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Structural Features of 4-Amino Antifolates Required for Substrate Activity with Mammalian Folylpolyglutamate Synthetase

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SUMMARY

The activity of a series of folic acid analogues as substrates for partially purified mouse liver folylpolyglutamate synthetase was determined and the effects of substituents on the binding to, and catalytic processes of, this enzyme were inferred. A 4-amino group improved substrate activity primarily by decreasing the apparent K_m while N^{10}-methyl substitution substantially diminished utilization as a substrate, again, by effects on K_m. Isosteric replacement of N-10 altered substrate activity. A free α-carboxyl group in the amino acid side chain was required for catalysis as was the presence of the side chain amide carbonyl group. Modification of the amino acid side chain length profoundly affected activity. Several observations were made that may be relevant to chemotherapy with folate antimetabolites: 1) 7-hydroxymethotrexate was a substrate for this enzyme; 2) substrate activity and substrate inhibition were observed with CB 3717, a potent inhibitor of thymidylate synthase; 3) potent classical dihydrofolate reductase inhibitors were identified that were either not substrates for mouse liver folylpolyglutamate synthetase (e.g., 4-amino-4-deoxy-N^{10}-methylpteroyl-L-α-aminoadipate) or were much better substrates than methotrexate for this enzyme (e.g., aminopterin); and 4) leucovorin and methotrexate appeared to be substrates for the same synthetase, but leucovorin saturated the reaction at much lower concentrations. These results have implications for the design of folylpolyglutamate synthetase inhibitors and for the selection of dihydrofolate reductase inhibitors that are either not polyglutamated or are efficiently polyglutamated *in vivo*.

INTRODUCTION

Mammalian cells efficiently incorporate exogenous folates into poly-γ-glutamate conjugates. Similarly, the intracellular folate pool in a variety of mammalian tissues *in vivo* has been shown to consist predominantly of folylpolyglutamates. These folylpolyglutamates are at least as good substrates as the corresponding monoglutamates with a number of purified folate-dependent enzymes (recently reviewed in Ref. 1). In addition, the accumulation of polyglutamates of dihydrofolate (but not of dihydrofolate itself) in methotrexate-treated L1210 cells indicates that folylpolyglutamates are the substrates for the folate-dependent reactions *in vivo* (2).

The question arises why these conjugates are made when the monoglutamate cofactors can also participate in the folate-dependent biosynthetic reactions in isolated enzyme systems. The information available to date favors the interpretation that polyglutamation is a mechanism to metabolically trap folates within the cell (3, 4). Thus, mouse leukemia cells that were preloaded with folylmonoglutamates rapidly lost these derivatives to the extracellular medium while these same cells quantitatively retained folylpolyglutamates for several hours (this topic has been reviewed in Ref. 4). In addition, mutant mammalian cells deficient in FPGS[1] could not form polyglutamates and were unable to accumulate folic acid

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[1] The abbreviations used are: FPGS, folylpolyglutamate synthetase; MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, methotrexate; D-MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-D-glutamic acid; PteGlu, folic acid; 4-NH_2-PteGlu, aminopterin; DCM, 3′,5′-dichloromethotrexate; 10-CH_3-PteGlu, 10-methylfolate; 7-OH-MTX, 7-hydroxymethotrexate; 6-(*R,S*)-5-CH_3-H_4PteGlu, 6-(*R,S*)-5-methyltetrahydrofolate; 6-(*R,S*)-5-CHO-H_4PteGlu, 6-(*R,S*)-5-formyltetrahydrofolate (leucovorin); MeAPA-Adi, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminoadipic acid; MeAPA-Pim, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminopimelic acid; MeAPA-Sub, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminosuberic acid; MeAPA-Gaba, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-4-aminobutyric acid; MeAPA-3-NH_2-Glu, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-DL-3-aminoglutaric acid; CB3717, *N*-[*p*-[*N*-(2-amino-4(3*H*)-oxoquinazolin-6-yl)methyl-*N*-propargyl]amino]benzoyl-L-glutamic acid; HPLC, high performance liquid chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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from the culture medium (3). It was of particular interest to us that these mutant cells were not viable under normal growth conditions (3); hence, the formation of noneffluxing polyglutamates seems to be a function that is essential for growing mammalian cell populations (4). It follows that FPGS is a promising new target enzyme for antifolate chemotherapy and that inhibition of FPGS would block the influx of folates into the pool of intracellularly retained cofactors. This approach would be different from, but not necessarily exclusive of, the use of antifolates that inhibit dihydrofolate reductase, whose cellular function is the re-utilization of existing folate pools. Based on this distinction, we have suggested that FPGS inhibitors would differ significantly from the classical folate antimetabolites typified by MTX (4).

