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Anion Specificity of the Jejunal Folate Carrier: Effects of Reduced Folate Analogues on Folate Uptake and Efflux

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Summary. We previously reported that ^{3}H-folate uptake by rabbit jejunal brush-border membrane (BBM) vesicles was markedly stimulated by an outwardly directed OH^- gradient (pH_{in} 7.7, pH_{out} 5.5), inhibited by anion exchange inhibitors (DIDS, SITS, furosemide), and saturable (folate K_m = 0.19 μM) suggesting carrier-mediated folate/OH^- exchange (or H^+/folate cotransport). In the present study, the anion specificity of this transport process was examined. Under conditions of an outwardly directed OH^- gradient, DIDS-sensitive folate uptake was *cis* inhibited (>90%) by reduced folate analogues: dihydrofolate (IC_{50} = 0.40 μM), folinic acid (IC_{50} = 0.50 μM), 5-methyltetrahydrofolate (IC_{50} = 0.53 μM), and (+)amethopterin (IC_{50} = 0.93 μM). In contrast, 10 μM (−)amethopterin had only a modest effect on folate uptake (18% inhibition) suggesting stereospecificity of the folate/OH^- exchanger. The nonpteridine compounds which are transported by the folate carrier in L1210 leukemic cells (phthalate, thiamine pyrophosphate, and PO_4^{-3}) did not inhibit jejunal folate uptake. Furthermore, folate uptake was not inhibited by SO_4^{-2} (4 mM) or oxalate (4 mM) thereby distinguishing this carrier from the previously described intestinal SO_4^{-2}/OH^- and oxalate/Cl^- exchangers. After BBM vesicles were loaded with ^{3}H-folate, the initial velocity of ^{3}H-folate efflux was stimulated by unlabeled folate in the efflux medium. The transstimulation of ^{3}H-folate efflux by unlabeled folate was furosemide (or DIDS) inhibitable and temperature sensitive. Half-maximal stimulation of furosemide-sensitive ^{3}H-folate efflux was observed with 0.25 ± 0.05 μM unlabeled folate, a concentration similar to the K_m for folate uptake. These data suggest that folate-stimulated ^{3}H-folate efflux is mediated by the folate/OH^- exchanger. With the exception of (−) amethopterin, reduced folate analogues also transstimulated furosemide-sensitive ^{3}H-folate efflux in a concentration-dependent manner suggesting stereospecific transport of these analogues by the folate/OH^- exchanger. In summary, folate transport by the jejunal folate/OH^- exchanger demonstrates both *cis* inhibition and transstimulation by reduced folate analogues, but not by other inorganic or organic anions suggesting bidirectional transport of folate and a high degree of anion specificity.

Key Words folate transport · anion exchange · methotrexate · intestinal electrolyte transport · vesicle transport

Introduction

We previously reported [17] that the initial velocity of folate uptake by rabbit jejunal, but not ileal BBM[1] vesicles, is markedly stimulated under conditions of an outwardly directed OH^- gradient (pH_{in} 7.7, pH_{out} 5.5). Under these pH conditions, folate is predominantly in the anionic form (pK_a's of 3.5 and 4.8, ref. 14) and uptake is inhibited by anion exchange inhibitors (DIDS, SITS, and furosemide) and is saturable (folate K_m = 0.19 μM). These data provide evidence for a carrier on the jejunal BBM that mediates folate/OH^- exchange (or phenomenologically indistinguishable H^+/folate cotransport[2]). Furthermore, these findings are consistent with the cardinal features of folate transport by intact intestine, i.e. the known presence of an outwardly directed OH^- gradient in vivo (acid microclimate, ref. 13), an acidic pH optimum for intestinal folate transport [21, 22], and the primary role of the jejunum in folate absorption [7].

In other normal mammalian tissues (liver, kidney, erythrocyte) and many tumor cell lines, entry of folate into the cell is mediated by a transport system with high affinity (K_m = 1 to 10 μM) for reduced folate analogues including (+)amethopterin (methotrexate) and low affinity (K_m > 100 μM) for folate [20]. However, studies using intact intestine [22] suggest that uptake of luminal folate is mediated by a single carrier with similar affinities for both reduced and nonreduced forms of folate. In the L1210 leukemic cell line, reduced folates are transported by an anion exchange mechanism with broad specificity for organic (phthalate, thiamine pyrophosphate) and inorganic (SO_4^{-2}, PO_4^{-3}) anions [6]. Studies in L1210 plasma membrane vesicles

[1] Abbreviations: BBM, brush border membrane; DIDS, 4, 4′-diisothiocyano-2,2′-disulfonic acid stilbene, disodium salt; SITS, 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid, disodium salt; TMA, tetramethylammonium; MES, 2-[N-morpholino]ethanesulfonic acid.

[2] For simplicity, this transport mechanism will hereafter be referred to as folate/OH^- exchange.

demonstrated that this transport system mediates both the influx and efflux of (+)amethopterin [23].

In the present study, we investigated whether reduced folate analogues are also transported by the jejunal folate/OH^- exchanger. The effects of a variety of anions on OH^- gradient-stimulated ^{3}H-folate uptake (*cis* inhibition) and ^{3}H-folate efflux (trans-stimulation) were studied in order to define the anion specificity of the jejunal folate/OH^- exchanger.

Materials and Methods

Preparation of BBM Vesicles

Jejunal BBM vesicles were prepared from fed New Zealand White male rabbits (4 to 5 lb) by a divalent cation precipitation method as previously described [17]. The purity of this membrane vesicle preparation was previously validated by enzyme marker studies which included quantitative recoveries of protein and enzyme activities [17].

