ABSTRACT

The cellular pharmacology of methotrexate was evaluated in freshly isolated rabbit hepatocytes in suspension with an analysis of drug metabolism by high-performance liquid chromatography. After exposure of hepatocytes at a cytocrit of 5% to 5 μM [3H]-methotrexate, intracellular 7-hydroxymethotrexate appears rapidly within the cell; within 15 sec, the level of 7-hydroxymethotrexate exceeds the level of intracellular methotrexate, although the latter has not achieved the dihydrofolate reductase binding capacity. Within 20 min, virtually all methotrexate is hydroxylated. There is minimal formation of methotrexate polyglutamyl derivatives even after exposure of cells to very high levels of methotrexate, and 7-hydroxymethotrexate polyglutamates do not accumulate in the cell at all after incubation with [3H]-7-hydroxymethotrexate. Because of the rapidity of the hydroxylation of methotrexate, transport of this agent could not be characterized. However, some aspects of the transport properties of 7-hydroxymethotrexate could be studied since the catabolite is neither bound nor metabolized in this system. Net 7-hydroxymethotrexate transport was reduced by the addition of 5-formyltetrahydrofolate. As observed for 4-aminopterin transport in other cell systems, net 7-hydroxymethotrexate transport was markedly stimulated by sodium azide, an inhibitor of energy metabolism.

The data suggest that hydroxylation of methotrexate proceeds at a rate at least comparable to the rate of association of the drug with dihydrofolate reductase and that transport of methotrexate into rabbit hepatocytes is slow relative to the rate of catabolism to the 7-hydroxy derivative. Rabbit hepatocytes may be a useful model for exploring methotrexate catabolism at the cellular level and may provide insights into the interaction between methotrexate and/or other 4-aminopterin derivatives and the human liver.

INTRODUCTION

The folate antagonist, methotrexate, is rapidly converted to the 7-hydroxy derivative by aldehyde oxidase, an enzyme found in both rabbit (21, 28) and human liver (20), which is apparently responsible for the high levels of this catabolite in human plasma after high-dose methotrexate therapy (4, 23). While 7-hydroxymethotrexate is only a weak inhibitor of dihydrofolate reductase (5) and was considered a detoxification product (28), recent studies raise the possibility that formation of this catabolite may have important pharmacological consequences (6, 7): (a) 7-hydroxymethotrexate shares the same membrane transport carrier as does methotrexate in tumor cells (7, 23) and when present in the extracellular compartment with methotrexate, suppresses both the influx and level of free methotrexate that accumulates in cells (7). This reduction in the intracellular monoglutamyl substrate level reduces the direct suppression of dihydrofolate reductase by methotrexate as well as subsequent polyglutamylation of methotrexate (7); (b) 7-hydroxymethotrexate is at least as good a substrate as methotrexate for folylpolyglutamate synthetase (6, 7, 25, 27), so that direct competition between these 2 compounds at the level of this enzyme is possible; and (c) because 7-hydroxymethotrexate polyglutamyl derivatives are retained within cells, and 7-hydroxymethotrexate tetraglutamate is a better inhibitor of dihydrofolate reductase than is the monoglutamate (6), these derivatives could themselves have pharmacological activity.

This paper describes studies that explore for the first time the cellular pharmacology of methotrexate in freshly isolated rabbit hepatocytes in suspension, a system that permits characterization of the 7-hydroxylation and polyglutamylation processes that also occur in human liver. This study demonstrates (a) very rapid catabolism of methotrexate to 7-hydroxymethotrexate in these cells, (b) the rapid release of 7-hydroxymethotrexate into the extracellular compartment, and (c) the slow metabolism of methotrexate to polyglutamyl derivatives. This report further characterizes the membrane transport of 7-hydroxymethotrexate and its interactions with 4-aminopterinates at the level of the transport carrier.

