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Na^+ and pH dependence of 5-methyltetrahydrofolic acid and methotrexate transport in freshly isolated hepatocytes *

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The dependence of the high-affinity transport systems for 5-methyltetrahydrofolic acid (5-CH_3-H_4PteGlu) and methotrexate on sodium ions and on pH was examined in freshly isolated rat hepatocytes. Previous studies indicated that transport of these folate derivatives was sodium-dependent. Experiments to determine the K_m for sodium of 5-CH_3-H_4PteGlu transport showed no dependence on extracellular sodium. However, uptake was sodium-dependent when hepatocytes were preincubated for 30 min in sodium-free medium, a treatment which resulted in an increase in the transmembrane pH gradient ($\Delta pH = pH_{out} - pH_{in}$) and a decrease in the uptake of 5-CH_3-H_4PteGlu. Uptake of methotrexate displayed a linear dependence on extracellular sodium ions. Uptake of 5-CH_3-H_4PteGlu increased linearly as the transmembrane pH gradient decreased; i.e., as the medium became more acid with respect to the cytosol. Lineweaver-Burk and Scatchard plots of 5-CH_3-H_4PteGlu uptake indicated an apparent K_m for H^+ of about 24 nM, equivalent to a pH of 7.6. Hill-plots suggested a stoichiometry of 1:1 for the interaction of protons with the 5-CH_3-H_4PteGlu transport system. Both the K_m and V_{max} for 5-CH_3-H_4PteGlu transport were increased at pH 5.5 compared to pH 7.4, suggesting that extracellular protons increased the number of and/or the activity of the membrane carrier. In contrast, methotrexate transport was maximal at pH 7 where the transmembrane pH gradient was zero. These results suggest the possibility that 5-CH_3-H_4PteGlu may be cotransported along with H^+ ions in hepatocytes, although they do not rule out a 'catalytic coupling' whereby protons interact with the carrier to stimulate substrate flux without concomitant H^+ transport

Introduction

Transport of 5-methyltetrahydrofolic acid (5-CH_3-H_4PteGlu) into isolated hepatocytes is mediated by two systems: a high-affinity component with a K_m of about 1 μM (1) and a low-affinity component with a K_m of about 13 mM [2]. Transport of the folate antagonist, methotrexate, in hepatocytes occurs via Na^+-dependent, high-affinity [3] and low-affinity [4] routes. However, the available evidence suggests that 5-CH_3-H_4PteGlu and methotrexate may be transported by different processes in liver cells [1–6]. Differences in the transport of these folate derivatives were as follows: (a) NaN_3, anaerobiosis, and ethanol stimulated uptake of 5-CH_3-H_4PteGlu; whereas, these same conditions inhibited methotrexate uptake and (b) methotrexate and 5-HCO-H_4PteGlu inhibited uptake of 5-CH_3-H_4PteGlu; whereas, high concentrations of 5-CH_3-H_4PteGlu or of 5-HCO-H_4PteGlu did not inhibit methotrexate uptake. In contrast, L1210 murine leukemia cells and other mammalian cells, including intestinal mucosal cells [7,8], apparently share the same transport system for all folate coenzymes [9–12]. Since antifolates are toxic both to the intestinal mucosa [13,14] and to the hepatocyte [15,16], it is important that we understand the mechanism(s) whereby naturally occurring folate coenzymes and antifolates are transported into cells. We have, therefore, continued our studies of the transport mechanism of folate coenzymes into isolated hepatocytes.

In the present paper we report a re-examination of the Na^+-dependence of 5-CH_3-H_4PteGlu and metho-

Abbreviations: PteGlu, pteroylglutamic acid, folic acid; H_4PteGlu, 5,6,7,8-tetrahydrofolic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione. pH_i, intracellular pH; pH_o, extracellular pH; $\Delta pH = pH_o - pH_i$.

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trexate transport into isolated hepatocytes. These studies indicated that, while methotrexate uptake increased with increasing extracellular Na^+ concentrations, uptake of 5-CH_3-PteGlu was independent of extracellular Na^+, unless hepatocytes were first incubated in Na^+-free medium. Thus, the Na^+-dependent uptake of 5-CH_3-H_4PteGlu, reported previously [1], was apparently not evidence for Na^+ cotransport but was due to a secondary process. Uptake of 5-CH_3-H_4PteGlu, but not of methotrexate, was a linear function of an imposed transmembrane pH gradient and analysis of the data by Hill-type plots suggested a H^+:5-CH_3-H_4PteGlu stoichiometry of 1:1 for interaction of protons with the carrier for 5-CH_3-H_4PteGlu. Also, kinetic analyses indicated an increased V_{max} and K_m for transport at extracellular pH 5.5 as compared to pH 7.4. These results suggest the possibility that 5-CH_3-H_4-PteGlu may be cotransported with H^+-ions in isolated hepatocytes.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN), weighing 200–400 g were used in all experiments. Rats were maintained on Wayne Lab-Blox and tap water fed ad libitum.

