A FUNCTIONAL, ACTIVE TRANSPORT SYSTEM FOR METHOTREXATE IN FRESHLY ISOLATED HEPATOCYTES

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SUMMARY: Methotrexate transport was studied in isolated rat liver cells. The process was found to be saturable with a K_t of 2.3 x 10^{-3}M and Vmax of 282 nmol/g wet wt. min. The system showed a requirement for sodium ions and it was sensitive to ouabain. Metabolic inhibitors, e.g., 2,4-dinitrophenol, anaerobic conditions, and deprivation of glucose, suppressed the uptake rate. Folic acid, but not folinic acid, was slightly inhibitory. It is suggested that methotrexate is transported in isolated hepatocytes by an active, sodium dependent process.

INTRODUCTION

This communication presents evidence demonstrating that methotrexate (MTX) transport in freshly isolated hepatocytes is a saturable, energy dependent process requiring extracellular sodium ions. Although a few reports have appeared in which isolated liver cells have been used to study the uptake of various compounds (1,2), to our knowledge, this is the first report of a functioning active transport system in freshly isolated hepatocytes. While Stege, et al (1) showed a time-dependent uptake of sulfobromophthalein in hepatocytes, they presented no evidence for the existance of a carrier. The findings of Ayala and Canonico (2) indicate an active transport of α-aminoisobutyric acid (AIB) in primary cultures of hepatocytes, but they found no time-dependent uptake of AIB in freshly isolated cells.

Transport of folates in cells isolated from other tissues has been examined previously only in intestinal epithelial cells. No time-

dependent uptake of folate was observed in intestinal cells isolated by hyaluronidase treatment. However, a folate binding protein (K_b = 3.98 x 10^{-5}M) was isolated from the brush border membrane (3). In intestinal epithelial cells isolated by a vibrational technique (4), glucose stimulated uptake of folic acid. No additional data was presented, however, to indicate that the uptake of folate was energy-dependent or carrier-mediated.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 170-250 g were used in all experiments. The rats were maintained on Wayne Lab-Blox and tap water fed ad libitum. Hepatocytes were isolated by modification of the method of Berry and Friend (5). The perfusion medium consisted of 10% saline-washed, aged human erythrocytes in calcium-free Krebs-Ringer solution (6) buffered with 25 mM HEPES (Sigma, St. Louis, Mo.). The pH was adjusted to 7.4 with NaOH and the solution equilibrated at 37°C with 100% oxygen. After anesthetizing the rat with Nembutal, the abdomen was opened, loose ligatures were laid around the inferior vena cava just above the renal veins and around the superior mesenteric and coeliac arteries, and the portal vein was cannulated. The perfusion pump was started and simultaneously the inferior vena cava was severed below the renal veins. The liver was perfused without recirculation at 14 ml/min for 3½ minutes during which the chest was opened and the inferior vena cava was cannulated via the right atrium. The ligatures around the lower part of the vena cava and the superior mesenteric and coeliac arteries were tightened. Perfusion to waste was continued for an additional 3½ minutes. The perfusate (approximately 80 ml) was recirculated and 12-20 mg of collagenase (type I, Worthington) was added to the perfusate in one ml of 0.9% saline. Perfusion was continued for 20 min. The liver was removed and placed in 50 ml of perfusion medium without red cells and gently minced with scissors. The resulting suspension was shaken at about 150 cycles/min for 10 min at 37°C in a 250 ml plastic erlenmeyer flask aerated with a stream of 100% O_2. The suspension was passed through nylon mesh to remove connective tissue. The resulting suspension consisted of a mixture of viable and damaged cells. The majority of damaged cells were removed by a selective centrifugation procedure based upon the observation that viable cells are more dense than the damaged ones. The cell suspension was centrifuged at 50g for one min and the supernatant fluid removed. Two layers of cells were apparent. The upper, lightly colored layer was gently suspended in 15 ml of incubation medium (K-R-HEPES, pH 7.4 containing 1.0% bovine serum albumin (BSA) or gelatin) and transferred to another centrifuge tube. The bottom, more darkly colored layer containing primarily intact cells was then suspended in 5 volumes of incubation medium, transferred to a plastic erlenmeyer flask and shaken slowly at 37°. Intact cells were harvested from the suspension of the original upper layer by centrifugation twice more and collection of the lower layer of the cell pellet. The suspension of "lower layer" cells was then centrifuged at 20 x g for one min and the supernatant discarded. This final pellet was washed once by centrifugation in 15 ml of medium and the cells finally were suspended in 20 volumes of incubation medium. This procedure routinely yielded 2-3 ml of packed cells of which >90% excluded trypan blue.