It is now apparent that polyglutamates of MTX are formed in both normal and neoplastic tissues after exposure to the drug either *in vivo* or in cell culture (see literature quoted in Ref. 5). These polyglutamates of MTX have been found by most investigators to be retained within cells longer than MTX itself and, as a result, with time, the drug bound to dihydrofolate reductase becomes progressively enriched in these polyglutamates (5). MTX has been shown to be cytotoxic only when the intracellular concentration of total drug exceeds the cellular content of dihydrofolate reductase (6), although only a slight molar excess is required for cytotoxicity. Hence, any factor that prolongs intracellular retention of free MTX must affect the length of inhibition of DNA synthesis following exposure to the drug. It has been well documented that the selective toxicity of MTX and related 4-amino antifolates for sensitive tumors correlates well with a substantially longer retention of drug in tumor cells at levels that exceed the dihydrofolate reductase content (reviewed in Ref. 7). Indeed, the therapeutic utility of MTX for a spectrum of murine tumors inversely correlates with the rate of loss of the drug from the tumor. In addition, the increase in lifespan of leukemic mice that resulted from treatment with various antifolates correlated well with the retention of these agents in tumor. It should be noted that the *toxicity* of 4-amino antifolates to mice also correlates with the retention of drug in intestinal cells at levels in excess of the content of dihydrofolate reductase.

A synthetase capable of adding glutamate to a variety of folates has been purified to homogeneity from bacteria (8, 9). FPGS has also been partially purified in this laboratory from mouse liver (10) and in other laboratories from rat and hog liver and from Chinese hamster ovary cells (reviewed in Refs. 1 and 10). Enzyme preparations from all mammalian sources studied to date have been characterized as having a fairly broad substrate specificity for reduced folates. However, there seems to be a major difference between bacterial and mammalian enzymes in the substrate activity of oxidized species such as folic acid and MTX, which are substrates for FPGS from mammalian sources, but not for those of bacterial origin.

In this paper, we compare the activity of various 4-amino antifolates as substrates for mouse liver FPGS and examine the role that each portion of the folate molecule plays in the binding to enzyme (as evidenced by the apparent K_m) and in the maximum velocity of the reaction. These studies have indicated structural features that would help maximize the binding of FPGS inhibitors to this enzyme and have suggested approaches to the design of new dihydrofolate reductase inhibitors that are either more efficiently metabolized to polyglutamates than MTX or, on the other hand, are not substrates for FPGS at all.

