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HEPATIC UPTAKE, INTRACELLULAR PROTEIN BINDING AND BILIARY EXCRETION OF AMETHOPTERIN*

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Abstract—Transport of the folate antagonist, amethopterin, from plasma to bile was studied in the isolated rat liver. The hepatic uptake of amethopterin was a saturable process with a K_m of 1.3 mM and V_{max} of 11.1 μmoles/hr/g liver. The uptake process was energy dependent and was inhibited by folate. The drug was bound to a soluble protein in the liver which was identified as dihydrofolate reductase. After an initial delay the biliary excretion of amethopterin paralleled the hepatic uptake and 80 per cent of the drug was recovered in the bile after a 5-hour perfusion. Chromatography of bile revealed no conjugation or metabolism of amethopterin during transcellular transport. The drug was 70–120 times more concentrated in the bile than in the perfusion medium; in separate experiments the amethopterin in bile was available for intestinal absorption and 25 per cent of the drug was excreted in the urine 48 hr after peroral administration. These studies suggest that hepatic uptake and biliary excretion of amethopterin is an active carrier-mediated process and may be dependent on two separate energy-requiring systems for the uptake and biliary excretion of the drug.

Treatment with high doses of amethopterin (Methotrexate; 4-amino-4-deoxy-10-methylpteroylglutamate) provides effective antifolate chemotherapy for certain types of solid tumors [1]. These very large doses may result in concentrations of 10^{-4} to 10^{-3}M amethopterin in the serum, leading to serious problems with toxicity [2, 3]. Elimination of the drug from the body has been generally regarded to be a function of renal glomerular filtration and active tubular transport [4], and 50–88 per cent of an oral dose is excreted in the urine by man in a 24-hr period. In a preliminary report of the present work attention was drawn to a possibly greater role for the liver in the excretion of amethopterin than has been generally appreciated [5]. This study was designed to probe into the mechanisms involved in the transport of amethopterin from plasma to bile in the isolated perfused rat liver system. This system permits direct study of the molecular events involved in the hepatic uptake, metabolism, and biliary excretion of amethopterin under a variety of experimental conditions.

MATERIALS AND METHODS

Materials. The following were obtained commercially: Sephadex G-25, G-75 and DEAE-Sephadex (Pharmacia); Aquasol (New England Nuclear); [3,5-^{3}H]amethopterin (12.4 Ci/m-mole; Amersham Searle); and unlabeled amethopterin (ICN Pharmaceuticals). Dihydrofolate was prepared by the method of Blakely [6].

Adult male Sprague–Dawley rats 250–400 g served as liver donors. They were fed a diet of Purina Laboratory Chow, containing 23% crude protein (Harrison-Riedy, Chula Vista, CA). Food was withheld for 40–48 hr prior to the experiment. Water was allowed *ad lib.*

Perfusion methods. The perfusion technique and apparatus were designed according to Miller *et al.* [7] with minor modifications. Briefly, rats were anesthetized with ether and anticoagulated with 2 mg sodium heparin intravenously. The abdominal cavity was exposed using a wide surgical incision and the portal vein and common bile duct, were isolated. After cannulating the portal vein and the bile duct, the liver was perfused *in situ* with oxygenated Krebs–Hensleit bicarbonate buffer, pH 7.4. Within 1 min after excision the liver was placed on a circular plexiglass disc resting on a platform and was connected to the circulating perfusion medium. A mixture of 95% O_2 and 5% CO_2 passed through the glass "thin film" oxygenator at a flow rate of 3.5 liter/min. The perfusate drained directly from the hepatic veins into the reservoir since the inferior vena cava was not cannulated.

The perfusion medium, 100 ml, contained Krebs–Hensleit bicarbonate buffer, saturated with 95% O_2 and 5% CO_2, 2.5% bovine serum albumin (Pentex Miles Laboratory, Kankakee, IL) which had been dialyzed overnight against the buffer, 400 mg sodium heparin, 2 ml TC amino acids Hela, 100 X† (Difco Laboratories, Detroit, MI) and 160 mg glucose. The medium was titrated with 3 N NaOH to pH 7.4. The appropriate concentration of tritium-labeled amethopterin was added to the perfusion medium at the outset to provide a radioactivity of 10,000–12,000 cpm/ml.