Vesicle ^{3}H-Folate Transport

Uptake of ^{3}H-folate by freshly prepared BBM vesicles was measured by a rapid Millipore filtration technique as previously described [17]. After preincubation for 2 hr at room temperature, membrane vesicles (10 μl) were added to 40 μl of reaction mixture (preincubated at 30°C) containing ^{3}H-folate and varying concentrations of unlabeled folate and incubated at 30°C. For influx studies, 6-sec uptakes were measured. Each uptake was terminated by the rapid addition of 3 ml of ice-cold, isotonic stop solution (10 mM Tris, 16 mM HEPES, pH 7.5; 182 mM K^+ gluconate) and immediate filtration through a 0.45-μm Millipore filter (HAWP).

A modification of this procedure was used for efflux studies. To load the inside of the vesicles with ^{3}H-folate, preincubated membrane vesicles (10 μl) were added to reaction mixture (40 μl; 30°C) containing 0.1 μM ^{3}H-folate. At one minute, a ninefold excess of efflux medium (450 μl; room temperature) was added to initiate efflux. Efflux was terminated by the addition of stop solution and filtration through Millipore filters as described above for uptake studies. When the efflux medium included 1 mM DIDS, the protocol was modified to keep vesicle protein concentration >0.65 mg protein/ml (*see* Fig. 4 legend). These changes were prompted by the finding that at protein concentrations lower than 0.65 mg/ml, 1 mM DIDS had nonspecific effects on vesicle content of ^{3}H-folate or ^{3}H-glucose when vesicles were loaded under OH^- or Na^+ gradient conditions, respectively (*data not shown*).

Filters were washed twice with 3 ml of ice-cold stop solution, dissolved in Hydrofluor® scintillation cocktail (National Diagnostics Inc., Somerville, N.J.), and counted on an LS 6800 liquid scintillation counter (Beckman Instruments Inc., Fullerton, Calif.).

Statistical Methods

Uptakes were performed in triplicate and efflux measurements in quadruplicate. Each experiment was repeated on at least three separate membrane preparations and the results expressed as the mean ± SE for all membrane preparations tested. Differences among means were tested for statistical significant ($P < 0.05$) using Student's paired t-test. For inhibition studies, the inhibitor concentration giving 50% inhibition (IC_{50}) was calculated by plotting 1/velocity versus [inhibitor] and extrapolating the regression line to the x-axis. The x-intercept was taken to represent the inverse of the IC_{50} [18] thus avoiding any assumptions regarding the mechanism of inhibition (i.e. competitive versus noncompetitive inhibition). The kinetics of efflux were analyzed from pooled data from multiple membrane preparations with a computerized weighted least-squares fit of the individual data points to a rectangular hyperbola as described previously [3].

Materials

^{3}H-folate (~32 Ci/mmol; in 1% ascorbic acid) was obtained from Amersham Corp. (Arlington Heights, Ill.). Furosemide was kindly provided by Hoechst-Roussel Pharmaceuticals (Somerville, N.J.) HEPES was purchased from United States Biochemical Co. (Cleveland, Ohio). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo.) and were of reagent grade or the highest purity available.

Results

Effects of Reduced Folate Analogues and Other Anions on OH^- Gradient-Stimulated Folate Uptake

Under conditions of an outwardly directed OH^- gradient (pH_{in} 7.8/pH_{out} 5.4), folate (0.1 μM) uptake measured at 1.8, 3.0, 4.8, and 6.0 sec was linear with time (*data not shown*) and the regression line passed through the origin implying that binding does not contribute significantly to uptake during the first 6 sec. Therefore, uptakes at 6 sec were used to estimate initial rates of folate uptake.

In the absence of folate analogues in the incubation medium, OH^- gradient-stimulated folate (0.1 μM) uptake was inhibited to 20% of control by 1 mM DIDS (Figs. 1*A* and 1*B*). Each of the reduced folate analogues also inhibited OH^- gradient-stimulated folate uptake in a dose-dependent manner (Fig. 1, upper lines). Moreover, folate uptake in the presence of 1 mM DIDS was not inhibited by reduced folate analogues over the range of concentrations tested (Fig. 1, lower lines). Analysis of the concentration dependence of inhibition of DIDS-sensitive folate uptake (defined as the difference between total uptake and uptake in the presence of DIDS) yielded an IC_{50} of 0.93, 0.50, 0.53, or 0.40 μM for (+)amethopterin, folinic acid, 5-methyltetrahydrofolate, or dihydrofolate, respectively (Fig. 1, insets).

In contrast to (+)amethopterin, (−)amethopterin (10 μM) only minimally inhibited OH^- gradient-stimulated folate uptake (Table, part A). Of the

