MINIREVIEW

Transport of Folates and Antifolates in Liver

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Abstract. **The characteristics and mechanisms of hepatic transport of folates and antifolate cancer drugs, for example, methotrexate, have been studied in perfused liver, isolated hepatocytes (in both freshly isolated cells and in primary cell culture), and membrane vesicles isolated from the basolateral membrane. Both naturally occurring folates and antifolates are taken up by the perfused liver and secreted into bile by apparently active processes, since these compounds are concentrated in liver and bile compared with the perfusate. Transport of the naturally occurring folate 5-methyltetrahydrofolate in isolated hepatocytes and basolateral membrane vesicles is via cotransport with hydrogen ions, is electroneutral, and is inhibitable by other reduced folates and by methotrexate. Transport of methotrexate is by a multispecific anion carrier, is electrogenic, and is not inhibitable by reduced folates (e.g., 5-methyl- and 5-formyltetrahydrofolate). Thus, the hepatocyte has separate systems for uptake of the naturally occurring, reduced folates and for the 4-amino-substituted antifolates.** [P.S.E.B.M. 1993, Vol 202]

The coenzyme forms of folic acid are necessary for many essential reactions in intermediary metabolism. These include providing one-carbon units for the synthesis of purines and thymidine, for the interconversion of serine and glycine, for the degradation of histidine and glycine, and for the methylation of homocysteine to regenerate methionine. Methionine, via *S*-adenosylmethionine, is necessary for a wide range of cellular methylation reactions, including methylation of DNA, RNA, and proteins. Folate antagonists, e.g., methotrexate, are used to treat cancer and rheumatoid arthritis. These "antifols" are toxic to normal tissues and hepatic injury is one aspect limiting the use of these drugs.

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Transport of both folates and antifolates into the cytoplasm across the plasma membrane is the first step in their assimilation by cells. Subsequent to cellular uptake, these compounds are converted to the polyglutamate forms by addition of several glutamic acid residues in γ-peptide linkage. These polyglutamates are better retained in the cell and are generally better substrates for folate-utilizing enzymes and, in the case of antifolates, better inhibitors of their respective target enzymes.

Hepatic transport of folate compounds, the subject of this review, is complex due to the polar nature of the hepatocyte. The hepatocyte takes up these compounds through the basolateral membrane from the portal vein or general circulation and secretes them into the bile through the canalicular membrane. The intestine may then reabsorb these compounds, thus giving rise to an enterohepatic circulation. The enterohepatic circulation of folates is beyond the scope of this review and the reader is referred to reviews of this topic (1, 2).

Background and Previous Reviews

The efficacy of the folate antagonists in cancer treatment has generated a large amount of work on the

mechanism of transport of these compounds and of the natural folate coenzymes in cancer cells and also in normal cells. The reader is referred to several excellent reviews which document studies in hematopoietic cells, choroid plexus, the small intestine, and kidney (3–6). Neoplastic cells generally express a single major folate transport pathway with high affinity for 5-substituted reduced folates and methotrexate and low affinity for folic acid, whereas normal cells have one or more transport systems that express differing relative affinities for folic acid, 5-substituted folates, and methotrexate (6).

It has recently been shown that a variety of cancer cell lines adapted for growth on low levels (n*M*) of folate express a glycosylphosphatidylinositol-linked membrane protein that binds folates. This protein is part of a receptor-mediated transport system for 5-methyltetrahydrofolate. This work has been done by Kamen and associates (7) in MA104 (monkey kidney epithelial) cells and by Antony and associates (8) in KB (human epidermoid carcinoma) cells. A recent article by Anderson *et al.* (9) and a review by Antony (10) summarize the current hypothesis regarding the role of this binding protein in folate transport in these cells. This protein has been cloned from KB cells (11, 12), Caco-2 cells (13), and human placenta (14). Of particular interest, Elwood (11) did not find mRNA for this protein in human liver on Northern blot analysis using radiolabeled cDNA probes to the KB protein, but he did in KB cells and placenta. More recent work by Weitman *et al.* (15) using monoclonal antibodies, as well as Northern blot analysis, confirmed the findings of Elwood (11) and extended them to show the presence of this binding protein in choroid plexus, lung, thyroid, and kidney. These investigators also found no evidence of the binding protein in liver, intestine, muscle, cerebellum, cerebrum, and spinal cord.