MATERIALS AND METHODS

Materials

PteGlu, MTX, and 6-(*R,S*)-5-CHO-H_4PteGlu were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without purification. Aminopterin was obtained from Lederle Laboratories (Pearl River, NY) as clinical grade drug and was purified prior to use by chromatography on DEAE-cellulose as was DCM. APA-Asp, *N*-[*p*-[*N*-(2,4-diaminoquinazolin-6-yl)methyl]aminobenzoyl]-DL-3-aminoglutaric acid, *N*-[*p*-[*N*-(2,4-diaminopyrido-(2,3-*d*)-pyrimidin-6-yl)methyl]aminobenzoyl]-DL-3-aminoglutaric acid, and *N*-[*p*-[*N*-(2,4-diaminoquinazolin-6-yl)methyl]amino]benzoyl-L-aspartic acid were obtained from the National Cancer Institute through Contract N01-CM-67065 (to K. K. C.) and were purified prior to use by DEAE-cellulose column chromatography. 10-CH_3-PteGlu was prepared by treatment of MTX with 1 N NaOH at 100°, recrystallized from HCl/NaCl (11), and purified by DEAE-cellulose column chromatography. 10-Deazaaminopterin and 10-thiofolic acid were generous gifts of Drs. J. I. DeGraw, Stanford Research Institute, and F. M. Sirotnak, Memorial-Sloan-Kettering Institute and of Dr. M. G. Nair, University of Southern Alabama, respectively. Homofolic acid was obtained from Dr. J. A. R. Mead of the National Cancer Institute and was found to be >98% pure by HPLC. *N*-*p*-[*N*-(2-Amino-4(3*H*)-oxoquinazolin-6-yl)methyl-*N*-propargyl]amino]benzoyl-L-glutamic acid (CB3717) was a generous gift of Dr. A. H. Calvert, Institute for Cancer Research, U.K., CB3717 and 10-thiofolate were purified by DEAE-cellulose chromatography prior to use. The syntheses of the α-*t*-butyl, α-*n*-butyl, and γ-*t*-butyl esters of the γ-monobenzylamide and γ-hydroxamate of MTX, of MeAPA-3-NH_2-Glu, and of *N*-[[[(2,4-diaminopteridin-6-yl)methyl]amino]benzyl]-L-glutamic acid (deoxoaminopterin) have been previously described (12–15). The syntheses of MeAPA-Gaba (16) and of MeAPA-Adi, MeAPA-Pim, and MeAPA-Sub (17) have been reported separately.

In the course of preparation of 4-amino-N^{10}-methyl pteroic acid from MTX using carboxypeptidase G_1, it was noticed that a small amount of starting material was resistant to repeated enzymatic digestion. This material was purified by DEAE-cellulose chromatography and was identified as D-MTX on the bases of its identical UV spectrum and chromatographic behavior, but opposite optical rotation compared with MTX. The contamination of commercial MTX with D-MTX has recently been reported by others (18).

Samples of 7-hydroxymethotrexate were kindly furnished by Drs. D. G. Johns, National Cancer Institute, and D. Farquhar, M.D. Anderson Hospital and Tumor Institute, and were prepared by enzymatic hydroxylation of MTX (19) and by chemical synthesis (20), respectively. These samples, which had been purified by recrystallization and chromatography (19, 20), had ultraviolet and NMR spectra characteristic of 7-OH-MTX and gave essentially a single peak (>98%) on reversed phase HPLC. The identity of these samples had been confirmed by mass spectrometry. A sample of 7-OH-MTX that had been purified by HPLC was kindly furnished by Drs. J. P. Cano and J. P. Sommadossi, INSERM, France. The results obtained with these two samples of 7-OH-MTX were not appreciably different.

Methods

Purification of compounds. Purification of folate analogues was usually performed on a 0.62 cm^2 × 35 cm column of DEAE-cellulose

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(Eastman Chemical Co., Rochester, NY) that was eluted with a linear gradient of ammonium acetate (0.01 to 2.5 M) with an initial mixer volume of 100 ml. [3,4-^{3}H]Glutamic acid (10–15 Ci/mmol) was purchased either from New England Nuclear (Boston, MA) or from ICN (Irvine, CA). Some batches of isotope required purification to reduce assay blanks to acceptable levels (150–350 cpm). For purification, 250–350 μCi were brought to dryness with a stream of N_2 and the residue was dissolved in 250 μl of 0.2 M Tris buffer, pH 8.5. This solution was passed onto a 1–2-mm column of acid-washed activated charcoal (Sigma Chemical Co.) held in a Pasteur pipet between two 0.5–1-cm layers of cellulose powder (Whatman, type CC41). The column was rinsed with 0.75 ml of 0.2 M Tris buffer, pH 8.5, and the eluted [^{3}H] glutamate was used without further treatment.

