

Transport of 5-Methyltetrahydrofolic Acid and Folic Acid in Freshly Isolated Hepatocytes*

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Transport of 5-methyltetrahydropteroylglutamic acid (5-CH₃-H₄PteGlu) was studied in hepatocytes isolated from rat liver by enzymatic dispersion. The hepatocytes maintained gradients for sodium and potassium ions ([Na⁺] = 28.1 meq/liter and [K⁺] = 133 meq/liter of cell water) during 90 min of incubation.

Uptake of 5-CH₃-H₄PteGlu was found to be a complex process. At low concentrations of substrate, uptake was saturable. At higher concentrations of substrate (up to 20 μ M), the uptake was not saturable. Both components were less active in the absence of extracellular sodium ions. The saturable component, both in the presence and absence of extracellular sodium, was energy-dependent; in both cases inhibition by sodium arsenate was observed and concentration of 5-CH₃-H₄PteGlu proceeded against an electrochemical gradient. Ion exchange chromatography indicated that PteGlu, but not 5-CH₃-H₄PteGlu, was metabolized significantly after 60 to 90 min of incubation. The accumulated 5-CH₃-H₄PteGlu was bound to macromolecules present in sonicated cell extracts to the extent of about 20%. Uptake of 5-CH₃-H₄PteGlu by the primary saturable component was inhibited by the structural analogs, methotrexate, 5-CHO-H₄PteGlu, and to a lesser degree, by PteGlu, whereas the secondary, nonsaturable component was unaffected by high concentrations of these compounds. Uptake of 5-CH₃-H₄PteGlu was stimulated, and efflux was inhibited by the metabolic inhibitor, sodium azide, suggesting that an energy-dependent efflux is operative. In addition, evidence is presented which suggests the conclusion that 5-CH₃-H₄PteGlu transport is dependent on the presence of intact disulfide bonds.

These studies show that 5-CH₃-H₄PteGlu transport in freshly isolated hepatocytes is a saturable, active process(es) composed of sodium-dependent and sodium-independent components and that efflux may also be an energy-dependent phenomenon.

Folate transport has been shown to be a carrier-mediated process in a number of different systems including bacteria (1-4), rabbit reticulocytes (5), the choroid plexus (6, 7), and L1210 leukemia cells (8). Intestinal absorption of naturally occurring folates appears to take place via passive diffusion (9). Transport of the folate analog, methotrexate, in L1210 cells has been studied most exhaustively (8). It has been shown to be a saturable, energy-dependent process exhibiting counter-transport and competition by PteGlu¹ and folinic acid.

Transport of folates in isolated mammalian tissue cells has been examined in intestinal epithelial cells. Time-dependent uptake of PteGlu was not observed in these intestinal cells which were isolated by hyaluronidase treatment. A folate binding protein ($K_b = 3.98 \times 10^{-9}$ M) was isolated, however, from the brush border membrane (10). In intestinal epithelial cells isolated by a vibrational technique (11), glucose stimulated uptake of PteGlu. No additional data was presented, however, to indicate that the uptake of folate was energy-dependent or carrier-mediated.

In a previous paper we reported that methotrexate is transported in hepatocytes by an active, carrier-mediated process dependent on metabolic energy and extracellular sodium ions (12). This paper describes the transport of 5-CH₃-H₄PteGlu in hepatocytes. Studies to be described suggest that 5-CH₃-H₄PteGlu is transported via processes which are carrier-mediated and dependent on metabolic energy.

MATERIALS AND METHODS

Animals—Male Sprague-Dawley rats (Harlan Industries) weighing 170 to 300 g were used for the isolation of hepatocytes. The rats were maintained on Wayne Lab-Blox and tap water fed *ad libitum*.

Hepatocyte Isolation and Incubation Techniques—Procedures for isolating hepatocytes and for performing transport experiments have been described in detail previously (12).

Briefly, the hepatocyte isolation procedure consists of a 20-min *in situ* perfusion of the liver with a collagenase-salts solution containing 10% washed human erythrocytes. The cells are dispersed by shaking at about 150 cycles/min at 37°C for 10 min. The viable cells are separated from debris and damaged cells by several centrifugations at 20 and 50 $\times g$.

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¹ The abbreviations used are: PteGlu, pteroylglutamic acid, folic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; H₂PteGlu, 5,6,7,8-tetrahydropteroylglutamic acid; H₄PteGlu, 7,8-dihydropteroylglutamic acid, dihydrofolic acid; 5-CHO-H₄PteGlu, folinic acid; 5-CH₃-H₄PteGlu, 5-methyltetrahydrofolic acid, 5-methyl-5,6,7,8-tetrahydropteroylglutamic acid; DNP, 2,4-dinitrophenol.

Uptake experiments were performed by adding radioactive substrates and/or other compounds to suspensions of cells in Krebs-Ringer solution buffered at pH 7.4 with 25 mM Hepes (Calbiochemicals) containing 1% (w/v) gelatin and 5.5 mM glucose. The uncapped vials were shaken in a gyrotory water bath at 37 or 25°C, as indicated, and duplicate 1- or 0.5-ml samples were taken at appropriate times. The samples were centrifuged in preweighed 1.5-ml plastic centrifuge tubes for 5 to 10 s in an Eppendorf 3200 centrifuge. The cell pellet was washed twice with 0.5-ml aliquots of ice-cold incubation medium (without gelatin). The tubes were weighed and 1.0 ml of Scintosol Complete (Isolab, Akron, Ohio) was added. Samples of incubation medium (0.1 ml) were counted similarly.