Transport of Folates into Liver Cells

Uptake and Biliary Secretion of 5-Methyltetrahydrofolate in Perfused Liver. Strum and Liem (16) used isolated, perfused rat liver to study uptake and biliary secretion of 5-methyltetrahydrofolate. They found that uptake into liver and secretion into bile was inhibited by metabolic poisons. 5-Methyltetrahydrofolate was concentrated in liver 1.3-fold and in bile 15- to 19-fold over the concentration in the perfusate. The apparent K_m was 450 μM. It has been reported that isolated hepatocytes also show a similar low-affinity carrier system for 5-methyltetrahydrofolate—K_m of 12.6 m*M* (17). These values are quite high considering that the concentration in rat plasma is about 0.3 μM and the physiologic significance of these findings is doubtful.

Transport of 5-Methyltetrahydrofolate into Isolated Hepatocytes and Purified Basolateral Membrane Vesicles. The isolated hepatocyte offers many advantages over perfused liver for transport studies, and freshly isolated and cultured hepatocytes have been used extensively for studying folate transport. Transport of 5-methyltetrahydrofolate, the predominant folate coenzyme in plasma, in freshly isolated hepatocytes was studied by Horne *et al.* (18). Uptake was found to be a complex process composed of a saturable system with an apparent K_m of 0.89 μM and a second system that was not saturable at concentrations up to 20 μM. Both of these systems were less active in the absence of Na^+ ions in the medium. 5-Methyltetrahydrofolate uptake was inhibited by the structural analogs 5-formyltetrahydrofolate, methotrexate, and, to a lesser extent, folic acid. Uptake was inhibited by 2,4-dinitrophenol, cyanide, and arsenate. Surprisingly, azide stimulated uptake of 5-methyltetrahydrofolate. This was attributed, at least in part, to inhibition of efflux by azide.

A recent study (19) showed that 5-methyltetrahydrofolate uptake into hepatocytes was dependent on a transmembrane hydrogen ion gradient, with uptake increasing linearly as the medium became more acid with respect to the cytosol. Scatchard plots of 5-methyltetrahydrofolate uptake versus hydrogen ion concentration indicated a K_m for H^+ of 24 n*M* (pH 7.6). Hill plots suggested a 1:1 stoichiometry for interaction of 5-methyltetrahydrofolate transport with protons. Both the apparent K_m and V_{max} for 5-methyltetrahydrofolate uptake were increased in hepatocytes incubated at extracellular pH 5.5 vs 7.4, which suggests an increased number and/or increased activity of the carrier. In this study, it was also shown that 5-methyltetrahydrofolate uptake was not dependent on sodium ions when the initial uptake rate was determined immediately after resuspending the cells in sodium-free medium. However, if hepatocytes were first preincubated in sodium-free medium for 30 min, uptake was dependent on sodium ions. Measurements of intracellular pH indicated that preincubation in sodium-free medium led to a decrease in the magnitude of the hydrogen ion gradient and, therefore, a decrease in the uptake of 5-methyltetrahydrofolate. Thus the sodium dependence reported previously (18) is apparently due to the inability of hepatocytes to properly regulate intracellular pH in the absence of extracellular sodium ions.

These findings in isolated hepatocytes have been confirmed and extended in transport studies using purified basolateral membrane vesicles (BLMV) isolated from liver by Percoll density-gradient centrifugation (20). In these BLMV, uptake of 5-methyltetrahydrofolate was similar when a 100-m*M* Na^+ gradient or a 100-m*M* K^+ gradient was imposed across the vesicle membrane, which proves that 5-methyltetrahydrofolate transport into the liver cell is independent of an Na^+

gradient. Uptake was, however, dependent on a gradient of hydrogen ions. The initial uptake rate was faster when an initial H^+ ion gradient was imposed across the membrane ($pH_{out} = 5.0$, $pH_{in} = 7.5$) and was faster at pH 5.0 (both out and in) than at pH 7.5 (out and in). In the presence of the H^+ ion gradient, uptake at 3–5 min was about 2-fold higher than at equilibrium (60 min)—the so-called "overshoot" phenomenon. No overshoot was seen when both inside and outside pH were the same (i.e., both at pH 5.0 or both at pH 7.5). Uptake was saturable at pH 5.0, both with or without an inwardly directed H^+ ion gradient (K_m = 0.58 and 0.36 μM, respectively). Interestingly, uptake was not saturable at pH 7.5. Uptake was inhibited by 5-formyltetrahydrofolate, methotrexate, and, to a lesser extent, folic acid under pH gradient conditions or pH = 5.0 (no gradient). These analogs were not inhibitory at pH 7.5 (no gradient), a finding that supports the lack of saturability at pH 7.5. Hill plots indicated a stoichiometry of 1:1 for the interaction of protons with 5-methyltetrahydrofolate transport in the BLMV. Uptake of 5-methyltetrahydrofolate was not inhibited by substrates (e.g., sulfate, oxalate, chloride) of a multispecific anion carrier present in liver (21). Transport of 5-methyltetrahydrofolate did not respond to a intravesicular electrical gradient. These same characteristics were seen for 5-methyltetrahydrofolate transport into human liver BLMV (D. W. Horne, unpublished observations). These data, taken together, strongly suggest that 5-methyltetrahydrofolate is taken up by the liver cell via cotransport with hydrogen ions.

