

Tissue-specific Synthesis of Methotrexate Polyglutamates in the Rat¹

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SUMMARY

Purified tritium-labeled methotrexate was administered to rats and Sephadex G-15 gel chromatography was used to study the formation of poly- γ -glutamyl metabolites of methotrexate in different tissues. These metabolites were recognized as tritium-labeled antifolate fractions and were identified by their response to serum pteroylpolyglutamate hydrolases and by cochromatography with authentic standards. After doses equivalent to 15 to 20 mg for man, rat liver and kidney were found to contain both 4-amino-10-methylpteroylglutamyl- γ -glutamic acid and 4-amino-10-methylpteroylglutamyl- γ -glutamyl- γ -glutamic acid. With one-third of the dose, only 4-amino-10-methylpteroylglutamyl- γ -glutamic acid was found. Synthesis of methotrexate polyglutamates in liver and kidney was limited to the interval immediately following methotrexate administration and appeared to occur by sequential addition of single glutamyl residues to so-called "free" intracellular methotrexate. No synthesis of methotrexate polyglutamates was demonstrated in small intestine or thymus. After formation, 4-amino-10-methylpteroylglutamyl- γ -glutamic acid disappeared from liver and kidney with a half-time of 6.5 days. The effect of these metabolites of methotrexate on cell metabolism is unknown.

INTRODUCTION

MTX³ (*N*-[*p*-{(2,4-diamino-6-pteridiny)methyl}methylamino}benzoyl]glutamic acid), an analog of the vitamin folic acid (pteroylglutamic acid) and a potent inhibitor of its metabolism, continues to be regarded as a potent and effective antineoplastic agent after more than 2 decades of use (6). Beneficial results have attended its use in the treatment of a wide variety of neoplastic diseases. Strikingly, its use in the treatment of acute lymphoblastic leukemia of childhood (11) and of choriocarcinoma (16), usually in combination with other agents, has resulted in

cures. In addition, it has proved of value in the treatment of psoriasis (19) and as an immunosuppressive agent (18).

As with other antineoplastic agents, the effectiveness of MTX has been limited by its toxicity toward normal tissues, particularly bone marrow and gastrointestinal mucosa, and by the inherent or acquired resistance of neoplastic tissues to its cytotoxic action. While the subsequent administration of folinic acid (DL-5-formyltetrahydrofolate) has been found to reduce host toxicity (8), permitting the use of exceedingly large doses of MTX (5), the problem of resistance remains unsolved. This has been thought to be due to one or another of 3 different mechanisms. (a) Resistance has been associated with a reduction in the permeability or active membrane transport of MTX into cells (7). (b) MTX administration has been found to result in the "induction" of synthesis of the target enzyme dihydrofolate reductase in human leukocytes and erythrocytes (3, 10). (c) Certain tissues have the capacity to metabolize MTX. Thus, oxidation of MTX is extensively carried out in rabbit liver and, to a lesser extent, in guinea pig liver but has not been detected in the livers of other species, including man (12). Again, certain bacteria, including those present in mouse intestine, contain an enzyme capable of hydrolyzing MTX to glutamic acid and 4-deoxy-4-amino-10-methylptericoic acid (14).

Recently, an additional mode of metabolism of MTX was discovered. Nair and Baugh (15) synthesized a series of poly- γ -glutamyl metabolites of MTX and with their aid were able to identify small amounts of MTX(G₁) and MTX(G₂) in rat tissues (2). Evidence of formation of similar metabolites of MTX in mouse liver was also reported (21). The present communication provides confirmation of the synthesis of MTX(G₁) and MTX(G₂) in rat liver and kidney and presents evidence that such synthesis is dose dependent, can be significant in amount, and is specific to certain tissues. Preliminary reports of some of these findings have been presented (21, 22).