Aqueous cell volumes were measured as described by LeCam and Freychet (13) except that inulin replaced polyethyleneglycol. It was found that the intracellular water content was 0.388 ± 0.013 ml/g wet weight. This value did not vary significantly from 10 to 60 min of incubation.

The Na^+ and K^+ content of hepatocytes were estimated as follows: At various incubation time intervals, cell samples were removed, washed twice with ice-cold 0.25 M sucrose, weighed, and digested overnight with concentrated HNO_3 . The samples were carefully neutralized with concentrated ammonia and analyzed for Na^+ and K^+ on an Instrumentation Laboratories flame photometer. The Na^+ content was 28.1 ± 1.4 meq/liter of cell water and the K^+ content was 133 ± 2.4 meq/liter of cell water. The values did not vary significantly during 90 min of incubation at 37°C. LeCam and Freychet (13) reported 130 to 150 meq/liter of K^+ , but they reported no values for Na^+ content.

Treatment of Kinetic Data—Preliminary experiments showed the presence of a primary saturable component as well as a linear, secondary component of transport of 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$. Thus the initial velocity of influx (v) may be related to extracellular concentration (s) as follows:

$$v = \frac{V_{\max} s}{K_m + s} + ks \quad (1)$$

where k is the first order rate constant of the secondary component. Equation 1 may be expanded and simplified to give Equation 2,

$$v = ks - K_m(v/s) + K_mk + V_{\max} \quad (2)$$

which is amenable to multiple linear regression on a desk-top computer. In addition, Lineweaver-Burk plots of the data, corrected for the linear components, were inspected to insure linearity.

Statistical Treatment of Data—All values are reported as the mean \pm standard error unless otherwise stated. Significance was judged by the t test for unpaired data.

Chemicals—[$G\text{-}^3\text{H}$]PteGlu (5Ci/mmol) was obtained from Amersham/Searle. (l)-5- $\text{CH}_3\text{-H}_4\text{[G-}^3\text{H]PteGlu}$ (5Ci/mmol, 98% radiochemical purity) was synthesized enzymatically from [$G\text{-}^3\text{H}$]PteGlu by the procedure of Horne *et al.* (14). [$\text{carboxy-}^{14}\text{C}$]Inulin was purchased from New England Nuclear. [$G\text{-}^3\text{H}$]PteGlu was purified by the chromatographic procedure used by Nixon and Bertino for 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ (15) except that a linear gradient of ammonium acetate from 0.2 to 3.0 M was employed. The radiochemical purity of the resulting [$G\text{-}^3\text{H}$]PteGlu was 96.7%. Unlabeled (l)-5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ was synthesized enzymatically (15) and (dl)-5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ was synthesized from (dl)- H_4PteGlu (Sigma) (16). Purification was achieved by elution from a column (2.5×75 cm) of Cellex-T (Bio-Rad) employing a linear gradient of ammonium acetate from 0.15 to 0.8 M (900 ml of each, containing 40 mM 2-mercaptoethanol). Fractions having the characteristic absorbance spectrum of 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ ($\lambda_{\max} = 290$ nm, $\lambda_{\min} = 245$ nm) and showing an absorbance ratio ($A_{290}:A_{245}$) of 3.4 to 3.9 were pooled and lyophilized to dryness. The white residue was dissolved in 40 mM 2-mercaptoethanol and stored under N_2 at -20°C . The product was homogeneous as judged by thin layer chromatography (17) and by its ultraviolet spectrum. Folinic acid was purchased from K & K Chemicals. PteGlu, methotrexate, ouabain, and dithioerythritol were from Sigma. All other chemicals were obtained commercially and were the highest grade available.

RESULTS

Time Course of 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ Uptake—Fig. 1 shows the time course of uptake of 0.25 μM 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ into hepatocytes in medium containing sodium chloride (Fig. 1a) and in medium containing choline chloride in place of sodium (Fig.

1b). In both cases, as shown in Fig. 1c for the controls, the uptake extrapolated to zero time failed to intersect the origin. This "zero-time" uptake was completed by the first sampling time (30 s) and probably represents rapid adsorption to the plasma membrane. Following the adsorption phase, the uptake is linear for the first 10 min then the rate slows and approaches the steady state at later times. This figure also

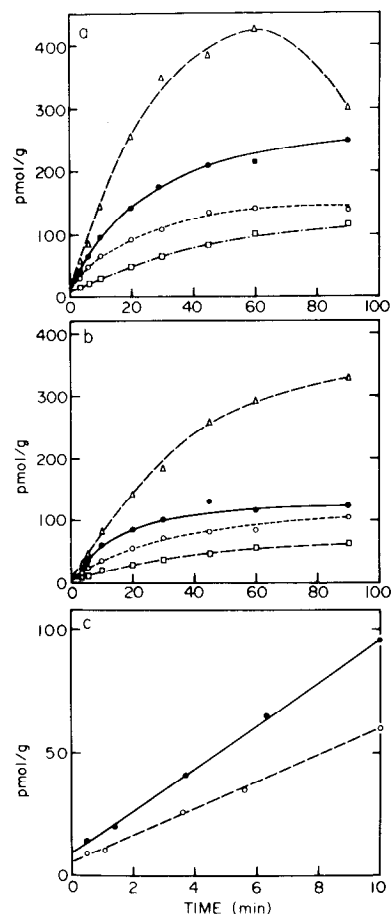


FIG. 1. Time course of 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ uptake in isolated hepatocytes. Labeled 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ and test compound were added to the cells simultaneously. The uptake of 0.25 μM 5- $\text{CH}_3\text{-H}_4\text{[G-}^3\text{H]PteGlu}$ was measured over the next 90 min in control cells (\bullet) and cells with 10 mM NaN_3 (Δ), 10 mM Na_2HAsO_4 (\circ), and 0.1 mM 5- $\text{CHO-H}_4\text{PteGlu}$ (\square). a, sodium-containing medium; b, choline-containing medium; c, expanded scale of uptake of control cells in sodium-containing (\bullet) and choline-containing medium (\circ). The results of a representative experiment are shown. Uptake was at 37°C.

