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pH Modulation of the Kinetics of Rabbit Jejunal, Brush-Border Folate Transport

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Summary. In jejunal brush-border membrane vesicles, an outwardly directed OH^- gradient (in > out) stimulates DIDS-sensitive, saturable folate (F) uptake (Schron, C.M. 1985. *J. Clin. Invest.* **76**:2030–2033), suggesting carrier-mediated folate: OH^- exchange (or phenomenologically indistinguishable H^+: folate cotransport). In the present study, the precise role of pH in the transport process was elucidated by examining F uptake at varying pH. For pH gradients of identical magnitude, F uptake (0.1 μM) was greater at lower (pH_{int}/pH_{ext}: 5.5/4.5) compared with higher (6.5/5.5) pH ranges. In the absence of a pH gradient, internal F *trans* stimulated DIDS-sensitive ^{3}H-folate uptake only at pH $\leq$ 6.0. Since stepwise increments in *internal* pH (4.5 $\rightarrow$ 7.5; pH_{ext} = 4.5) stimulated F uptake, an inhibitory effect of higher *internal* pH was excluded. In contrast, with increasing external pH (4.35 $\rightarrow$ 6.5; pH_{int} = 7.8), a 50-fold decrement in F uptake was observed (H^+ K_m = 12.8 $\pm$ 1.2 μM). Hill plots of these data suggest involvement of at least one H^+ (OH^-) at low pH (monovalent F^- predominates) and at least 2 H^+ (OH^-) at high pH (divalent F^{-2} predominates). Since an inside-negative electrical potential did not affect F uptake at either pH_{ext} 4.55 or 5.8, transport of F^- and F^{-2} is electroneutral. Kinetic parameters for F^- and F^{-2} were calculated from uptake data at pH_{ext} 4.55 and 5.0. Comparison of predicted *vs.* experimentally determined kinetic parameters at pH_{ext} 5.8 (K_m = 1.33 *vs.* 1.70 μM; V_{max} = 123.8 *vs.* 58.0 pmol/mg prot min) suggest that increasing external pH lowers the V_{max}, but does not affect the K_m for carrier-mediated F transport. These data are consistent with similar K_i's for sulfasalazine (competitive inhibitor) at pH_{ext} 5.35 and 5.8 (64.7 and 58.5 μM, respectively). In summary, the jejunal F carrier mediates electroneutral transport of mono- and divalent F and is sensitive to external pH with a H^+ K_m (or OH^- IC_{50}) corresponding to pH 4.89. External pH affects the V_{max}, but not the K_m for carrier-mediated F uptake suggesting a reaction mechanism involving a ternary complex between the outward-facing conformation of the carrier and the transported ions (F and either OH^- or H^+), rather than competitive binding that is mutually exclusive.

Key Words anion exchange · intestinal transport · vesicle transport

Introduction

The monoglutamyl form of dietary folate is absorbed predominantly in the jejunum by a saturable process that is maximal at a luminal pH between 5.5 and 6.0 [19, 20]. When the luminal pH exceeds 6.5, concentrative folate uptake is not observed [20]. Studies in membrane vesicles [13, 17] confirmed these observations leading to the suggestion that the transport process, or the carrier itself, is pH sensitive. However, since the pH of the enterocyte interior is $\approx$6.8–7.1 [7, 18], these data are also compatible with the hypothesis that an outwardly directed OH^- gradient (or inwardly directed H^+ gradient) is required for carrier-mediated folate transport. As discussed below, these two interpretations are not mutally exclusive.

Previous studies [14] demonstrated that in rabbit jejunal BBM[1] vesicles an outwardly directed OH^- gradient (pH_{int} 7.7, pH_{ext} 5.5) markedly stimulates the initial velocity of folate uptake compared with uptake in the absence of a pH gradient (pH_{int} 5.5, pH_{ext} 5.5). Moreover, an outwardly directed OH^- gradient is required for the transient accumulation of folate at concentrations greater than at equilibrium ("overshoot"). Under these pH conditions, folate is predominantly in the anionic form [6, 12][2] and uptake

[1] *Abbreviations:* BBM, brush-border membrane; F^-, monovalent folate; F^{-2}, divalent folate; and TMA, tetramethylammonium.

[2] Folate consists of a pteridine ring to which is attached *p*-aminobenzoic acid and L-glutamic acid. A peptide bond joins the carboxyl group of *p*-aminobenzoic acid to the α-amino group of L-glutamic acid. At very low pH, the nitrogens at positions 5, 10, and 1 on the pteridine ring are protonated, which confers positive charge on the molecule (pK_a's = < -1.5, 0.20, and 2.35, respectively corresponding to $[H^+]$'s of 31.6, 0.63, and 0.00446 M, respectively; *see* Fig. 4 legend and Table II from ref. [12]). At the opposite pH extreme, amidization of the nitrogen at position 3 places a negative charge on the pteridine ring at N(3)/C(4) (pK_a = 8.38; ref. [12]). Over the pH range used in the present study (pH 4.35 $\rightarrow$ 7.8), no positive or negative charge is present on the pteridine ring. Although the folate pK_a's for the α- and γ-carboxyl groups on L-glutamic acid have not been measured, pK_a's for the corresponding groups on methotrexate and 5-formyltetrahydrofolate are 3.36 and 3.1 (α-carboxyl) and 4.7 and 4.8 (γ-carboxyl), respectively (*see* ref. [12] for summary). Since even

is inhibited by anion transport inhibitors (DIDS, SITS, and furosemide) and is saturable. These data provide evidence for carrier-mediated folate: OH^- exchange (or phenomenologically indistinguishable H^+: folate cotransport) and suggest that the transmembrane pH gradient in vivo (acid microclimate, ref. [9]) drives uphill folate transport under physiologic conditions.