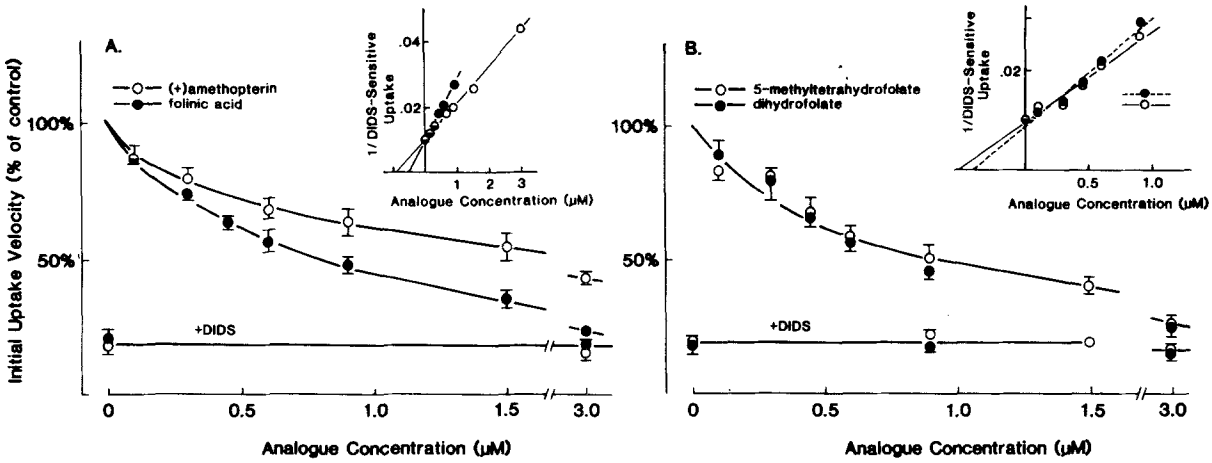

Fig. 1. Effects of reduced folate analogues on OH^- gradient-stimulated folate uptake. Vesicles were preincubated for 2 hr at room temperature in (final concentrations): 70 mM Tris, 70 mM HEPES, pH 7.8; and 250 mM mannitol. Uptakes (measured at 6 sec) were initiated by adding 10 μl of vesicles to 40 μl of reaction medium at 30°C [final concentrations: 16 mM Tris, 20 mM HEPES, 104 mM MES, pH 5.4; 250 mM mannitol, 0.1 μM ^{3}H-folate, and varying concentrations of reduced folate analogues (0 to 3.0 μM) with or without 1 mM DIDS]. Vesicle folate uptake at 6 sec was 0.634 ± 0.062 pmol/mg protein. Insets: Dixon plots of DIDS-sensitive folate uptake under OH^- gradient conditions. Symbols depict means ± SE for at least three separate membrane preparations. Where error bars are not shown, they are included within the symbol

three components of the folate molecule [pteridine (pterine) ring, para-aminobenzoic acid, L-glutamic acid], only extremely high concentrations of para-aminobenzoic acid inhibited folate uptake (Table, A).

Recently, separate carriers mediating SO_4^{-2}/OH^- (SO_4^{-2} K_m = 0.475 mM, Ref. 15), oxalate/Cl^- (oxalate K_m = 0.566 mM, ref. 8), and Cl^-/HCO_3^- [10] exchange have been described in BBM vesicles from rabbit ileum. To distinguish the folate/OH^- exchanger from these carriers, the effects of SO_4^{-2}, oxalate, or Cl^- on OH^- gradient-stimulated folate uptake were examined (Table, B). Jejunal folate uptake was not inhibited by SO_4^{-2} (4 mM) or oxalate (4 mM). Although folate uptake was mildly reduced by 40 mM Cl^- (18.5% inhibition), this concentration of Cl^- is ~10-fold greater than the K_m of Cl^- for the intestinal Cl^-/HCO_3^- exchanger (Cl^- K_m = 3.5 mM, ref. 10).

In the L1210 leukemic cell line [6], a wide variety of inorganic and organic anions are potent inhibitors of reduced folate transport, e.g. SO_4^{-2}, PO_4^{-3}, phthalate, and thiamine pyrophosphate (K_i's = 0.54 mM, 0.40 mM, 20 μM, and 3 μM, respectively). In contrast, in jejunal BBM vesicles, these anions did not inhibit OH^- gradient-stimulated folate uptake (Table, B and C).

Folate-Stimulated ^{3}H-Folate Efflux

When BBM vesicles were loaded with ^{3}H-folate under OH^- gradient conditions, the uptake of ^{3}H-folate at 1 min was inhibited by 1 mM DIDS to 28% of control (Fig. 2A, open triangle). These data suggest

Table. Effects of anions on folate uptake under OH^- gradient conditions[a]

		[Anion] (mM)	Folate uptake (% control)
A. Other folate analogues and components of folate molecule:			
(−)amethopterin	(n = 5)	0.01	85.7 ± 1.8 (P < 0.005)
p-aminobenzoic acid	(n = 4)	1	88.9 ± 3.0 (P < 0.05)
	(n = 3)	25	53.5 ± 0.7 (P < 0.001)
L-glutamic acid	(n = 3)	1	103.0 ± 6.9
pterine	(n = 3)	0.1	98.7 ± 1.9
B. Ligands for other intestinal anion exchangers:			
Cl^-	(n = 3)	40	81.5 ± 2.6 (P < 0.02)
SO_4^{-2}	(n = 3)	4	100.5 ± 3.3
oxalate	(n = 3)	4	87.4 ± 4.8
C. Anions transported by the L1210 folate carrier:			
PO_4^{-3}	(n = 3)	20	105.7 ± 2.1
phthalate	(n = 4)	1	88.8 ± 4.6
thiamine pyrophosphate	(n = 3)	1	104.6 ± 3.7

[a] Folate (0.1 μM) uptake was determined at 6 sec in the presence or absence (control) of different anions. Each anion was tested on at least three separate membrane preparations and the results expressed as % of control (P values indicate significant differences between control uptake rates and uptake in the presence of anion). When the concentration of anion exceeded 1 mM, isosmolality was maintained by adjusting the mannitol concentration in the reaction medium. Buffer composition as in the legend to Fig. 1.