MATERIALS AND METHODS

Groups of 3 to 5 male adult Sprague-Dawley rats were housed in wire-bottomed metabolic cages and fed a commercial diet (laboratory chow; Ralston Purina Co. of Canada Ltd., Woodstock, Ontario, Canada) and water *ad libitum*. [³H]MTX was usually administered s.c. in 1, 3, or 9 doses. At intervals after the final dose, animals were anesthetized with ether, blood was withdrawn from a jugular vein, and organs were removed. Liver, kidney, and thymus were rapidly weighed and homogenized in cold 100

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³The abbreviations used are: MTX, methotrexate; MTX(G₁), methotrexate monoglutamate; MTX(G₂), methotrexate diglutamate; [³H]MTX, [³H]-[3',5'-9(n)-³H]methotrexate.

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mm sodium phosphate buffer containing ascorbic acid, 150 mg/100 ml, final pH 7.0. The small intestine was rinsed with 0.9% NaCl solution and blotted dry, and the proximal one-third was weighed and homogenized as described above. Homogenates were heated for 5 min in boiling water and centrifuged, and the supernatants were stored. The preparation and heating of extracts were completed in less than 20 min, since studies revealed that hydrolysis of pteroylpolyglutamates in unheated rat liver extracts did not commence within this interval of time (unpublished data). Blood samples were mixed with an equal volume of 0.5 N perchloric acid (Allied Chemical Co., Morristown, N. J.) and centrifuged, and the supernatants were stored.

Extracts were initially stored at 4° to avoid possible hydrolysis of poly- γ -glutamyl metabolites (4), but these proved unstable. Thereafter, extracts were stored at -20°, following the demonstration that hydrolysis did not occur with freezing. The ^3H in extracts and in chromatographic fractions was counted in a Packard Tri-Carb Model 2425 liquid scintillation spectrometer using a commercial scintillant (Aquasol; New England Nuclear, Boston, Mass.). Results were expressed as ng MTX or MTX equivalent per g tissue or ml blood.

Gel chromatography was carried out using Sephadex G-15 gel (Pharmacia Co., Uppsala, Sweden) and 0.9- x 55-cm columns (20). The gel was swelled in boiling water for 1 hr and columns were poured by gravity. Columns were eluted at room temperature with 25 mM sodium phosphate buffer containing 200 mM mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.), final pH 7.0, at a flow rate of 20 to 25 ml/hr, collecting 1.7- to 2.0-ml fractions. Later, 100 mM NaCl were substituted for mercaptoethanol without altering the elution of MTX or its metabolites. Void and total column volumes were determined with hemoglobin and tritiated water and usually corresponded to Tubes 7 and 16, respectively.

Folate was assayed using *Lactobacillus casei* (American Type Cell Culture 7469) (1, 23). To provide a greater contrast in chromatograms between regions of growth of *L. casei* and regions of inhibition of growth, due to the presence of MTX and its metabolite(s), folic acid, 0.5 ng, was added to all assay tubes.

Hydrolysis of poly- γ -glutamyl metabolites of MTX in liver extracts was carried out using fresh human serum as a source of pteroylpolyglutamate hydrolase(s) as previously described for the hydrolysis of pteroylpolyglutamates in human liver (20). Equal volumes of fresh human serum and of pooled chromatographic fractions were mixed and incubated for 90 min at 37°. With such treatment, complete hydrolysis of tritium-labeled liver pteroylpolyglutamates to oligoglutamates and monoglutamates has been demonstrated when the pH was 4.6, while no hydrolysis occurred when incubation was carried out at pH 7.0 (unpublished data).

[^3H]MTX was obtained as the sodium salt from Amer-sham/Searle Co., Don Mills, Ontario, Canada, and was mixed with commercial MTX (Lederle Products Department, Cyanamid of Canada Ltd., Montreal, Quebec, Canada) to obtain a specific activity of 1 or 2 $\mu\text{Ci}/\mu\text{g}$. This mixture was purified by gel chromatography prior to use (Chart 1). *p*-Aminobenzoyl-L-glutamic acid was obtained

from Nutritional Biochemical Corp., Cleveland, Ohio. MTX(G_1) and MTX(G_2) were obtained as gifts from Dr. Nair and Dr. Baugh (15). 4-Deoxy-4-amino-10-methyl-ptericoic acid was a gift from Dr. Y. S. Shin (17). The chromatographic behavior of standard materials was determined spectrophotometrically in a Beckman DK2 recording spectrophotometer.

