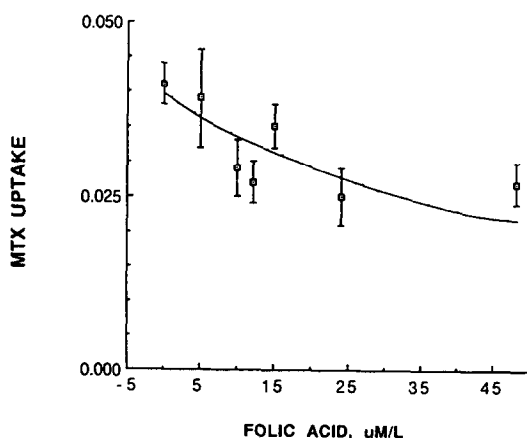

Fig. 6. Effect of folic acid on the initial rate of uptake of MTX into the mucosa of the organ-cultured human small intestine at pH 5.5. Biopsy specimens were organ-cultured at pH 5.5. After 20 min, the medium was removed and the tissue was cultured for 10 min in a medium of the same pH, containing [^{3}H]MTX (0.1 μmol/L), inulin[^{14}C]-carboxylic acid and folic acid at various concentrations. Each data point represents a mean of 6 experiments, with the exception of that with a folic acid concentration of 0 μmol/L, where N = 12. Error bars indicate SEM. The line is a least-squares fit of an exponential equation of the first order to the data.

in the tissue was due exclusively to preferential binding of MTX to the enzyme at acidic pH conditions, it would be anticipated that the ratio of ^{3}H counts in the intracellular fluid to the medium would be > 1.0 not only in the mucosa of the small intestine, but in the cecum as well. When the mechanism of the effect of pH on the transport of

MTX was explored, another difference between MTX and folic acid emerged: whereas the effect of medium pH on the initial rate of uptake of folic acid was maintained even at pharmacological concentrations of 100 μM, that effect was evident for MTX only at a low concentration of 0.1 μM and was abolished at 10 μM. A kinetic analysis of initial rates of uptake of folic acid versus medium H$^+$ concentrations revealed two proton-dependent carriers for folic acid while for MTX, only one carrier was demonstrable. Yet another difference between the transport of MTX and folic acid was evident when the kinetics of transport of these folates were investigated by determining the initial rates of uptake of these folates as a function of their respective concentrations in the medium. In the case of folic acid, a classical pattern of Michaelis–Menten kinetics was evident at pH 5.5 as well as at 7.5. The lower initial rates of uptake of folic acid at pH 7.5 with respect to 5.5 were due to reduced affinity of transport at pH 7.5, manifested by a two-fold higher K_m with no appreciable change in V_{max}. These results were consonant with findings in the rat intestine, using the influx chamber technique [32]. By contrast, uptake of MTX into the organ-cultured mucosa of the human small intestine exhibited a kinetic pattern distinct from that of folic acid. At pH 5.5, the relationship between the initial rates of transport of MTX and concentrations of MTX in the medium was not hyperbolic as in the case of folic acid. Rather, this relationship was sigmoidal and therefore suggestive of an allosteric effect. Indeed, fitting of the Hill's model to the data yielded a Hill's coefficient significantly greater than one, indicating a positive cooperativity effect in uptake of MTX. In contrast to the sigmoidal relationship between MTX transport and medium concentration of MTX observed at pH 5.5, at pH 7.5 this relationship was linear. This may imply either uptake of MTX by passive diffusion

or by facilitated diffusion through a carrier with a very low affinity. The most interesting finding in the present study was the pattern of inhibition of the initial rate of transport of MTX by folic acid. In a previous study, we have shown that MTX competitively inhibited the transport of folic acid both in the small intestine as well as in the cecum, and the inhibition constant was pH-dependent in the former but not in the latter [12]. Competitive inhibition of transport of folic acid and 5-methyltetrahydrofolate has been previously described in various preparations of rat intestine [9, 10, 12, 22, 23] and in brush-border membrane vesicles of the human small intestine [11]. These findings indicated that MTX interacted with the carrier for folic acid in the luminal membrane. However, in order to prove that the two folates share a common carrier, it was necessary to show that folic acid competitively inhibited MTX transport as well. In the present study, we have shown that in the presence of folic acid at increasing concentrations, the initial rate of uptake of MTX decreased exponentially to plateau at a value about 63% of that in the absence of folic acid. Thus, two distinct fluxes of MTX were discernible: a folic acid-sensitive flux whose contribution was about one-third of the total flux, and a folic acid-insensitive flux. From the data presented in Fig. 6, the concentration of folic acid required to inhibit the folic acid-sensitive flux by 50% was calculated to be $9.6 \mu M$. This value is in the same range as the K_m ($15.76 \mu M$) for transport of folic acid in the organ-cultured mucosa of the human small intestine under the same conditions [12]. The demonstration in the present study of a folic acid-insensitive flux as a major component of the total flux of MTX in the human proximal small intestine indicates a heterogeneity of the transport of MTX. Further studies using naturally occurring folate derivatives will clarify the existence of folic acid-insensitive pathways for their absorption in the human intestine.

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