TABLE I

Influence of various inhibitors on influx of 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$

Hepatocytes were incubated as described in Fig. 1. After 60 min of incubation samples of cells and medium were assayed to determine the concentration distribution of labeled 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ in cells incubated in Na^+ - and choline-containing media. The values are corrected for binding to intracellular proteins.

Addition	Concentration mM	Distribution ratio	
		Na^+	Choline
None		1.6	0.8
NaN_3	10.0	3.3	2.2
Na_2HAsO_4	10.0	1.0	0.6
5- $\text{CHO-H}_4\text{PteGlu}$	0.1	0.8	0.4

demonstrates that sodium azide had an unexpected stimulatory effect on uptake, whereas sodium arsenate and 5-CHO-H₄PteGlu were both inhibitory. This pattern was true in both sodium- and choline-containing media. In Table I, the tissue to medium concentration ratios (*T/M*) are listed for the various treatments of Fig. 1. It can be seen that the uptake of 5-CH₃-H₄PteGlu in control cells takes place against an electrochemical gradient in the presence or absence of sodium since the measured *T/M* exceeds the expected *T/M* of 0.1 predicted by the transmembrane potential (see "Discussion"). Furthermore, the *T/M* value in the presence of 10 mM sodium azide is about 2 to 3 times that of the control cells in the presence and absence of Na⁺, respectively. In contrast, the addition of sodium arsenate or 5-CHO-H₄PteGlu to the medium led to *T/M* values below those of the control.

Metabolism and Intracellular Binding of 5-CH₃-H₄PteGlu—In order to assess the extent of metabolism of 5-CH₃-H₄PteGlu by isolated hepatocytes the cells were incubated for 90 min at 37°C with 5-CH₃-H₄[G-³H]PteGlu at a concentration of 0.25 μM. The cells were separated from the medium, washed twice with ice-cold buffer and resuspended in a solution of 1% sodium ascorbate. This suspension was immediately placed in a boiling water bath for 10 min and the precipitated protein was removed by centrifugation. The washed pellet contained about 3% of the radioactivity associated with the cells. Unlabeled 5-CH₃-H₄PteGlu was added as marker and the supernatant was chromatographed on a DEAE-Sephadex A-25 column according to Nixon and Bertino (18). The results appear in Fig. 2. The ultraviolet absorbing peak eluting at about tube 95, and having the characteristic spectrum of 5-CH₃-H₄PteGlu, was associated with the major peak of radioactivity (88% of that eluted from the column). Recovery of radioactivity from the column was greater than 98%. When cells were incubated similarly with 16 and 50 nM 5-CH₃-H₄PteGlu, 95 and 89%, respectively, of the radioactivity eluting from the columns was coincident with authentic 5-CH₃-H₄PteGlu.

In a separate experiment, hepatocytes were incubated with 0.25 μM 5-CH₃-H₄[G-³H]PteGlu at 37°C for 90 min. The cells were separated, washed as above, and suspended in ice-cold

incubation buffer without gelatin and containing 0.04 M 2-mercaptoethanol. The suspension was sonicated for 15 s and centrifuged at 12,000 rpm in a Brinkmann microcentrifuge for 2 min to precipitate cell debris. The washed pellet contained less than 5% of the total radioactivity. The supernatant (0.5 ml) was placed in one chamber and 0.5 ml of cold buffer was placed in the other chamber of a small lucite equilibrium dialysis apparatus. After dialysis for 16 h in the cold, the two compartments were sampled and from the distribution of radioactivity it was determined that about 23% of the 5-CH₃-H₄[G-³H]PteGlu was bound to intracellular macromolecules. Also, these results are comparable to data obtained from the efflux experiments described in Fig. 8 where, for control cells, all but 19.3% (*n* = 3) of the accumulated 5-CH₃-H₄PteGlu was exchangeable.

Thus, under the conditions of the present study, isolated hepatocytes did not significantly metabolize 5-CH₃-H₄PteGlu. Also, at the concentrations studied, only about 20% of the accumulated radioactivity was bound to intracellular macromolecules. When this binding was taken into account, hepatocytes, incubated for 90 min at 37°C in the presence of 15 nM 5-CH₃-H₄PteGlu, concentrated the 5-CH₃-H₄PteGlu about 6-fold when compared to the medium.

Time Course of PteGlu Uptake—Fig. 3 depicts the time course of uptake of 0.06 μM [G-³H]PteGlu into hepatocytes in sodium-containing medium. As with 5-CH₃-H₄PteGlu, there is a rapid adsorption phase which appeared to be complete by 30 s. Following this phase uptake at 25°C appeared linear for approximately 2.5 min. The rate approached zero at about 15 min and began to decline thereafter. At about 70 min there was again a net transport of radioactivity into the cells. At 37°C a similar pattern was observed, but the time sequence of events was shortened. The decline after 15 min (at 25°C) was interpreted to be due to PteGlu metabolites effluxing from the cell faster than PteGlu was being taken up. The data of Fig. 4 lends support to this hypothesis. Here the efflux component again seen in the control cells is not apparent in cells preincubated with methotrexate to curtail metabolism of PteGlu. Also, DEAE-Sephadex analysis (not shown) (18) of hepatocyte extracts and medium incubated for 90 min at 25°C showed the presence of radioactivity eluting at the positions of 10-CHO-H₄PteGlu and 5-CH₃-H₄PteGlu as well as PteGlu.