It is appropriate to question the physiologic significance of the suggestion that 5-methyltetrahydrofolate is cotransported into the liver cell along with protons, because the extracellular fluid is, it is assumed, maintained at about pH 7.4. The manner in which a transmembrane H^+ gradient across the liver cell could be maintained is not known. However, two separate systems could be involved. The first is the Na^+/H^+ antiporter (22), which is present in liver basolateral membranes. The second system is composed of a plasma membrane NADH oxidoreductase (23, 24) and a *b*-type cytochrome (25), which suggests the possibility of an electron transport system that may also translocate protons across the plasma membrane. In this regard, 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 (26, 27). Therefore, the hepatocyte need not maintain a bulk-phase hydrogen ion gradient to energize transport systems; the high proton conductivity of the phospholipid membrane could result in the delivery of protons extruded via either the Na^+/H^+ antiporter or the NADH oxidoreductase system directly to the membrane carrier, in effect, generating a "localized" hydrogen-ion gradient that could be utilized for active transport of solutes.

Effect of Aging on 5-Methyltetrahydrofolate Transport into Hepatocytes. The effect of aging on 5-methyltetrahydrofolate transport and metabolism has been studied in isolated rat liver cells (28). The initial rate of uptake and the ability to accumulate 5-methyltetrahydrofolate was significantly decreased in hepatocytes from 24-month-old rats as compared with rats aged 3 or 12 months. Neither the total folate level nor the distribution of the individual coenzymes was affected by age, which suggests that membrane transport is not the limiting factor in hepatic folate assimilation.

Effect of Alcohol on 5-Methyltetrahydrofolate Transport into Hepatocytes. Ethanol is known to interfere with folate metabolism (2) and to affect folate uptake into hepatocytes. Ethanol was shown to stimulate uptake of 5-methyltetrahydrofolate (by about 40% over control) in isolated hepatocytes (29). The effect was specific for 5-methyltetrahydrofolate, because ethanol resulted in inhibition of uptake of methotrexate and folic acid. This stimulation required ethanol metabolism since it required time for the effect to be seen (maximal stimulation at 50 min of incubation) and was abolished by the alcohol dehydrogenase inhibitor, pyrazole (30).

Transport of 5-Methyltetrahydrofolate into Hepatoma Cells. Galivan (31) has studied the transport of 5-methyltetrahydrofolate into H35 hepatoma cells. Uptake of 5-methyltetrahydrofolate obeyed saturation kinetics: K_m = 2.5 μM and V_{max} = 1.9 pmol/min/mg protein. The H35 cells accumulated 5-methyltetrahydrofolate some 5-fold over the medium concentration (2 μM). Methotrexate was a competitive inhibitor of 5-methyltetrahydrofolate transport, with a K_i = 10.4 μM. An H35 cell line that was resistant to methotrexate growth inhibition showed greatly decreased uptake and accumulation of 5-methyltetrahydrofolate and methotrexate. Inhibitor studies showed that 500 μM 5-methyltetrahydrofolate or 500 μM methotrexate did not inhibit uptake of 10 μM methotrexate or 10 μM 5-methyltetrahydrofolate, respectively which indicates that the resistant cell line had lost the folate carrier. This is supported by the fact that 5-formyltetrahydrofolate caused transstimulation of 5-methyltetrahydrofolate uptake in the parent H35 cell line but not in the resistant line.

Transport of Antifolates into Liver Cells

Uptake and Biliary Secretion of Methotrexate in Perfused Liver. Transport of the antifolate drug methotrexate from blood to bile has been studied using the isolated perfused liver. Strum and Liem (32) found that hepatic uptake was saturable with a K_m of 1.3 mM. Uptake was energy dependent and inhibited by folic acid. Methotrexate was concentrated in the liver 4.6

times and in the bile from 70 to 120 times that in the perfusate. Subsequently, it was shown that the unconjugated bile acids, cholate and deoxycholate, inhibited liver uptake by about 75% and biliary secretion of methotrexate by about 95% (33). Deutsch and Kolhouse (34) used an *in vivo* model with systemic administration of compounds to show that methotrexate uptake into liver apparently is composed of two system one inhibitable by cholate and the other inhibitable by unlabeled methotrexate or folic acid. They ascribed the biliary output of methotrexate to the cholate-sensitive hepatic uptake.