Hepatocyte isolation and uptake experiments. Hepatocytes were isolated by our modification [4] of the procedures of Berry and Friend [17]. Greater than 95% of these cells excluded Trypan blue and maintained normal gradients for Na^+ and K^+ [1]. Unless otherwise indicated, the incubation medium for transport experiments contained the following: NaCl, 125 mM; KCl, 5 mM; $CaCl_2$, 2.7 mM; KH_2PO_4, 1.25 mM; $MgSO_4$, 1.25 mM; Hepes, 25 mM; glucose, 5.5 mM; and gelatin, 1% (w/v). [^{14}C]Inulin or [^{3}H]inulin, as appropriate, were used to correct for trapped, extracellular substrate. In experiments in which extracellular pH (pH_o) was varied, Mes (25 mM) replaced Hepes as the buffering agent at pH of 6.5 or less. Uptake experiments were performed by adding 5-CH_3-H_4-[3′,5′,7,9-^{3}H]PteGlu, [3′,5′,7,9-^{3}H]methotrexate or α-amino[*methyl*-^{3}H]sobutyric acid and other compounds, as indicated, to cell suspensions. These were incubated in unstoppered flasks in a gyrotory water bath at 37 °C. At appropriate time intervals, samples were centrifuged in 1.5-ml plastic tubes in an Eppendorf 3200 centrifuge for 15 s. The incubation medium was removed and 1 ml of Scintisol (Isolabs, Inc. Akron, OH) was added. The samples (and aliquots of medium) were analyzed for ^{3}H and ^{14}C by dual-label counting in a Tm Analytic Betatrac model 6895 liquid scintillation counter.

Measurement of intracellular pH. Intracellular pH (pH_i) was determined by the distribution of the weak acid, 5,5-dimethyloxazolidine-2,4-dione (DMO). This procedure has been thoroughly discussed by McGivan [18] and by Fafournoux et al. [19] who concluded that the distribution of DMO estimates a pH closely approximating that of the cytosol. In preliminary experiments we determined that the distribution of 1.1 μM [^{14}C]DMO had reached equilibrium in hepatocytes after about 5 min of uptake. In these experiments, [^{3}H]inulin was used to correct for trapped extracellular water. Also, 0.25 μM unlabeled 5-CH_3-H_4PteGlu or methotrexate was included when [^{14}C]DMO was used to estimate intracellular pH and 1.1 μM unlabeled DMO was included when 5-CH_3-H_4PteGlu or methotrexate uptake was estimated. The apparent intracellular pH was calculated as described [18,19].

Materials. [3′,5′,7,9-^{3}H]PteGlu (5 Ci/mmol) and [3′,5′,7,9-^{3}H]methotrexate (10 Ci/mmol) were obtained from Amersham. α-Amino[*methyl*-^{3}H]isobutyric acid (10 Ci/mmol), [2-^{14}C]DMO (49.9 mCi/mmol), [*carboxy*-^{14}C]inulin and [^{3}H]inulin were from New England Nuclear. (6*S*)-5-CH_3-H_4-[^{3}H]PteGlu, unlabeled (6*S*)-5-CH_3-H_4PteGlu, and methotrexate were synthesized and/or purified as previously outlined [1]. Labeled 5-CH_3-H_4PteGlu and methotrexate were both diluted to 750 mCi/mmol specific radioactivity and stored in 40 mM 2-mercaptoethanol at −70 °C under an argon atmosphere. These radioisotopes were of greater than 98% purity as judged by HPLC [20] and were checked at weekly intervals and re-purified if necessary. Labeled inulin derivatives were purified by chromatography on Bio-Gel P-6, using distilled water for elution, prior to use.

Results

Na^+-Dependence of 5-CH_3-H_4PteGlu and methotrexate transport

Previous results [1–3] had indicated that transport of both 5-CH_3-H_4PteGlu and methotrexate was decreased when extracellular Na^+ was replaced by other cations. We, therefore, performed experiments to determine the Michaelis-Menten kinetic parameters for Na^+ for transport of these substrates. In these experiments, hepatocytes, isolated in medium containing 125 mM Na^+, were washed twice in medium in which choline chloride replaced NaCl, resuspended in medium with varying Na^+ concentrations (maintaining the osmolarity by substituting choline chloride), and the initial rate of uptake of substrate was determined immediately. The results are shown in Fig. 1. Under these conditions, 5-CH_3-H_4PteGlu uptake showed essentially no dependence on the extracellular Na^+ concentration. Methotrexate uptake increased linearly as the extracellular Na^+ concentration increased. On the other hand, transport of α-aminoisobutyric acid displayed a Michaelis-Menten-type dependence on extracellular Na^+ concentration as reported previously in isolated hepatocytes [21].

Since earlier studies [1] had indicated that uptake of 5-CH_3-H_4PteGlu demonstrated a Na^+-dependent com-

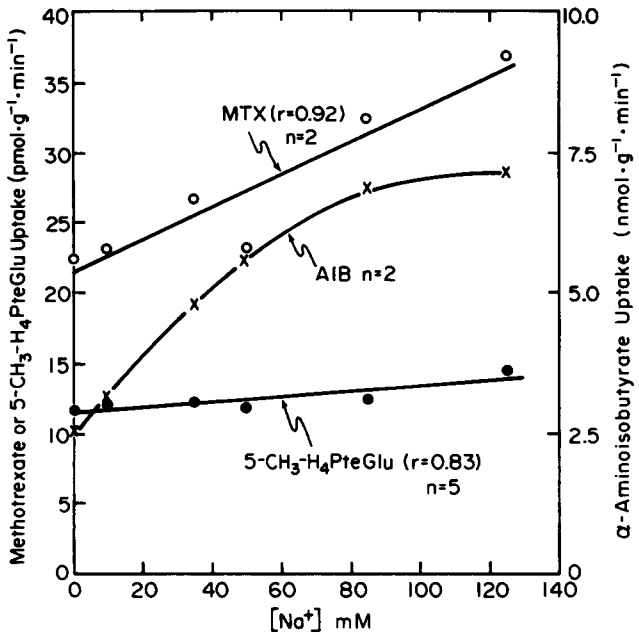

Fig. 1. Uptake of 5-CH_3-H_4PteGlu, methotrexate, and α-aminoisobutyrate as a function of extracellular Na^+ concentration. Freshly isolated hepatocytes were washed twice by centrifugation at 50 × *g* and resuspension in Na^+-free incubation medium (pH 7.4, choline replaced sodium). The cells were then resuspended in medium in which the proportions of NaCl and choline chloride were varied to give the indicated concentrations of sodium. The initial transport rate of 0.25 μM 5-CH_3-H_4PteGlu or methotrexate (MTX) was determined after 7.5 min of incubation at 37° C. Uptake of 0.1 mM α-aminoisobutyrate (AIB) was measured after 4 min. The number of replicates is given by *n* and *r* is the least-squares correlation coefficient.