Since there was significant metabolism of PteGlu evident as early as 15 min at 25°C, and probably at earlier times as well, the concentration dependence of transport was not examined in this study.

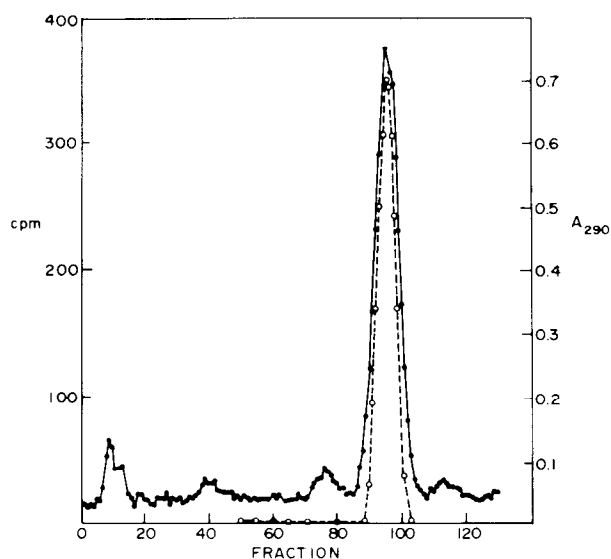


FIG. 2. DEAE-Sephadex chromatography of an extract of hepatocytes incubated for 90 min with 0.25 μM labeled 5-CH₃-H₄PteGlu. —, radioactivity (background not subtracted); ---, absorbance of marker 5-CH₃-H₄PteGlu. Details of the experiment are in the text. Uptake was at 37°C.

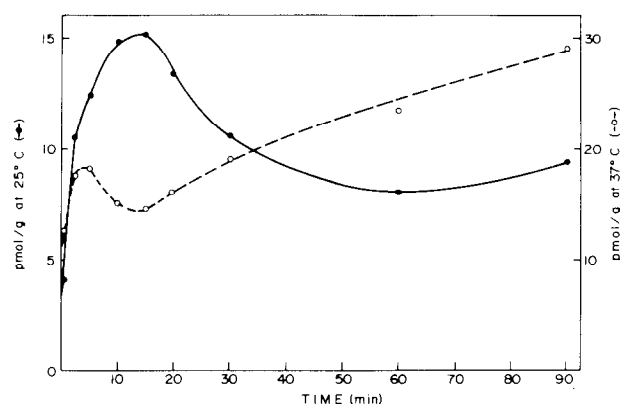


FIG. 3. Time course of uptake of PteGlu at 25°C (—) and at 37°C (---) at an extracellular concentration of 0.061 μM. The data at 25 and 37°C represent the average of at least two experiments.

Concentration Dependence of 5-CH₃-H₄PteGlu Transport—Fig. 5a shows that uptake of 5-CH₃-H₄PteGlu as a function of extracellular concentration appears to be composed of at least two components, a primary, saturable system evident at concentrations below about 5 μ M and a secondary component that remains linear in the concentration range tested. This

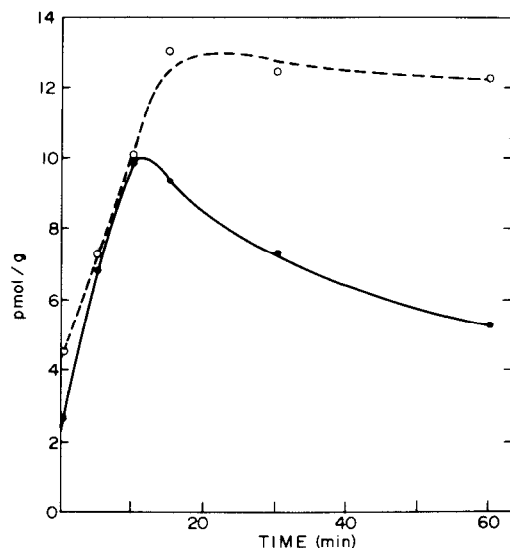


FIG. 4. Time course of PteGlu uptake in cells preincubated with 0.56 mM methotrexate (---) and in control cells (—). Cells were incubated for 15 min at 37°C with or without methotrexate, washed twice with fresh medium, and incubated for an additional 20 min at 37°C to allow unbound methotrexate to efflux from the cells. The cells were then washed once, again resuspended in fresh medium and incubated at 25°C with 45 nM [G -³H]PteGlu.