Transport of Methotrexate into Isolated Hepatocytes and Purified Basolateral Membrane Vesicles. Transport of methotrexate into isolated liver cells was found to be via an energy-requiring process(es). There is apparently a low-affinity system (K_m = 2.3 mM) (35) and a high-affinity system (K_m = 5.9 μM) (36). Both transport systems were inhibited by metabolic poisons, anaerobiosis, and removal of glucose from the incubation medium. Both systems were diminished by ouabain and replacement of sodium with choline. Maximal uptake of methotrexate was seen at pH $\approx$ 7.0 (19).

The specificity of the methotrexate transport systems is of particular interest. Neither the low- nor high-affinity systems were inhibited by 100- to 1000-fold excess of folic acid, 5-formyltetrahydrofolate, or 5-methyltetrahydrofolate, whereas unlabeled methotrexate and aminopterin similarly inhibited uptake of the drug. These inhibition patterns indicate that the 4-amino group of the pteridine moiety of methotrexate and aminopterin is a structural determinant in the specificity of these transport systems. Furthermore, these findings show that the hepatocyte has a separate route(s) for uptake of methotrexate and the naturally occurring, reduced folates (e.g., 5-methyl- and 5-formyltetrahydrofolate), since methotrexate markedly inhibits 5-methyltetrahydrofolate uptake (*vide supra*) whereas 5-methyltetrahydrofolate does not inhibit methotrexate uptake.

Gewirtz *et al.* (37) found that bile salts and various organic anions were potent inhibitors of methotrexate influx. At 100 μM concentrations, taurocholate, cholate, bromsulfophthalein, and rose bengal inhibited uptake of 1 μM methotrexate by 70–90%. The K_i for taurocholate inhibition was 20 μM and high concentrations of methotrexate inhibited uptake of taurocholate, which suggest that these two compounds may share a transport site(s), at least in part. Similarly, the organic anion probenecid was found to competitively inhibit methotrexate uptake ($K_i \approx$ 100 μM) into hepatocytes (38). These findings would indicate that the methotrexate transport system has a relatively broad specificity for organic anions. In this regard, it is not known why methotrexate uptake is not inhibited by the reduced folates when they are, indeed, organic anions—except that they lack the 4-amino group which methotrexate and aminopterin both possess (*vide supra*).

The addition of dibutyryl cyclic AMP (Bu_2cAMP) or isobutyl methyl xanthine to hepatocytes stimulated efflux of accumulated, freely exchangeable drug and of a small portion of apparently intracellularly bound drug (39). The efflux induced by Bu_2cAMP was energy dependent, leading the authors to suggest that this agent may induce a "secretory" pathway whereby the drug is released into the capillary sinusoid and/or the bile canaliculus. The mechanism whereby Bu_2cAMP or isobutyl methyl xanthine induces this efflux phenomenon was unclear, since other compounds that raise cAMP levels (glucagon and isoproterenol) were without effect on methotrexate efflux.

A role for reduced sulfhydryl groups in methotrexate transport in isolated hepatocytes was suggested by inhibition by *p*-chloromercuriphenylsulfonate (*p*CMS) (36) and iodoacetamide (35). This role was confirmed by studies showing that reduced glutathione (GSH) stimulated methotrexate transport (40). Stimulation of uptake required preincubation with GSH and was dependent on GSH concentration increasing as the extracellular GSH concentration increased up to 5 mM. As one would expect, added GSH reversed the inhibition by pCMS. In a subsequent study (41), rats were fasted for 24 to 28 hr or treated with phorone 2 hr before hepatocyte isolation. Both treatments deplete cellular GSH, but, interestingly, titration with [^{203}Hg]pCMS showed up to three times greater amounts of free -SH groups on external membranes from hepatocytes from fasted and phorone-treated rats. The increased number of external -SH groups was associated with a decrease in the methotrexate transport K_m (1.2 μM vs 4 μM in the experimental and control groups, respectively), while the transport V_{max} was unchanged. This dependence on GSH was also demonstrated by diurnal changes in the transport K_m and cellular GSH levels—the K_m decline paralleled the decline in cellular GSH levels (42). These findings suggest that the redox state of plasma membrane -SH/-S-S- groups may control the methotrexate transport system in the hepatocyte.