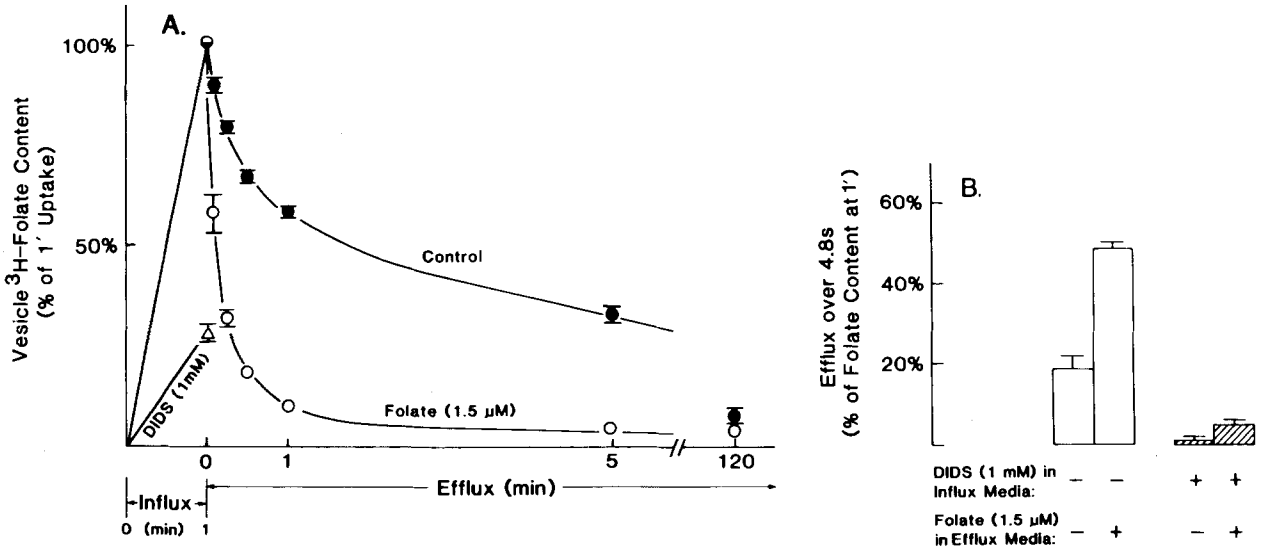

Fig. 2. Effects of external folate on efflux of ^{3}H-folate. (*A*) Folate influx was initiated under the same conditions as described in the legend to Fig. 1. Open triangle represents folate uptake under these conditions, but in the presence of 1 mM DIDS. At 1 min, efflux medium (450 μl) was added and efflux was allowed to proceed at room temperature. Efflux medium contained 250 mM mannitol, 16 mM Tris, 20 mM HEPES, and 104 mM MES (pH 5.4), with (open circles) or without (filled circles) unlabeled folate (1.5 μM). Efflux is expressed as % of uptake at 1 min. (2.25 ± 0.18 pmol/mg protein). (*B*) Vesicles were loaded with ^{3}H-folate under OH^- gradient conditions (pH 7.8 inside, pH 5.4 outside) in the presence (hatched bars) or absence (open bars) of 1 mM DIDS. At 1 min, efflux medium with or without 1.5 μM folate was added and vesicle ^{3}H-folate content was measured 4.8 sec later. The amount of ^{3}H-folate that was released over 4.8 sec was calculated as the difference between vesicle ^{3}H-folate content at the end of the 1 min influx period (2.91 ± 0.66 pmol/mg protein) and vesicle ^{3}H-folate content 4.8 sec after addition of efflux medium

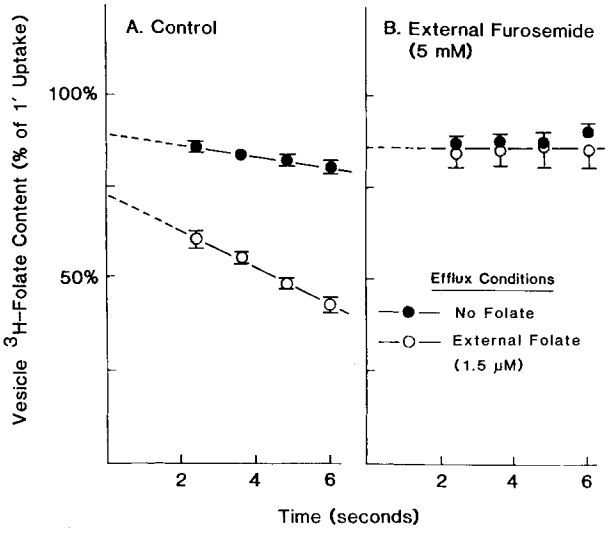

Fig. 3. Effects of furosemide on ^{3}H-folate efflux. After a 1-min influx under OH^- gradient conditions (2.46 ± 0.28 pmol/mg protein), efflux was initiated by the addition of efflux medium with or without 5 mM furosemide. Efflux conditions as in the legend to Fig. 2

that at least 72% of uptake at 1 min is mediated by the folate/OH^- exchanger. After a 1 min influx under OH^- gradient conditions, a ninefold excess of efflux medium was added and vesicle ^{3}H-folate content was measured at varying time points thereafter (Fig. 2*A*). With folate (1.5 μM) in the efflux medium (Fig. 2*A*, open circles), efflux of ^{3}H-folate was more rapid compared with efflux in the absence of added folate (filled circles). The initial rate of ^{3}H-folate efflux (over 4.8 sec, vide infra) was significantly stimulated by 1.5 μM folate in the efflux medium (Fig. 2*B*, open bars). In contrast, when the influx medium included 1 mM DIDS, only a small increase in efflux was observed with external folate (Fig. 2*B*, hatched bars). These findings suggest that if uptake in the presence of DIDS is mediated by contaminating vesicles without the folate/OH^- exchanger, this component of uptake cannot account for folate-stimulated ^{3}H-folate efflux.

In order to determine whether the folate carrier mediates folate-stimulated ^{3}H-folate efflux, the effects of inhibitors of folate/OH^- exchange on ^{3}H-folate efflux were examined. The addition of folate (1.5 μM) to the efflux medium increased the initial velocity (more negative slope) of ^{3}H-folate efflux (Figs. 3*A* and 4*A*). This effect was completely inhibited by the addition of 5 mM furosemide or 1 mM DIDS to the efflux medium (Figs. 3*B* or 4*B*, respectively). These concentrations of furosemide and DIDS inhibit OH^- gradient-stimulated ^{3}H-folate (0.1 μM) uptake by 91% (*data not shown*) and 84% [17], respectively. At these inhibitor concentra-