MATERIALS AND METHODS

Materials. [3',5',7-3H]Methotrexate was synthesized by Amersham-Searle (Arlington Heights, IL) and purified by HPLC as described previously (12). Unlabeled methotrexate, and aminopterin obtained from the United States-France (National Cancer Institute-Institut National de la Santé et de la Recherche Médicale) Cancer Program (G50111). Present address: INSERM SC16, 27 Boulevard Jean Moulin, 13385 Marseille, Cedex 5, France.

The abbreviations used are: HPLC, high-performance liquid chromatography; 4-NH2-10-CH3-PteGlu2 and 4-NH2-10-CH3-PteGlu4 were kindly supplied by Dr. C. M. Baugh (University of South Alabama, Mobile, AL) and used without further purification. Unlabeled 7-hydroxymethotrexate was obtained by direct 7-hydroxylation from methotrexate after incubation with a crude preparation of fresh mature rabbit liver and purified by DEAE-cellulose chromatography as described previously (7, 18). Radiolabeled 7-hydroxymethotrexate was prepared by the same technique using a highly purified aldehyde oxidase, followed by HPLC purification (7, 8). Bio-Gel P-6 (200 to 400 mesh) and Bio-Gel P-60 were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were reagent grade.

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Preparation of Hepatocyte Suspensions. Hepatocytes in suspension were prepared from male New Zealand white rabbits, weighing 0.6 to 1.5 kg, by a modification (12) of the collagenase perfusion technique of Berry and Friend (1). Before experimentation, animals were anesthetized by injection of pentobarbital (45 mg/kg) in the marginal vein of the ear. Trypan blue exclusion was determined at the beginning and end of experiments and was 90% or greater.

Incubation Conditions. All experiments were performed at 37° in Waymouth's medium (Grand Island Biological Co., Grand Island, NY) in specially designed flasks (17). The pH was maintained at 7.4 by passing warm and humidified 95% O2:5% CO2 over the cell suspension. Radio-labeled compounds were added following which portions of the cell suspension were injected into 10 ml of a 0.85% NaCl solution at pH 7.4. Cells were separated by centrifugation at 1000 × g for 60 sec and then washed twice with the same solution. The washed pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare, dried overnight at 70°, weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, CA), and then digested in 0.25 ml of 1 N HCl, dried overnight at 70°, weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, CA), and then digested in 0.25 ml of 1 N HCl, dissolved in 3 ml of Ready-Solve scintillation fluor (Beckman Instruments, Fullerton, CA), and radioactivity was measured in a liquid scintillation spectrometer.

Analysis of Intracellular and Extracellular Radiolabel by HPLC. Cell pellets were resuspended in 0.6 ml of 10 mM sodium ascorbate (pH 7.2) and boiled for 10 min following which 50% trichloroacetic acid was added to achieve a final concentration of 10%. Protein debris was separated by centrifugation at 15,000 × g for 10 minutes, and 0.7 ml of the supernatant was passed through a minicolumn of Bio-Gel P-60 equilibrated with 1 M KOH and 0.35 ml of 1 M KH2PO4 (pH 7.0). Samples of the medium were chromatographed directly without extraction. Analyses were performed with an Altex Model 332 gradient liquid chromatography equipped with a Model 210 injector and a 5-μm-particle-size octadecyl C18 column (25 cm × 4.5 mm; IBM Instruments). The resolution of the compounds was achieved by a modification of the procedure of Fry et al. (11). Each analysis for methotrexate and its metabolites consisted of linear gradients of 0 to 30% in 2.5 min, 30 to 62% in 10 min, and 62 to 100% in 1 min of 0 to 30% in 2.5 min, 30 to 62% in 10 min, and 62 to 100% in 1 min of 5% acetonitrile in 0.1 M sodium acetate buffer at pH 5.5. The flow rate was 2 ml/min, and the temperature was ambient. One-ml fractions were collected and examined for radioactivity as described above. Authentic standards of methotrexate, 7-hydroxymethotrexate, 4-NH2-10-CH3-PteGlu2 and -PteGlu3 were included in each sample and monitored with a UV detector at 254 nm. This chromatographic technique permits resolution of methotrexate, 4-NH2-10-CH3-PteGlu2, 4-NH2-10-CH3-PteGlu3, and 7-hydroxymethotrexate (Chart 1).