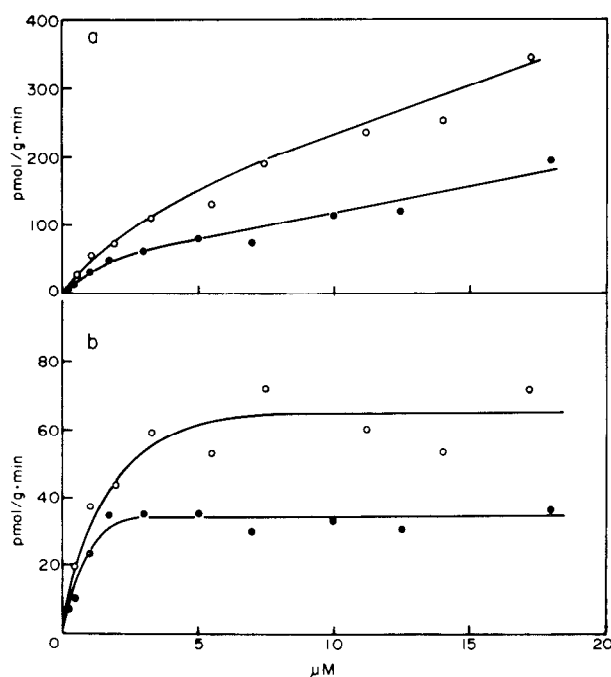


FIG. 5. Concentration dependence of 5-CH₃-H₄PteGlu transport into hepatocytes. Uptake was measured at 37°C by sampling the cells immediately (<2 s) after adding substrate and after 5 min of incubation. Initial uptake velocities were corrected for adsorption by taking the difference between these two values. *a*, total transport; *b*, uptake after correction for nonsaturable component; cells incubated in Na⁺-containing medium (○) and in choline-containing medium (●). The figure depicts a single, representative experiment.

observation was true for both sodium- and choline-containing media. Further, the slopes (*k*) of the linear, secondary component in the presence of sodium and choline (see Table II), were 0.041 min⁻¹ and 0.015 min⁻¹, respectively. These values are significantly different (*p* < 0.005). In Fig. 5b the linear components of transport have been subtracted from the net uptake rate. The resulting plots demonstrate that transport of 5-CH₃-H₄PteGlu is a saturable process in the presence or absence of sodium, although when sodium is omitted the initial velocity is decreased.

Table II lists the various kinetic parameters for 5-CH₃-H₄PteGlu transport both in the presence and absence of sodium. In choline-containing medium the apparent *K_m* was 0.83 μ M and the calculated *V_{max}* was 26.9 pmol/g·min. In the presence of sodium these values were 0.89 μ M and 56.9 pmol/g·min, respectively. There was no significant difference in the apparent *K_m* values in the presence or absence of sodium, whereas *V_{max}* in the absence of sodium was about half the value with sodium.

Fig. 6 demonstrates that the addition of 50 μ M 5-CHO-H₄PteGlu completely abolished the initial saturable component of 5-CH₃-H₄PteGlu uptake both in the presence or absence of sodium, while the secondary linear component was unaffected. Furthermore, in other experiments not shown here, carried out in the presence of sodium, the addition of 100 μ M 5-CHO-H₄PteGlu or 100 μ M methotrexate also abolished the initial saturable component without affecting the second component. When 500 μ M PteGlu was included, the uptake of 0.25 μ M 5-CH₃-H₄PteGlu was inhibited only about 40%. Therefore, initial rates determined in the presence of at least 50 μ M 5-CHO-H₄PteGlu reflect the entry of 5-CH₃-H₄PteGlu into the cells by the secondary, linear component of the uptake process.

In Fig. 7 the initial rate of uptake of 5-CH₃-H₄PteGlu at 0.25 μ M in sodium-containing medium was measured at various extracellular concentrations of methotrexate. Uptake in the presence of 100 μ M 5-CHO-H₄PteGlu was used to correct for the secondary, nonsaturable entry into the cells. The results indicate the *K_i* for methotrexate in sodium-containing medium is 5.5 μ M.

Effect of Inhibitors on 5-CH₃-H₄PteGlu Uptake—Table III shows the effects of various inhibitors on 5-CH₃-H₄PteGlu uptake. As shown, DNP at 0.1 mM had no significant effect on uptake in the presence of Na⁺ but actually stimulated uptake in choline-containing medium. DNP at 1.0 mM led to about 60% inhibition in the presence of choline, whereas in sodium-containing medium the inhibition was about 90%. Sodium cyanide and sodium arsenate at 10 mM were potent inhibitors regardless of the presence or absence of sodium in the medium.

TABLE II

Kinetic parameters for 5-CH₃-H₄PteGlu transport

The kinetic constants *K_m*, *V_{max}*, and *k* (the first order rate constant) were estimated as described under "Treatment of Kinetic Data" under "Materials and Methods." Binding of 5-CH₃-H₄PteGlu to the cells was estimated by terminating incubation immediately after adding the labeled 5-CH₃-H₄PteGlu. These values were used to correct initial velocities for binding. Numbers in parentheses are number of determinations. Values are reported as mean \pm standard error. Numbers with the same superscript are significantly different, *p* < 0.05.

Major cation in medium	<i>K_m</i> μ M	<i>V_{max}</i> pmol/g·min	<i>k</i> min ⁻¹
Sodium	0.89 \pm 0.19 (6)	56.9 ^a \pm 8.2 (6)	0.041 ^b \pm 0.005 (6)
Choline	0.83 \pm 0.39 (5)	26.9 ^a \pm 3.6 (5)	0.015 ^b \pm 0.003 (5)

FIG. 6. Concentration dependence of 5-CH₃-H₄PteGlu uptake. Initial velocities were determined as in Fig. 5. *a*, uptake in Na⁺ medium; *b*, uptake in choline medium. Cells were incubated in the presence of 50 μ M 5-CHO-H₄PteGlu (●) or with no addition (○), control.