ponent, experiments were performed to delineate the apparent discrepancy with the finding reported above. In our previous studies [1], hepatocytes were preincubated in Na^+-free medium for 30 to 45 min prior to determining the uptake of 5-CH_3-H_4PteGlu. Therefore, we performed an experiment to assess the possibility that this preincubation might explain our findings. When hepatocytes were preincubated in Na^+-free medium (either KCl or choline chloride replaced NaCl) for 30 min, 5-CH_3-H_4PteGlu uptake increased from 5 to 12 pmol/g per min (KCl experiments) and from 8.4 to 13.1 pmol/g per min (choline chloride experiments) at 0 and 125 mM extracellular Na^+, respectively. In these experiments, with no Na^+-free preincubation period, similar uptakes, to those seen in Fig. 1, were observed whether NaCl was replaced with KCl or choline chloride.

pH-Dependence of 5-CH_3-H_4PteGlu and methotrexate transport in hepatocytes

Since all previous studies of folate transport in isolated hepatocytes had been carried out at pH 7.4 [1–6] and because of the pH dependence of folate transport in the intestine [7,8], the pH dependence of 5-CH_3-H_4PteGlu uptake into hepatocytes was examined. The initial uptake velocity for 5-CH_3-H_4PteGlu was greater at an extracellular pH of 5.5 than 7.4 (22.1 ± 1.9 vs. 10.0 ± 1.3 pmol/g per min, respectively, $P < 0.01$, $n = 3$). However, after 60 min, the uptake at both pH was identical (approximately 500 pmol/g, data not shown). These experiments were performed at an initial extracellular glucose concentration of 5.5 mM. Intracellular pH [pH_i] measurements (Fig. 2A), suggested that hepatocytes were unable to maintain a stable pH_i at this glucose level. However, when the extracellular glucose concentration was doubled (11.1 mM), hepatocytes apparently maintained stable pH_i from 5 to 40 min of incubation (Fig. 2B). Under these conditions, hepatocytes maintained a rather large pH gradient across the plasma membrane as shown in Fig. 3.

Fig. 4 shows the uptake of 5-CH_3-H_4PteGlu and of methotrexate as a function of pH_o. In this case (Fig. 4A), not only was the initial rate of uptake of 5-CH_3-H_4PteGlu higher at pH 5.5 than at pH 7.4 (18.0 ± 1.5 vs. 7.4 ± 0.6 pmol/g per min, respectively, $P < 0.001$, $n = 8$) but the accumulation of this substrate was increased, as evidenced by the uptake at 60 min (542 ± 23 vs. 323 ± 7 pmol/g per min, pH 5.5 vs. pH 7.4,

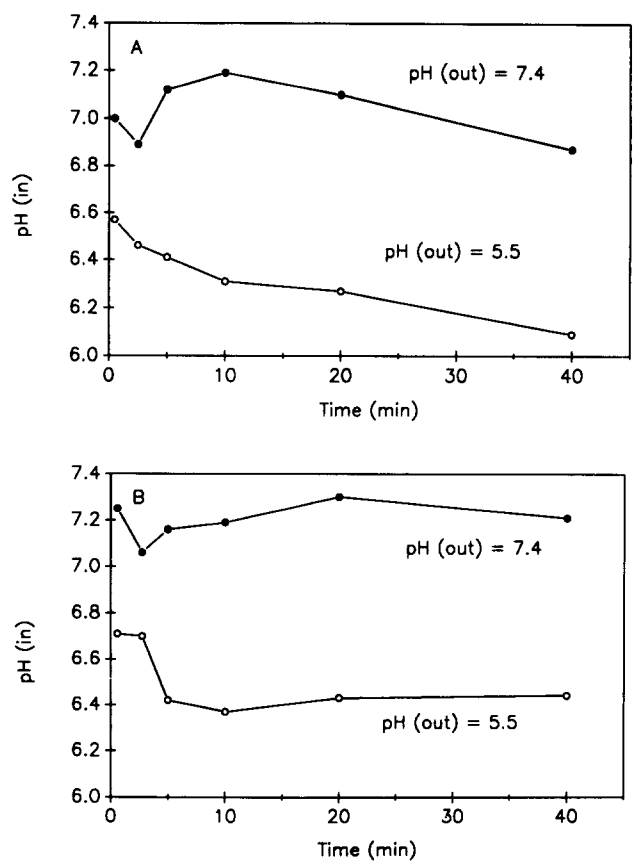

Fig. 2. Effect of extracellular glucose concentration on intracellular pH. Intracellular pH was measured by the distribution of DMO at various time intervals at extracellular pH of 5.5 or 7.4 as indicated. (A) Initial extracellular glucose concentration was 5.55 mM. (B) Initial glucose concentration was 11.1 mM. A representative experiment is shown.