In our previous studies [14], vesicle ^{3}H-folate uptake was measured under conditions of constant external pH and varying internal pH. While these conditions were optimal for elucidating the role of the transmembrane pH gradient in energizing folate transport, effects of external pH on folate uptake apart from the transmembrane pH gradient were not assessed. Such modulating effects of external pH might be expected from our previously proposed model of folate transport, i.e., folate: OH^- exchange or H^+: folate cotransport [14]. For example, if OH^- ions compete with folate for a mutually exclusive binding site, raising external pH would increase the folate K_m (competitive inhibitor). Alternatively, the binding of H^+ (or OH^-) to a separate binding site on the transporter might affect the folate K_m and/or V_{max}. The present study assessed these possible effects of external pH in modulating the kinetics of carrier-mediated folate transport.

Materials and Methods

Preparation of BBM Vesicles

Jejunal BBM vesicles were prepared from fed New Zealand White male rabbits (4–5 lb) by a divalent cation precipitation method as previously described [14]. This membrane preparation was fully validated previously and is enriched 12.6-fold relative to homogenate in the brush-border enzyme marker, lactase, but not in markers for the basolateral membrane, endoplasmic reticulum, or mitochondria [14].

Vesicle ^{3}H-Folate Transport

Vesicle uptake of ^{3}H-folate was measured by a rapid Millipore filtration technique as previously described [14]. Briefly, freshly prepared BBM vesicles were preincubated at room temperature for 2 hr and placed on ice until used for uptake studies (<2–3 hr). An aliquot of membrane vesicles (5–10 μl) was added to an isosmotic reaction mixture (40–95 μl; preincubated at 30°C) containing ^{3}H-folate and varying concentrations of unlabeled folate. Uptake at 30°C 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, presoaked in 100 mM K^+ gluconate). Filters were washed twice with 3 ml of ice-cold stop solution, dissolved in ReadySolv scintillation cocktail (Beckman Instruments, Fullerton, CA), and counted in a LS 6800 liquid scintillation counter (Beckman Instruments).

Statistical Methods

Uptakes were performed in triplicate 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 significance ($P < 0.05$) using Student's paired t test. Kinetic parameters were determined for pooled data from multiple membrane preparations using a computerized, weighted least-squares fit of the individual data points to a rectangular hyperbola as described previously [2].

Materials

^{3}H-folate (~20–30 Ci/mmol in 1% ascorbic acid) was obtained from Amersham (Arlington Heights, IL). TMA_2folate was prepared by combining TMAOH and folic acid in a 2 : 1 molar ratio (final pH = 7.0). Furosemide was kindly provided by Hoechst-Roussel Pharmaceuticals (Somerville, NJ). HEPES was purchased from United States Biochemical (Cleveland, OH). All other chemicals were from Sigma Chemical (St. Louis, MO) and were of reagent grade or of the highest purity available.

Results

Effects of varying pH on ^{3}H-Folate Uptake

Folate (0.1 μM) uptake at 2.4 sec was measured in the presence of different pH gradients of identical magnitude (1 pH unit) with or without 1 mM DIDS. At this concentration of DIDS, the equilibrium ^{3}H-glucose uptake was not affected (*data not shown*) thereby excluding a nonspecific effect of this membrane transport inhibitor on vesicle integrity. At higher pH ranges, DIDS-sensitive folate uptake was less compared with uptake at lower pH ranges (Fig. 1*A*). Since DIDS-sensitive folate uptake was stimulated by stepwise increments in internal pH at constant external pH (Fig. 1*B*), inhibition by increasing internal pH is unlikely to account for the decrement in folate uptake at higher pH ranges.

Lack of equilibration between vesicle interior and preincubation buffer at higher pH, or more rapid dissipation of the pH gradient, might also explain diminished folate uptake. Therefore, in the absence

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the elimination of the pteridine ring (*p*-aminobenzoylglutamic acid) only minimally influences the pK_a's of the α- and γ-carboxyl groups (pK_a's = 3.76 and 4.83, respectively; see Table I, ref. [6]), the relatively minor differences in pteridine ring structure between different folate species is unlikely to significantly affect α- and γ-carboxyl group dissociations. Thus, we can infer pK_a values of 3.4 (range: 3.1–3.76) and 4.8 (range: 4.7–4.83) for the α- and γ-carboxyl groups of folate, respectively.

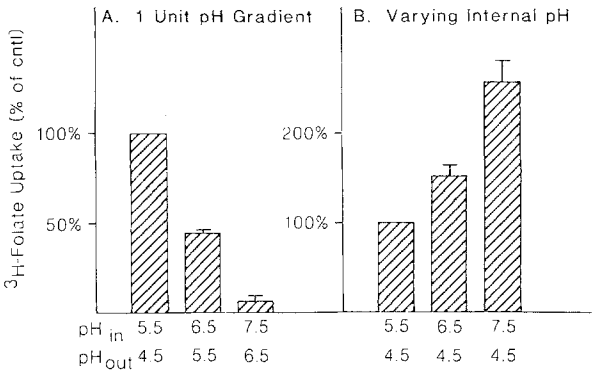

Fig. 1. Effects of varying pH conditions on folate uptake. Vesicles were preincubated for 2 hr at room temperature in (final concentrations in mM): 150 mannitol, 50 K^+ gluconate, and pH 7.5 (55 Tris, 85 HEPES), pH 6.5 (32 Tris, 74 HEPES, 34 MES), or pH 5.5 (20 Tris, 23 HEPES, 97 MES) buffer. Uptakes measured at 2.4 sec were initiated by adding vesicles to reaction medium at 30°C with or without 1 mM DIDS. Final composition of reaction medium was 150 mM mannitol, 50 mM K^+ gluconate, 0.1 μM ^{3}H-folate, and pH 5.5, 6.5, or 4.5 (3 mM Tris, 5 mM HEPES, 132 mM MES) buffer as indicated on the abscissa. The DIDS-sensitive component of uptake (hatched bars) was calculated as the difference between total uptake and uptake in the presence of 1 mM DIDS and averaged 98.7 ± 1.7% of total uptake. Under pH_{in} 5.5/pH_{out} 4.5 conditions, DIDS-sensitive uptake at 2.4 sec was 0.298 ± 0.081 pmol/mg protein