Preparation of FPGS from mouse tissues and rat liver. B6D2F1 female mice were obtained from Simonsen Laboratories (Gilroy, CA) and were fed standard laboratory chow *ad libitum*. Typically, 30 mice were used for a preparation of mouse liver enzyme. Mice were sacrificed by cervical dislocation and the livers were perfused *in situ* with ice-cold 20 mM Hepes, pH 7.4, containing 0.25 M sucrose. Livers were removed, weighed, and homogenized in 2 volumes of this same buffer containing 50 mM α-thioglycerol using a motor-driven Teflon pestle. The homogenate was centrifuged for 1 hr at 165,000 × *g* and the supernatant was brought to 30% saturation with $(NH_4)_2SO_4$ at 0°. After 1 hr, the precipitated protein was centrifuged at 15,000 × *g* for 20 min, washed with 50% $(NH_4)_2SO_4$, and dissolved in a minimal volume of homogenization buffer; residual insoluble material was removed by centrifugation. The supernatant protein was precipitated with 50% $(NH_4)_2SO_4$ and was stored until use in this form. $(NH_4)_2SO_4$ was removed prior to assay by Sephadex G-25 chromatography. We have previously shown that 90% of the conjugase activity found in crude homogenates of mouse liver was discarded by this fractionation (10). Conjugase activity was not detectable (<0.02 nmol/hr/mg of protein) in this $(NH_4)_2SO_4$-precipitated protein fraction when assayed under the conditions of the FPGS assay (10). Equivalent rat liver FPGS was prepared from female adult Sprague-Dawley rats using this same procedure. Mouse kidneys (100 kidneys/enzyme preparation) were used as a source of FPGS for some experiments. The same procedure was used as described above for mouse liver, except that the kidneys were not perfused *in situ* prior to excision. Mouse L1210 leukemia cells were harvested from the peritoneal cavities of female B6D2F1 mice 7 days after the injection of 10^6 leukemic cells intraperitoneally. The L1210 cells were washed once with ice-cold phosphate-buffered isotonic saline, suspended in 2 volumes of 20 mM Hepes buffer, pH 7.4, containing 0.25 M sucrose and 25 mM α-thioglycerol and were disrupted with four 15-sec bursts of sonic oscillation. The resultant suspension was centrifuged and a 30% $(NH_4)_2SO_4$ pellet was prepared as described above.

FPGS assay. Compounds were incubated with FPGS for 60 min at 37° in a mixture containing, in addition to a folate or a folate analogue (0–1000 μM), 1 mM [^{3}H]glutamic acid (4 mCi/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM α-thioglycerol, and 200 mM Tris, pH 8.6, in a total volume of 0.25 ml. [^{3}H]Folyloligoglutamyl product was isolated from incubation mixtures by adsorption onto acid-washed activated charcoal (Sigma Chemical Co.) which had been treated with Dextran T-70 (25 mg/g charcoal; Pharmacia, Inc., Uppsala, Sweden). The charcoal was washed extensively to remove unreacted glutamate and the product was eluted from the charcoal with ethanolic ammonia. The characteristics of this assay have been described in greater detail separately (21). In the experiments reported here, there were 3.3–4.1 cpm/pmol of glutamate incorporated into product and the FPGS used had a typical specific activity of 1.2 nmol of product formed per hr/mg of protein. Negligible reaction (<3%) was observed in the absence of folate, ATP, or $MgCl_2$. The blanks used in these experiments contained all components of the reaction mixture including protein but lacked folate; blanks were incubated for 1 hr at 37°. The reaction catalyzed by this $(NH_4)_2SO_4$-precipitated fraction was previously shown to be linear with time and protein (21) and the products isolated by our procedure have been characterized as folyloligoglutamates by chromatography on

