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Uptake of 5-methyltetrahydrofolate into PC-3 human prostate cancer cells is carrier-mediated

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Abstract

Uptake of 5-methyltetrahydrofolate into the PC-3 human prostate cancer cells was linear for the first 60 min. There was no difference in the initial rate of uptake in cells incubated in folate-free medium for 24 or 48 hr compared to control cells grown in folate-containing medium. The initial rate of 5-methyltetrahydrofolate uptake showed little dependence on extracellular pH and it was independent of extracellular sodium ions. Transport of 5-methyltetrahydrofolate into PC-3 cells was saturable – K_m = 0.74 μM and V_{max} = 7.78 nmol/10^9cells/min and these kinetic constants were not different in cells incubated for 24 hr in folate-free medium (K_m = 0.80 ± 0.22, V_{max} = 8.52 ± 0.50; P = 0.09, N = 3). Uptake of 5-methyltetrahydrofolate was inhibited by structural analogs with the K_i values being 0.50, 1.79, and 31.8 μM for 5-formyltetrahydrofolate, methotrexate, and folic acid, respectively. Uptake of 5-methyltetrahydrofolate was inhibited by the energy poisons, sodium cyanide, sodium arsenate, *p*-chloromercuriphenylsulfonate, and sodium azide. Uptake was inhibited by increasing concentrations of sulfate and phosphate ions, suggesting that 5-methyltetrahydrofolate may be transported by an anion-exchange mechanism. These results show that 5-methyltetrahydrofolate is transported into PC-3 prostate cancer cells by a carrier-mediated process.

Keywords: 5-methyltetrahydrofolate; Transport; PC-3 cells; Prostate; Prostate cancer cells

1. Introduction

Folic acid and its derivatives play a central role in metabolism. These coenzymes provide one-carbon units for the synthesis of thymidine, purines, methionine, and glycine and for methylation of DNA and RNA. Deranged folate metabolism has been implicated in numerous diseases including megaloblastic anemia [1], carcinogenesis [2], cardiovascular disease [3], and in the occurrence of neural tube defect pregnancies [4].

Heston and colleagues have recently discovered a protein which they named prostate-specific membrane antigen (PSMA) [5]. This protein is expressed in normal and malignant prostate cells [6]. PSMA was shown to have folylpolyglutamate hydrolase activity [5] and Heston suggested that this activity may result in decreased cellular folate coenzyme levels in prostate and that this could contribute to cancer of the prostate. This protein is expressed also in the small intestine where it functions to hydrolyze folylpolyglutamates to the monoglutamates prior to absorption by the enterocytes [7]. These findings have kindled an interest in folate transport and metabolism in the prostate and in prostate cancer cells.

Because mammals are not able to biosynthesize folates, they have evolved protein carriers which transport these essential compounds into cells. Several folate transport systems have been described in mammalian cells, including L1210 leukemia cells, fibroblasts, erythroid cells, hepatocytes, and enterocytes [8, 9].

Transport of the antifolate, methotrexate, into PC-3 human prostate cancer cells has been shown to be a carrier-mediated, energy-dependent process [10]. In the present paper we have investigated the transport of 5-methyltetrahydrofolate, the predominant folate in the blood, into PC-3 prostate cancer cells. We report that this transport system is independent of extracellular sodium ions, is carrier-mediated being inhibited by structural analogs and structurally unrelated organic anions, and is energy-dependent. The fact that methotrexate uptake into PC-3 cells was upregulated by prior incubation for 24 h in folate-free medium [10] while

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uptake of 5-methyltetrahydrofolate was not similarly upregulated suggests that there may be one system for transporting 5-methyltetrahydrofolate and methotrexate into the cells and an additional system which transports methotrexate (and is upregulated in folate deficiency) but does not transport 5-methyltetrahydrofolate into the cells.