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†TC Amino Acids Hela, 100 X is a hundred fold concentration of the original chemically defined basal Hela medium of Eagle (Science, **122**, 501 (1955).

The liver was perfused at 37°, with a flow rate of 35–45 ml/min with continuous recycling of the perfusate. Each perfusion was continued for 2–5 hr; samples (1 ml) of the perfusate were withdrawn at time 0 and at 10, 20 or 30 min intervals thereafter and dissolved in 10 ml Aquasol for radioactive counting in a Beckman LS-233 liquid scintillation spectrometer. The bile was collected at the end of the first and second hr; the bile flow was 0.4–1.0 ml per hour. The bile sample was diluted to 1.5 ml with distilled water and a 50 μl aliquot was monitored for radioactivity.

In experiments previously described, the pH of the perfusion medium was monitored hourly and was found to vary minimally over a 5-hr period, the portal pressure was constant at 13–14 cm of water, and the ultrastructure of the hepatic tissue, as observed by electron microscopy, was preserved [8].

Identification of amethopterin by chromatography. Identification of amethopterin was performed using procedures by Nixon and Bertino [9] and Oliverio [10]. Samples of bile or urine were admixed with 2 μmoles of unlabeled amethopterin and applied to a 1.0 × 14 cm column of DEAE-Sephadex which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Elution was accomplished with a linear gradient of 0.1 M–0.8 M phosphate buffer at the same pH. Fractions (5 ml) were collected and monitored for both radioactivity of the labeled amethopterin in the sample and absorbance at 302 nm; the peak tubes were monitored for the characteristic ultraviolet absorbance spectrum of amethopterin.

Protein binding of amethopterin. Following a 2-hr perfusion study with 1 μM amethopterin, the entire liver was homogenized using a Teflon-glass, motor driven homogenizer and the supernatant was recovered after centrifugation at 100,000 *g* for 60 min at 2°. The supernatant was assayed for radioactivity and protein binding was determined by chromatographic and dialysis techniques. In one type of experiment, protein binding was assessed by elution of hepatic cytosol from a 2 × 25 cm column of Sephadex G-25 using 0.05 M potassium phosphate buffer, pH 7.0. Sixty fractions (3 ml each) were collected and monitored for radioactivity and absorbance at 280 nm. A second technique involved determination of the nondialysable fraction of the cytosol using a dialysis membrane with a 24 Å pore size; experiments were performed at 25° with constant stirring over a 6-hr period. Hourly aliquots of the dialysate were monitored for radioactivity to confirm that equilibrium had been reached within the 6-hr period. The dialysant was monitored for radioactivity at the end of the experiment. A control dialysis was performed with amethopterin in bile. In a third type of experiment, the cytosol was applied to a 3 × 80 cm column of Sephadex G-75 equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, and eluted with the same buffer. Elution was performed using a pump driven flow system which permitted accurate timed collections of constant vol. with a flow rate of 15 ml/hr. The column was previously calibrated for mol. wt determinations [11] with the following proteins (mol. wt in parentheses): cytochrome *c* (11,700), myoglobin (17,200), ovalbumin (43,000), bovine serum albumin (66,000) and hexokinase (102,000). The void vol. was determined using blue dextran and for each protein the relative mobility (ratio of its elution vol. to the void vol.) was determined. Fractions (7.5 ml) were collected and monitored for radioactivity and absorbance at 280 nm. In a separate experiment, liver cytosol without prior exposure to amethopterin was fractionated by Sephadex G-75 under identical conditions and the samples collected were monitored for absorbance at 280 nm and for dihydrofolate reductase activity. Enzymatic activity was assayed spectrophotometrically by a modification of the technique of Mathews and Huennekens [12]. One unit of activity was defined as the amount required to reduce 1 μmole of dihydrofolate/min under these conditions.

Intestinal absorption of amethopterin in bile. The intestinal absorption of amethopterin in bile was investigated by collecting bile during hepatic perfusion with amethopterin and administering the bile to litter mates by peroral intragastric infusion. The concentration of amethopterin in the bile was determined by radioactive counting. A calculated dose of 50 μg/kg or 1.5 mg/kg amethopterin in the bile was mixed with 0.5–1.0 ml of normal saline and then instilled into the stomach of four rats under light ether anesthesia using a soft tygon tube connected to a 1 ml tuberculin syringe. Identical experiments were performed using [^{3}H]amethopterin diluted in saline. The animals were housed individually in metabolic cages and urine from each animal was collected at 4, 8, 24 and 48 hr. The urine was monitored for radioactivity and two separate pools of urine, one from the 0–8 hr time period and the second from the 0–24 hr time period, were chromatographed on DEAE-Sephadex.