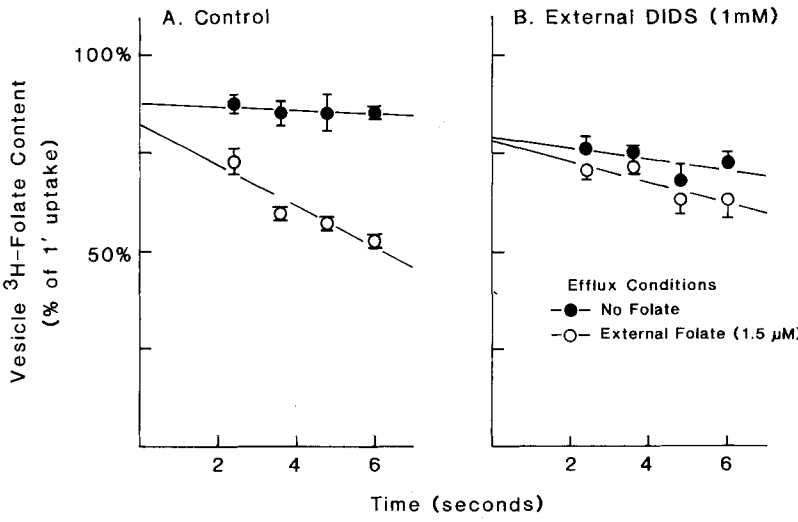

Fig. 4. Effects of DIDS on ^{3}H-folate efflux. Influx of ^{3}H-folate was initiated by adding 10 μl of vesicles preincubated in pH 7.8 buffer to 35 μl of reaction medium (final pH = 5.4). Buffer conditions as in Fig. 1 legend. At 1 min, 270 μl of efflux medium (pH 5.4, *see* Fig. 2 legend) with or without 1 mM DIDS was added. These modifications in the experimental protocol kept vesicle protein concentration >0.65 mg protein/ml thereby avoiding nonspecific effects of 1 mM DIDS (*see* Materials and Methods, *data not shown*)

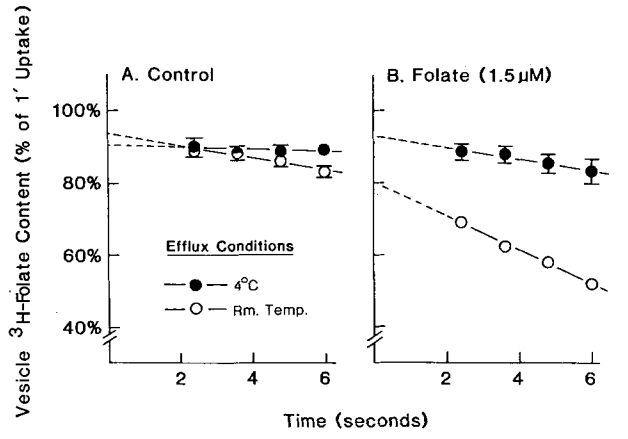

Fig. 5. Effects of temperature on ^{3}H-folate efflux. Vesicles were loaded with ^{3}H-folate under OH^- gradient conditions (2.25 ± 0.26 pmol/mg protein at 1 min) and efflux was initiated by the addition of efflux medium at room temperature (open circles) or 4°C (filled circles). Efflux conditions as in the legend to Fig. 2

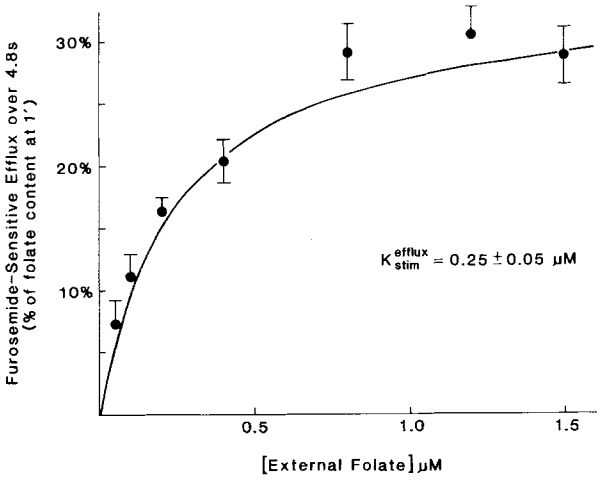

Fig. 6. Kinetics of folate-stimulated ^{3}H-folate efflux. After influx under OH^- gradient conditions for 1 min (3.27 ± 0.53 pmol/mg protein), vesicle ^{3}H-folate content was determined at 4.8 sec in the presence or absence of 5 mM furosemide as the folate concentration in the efflux medium was varied from 0.05 to 1.50 μM. The furosemide-sensitive component of ^{3}H-folate efflux was calculated as the difference between vesicle ^{3}H-folate content in the presence or absence of 5 mM furosemide. A weighted, least-squares fit of the individual data points was performed on a computer yielding the depicted rectangular hyperbola and K_{stim}^{efflux}. Symbols depict means ± SE of individual data points

tions, the glucose space was not affected (*data not shown*) thereby excluding a nonspecific effect of these inhibitors on vesicle integrity.

The initial rates of ^{3}H-folate efflux were linear through 6 sec in the presence or absence of external folate or inhibitors (Figs. 3 and 4). However, if the apparent folate content prior to efflux is estimated by extrapolating the line representing vesicle folate content versus time to the *y*-axis, the *y*-intercept is significantly less than the 1-min uptake value (Figs. 3 and 4). This discrepancy between the apparent vesicle ^{3}H-folate content at the initiation of efflux (*y*-intercept) and the 1-min uptake value suggests debinding or an extremely rapid initial component of efflux. To distinguish between these two possibilities, efflux of ^{3}H-folate was compared at room temperature and at 4°C (Fig. 5). In the absence of folate, ^{3}H-folate efflux was unaffected by temperature suggesting that the difference between the *y*-intercept and the 1-min uptake value is secondary to debinding. In contrast, folate-stimulated ^{3}H-folate efflux was markedly reduced by low temperature.