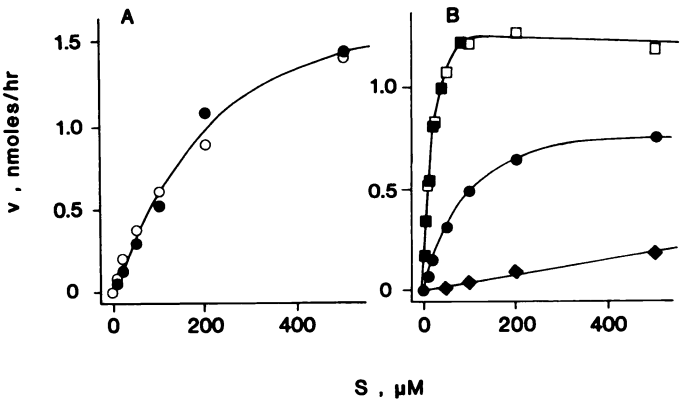

FIG. 1. *Similar substrate activities of PteGlu (●) and MTX (○) for mouse liver FPGS (A) and opposing influences of 4-amino and 10-methyl substituents on the substrate activity of PteGlu (B)*

The indicated concentrations of substrates were incubated with enzyme for 1 hr at 37° and products were isolated as described in Materials and Methods. In B, the product formed by mouse liver FPGS using either PteGlu (●), 4-NH_2-PteGlu (□ or ■, different experiments), or 10-CH_3-PteGlu (♦) was measured as described in the text. Each symbol in this and all subsequent figures represents the mean of duplicate assays from a representative experiment.

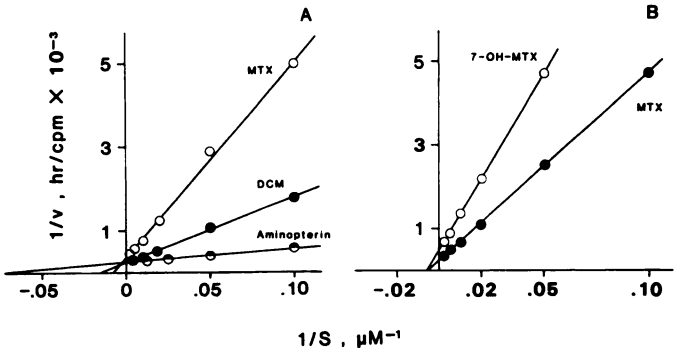

FIG. 2. *The relative substrate activities of several chemotherapeutically relevant 4-amino antifolates for mouse liver FPGS*

DEAE-cellulose, Sephadex G-25, and reverse phase HPLC (10, 21). No more than 20% of the folyl substrate was consumed during the 1-hr incubation in these experiments.

In previous work, we have measured the efficiency of adsorption of the products of the FPGS reaction onto charcoal, the efficiency of elution from charcoal, and the overall recovery of product under our conditions (21). Using either MTX or PteGlu or $PteGlu_3$ as a substrate at 500 μM, adsorption and recovery of ^{3}H-product were nearly quantitative (>96 and >84%, respectively) (21). A single elution of the charcoal allowed the recovery of the same percentage of all three of these oxidized pteridines (72–76%) (21). We have also studied the recovery of product from charcoal as a function of the concentration of folyl substrate using CB3717, 10-CH_3-PteGlu, and a pyridopyrimidine not discussed in this article. In all cases, concentrations of substrate up to 1000 μM did not diminish recovery of product. At higher concentrations, recovery progressively decreased; for instance, at 4.5 or 10.5 mM 10-CH_3-H_4PteGlu, recovery was 35 and 65% lower than that seen at 500 μM 10-CH_3-H_4PteGlu. As a result, all saturation curves used in these experiments were restricted to substrate concentrations below 1 mM.