Intracellular methotrexate and methotrexate polyglutamyl derivatives were quantitated by evaluating the percentage of each derivative from the chromatographic analysis and the total radiolabel in each cell pellet as determined in units of nmol per g dry weight. In these calculations, the data were corrected for the loss of 3H at position 7 of the pteridine molecule that occurs with the hydroxylation of methotrexate. This was based upon a nuclear magnetic resonance spectroscopic analysis of a typical batch of methotrexate, provided by Amersham, which indicated that 53.6% of the label is at position 7 of the molecule.

Intracellular Space Determination. A portion of the hepatocyte suspension was incubated under the same conditions as described above with [carboxy-14C]inulin for 5 to 10 min. Intra- and extracellular volumes were determined on unwashed pellets using [14C]inulin as an extracellular marker and gravimetric determination of cell wet and dry weights as described previously (3). The rabbit hepatocyte dry weight: wet weight ratio was 0.239 ± 0.039 (S.D.); the extracellular water: wet weight ratio was 0.346 ± 0.129 μl/mg; and intracellular water: dry weight ratio was 2.131 ± 0.448 μl/mg (n = 6), similar to the values reported by Gewirtz et al. (14) for rat hepatocytes.

Intracellular Binding to Macromolecules. Cell pellets were resuspended in 1.5 ml of ice-cold buffer consisting of 0.05 M citrate (pH 6.0), 0.15 M KCl, 0.05 M mercaptoethanol, 1 mM EDTA, and 0.1 mM NADPH. The cell suspension was subjected to sonic oscillation until 95% of the cells were broken and then centrifuged at 40,000 × g for 30 min. One ml of the supernatant was passed through a minicolumn of Bio-Gel P-6 by centrifugation. As described previously (9, 11), this procedure permits isolation of the enzyme-ligand complex from the free drug which is retained completely within the column. The enzyme-ligand complex was also chromatographed on a column (1 x 50 cm) of Bio-Gel P-60 equilibrated with the above buffer and calibrated with appropriate molecular weight markers.

RESULTS

Analysis of Intracellular 4-Aminoantifolate Composition after Exposure of Hepatocytes to Methotrexate. The time course of appearance of methotrexate, 7-hydroxymethotrexate, and methotrexate polyglutamyl derivatives in the intracellular water of hepatocytes after exposure to 5 μM [3H]methotrexate is illustrated in Chart 2A. There was a rapid initial increase in total cell 3H, the major portion of which represented 7-hydroxymethotrexate, following which the rate of 3H uptake fell to a very slow constant velocity. 7-Hydroxymethotrexate far exceeded the methotrexate level at the first measurement (5 min) and remained in excess of the methotrexate level over the following hour of observation. The intracellular methotrexate level also rose rapidly initially but achieved only a value that was below the dihydrofolate reductase binding capacity of 1.99 ± 0.67 μmol/liter of intracellular water (n = 5). There was a slow rise in intracellular methotrexate polyglutamyl derivatives that paralleled the small increment in total 3H over 20 to 60 min. No 7-hydroxymethotrexate polyglutamyl derivatives were detected in the cells as confirmed by incubation of each radiolabeled derivative with chicken pancreas conjugase (2) which resulted in quantitative reversion of each derivative to an elution time identical to that of methotrexate.