2. Materials and methods

2.1. Materials

(6S)-5-methyl-[3′,5′,7,9-^{3}H]tetrahydrofolate (5-CH_3-H_4 PteGlu)[2] was purchased from Moravek Biochemicals (Brea, CA). Methotrexate, (6RS)-5-methyl- and (6RS)-5-formyltetrahydrofolate, folic acid, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 4-morpholineethane sulfonic acid (MES), and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), probenecid, *p*-chloromercuriphenylsulfonate (pCMPS), and dexamethasone were from Sigma Chemical Company. All other chemicals were reagent grade and were purchased from commercial sources.

2.2. Cell culture

Human prostate cancer cells, PC-3 (passage 19) were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone Labs), 1% (v/v) penicillin/streptomycin (5000 U/mL and 5000 μg/mL), and dexamethasone (1 ng/mL). Cells were maintained at 37°C in T-75 flasks (Costar) in a humidified 5% CO_2 atmosphere and subcultured at 1:6 dilution by trypsinization with 0.25% trypsin, 1 mM EDTA (Gibco-BRL). For transport experiments, cells were plated onto 6-well plates (Costar) at a concentration of 3×10^5 cells/well. For some experiments, cells were washed with folate-free RPMI 1640 and incubated in this medium for 24 or 48 hr prior to measuring uptake of 5-methyltetrahydrofolate as described below.

2.3. Uptake experiments

Cell monolayers on the 6-well plates were washed with the appropriate buffer: HBS (135 mM NaCl, 5 mM KCl,2.5 mM $CaCl_2$, 0.6 mM $MgSO_4$, 6 mM glucose, and 10 mM HEPES, pH 7.4) or MBS (MES replaced HEPES at pH 6.5 and below). In the experiments for determining the effects of sulfate and phosphate ion concentration on uptake the appropriate concentrations of potassium sulfate or potassium phosphate were added, isoosmotically, to Hepes—sucrose—MgO buffer (20 mM Hepes, 235 mM sucrose, pH adjusted to 7.4 with MgO). [^{3}H]-5-methyltetrahydrofolate was added at the onset of the experiments and uptake was terminated at the appropriate time by placing the 6-well plates on ice, aspirating the medium and rapidly washing them two times with 3 mL of ice-cold incubation medium. The cells were lysed with 1 mL of 10 mM EDTA, pH 12 and sonicated for 10 s. Aliquots of the lysate were counted for radioactivity in a liquid scintillation counter. Cell numbers were determined on parallel wells after trypsinization. Results are expressed as nmol or pmol per 10^9 cells per unit time. Curves were fitted and Michaelis-Menten kinetic parameters (K_m and V_{max}) were determined using GraphPad Prism version 3.02 for Windows 95 and statistical significance determined using GraphPad Instat version 3.05 for Windows 95 using one-way ANOVA with post-test significance determined by the Dunnett Multiple Comparisons Test (both programs were from GraphPad Software, San Diego, CA).

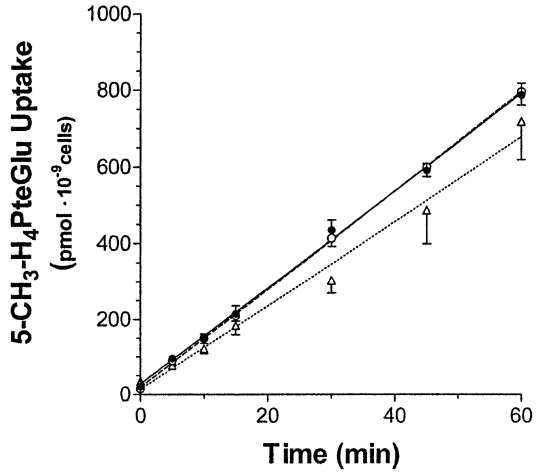

Fig. 1. Transport of 5-methyltetrahydrofolate into PC-3 cells vs. time. Uptake of 0.014 μM [^{3}H]-5-methyltetrahydrofolate was determined, at the times indicated, as described in Materials and Methods. Closed circles (●) represent uptake in cells grown in normal RPMI 1640 which contains 2.2 μM folate (N = 6). Open circles (○) and open triangles (Δ) represent uptake in cells pre-incubated for 24 h (N = 6) or 48 h (N = 2), respectively, in folate-free RPMI 1640 prior to measuring uptake. Results are mean ± SEM (error bars).