Methotrexate transport has been studied in primary cultures of hepatocytes. Galivan (43) showed that after 24 hr in culture, hepatocytes accumulated methotrexate (over a 2-hr incubation) to a much higher level than that in cells cultured for 48 or 72 hr. Furthermore, uptake after 24 hr of culture was inhibited by azide. This is in accord with the inhibition of methotrexate uptake by azide reported in freshly isolated hepatocytes (30, 36). However, after 48 or 72 hr of culture, methotrexate uptake was greatly diminished and was no longer sensitive to azide inhibition. Subsequently, it was shown that inclusion of glucagon, hydrocortisone, and tocopherol in the culture medium resulted in in-

creased uptake and at least partial restoration of azide sensitivity in cells cultured for 48 hr. These results show that hepatocytes cultured in minimal medium lost the native methotrexate transport system and that this can be ameliorated by adding factors, such as hormones and antioxidants, which the cells normally encounter *in vivo* (44).

We have utilized purified membrane vesicles isolated from the BLMV from rat liver to study methotrexate transport into the liver cell. Uptake of methotrexate into the vesicles was not affected by imposition of a sodium ion gradient across the vesicular membrane. Uptake displayed a broad pH optimum—no significant difference in uptake was seen at extravesicular pH ranging from 5.5 to 7.5. Uptake was not stimulated nor was there an overshoot (as seen with 5-methyltetrahydrofolate uptake, see above) when a hydrogen ion gradient was imposed across the membrane. Transport was saturable K_m = 0.15 μM, V_{max} = 11.4 pmol/10 sec/mg protein. Uptake of methotrexate was not inhibited by 5-methyltetrahydrofolate or 5-formyltetrahydrofolate, in agreement with findings in isolated hepatocytes (see above). Uptake was weakly inhibited by folic acid. Uptake of methotrexate was also inhibited by several structurally unrelated anions: ATP and ADP (but not adenosine), chloride, sulfate, and oxalate. There was no transstimulation of uptake in vesicles preloaded with anions (ADP, sulfate, or oxalate), such that a 5-fold gradient of the anions (inside > outside) existed across the membrane. Methotrexate uptake was electrogenic, as shown by stimulation of uptake by an inside positive electrical potential induced by valinomycin and an inwardly directed potassium gradient. These results suggest that methotrexate is transported into the hepatocyte as an anionic species via an electrogenic, carrier-mediated system (45).

The lack of sodium dependency seen in the BLMV transport of methotrexate and the apparent sodium dependence reported in hepatocytes (35, 36) are difficult to reconcile. The hepatocyte is a much more complex system than the isolated BLMV. The hepatocyte has both the basolateral membrane, through which substances are transported from and into the circulation, and a canalicular membrane, through which substances (including methotrexate) are secreted into the bile. Transport characteristics determined in the isolated hepatocyte could reflect a combination of the properties of systems in both membranes. In this regard, it has been shown that the canalicular membrane contains a sodium-dependent system for transport of glutamate (46) and glycine (47). In addition, the hepatocyte maintains an electrical potential across the plasma membrane (about 32 mV, cytosol negative with respect to the medium) and it is known that replacement of sodium in the medium with potassium and ouabain results in perturbation of the electrical gradient (48, 49). Since methotrexate transport is electrogenic, perturbation of the electrical gradient should affect uptake of this compound. Indeed, Gewirtz *et al.* (39) have shown that Bu_2cAMP caused enhanced efflux of methotrexate from hepatocytes and cAMP is known to cause a hyperpolarization (15–20 mV more negative) of the hepatocyte membrane (49). Since methotrexate transport is electrogenic, the more negative electrical potential could result in enhanced efflux of methotrexate because it is transported across the membrane as a negatively charged species.

Transport of Methotrexate in Hepatoma Cells. Methotrexate transport into H35 hepatoma cells has been shown to be a saturable process with K_m of 5.2 μM. Uptake was inhibited by 5-methyltetrahydrofolate, a finding that is quite different from the case of normal hepatocytes wherein 5-methyltetrahydrofolate or 5-formyltetrahydrofolate did *not* inhibit methotrexate uptake (35, 36). Thus it would appear that the transformation from normal hepatocyte to the neoplastic state resulted in changes in the transport systems such that H35 cells have a single system for transport of methotrexate and the reduced folates similar to that generally seen in neoplastic cells (6).