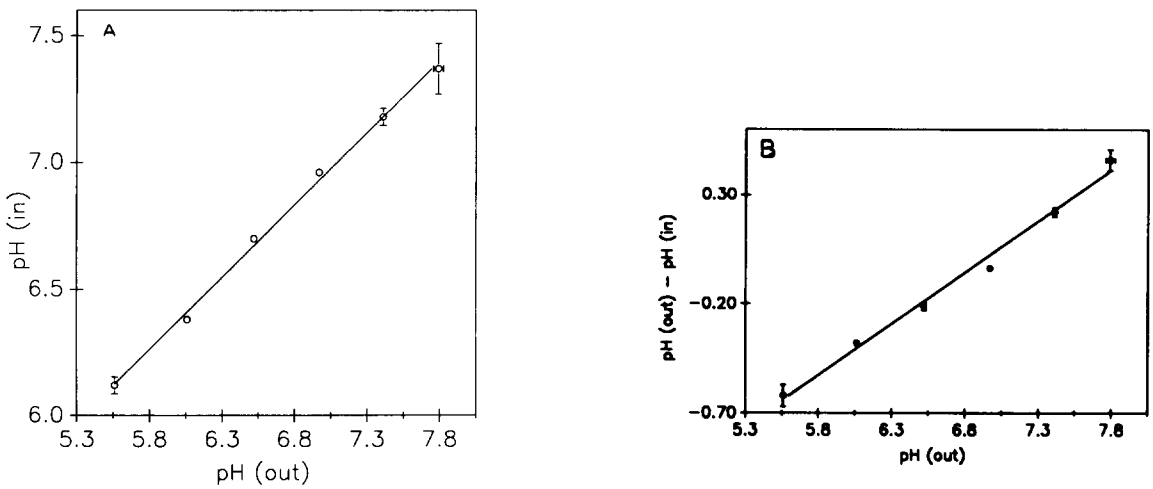

Fig. 3. Intracellular pH as a function of extracellular pH in isolated hepatocytes (A). Intracellular pH (pH_i) was measured by the distribution of DMO and extracellular pH (pH_o) with the pH meter. The results are the means of at least four experiments. Bars indicate standard errors. (B) Transmembrane pH gradient ($pH_o - pH_i$) vs. pH_o.

respectively, $P < 0.01$). On the otherhand, as shown in Fig. 4B, the initial rate of uptake of methotrexate was not significantly different at either extracellular pH (41.2 ± 0.7 vs. 37.0 ± 2.6 pmol/g per min at pH 5.5 vs. pH 7.4, respectively, $P > 0.2$, $n = 3$). Also, in contrast to 5-CH_3-H_4PteGlu transport, the accumulation of

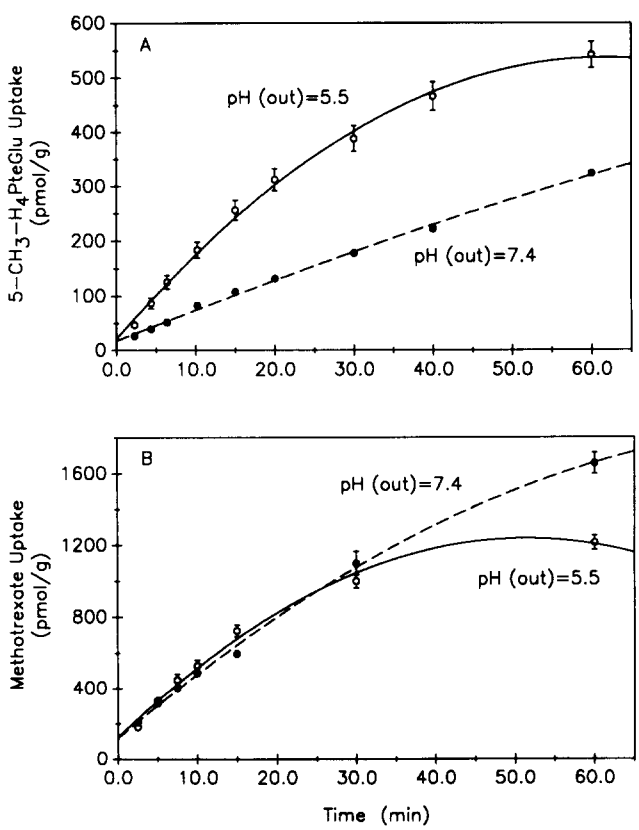

Fig. 4. Effect of extracellular pH on uptake of 5-CH_3-H_4PteGlu and methotrexate. Hepatocytes isolated in media at $pH_o = 7.4$ were washed once and resuspended in incubation medium (extracellular glucose concentration was 11.1 mM) buffered with either 25 mM Hepes (pH 7.4) or 25 mM Mes (pH 5.5). (A) Uptake of 0.25 μM 5-CH_3-H_4PteGlu ($N = 8$). (B) Uptake of 0.18 μM methotrexate ($N = 3$).