3. Results

3.1. Uptake of 5-methyltetrahydrofolate

Uptake of 5-methyltetrahydrofolate into PC-3 cells was determined in cells grown in normal medium which contains 2.2 μM folic acid and in cells which had been pre-incubated 24 and 48 h in folate-free medium prior to uptake. Fig. 1 shows that uptake of 5-methyltetrahydrofolate was linear for 60 min. There was a small (~20 pmol · 10^{-9}cells), apparent uptake at zero-time which probably represents rapid binding of substrate to the plasma membrane. Fig. 1 illustrates that pre-incubation of the cells for 24 or 48 h in medium without folate had no significant

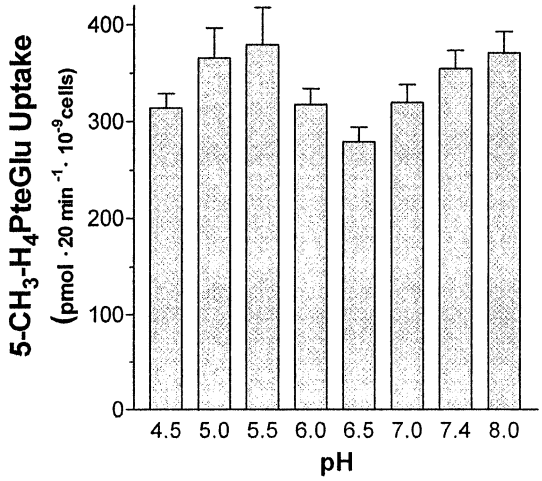

Fig. 2. pH dependence of 5-methyltetrahydrofolate transport into PC-3 cells. PC-3 cells were incubated in uptake medium at the indicated pH for 20 min with 0.014 μM 5-methyltetrahydrofolate. Results are mean ± SEM (error bars) for three separate experiments.

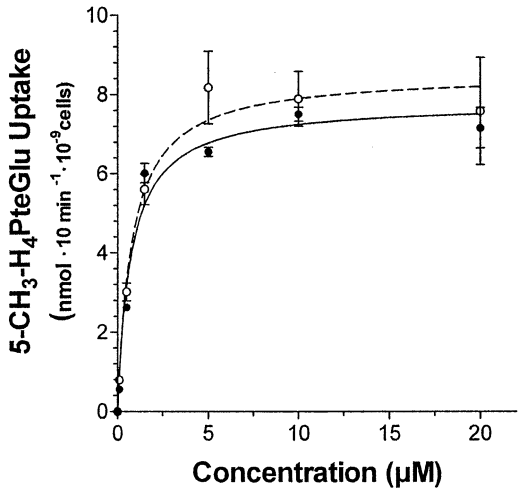

Fig. 3. Concentration dependence of 5-methyltetrahydrofolate transport into PC-3 cells. Uptake of 5-methyltetrahydrofolate was determined at 10 min in medium containing the indicated concentrations of labeled 5-methyltetrahydrofolate. Closed circles (●) represent uptake in cells grown in normal RPMI 1640 which contains 2.2 μM folate. Open circles (○) represent uptake in cells pre-incubated for 24 h in folate-free RPMI 1640 prior to uptake. Results are mean ± SEM (error bars) for three separate experiments.

effect on the initial rates of uptake – 12.7 ± 0.33, 12.9 ± 0.35, and 11.1 ± 0.82 pmol · min^{-1} · 10^{-9}cells, normal and 24 h and 48 h in folate-free medium, respectively ($P > 0.9$).

Fig. 2 shows the initial rate of uptake of 5-methyltetrahydrofolate at different initial incubation medium pH values. Uptake showed little change at pH values ranging from 4.5 to 8.0. All further experiments were carried out at the physiological pH of 7.4.