RESULTS

Sephadex G-15 Gel Chromatography of MTX

Chart 1 shows chromatograms of [^3H]MTX before and after purification. MTX bound tightly to the gel, appearing in about 2 column volumes. The contaminant in Tube 12 (Chart 1A) cochromatographed with para-aminobenzoyl-L-glutamic acid. The identity of other impurities was not determined. 4-Deoxy-4-amino-10-methylptericoic acid eluted in Tube 88 and appeared to be a contaminant of unlabeled MTX but not of [^3H]MTX. A number of contaminants of unlabeled MTX was detected spectrophotometrically. None appeared to correspond to contaminants present in [^3H]MTX. When unpurified [^3H]MTX was administered to rats, chromatograms of liver, kidney, and thymus revealed the presence of materials corresponding to 2 contaminant fractions (Chart 1A, Tubes 15 and 44) in these tissues. This was particularly striking when tissues were examined several days after injection. These contaminants were not detected following administration of purified [^3H]MTX (Chart 1B). Purified [^3H]MTX was stored at 4° and remained pure for as long as 2 months under these conditions (Chart 1B). At the same time, there appeared to be no exchange of the tritium label with the aqueous storage medium.

Identification of MTX(G_1) in Rat Liver and Kidney

A typical Sephadex G-15 gel chromatogram of a heated extract of rat liver is shown in Chart 2. This extract was prepared from a 556-g rat 24 hr after the last of 5 daily i.p. injections of 20 μg [^3H]MTX. Two major tritium-labeled fractions were present: MTX (Tube 43) and an unknown material (Tube 21). Both fractions were inhibitory to growth of *L. casei*. A similar chromatogram was obtained from an extract of kidney from the same animal.

When folic acid was added to assay tubes containing inhibitory quantities of the unknown material, growth of *L. casei* was restored, demonstrating that this tritium-labeled material was a folate antagonist. To determine whether this material was a poly- γ -glutamyl metabolite of MTX, aliquots of the material were incubated with fresh human serum [as a source of pteroylpolyglutamate hydrolase(s)] for 90 min at 37°. The mixture was then heated to coagulate protein and the centrifuged supernatant was rechromatographed. No change in the Sephadex G-15 chromatographic behavior of the unknown material occurred when incubation was carried out at pH 7. However, after incubation at pH 4.6, the unknown material was found to chromatograph in the position of MTX. These results demonstrated that the

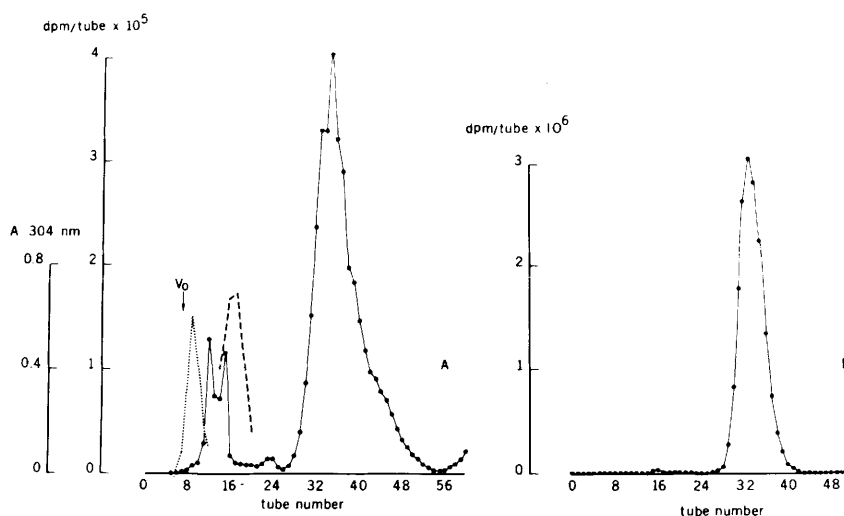


Chart 1. Sephadex G-15 gel chromatograms of $[^3\text{H}]\text{MTX}$ before and after purification. *A*, cochromatography demonstrated that $\text{MTX}(G_1)$ (---) and $\text{MTX}(G_2)$ (....) were not contaminants of $[^3\text{H}]\text{MTX}$. See text for further details. *B*, chromatogram of purified $[^3\text{H}]\text{MTX}$ after storage at 4° for 30 days. An identical chromatogram was obtained after 60 days. V_0 , void volume.