Identification of Membrane Proteins Involved in Transport of Folates and Antifolates in Liver. Zamierowski and Wagner (50) were the first to find evidence for folate-binding proteins in liver plasma membranes. Rats were injected with [^{3}H]folic acid and subcellular fractions were prepared from liver at various times. At 30 min after injection, labeled folic acid was found in association with the plasma membrane fraction. Da Costa and Rothenberg (51) have identified two forms of folate-binding protein (FBP) by gel filtration of Triton X-100 extracts of rat liver plasma membranes labeled noncovalently with [^{3}H]folic acid. Gel filtration in Triton X-100 containing buffers gave M_r of 55,000 and 100,000. These proteins were not purified, nor was sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed, so that the subunit molecular weights are not known. The relative affinities of the FBP were folic acid > dihydrofolic acid ≫ 5-methyltetrahydrofolate >>> methotrexate and 5-formyltetrahydrofolate. This FBP probably does not function in the transport of folates into the liver cells as it had no detectable affinity for methotrexate or 5-formyltetrahydrofolate, both of which are excellent inhibitors of 5-methyltetrahydrofolate uptake in liver cells (18). Holm *et al.* (52) have identified two FBP in Triton X-100 extracts of human liver membranes precipitated with $CaCl_2$. Gel filtration gave M_r of 25,000 and 100,000. Immunoblots using rabbit anti-human milk FBP of sodium dodecyl sulfate gels of the 25,000 and 100,000 M_r gel filtration peaks showed a single band at M_r of 65,000. No explanation for this finding was given. The relevance of these proteins to folate transport is unknown.

We have recently utilized *N*-hydroxysulfosuccinimide-activated [^{3}H]5-methyltetrahydrofolate (NHSS-MTHF) or [^{3}H]methotrexate (NHSS-MTX), prepared according to the methods of Henderson and Zevely (53), to covalently label a membrane protein in BLMV from rat liver. Treatment of BLMV with NHSS-MTHF resulted in inhibition of uptake of 5-methyltetrahydrofolate into the vesicles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of extracts of vesicles covalently labeled with NHSS-MTHF showed one major band at M_r of 35,000, whereas treatment with NHSS-MTX labeled the same band plus additional bands at about 50,000, 100,000, and 115,000. The 5-methyltetrahydrofolate band (35,000) was specifically labeled, since inclusion of unlabeled 5-methyltetrahydrofolate in the reaction mixture competitively inhibited labeling. The NHSS-MTX-labeled bands were specifically labeled because unlabeled methotrexate inhibited labeling; however, unlabeled 5-methyltetrahydrofolate did not inhibit labeling of the ≈100,000 bands by NHSS-MTX, but it did inhibit labeling of the 35,000 band. These labeling patterns are consistent with the transport characteristics for these compounds, since methotrexate inhibited 5-methyltetrahydrofolate uptake whereas 5-methyltetrahydrofolate did not inhibit methotrexate uptake in hepatocytes or BLMV (D. W. Horne and K. A. Reed, unpublished observations).

This identification of liver basolateral membrane proteins that bind 5-methyltetrahydrofolate and methotrexate with the same specificity as the individual transport systems will allow us to purify these carrier proteins. We will then be able to obtain specific antibodies and amino acid sequence information which may be used in cDNA cloning of the carriers and, thus, the amino acid sequence to be inferred. Antibodies and DNA probes should permit us to estimate the amount of these carriers in liver and to determine whether the same or antigenically similar proteins are expressed in other tissues and to explore the regulation of expression in both normal and neoplastic cells.