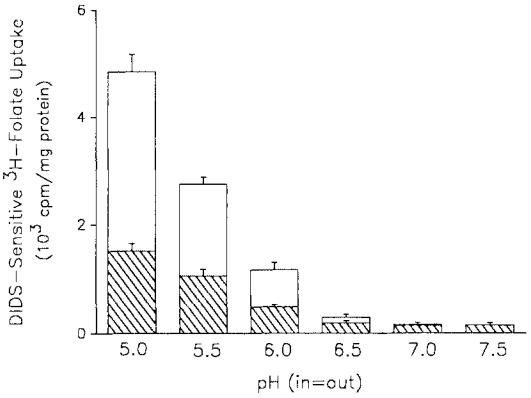

Fig. 2. Folate stimulated ^{3}H-folate uptake under different pH conditions. Vesicles were preincubated in 140 mM Tris/HEPES/MES buffer, 150 mM mannitol, 50 mM K^+ gluconate with (open bars) or without (hatched bars) 5 μM TMA_2folate. Uptakes measured at 6 sec were initiated by adding 5 μl of vesicles to 95 μl of reaction medium with or without 1 mM DIDS (final composition: 140 mM Tris/HEPES/MES buffer, 150 mM mannitol, 50 mM K^+ gluconate, 0.25 μM TMA_2folate, and 0.035 μM ^{3}H-folate). The ratios of Tris/HEPES/MES were 40/82/18, 35/24/81, and 9/13/118 mM for pH 7.0, 6.0, and 5.0 buffers, respectively. *See* Fig. 1 legend for composition of other buffers

of a pH gradient, an outwardly directed folate gradient (in/out: 5/0.285 μM) was used to drive ^{3}H-folate uptake (Fig. 2). Under these conditions, the folate: OH^- exchanger (or H^+: folate cotransporter) mediates $folate_{int}$: $folate_{ext}$ exchange [15]. DIDS-sensitive, folate gradient-stimulated ^{3}H-folate uptake was greater at pH 5.0, than at pH 6.0 and was not detectable at pH ≥ 6.5 (open bars, Fig. 2). Even in the absence of internal folate (hatched bars, Fig. 2), DIDS-sensitive ^{3}H-folate uptake was 10-fold greater at low compared with high pH. These data suggest that in the absence of a pH gradient, folate uptake is carrier mediated (i.e., DIDS sensitive) and is optimal at low pH.

Since high internal pH did not inhibit folate uptake (Fig. 1*B*), the effect of varying external pH on DIDS-sensitive folate uptake was examined (Fig. 3). With increasing external H^+ concentration (pH_{ext} 6.5 → 4.35), folate (0.08 μM) uptake was saturable. For pH values 5.5 → 4.35, a computer-derived [2], weighted least-squares fit of the individual data points yielded a rectangular hyperbola and a H^+ K_m (or OH^- IC_{50}) corresponding to pH 4.89 ± 0.04. At high external pH (pH 6.5 → 5.65), the Hill coefficient (n_{app}) exceeded 1.0 (*vide infra*) and consequently, the plot of folate uptake *versus* H^+ concentration did not follow Michaelis-Menton kinetics over this pH range. All of the individual data points and the

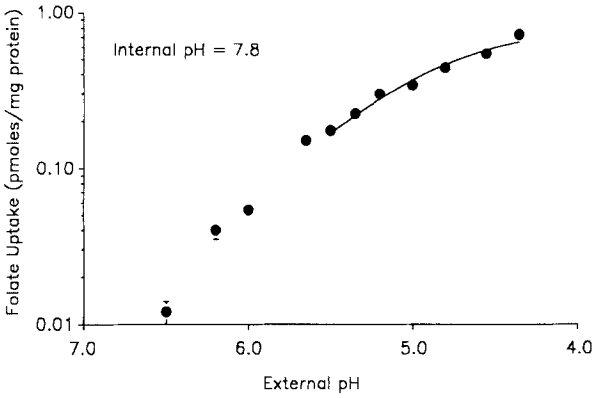

Fig. 3. Effect of varying external pH on pH gradient-stimulated folate uptake. Vesicles were preincubated in (final composition in mM): pH 7.8 buffer (70 Tris, 70 HEPES) and 250 mannitol. Reaction medium consisted of varying proportions of Tris/HEPES/MES to achieve pH conditions ranging from pH 4.3 → 6.5, 250 mM mannitol, and 0.08 μM TMA_2folate. Folate uptake was determined at 1.8 sec in the absence or presence of 1 mM DIDS. The DIDS-sensitive component of uptake was 93.0 ± 1.1% of total uptake. A computer-derived [2], weighted, least-squares fit of the individual data points for pH values between 5.5 and 4.35 yielded a rectangular hyperbola and a folate V_{max} of 0.944 pmol · mg protein^{-1} · 1.8 sec (*see* text for details). Symbols depict the means ± SE of individual data points

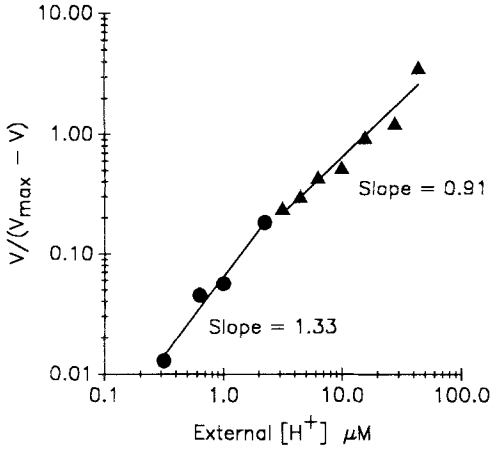

Fig. 4. Hill plot of folate uptake. $V/(V_{max} - V)$ values are plotted on the ordinate using uptake data from Fig. 3. The lines connecting the points of this log-log plot are regression lines from which slopes were calculated. Since the decades on both axes are the same size, the slopes are equivalent to the Hill coefficients (*see* p. 372, ref. [16]). For each animal tested ($n = 6$), a V_{max} value was calculated (*see* Fig. 3 legend for method) and regression lines were determined from log-log plots of $V/(V_{max} - V)$ *vs.* $[H^+]$. In arriving at all slopes, the R^2 (coefficient of determination) was 87% or more indicating a good linear fit. The slopes of the two regression lines were compared by a paired *t* test using the SAS (Statistical Analysis System) computer package and were statistically different ($P = 0.0296$)

computer fit of the pH 5.5 → 4.35 data (solid line) are plotted in log-log format in Fig. 3.