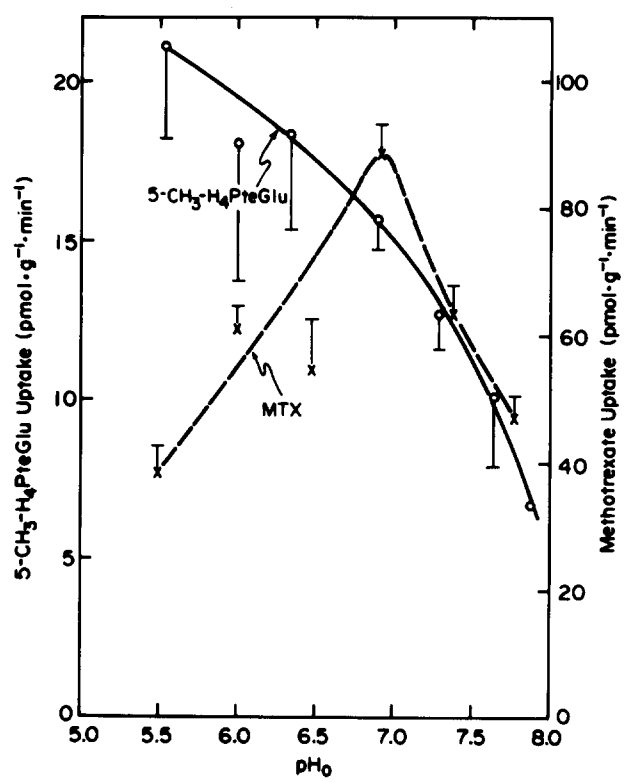

Fig. 5. Effect of extracellular pH on transport of 5-CH_3-H_4PteGlu and methotrexate. The initial rates of uptake of 0.23 μM 5-CH_3-H_4PteGlu (○———○) and 0.45 μM methotrexate (×- - -×) were determined over the first 7.5 min of incubation. The extracellular pH (pH_o) was measured with a pH meter on the supernatant after centrifugation of the cell suspension. For experiments at pH_o 5.5 to 6.5, 25 mM Mes was the buffer agent; 25 mM Hepes was used at pH_o 7.0 to 8.0. The Na^+ concentration in the incubation medium was 125 mM. The results are the average of three separate experiments.

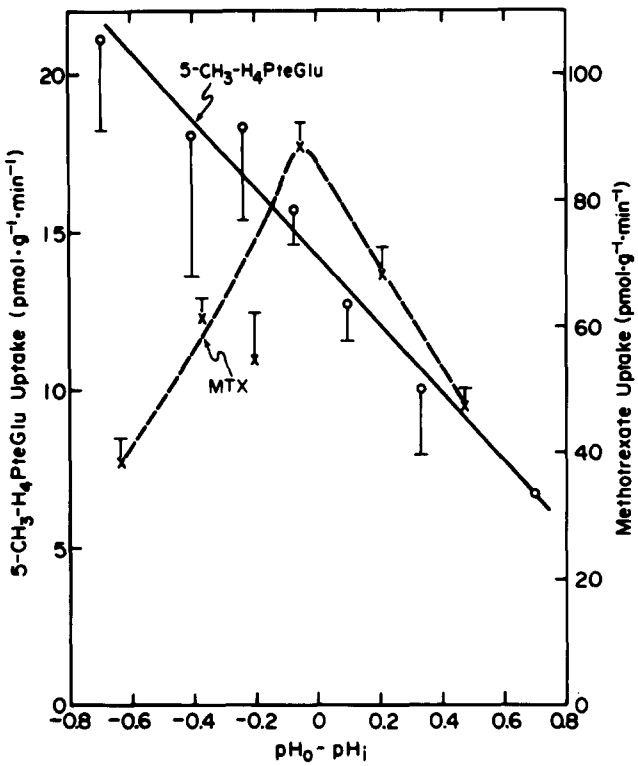

Fig. 6. Effect of transmembrane pH gradient on uptake of 5-CH_3-H_4PteGlu and methotrexate. Intracellular pH (pH_i) was measured by the distribution of DMO, as described in Materials and Methods. Extracellular pH was measured with the pH meter. Other conditions were as described in Fig. 5. (o——o) 5-CH_3-H_4PteGlu uptake; (×---×) methotrexate uptake.

methotrexate by hepatocytes at 60 min was less at an extracellular pH of 5.5 than at pH 7.4 (1.21 ± 0.04 vs. 1.66 ± 0.07 nmol/g per min, respectively, $P < 0.01$).

In further experiments, the effect of varying extracellular pH (pH_o) on the initial rate of uptake of 5-CH_3-H_4PteGlu and of methotrexate was examined. Fig. 5 illustrates the dependence of 5-CH_3-H_4PteGlu and methotrexate uptake on pH_o. The initial velocity of methotrexate transport showed a distinct optimum at about pH 7. In contrast, the initial rate of 5-CH_3-H_4PteGlu transport increased as the extracellular pH decreased from 7.9 to 5.5. The Lineweaver-Burk plot of 5-CH_3-H_4PteGlu transport vs. $[H^+]$ (not shown) yielded a straight line relationship from which a half-maximal uptake at $[H^+] = 23.9$ nM (equivalent to pH = 7.62) was calculated.

In these experiments the distribution of the weak acid, DMO, was used concurrently to estimate the intracellular pH (pH_i), and, along with the measured extracellular pH (pH_o), this data allows the calculation of the transmembrane pH gradient ($pH_o - pH_i$). In fig. 6, the initial rate of uptake of 5-CH_3-H_4PteGlu and of methotrexate is plotted vs. the transmembrane pH gradient. The results indicate that maximal uptake of methotrexate occurred when the pH gradient was essentially zero. In contrast, uptake of 5-CH_3-H_4PteGlu increased linearly as the transmembrane pH gradient decreased; i.e., as the medium became more acid with respect to the cytosol.

These experiments also shed light on the question of Na^+-dependence of 5-CH_3-H_4PteGlu uptake. When hepatocytes were incubated in Na^+-free medium for 45 min, pH_o was 7.24 ± 0.03 and pH_i was 6.78 ± 0.05, $n = 3$, $\Delta pH = +0.46$; in Na^+-containing medium pH_o was 7.33 ± 0.02 and pH_i was 7.28 ± 0.06, $n = 6$, $\Delta pH = +0.05$. Inspection of Fig. 6 indicates that uptake of 5-CH_3-H_4PteGlu was greater at the lower transmembrane pH gradient.