unknown material was a poly- γ -glutamyl metabolite of MTX and not a degradation product. The identity of this material was established by cochromatography with authentic standards. As shown in Chart 3, the unknown material in extracts of rat liver and kidney appeared after $\text{MTX}(G_2)$ and cochromatographed with $\text{MTX}(G_1)$.

Synthesis of MTX Polyglutamates in Rat Tissues. Table 1 contains results of 2 studies carried out to determine the effect of time after injection of $[^3\text{H}]\text{MTX}$ on the synthesis of MTX polyglutamates in rat liver. No increase in concentration of $\text{MTX}(G_1)$ appeared to occur in the interval between the earliest time studied (1 and 4 hr postinjection, respectively) and 24 hr. These results indicated that synthesis of $\text{MTX}(G_1)$ had essentially ceased by the time initial samples were studied, being limited to an interval of time immediately following injection. At the same time, these results demonstrated that the time of examination of tissues following injection was not a critical factor in assessing differences of MTX polyglutamate synthesis.

In a series of experiments, the concentration of $\text{MTX}(G_1)$ in liver was measured after administration of $[^3\text{H}]\text{MTX}$ at a dose of $80 \mu\text{g}/\text{kg}$. With this dose, the concentration of $\text{MTX}(G_1)$ was greater with 3 divided doses than with a single dose and was greater after parenteral than after p.o. administration. These observations were consistent with the hypothesis that synthesis of MTX polyglutamates was limited to intervals during which the extracellular concentration of MTX was high. Maximum concentrations of MTX polyglutamates for a given dose of $[^3\text{H}]\text{MTX}$ were found with s.c. administration in divided doses.

Synthesis of MTX polyglutamates was dose dependent and tissue specific. At a dose of $80 \mu\text{g}/\text{kg}$, no synthesis of $\text{MTX}(G_1)$ was detected in either small intestine or thymus (Chart 4 and Table 2). In contrast, this dose resulted in significant concentrations of $\text{MTX}(G_1)$ in both liver and kidney (Table 2). A 3-fold increase in the dose of $[^3\text{H}]\text{MTX}$ yielded a higher concentration of $\text{MTX}(G_1)$ in liver, and this

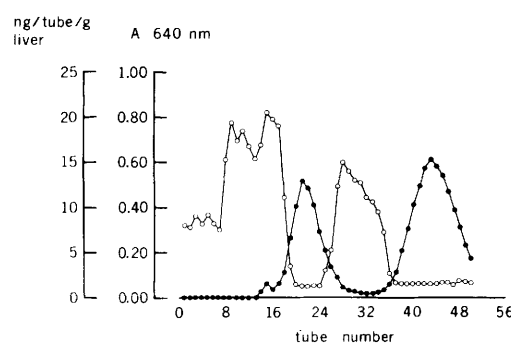


Chart 2. Sephadex G-15 gel chromatogram of an extract of rat liver prepared after administration of $[^3\text{H}]\text{MTX}$. Folate in fractions was assayed with *L. casei* (O) and ^3H was counted (\bullet). See text for details.

was accompanied by the appearance of an additional tritium-labeled fraction. This material was inhibitory to growth of *L. casei* and was identified as $\text{MTX}(G_2)$ by cochromatography with authentic standards (Chart 5). At the highest doses tested, both rat liver and kidney were found to contain $\text{MTX}(G_1)$ and $\text{MTX}(G_2)$ in significant amounts. In contrast, synthesis of MTX polyglutamates in rat small intestine did not occur, except possibly in trace amounts following the highest dose used (Table 2).