The external folate concentration required for half-maximal stimulation of ^{3}H-folate efflux was determined by incubating the vesicles with varying folate concentrations in the efflux medium and measuring efflux at 4.8 sec (Fig. 6). The furosemide-sensitive component of folate-stimulated ^{3}H-folate

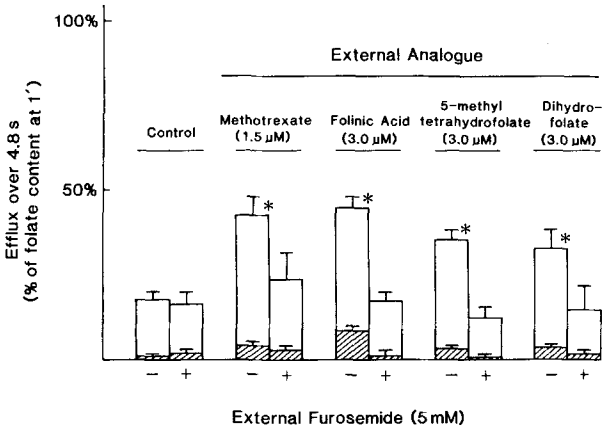

Fig. 7. Effects of reduced folate analogues on ^{3}H-folate efflux. ^{3}H-folate efflux (over 4.8 sec) was examined in the absence (control) or presence of reduced folate analogues after vesicles were loaded with ^{3}H-folate under OH^- gradient conditions with (hatched bars) or without (open bars; 2.50 ± 0.16 pmol/mg protein) 1 mM DIDS. The presence or absence of 5 mM furosemide in the efflux medium is indicated by the +/− symbols along the ordinate. (* $P < 0.05$ when compared with efflux under control conditions.)

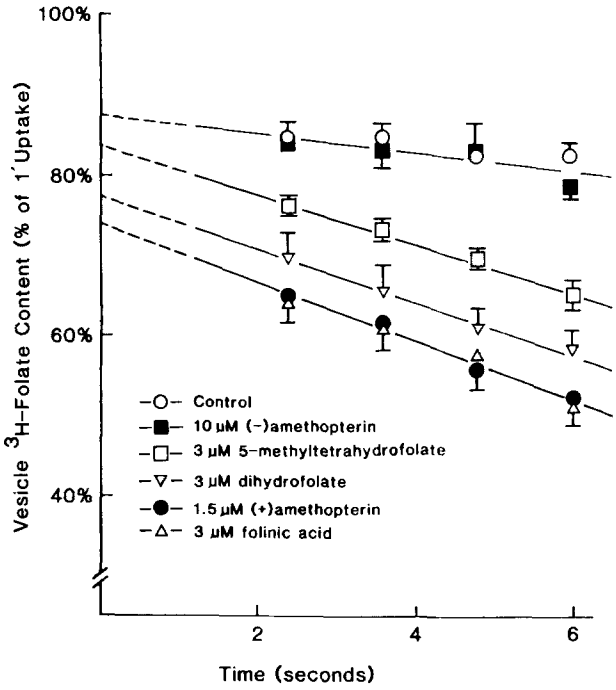

Fig. 8. Effects of reduced folate analogues on the initial rate of ^{3}H-folate efflux. After vesicles were loaded with ^{3}H-folate under OH^- gradient conditions (2.21 ± 0.25 pmol/mg protein at 1 min), vesicle ^{3}H-folate content was measured 2.4, 3.6, 4.8 or 6.0 sec following addition of efflux medium with or without reduced folate analogues at the concentrations indicated

efflux, calculated as the difference between total efflux and efflux in the presence of 5 mM furosemide, was a saturable function of folate concentration. A computer-derived [3], weighted least-squares fit of the individual data points yielded a rectangular hyperbola and a folate K_{stim}^{efflux} of 0.25 ± 0.05 μM.

Effects of Reduced Folate Analogues on ^{3}H-Folate Efflux

To determine if reduced folate analogues are also transported by the folate carrier, the effects of these analogues on ^{3}H-folate efflux were assessed. Folate analogues which inhibited OH^- gradient-stimulated folate uptake also stimulated ^{3}H-folate efflux (measured at 4.8 sec) by a furosemide-sensitive mechanism (Fig. 7, open bars). This stimulation of ^{3}H-folate efflux was not secondary to efflux from vesicles loaded with ^{3}H-folate under OH^- gradient plus DIDS conditions (Fig. 7, hatched bars), suggesting that the stimulation of ^{3}H-folate efflux did not occur from vesicles lacking the folate/OH^- exchanger. Furthermore, with the exception of (−) amethopterin, a weak inhibitor of OH^- gradient-stimulated folate uptake, reduced folate analogues stimulated the initial rate of ^{3}H-folate efflux (Fig. 8). This transstimulation of ^{3}H-folate efflux by reduced folate analogues was concentration-dependent with half-maximal transstimulation at 0.25 to 0.5 μM (Fig. 9).

Discussion

In this study of the anion specificity of the jejunal folate/OH^- exchanger, our findings suggest that this transporter mediates both the influx and efflux of folate (bidirectional transport). In addition, the present study suggests that the folate/OH^- exchanger also transports reduced folate analogues [(+)amethopterin, 5-methyltetrahydrofolate, folinic acid, and dihydrofolate]. Unlike the carrier for reduced folates in L1210 cells (leukemic cell line), the jejunal folate/OH^- exchanger exhibits a high degree of anion specificity. The specificity of this exchanger for folate and its analogues distinguishes this transport mechanism from other intestinal anion transporters.

In ^{3}H-folate efflux experiments, jejunal BBM vesicles were loaded with ^{3}H-folate in the presence of an outwardly directed OH^- gradient. These conditions favor loading of ^{3}H-folate into those BBM vesicles having the DIDS-sensitive folate/OH^- exchanger. The DIDS-insensitive uptake under these conditions is a relatively minor (28%) component of total uptake and can be accounted for by passive diffusion of folate into BBM vesicles or uptake by contaminating membrane vesicles.