Turner and Moran [22] used the 'activation method' to determine the stoichiometry of the Na^+-dependent renal outer cortical brush-border D-glucose transport

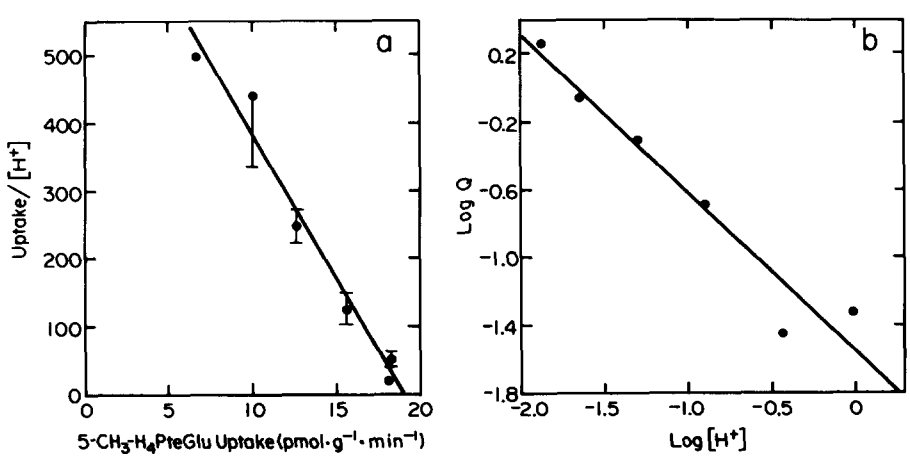

Fig. 7. Stoichiometry of H^+/5-CH_3-H_4PteGlu in hepatocytes. The data of fig. 6 for 5-CH_3-H_4PteGlu uptake have been re-plotted. (A) Scatchard-type plot. A least-squares fit of the data yielded slope ($K_{0.5}$) of 23.3 nM and x-intercept (F_∞), the initial rate of uptake at saturating H^+ concentration) of 18.9 pmol·g^{-1}·min^{-1}, $r = 0.98$. (B) Log-log (Hill-type) plot. The quantity Q is given by $Q = (F_\infty - F)/F$, where F is the initial rate at a given H^+ concentration (F, F_∞, Q, and $K_{0.5}$ are as defined by Turner and Moran [22]). The least-squares fit gave x-intercept ($K_{0.5}$) of 21.9 nM and the slope (stoichiometry) of 0.94, $r = 0.97$.

system. This method is based on the stimulation of solute uptake by increasing concentrations of the activator (Na^+). The data were analyzed by Hill-type plots, in which the slope indicated the stoichiometry of the activator–substrate interaction. For these relationships to hold true, either the electrical potential difference across the membrane must be zero or the transport of the substrate must be electrically neutral. The initial rate of 5-CH_3-H_4PteGlu uptake was unaffected by 5 μg/ml of valinomycin (20.3 ± 1.9 vs. 28.9 ± 3.3 pmol/g per min, $P > 0.23$, $n = 3$, control vs. valinomycin, respectively) a concentration which severely depolarized the hepatocyte membrane electrical potential [23]. Thus, with this conclusion that 5-CH_3-H_4PteGlu uptake in hepatocytes is electroneutral, we may apply the activation method to determine the stoichiometry of coupling of protons with 5-CH_3-H_4PteGlu transport. Fig. 7A shows a Scatchard-type plot of 5-CH_3-H_4PteGlu uptake at various extracellular concentrations of H^+. A least-squares fit of the data indicated a slope (K_m) of 23.3 nM (equivalent to pH = 7.63), in excellent agreement with the Lineweaver-Burk analysis of the data. From the slope of the Hill-type plot of the data (Fig. 7B), a stoichiometry for H^+/5-CH_3-H_4PteGlu of 0.94 was calculated, indicating a 1 : 1 interaction of protons with the carrier system for 5-CH_3-H_4PteGlu in hepatocytes.

Effect of extracellular pH on kinetics of 5-CH_3-H_4PteGlu transport

Fig. 8 shows the uptake of 5-CH_3-H_4PteGlu as a function of concentration at pH_o 5.5 and 7.4. Uptake was composed of two processes; one saturable at low concentrations, the other not saturable up to 20 μM.

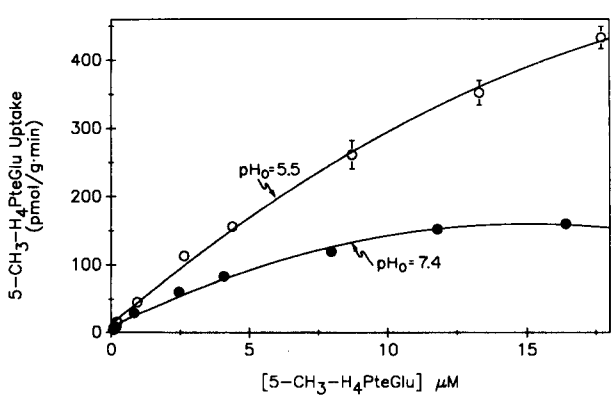

Fig. 8. Concentration dependence of 5-CH_3-H_4PteGlu uptake at extracellular pH 5.5 and 7.4. The initial rate of transport was determined at 37 °C by measuring uptake at 8 min after the start of incubation. Non-specific binding (145 ± 5 pmol · g^{-1} · μM^{-1}, determined in earlier experiments as the intercept of uptake vs. zero time at various concentrations of 5-CH_3-H_4PteGlu) was subtracted from all values. $pH_o = 7.4$, $N = 2$; $pH_o = 5.5$, $N = 3$. Results are means ± S.E.

