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Renal Tubular Transport of Methotrexate in the Rhesus Monkey and Dog[1]

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ABSTRACT

The mechanism and localization of renal transport of methotrexate (MTX) were studied in the rhesus monkey and the dog. It was found that in both animals MTX was bound with plasma protein in a range of 50 to 68% varying with the MTX plasma concentration. Paper chromatographic analysis showed that a negligible amount of MTX was metabolized. The excretion of MTX in rhesus monkey was mainly by tubular secretion which was blocked by probenecid, but in the dog a bidirectional transport mechanism for MTX was indicated. Tubular secretion was localized in the proximal tubules, and a tubular reabsorptive process was in the distal section. Simultaneous administration of folic acid blocked the tubular reabsorption of MTX, resulting in an increase of renal excretion. Maximum tubular excretory capacity determination showed that a maximum tubular excretory capacity value of approximately 5 μmol/100 ml of glomerular filtrate was observed in the rhesus monkey at a plasma concentration of 0.07 mM and a value of 2 μmol/100 ml of glomerular filtrate for the dog. Studies with renal cortical slice technique also indicated that the monkey kidney can accumulate greater amounts of MTX than can the dog kidney.

INTRODUCTION

MTX,[4] an antifolate agent, has been a useful antineoplastic agent in the treatment of cancer in experimental animals as well as in humans. The effectiveness of this agent is dependent on the dose administered and the rate of elimination. In their early studies in humans, Henderson *et al.* (9, 18) reported that the renal clearance ratio between MTX and inulin was >1 and that this ratio decreased in the presence of *p*-aminohippuric acid or salicylic acid, indicating tubular secretion. Bourke *et al.* (2) also demonstrated that the tubular secretion of MTX in the monkey can be inhibited by probenecid. Other studies showed that folic acid was found to be reabsorbed in dog experiments (7, 9), and the clearance of MTX in the dog was below the clearance of inulin (18). Two questions, therefore, have been raised. (*a*) Is there a species difference in the renal handling of MTX? Is there tubular secretion of this agent in humans and monkey but tubular reabsorption only in the dog? (*b*) Would folic acid compete for the tubular reabsorptive process of MTX, indicating that both compounds share the same or similar reabsorptive mechanism? This investigation has resolved these questions and found that there is a bidirectional transport mechanism for MTX in the dog that is similar to that observed with other organic acids (14).

MATERIALS AND METHODS

Animals. Female rhesus monkeys, 3.8 to 4.8 kg, and mongrel dogs, 6 to 12 kg, were used. The rhesus monkeys were kindly supplied by Dr. Theodore Lawwill of the Department of Ophthalmology of this Institute after his experimentations studying the effect of light exposures such as argon and dye lasers on monkey eyes. The animals had been quarantined for at least 3 months in the Animal Care Center. They were well nourished and appeared in very good health. They had received beams of light on their eyes, but no direct systemic effect was observed.

Methods. The animal was anesthetized with pentobarbital. After surgical removal of both eyeballs for electron microscopic examination, cannulations of a jugular vein and a small branch of the femoral artery were performed, and both ureters were catheterized. Inulin and MTX were administered i.v. by a priming dose and sustaining infusion. Mannitol was added to the infusion fluid to maintain the urine flow between 1.0 to 2.0 ml/min. Sodium acetate was given to some of the animals at a dose of 2 mmol/kg. Following a period of 10 to 20 min, which was allowed for a steady state to be reached, urine samples were collected for 10-min periods, and plasma samples were obtained at the beginning and end of these periods. The plasma levels of either inulin or MTX stayed constant during collection periods. The experimental procedure has been described in our previous publications (11–13, 15). In several of the experiments, the blood pressure of a femoral artery was monitored with a transducer. In the experiments for *Tm* determination, the priming doses and sustaining infusion rates of MTX were increased stepwise at 0.5-hr intervals until the animal showed signs of intoxication such as nausea and vomiting. Stop-flow experiments were performed in dogs only. Mannitol and mammalian Ringer's solution were infused into the animal until the urine flow reached 6 ml/min. The left ureter was then clamped for 4 min, and upon release a urine sample was collected every 5 sec for 30 consecutive samples. The details of experimentation were the same as those described previously (12, 14). Sodium concentration in the urine samples collected was not determined because in this investigation no attempt was made to study the effect of urinary sodium ion on the tubular transport mechanism of MTX. In renal cortical slice studies, the kidneys were removed from the anesthetized animal and decapsulated. A Stadie-Riggs microtome was used to slice the cortices. Several slices with a total weight of approximately 200 mg were put in each of a series of vials containing 4 ml of sodium acetate-Taggart-Ringer solution with MTX added in various concentrations (5, 13, 16). Each vial was oxygenated and incubated for 1 hr in a Dubnoff shaker at 25°. The slices were

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[4] The abbreviations used are: MTX, methotrexate; *Tm*, maximum tubular excretory capacity; GFR, glomerular filtration rate; i.a., intraaortally.