To determine the stoichiometry of folate transport with respect to H^+ (or OH^-), a Hill plot was constructed from the uptake data in Fig. 3. At low external pH (4.35 → 5.5, Fig. 4), the Hill plot was linear with a slope of 0.91 ($=n_{app}$). If transport of monovalent folate (F^-) predominates over this pH range (pK_a's = 3.4, 4.8)[2], the Hill coefficient (n_{app}) of ≤1 suggests that F^- is cotransported with one H^+ (or exchanges for one OH^-). In contrast, at high external pH (pH 5.65 → 6.5, Fig. 4), the apparent Hill coefficient of 1.33 suggests that 2 H^+ (or 2 OH^-) are transported with divalent folate (F^{-2}), the predominant folate species at this pH.

If these predictions of the stoichiometry of folate transport are correct, folate uptake under pH gradient conditions should be electroneutral at both low and high external pH. To test this hypothesis, an inside-negative potential was established by exposing the vesicles to an outwardly directed K^+ gradient in the presence of the K^+ ionophore, valinomycin. Uptake under these conditions was similar to uptake in the absence of an electrical potential ($[K^+]$ in/out = 50/50 mM plus valinomycin) at both low and high external pH (Fig. 5*A*). In contrast, in the presence of identical K^+ (in > out) and pH gradients, Na^+-stimulated glucose uptake, which was previously shown to be electrogenic [10], was enhanced 2.5-fold confirming that an inside-negative electrical potential was indeed established under these conditions (Fig. 5*B*). In aggregate, these data suggest electroneutral $F^- : OH^-$ exchange (or $H^+ : F^-$ cotransport) at low external pH and $F^{-2} : 2OH^-$ exchange (or $2H^+ : F^{-2}$ cotransport) at high external pH.

Since the kinetic parameters (K_m, V_{max}) for the transport of F^- and F^{-2} may differ, the analysis of folate kinetics takes into account the effect of external pH on the F^-/F^{-2} ratio (*vide infra*). The kinetics of folate uptake were determined by incubating membrane vesicles with varying folate concentrations under conditions of constant internal pH (7.8) and varying external pH (4.55, 5.0, or 5.8) in the presence or absence of 1 mM DIDS. The time points used for the uptakes were within the linear portions of the initial uptake rates and the regression lines passed through or near the origin, implying that under these conditions binding does not contribute significantly to uptake (*data not shown*). The DIDS-sensitive component of folate uptake, calculated as the difference between total uptake and uptake in the presence of 1 mM DIDS, was saturable (Fig. 6). Computer-derived, weighted least-squares fits of the individual data points yielded rectangular hyperbolas (Fig. 6) and values for folate K_m and V_{max} as depicted in panel *A* of the Table.

If increasing external pH from 4.55 to 5.0 does not affect the folate carrier, the apparent change in folate K_m or V_{max} between pH 4.55 and 5.0 would have to be explained by differing proportions of F^- and F^{-2} with different kinetic parameters for the two folate species. Since the ratio of F^-/F^{-2} is known at external pH 4.55 and 5.0, two simultaneous equations with two unknowns can be used to calculate the K_m (or V_{max}) for F^- and F^{-2} (panel B, Table). Knowing these kinetic parameters for F^- and F^{-2}, the folate K_m and V_{max} can be predicted at other external pH values, e.g., pH 5.8 (panel *C*, Table). Since the predicted folate K_m (1.33 μM) closely approximates the experimentally determined value (1.70 ± 0.27 μM; panel *A*, Table), these data suggest that the affinity of folate for the carrier is not affected by increasing external pH. In contrast, the predicted V_{max} (123.8 pmol · mg protein^{-1} · min^{-1}) is twofold greater than the experimentally determined V_{max} (58.0 ± 5.6 pmol · mg protein^{-1} · min^{-1}; panel *A*, Table), suggesting that increasing external pH lowers the V_{max} for carrier-mediated folate transport.

If this analysis of the data is correct, then the K_i for a competitive inhibitor of folate transport, e.g., the monovalent anion sulfasalazine [11, 21], should be the same irrespective of the external pH, pro-