RESULTS

General characteristics of amethopterin transport. The time-dependent disappearance of 1 μM amethopterin from the perfusion medium is illustrated in Fig. 1. The rate of uptake (11.4 nmoles/hr/g liver) was linear for the first 20 min and decreased gradually over the 5-hr period. After 5 hr, 96% of the amethopterin was removed from the perfusion medium; 81% was recovered in the bile. Of the

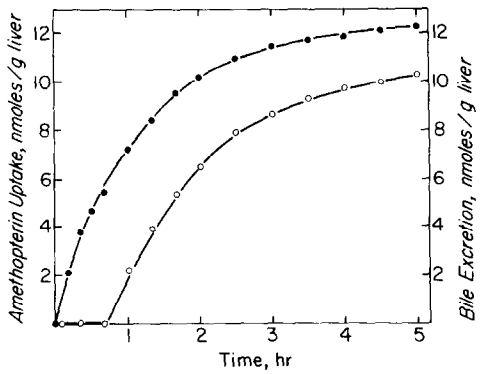

Fig. 1. Hepatic uptake and biliary excretion of amethopterin during perfusion of isolated rat liver. Initial concentration of amethopterin, 1.0 μM. Perfusion procedure as described in Methods. (●) hepatic uptake; (○) biliary excretion.

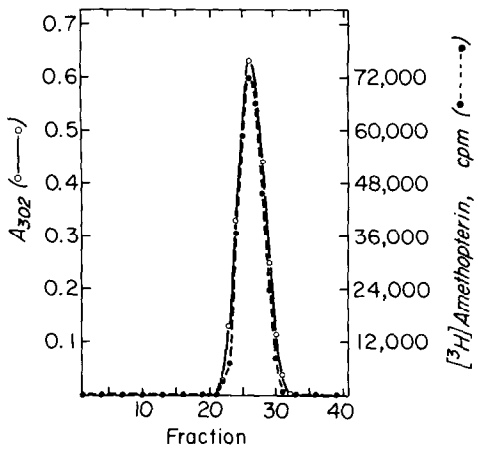

Fig. 2. Chromatographic identification of amethopterin collected in bile during 2 hr perfusion of rat liver with 1 μM amethopterin (cf. Fig. 1). Unlabeled amethopterin was added to the bile sample as a marker and chromatography was performed as described in Methods.

remaining 15%, the hepatic cytosol contained 7%, while 8% was not recovered and was presumably in the insoluble portion of the liver. The appearance of the drug in the bile occurred after a 40 min lag which is probably due to a combination of retention of the drug by dihydrofolate reductase and the transit time required to cross the hepatic cells and enter the biliary channels. Unlike folate which is metabolized to some extent during transit through the liver [5, 13], amethopterin passed through unchanged. This was demonstrated by an experiment in which the bile excreted during a 2-hr period was chromatographed on DEAE-Sephadex (Fig. 2). Monitoring the effluent for radioactivity and absorbance revealed only a single peak of material whose position in the elution profile and characteristic absorbance maximum (302 nm) indicated that it was unmetabolized amethopterin. Greater than 98 per cent of the total radioactivity was associated with this peak.

In the absence of bovine serum albumin in the perfusion medium, a slightly increased rate of initial uptake (15 nmoles/hr/g liver) was observed, indicating that dissociation of the drug from albumin is of minor regulatory significance.

Effect of concentration. The effect of concentration upon the initial rate of amethopterin uptake was studied over a concentration range from 0.1 to 2.0 mM (Fig. 3). As shown in Fig. 1, the rate of uptake was nearly linear over the first 20 min and measurement prior to this time should approximate the initial rate of transport. From the plot in Fig. 3 a K_m of 1.3 mM and V_{max} of 11.1 μmoles/hr/g liver were determined. The insert in Fig. 3 represents the least squares best fit for the double reciprocal plot.

