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ENZYMATIC REDUCTION AND METHYLATION OF FOLATE FOLLOWING pH-DEPENDENT, CARRIER-MEDIATED TRANSPORT IN RAT JEJUNUM

WILLIAMSON B. STRUM

Department of Medicine, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037 (U.S.A.)

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Summary

Intestinal transport of [^{3}H]folate was studied using everted sacs of rat jejunum. The proximal small intestine transports folate against a concentration gradient by a system which is saturable, pH-dependent, energy-dependent, sodium-dependent, sensitive to temperature, and appears to be a common transport system for folate compounds. Chromatographic analysis of folate compounds in the serosal compartment after a 60 min incubation with folate in the mucosal medium in sodium phosohate buffer indicated that metabolism of folate to 5-methyltetrahydrofolate was extensive at pH 6.0 and negligible at pH 7.5. The percent conversion of folate to 5-methyltetrahydrofolate at pH 6.0 was reduced by increasing the concentration of folate in the mucosal medium, thus indicating saturation of the reduction and methylation process. These findings indicate that folate transport in rat jejunum occurs by an energy-dependent, carried-mediated system and that both folate transport and intestinal conversion of folate to 5-methyltetrahydrofolate are pH-dependent.

Introduction

The mechanism of intestinal transport of folate compounds is a complex subject that has been under study for almost two decades. The observations that folate transport in other mammalian cells occurs by an active, carrier-mediated process [1—3], the reports of congenital folate malabsorption [4,5] and the characterization of a folate-binding protein in rat intestinal brush

Abbreviation: pCMS, *p*-chloromercuriphenylsulfonate.

border [6] suggested that a carrier mechanism should be responsible for folate transport in the intestine. However, reports in the literature were interpreted as consistent with both active and passive mechanisms (reviewed by Rosenberg [7]). This controversy was primarily a result of the influence of pH on intestinal transport of folate compounds [8—11] and recent studies from this laboratory have described a pH-dependent, carrier-mediated transport system for the folate analog, amethopterin, in proximal rat intestine [12]. Metabolism of folate to reduced coenzyme forms is known to occur during intestinal transport [13,14], but this process has not been evaluated in light of the apparent importance of pH on the transport system. The present study was performed to extend prior studies concerning the influence of pH on the mechanism of folate transport, to compare these findings with the intestinal transport of amethopterin, and to determine the effect of optimal conditions of transport on the enzymatic reduction and methylation of the vitamin during transmural intestinal transport.

Materials and Methods

The following materials were obtained commercially: DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ); Aquasol (New England Nuclear, Boston, MA); [3′,5′9-$^3H_3$]folic acid (500 Ci/mol; Amersham/Searle Corp., Des Plaines, IL); unlabeled folate (Calbiochem, La Jolla, CA) and amethopterin (ICN Pharmaceuticals, Plainview, NY); *p*-aminobenzoyl-L-glutamate (Sigma, St. Louis, MO). 10-Formylfolate was prepared by the method of Blakley [15]. 5-Methyltetrahydrofolate and 5-formyltetrahydrofolate were gifts from Dr. J. Whiteley. Preliminary chromatographic studies of [^{3}H]folate on DEAE-Sephadex indicated that the compound was greater than 98% radiochemically pure.

Folate transport in everted intestinal sacs. Male, albino Sprague-Dawley rats weighing 180—220 g were fasted for 18 h and anesthetized lightly with ether; the intestinal segments were washed with saline at 4°C and removed. Everted sacs were prepared from 6-cm segments of intestine, as described previously [12,16]. Results were expressed as pmol of folate transported/min per sac at 37°C after a 45-min incubation; values were corrected to 0.5 g wet weight of the empty sac. All determinations were performed at least in quadruplicate. Viability of the everted sacs has been demonstrated in prior studies [12].

The incubation solution contained 110 mM NaH_2PO_4, 35.7 mM NaCl, 5.5 mM KCl and 1.8 mM $MgSO_4$. When desired, the pH was varied over the range 5.5—7.5 by the addition of 1 N NaOH; the pH of the buffer did not change during the 45—60-min incubations. A sodium-free medium was obtained by substituting equimolar concentrations of potassium or ammonium for sodium in the incubation buffer. Kinetic constants were determined as described previously [12].