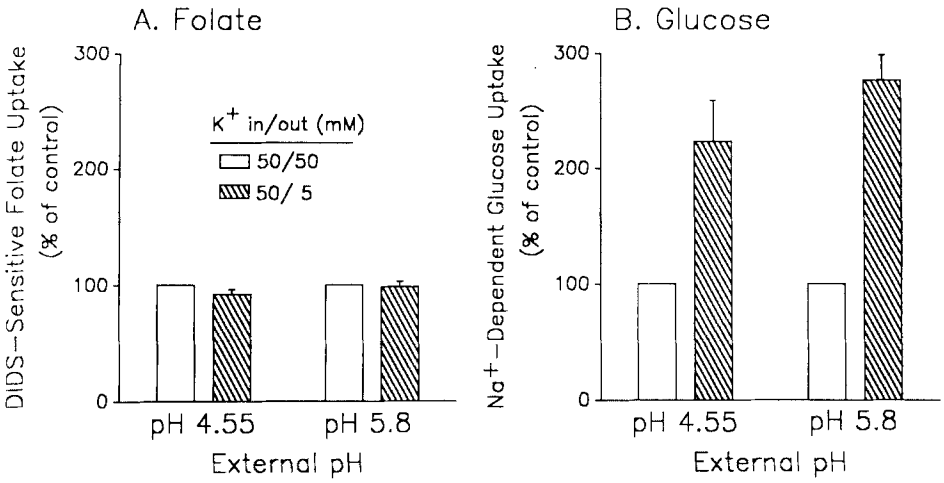

Fig. 5. Effects of inside-negative electrical potential on folate or glucose uptake. (*A*) Vesicles were preincubated in pH 7.8 buffer (*see* Fig. 3 legend), 150 mM mannitol, 50 mM K^+ gluconate, and valinomycin (10 μg/mg protein for 30 min). Reaction medium consisted of (in mM): pH 4.55 (3.5 Tris, 4.5 HEPES, 132 MES) or pH 5.8 (32 Tris, 17 HEPES, 91 MES) buffer, 0.1 μM ^{3}H-folate, and either 150 mannitol/50 K^+ gluconate (voltage-clamped control, open bars) or 240 mannitol/5 K^+ gluconate (inside-negative potential, hatched bars). Folate uptake was measured at 1.2 (pH 4.55) or 2.4 sec (pH 5.8) in the absence or presence of 1 mM DIDS. Under voltage-clamped conditions, folate uptake was 0.204 ± 0.023 or 0.079 ± 0.029 pmol/mg protein at pH_{ext} 4.55 or 5.8, respectively. (*B*) The pH buffers, K^+ gluconate gradients, and time points for uptake measurements were identical to *A*. When vesicles were used with pH 4.55 reaction medium, the preincubation buffer included 275 mM mannitol. The pH 4.55 reaction medium consisted of 0.01 mM ^{3}H-glucose and either 130 mM Na or TMA gluconate. In the pH 5.8 reaction medium, 60 mM Na (or TMA) chloride substituted for 130 mM Na (or TMA) gluconate. The mannitol concentration in the reaction medium was adjusted to maintain isosmolality. Under voltage-clamped conditions, glucose uptake was 15.5 ± 2.9 or 11.8 ± 3.1 pmol/mg protein at pH_{ext} 4.55 or 5.8, respectively

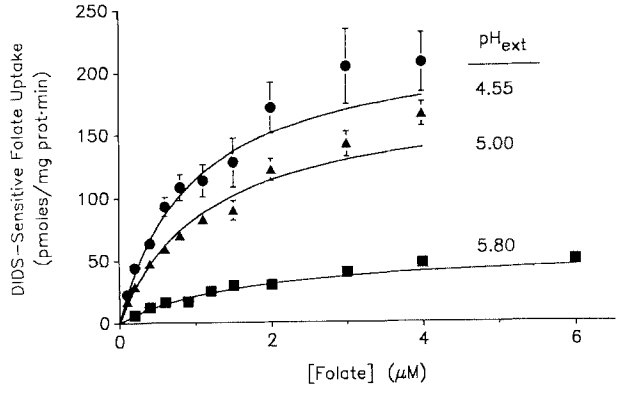

Fig. 6. Kinetics of folate uptake. Under pH gradient conditions [pH_{int} = 7.8; pH_{ext} = 4.55 (●), 5.0 (▲), or 5.8 (■)], folate uptake was measured in the presence or absence of 1 mM DIDS at 1.8, 2.4, or 4.8 sec, respectively. Buffer composition was identical to that described in the legends to Figs. 2, 3, and 5, except for the replacement of 150 mM mannitol/50 mM K^+ gluconate by 250 mM mannitol in the preincubation and reaction media. Calculation of DIDS-sensitive component of uptake and computer fit to generate depicted rectangular hyperbolas (solid lines) are described in the legends to Figs. 1, 3, and 5

vided the competitive inhibitor shares the same binding site with folate. Under pH gradient conditions, sulfasalazine inhibited DIDS-sensitive folate uptake in a dose-dependent manner. At external pH 5.35 or 5.8 (Fig. 7*A* or *B*, respectively), Dixon plots of uptake with 0.1 or 1.4 μM folate gave linear regression lines that intersected above the abscissa, a finding consistent with competitive inhibition. The derived K_i at pH 5.35 was similar to the value at pH 5.8 (64.7 *vs.* 58.5 μM, respectively).

Discussion

In our previous studies, we presented evidence for a carrier on jejunal BBM vesicles that mediates folate: OH^- exchange or phenomenologically equivalent, H^+: folate cotransport [14]. These studies suggest that the pH gradient across the BBM is the driving force for energizing uphill folate transport. However, if the transmembrane pH gradient is the sole determinant of carrier-mediated folate uptake, uptake should be similar with the same pH gradient at high or low pH ranges. In the present study, we found that DIDS-sensitive folate uptake was greater at low compared with high pH ranges. While these data are most consistent with a pH effect indepen-

Table. Analysis of folate kinetics at varying external pH[a]

A. Experimental data	B. Kinetic parameters for F^- and F^{-2}	C. Predicted parameters at pH_{ext} 5.80
	Kinetic parameters at external pH 4.55 and 5.0 are weighted averages of values for F^- and F^{-2} (Eqs. (B.1) and (B.2) for pH 4.55 and 5.0, respectively)	
pH_{ext} K_m (μM)	pH_{ext}	Predicted K_m =
4.55: 0.954 ± 0.148	1) $0.64x + 0.37y = 0.954$ μM 4.55	$0.09 \cdot x + 0.91 \cdot y$
5.00: 1.127 ± 0.134	2) $0.39x + 0.61y = 1.127$ μM 5.00	
5.80 1.703 ± 0.274[b]		$0.09 \cdot 0.705$ μM + $0.91 \cdot 1.396$ μM
	Solving for x and y	
	$x = F^-$ $K_m = 0.705$ μM	= 1.334 μM
	$y = F^{-2}$ $K_m = 1.396$ μM	
pH_{ext} V_{max}	pH_{ext}	Predicted V_{max}
4.55: 223.7 ± 20.0	1) $0.64x + 0.36y = 223.7$ 4.55	$0.09 \cdot x + 0.91 \cdot y$
5.00: 178.3 ± 12.7	2) $0.39x + 0.61y = 178.3$ 5.00	
5.80: 58.0 ± 5.6 [c]		$0.09 \cdot 289.1 + 0.91 \cdot 107.4$
	Solving for x and y	
	$x = F^-$ $V_{max} = 289.1$	= 123.8
	$y = F^{-2}$ $V_{max} = 107.4$	