3.2. Uptake of 5-methyltetrahydrofolate vs. concentration in PC-3 cells

The initial velocity of 5-methyltetrahydrofolate transport into PC-3 cells was measured after 10 min as a function of increasing substrate concentration. Transport was determined in cells grown in normal medium and in cells pre-incubated ~24 h in folate-free medium. The results are shown in Fig. 3 and indicate that there was no effect of pre-incubation in folate-free medium in confirmation of the results reported in Fig. 1. The Michaelis-Menten kinetic parameters were determined by non-linear regression curve fitting of the data to the standard equation using GraphPad Prism. For cells grown in normal medium and cells pre-incubated 24 h in folate-free medium, respectively, K_m = 0.74 ± 0.12 and 0.80 ± 0.22 μM ($P = 0.91$, $N = 3$) and V_{max} = 7.78 ± 0.25 and 8.52 ± 0.50 nmol · min^{-1} · 10^{-9}cells ($P = 0.09$, $N = 3$).

3.3. Effect of structural analogs on 5-methyltetrahydrofolate transport in PC-3 cells

Transport of 5-methyltetrahydrofolate into PC-3 cells was inhibited by the structural analogs, 5-formyltetrahydrofolate (Fig. 4A), methotrexate (Fig. 4B), and folic acid (Fig. 4C). These analogs caused a concentration dependent inhibition of 5-methyltetrahydrofolate uptake. The insets in Fig. 4 show the Dixon plot of the data. Assuming that the observed inhibition is competitive, these plots may be used to calculate an apparent inhibition constant, K_i. This value is determined by the intersection of the line $1/V_{max}$ (dotted line in insets) with the plot of $1/v_i$ vs. concentration of inhibitor. The K_i for 5-formyltetrahydrofolate was 0.50 μM, for methotrexate it was 1.79 μM, and for folic acid 31.87 μM.

3.4. Effect of sulfate and phosphate ions on 5-methyltetrahydrofolate uptake into PC-3 cells

The effects of the structurally unrelated anions, sulfate and phosphate, on uptake of 5-methyltetrahydrofolate are shown in Fig. 5. Uptake decreased as the anion concentration increased up to 20 mM with the maximal inhibition being 60% for sulfate and 53% for phosphate ions.

3.5. Effect of inhibitors on 5-methyltetrahydrofolate uptake into PC-3 cells

The effects of various inhibitors on the transport of 5-methyltetrahydrofolate into PC-3 cells were tested. The results are shown in Table 1. The cells were incubated with the inhibitors for 60 min prior to measuring 5-methyltetrahydrofolate uptake, except for probenecid and DIDS which were added at the same time as the labeled substrate. When sodium chloride in the medium was replaced with choline chloride there was no effect on uptake, indicating that