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then removed from the incubation medium, weighed, and analyzed for MTX concentration. The rate of uptake was calculated in terms of μmol/g of tissue wet weight.

Materials. Inulin used as the marker of the GFR was given to the animal at a dose of 100 mg/kg and a sustaining infusion rate of 1 mg/kg/min. In the stop-flow experiments, *carboxyl*-[^{14}C]inulin (New England Nuclear, Boston, Mass.) was added to the nonlabeled compound as a tracer. Sodium MTX (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y.) was diluted with Ringer's solution to 10 mM with [^{3}H]MTX (Amersham Corporation, Chicago, Ill.) and added as a tracer. Paper chromatography showed identical spots for the labeled and nonlabeled compounds (1).

Analysis. Inulin was determined by the method of Walser *et al.* (26). The radioactivity of [^{14}C]inulin and [^{3}H]MTX was determined by pipeting a 0.1-ml sample in 10 ml Bray's scintillation fluid and counting in an automatic liquid scintillation counter (3). Protein binding of MTX was determined by the method of Toribara *et al.* (25). Separation of metabolites of MTX was performed by paper chromatography in 0.5% sodium carbonate solution and observed under UV (1).

RESULTS

Protein Binding and Paper Chromatographic Analysis. In experiments with both monkey and dog, protein binding determinations were performed on plasma, and it was found that MTX bound to protein in a range of 50 to 68%, varying with the MTX plasma concentration. The urine samples were subjected to paper chromatographic analysis. The standard MTX was used as a control and gave an R_F of 0.69. In monkey and dog urine samples, 90 to 95% of radioactivity can be recovered from the spot corresponding to that of the standard MTX spot. Since Jacobs *et al.* (15) and Henderson *et al.* (8) had also reported that little or no detectable amount of MTX metabolites was found in human, monkey, or dog urine, it can be assumed that the urine samples contained the free MTX form almost entirely.

Tubular Secretion and Tubular Reabsorption of MTX. Four rhesus monkeys were given MTX at doses of either 5 or 10 μmol/kg plus sustaining infusion throughout the experiment. Blood pressure was maintained at a steady level during the 2-hr experimental period. The MTX concentration in the plasma fluctuated very little, and the GFR measured by inulin clearance was quite constant except in 2 experiments in which the clearance was decreased after folic acid infusion. Results are summarized in Table 1. The clearance of MTX was greater than that of inulin regardless of its protein-binding factor. When the excretory rate (*T*) value was calculated, a positive *T* was obtained, indicating tubular secretion. In Monkeys 1 and 2, probenecid solution (1.0 g/100 ml) was infused i.a. at a point close to the renal arterial branching at a rate of 0.1 ml/min. Infusion was done in this manner to demonstrate a direct effect of probenecid on the tubular transport without provoking a systemic hemodynamic effect or fluid-loading effect. The C_{MTX}/C_{in} decreased to a ratio of less than 1, and the *T* value was reduced although the GFR remained fairly constant. Since probenecid did not significantly affect GFR (C_{in}), the decrease in the ratio of C_{MTX}/C_{in} was due to a decrease in MTX excretion rate rather than due to a change in urinary flow. Since sodium acetate has been shown to augment the secretory rate of most organic acids (14, 19), in Monkeys 3 and 4 sodium acetate was given i.v. at a dose of 2 mmol/kg prior to administration of MTX. After 2 control periods of urine collection, a 2.5 g/100 ml folic acid solution was infused i.a. at the rate of 0.1 ml/min causing a decrease in C_{in} but only a slight increase or no change in the *T*-value. Three dogs were also used in the same type of experiment. Dog 5 was given MTX in a dose of 5 μmol/kg and sustaining infusion, and the calculated *T*-value (after consideration of the free and protein-binding fraction) was initially negative and changed gradually to positive. In 2 other dogs, sodium acetate was administered at a dose of 2 mmol/kg together with inulin, and the calculated *T*-value of MTX was positive throughout the experiments. When folic acid was given to the animals at a dose of 200 mg/kg, the inulin clearance dropped markedly, but the *T*-value of MTX was increased significantly resulting in a C_{MTX}/C_{in} ratio of approximately unity This indicates that folic acid enhances renal excretion of MTX either by increasing secretory rate or reducing the tubular reabsorption.