Chart 2B illustrates the extracellular 3H composition under the same conditions as described above (cytocrit 5%). The appearance of methotrexate and the appearance of 7-hydroxymethotrexate are very rapid. Only a very low level of unchanged methotrexate (0.53 ± 0.15 μmol/liter of extracellular water; n = 3) was detectable after 30 min. Aside from the very low levels of methotrexate bound to dihydrofolate reductase and converted to polyglutamyl derivatives, conversion of methotrexate to 7-
7-HYDROXYLATION OF METHOTREXATE IN RABBIT HEPATOCYTES

Chart 2. Intracellular (A) and extracellular (B) 4-aminoantifolate composition after exposure of hepatocytes to 5 μM [3H]methotrexate (MTX). Each symbol in A represents the mean ± S.D. (bars) of 4 different experiments. 7-OH-MTX, 7-hydroxymethotrexate; MTX-PG, methotrexate polyglutamyl derivatives.

Chart 3. Time course of uptake and metabolism of 5 μM methotrexate (MTX) over 15 to 120 sec. Bound intracellular "H was assessed by gel-filtration analysis as described under "Materials and Methods." 7-OH-MTX, 7-hydroxymethotrexate.

hydroxymethotrexate was about 90% complete by this time. The hydroxylation of methotrexate occurs entirely in the intracellular compartment; there is no extracellular aldehyde oxidase activity. Hence, when hepatocytes were incubated for 15 min following which the cells were separated from the medium by centrifugation, 7-hydroxymethotrexate was added to the supernatant and incubation at 37° was continued for 30 min, no metabolites of methotrexate were detected.

Chart 3 focuses on the time course of accumulation and metabolism of methotrexate over 15 to 120 sec after addition of the drug. Within 15 sec, the intracellular level of 7-hydroxymethotrexate exceeded that of methotrexate and increased at a rate faster than that of methotrexate. Gel filtration followed by HPLC analysis of the drug-protein complex indicated that all methotrexate that appears within the cells over this interval is bound; no bound 7-hydroxymethotrexate could be detected. Bio-Gel P-60 chromatography indicates that the drug-protein complex elutes with the same pattern as does dihydrofolate reductase suggesting that this enzyme is the binding site for methotrexate.

Relationship between Extracellular Methotrexate Level and Intracellular Levels of Methotrexate and Its Metabolites. Intracellular composition after a 30-min exposure of hepatocytes to 5, 25, 50, and 100 μM [3H]methotrexate is analyzed in Chart 4, conditions in which intracellular radiolabel was at or near constant levels. The intracellular increase in total "H as extracellular methotrexate was increased is accounted for largely by the increase in 7-hydroxymethotrexate. There was a much smaller increase in methotrexate and methotrexate polyglutamyl derivatives. Hence, as the extracellular methotrexate concentration was increased from 5 to 100 μM, there was a 12-fold increase in the formation of 7-hydroxymethotrexate but only a 3-fold increase in methotrexate and its polyglutamyl derivatives. No free methotrexate could be detected at methotrexate concentrations below 25 μM. When extracellular methotrexate was increased from 50 to 100 μM, there was a 10-fold increase in free drug. However, this was accompanied by only a 1.6-fold increase in methotrexate polyglutamyl derivatives.

Intracellular Retention of Methotrexate and Its Derivatives. Chart 5A illustrates the efflux properties of radiolabeled intracellular constituents after hepatocytes were loaded for 30 min in the presence of 5 μM [3H]methotrexate, conditions in which methotrexate, 7-hydroxymethotrexate, and methotrexate polyglutamyl derivatives represented 29, 62, and 9% of the total radiolabel in the cells. Bound methotrexate, 7-hydroxymethotrexate, and methotrexate polyglutamyl derivatives accounted for 29, 62, and 9% of the total radiolabel in the cells.
7-HYDROXYLATION OF METHOTREXATE IN RABBIT HEPATOCYTES

Chart 5. Efflux properties for methotrexate (MTX) and its metabolites. In A, cells were exposed to 5 μM [3H]-methotrexate for 30 min, separated by centrifugation, washed twice in methotrexate-free buffer, and resuspended in drug-free medium. At the indicated times, portions of the cells were analyzed for intracellular constituents as described under "Materials and Methods." In B, cells were exposed to 5 μM [3H]-7-hydroxymethotrexate for 30 min, diluted 10-fold in fresh drug-free medium, and the efflux of 7-hydroxymethotrexate (7-OH-MTX) was monitored. MTX-7G, methotrexate polyglutamate derivatives.