Effect of inhibitors. Inhibitors of aerobic and anaerobic glycolysis added to the perfusion medium at the outset produced profound decreases in the rate of uptake and the results are illustrated in Fig. 4. Of special interest was the effect of iodoacetate, a diffusible sulfyhydryl group inhibitor [14], which caused a minimal decrease in uptake during the initial 30 min but subsequently led to release of amethopterin back into the perfusion medium. When the concentration of iodoacetate was decreased to 5 mM and 1 mM a progressively lesser effect on amethopterin uptake was observed (data not shown).

The inhibition from iodoacetate is considerably different from that of *p*-chloromercuriphenylsulfonate, a cell surface sulfhydryl inhibitor [15], the effect of which is also shown in Fig. 4. The biliary excretion of amethopterin (not shown) was reduced to 13 per cent by the former and to 1 per cent by the latter inhibitor over a 2-hr period.

Effect of analog. Increasing concentrations of folate from 1 mM to 1.75 mM resulted in a proportionate decrease in the rate of amethopterin (1 μM) uptake (Fig. 5), thus indicating inhibition probably at the cell surface. A curve showing the uptake of amethopterin in the absence of folate is presented in Fig. 4.

Intracellular protein binding of amethopterin. Binding of amethopterin to intracellular proteins was studied by three methods. First, the cytosol (100,000 g supernatant) of rat liver homogenate after amethopterin perfusion was applied to a column of Sephadex G-25 and 92% of the radioactivity in the effluent was recovered in the early fractions (24–36 ml) which corresponded to the protein peak (280 nm absorbance).

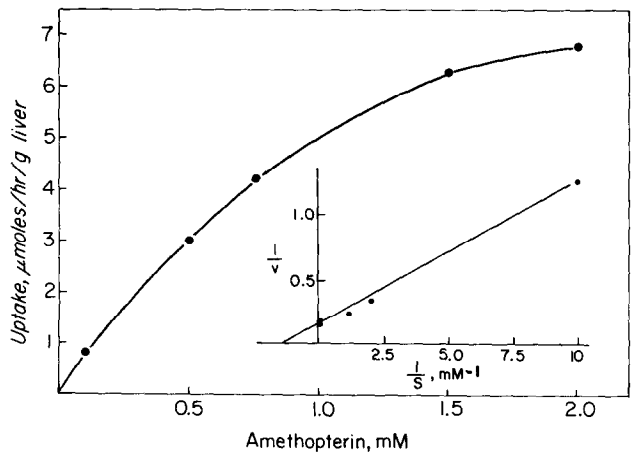

Fig. 3. Effect of concentration of amethopterin (S) on hepatic uptake. Velocity (v) is expressed as the initial uptake in μmoles/hr/g liver by the standard perfusion procedure. Inset, plot of $1/v$ vs. $1/S$.

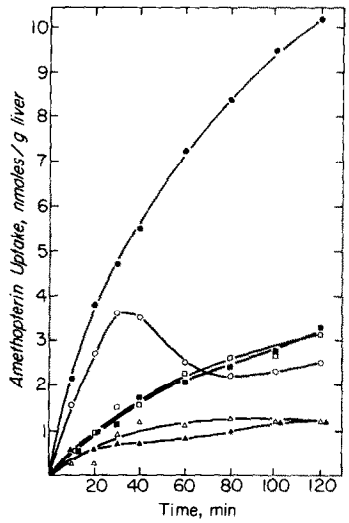

Fig. 4. Effect of metabolic inhibitors on amethopterin uptake during 2 hr perfusion. Initial concentration of amethopterin, 1.0 μM. (●) no inhibitor; (○) 10 mM iodoacetate; (■) 0.75 mM dinitrophenol; (□) 10 mM ouabain; (△) 10 mM *p*-chloromercuriphenylsulfonate; (▲) anaerobiosis.