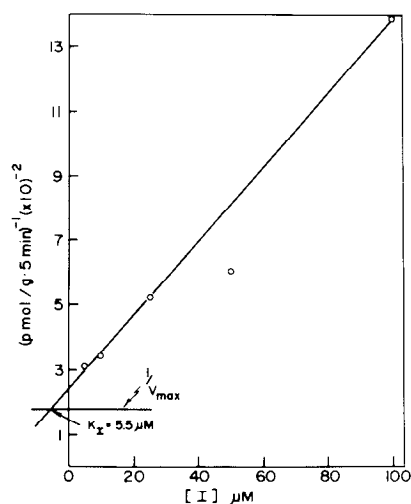
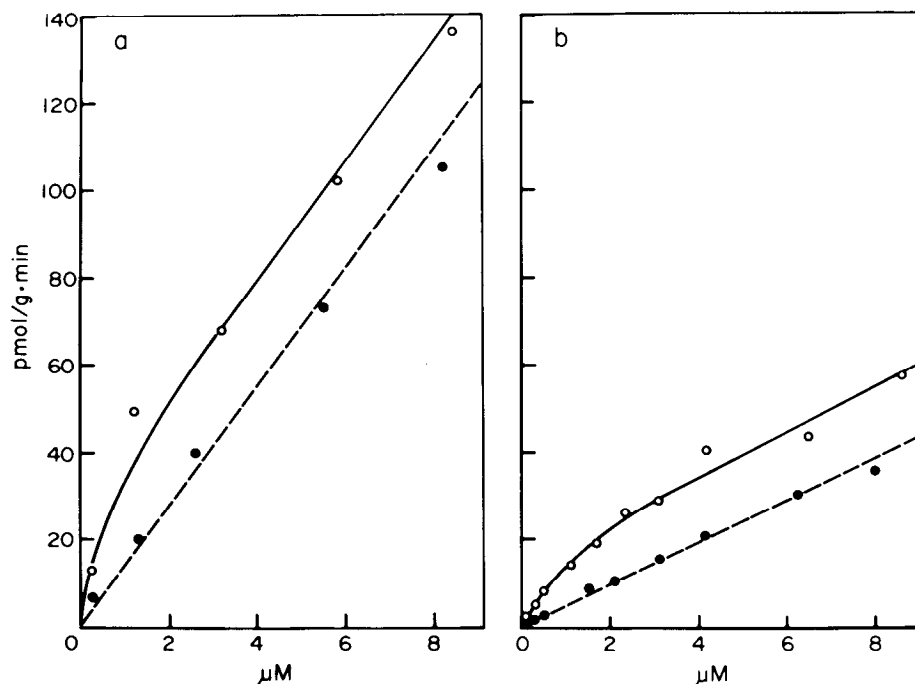


FIG. 7. Inhibition of 5-CH₃-H₄PteGlu transport by methotrexate in Na⁺-containing medium. Initial velocities were determined as in Fig. 5. Dixon plot (19) of initial velocity versus concentration. The concentration of 5-CH₃-H₄PteGlu was 0.25 μ M and uptake in the presence of 100 μ M 5-CHO-H₄PteGlu was used to correct for non-saturable entry into the cells. The points are the average of two separate experiments. The line $1/V_{\max}$ is from the estimated V_{\max} (Table II) for transport.

Sodium azide, however, at 10 mM stimulated uptake of 5-CH₃-H₄PteGlu with or without sodium in the medium. Ouabain, an inhibitor of the sodium, potassium pump, was slightly inhibitory at 1.0 mM. Ouabain was added to the incubation medium in ethanol and the final ethanol concentration was 1%. In sodium-containing medium the addition of 2% ethanol by itself had no significant effect, whereas in choline-containing medium ethanol was slightly stimulatory. Dithioerythritol at 10 mM was inhibitory in either medium. Addition of 10 mM sodium chloride to cells incubated in choline-containing medium had no effect on uptake. Removal of glucose from the sodium-containing medium had no effect. One might expect

TABLE III

Influence of inhibitors on 5-CH₃-H₄PteGlu transport

Hepatocytes were preincubated for 45 min at 37°C in the presence of inhibitor in both Na⁺- and choline-containing media. Labeled 5-CH₃-H₄PteGlu was added at a concentration of 0.25 μ M and uptake was allowed to proceed for 5 min. Inhibition by 0.1 mM 5-CHO-H₄PteGlu was determined by adding this compound to the cells at the same time as the labeled 5-CH₃-H₄PteGlu. Each value represents the mean calculated from the number of determinations in parentheses.

Addition or omission	Concentration	% Control	
		Na ⁺	Choline
	<i>mM</i>		
2,4-Dinitrophenol	0.1	95.2 (10)	123 (9) ^a
	1.0	9.5 (7) ^a	39.6 (1)
NaCN	1.0	81.8 (5) ^a	
	10.0	59.6 (8) ^a	49.3 (5) ^a
NaN ₃	1.0	100 (6)	
	10.0	130 (8) ^a	134 (5) ^a
Na ₂ HAsO ₄	1.0	82.2 (6) ^a	
	10.0	53.4 (8) ^a	56.5 (5) ^a
Ouabain	0.1	104 (2)	
	1.0	89.4 (11) ^a	
Ethanol	2% (v/v)	103 (2)	115 (3) ^a
Dithioerythritol	10.0	68.1 (2) ^a	56.6 (2) ^a
NaCl	10.0		98.6 (2)
Glucose		101 (2)	
5-CHO-H ₄ PteGlu	0.1	28.0 (11) ^a	31.5 (9) ^a

^a Significantly different from control, $p < 0.05$.

these cells to have sufficient glycogen stores since the level of glycogen in cells isolated by a similar procedure was high (20) and glycogen could be utilized for energy in the absence of extracellular glucose. 5-CHO-H₄PteGlu at 0.1 mM led to about 70% inhibition of uptake.