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1. Hillman RS, Steinberg SE. The effects of alcohol on folate metabolism. Annu Rev Med **33**:345–354, 1982.
2. Weir DG, McGing PG, Scott JM. Folate metabolism, the enterohepatic circulation and alcohol. Biochem Pharmacol **34**:1–7, 1985.
3. Goldman ID. The characteristics of the membrane transport of amethopterin and the naturally occurring folates. Ann NY Acad Sci **186**:400–422, 1971.
4. Henderson GB. Transport of folate compounds into cells. In: Blakley RL, Whitehead VM, Eds. Folates and Pteridines: Vol. 3. Nutritional, Pharmacological, and Physiological Aspects. New York: John Wiley & Sons, p207, 1986.
5. Henderson GB. Folate-binding proteins. Annu Rev Nutr **10**:319–335, 1990.
6. Ratnam M, Freisheim JH. Proteins involved in the transport of folates and antifolates by normal and neoplastic cells. In: Picciano MF, Stokstad ELR, Gregory JF III, Eds. Folic Acid Metabolism in Health and Disease. New York: Wiley-Liss, Inc., p91, 1990.
7. Kamen BA, Wang M-T, Streckfuss AJ, Peryea X, Anderson RGW. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. J Biol Chem **263**:13602–13609, 1988.
8. Antony AC, Kane MA, Portillo RM, Elwood PC, Kolhouse JF. Studies of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. J Biol Chem **260**:14911–14917, 1985.
9. Anderson RGW, Kamen BA, Rothberg KG, Lacey SW. Potocytosis: Sequestration and transport of small molecules by caveolae. Science **255**:410–411, 1992.
10. Antony AC. The biological chemistry of folate receptors. Blood **79**:2807–2820, 1992.
11. Elwood PC. Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells. J Biol Chem **264**:14893–14901, 1989.
12. Sadasivan E, Rothenberg SP. The complete amino acid sequence of a human folate binding protein from KB cells determined from the cDNA. J Biol Chem **264**:5806–5811, 1989.
13. Lacey SW, Sanders JM, Rothberg KG, Anderson RGW, Kamen BA. Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. J Clin Invest **84**:715–720, 1989.
14. Ratnam M, Marquardt H, Duhring JL, Freisheim JH. Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. Biochemistry **28**:8249–8254, 1989.
15. Weitman SD, Lark RH, Coney LR, Fort DW, Frasca V, Zurawski VR Jr, Kamen BA. Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. Cancer Res **52**:3396–3401, 1992.
16. Strum WB, Liem HH. Hepatic uptake, intracellular accumulation, and biliary secretion of 5-methyltetrahydrofolate. Res Commun Chem Pathol Pharmacol **30**:493–507, 1980.
17. Horne DW. Studies on the mechanism of folate transport in isolated hepatocytes. In: Cooper BA, Whitehead VM, Eds. Chemistry and Biology of Pteridines 1986. Berlin-New York: Walter de Gruyter & Co., p559, 1986.
18. Horne DW, Briggs WT, Wagner C. Transport of 5-methyltetrahydrofolic acid and folic acid in freshly isolated hepatocytes. J Biol Chem **253**:3529–3535, 1978.
19. Horne DW. Na^+ and pH dependence of 5-methyltetrahydrofolic acid and methotrexate transport in freshly isolated hepatocytes. Biochim Biophys Acta **1023**:47–55, 1990.
20. Horne DW, Reed KA, Said HM. Transport of 5-methyltetrahydrofolic acid in basolateral membrane vesicles of rat liver. Am J Physiol **262**:G150–G158, 1992.
21. Hugentobler G, Meier PJ. Multispecific anion exchange in basolateral (sinusoidal) rat liver plasma membrane vesicles. Am J Physiol **251**:G656–G664, 1986.
22. Arias IM, Forgac M. The sinusoidal domain of the plasma membrane of rat hepatocytes contains an amiloride-sensitive Na^+/H^+ antiport. J Biol Chem **259**:5406–5408, 1984.
23. Goldenberg H, Crane FL, Morre DJ. NADH oxidoreductase of mouse liver plasma membranes. J Biol Chem **254**:2491–2498, 1979.
24. Crane FL, Low H. NADH oxidation in liver and fat cell plasma membranes. FEBS Lett **68**:153–156, 1976.
25. Jarasche E-E, Kartenbeck J, Bruder G, Fink A, Morre DJ, Franke WW. B-type cytochromes in plasma membranes isolated from rat liver, in comparison with those of endomembranes. J Cell Biol **80**:37–52, 1979.