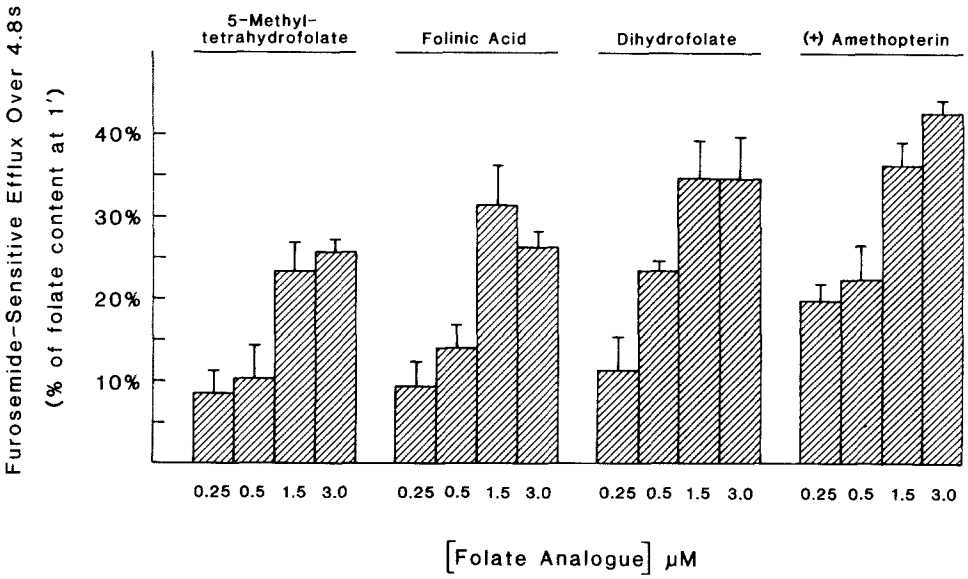

Fig. 9. Concentration dependence of stimulation of ^{3}H-folate efflux by reduced folate analogues. After vesicles were loaded with ^{3}H-folate (2.23 ± 0.22 pmol/mg protein), efflux (over 4.8 sec) was determined in the presence or absence of 5 mM furosemide as the folate analogue concentration was varied from 0.25 to 3.0 μM. The furosemide-sensitive component of ^{3}H-folate efflux was calculated as the difference between vesicle ^{3}H-folate content in the presence or absence of 5 mM furosemide

^{3}H-folate efflux was initiated by the addition of a ninefold excess of efflux medium. With unlabeled folate in the efflux medium, ^{3}H-folate efflux was stimulated compared with efflux in the absence of unlabeled folate (transstimulation). Although an inwardly directed anion (i.e. folate) gradient may induce an inside-negative electrical potential, two lines of evidence suggest that this is unlikely to account for folate-stimulated ^{3}H-folate efflux. First, an inside-positive electrical potential did not stimulate ^{3}H-folate uptake by rabbit jejunal BBM vesicles [17] suggesting that vesicle folate transport is potential insensitive. Second, while 1.5 μM (+)amethopterin stimulated ^{3}H-folate efflux, 10 μM (−) amethopterin did not, suggesting that the mere presence of an inwardly directed anion gradient is not sufficient to stimulate ^{3}H-folate efflux. The observation that lowering the temperature of the efflux medium markedly reduced folate-stimulated ^{3}H-folate efflux is consistent with efflux by a carrier-mediated mechanism, but does not exclude debinding which may also be temperature dependent. Since efflux from vesicles loaded in the presence of DIDS accounted for only a small component of folate-stimulated ^{3}H-folate efflux, these data suggest that folate-stimulated efflux occurs predominantly from vesicles which take up folate via the folate/OH⁻ exchanger. This general strategy of loading vesicles with ^{3}H-folate via a specific transport mechanism should be applicable to the performance of efflux studies with other substrates when contaminating or other BBM membrane vesicles are present which do not possess the transport system of interest.

Two experiments provide evidence that the folate/OH⁻ exchanger mediates folate-stimulated ^{3}H-folate efflux. First, the initial velocity of folate-stimulated ^{3}H-folate efflux was inhibited by furosemide or DIDS, both of which inhibit folate/OH⁻ exchange [17]. Second, the concentration of unlabeled folate required to half-maximally stimulate ^{3}H-folate efflux (0.25 ± 0.05 μM, Fig. 6) approximates the folate K_m for uptake via the folate/OH⁻ exchanger (0.19 ± 0.02 μM, ref. 17). Together with the results of previous studies [17], these data suggest that in vitro, the folate/OH⁻ exchanger mediates both folate influx and efflux (bidirectional transport). Since freeze-fracture electron microscopy demonstrated that 79% of the jejunal BBM vesicles are oriented right-side-out (*data not shown*), vesicle uptake of folate is analogous to uptake of luminal folate by the intact enterocyte. In vivo, the outwardly directed OH⁻ gradient across the jejunal BBM (intracellular pH = 6.8, luminal pH = 5.8; refs. 11 and 13, respectively) favors uptake via the exchanger, thereby driving the intestinal absorption of this vitamin.

The effects of reduced folate analogues on ^{3}H-folate efflux were assessed in order to determine in vitro whether these analogues are also transported by the folate/OH⁻ exchanger. With the exception of (−)amethopterin, the reduced folate analogues, i.e. (+)amethopterin, 5-methyltetrahydrofolate, dihydrofolate, and folinic acid, stimulated ^{3}H-folate ef-

flux by a saturable, furosemide-sensitive mechanism. Each of these analogues also *cis* inhibited OH^- gradient-stimulated folate uptake (IC_{50}'s = 0.4 − 0.9 μM). *Cis* inhibition of folate uptake and transstimulation of folate efflux by the reduced folate analogues provide compelling evidence that these analogues are transported by the folate/OH^- exchanger. The lack of stimulation of ^{3}H-folate efflux by (−)amethopterin is consistent with the relatively weak inhibition of OH^- gradient-stimulated folate uptake by this compound and implies stereospecificity of the transport mechanism.

Studies with intact rat intestine also suggest the presence of a folate carrier with similar affinities for reduced and nonreduced forms of folate. Using intestinal loops perfused in vivo [2], the absorption of (+)amethopterin was inhibited by unlabeled folate (K_i = 1.28 μM). Similarly, using everted gut sacs, the uptake of 0.1 μM ^{3}H-folate (K_m = 4.4 μM) was inhibited by unlabeled reduced folate analogues, i.e. folinic acid, (+)amethopterin, and 5-methyltetrahydrofolate (K_i = 9, 20, and 24 μM, respectively; ref. 22).