***Tm* Determination.** When the priming dose and infusion rate of MTX was increased stepwise every 30 min, a *Tm* could be reached at high plasma concentration. It was found that the monkey can tolerate MTX better than dogs; therefore, a larger dose of MTX can be administered to the monkey. In the experiment with one monkey, a total accumulated dose of 28 μmol/kg of MTX was given plus a sustaining infusion rate of 1.26 μmol/min, purposely calculated to be enough to replace the amount lost via the kidney. Data obtained from this animal are presented in the upper curve of Chart 1. A *T*-value of approximately 5 μmol/100 ml of glomerular filtrate is being reached at a plasma concentration of 0.07 mM MTX. The lower curve presents the data obtained from 2 dogs. The *T*-value gradually leveled off at the plasma concentration of 0.06 mM. If a *Tm* value is considered to have been reached at this level, it is approximately 2 μmol/100 ml of glomerular filtrate which is much lower than that observed in the monkey.

Localization of Tubular Transport. Stop-flow experiments were performed in 4 male dogs. MTX was given to each animal at a dose of either 5 or 10 μmol/kg plus sustaining infusion. The first set of urine collections served as a control. Thirty min after folic acid, 200 mg/kg, was infused into the animal, a second set of urine collections was obtained from the same kidney. Chart 2 presents typical results obtained from one of the stop-flow experiments. In 2 other dog experiments, the results were practically the same. As shown here, at the portion corresponding to the distal tubules, the ratio of U/P_{MTX} to U/P_{in} was below one, and at the proximal tubular portion, the ratio was between 1 and 2, indicating that secretion is localized in the proximal tubules. Folic acid raised the ratio of U/P_{MTX} to U/P_{in} above unity in all urine samples, especially in the distal tubular portion, indicating that tubular reabsorption occurred mainly in that portion.

MTX Uptake by Renal Cortical Slices. When cortical slices of monkey or dog kidneys were incubated at MTX-Taggart-Ringer solution at 25°, there was a larger quantity of MTX accumulated in the monkey kidney than in the dog. Results are presented in Chart 3*A*. Kinetic analysis of these data, as shown in Chart 3*B*, was then performed in calculating the K_m and V_{max}. It was found that the K_m values were 0.05 mM for the monkey kidney and 1.25 mM for the dog kidney; the V_{max} values were 0.45 and 0.83 μmol/g, respectively.