Fate of $\text{MTX}(G_1)$ in Rat Liver and Kidney

To determine whether $\text{MTX}(G_1)$ persisted in liver and kidney, groups of rats received 4 daily s.c. injections of $[^3\text{H}]\text{MTX}$, $20 \mu\text{g}/\text{kg}$, and were sacrificed 1, 4, 7, and 11 days after the last dose. Initial chromatograms revealed that $\text{MTX}(G_1)$ comprised 49.3 and 19.6% of the ^3H in pooled extracts of liver and kidney, respectively. Succeeding chromatograms of extracts revealed that $\text{MTX}(G_1)$ disappeared exponentially at a similar rate from both organs (Chart 6). No $\text{MTX}(G_2)$ was formed during this interval of time. In a

2nd study, rats received a single injection of [³H]MTX, 80 μg/kg, and extracts of their livers and kidneys were pooled and chromatographed 26 days later (Table 1). During this interval, there was a 23.4% fall in total liver MTX from 1.88 ± 0.30 to 1.44 ± 0.12 μg (mean ± S.D.). After 26 days, 92% and 96% of the remaining ³H in liver and kidney, respectively, chromatographed as MTX. MTX(G₁) was not identified in these chromatograms. These results demonstrated that MTX(G₁) did not persist in rat tissues in contrast to MTX itself. Nor was it converted to MTX(G₂) with time.

DISCUSSION

The apparent preferential and progressive accumulation in rat tissues of 2 tritium-labeled contaminant materials present in [³H]MTX rendered mandatory the purification of [³H]MTX prior to use. This was easily achieved by gel chromatography, pooling the large [³H]MTX peak but discarding the final tubes of the peak that contained 1 of the offending contaminants. The product remained stable for as long as 2 months in aqueous medium at 4° and was calculated to be 99.7% pure when rechromatographed on a

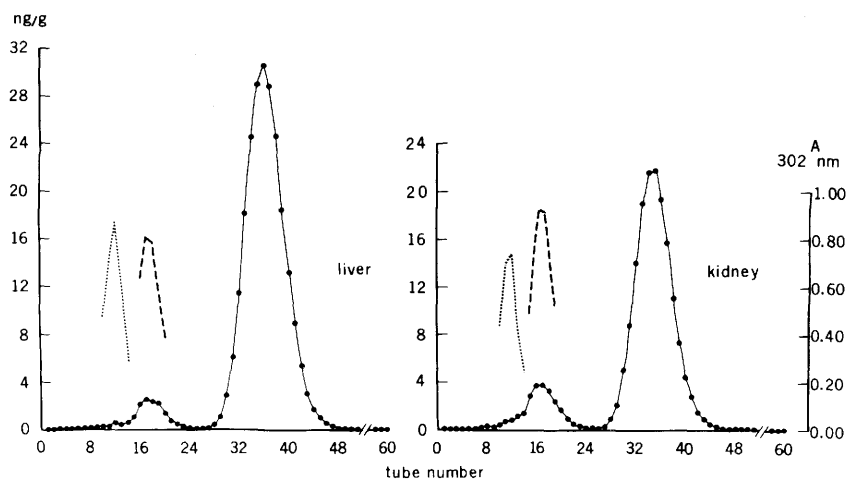


Chart 3. Cochromatography of extracts of rat liver and kidney with MTX(G₁) (---) and MTX(G₂) (.....).

Table 1

The effect of time after injection on synthesis of MTX(G₁) in rat liver

Rats were given s.c. injections of [³H]MTX, 80 μg/kg, and liver extracts were prepared at intervals thereafter. Pools of extracts from each time interval were chromatographed on Sephadex G-15. Results are expressed as ng MTX or ng equivalents of MTX per g liver.

Study	Time	No. of rats	MTX(G ₁) (ng/g)	MTX (ng/g)	Total (ng/g)	Total (ng/liver)	Blood (ng/ml)
A	4 hr	3	73	143	219		4.1
	8 hr	3	65	148	214		3.3
	26 hr	4	83	150	236		4.2
B	1 hr	3	33	216	250	1827	8.9
	24 hr	3	35	229	265	1942	4.5
	26 ^a days	4	0	98	107	1440	0.0

^a Rats killed at 26 days were part of Study B.