The stimulation of uptake by azide could be due to an inhibition of efflux of accumulated material as shown by Goldman (21) for methotrexate uptake by L1210 cells. To examine this hypothesis hepatocytes were incubated for 15 min with labeled 5-CH₃-H₄PteGlu in the presence of sodium

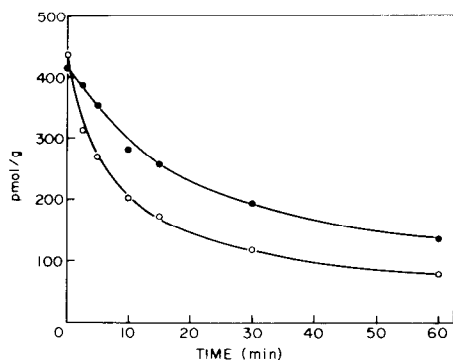


Fig. 8. Inhibition of efflux of 5-CH₃-H₄PteGlu from isolated hepatocytes in Na⁺-containing medium. The cells were incubated for 15 min in the presence of 1.66 μ M 5-CH₃-H₄-(G-³H)PteGlu and 10 mM sodium azide. The cells were washed once in 5-CH₃-H₄PteGlu- and azide-free medium at 37°C, resuspended in medium with (●) or without (○) 10 mM azide, and the accumulated 5-CH₃-H₄PteGlu was allowed to efflux from the cells. The results are the average of three determinations and are reported as picomoles per g of labeled 5-CH₃-H₄PteGlu retained by the cells at the given time.

azide. The cells were washed, resuspended in fresh medium with or without azide, and the accumulated 5-CH₃-H₄PteGlu was allowed to efflux from the cells. Fig. 8 shows that, in the presence of azide, efflux was inhibited as compared to control cells. Assuming that the initial rate of efflux is represented by the first 5 min of the process, the initial rate of efflux in medium containing azide was about half that of the control (12.7 ± 2.2 and 29.8 ± 4.7 pmol/g·min, respectively; $n = 3$, $p < 0.03$).

DISCUSSION

The present study indicates that the uptake of 5-CH₃-H₄PteGlu in freshly isolated rat hepatocytes is energy-dependent and saturable. The data further show that the initial rate of uptake can be divided into at least two components. One component was linear over the concentration range tested. However, the rate constant for this linear, secondary process varied significantly from 0.041 min^{-1} to 0.015 min^{-1} in medium containing, respectively, sodium or choline as the major cation. This component was not affected by the structural analogs 5-CHO-H₄PteGlu (100 μ M), methotrexate (100 μ M), or PteGlu (500 μ M). These properties suggest that the linear component of the uptake process represents either transport by a second, low affinity transport mechanism which is operating far below the K_m for 5-CH₃-H₄PteGlu or that 5-CH₃-H₄PteGlu is entering the cell by passive diffusion. The fact that this secondary, linear component is sensitive to the presence of sodium ions may favor the former interpretation.

By subtracting the initial velocities determined in choline-containing medium (the sodium-independent component) from those determined in sodium-containing medium (both components) the kinetic parameters for the sodium-dependent component(s) may be estimated. Preliminary observations indicate a K_m of $0.66 \pm 0.32 \mu$ M, a V_{\max} of 27.0 ± 9.5 pmol/g·min, and a k of $0.222 \pm 0.003 \text{ min}^{-1}$ ($n = 4$). These values are not significantly different from those for the sodium-independent component. Nevertheless both systems appear able to concentrate 5-CH₃-H₄PteGlu. Using the reported membrane potential of about 30 mV for isolated hepatocytes (22) and assuming that 5-CH₃-H₄PteGlu is a dianion at physiological pH, the Nernst equation allows the calculation of the distribution ratio for passive diffusion (about 0.1) in the presence of sodium. Assuming a similar ratio in choline-containing me-

dium, for control cells in the presence or absence of sodium the measured distribution ratio exceeded the expected value of 0.1 by at least 8-fold (Table I). Thus both the sodium-dependent and -independent components are probably energy-dependent. These findings were confirmed by the inhibition seen with sodium arsenate (Fig. 1 and Table III) a compound which inhibits ATP formation as a result of both respiration and glycolysis. Both sodium-dependent and -independent active transport systems for cycloleucine have been observed in hepatocytes (13).

The K_m value for transport of 5-CH₃-H₄PteGlu (about 0.9 μ M) in isolated hepatocytes by the saturable components agrees well with those reported in other mammalian systems. Goldman (8) reported K_m values for methotrexate of 5 μ M and for 5-CH₃-H₄PteGlu of 1 μ M in L1210 cells. Chen and Wagner (6) reported a K_m for 5-CH₃-H₄PteGlu of 0.9 μ M in hog choroid plexus. These reported K_m values are of the same order of magnitude as the concentration of folates in the serum, about 0.1 μ M (23).

It is known that the concentration of folate derivatives in the liver of the rat is much higher than in the serum, 20 μ g/g of liver and about 62 ng/ml of serum (23). Also, it is known that polyglutamate derivatives are the predominant forms in the liver (23). Since these forms of folate have been shown to be impermeable to human marrow cells (24) and, in liver, they are mostly bound to intracellular proteins (25, 26), Brown *et al.* (27) have postulated that formation of polyglutamates may be one mechanism for keeping folate compounds in the cells. In the present study we have shown that 5-CH₃-H₄PteGlu, the major form of folate in serum (23), is concentrated by hepatocytes. Thus there are at least two mechanisms by which the liver may retain folates.