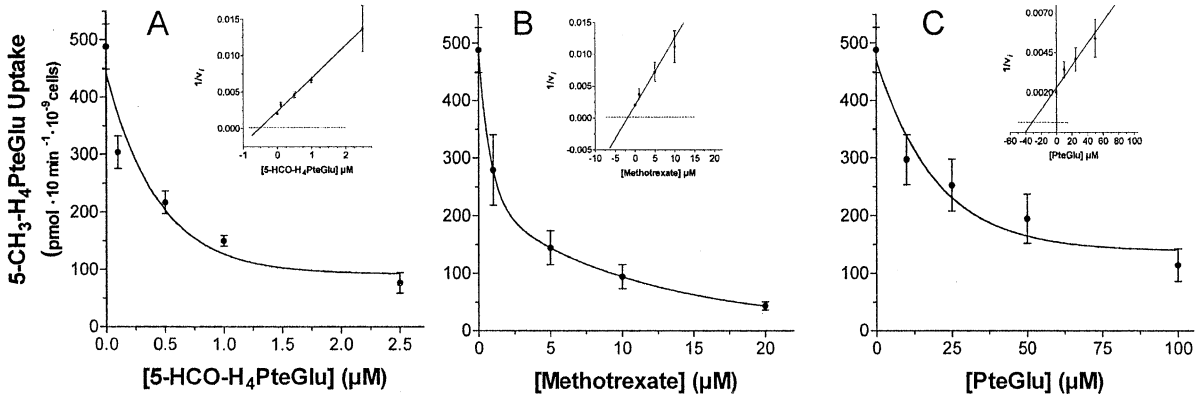

Fig. 4. Effect of structural analogs on 5-methyltetrahydrofolate transport into PC-3 cells. Uptake of 0.028 μM 5-methyltetrahydrofolate was determined at 10 min in incubation medium with increasing concentrations of the structural analogs, 5-formyltetrahydrofolate (panel A), and methotrexate (panel B), and folic acid (panel C). Insets in each panel show a Dixon plot of the data. The dashed line in the insets represents $1/V_{max}$ and the point where the lines for the inhibitors cross this line represents $-K_i$. Results are the mean ± SEM (error bars) for three separate experiments.

5-methyltetrahydrofolate transport is not sodium-dependent. Uptake of 5-methyltetrahydrofolate was inhibited by the energy inhibitors sodium azide, sodium cyanide, and sodium arsenate. The sulfhydryl inhibitor, *p*-CMPS was also an inhibitor of 5-methyltetrahydrofolate transport into the cells.

The uptake of 5-methyltetrahydrofolate into the PC-3 cells was inhibited by the structurally unrelated anions – probenecid and DIDS – as shown also in Table 1.

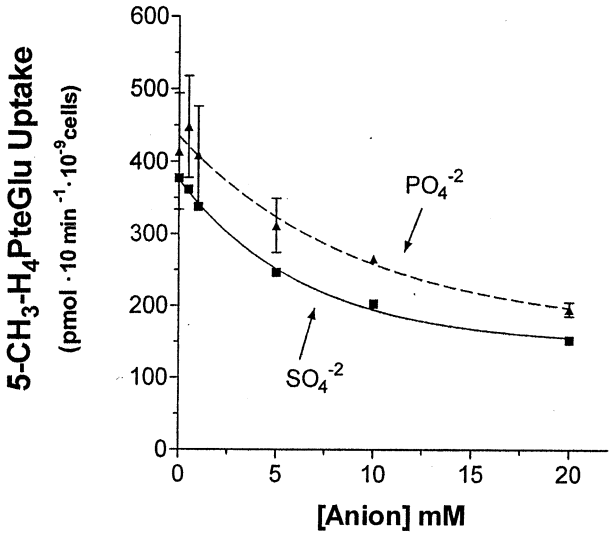

Fig. 5. The effects of increasing concentrations of sulfate and phosphate ions on 5-methyltetrahydrofolate uptake into PC-3 cells. Cells were incubated for 25 min in medium of differing concentrations of the anions, as shown, in Hepes—sucrose—MgO buffer as described in Material and Methods. [^{3}H]-5-methyltetrahydrofolate (0.028 μM) was added and uptake was determined after 10 min incubation. Results are mean and range of two experiments for phosphate and a representative experiment for sulfate.

4. Discussion

Folate coenzymes are necessary for the synthesis of purines, thymidine, and certain amino acids. These coenzymes are especially important for the rapid growth of cancer cells, and, because mammalian cells can not synthesize folates, transport systems in the plasma membrane are required for cells to take up these coenzymes. Therefore, we undertook the present study to investigate the mechanism whereby 5-methyltetrahydrofolate (the predominant folate derivative in blood plasma) is transported into human PC-3 prostate cancer cells. Transport of 5-methyltetrahydrofolate into the PC-3 cells was linear over 60 min of uptake (Fig. 1). We found that uptake of 5-methyltetrahydrofolate was not

Table 1
Effect of Inhibitors on 5-Methyltetrahydrofolate Uptake into PC-3 Cells[a]

Inhibitor	5-Methyltetrahydrofolate Uptake (pmol · 20 min^{-1} · 10^{-9}cells)
Control (9)	661 ± 80
$-Na^+$ (2)[b]	685 ± 9
NaN_3, 10 mM (5)	299 ± 36[c]
NaCN, 10 mM (6)	16.2 ± 1.7[c]
$NasO_4$, 10 mM (5)	386 ± 76[c]
p-CMPS, 1 mM (6)	26.6 ± 3.9[c]
Probenecid, 1 mM (3)	314 ± 7[c]
DIDS, 1 mM (3)	48.3 ± 3.8[c]