26. Prats M, Teissie J, Tocanne J-F. Lateral proton conduction at lipid-water interfaces and its implications for the chemiosmotic-coupling hypothesis. Nature **322**:756–758, 1986.
27. Teissie J, Prats M, Soucaille P, Tocanne J-F. Evidence for conduction of protons along the interface between water and a polar lipid monolayer. Proc Natl Acad Sci USA **82**:3217–3221, 1985.
28. Horne DW, Patterson D, Said HM. Aging: Effect on hepatic metabolism and transport of folate in the rat. Am J Clin Nutr **50**:359–363, 1989.
29. Horne DW, Briggs WT, Wagner C. Ethanol stimulates 5-methyltetrahydrofolate accumulation in isolated hepatocytes. Biochem Pharmacol **27**:2069–2074, 1978.
30. Horne DW, Briggs WT, Wagner C. Studies on the transport mechanism of 5-methyltetrahydrofolic acid in freshly isolated hepatocytes: Effect of ethanol. Arch Biochem Biophys **196**:557–565, 1979.
31. Galivan J. 5-Methyltetrahydrofolate transport by hepatoma cells and methotrexate-resistant sublines in culture. Cancer Res **41**:1757–1762, 1981.
32. Strum WB, Liem HH. Hepatic uptake, intracellular protein binding, and biliary excretion of amethopterin. Biochem Pharmacol **26**:1235–1240, 1977.
33. Said HM, Strum WB, Hollander D. Inhibitory effect of unconjugated bile acids on the enterohepatic circulation of methotrexate. J Pharmacol Exp Ther **213**:660–664, 1984.
34. Deutsch JC, Kolhouse JF. Structural specificity of mechanisms controlling the hepatic uptake and biliary output of methotrexate in the rat. J Pharmacol Exp Ther **250**:221–226, 1989.
35. Horne DW, Briggs WT, Wagner C. A functional, active transport system for methotrexate in freshly isolated hepatocytes. Biochem Biophys Res Commun **68**:70–76, 1976.
36. Gewirtz DA, White JC, Randolph JK, Goldman ID. Transport, binding, and polyglutamation of methotrexate in freshly isolated rat hepatocytes. Cancer Res **40**:573–578, 1980.
37. Gewirtz DA, Randolph JK, Goldman ID. Potent bile salt and organic anion inhibition of methotrexate uptake and accumulation in the freshly isolated rat hepatocyte. Cancer Res **40**:1852–1857, 1980.
38. Gewirtz DA, Plotkin JH, Randolph JK. Interaction of probenecid with methotrexate transport and release in the isolated rat hepatocyte in suspension. Cancer Res **44**:3846–3850, 1984.
39. Gewirtz DA, Randolph JK, Goldman ID. Efflux in isolated hepatocytes as a possible correlate of secretion *in vivo*: Induced exit of the folic acid analog, methotrexate, by dibutyryl cyclic AMP or isobutyl methyl xanthine. Biochem Biophys Res Commun **101**:366–374, 1981.
40. Leszczynska A, Pfaff E. Activation by reduced glutathione of methotrexate transport into isolated rat liver cells. Biochem Pharmacol **31**:1911–1918, 1982.
41. Leszczynska-Bisswanger A, Pfaff E. Effect of depletion of cellular glutathione on methotrexate influx, efflux and retention in hepatocytes. Biochem Pharmacol **34**:1627–1634, 1985.
42. Leszczynska-Bisswanger A, Pfaff E. Diurnal variation of methotrexate transport and accumulation in hepatocytes—A consequence of variations in cellular glutathione. Biochem Pharmacol **34**:1635–1638, 1985.
43. Galivan J. The effect of age in culture on the uptake and metabolism of methotrexate by primary cultures of hepatocytes. Res Commun Chem Pathol Pharmacol **24**:571–582, 1979.
44. Galivan J. Transport of methotrexate by primary cultures of rat hepatocytes: Stimulation of uptake *in vitro* by the presence of hormones in the medium. Arch Biochem Biophys **206**:113–121, 1981.
45. Horne DW, Reed KA. Transport of methotrexate in basolateral membrane vesicles from rat liver. Arch Biochem Biophys **298**:121–128, 1992.
46. Ballatori N, Moseley RH, Boyer JL. Sodium gradient-dependent L-glutamate transport is localized to the canalicular domain of liver plasma membranes: Studies in rat liver sinusoidal and canalicular membrane vesicles. J Biol Chem **261**:6216–6221, 1986.
47. Moseley RH, Ballatori N, Murphy SM. Na^+-glycine cotransport in canalicular liver plasma membrane vesicles. Am J Physiol **255**:G253–G259, 1988.
48. Hoek JB, Nicholls DG, Williamson JR. Determination of the mitochondrial protonmotive force in isolated hepatocytes. J Biol Chem **255**:1458–1464, 1980.
49. Moule SK, McGivan JD. Regulation of the plasma membrane potential in hepatocytes—Mechanism and physiological significance. Biochim Biophys Acta Rev Biomembr **1031**:383–397, 1990.
50. Zamierowski MM, Wagner C. Identification of folate binding proteins in rat liver. J Biol Chem **252**:933–938, 1977.
51. da Costa M, Rothenberg SP. Characterization of the folate-binding proteins associated with the plasma membrane of rat liver. Biochim Biophys Acta **939**:533–541, 1988.
52. Holm J, Ingemann Hansen S, Hoier-Madsen M. High-affinity folate binding in human liver membranes. Biosci Rep **11**:139–145, 1991.
53. Henderson GB, Zevely EM. Affinity labeling of the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells by treatment with an N-hydroxysuccinimide ester of [^{3}H]methotrexate. J Biol Chem **259**:4558–4562, 1984.