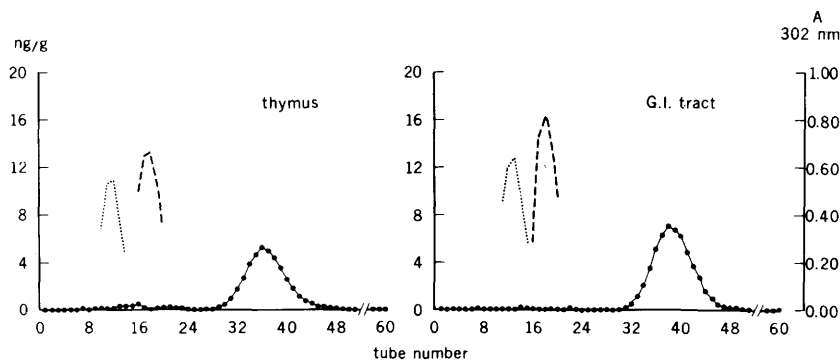


Chart 4. Cochromatography of extracts of thymus and small intestine with MTX(G₁) (---) and MTX(G₂) (.....). G.I., gastrointestinal.

number of occasions. Even with this purity, however, a problem could still be encountered. Thus, in a recent study, a rat received 75 mg of MTX containing 600 μCi of purified ^3H MTX in a 6-hr infusion. Greater than 90% of the tritium in liver, kidney, small intestine, and brain chromatographed as a contaminant (Chart 1A, Tube 15) and appeared to originate from the 1- μCi amount of this material present in the infusate. Should this material be present as a contaminant in unlabeled MTX, it is interesting to speculate on its effect on tissues of patients treated with very large doses of MTX (5).

These studies provided confirmation of the synthesis of poly- γ -glutamyl metabolites of MTX in rat tissues (2). In addition, the findings delineated a number of specific features concerning this metabolism. Unlike the synthesis of pteroylpolyglutamates from folic acid in mammalian tissues, in which successive glutamyl residues are added to folate to yield predominantly pteroylpentaglutamates and pteroylhexaglutamates after 24 to 48 hr and, with further time, some pteroylheptaglutamates (13), no evidence of progressive addition of glutamyl residues to MTX with time

was demonstrated. Rather, synthesis of MTX polyglutamates appeared to be a phenomenon of short duration, limited to the interval immediately following ^3H MTX administration. The quantity of MTX polyglutamates synthesized was clearly dose dependent. In addition, with the same dose, a greater synthesis was seen with parenteral than with p.o. administration and with the use of divided doses. At the highest doses studied, there was formation of both MTX(G_1) and MTX(G_2) in rat liver and kidney, while at lower doses only MTX(G_1) was found. These observations suggested that synthesis of MTX polyglutamates occurred by sequential addition of single glutamyl residues to so-called free intracellular MTX (9). It appeared that MTX bound to dihydrofolate reductase was not a substrate for MTX polyglutamate synthesis. It was not clear why MTX(G_2) formation occurred with large divided doses of MTX but did not arise with time from MTX(G_1) after this had been formed in liver and kidney. Possibly, MTX(G_1) became bound within the cell soon after its formation and, in that state, was no longer a substrate for addition of further glutamyl residues.

The persistence of MTX in tissues has been attributed to its pseudoirreversible binding to the enzyme dihydrofolate reductase with stabilization of the complex (10). Such persistence was demonstrated in rat liver and kidney in the