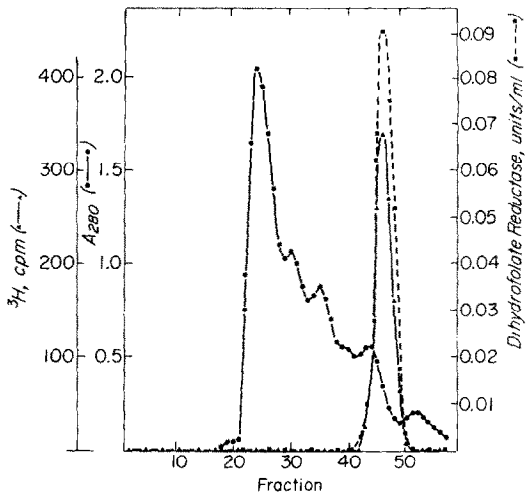

Fig. 6. Filtration of hepatic cytosol preparation through Sephadex G-75. Cytosol recovered and chromatography performed as described in Methods. Protein was measured by absorbance at 280 nm, and [^{3}H]amethopterin was determined by radioactive counting. The dashed line indicates dihydrofolate reductase activity measured by spectrophotometric assay and expressed as units/ml in each fraction.

The remaining 8 per cent of the radioactivity was recovered in a single, broad peak in the 120–170 ml fractions. Secondly, dialysis of the cytosol was performed over a 6-hr period during which equilibration with the dialysate was obtained and 72 per cent of the radioactivity representing protein-bound amethopterin was retained in the dialysant. A control dialysis of amethopterin in bile resulted in 2 per cent retention in the dialysant after 6-hr dialysis. Since the first two techniques indicated that amethopterin was probably protein-bound, the third technique was employed to determine which cytoplasmic protein was responsible for binding. The cytosol was fractionated on Sephadex G-75 and greater than 95 per cent of the radioactivity in the effluent was recovered at a position in the elution profile which corresponded to a mol. wt of approximately 20,000 (Fig. 6). The radioactivity co-chromatographed with dihydrofolate reductase which was assayed in a separate experiment in which amethopterin had been omitted and is consonant with the observed mol. wt of hepatic dihydrofolate reductase of ca. 20,000 [16].

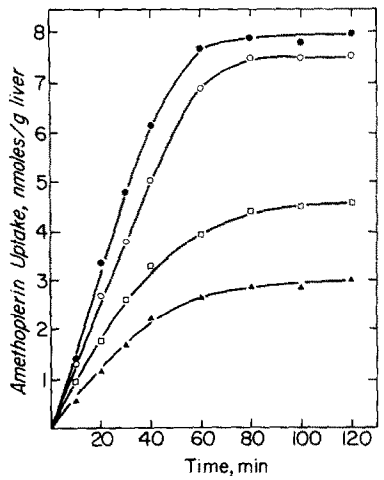

Fig. 5. Effect of folate on amethopterin transport during 2 hr perfusion. Initial concentration of amethopterin, 1 μM. Folate concentration was varied: (●) 1 mM; (○) 1.25 mM; (□) 1.50 mM; (▲) 1.75 mM.

Amethopterin excretion in bile and subsequent intestinal absorption. The appearance of amethopterin in bile began after a 40 min lag period (cf. Fig. 1). Amethopterin from the perfusion medium was concentrated in the bile from 70 to 120-fold and 58–72 per cent was excreted into the bile in 2 hr and 70–81 per cent in 5 hr, as compared to the initial concentration in the perfusion medium. The oral administration of bile containing [^{3}H]amethopterin by gastric lavage to litter mates resulted in the cumulative recovery of approximately 25 per cent of the drug in the urine after 48 hr for both a 50 μg/kg and 1.5 mg/kg dose (Table 1). Similar results were obtained when [^{3}H]amethopterin was administered

Table 1. Urinary excretion of [^{3}H]amethopterin by rats* (Cumulative % of administered dose)

Experiment number	Dose per kg	hr 4	8	24	48
1	50 μg†	10	14	24	27
2	50 μg†	14	17	29	34
3	1.5 mg†	11	15	20	22
4	1.5 mg†	9	12	22	26
5	1.5 mg‡	9	11	16	26
6	1.5 mg‡	10	14	16	25

* The dose was administered to litter mates by intragastric instillation through a peroral tube.

† The drug was contained in bile collected from perfusion of rat liver with amethopterin and diluted with saline.

‡ The drug was contained in saline.

in saline (1.5 mg/kg). Chromatography of the urine on DEAE-Sephadex (see Methods) indicated that the majority of the radioactivity co-chromatographed with that of unlabeled amethopterin; 99 per cent of the radioactivity from the 0–8 hr time period and 73 per cent of the radioactivity from the 0–24 hr time period were associated with the amethopterin peak.