[a] PC-3 cells were pre-incubated for 60 min with the substances shown prior to measuring uptake except for probenecid and DIDS which were added to the incubation medium at the same time as the [^{3}H]-5-methyltetrahydrofolate. In either case, 5-methyltetrahydrofolate uptake was measured over a 20 min time period. The values represent the mean ± SEM of the number of experiments in parentheses. The concentration of 5-methyltetrahydrofolate was 0.028 μM.

[b] Choline chloride replaced sodium chloride in the sodium-free incubation medium.

[c] $p < 0.01$ when compared to Control.

dependent on a sodium ion gradient because uptake was unchanged when choline replaced sodium in the uptake medium (Table 1). The pH profile for 5-methyltetrahydrofolate uptake (Fig. 2) shows that there is little difference in uptake from pH 4.5 to 8.0. This is decidedly different from the pH profile for methotrexate uptake in PC-3 cells [10] and in LNCaP cells [11] – methotrexate uptake in these prostate cancer cells decreased markedly as the medium pH increased from pH 4.5 and uptake was near the minimum at physiological pH. It might be argued that the difference in pH dependence of 5-methyltetrahydrofolate and methotrexate transport in PC-3 cells could be explained by differential concentrations of species with differing net charge as the extracellular pH is varied. However, inspection of the dissociation constants for these folate derivatives does not support this conclusion (methotrexate: N-1, pK_a 5.71; α-carboxyl, 3.36; γ-carboxyl, 4.70; N-5, $<$ -1.5; N-10, 0.05 and 5-methyltetrahydrofolate: N-1, 1.24; α-carboxyl, 3.5; γ-carboxyl, 4.8; N-5, 5.2; N-3/O-4 amide, 10.8 [12-15]. At the extracellular pH values examined in the present study for 5-methyltetrahydrofolate and for methotrexate [10], both compounds would have similar charge. It is possible that protonation of N-1 of methotrexate (pK_a 5.71) might result in increased uptake of this derivative due to the positive charge at N-1 interacting with the carrier, whereas protonation of N-5 of 5-methyltetrahydrofolate (pK_a 5.2) may not result in such interaction. Confirmation of this awaits a three-dimensional structure for the carrier protein.

The pH profile for methotrexate uptake into L1210 murine leukemia cells [16] is different from the profile for either methotrexate or 5-methyltetrahydrofolate in PC-3 cells. In the L1210 cells, methotrexate uptake displayed a broad optimum at about pH 7.5 with uptake decreasing quickly to a minimum at pH 6.5 to 5.0. In isolated hepatocytes, we found that methotrexate uptake showed a sharp optimum at approximately pH 7, whereas uptake of 5-methyltetrahydrofolate showed a smooth increase from pH ~8.0 to 5.5 [17]. Uptake of folates in the intestine resembles uptake of 5-methyltetrahydrofolate in liver in that uptake was maximal at low pH ([18, 19]. It is interesting that Kumar et al. [20] have shown that expression of the reduced folate carrier (RFC) in IEC-6 intestinal epithelial cells but not in X*enopus laevis* oocytes, resulted in increased uptake of folates at low pH. These findings have been confirmed by Rajgopal et al. [21] in IEC-6 cells and they further showed that expression of the RFC in L1210 cells only resulted in uptake at physiological pH. These results suggest that post-translational modifications(s) of the RFC protein may be involved with the pH dependence of folate transport. It is also interesting that Sharina et al. [22] have mutated the RFC and they have found that several amino acid substitutions resulted in insensitivity to inhibition of uptake by chloride ion. Thus, whether differences in the pH dependence may be due to sequence differences and/or post-translational modifications requires further studies.