Uptake experiments were performed by adding [^{3}H]-methotrexate and/or other compounds to the cell suspension. The uncapped vials were shaken in a

Gyrotory water bath at 37°C for the times indicated. One ml samples were added to pre-weighed 1.5 ml plastic centrifuge tubes and centrifuged for 10 sec in an Eppendorf 3200 centrifuge (Brinkmann Industries). The medium was retained and the cell pellet was washed twice with 0.5 ml of ice-cold medium (without gelatin). The tubes were weighed and 1.0 ml of Scintisol-complete (Isolab, Akron, Ohio) was added. Samples of incubation medium (0.1 ml) were counted similarly. Potassium was assayed using an Instrumentation Laboratories flame photometer. [^{14}C] inulin and [^{3}H] H_2O spaces were determined essentially by the method of Quistorff, et al. (7). [3',5',9n-^{3}H] methotrexate was purchased from Amersham-Searle. It was purified by DEAE-cellulose chromatography (8). [Carboxy-^{14}C] inulin and [^{3}H] H_2O were obtained from NEN. [^{14}C] inulin was purified by elution from a BioGel P-6 column with water. Unlabeled MTX was from N.B. Co. Folinic acid was purchased from K&K Chemicals.

RESULTS AND DISCUSSION

Figure 1 illustrates the time course of MTX uptake into isolated hepatocytes. At an extracellular concentration of 1.34 μM the uptake of MTX was linear for 15 min. At this point the cells had taken up only about 7% of the added MTX.

To exclude the possibility of intracellular metabolism of MTX, an experiment was performed in which hepatocytes were incubated for 60 minutes in medium containing 1.2 μM [^{3}H] MTX. The washed cells were sonicated and the debris was removed by centrifugation. The supernatant was chromatographed on a DEAE-cellulose column as previously described. The sole peak of radioactivity corresponded to authentic unlabeled MTX. The cell debris contained only about 1% of the total activity. Thus, during the

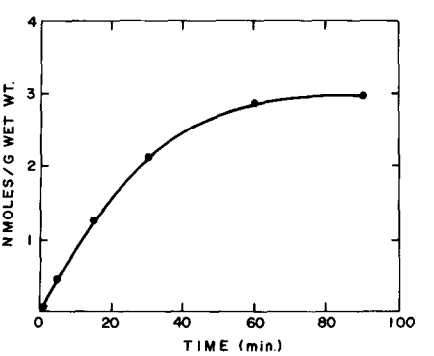

Figure 1. The time course of MTX uptake in isolated hepatocytes. Extracellular [MTX] = 1.3 μM.

incubation periods normally employed in these studies no significant metabolism of MTX had occurred. In a similar experiment using a concentration of 0.61 μM [^{3}H] MTX, the sonicated cell extract was chromatographed on a 0.9 x 11 cm column of Biogel P-6 eluted with 0.05M, pH 7.2 phosphate buffer. Only 7.5% of the MTX taken up by the cells was bound to intracellular proteins. Furthermore, dialysis of the sonicated extract against 10 volumes of medium at 4^{0}C for 24 hrs indicated 20.5% binding. Under these conditions, the tissue to medium concentration ratio (T/M) was 7.29. If the bound MTX fraction (as estimated by dialysis) is taken into account, the T/M was 5.80. This finding indicates that MTX is concentrated by hepatocytes against a gradient and constitutes evidence for an active carrier-mediated transport system.

Since the rate of MTX uptake appears linear for at least 15 min, measurement prior to this time should approximate the initial rate of transport. As shown in Fig. 2, the rate of MTX uptake, measured over the first 5 min, approached saturation at high extracellular concentrations of

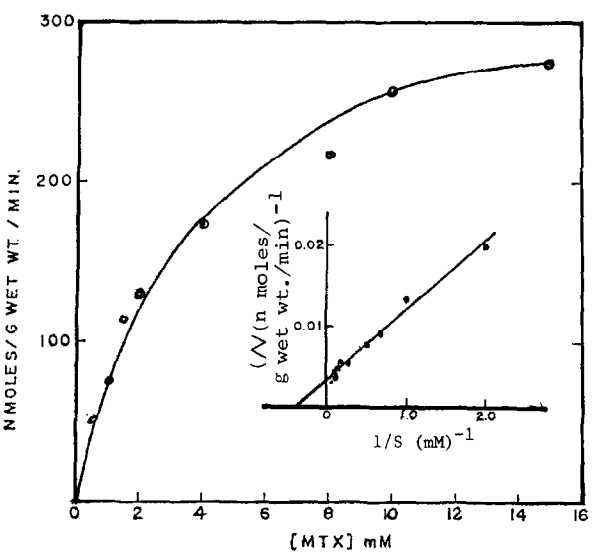

Figure 2. The relationship between the initial MTX uptake velocity and extracellular MTX concentration. In the reciprocal plot (inset) the line was fitted by the method of least squares.