[a] A computer fit of the individual data points in Fig. 6 gave folate K_m and V_{max} values as shown in *A*. If external pH does not affect folate kinetics, then the K_m (or V_{max}) at any pH is the weighted average of K_m (or V_{max}) values for F^- and F^{-2}. Kinetic parameters for F^- (x) and F^{-2} (y) were calculated using simultaneous equations based on known ratios of F^- and F^{-2} at pH 4.55 and 5.0 (*B*). These kinetic parameters (x,y) were used to predict the folate K_m (or V_{max}) at pH 5.8 (*C*).

[b] Significantly different from K_m at pH_{ext} 4.55 based on a two-tailed Z test, ($Z = 2.405$, $P = 0.016$).

[c] Significantly different from V_{max} values at pH_{ext} 4.55 and 5.0 based on a two-tailed Z test (Z values = 7.98 and 8.68, respectively; $P < 0.0002$).

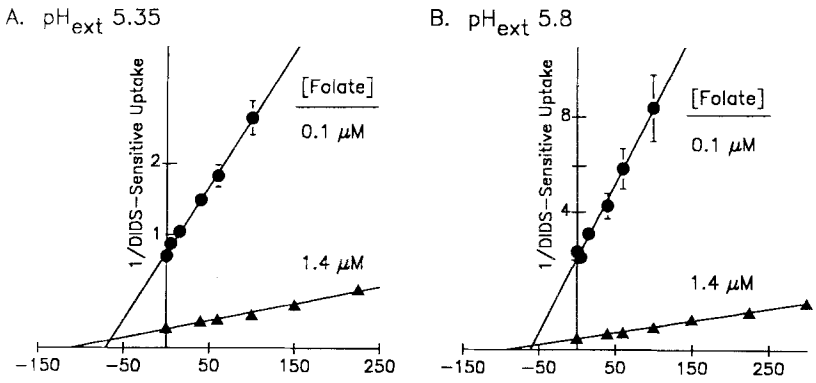

Fig. 7. Dixon plots of sulfasalazine inhibition of folate uptake. Uptake of 0.1 μM (circles) or 1.4 μM (triangles) folate was measured at 6 sec in the absence or presence of 1 mM DIDS as the sulfasalazine concentration was varied from 0 → 300 μM. The preincubation buffer was pH 7.8 and the reaction medium was either pH 5.35 [16 mM Tris, 20 mM HEPES, 104 mM MES; (*A*)] or pH 5.8 (*B*). *See* the legends to Figs. 3 and 5 for buffer composition

dent of the transmembrane pH gradient, two alternative explanations need to be considered.

First, the vesicles might be more permeable to H^+ (or OH^-) at high pH ranges allowing for more rapid dissipation of the pH gradient. Since the folate uptake rate was linear for 6.0 sec at pH_{ext} 5.8, but only 2.4 sec at pH_{ext} 4.55 (*data not shown*), this explanation is unlikely. Moreover, initial uptake rates were determined at 2.4 sec, a time point within the linear portion of the initial uptake rate. Thus, true initial rates were obtained at both low and high pH ranges.

Second, an accurate comparison of folate uptake at high *versus* low pH ranges requires that the desired internal pH be achieved during the preincubation period. Although the pH_{int} was not directly measured, the experimental conditions were chosen to enhance the likelihood of equilibration between vesicle interior and preincubation buffer. Specifically, all buffers included 50 mM K^+ gluconate.

These conditions were chosen based on studies using tracheal epithelial vesicles [8]. When tracheal vesicles are incubated in buffers *without* added salt, an inside-negative electrical potential (presumably secondary to intramembranous proteins with fixed negative charges) stimulates uptake of protons rendering the vesicle inside-acid compared with the incubation buffer. Since the magnitude of this effect is greatest in alkaline buffers, uptakes requiring a high internal pH are more affected than uptakes at low pH. In tracheal vesicles, the inside-negative electrical potential is abolished by adding various salts including 50 mM K^+ gluconate to the incubation buffer. If similar phenomena are operative in jejunal BBM vesicles, our experimental conditions would avoid the inside-negative electrical potential that might otherwise result in intravesicular acidification relative to the incubation buffer.

To validate that the decrease in carrier-mediated folate uptake at high pH ranges was secondary to alkaline pH conditions, an outwardly directed folate gradient was used to drive ^{3}H-folate uptake in the absence of a pH gradient ($folate_{int}$: ^{3}H-$folate_{ext}$ exchange). The decrement in DIDS-sensitive, folate-stimulated ^{3}H-folate uptake with increasing pH is consistent with an inhibitory effect of high pH. Since increasing internal pH *stimulates* folate uptake (external pH constant; Fig. 1*B*), the decrement in uptake at high pH ranges is most likely secondary to high external, not internal pH. Alternatively, with increasing internal pH, the driving force for folate uptake is enhanced thereby masking an inhibitory effect of high internal pH. To rule out the latter possibility, folate uptake was measured as a function of varying internal pH. With increasing internal [OH^-], folate uptake was saturable with half-maximal uptake at pH_{int} 6.09 ± 0.03 (*data not shown*). More importantly, uptakes between pH_{int} 7.5 and 8.2 closely approximated the values predicted from a computer-derived rectangular hyperbola (105–112% of expected), suggesting that high internal pH does not inhibit folate uptake.