TABLE I

Kinetic parameters for 5-CH_3-H_4PteGlu transport

The kinetic parameters K_m and V_{max} were estimated according to Wilkinson [24] and the first-order rate constant, k, was estimated from the slope of the linear component of transport from the data of Fig. 8. Values are reported as means ± standard error.

Extracellular pH	K_m (μM)	V_{max} (pmol · g^{-1} · min^{-1})	k (pmol · g^{-1} · μM^{-1})
5.5 ($n = 3$)	2.45 ± 0.16 [a]	121 ± 12.6 [b]	20.7 ± 0.7 [c]
7.4 ($n = 2$)	0.93 ± 0.01 [a]	58.4 ± 0.7 [b]	6.75 ± 0.20 [c]

[a] $P < 0.01$; [b] $P < 0.05$; [c] $P < 0.001$.

The linear component was subtracted from the total uptake to determine the contribution of the saturable system. Kinetic parameters were then determined according to Wilkinson [24]. Table I shows that the apparent K_m and V_{max} for 5-CH_3-H_4PteGlu transport were increased at extracellular pH of 5.5 as compared with pH 7.4.

Discussion

In the presents study, we re-examined the Na^+-dependence of uptake of 5-CH_3-H_4PteGlu and of methotrexate into isolated hepatocytes via the high-affinity transport systems. This was done when the results of experiments designed to ascertain the Michaelis-Menten kinetics with respect to Na^+ suggested that 5-CH_3-H_4PteGlu transport was not Na^+-dependent, conflicting with our previous report [1]. We, therefore, carried out experiments to clarify this situation. Uptake of α-aminoisobutyric acid was included in this study as a control, since transport of this amino acid was known to exhibit a Michaelis-Menten kinetic dependence on Na^+ [21].

Transport of α-aminoisobutyric acid displayed Michaelis-Menten kinetics (Fig. 1). In similar experiments, uptake of methotrexate displayed a linear dependence on extracellular Na^+ concentration (Fig. 1). Our previous results [4] and that of Gewirtz et al. [3] indicated significant inhibition of methotrexate uptake into hepatocytes when extracellular sodium was omitted or when ouabain was included in the incubation medium, further strengthening the conclusion that methotrexate transport is dependent on extracellular Na^+. Fig. 1 indicates that transport of 5-CH_3-H_4PteGlu into hepatocytes displayed essentially no dependence on extracellular Na^+. As noted above, this finding conflicts with our previous conclusion [1]. However, in that paper [1] we reported that ouabain was only minimally inhibitory (approx. 10% inhibition at 1.0 mM), whereas ouabain inhibited methotrexate uptake by 77% [4]. The

lack of ouabain sensitivity and the data of Fig. 1, suggest that transport of 5-CH_3-H_4-PteGlu may be independent of Na^+ in isolated hepatocytes. Furthermore, direct measurements showed that preincubation in Na^+-free medium caused an increase in the transmembrane pH gradient which would result in decreased uptake of 5-CH_3-H_4PteGlu (see Fig. 6). These results suggest that preincubation in Na^+-free medium resulted in lowered uptake by changing the pH gradient.

Since we were unable to confirm that the Na^+ gradient was responsible for energization of the active transport of 5-CH_3-H_4PteGlu in hepatocytes we decided to explore other mechanisms. It is known that a transmembrane pH gradient apparently energizes uptake of folates in intestinal brush-border membrane vesicles from rabbits [25] and humans [26] and in rat kidney brush-border membranes [27]. A pH gradient was also implicated in the uphill transport of unconjugated bile acids in isolated basolateral membrane vesicles from rat liver [28]. We, therefore, decided to examine the pH-dependence of 5-CH_3-H_4PteGlu transport into hepatocytes.

Initial experiments indicated that the initial velocity of uptake of 5-CH_3-H_4PteGlu into hepatocytes was greater at pH_o of 5.5 than at 7.4. However, the uptake at 60 min of incubation was similar at either pH, suggesting that the transmembrane pH gradient had no effect on the accumulation of this substrate. Subsequent experiments, in which DMO was utilized to measure intracellular pH, Fig. 2A, indicated that hepatocytes could not maintain a stable pH_i at pH_o of 5.5 and at an extracellular glucose concentration of 5.5 mM. However, when the extracellular glucose concentration was increased to 11.1 mM (Fig. 2B and Fig. 3), hepatocytes maintained a stable pH_o for at least 40 min of incubation. Thus, all subsequent experiments were performed using an initial extracellular glucose concentration of 11.1 mM.

Fig. 4A shows that at 11.1 mM glucose, not only was the initial rate of 5-CH_3-H_4PteGlu transport increased at pH_o 5.5, the hepatocytes also apparently maintained a greater concentration gradient for 5-CH_3-H_4PteGlu. Methotrexate uptake into hepatocytes at pH_o of 5.5 and 7.4 showed similar initial rates of transport (see Fig. 4B and Results). Also, uptake of methotrexate at 60 min was less at pH_o 5.5 than at 7.4, unlike that for 5-CH_3-H_4PteGlu (see Results). These results point out yet more differences (see the Introduction) in the transport characteristics of these two folate derivatives in hepatocytes. The reason(s) for separate transport systems in hepatocytes for reduced folates (i.e., 5-HCO- and 5-CH_3-H_4PteGlu) and methotrexate is not known. However, the liver is constantly exposed to 5-CH_3-H_4PteGlu via the portal vein due to the reduction and methylation of absorbed folates [29] and due to the enterohepatic circulation of folates [30]. Thus, the hepatocyte may have evolved a transport system with greater specificity for the reduced, one-carbon substituted folate coenzymes.