MTX. The insert of Fig. 2 represents the least squares best fit for the double reciprocal plot. From this plot, the observed K_t for MTX was 2.4 x 10^{-3}M. The apparent Vmax was 282 nmol/min·g wet wt. The observed K_t was much higher than expected based on observations from other cellular systems. Goldman (9) reported K_t values of 3-10 μM for MTX in L1210 cells and about 2 μM in rabbit reticulocytes (10). In this concentration range, hepatocytes demonstrated a linear dependence of the initial MTX uptake velocity (v) on concentration such that

$$v = k\ [MTX]$$

In four separate experiments (n=27), the calculated value of k was 9.66 (± SE 0.48) x 10^{-5} 1/min·g wet wt over the concentration range from 0.07 to 100 μM. The low affinity of the carrier for MTX could reflect damage to a carrier protein by a proteolytic enzyme in the crude collagenase and/or by mechanical manipulations during the isolation procedures. However, the cells were >90% viable as judged by exclusion of trypan blue. The potassium level was 61.6 mEq/g compared to 80.9 mEq/g for whole liver, a loss of about 24% of the K^+ by isolated hepatocytes. This loss may be compared to a 60% K^+ loss by hepatocytes seen by Berry and Friend (5). Also, the ratio of [^{14}C] inulin to [^{3}H] H_2O spaces was 3.1 (range 2.7-3.6), a value essentially in agreement with that of Quistorff, et al. (7).

Since serum folates may be bound to albumin (11), the BSA used in the incubation medium could affect the uptake of MTX by lowering its free concentration. This appears not to be the case. The k values calculated for MTX uptake with BSA and gelatin in the incubation medium were 10.2 (± SEM 0.63) and 8.53 (± SEM 0.58) x 10^{-5} 1/g wet wt·min, respectively (p=0.1). Thus, any binding of MTX to BSA did not significantly effect MTX uptake by hepatocytes.

The effect of inhibitors and various incubation conditions on MTX transport is summarized in Table 1. The uptake of MTX was dependent on the availability of metabolic energy. Incubation under an atmosphere of N_2 led

to a 75% inhibition while the omission of glucose and O_2 resulted in an 88% inhibition of MTX uptake. The uncoupling agent (2,4-dinitrophenol) and 2-heptyl-4-hydroxyquinoline-N-oxide (HHQNO), an inhibitor of oxidative phosphorylation, were also inhibitory (84% and 68% inhibition, respectively). MTX transport was also dependent on sodium ions in the medium. Replacing Na^+ with Tris HCl resulted in 65% inhibition of MTX uptake by hepatocytes. Ouabain, which is known to inhibit the sodium, potassium pump, was also inhibitory. The sulfhydryl inhibitor, iodoacetamide, was relatively ineffective. Table 1 also shows that the transport of MTX is slightly inhibited by folic acid but not by folinic acid. Any conclusions regarding the sharing of common transport systems for the various forms of folate, however, must await studies in which direct measurement of the transport of the folate forms is carried out. Such studies are now in progress.

TABLE 1. Effect of Inhibitors and Various Incubation Conditions on MTX Transport by Isolated Hepatocytes

Addition or Omission	Conc. (mM)	Percent of Control	
None	--	100	(n=3)
$NaHAsO_4$	1.0	66.0	(n=3)
Iodoacetamide	1.0	59.7	(n=3)
HHQNO	0.1	31.7	(n=5)
2,4-dinitrophenol	1.0	16.1	(n=5)
Folic acid	1.0	80.3	(n=6)
Folinic acid	1.0	97.7	(n=6)
Ouabain	1.0	23.1	(n=4)
Ouabain	0.06	87.0	(n=2)
Tris HCl ($-Na^+$)	--	35.9	(n=2)
$-O_2$	--	25.4	(n=4)
$-O_2$-glucose	--	12.3	(n=2)
-glucose	--	39.8	(n=2)

Hepatocytes were isolated as described in Methods. The cells were washed once and resuspended in the appropriate test medium. The cells were pre-incubated for 10 min, [^{3}H] MTX was added at 0.5-1.5 μM and incubation continued for an additional 10 min. Results are expressed as % of control carried out at the same time.

Our results may be compared with those of Goldman in L1210 leukemia cells and Erlich ascites tumor cells (9) and of Bobzien and Goldman (10) in rabbit reticulocytes. In these systems, MTX transport was shown to be saturable and energy dependent but independent of extracellular sodium ions. Chen and Wagner (12) have shown that the choroid plexus has a carrier for 5-methyltetrahydrofolic acid but the system required neither energy nor sodium ions. Ruben, et al. (13) reported that the choroid plexus actively transports MTX by a mechanism independent of sodium. Thus, transport of MTX in isolated hepatocytes differs from folate transport in other systems in the requirement for extracellular sodium ions.

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