Identification of folate compounds in serosal fluid by chromatography. Identification of folate compounds was performed using chromatographic procedures described previously [16—18]. To preserve reduced folate compounds produced during the experiment, 10 mM ascorbate was added to the incubation medium prior to the adjustment of pH. Serosal fluid was combined

from six everted sacs (total volume 6 ml), admixed wity unlabelled marker compounds (2 μmol folate and 5-methyltetrahydrofolate; 1 μmol *p*-aminobenzoyl-L-glutamate) and applied to a 1.0 X 15 cm column of DEAE-Sephadex that had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM mercaptoethanol. A small amount of 2-amino-4-hydroxypteridine was identified as a contaminant in the unlabeled folate. The column was eluted with a linear gradient (0.1—0.8 M) of potassium phosphate buffer, pH 7.0. Fractions (6 ml) were collected and monitored for both radioactivity and absorbance at 280 nm, and the absorbance spectra of the peak tubes were obtained. The results obtained from two or more experiments for each condition were in close agreement.

Results

General features of folate transport

The time-dependent appearance of radiolabel from 0.1 μM folate in the mucosal medium to the serosal compartment of everted jejunal sacs at pH 6.0 and 7.0 is shown in Fig. 1. After an initial lag of approximately 10 min the rate of transport was linear for at least 50 min at a rate of 1.1 pmol/min per sac at pH 6.0 and 0.3 pmol/min per sac at pH 7.0. The addition of a low concentration of amethopterin (50 nM) to the incubation medium, which almost completely blocks folate reduction (vide infra), did not alter the transport rate at pH 6.0. The time course was similar over the concentration range of 10^{-7}—$2 \cdot 10^{-5}$ M, and the rate of transport was consonant with that for amethopterin at the same concentration [12]. The effect of pH on the rate of folate transport is illustrated in more detail in the inset in Fig. 1. A pronounced optimum in the rate of transport was observed at pH 6.0. The system was saturable with a K_m value for folate transport of 4.4 μM and V of 61 pmol/min per sac at pH 6.0. The system remained saturable at pH 7.0 ($K_m \approx 16$ μM and $V \approx 63$ pmol/min per sac) but was not saturable at pH 7.5 at folate concentrations up to $5 \cdot 10^{-5}$ M.

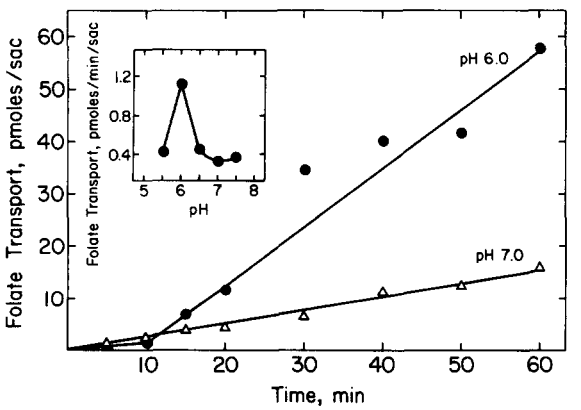

Fig. 1. Time-dependent appearance of folate (0.1 μM) in the serosal compartment of everted jejunal sacs. Standard assay conditions were used as described under Materials and Methods. Insert shows the effects of pH on the folate transport rate under the same conditions.

When folate was present at equal initial concentrations of 0.1 μM on both the mucosal and serosal sides of jejunal sacs at pH 6.0, folate accumulated on the serosal side (serosa/mucosa ratio = 2.1) after a 45 min incubation. Accumulation against a gradient decreased with increasing pH until it was less than one at pH 7.0 and above. Accumulation against a gradient was not observed in ileal sacs under identical conditions. These results are in close agreement with those described for amethopterin [12]. In order to take into account the probability that the molecular species of folate is not the same on both sides of the intestine, the experiments at pH 6.0 were repeated with 50 nM amethopterin in the mucosal medium, and the results were the same.