The external [OH^-] required to half-maximally inhibit folate uptake, or the [H^+] required to half-maximally stimulate folate uptake, corresponds to a pH of 4.89. These data suggest that transport is critically dependent on an amino acid residue having a pK_a between 4.5 and 5.0. This pH range might indicate a carboxyl containing amino acid, i.e., an aspartic or glutamic acid. Others have shown that glutamate residues are required for the transport activity of the erythrocyte Band 3 protein (anion exchanger mediating Cl^- : HCO_3^- exchange, ref. [4]) and the bacterial H^+ : lactate cotransporter [5]. Kaback [5] proposed that the glutamate residue in the bacterial H^+ : lactate cotransporter participates in a charge relay system that facilitates the translocation of protons across the membrane. If future studies identify a class of pH-dependent transport processes that require carboxyl containing amino acids, these data would suggest a common transport mechanism.

The slopes of the Hill plots, or Hill coefficients (n_{app}), gave estimates of the stoichiometry of folate transport with respect to external H^+ (OH^-) ions. Since the Hill coefficient depends on the number of H^+ (or OH^-) binding sites and the strength of the interactions between the binding sites, the n_{app} may underestimate the actual number of sites [16]. In practice, the next highest integer above the n_{app} gives the *minimum* number of possible H^+ (or OH^-) binding sites. Since folate uptake was electroneutral over the entire pH range, the n_{app} values of 0.91 (pH 4.35 → 5.5) and 1.33 (pH 5.65 → 6.5) are most consistent with $H^+ : F^-$ (or $F^- : OH^-$ exchange) and $2H^+ : F^{-2}$ (or $F^{-2} : 2OH^-$ exchange) cotransport at low and high pH, respectively. Other combinations that maintain electroneutrality are also possible, e.g., $2H^+ : 2F^-$ and $4H^+ : 2F^{-2}$ cotransport at low and high pH, respectively.

Our analysis of folate kinetics at varying external pH takes into account the possibility that both F^- and F^{-2} are transported, but with different kinetic parameters. By assuming that varying external pH (4.55 → 5.0) does not affect the folate K_m or V_{max}, we were able to calculate theoretical K_m and V_{max} values for F^- and F^{-2}. Since these kinetic parameters for F^- and F^{-2} correctly predict the folate K_m at pH 5.8, but not the V_{max}, the assumption that external pH does not affect folate kinetics is valid only for the K_m. Thus, the ≈twofold effect of external pH on the experimentally determined K_m over the pH range tested (pH 4.55 → 5.8; panel *A*, Table) is likely due to a change in the relative proportion of F^- to F^{-2}. Furthermore, if F^- and F^{-2} are transported, the calculated K_m values for F^- and F^{-2} are likely to be correct, whereas the V_{max} values for F^- and F^{-2} do not accurately reflect the effect of increasing external pH.

While the data are most consistent with transport of both F^- and F^{-2}, other possibilities have to be considered. For example, if F^{-2} is exclusively transported by $2H^+ : F^{-2}$ cotransport, poor cooperativity between H^+ binding sites at low pH could account for the n_{app} value of 0.91. Alternatively, if only F^- is transported, $2H^+ : 2F^-$ cotransport at high pH would maintain electroneutrality and explain the n_{app} value of 1.33.

The kinetics data were reanalyzed assuming that the carrier transports only F^{-2} or F^-. At pH 5.8, the concentration of F^{-2} is 2.5-fold greater than at pH 4.55. If F^{-2} were exclusively transported, and as-

suming no effect of external pH on folate kinetics, the apparent K_m at pH 5.8 should be 2.5-fold lower than at pH 4.55. Since the apparent K_m at pH 5.8 is actually greater than the K_m at pH 4.55 (1.703 and 0.954 μM, respectively), this would require that increasing external pH lowers the affinity of the carrier for F^{-2}. While theoretically possible, this contradicts the finding that external pH does not affect the affinity of sulfasalazine, a monovalent competitive inhibitor [11, 21], for the carrier. If F^- is the transported species, a similar analysis of the data would lead to the conclusion that raising external pH increases the affinity of F^- for the carrier. Such an effect of increasing external pH on F^- transport is difficult to conceptualize in terms of a transport model and contradicts the K_i data for sulfasalazine. Therefore, it is unlikely that the carrier transports only one folate species.

The transport affinity (K_m) of folate was measured under conditions of varying external pH. While the K_m is a measure of intrinsic binding affinity, the complete equation for K_m includes terms for the translocation rates of the loaded carrier between outward (X_o) and inward (X_i) facing conformations [3]. Theoretically, increasing external pH might have raised the intrinsic binding affinity of folate 10-fold. If this were accompanied by either a 10-fold decrease in the translocation rate from $X_o \rightarrow X_i$, or a 10-fold increase in translocation rate from X_i to X_o, then the experimentally determined folate K_m would be *unchanged*. Intuitively, such equal and opposite effects of external pH seem unlikely. Thus, the most likely interpretation of the kinetics data is that external pH does not affect the intrinsic binding affinity of folate for the carrier.

Since increasing external pH lowers the folate V_{max} without affecting the K_m, our data excludes the possibility that folate and OH^- ions compete for a mutually exclusive binding site on the carrier. Such a mechanism would predict an increase in folate K_m with increasing [OH^-]. If OH^- ions are the transported species, the data is most consistent with a model in which folate and OH^- ions are bound to an outward-facing conformation of the carrier forming a ternary complex (Fig. 8). According to this scheme, the OH^--loaded, outward-facing conformation of the carrier binds external folate (*step 2*) prior to releasing OH^- (*step 3*). In such transport schemes, increasing external [OH^-] affects either the folate K_m or V_{max} depending on the various rate constants of the reaction (*see* product inhibition of bi-bi systems [16]). Similar models assuming H^+ : folate cotransport may also be constructed. As with other pH-dependent transport processes, analysis of kinetics does not distinguish between OH^- and H^+ mechanisms. For example, although amiloride-sensitive, pH gradient-stimulated Na^+ transport is usually referred to as $Na^+ : H^+$ exchange, the data is equally compatible with $Na^+ : OH^-$ cotransport [1].