The use of inhibitors to deduce information about metabolic processes in intact cellular systems is less than satisfactory. Among the factors which must be considered are the penetration of the inhibitors themselves, and multiple modes of action which these compounds can exert (28). Nevertheless, their utility has been demonstrated in innumerable studies and useful information can be obtained provided one is aware of their limitations. The use of a group of inhibitors in these experiments has permitted us to make a number of tentative conclusions. Hepatocytes apparently have an active system for efflux of 5-CH₃-H₄PteGlu. When cells are incubated with sodium azide, an inhibitor of electron transport, there was a stimulation of uptake both with and without sodium (see Fig. 1 and Table III). The phenomenon might be explained by a direct stimulation of the influx process only; however, it is more likely that azide inhibits both influx and efflux, but that it inhibits the latter to a larger degree. Indeed, the results (see Fig. 8) support the conclusion that efflux of 5-CH₃-H₄PteGlu is inhibited by azide. Azide also inhibits efflux of methotrexate in L1210 cells as reported by Goldman (8). His explanation for this phenomenon is that influx of methotrexate is coupled to energy reserves and is relatively insensitive to metabolic inhibitors, while the efflux component is highly dependent upon energy metabolism. In transport of 5-CH₃-H₄PteGlu by hepatocytes, arsenate (Fig. 1 and Table III) was inhibitory, whereas azide was stimulatory in both sodium-containing and choline-containing media. Since arsenate inhibits glycolytic phosphorylation as well as uncoupling phosphorylation from mitochondrial electron transport, while azide inhibits primarily electron transport, it is interesting to speculate that, for the most part, glycolysis can provide the energy reserves coupled to 5-CH₃-H₄PteGlu influx, but that

active efflux depends on the high level of ATP generated by oxidative metabolism.

The inhibition of 5-CH₃-H₄PteGlu uptake by sodium cyanide was contrary to the stimulation shown by azide. Since both compounds inhibit electron transport, some other action must explain this difference. Indeed, it is known that cyanide will disrupt disulfide bonds (29). Also, as shown in Table III, dithioerythritol led to significant inhibition of 5-CH₃-H₄PteGlu uptake. It is known that dithioerythritol will also lead to the disruption of disulfide bonds (28, 30). Thus, it appears that 5-CH₃-H₄PteGlu transport is dependent on intact disulfide bonds. Whether this is a specific effect on the carrier(s) or a general effect on the membrane is not clear.

5-CH₃-H₄PteGlu uptake was inhibited by structural analogs such as methotrexate (Fig. 7), 5-CHO-H₄PteGlu (Table III), and, to a lesser extent, PteGlu. The K_i value for methotrexate in the presence of sodium was estimated to be 5.5 μ M which corresponds well with the K_m obtained for methotrexate transport in L1210 cells by Goldman (8). The effects of inhibitors on the transport of methotrexate in hepatocytes reported by us previously (12), however, differ from the effects of the same inhibitors on 5-CH₃-H₄PteGlu transport reported here. Transport of methotrexate at 0.5 to 1.5 μ M was inhibited more strongly by ouabain (about 75 versus 10%) and by removal of glucose (60% versus no inhibition). Although 50 μ M 5-CHO-H₄PteGlu completely abolished the primary saturable component of 5-CH₃-H₄PteGlu uptake, at 1000 μ M it had no effect on methotrexate uptake. In the presence of sodium the K_m for methotrexate uptake was 2.2 mM (12) while the K_m for 5-CH₃-H₄PteGlu uptake was 0.9 μ M (Table III). Thus it appears that transport of 5-CH₃-H₄PteGlu and methotrexate in hepatocytes take place by different systems. The primary, saturable component for 5-CH₃-H₄PteGlu uptake appears to be a high affinity system which operates to transport this natural form of folate under physiological conditions. This is susceptible to inhibition by methotrexate. The secondary, nonsaturable component may reflect a low affinity system as discussed earlier. This latter system may be relatively nonspecific and be responsible for the transport of synthetic anions such as methotrexate.

The effects of DNP deserve special comment. At a concentration of 1.0 mM, DNP was more inhibitory than any other compound tested in the presence of sodium. It was even more inhibitory than 0.1 mM 5-CHO-H₄PteGlu which completely abolishes the primary saturable components of transport at this concentration (Fig. 6 and text). This indicates that the secondary, nonsaturable component is also susceptible to the action of DNP. In the absence of sodium, 1.0 mM DNP was less inhibitory, suggesting that it is the sodium-dependent uptake component which is more sensitive to inhibition by DNP.

Because of the existence of multiple uptake systems for 5-CH₃-H₄PteGlu which are saturable and nonsaturable in the concentration range tested, together with varying dependence on the presence of sodium, and the probable existence of an energy dependent efflux system in this hepatocyte preparation, the specific actions of the different inhibitors are difficult to sort out. For example, 0.1 mM DNP and 2% ethanol have no effect on uptake in the presence of sodium, but are stimulatory

in the absence of sodium. It is probable that they act similar to azide which inhibits efflux (Fig. 8 and Ref. 20). Varying actions of the inhibitors on different components of the uptake system may occur, and it is difficult to ascribe any observation to one specific component of this system. The present paper provides a general description of the characteristics of transport of 5-CH₃-H₄PteGlu by hepatocytes. A detailed analysis of the various components which contribute to this transport system is presently underway.

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