The time course of 7-hydroxymethotrexate efflux is illustrated in more detail in Chart 5B. Cells were loaded for 30 min in the presence of 5 μM [3H]-7-hydroxymethotrexate, then diluted with 10 volumes of fresh drug-free medium at 37°C, conditions under which intracellular 7-hydroxymethotrexate leaves the cells by 20 min. This technique permits measurements of initial rates of efflux that could be perturbed by the usual washing and centrifugation procedures (13). Based upon the decline of intracellular drug over the first 2 min during which only 25% of intracellular 7-hydroxymethotrexate leaves the cells, the efflux rate constant was computed to be 0.186 ± 0.049/min (n = 3).

Analysis of 7-Hydroxymethotrexate Transport. Methotrexate and 7-hydroxymethotrexate share the same uphill transport carrier in Ehrlich and other tumor cells (7, 23). Because of the rapid metabolism of methotrexate in rabbit hepatocytes and the technical difficulty in the accurate measurement of initial uptake rates (see Chart 3), properties for this transport process were characterized with 7-hydroxymethotrexate.

When hepatocytes were incubated for 30 min with 5 μM 7-[3H]-hydroxymethotrexate and 5 mM L-glutamine, no polyglutamyl derivatives were detected and no intracellular 7-hydroxymethotrexate was bound, as assessed by gel filtration. 7-Hydroxymethotrexate rapidly achieved (20 min) a steady-state intracellular level of 2.08 ± 0.38 μM (Chart 6; n = 4), comparable to the 7-hydroxymethotrexate level of 2.18 ± 0.32 μM (Chart 2; n = 5) obtained after a 30-min exposure to 5 μM [3H]-methotrexate, conditions in which virtually all extracellular methotrexate was catabolized to this derivative (Chart 2). When cells at the steady state with 5 μM [3H]-7-hydroxymethotrexate were then exposed to 10 mM of sodium azide, there was a rapid and marked augmentation of net drug uptake resulting in a 2- to 3-fold increase by 30 min (Chart 6). The addition of 50 μM 5-formyltetrahydrofolate resulted in a net efflux of 7-hydroxymethotrexate from cells (not shown) consistent with a common carrier mechanism for these compounds (16). Nonlabeled methotrexate (50 μM) also induced a net efflux of 7-[3H]hydroxymethotrexate from the hepatocytes; however, interpretation of this result is complicated by the rapid intracellular formation of the nonlabeled catabolite followed by its rapid efflux and extracellular accumulation with subsequent inhibition of influx of the labeled 7-hydroxymethotrexate into the cells.

DISCUSSION

With high-dose methotrexate regimens there is appreciable metabolism to the 7-hydroxy derivative with plasma concentration ratios of 7-hydroxymethotrexate to methotrexate that range from 30:1 to 1:1 (4, 23). This catabolite is a very poor inhibitor of mammalian dihydrofolate reductase (5, 6) and has been considered to be inactive (27). However, recent studies indicate that 7-hydroxymethotrexate has a high affinity for the methotrexate-tetrahydrofolate cofactor transport carrier (7) and thereby limits both the rate of entry of methotrexate into cells and the free intracellular methotrexate level that is achieved. This interaction depresses the inhibitory effect of methotrexate on tetrahydrofolate-dependent thymidylate synthase and slows the rate of methotrexate polyglutamylation in cells (7). Furthermore, 7-hydroxymethotrexate has been shown to be a very good substrate for the folylpolyglutamate synthetase (6, 8, 25, 26), and finally, the 7-hydroxymethotrexate tetraglutamate derivative, which is retained within Ehrlich cells, binds to and is a much more potent inhibitor of dihydrofolate reductase than is the 7-hydroxymono glutamate (6).