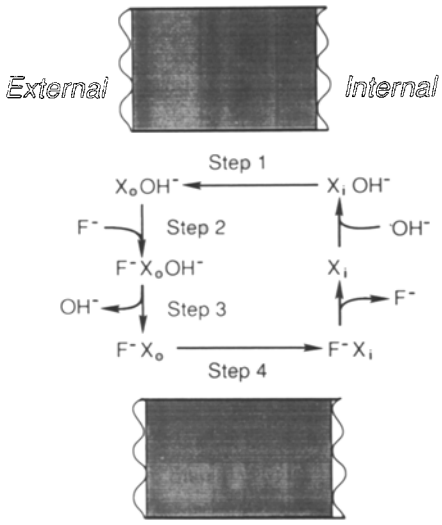

Fig. 8. Model of folate: OH^- exchange in which folate and OH^- ions are bound to an outwardly facing conformation of the carrier forming a ternary complex ($F^- \cdot X_o \cdot OH^-$)

In summary, pH sensitivity of the carrier *per se* is not required to account for the observation that pH gradients of identical magnitude are not equally effective in driving folate uptake. Instead, a transport mechanism involving either folate : OH^- exchange or H^+ : folate cotransport can fully account for the effects of external pH on vesicle folate uptake. Furthermore, our data excludes competition between OH^- and folate ions for a mutally exclusive binding site and argues for electroneutral transport of monovalent and divalent folate by the carrier. A more complete model of jejunal folate transport will require detailed structural information regarding the carrier protein.

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References

1. Aronson, P.S. 1985. Kinetic properties of the plasma membrane $Na^+ - H^+$ exchanger. *Annu. Rev. Physiol.* **47**:545–560
2. Cleland, W.W. 1979. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**:103–138
3. Fröhlich, O., Gunn, R.B. 1986. Erythrocyte anion transport: The kinetics of a single-site obligatory exchange system. *Biochim. Biophys.Acta.* **864**:169–194
4. Jennings, M.L., Anderson, M.P. 1987. Chemical modifica-

tion and labeling of glutamate residues at the stilbenedisulfonate site of human red blood cell band 3 protein. *J. Biol. Chem.* **262:**1691–1697
5. Kaback, H.R. 1988. Site-directed mutagenesis and ion-gradient driven active transport: On the path of the proton. *Annu. Rev. Physiol.* **50:**243–256
6. Kallen, R.G., Jencks, W.P. 1966. The dissociation constants of tetrahydrofolic acid. *J. Biol. Chem.* **241:**5845–5850
7. Kurtin, P., Charney, A.N. 1984. Intestinal ion transport and intracellular pH during acute respiratory alkalosis and acidosis. *Am. J. Physiol.* **247:**G24–G31
8. Langridge-Smith, J.E., Dubinsky, W.P. 1986. Relationship of the Donnan potential to the transmembrane pH gradient in tracheal apical membrane vesicles. *J. Membrane Biol.* **94:**197–204
9. Lucas, M.L., Blair, J.A. 1978. The magnitude and distribution of the acid microclimate in proximal jejunum and its relation to luminal acidification. *Proc. R. Soc. London A* **200:**27–41
10. Murer, H., Hopfer, U. 1974. Demonstration of electrogenic Na^+-dependent D-glucose transport in intestinal brush border membranes. *Proc. Natl. Acad. Sci. USA* **71:**484–488
11. Nygård, B., Olofsson, J., Sandberg, M. 1966. Some physicochemical properties of salicylazosulphapyridine, including its solubility, protolytic constants and general spectrochemical and polarographic behaviour. *Acta Pharm. Suec.* **3:**313–342
12. Poe, M. 1977. Acidic dissociation constants of folic acid, dihydrofolic acid, and methotrexate. *J. Biol. Chem.* **252:**3724–3728
13. Said, H.M., Ghishan, F.K., Redha, R. 1987. Folate transport by human intestinal brush-border membrane vesicles. *Am. J. Physiol.* **252:**G229–G236
14. Schron, C.M., Washington, C., Jr., Blitzer, B.L. 1985. The transmembrane pH gradient drives uphill folate transport in rabbit jejunum: Direct evidence for folate/hydroxyl exchange in brush border membrane vesicles. *J. Clin. Invest.* **76:**2030–2033
15. Schron, C.M., Washington, C., Jr., Blitzer, B.L. 1988. Anion specificity of the jejunal folate carrier: Effects of reduced folate analogues on folate uptake and efflux. *J. Membrane Biol.* **102:**175–183
16. Segel, I.H. 1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. pp 360–375. John Wiley and Sons, New York
17. Selhub, J., Rosenberg, I.H. 1981. Folate transport in isolated brush border membrane vesicles from rat intestine. *J. Biol. Chem.* **256:**4489–4493
18. Shimada, T., Hoshi, T. 1987. Role of Na^+/H^+ antiport in intracellular regulation by rabbit enterocytes. *Biochim. Biophys. Acta* **901:**265–272
19. Smith, M.E., Matty, A.J., Blair, J.A. 1970. The transport of pteroylglutamic acid across the small intestine of the rat. *Biochim. Biophys. Acta* **219:**37–46
20. Strum, W.B. 1979. Enzymatic reduction and methylation of folate following pH-dependent, carrier-mediated transport in rat jejunum. *Biochim. Biophys. Acta* **554:**249–257
21. Zimmerman, J., Selhub, J., Rosenberg, I.H. 1985. Drug-folate interactions in intestinal folate absorption: Comparison of sulfasalazine, phenytoin and salicylates. *Gastroenterology* **88:**1643

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