Data analysis. The substrate and inhibitor activity of each compound was tested by measuring the rate of the FPGS reaction with 500 μM compound in the absence and presence of 500 μM PteGlu, respectively. The activity of each enzyme preparation was tested simultaneously with 500 μM PteGlu as a standard. Compounds that showed signifi-

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TABLE 1

A quantitative comparison of the activity of several folate analogues as substrates for mouse liver FPGS

Full substrate saturation curves were run for each of the compounds listed using duplicate assays for 5–6 concentrations on the range of 0.2–3 apparent K_m values. The values listed were derived from analysis of the data by the method of Cleland (22).

Compound	n[a]	Apparent K_m			V_{max}	$(V_{max})^b/(K_m)$
		μM	Relative to PteGlu[3]	Relative to MTX		
PteGlu	49	140 ± 47	1.0	0.92 ± 0.10[c]	1.0	1.0
MTX	12	166 ± 49	1.10 ± 0.12[c]	1.0	0.99 ± 0.16[c]	0.90 ± 0.05[c]
Aminopterin	4	17.6 ± 5.8	0.21 ± 0.04[c]	0.15 ± 0.08	1.52 ± 0.15	12.4 ± 5.0[d]
DCM	2	56 ± 7		0.43 ± 0.01	1.58 ± 0.28	3.7 ± 0.6[d]
10-CH_3-PteGlu[e]	2					0.048 ± 0.001
7-OH-MTX	3	140 ± 54		0.80 ± 0.19	0.47 ± 0.04[d]	0.62 ± 0.16[d]
10-Deazaaminopterin	2	144 ± 4	1.20 ± 0.30		1.16 ± 0.18	0.95 ± 0.05
Homofolate	2	250 ± 58	1.87 ± 0.35		1.01 ± 0.12	0.54 ± 0.04
10-Thiofolate	2	88 ± 40	0.54 ± 0.06		0.58 ± 0.08	1.08 ± 0.02
CB3717	2	40 ± 2[f]	0.39 ± 0.01[f]		0.88 ± 0.01[f]	2.26 ± 0.04
MeAPA-Pim	2	430 ± 170	2.35 ± 0.28		0.42 ± 0.11	0.18 ± 0.02

[a] Number of experiments used for each estimate.

[b] In order to standardize the results between experiments, a saturation curve was run for PteGlu or MTX in each experiment and the apparent K_m, V_{max}, and their ratio, the apparent first order rate constant, were expressed relative to these controls. It should be noted that relative K_m values were calculated by dividing the apparent K_m for the compound by that of the control in each experiment and by then averaging the values obtained. Unless otherwise specified, PteGlu was used as a control.

[c] This value was derived from only two experiments.

[d] Relative to a control of MTX.

[e] The reaction velocity at the highest concentrations permitted by the charcoal assay was too low to allow an accurate determination of V_{max} or K_m for 10-CH_3-PteGlu. However, the relative first order rate constants of this compound and PteGlu could be estimated from the initial slopes of plots such as Fig. 1B.

[f] Extrapolated using only low concentration points; see, for instance, Fig. 3.

TABLE 2

Activity of methotrexate analogues modified in the side chain as substrates and inhibitors of FPGS

The listed compounds were incubated with FPGS under standard conditions for 1 hr at 37° and the product of the reaction was isolated by adsorption onto charcoal. For details, see text. The FPGS activity of the enzyme preparations used was measured for a PteGlu control (500 μM). Each compound was tested at 500 μM in duplicate in each of two experiments. The ability of these compounds to inhibit FPGS was tested by incubating enzyme with 500 μM PteGlu in the presence of 500 μM compound (PteGlu + compound).