Table 1

Summary of renal excretion of MTX in monkey and dog

Animal	Wt (kg)	Sex	MTX dose: Prime (μmol/kg)	MTX dose: Infusion (μmol/min)	C_{in}[a] (ml/min)	C_{MTX} (ml/min)	MTX: Plasma concentration: Total (mM)	MTX: Plasma concentration: $P \cdot F_w$ (mM)	U (μmol/ml)	V (ml/min)	T (μmol/100 ml GFR)
Monkey 1	3.86	F	10	0.39	12.7	23.1	0.010	0.003	0.58	0.36	+1.4
				0.39	13.9	13.9	0.012	0.004	0.46	0.35	+0.82
				Probenecid (1%): i.a. infusion (0.1 ml/min)							
				0.39	15.77	11.7	0.013	0.0042	0.37	0.41	+0.53
				0.39	15.75	12.0	0.014	0.0046	0.37	0.46	+0.61
Monkey 2	4.2	F	5	0.22	20.5	71.4	0.003	0.001	0.65	0.34	+1.0
				0.22	23.1	54.8	0.0027	0.0009	0.30	0.50	+0.55
				Probenecid (1%): i.a. infusion (0.1 ml/min)							
				0.22	22.7	22.4	0.0035	0.0012	0.093	0.85	+0.23
				0.22	28.1	19.4	0.0047	0.0016	0.11	0.85	+0.17
Monkey 3				*Sodium acetate (2 mmol/kg): i.v. prior to MTX*							
	4.8	F	5	0.24	15.2	94.4	0.001	0.0005	0.12	0.78	+0.57
				0.24	15.5	78.0	0.0011	0.0005	0.098	0.80	+0.45
				Folic acid (2.5%): i.a. infusion (0.1 ml/min)							
				0.24	6.33	8.4	0.007	0.0035	0.11	0.53	+0.59
				0.24	6.55	12.3	0.0075	0.0035	0.12	0.75	+1.027
				0.24	5.97	10.6	0.0071	0.0036	0.090	0.83	+0.85
Monkey 4				*Sodium acetate (2 mmol/kg): i.v. prior to MTX*							
	4.3	F	10	0.43	8.46	43.6	0.0043	0.002	0.171	1.10	+2.0
				0.43	8.75	32.0	0.0055	0.0028	0.160	1.10	+1.7
				Folic acid (2.5%): i.a. infusion at 0.1 ml/min							
				0.43	6.10	11.5	0.0137	0.007	0.18	0.85	+1.9
				0.43	6.06	11.7	0.0132	0.0066	0.16	0.98	+1.89
				0.43	6.52	11.0	0.0126	0.006	0.119	1.17	+1.49
Dog 5	11.3	M	5	0.55	86.4	40	0.0135	0.0075	1.35	0.40	−0.11
				0.55	68.8	36.4	0.011	0.0061	0.46	0.87	−0.03
				0.55	68.9	34.6	0.011	0.0061	1.05	0.36	−0.08
				0.55	92.9	50	0.011	0.006	1.37	0.40	−0.045
				0.55	74.1	46	0.011	0.006	1.25	0.40	+0.035
				0.55	57.8	41	0.01	0.006	0.98	0.46	+0.135
Dog 7				*Sodium acetate (2 mmol/kg): i.v. prior to MTX*							
	6.4	M	10	0.64	20.4	13.5	0.026	0.012	0.158	2.25	+0.52
				0.64	20.6	13.5	0.020	0.009	0.112	2.43	+0.384
				Folic acid i.v. infused (200 mg/kg)							
				0.64	8.9	8.6	0.032	0.0147	0.184	1.50	+1.60
				0.64	9.1	9.4	0.034	0.0152	0.203	1.55	+1.91
				0.64	8.14	10.25	0.034	0.0152	0.233	1.50	+2.76
Dog 14				*Sodium acetate (2 mmol/kg): i.v. prior to MTX*							
	4.8	F	20	1.0	30.7	16.6	0.058	0.0278	0.54	1.79	+0.35
				1.0	28.2	16.8	0.051	0.024	0.34	1.88	+0.56
				Folic acid i.v. infused (200 mg/kg)							
				1.0	20.4	14.5	0.045	0.021	0.34	1.91	+1.10
				1.0	7.2	7.1	0.044	0.021	0.23	1.35	+2.19
				1.0	5.7	4.4	0.050	0.024	0.24	0.93	+1.53
				1.0	4.8	4.6	0.057	0.028	0.23	1.15	+2.74

[a] C_{in}, inulin clearance = $U_{in} \cdot V/P_{in}$; C_{MTX}, MTX clearance = $U_{MTX} \cdot V/P_{MTX}$; $P \cdot F_w$, unbound fraction of MTX in plasma; U, urinary concentration (μmol/ml); V, urinary volume per min (ml/min); $T = U \cdot V - P \cdot F_w \cdot C_{in}/[C_{in}]$ (μmol/100 ml GFR).