When the temperature of the incubation medium was reduced from 37 to 27°C, Q_{10} values of 7.2 and 2.3 were observed at pH 6.0 and 7.0, respectively. The removal of sodium from the incubation medium prevented accumulation of folate against a chemical gradient and led to a dramatic decrease in the rate of mucosa-to-serosa folate transport; inhibition of 62% and 77% was observed at pH 6.0 when potassium or ammonium was substituted for sodium. Although less at the higher pH the inhibitor effect of a sodium-free mediumwas demonstrable at pH 6.0 and 7.0. The addition of 10 mM glucose to the sodium phosphate incubation medium at pH 6.0 had no effect on the serosal accumulation of folate against a gradient in either jejunal or ileal sacs and did not affect the rate of folate transport from the mucosal-to-serosal compartment.

Effect of inhibitors

The addition of hydroxylamine, dinitrophenol, azide, fluoride, iodoacetate and *p*-chloromercuriphenylsulfonate (PCMS) to the mucosal medium, the addition of ouabain to the serosal medium, and anaerobiocity produced dramatic decreases in folate transport at pH 6.0 (Table I). Parallel experiments at pH 7.0 demonstrated minimal or no inhibition (Table I). Folate transport was inhibited competitively by other folate compounds including amethopterin ($K_i \approx 20$ μM), 5-methyltetrahydrofolate ($K_i \approx 24$ μM), 5-formyltetrahydro-

TABLE I

EFFECT OF METABOLIC INHIBITORS ON FOLATE TRANSPORT ACROSS EVERTED INTESTINAL SACS

Tranport of folate at pH 6.0 and 7.0 at 37°C using the standard assay technique as described under Materials and Methods. Results are expressed as a percentage of a control containing no inhibitor. Initial folate concentration, 0.1 μM.

Condition	Concentration (mM)	Transport	
		pH 6.0	pH 7.0
No addition		100	100
Anaerobic		19	100
Hydroxylamine	1	35	98
Dinitrophenol	1	26	126
Azide	10	29	111
Fluoride	1	28	101
Iodoacetate	1	37	85
p-Chloromercuriphenylsulfonate	0.1	21	92
Ouabain	10	36	100

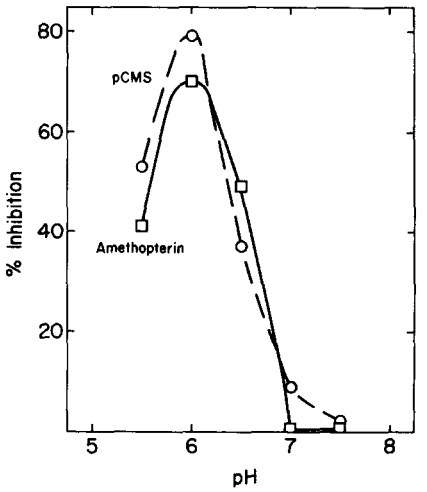

Fig. 2. Effect of pH on inhibition of folate (0.1 μM) transport by amethopterin and *p*-chloromercuriphenylsulfonate. Standard assay conditions were used except that the pH of the buffer was varied from 5.5 to 7.5, as described under Materials and Methods. ○, pCMS, 0.1 mM; □, amethopterin, 50 μM.

folate ($K_i \approx 9$ μM), and 10-formylfolate ($K_i \approx 18$ μM). Further assessment of the inhibition process was obtained by determining the effect of amethopterin (50 μM) and *p*-chloromercuriphenylsulfonate (0.1 mM) over the pH range of 5.5—8.5 (Fig. 2). Maximum inhibition occurred at pH 6.0 and inhibition was negligible at pH 7.0 and above for either compound.