We performed additional experiments to examine the dependence of 5-CH_3-H_4PteGlu and of methotrexate transport on extracellular pH and on the transmembrane pH gradient ($pH_o - pH_i$). Fig. 5 shows that methotrexate uptake was maximal at pH_o about 7, whereas, uptake of 5-CH_3-H_4PteGlu increased as the pH_o decreased to 5.5. A Lineweaver-Burk analysis of the data for 5-CH_3-H_4PteGlu transport indicated a Michaelis-Menten dependence on the extracellular $[H^+]$ and yielded a '$K_m^{H^+}$' of 23.9 nM (equivalent to pH_o = 7.62). Fig. 6 depicts the data plotted as a function of the transmembrane pH gradient. This analysis indicated that, while 5-CH_3-H_4PteGlu displayed a linear dependence on the pH gradient, methotrexate uptake showed a biphasic dependence. It might be argued that these differences in methotrexate and 5-CH_3-H_4PteGlu transport could be explained by differential concentrations of species with differing charge as the extracellular pH is varied. Inspection of the acid dissociation constants for these two folate derivatives (methotrexate: N-1, pK_a 5.71; α-carboxyl, 3.36; γ-carboxyl, 4.70; N-5, < -1.5; N-10, 0.50 and 5-CH_3-H_4PteGlu: N-1, 1.24; α-carboxyl, 3.50; γ-carboxyl, 4.80; N-5, 5.20; N-3/O-4 amide, 10.8; Refs. 31–34) does not bear out this supposition. At the extracellular pH values examined in the present study, both derivatives would have similar charge. However, protonation of N-1 of methotrexate ($pK_a = 5.71$) could result in decreased uptake leading to the biphasic dependence on pH (Fig. 6), while protonation of N-5 of 5-CH_3-H_4PteGlu ($pK_a = 5.2$) may not.

Further analysis of the data by the 'activation method' [22], see Fig. 7, suggested a coupling stoichiometry of 1 : 1 for H^+ : 5-CH_3-H_4PteGlu. This result suggests that this folate coenzyme may be cotransported with hydrogen ions in the hepatocyte. In support of this contention, our data indicated that the maximal transport velocity (V_{max}) was doubled at extracellular pH 5.5 as compared to 7.4 (Fig. 8, Table I, and Results). This suggests that the acidic pH either increased the number of active carriers or increased the rate of transport. The 'activation method' in its simplified form as applied by Turner and Moran [22] assumes that uptake of the substrate is electroneutral; i.e., that transport does not respond to a transmembrane electrical potential gradient. Our data (see Results) suggested that 5-CH_3-H_4PteGlu uptake into hepatocytes was unaffected by valinomycin concentrations which led to a severe depolarization of the hepatocyte electrical membrane potential [23]. As pointed out by Turner and Moran [22], the 'activation method' does not distinguish between an 'energetic' coupling (i.e., cotransport) or a 'catalytic' coupling (in which H^+ interacts with the carrier to stimulate substrate flux without concomitant H^+ transport). Such information awaits experiments in

isolated hepatocyte membrane vesicles underway in our laboratory.

In intestinal mucosal cells, experiments have demonstrated an acid microclimate [35,36], such that the pH adjacent to the brush border cell membrane is maintained at about pH 5.5. Folate coenzyme transport, in these cells, has been shown to be dependent on this low-extracellular pH, both in gut sacs [7,8] and in isolated brush-border membrane vesicles [25,26]. pH-gradient-dependent uptake of unconjugated bile acids has been demonstrated in basolateral membrane vesicles isolated from rat liver [28]. The existence of and the manner in which a transmembrane pH gradient might be maintained in hepatocytes is unknown. However, it has been shown that liver cell plasma membranes contain an NADH oxidoreductase [37,38] and a *b*-type cytochrome [39], suggesting the possibility of an electron transport system and, further, the possibility of proton translocation across the plasma membrane. Indeed, it has been shown that protons diffuse along the interface between water and phospholipid monolayers some 20-times faster than in the bulk aqueous phase [40,41]. Therefore, hepatocytes need not maintain a bulk-phase H^+ gradient in order for transport systems to be energized via H^+ cotransport. The high proton conductivity of the phospholipid membrane relative to diffusion into the bulk water phase could result in the delivery of extruded protons directly to the carrier, in effect, generating a 'localized' pH gradient across the plasma membrane which could be utilized for the active transport of solutes into the hepatocyte.

It should be noted that Kamen and associates [42,43] and Kolhouse and associates [44] have described a high-affinity system for uptake of folates in monkey kidney cells and human KB cells, respectively, cultured on limiting levels of folates. These investigators suggest that folate uptake in these cell lines may be via receptor-mediated endocytosis. In these cells there is apparently a single system for uptake of all folate derivatives. Isolated hepatocytes, on the other hand, have transport systems which discriminate between 5-CH_3-H_4PteGlu and methotrexate as shown by Goldman and associates [3] and by our group [1,4,5]. Furthermore, 5-CH_3-H_4PteGlu transport in hepatocytes is stimulated by sodium azide [1] and anaerobiosis [5], factors which inhibit receptor-mediated endocytosis. Therefore, receptor-mediated endocytosis should not significantly contribute to 5-CH_3-H_4PteGlu transport into hepatocytes.

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