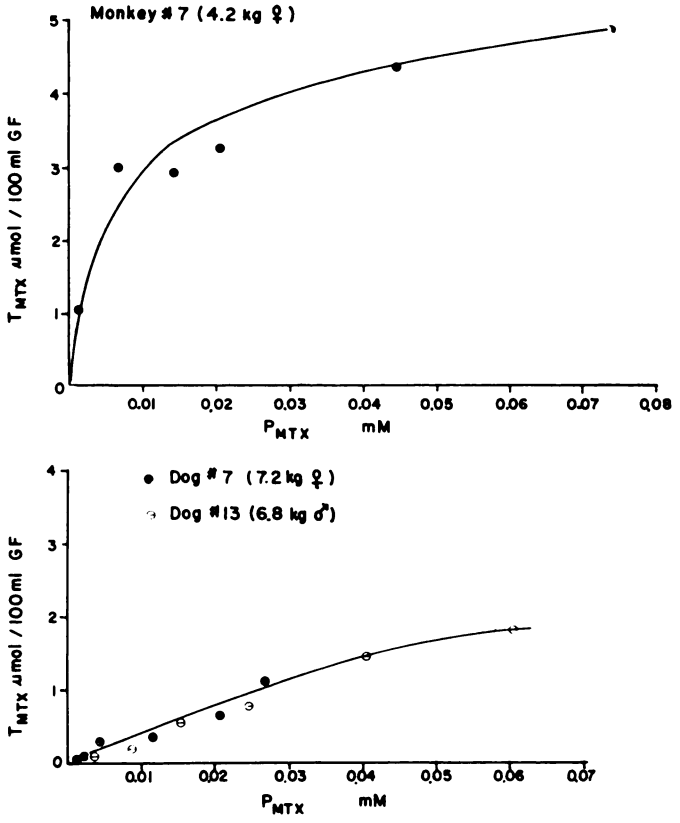

Chart 1. Tm determination of MTX in monkey and in dog. *Ordinate*, *T*-value in terms of µmol/100 ml glomerular filtrate calculated from the formula: $T = UV - P \cdot F_w \cdot C_{in}$ (F_w is the protein-free fraction) divided by the value of glomerular filtration rate.

DISCUSSION

MTX is a weak organic acid with pK_a values of 4.84 and 5.51. It contains an acylamino group, —CONH—, and 2 anionic groups, COO^-. Based on our previous investigations on renal handling of organic acids and amino acids, MTX would be favorable for secretion by the renal tubules (12, 16, 17). Data presented in this study with monkeys support this conclusion and confirm the results by Henderson *et al.* (9, 18) and Bourke *et al.* (2) suggesting that in rhesus monkey MTX excretion is handled mainly by tubular secretion which can be blocked by probenecid. Although the dog is physiologically very close to the human and monkey, certain differences do exist. In their early investigation with dogs, Liegler *et al.* (18) reported that the clearance ratio of MTX and inulin was <1, suggesting that there is tubular reabsorption of MTX instead of secretion. As shown in Table 1, the MTX clearance in the dog was lower than that of inulin in almost every experiment, but considering only the unbound fraction of MTX being filtered through the glomeruli, the *T*-value of MTX is then calculated and is found to be positive except in Dog 5 when it received no sodium acetate prior to the administration of MTX, and the calculated *T*-value was first negative and then gradually changed to positive. These results demonstrate that in the dog there is a bidirectional tubular transport, reabsorption, and secretion of MTX, whereby the rate of reabsorption is probably greater than that of secretion when the plasma concentration and the urine flow rates are low.

The effect of sodium acetate on tubular transport of organic acids has been well documented in both *in vivo* (14, 19) and *in vitro* studies (5). With the data presented in Table 1, one can compare the clearance ratios (C_{MTX}/C_{in}) in the control period of Monkey 1 with Monkey 4 and Monkey 2 with Monkey 3, since each pair received a comparable dose of MTX. These ratios ranged from 1.0 to 1.8 in the absence of acetate to 3.6 to 5.0 in the presence of acetate for Monkeys 1 and 4 and from 2.4 to 3.5 to 5.0 to 6.2 for Monkeys 2 and 3, suggesting that acetate increases the tubular secretory rate of MTX.

Actually, the bidirectional transport of many organic and amino acids has been well demonstrated by us (14, 27), wherein the reabsorption and secretion probably operate by different mechanisms. Since folic acid, a MTX analog, has been reported to be reabsorbed in the dog (7), it would be expected that folic acid would compete for the tubular reabsorptive process of MTX, resulting in an increase of MTX excretion. Such competition can be shown in both *T*-value determinations and stop-flow experiments. As shown in Chart 2, at the section corresponding with the distal tubules, the ratio of U/P_{MTX} to U/P_{in} is below 1, indicating the site of reabsorption. With the simultaneous infusion of folic acid, the ratio increases to 2 or higher indicating that reabsorption has been partially blocked. Liegler *et al.* (18) speculated that the reabsorption of MTX in the dog is probably by a special reabsorptive