Enzymatic reduction and methylation of folate

The metabolism of folate during intestinal transport at pH 6.0 and 7.5 was compared by experiments in which six jejunal sacs were incubated for 60 min with [^{3}H]folate followed by chromatography of the combined serosal fluids on DEAE-Sephadex. Greater than 95% of the total radioactivity applied to the column recovered in the eluate fractions. A representative experiment is shown in Fig. 3 for pH 6.0. Monitoring the effluent for absorbance revealed a peak with the void volume (fractions 5—12) followed by four well-defined peaks with absorbance spectra identical to 2-amino-4-hydroxypteridine (fractions 14—18) and the unlabeled markers, *p*-aminobenzoyl-L-glutamate (fractions 22—28), 5-methyltetrahydrofolate (fraction 40—50) and folate (fractions 58—70). Small amounts of radioactivity in the early fractions (10—20) may represent other folate forms; 10-formyl folates and other reduced folate forms have been identified in this region of the elution profile using a similar technique [18,19]. With 0.1 μM [^{3}H]folate in the incubation medium, the majority of tritium label co-chromatographed with either folate (36%) or 5-methyltetrahydrofolate (42%), while less than 25% was catabolized to 2-amino-4-hydroxypteridine, *p*-aminobenzoyl-L-glutamate, or unidentified compounds seen in the void volume (Fig. 3). When amethopterin (50 nM) was added to the incubation medium containing 0.1 μM [^{3}H]folate at pH 6.0, only 12% of the radioactivity recovered was identified as 5-methyltetrahydrpfpolate while 71% remained as unchanged folate and the remainder was distributed in the early peaks; these

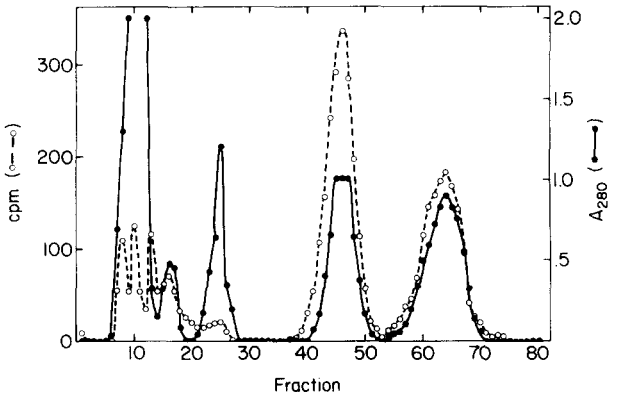

Fig. 3. Chromatographic identification of radioactivity recovered in the serosal compartment of everted jejunal sacs after 60 min of exposure to 0.1 μM [^{3}H]folate in the incubation medium, pH 6.0. Unlabeled folate, 5-methyltetrahydrofolate, and *p*-aminobenzoyl-L-glutamate were added to the serosal sample as markers and chromatography was performed as described under Materials and Methods.

results probably reflect a smaller pool of tetrahydrofolate available for methylation owing to inhibition of dihydrofolate reductase by amethopterin.

Under identical conditions at pH 7.5 (Fig. 4), the tritium label in the serosal medium co-chromatographed with folate (86%) (fractions 46—56), but not with 5-methyltetrahydrofolate (1%) (fractions 38—44); the remainder of the tritium label was recovered in the *p*-aminobenzoyl-L-glutamate (fractions 22—27) and void volume (fractions 9—16). The total amount of folate compounds was decreased by approximately four-fold; the appearance of a greater amount of total folate in the serosal compartment at pH 6.0 is probably a reflection of the more rapid transport rate at this pH (cf. Fig. 1).

When the concentration of folate in the mucosal medium at pH 6.0 was increased to 0.5 μM, the percent of 5-methyltetrahydrofolate recovered in the serosal compartment decreased to approximately 36% and folate increased to

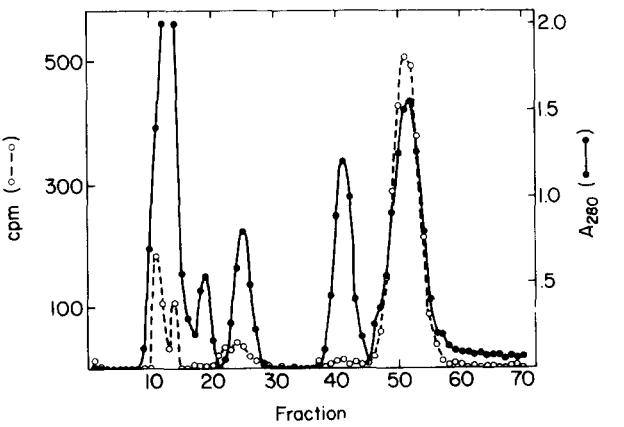

Fig. 4. Chromatographic identification of radioactivity recovered in the serosal compartment of everted jejunal sacs after 60 min of exposure to 0.1 μM [^{3}H]folate in the incubation medium, pH 7.5. The experiment was performed in a manner identical to that shown in Fig. 3 except for the change in pH.