DISCUSSION

This study was designed to analyze the mechanisms involved in the transport of amethopterin from plasma to bile utilizing an isolated perfused rat liver system. Amethopterin uptake occurred via a saturable process with a K_m of 1.3 mM and V_{max} of 11.1 μmoles/hr/g liver. The uptake was enhanced by the removal of albumin from the perfusion medium. Studies with metabolic inhibitors demonstrated that energy is required for the transport process. *p*-Chloromercuriphenylsulfonate, a cell surface sulfhydryl inhibitor, almost completely blocked uptake, whereas iodoacetic acid, a diffusible sulfhydryl inhibitor, did not affect the initial uptake (Fig. 4) but caused a release of amethopterin back into the perfusion solution. Bile excretion was reduced by both inhibitors thus suggesting that there are cell surface sites sensitive to *p*-chloromercuriphenylsulfonate and intracellular active sites for excretion sensitive to iodoacetic acid. Inhibition by folate was demonstrated to affect the rate of uptake probably at the cell surface, suggesting that the uptake system may not be able to completely distinguish between amethopterin and folate. These results are in general agreement with those of Horne *et al.* [17], using isolated hepatocytes, who found a K_m for amethopterin uptake of 2.4 mM and an apparent V_{max} of 282 nmoles/min/g wet weight. Their system required sodium ions and was sensitive to ouabain and metabolic inhibitors; folate, but not folinate, was slightly inhibitory. In contrast, in L1210 murine leukemia cells amethopterin shares a common transport system with 5-methyltetrahydrofolate and 5-formyltetrahydrofolate while folate appears to utilize a separate system [18, 19].

The present study raises the possibility that amethopterin uptake and excretion involve at least two completely separate steps: the first being a rapid uptake process at the membrane surface which is sensitive to *p*-chloromercuriphenylsulfonate, dinitrophenol, anaerobiosis, ouabain and folate, but not to iodoacetic acid; the second being a slower process which is inhibited by iodoacetic acid but involves minimal direct interaction with the membrane surface and may control biliary excretion. Sulfhydryl agents have been used in other systems to discriminate between modes of transport; these include the two mechanisms for the sugar-transporting system in yeast [14] and the separate transport systems for folate and amethopterin in L1210 murine leukemia cells [19].

In humans, amethopterin that is carried in the ·ma to the sinusoidal surface of the liver cells is ximately 70 per cent bound to plasma pro- ·]. The present studies show the drug to be ·en up by the rat liver, and after 2 hr, 95 ·e remaining amethopterin that gained ·tracellular compartment was bound ·n identified as dihydrofolate reductase by Sephadex G-75 chromatography. The role of this enzyme in the uptake and excretion of amethopterin is not known but it is not likely to have a specific transport function. Ion exchange chromatography of bile revealed no conjugation or metabolism of excreted amethopterin after transhepatic transport. Excretion of amethopterin occurred against a large concentration gradient and is probably an energy requiring process. After biliary excretion amethopterin is available for intestinal reabsorption which provides a route of re-entry into the plasma. This enterohepatic circulation may then contribute significantly to blood levels of the drug, especially if renal failure is coexistent. These studies extend prior experiments by Henderson *et al.* [21] who showed 2–18 per cent cumulative urinary excretion of the drug over a 5-hr period in rats when the drug was in solution in bile, and by Bischoff *et al.* [22] who demonstrated liver to plasma and bile to plasma ratios in mice of 10:1 and 300:1, respectively. These studies also show that amethopterin catabolism, as demonstrated by urinary metabolites of amethopterin, increases with time although the degree of degradation was not as extensive as the 87 per cent reported in rats by Zaharko *et al.* [23] during the 6–12 hr time period. Amethopterin metabolism is attributed to enzymatic cleavage by bacteria during enterohepatic circulation [24].

In summary, these studies permit the tentative conclusion that the factors regulating the movement of amethopterin from plasma to bile include the release of the drug from albumin, probable uptake by a specific cell surface receptor, intracellular binding to dihydrofolate reductase, and possibly a second receptor responsible for excretion into bile. The single major determinant affecting amethopterin uptake and excretion by the liver appears to be the concentration of the drug in the plasma, thus emphasizing the importance of hepatic competence in preventing toxicity during high dose amethopterin treatment. Characterization of the kinetics and functional capacity of each of the steps involved in the uptake and excretion will be necessary for a complete understanding of the mechanisms involved.

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