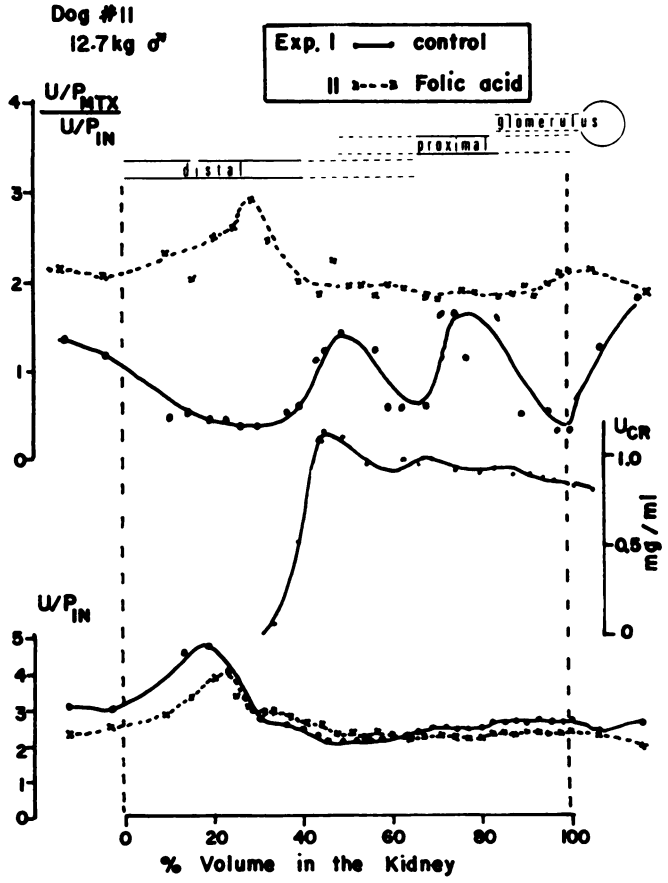

Chart 2. Studies of MTX excretion pattern by stop-flow technique. MTX was administered to the dog by a priming dose of 5 µmol/kg and a sustaining infusion (1.5% of that dose per min). After the first experiment (——), folic acid was simultaneously administered to the animal at a dose of 200 mg/kg, and 30 min later the second experiment (– – – –) was performed.

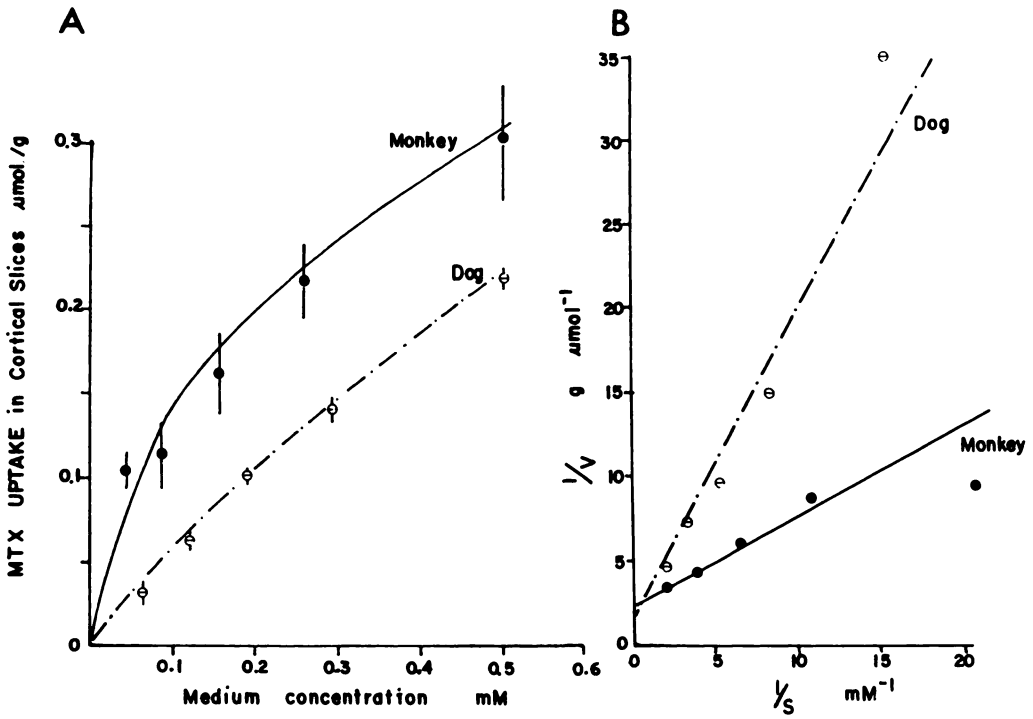

Chart 3. *A*, MTX uptake by renal cortical slices at 25°. Each *point* is the average of 3 (monkey) or 4 (dog) experiments. *Bars*, S.E. *B*, data plotted by double reciprocal method. The lines were drawn subjectively.