46%. At pH 7.5 and the higher initial level of folate the total amount of folate compounds in the serosal medium was approximately four-fold less, but again, the labeled compound recovered was folate, rather than 5-methyltetrahydrofolate. Thus, maximal intestinal biosynthesis of 5-methyltetrahydrofolate from folate occurs only when the external environmental is acidic and at which time the transport rate is optimized.

In ileal sacs exposed to 0.1 μM [^{3}H]folate at pH 6.0, 21% of the recovered radioactivity was 5-methyltetrahydrofolate while 42% remained as unchanged folate and 38% was distributed equally in the early peaks. These results indicate that the reduction and methylation of folate is less in the ileum but occurs throughout the entire intestine.

Discussion

The present study demonstrates that an active transport system for folate is readily observed at pH 5.5—6.5. In addition to folate transport being maximal at pH 6.0, this conclusion is supported by the following evidence obtained at this pH: (1) accumulation of folate against a chemical gradient; (2) conformation to saturation kinetics; (3) inhibition of transport by folate analogs; (4) inhibition of transport by anoxia and metabolic poisions; (5) temperature sensitivity; (6) sodium-dependence, and (7) specificity for the jejunum. All of these processes are markedly pH-dependent and are less apparent or not detectable at pH 7.0 and above. These studies are further supported by the demonstration that the folate analog, amethopterin, is transported by a very similar, if not identical, system [12] and are of particular interest since the pH at the absorptive surface of the jejunal cells is 5.7—6.5 by direct measurement in both human and rat intestine [20].

The differences between the intestinal transport of folate and amethopterin in the rat include the following: folate is more sensitive to temperature at pH 6.0 (Q_{10} = 7.2 in contrast to Q_{10} = 2.6 for amethopterin); folate is metabolized during transport, although this does not appear to affect the transport rate [13,14]; and the inhibition constants for folate analogs are higher for folate transport than for amethopterin [12].

5-Methyltetrahydrofolate is the primary circulating and storage form of folate compounds [20]. Partial conversion of folate to 5-methyltetrahydrofolate during intestinal transport has been demonstrated in rat intestinal preparations in bicarbonate buffer with added ascorbate [13,14,22] and indirectly in man [23,24]. The present results indicate that intestinal conversion of folate to 5-methyltetrahydrofolate, like, the transport process, is pH-dependent. With an incubation medium at pH 6.0 containing 100 nM folate, newly synthesized 5-methyltetrahydrofolate is the major folate component in the serosal compartment after a 60 min exposure. This process is completely inhibited at pH 7.5. The apparent discrepancy with other reports showing folate reduction and methylation at pH 7.4 [13,14,22] may be the result of acidification by ascorbate in excess of the buffer capacity. These results would also explain the inability of other investigators to demonstrate this metabolic process in an alkaline medium [8]. The reaons for this pH effect are not known, but they may reflect in part that dihydrofolate reductase, probably

the rate-limiting enzyme in the reduction and methylation process, has an acidic pH optimum [25]. These and other studies indicate that the reduction and methylation process is maximal in the jejunum but occurs throughout the small intestine [13,14], is pH-dependent, is time and concentration dependent [13,14], does not appear to affect the transport rate [13,14] and is not demonstrable in the mucosal medium [13,14].

In light of these and other recent experiments [8—12], many of the inexplicable findings related to intestinal folate transport can probably be resolved. The discrepancies appear to be related to using an incubation pH of 7.0 or higher, a bicarbonate buffer which becomes more alkaline during the incubation [9], a folate concentration above the capacity of the active transport system, or a combination of these factors. The results cannot be explained by non-ionic diffusion since folate and amethopterin are affected in the same manner [8,12], although the pK_a for folate (8.38) is considerably higher than that for amethopterin (5.71) [26]. Cumulative data suggest that the mechanism of intestinal folate transport can be defined as: (1) a rapid, carrier-mediated transport system which operates at an acid pH when the folate concentration is 10^{-5} M or less, and (2) a slower, low affinity or passive diffusion process which prevails in an alkaline pH or at substrate concentrations of 10^{-5} M or higher [9].

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