The present findings extend those of an early study [19] using rat intestinal BBM vesicles in which ^{3}H-(+)amethopterin uptake was saturable (K_m = 1.5 μM) and competitively inhibited by folate (K_i = 0.6 μM) or 5-methyltetrahydrofolate (K_i = 1.35 μM). Although preloading the vesicles with unlabeled folate or reduced folate analogues transstimulated ^{3}H-(+)amethopterin uptake (measured at 0.5, 1.0, and 7.5 minutes), the effects of temperature, membrane transport inhibitors, or increasing concentrations of unlabeled transstimulating anion on the initial rate of ^{3}H-(+)amethopterin uptake were not examined. However, assessing the effects of such experimental manipulations on isotope flux is necessary to demonstrate that uptake and efflux are mediated by the same transport mechanism. Since in our studies the concentration of external folate required to half-maximally stimulate ^{3}H-folate efflux approximated the folate K_m for uptake and inhibitors of the folate carrier inhibited folate-stimulated ^{3}H-folate efflux, these data suggest that transstimulation of efflux by folate is mediated by the folate/OH^- exchanger.

All folates consist of three major structural components: a pteridine ring, a para-aminobenzoic acid moiety, and a L-glutamic acid residue. Although folate uptake was not affected by glutamate or pterine (pteridine ring), very high concentrations of *p*-aminobenzoic acid were inhibitory (46.5% inhibition at 25 mM). The lack of effect of pterine on folate uptake is consistent with the results of a previous study [1] which showed only a slight rise in serum or urine levels of tetrahydrobiopterin following oral administration of this compound to human volunteers.

Recently, several anion exchangers have been reported on the intestinal brush border, i.e. Cl^-/HCO_3^- [10, 12], SO_4^{-2}/OH^- [15, 16], and oxalate/Cl^- exchange [8, 9]. In all but one study [12], rabbit ileal BBM vesicles were used to characterize these various anion exchange processes. Since our initial studies localized folate/OH^- exchange to rabbit jejunal and not ileal BBM vesicles [17], the possibility that OH^- gradient-stimulated folate uptake was mediated by one of these anion exchangers was unlikely. Nevertheless, the effects of these anions on OH^- gradient-stimulated folate uptake were examined at concentrations far greater than the K_m's of these anions for their respective transporters.

The lack of effect of 4 mM SO_4 (K_m = 0.475 mM, ref. 15) or 4 mM oxalate (K_m = 0.566 mM, ref. 8) on folate uptake suggests that the carrier-mediating folate/OH^- exchange is a separate transporter from the SO_4^{-2}/OH^- or oxalate/Cl^- exchangers. An unexpected finding was inhibition of OH^- gradient-stimulated folate uptake by Cl^- (18.5% inhibition with 40 mM TMAC1, Table) without an effect on DIDS-insensitive uptake (*data not shown*). Since the initial uptake rate for folate remained linear through 6 sec in the presence of 40 mM TMAC1 (*data not shown*), the inhibition by TMAC1 was not secondary to a Cl^--induced dissipation of the pH gradient. Furthermore, 40 mM TMA gluconate did not inhibit OH^- gradient-stimulated folate uptake (*data not shown*) suggesting that neither TMA^+, nor increased ionic strength could account for the inhibition by TMAC1. In aggregate, these data suggest that Cl^- has an effect on carrier-mediated folate uptake, but only at concentrations far in excess of the Cl^- K_m (=3.5 mM, ref. 10) for the Cl^-/HCO_3^- exchanger.

The jejunal folate/OH^- exchanger has many features in common with the folate transporter from the L1210 mouse leukemic cell line, the mammalian system most extensively studied to date [4]. The L1210 folate carrier is a high-affinity transport system with K_m's for reduced folates in the 1- to 3 μM range [4]. Many anions competitively inhibit and transstimulate (+)amethopterin uptake by L1210 cells suggesting an anion exchange process [4]. Moreover, the L1210 folate transporter is DIDS-sensitive [5] and inhibited by high concentrations of Cl^- (K_i = 28.2 mM, ref. 23). Finally, in vitro, the L1210 folate transporter mediates the bidirectional transport of (+)amethopterin [23].

However, the jejunal folate/OH^- exchanger differs significantly from the L1210 folate transporter with respect to three important characteristics. First, the jejunal folate carrier has equal affinities for reduced and nonreduced forms of folate. In contrast, for the L1210 folate transporter, the K_m's for reduced folate analogues are in the 1- to 3-μM range compared with a folate K_m of 200 μM [4]. Second,

the nonpteridine compounds (e.g. thiamine pyrophosphate, phthalate, SO_4^{-2}, and PO_4^{-3}) that are transported by the L1210 folate transporter [6] do not inhibit folate uptake mediated by the jejunal folate/OH^- exchanger. Finally, in L1210 cells, lowering the pH of the incubation medium (i.e. imposing an outwardly directed OH^- gradient) only modestly affects the kinetics of (+)amethopterin uptake suggesting that the transport process is relatively pH insensitive (G.B. Henderson, *personal communication*). In contrast, in jejunal BBM vesicles, an outwardly directed OH^- gradient drives the uphill transport of folate by carrier-mediated folate/OH^- exchange [17].

In summary, the present study provides evidence that the jejunal folate/OH^- exchanger mediates the transport of folate and reduced folate analogues, but not other organic or inorganic anions, thereby distinguishing this transporter from either the L1210 folate transporter or other intestinal anion exchangers. The concordance of these results with in vivo observations on intestinal folate transport further strengthens the hypothesis that carrier-mediated folate/OH^- exchange is the mechanism for intestinal absorption of folate under physiological conditions. Finally, the demonstration that ^{3}H-folate efflux is mediated by the folate/OH^- exchanger provides a system to rapidly and conveniently test in vitro whether other folate analogues are transported by the jejunal folate carrier.

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