mechanism. Our results suggest that such a reabsorptive mechanism is shared also by folic acid. As in the monkey kidney, little or no tubular reabsorption of MTX can be detected. In the experiment with the i.a. infusion of probenecid, the *T*-value of MTX was markedly reduced but never decreased to a negative value. Administration of folic acid to the rhesus monkey caused no great augmentation of the excretory rate of MTX as compared with the dog experiment. Therefore, it may be concluded that MTX elimination in the monkey is handled mainly by a secretory mechanism but that in the dog it is handled by both secretory and reabsorptive processes.

An unforeseen adverse effect of folic acid administration was the marked decrease in GFR during both the i.a. infusions in monkeys and i.v. infusions in dogs. Folic acid has been reported to be nontoxic in man; however, massive doses when given to rats can produce renal toxicity (10). This renal toxicity is due to the low solubility of folic acid, resulting in the precipitation of crystalline folic acid within the tubules. If this precipitation did occur in our experiments, there appears to be little effect on the tubular secretion of MTX. As shown in Table 1 and in Chart 2, the ratio of C_{MTX}/C_{in} or U/P_{MTX} to U/P_{in} was initially <1 but increased to >1 during the infusion of folic acid. This increase is probably not due to a change in urinary volume because folic acid caused a decrease in GFR resulting in a decreased urinary volume. In the stop-flow experiments in the dog, the urinary volume was maintained at 6 ml/min throughout the experimental period. Administration of folic acid had no effect on the pattern of U/P_{in}, as shown in Chart 2; however, the ratio of U/P_{MTX} to U/P_{in} increased. Since this ratio normalized the effect of water reabsorption and any changes in urinary volume on C_{in}, it can be concluded that the effect of folic acid on MTX excretion is to inhibit MTX absorption competitively.

MTX and folic acid contain the glutamic acid moiety, a diacidic amino acid. The tubular reabsorption of glutamic acid has been investigated in the dog (21). To determine whether the reabsorptive mechanism for MTX and folic acid in the dog is similar to that for amino acids requires further investigation.

In vitro studies of MTX uptake have shown that monkey cortical slices can accumulate more MTX than can dog cortical slices. Since the renal cortical slice technique has been used to characterize the secretion of organic acids (13, 16) as well as the reabsorption of amino acids (22, 23) in renal tubular cells, our data can only support the conclusion that the monkey kidney can rapidly transport MTX from the medium to the extracellular fluid into the cells. Thus, in these experiments, it is difficult to separate the direction of transport whether reabsorption or secretion.

As a result of the widespread use of MTX in cancer chemotherapy, nephrotoxicity has been well recognized in humans (6, 14, 20). If this toxicity is directly correlated to the amount of MTX accumulated inside the tubular cells, then the dog is not a typical model for studying MTX nephrotoxicity in humans. Rubin *et al.* (24) found that [^{3}H]MTX was taken up against a concentration gradient in rabbit cortical slices. This accumulation of [^{3}H]MTX was inhibited by ouabain, a Na^+-K^+-ATPase inhibitor, and also by the metabolic inhibitors, 2,4-dinitrophenol and iodoacetate. However, in these studies, [^{3}H]MTX was used only at low concentrations (10^{-8} M). Because of the lack of additional investigations using higher concentrations of MTX, it is difficult to predict possible species differences in MTX accumulation among the monkey, dog, and rabbit.

In conclusion, our data show clearly that the renal excretion of MTX is handled by a bidirectional transport mechanism, namely, tubular secretion and tubular reabsorption. However, in the rhesus monkey, the tubular secretion of MTX is the predominant process. This secretion of MTX is competitively inhibited by probenecid, augmented by sodium acetate, and mediated by an organic acid transport process. In the dog, the

tubular reabsorption of MTX is predominant and probably occurs via a folic acid reabsorptive process.

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