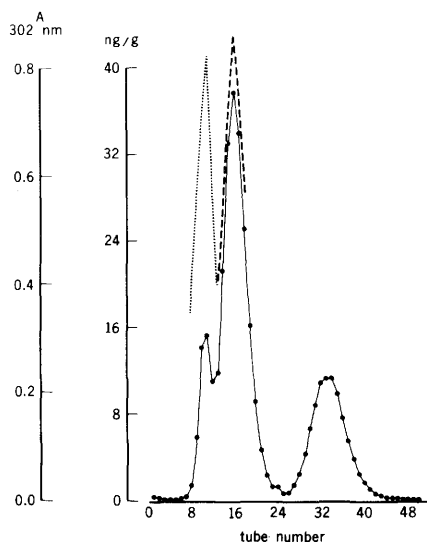


Chart 5. Chromatography of a liver extract with MTX(G_1) (---) and MTX(G_2) (····). The rat received ^3H MTX, 240 $\mu\text{g}/\text{kg}$, in 9 divided s.c. doses at 15-min intervals and was killed 2 hr later (see Table 2).

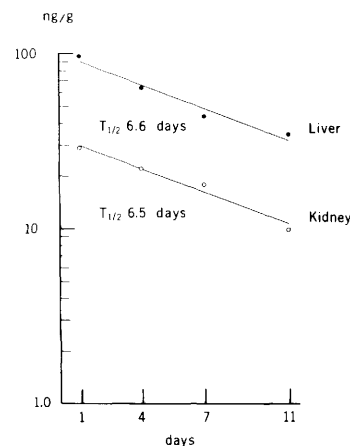


Chart 6. Levels of MTX(G_1) in pooled extracts of rat liver and kidney at intervals following the last of 4 daily s.c. injections of ^3H MTX, 20 $\mu\text{g}/\text{kg}$. $T_{1/2}$, half-time.

Table 2
Levels of MTX, MTX(G_1), and MTX(G_2) in rat tissues following s.c. ^3H MTX

The dose was divided and administered in 3 or 9 injections of about 27 $\mu\text{g}/\text{kg}$ at 15- or 30-min intervals, and animals were killed 4 hr after the 1st dose. Results are expressed as ng MTX or equivalent per g tissue.

Dose	No. of rats	Liver (ng/g)			Kidney (ng/g)			Small intestine (ng/g)		Thymus (ng/g)	
		MTX(G_2)	MTX(G_1)	MTX	MTX(G_2)	MTX(G_1)	MTX	MTX(G_1)	MTX	MTX(G_1)	MTX
88	1	0.0	90.3	155.6	0.0	30.2	216.0	0.0	73.8	0.0	50.9
77	3	0.0	22.0	233.7	0.0	21.5	216.7	0.0	61.0	0.0	29.1
80	3	2.5	60.9	190.4	0.0	20.0	178.8	0.0	49.5	0.0	37.3
240	1	37.0	143.6	70.6	3.2	28.2	173.1	0.0	73.4		
241	1	44.2	77.9	102.8	3.7	22.0	179.9	0.0	128.7		
287	1	17.0	103.6	139.8	19.9	59.6	314.3	0.0	368.5		
298	1	59.3	228.8	142.9	18.1	58.5	291.4	2.0	150.7		

present studies. In contrast, the rate of disappearance of MTX(G₁) from rat liver and kidney appeared to be about 8 times faster than that of MTX. Whether this resulted from loss of a specific population of cells containing high levels of MTX(G₁), from degradation and/or release of MTX(G₁) from cells, or from conversion of MTX(G₁) to MTX was not determined. Thus, it was not clear whether the poly-γ-glutamyl metabolites of MTX served as an intracellular reservoir of MTX or not.

The apparent inability of small intestine and thymus to synthesize poly-γ-glutamyl metabolites of MTX may have been due to their failure to accumulate high levels of intracellular MTX. In support of this was the finding of small amounts of MTX(G₁) in small intestine with the highest dose administered. However, synthesis was not seen when MTX was fed with the intent of ensuring exposure of the small intestine to high levels of the drug. Whatever be the mechanism, these differences in ability to synthesize MTX polyglutamates were seen with doses of MTX within the usual therapeutic range used in man. Since small intestine and, presumably, thymus (18) differ markedly from liver and kidney in their susceptibility to MTX intoxication, it might be speculated that synthesis of MTX polyglutamates serves to protect cells from the lethal effect of this drug. However, at the present time, the effect of these metabolites of MTX on cell metabolism is unknown.

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