Transport of some substrates may be regulated by dietary levels of that substrate [23]. For example, uptake of biotin [24] and riboflavin [25, 26] are regulated by levels of the vitamin. The intestinal transporter for folate has recently been shown to be upregulated in folate deficiency [27]. We also showed that uptake of methotrexate into PC-3 cells was upregulated by pre-incubation for 24 h in folate-free medium [10]. We therefore repeated this experiment in the present study of 5-methyltetrahydrofolate transport into PC-3 cells. We found no evidence for upregulation of 5-methyltetrahydrofolate transport since pre-incubation for 24 or 48 h in folate-free medium had no effect on uptake as shown in Figs. 1 and 3. Thus, it appears that regulation of transport of these two folate derivatives is different in the PC-3 cells.

Additional evidence for carrier-mediated transport of 5-methyltetrahydrofolate into PC-3 cells is provided by inhibition by structural analogs. The apparent inhibition constant, K_i for 5-formyltetrahydrofolate and methotrexate (0.50 and 1.79 μM, respectively) was similar to the K_m (0.74 μM) for 5-methyltetrahydrofolate uptake. Folic acid was much less inhibitory (K_i = 32 μM). This inhibition pattern – the reduced folates and methotrexate displaying similar affinity for the carrier and folic acid displaying much less affinity – is seen for 5-methyltetrahydrofolate uptake into the hepatocyte basolateral membrane vesicles [28], in L1210 cells [29], and in PC-3 cells [[10] and this communication]. The only carrier which shows equal affinity for folic acid and the reduced folates is in the small intestine [8].

Transport of methotrexate into L1210 leukemia cells is known to be via an ion-exchange mechanism and uptake is inhibited by a number of anions [30-33]. Fig. 5 shows that the initial rate of uptake of 5-methyltetrahydrofolate decreased as the concentration of sulfate or phosphate in the medium increased. It would appear that 5-methyltetrahydrofolate uptake into PC-3 cells may also be via an ion-exchange mechanism. We saw a similar inhibition of uptake of methotrexate by chloride ion in PC-3 cells [10] and by sulfate and phosphate ions in LNCaP cells [11]. These results are in contrast to methotrexate uptake into rat liver basolateral membrane vesicles (BLMV) [34] and to 5-methyltetrahydrofolate uptake into these vesicles [35]. Methotrexate transport in BLMV was inhibited by a number of structurally unrelated anions; however, when uptake was measured with a five-fold anion gradient (inside greater than outside) there was no trans-stimulation of methotrexate uptake into the vesicles ruling out an ion-exchange mechanism [34]. Uptake of 5-methyltetrahydrofolate into the BLMV showed no significant inhibition by structurally unrelated anions [35].

Uptake of 5-methyltetrahydrofolate into PC-3 cells was inhibited by metabolic poisons and by the sulfhydryl agent, *p*-CMPS. The metabolic poisons, azide and cyanide inhibited uptake of 5-methyltetrahydrofolate into the PC-3 cells (Table 1). This is similar to methotrexate uptake in these cells [10]. In contrast, methotrexate uptake in LNCaP cells

was not inhibited by these metabolic poisons [11] and uptake of 5-methyltetrahydrofolate in hepatocytes [36] and methotrexate in L1210 cells [37] was stimulated by azide. This was attributed to an active efflux system for the folates which was inhibited by the metabolic effects of azide whereas the uptake of the folates was much less affected by reducing energy stores. Thus, net uptake of folates in cells is a balance between uptake and efflux and the differences in susceptibility to metabolic poisons is complex. For 5-methyltetrahydrofolate uptake into PC-3 cells it would appear that the energy-dependent active efflux system does not contribute significantly to net uptake.

In conclusion, the results of our studies suggest that 5-methyltetrahydrofolate is transported into PC-3 cells by an energy-dependent, carrier-mediated system. Uptake is independent of extracellular sodium ions. Uptake was inhibited by increasing concentrations of sulfate and phosphate ions, suggesting the possibility that 5-methyltetrahydrofolate uptake is via an exchange for intracellular anions.

Acknowledgments

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