Because of the potential importance of the 7-hydroxy catabolite of methotrexate and its known site of production in the liver, studies were undertaken to characterize the hydroxylation in freshly isolated rabbit hepatocytes which like the human liver (20) contain appreciable levels of aldehyde oxidase and are therefore capable of this reaction (21, 28).

Catalysis of methotrexate to the 7-hydroxy derivative is very
rapid in hepatocytes. At a cytotic of about 5% and with an extracellular methotrexate concentration of 5 μM, essentially all methotrexate was hydroxylated within 20 min except for a small portion bound to dihydrofolate reductase within the cells, and an even smaller portion which is metabolized to polyglutamyl derivatives. At 20 min, 7-hydroxymethotrexate is at near maximum levels in the intracellular and extracellular water.

In rabbit hepatocytes, the association of methotrexate with aldehyde oxidase proceeds at a rate comparable to the rate of binding of methotrexate to dihydrofolate reductase, since hydroxylolation occurs prior to saturation of this enzyme by methotrexate. Likewise, the very low levels of free methotrexate in cells over a broad extracellular concentration range suggests that transport of the drug is slow relative to hydroxylation. The rapidity of hydroxylation complicates the characterization of the transport properties for methotrexate. However, aspects of the transport of 7-hydroxymethotrexate which is neither bound nor further metabolized in these cells could be evaluated. These studies indicate rapid transport of 7-hydroxymethotrexate by a mechanism similar to that used by 4-aminoantifolates and 5-formyltetrahydrofolate. Hence, when cells at steady state with 7-hydroxymethotrexate were exposed to 5-formyltetrahydrofolate, there is rapid net efflux of 7-hydroxymethotrexate. Furthermore, as observed previously for both methotrexate and 7-hydroxymethotrexate in Ehrlich and other tumors (7, 10, 15), net transport of 7-hydroxymethotrexate is stimulated by sodium azide in rabbit hepatocytes. This is attributed to inhibition of an energy-dependent exit process which limits the accumulation of free drug (10, 15, 16).

Only negligible levels of methotrexate polyglutamyl derivatives were formed in rabbit hepatocytes which is consistent with the absence of methotrexate polyglutamylates in rabbit liver in vivo (28). While this might be attributed to rapid hydroxylation limiting the methotrexate substrate available for polyglutamylation, a 20-fold increase of the extracellular methotrexate level which increases free intracellular drug resulted in only a small increment in the accumulation of methotrexate polyglutamyl derivatives, negligible in comparison to the levels of 7-hydroxymethotrexate formed. Furthermore, no 7-hydroxymethotrexate polyglutamyl derivatives were formed after exposure to methotrexate. Indeed, no 7-hydroxymethotrexate polyglutamyl derivatives were detected even after cells were exposed directly to 7-hydroxymethotrexate when the lack of polyglutamylation of the 7-hydroxy derivative could not be attributed to competition with methotrexate at the level of the folylpolyglutamate synthetase. The basis for this observation is unclear since rabbit liver contains appreciable levels of folylpolyglutamate synthetase (28). Reduced polyglutamylation could be attributed to high intracellular levels of folate substrates that compete with the antifolates for the synthetase and/or high conjugate activity which hydrolyzes polyglutamylates rapidly after they are formed. It would be expected that the liver of the rabbit would store folates as polyglutamates for subsequent release and utilization by peripheral folate-dependent tissues, and hence would form 4-aminoantifolate polyglutamylates as well. Methotrexate polyglutamates have been detected in human liver (19), and they form rapidly in rat hepatocytes (13, 14); it is of interest that the latter organ, however, produces only negligible levels of 7-hydroxymethotrexate while humans rapidly oxidize methotrexate in vivo (4, 25).

These data raise the possibility that the rabbit hepatocyte system may be a useful model for understanding this catabolic pathway for methotrexate and supports the critical role of the hepatocyte as a major element in the clearance of methotrexate from the circulation.

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Characteristics of the Formation and Membrane Transport of 7-Hydroxymethotrexate in Freshly Isolated Rabbit Hepatocytes

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