Compound	Exp.	FPGS activity				
		PteGlu control	Compound	Substrate activity (mean)	PteGlu + compound	Inhibition (mean)
		nmol/hr		%	*nmol/hr*	%
D-MTX	1	0.79 ± 0.06	0.018 ± 0.002	1.9	0.66 ± 0.004	15.7
	2	1.18 ± 0.004	0.016 ± 0.004		0.99 ± 0.03	
MTX γ-*t*-butylester	1	1.40 ± 0.01	<0.001[a]	<0.1	1.14 ± 0.02	18.5
	2	1.24 ± 0.02	<0.001[a]		1.01 ± 0.05	
MTX γ-monobenzylamide	1	1.40 ± 0.01	<0.001[a]	<0.1	1.09 ± 0.04	19.2
	2	1.24 ± 0.02	<0.001[a]		1.03 ± 0.07	
MTX γ-monohydroxamate	1	1.40 ± 0.01	0.11 ± 0.02	6.7	1.26 ± 0.03	12.2
	2	1.24 ± 0.02	0.07 ± 0.01		1.06 ± 0.02	
MTX α-*t*-butylester	1	0.95 ± 0.02	<0.005[a]	0.9	0.76 ± 0.4	22.4
	2	1.41 ± 0.05	0.025 ± 0.001		1.06 ± 0.01	
MTX α-*t*-butylester	1	0.95 ± 0.02	<0.005[a]	1.8	0.78 ± 0.008	19.6
	2	1.41 ± 0.05	0.05 ± 0.006		1.11 ± 0.02	
MeAPA-Gaba	1	1.30 ± 0.02	0.001 ± 0.006	<0.1	1.13 ± 0.01	11.0
	2	1.00 ± 0.03	−0.003 ± 0.01		0.91 ± 0.001	
Deoxoaminopterin	1	0.79 ± 0.006	0.02 ± 0.003	1.9	0.71 ± 0.02	12.9
	2	1.96 ± 0.06	0.009 ± 0.001		1.65 ± 0.02	

[a] For several compounds, product formation was not detectable. For these cases, the minimal detectable amount in that experiment is shown as an upper limit value.

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cantly substrate activity were analyzed further as described in the legends of Table 1 and Fig. 1. Duplicate assays were performed for each condition in each experiment and experiments were performed at least twice. Data were analyzed by weighted nonlinear regression fitting to a rectangular hyperbola using a standard statistical program for enzyme data previously described by Cleland (22). This FORTRAN program was run on a Digital Equipment Corporation PDP 11/45 computer.

RESULTS

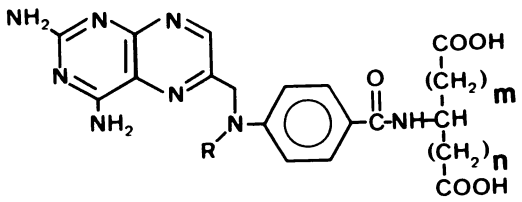
Substrate activity of MTX. Mouse liver FPGS added [^{3}H]glutamate to MTX, although moderately high concentrations of drug were required to saturate the reaction (Fig. 1A). Substrate saturation experiments showed no detectable deviation from a rectangular hyperbola and a strictly linear double reciprocal plot was observed (Fig. 2). The activity of MTX as a substrate for this reaction was equivalent to that of PteGlu, with half-maximal reaction velocities requiring essentially the same concentration for both compounds (Fig. 1 and Table 1). However, the equivalence of MTX and PteGlu as substrates for this enzyme appeared to be due to a cancellation of the opposing influences of the 4-amino and N^{10}-methyl groups that distinguish these two compounds. Thus, 4-NH_2-PteGlu saturated the FPGS reaction at substantially lower substrate concentrations than PteGlu and reached a maximum velocity that was, on the average, 52% higher than that of PteGlu (Fig. 1B and Table 1). On the other hand, the presence of a methyl group at N^{10} profoundly diminished the ability of FPGS to utilize the molecule as a substrate. 10-CH_3PteGlu was a poor substrate compared to PteGlu (Fig. 1B and Table 1).

Substrate activity of some clinically encountered 4-amino antifolates. A direct comparison of aminopterin and MTX in a series of experiments revealed that aminopterin was 10–15 times better as a substrate for mouse liver FPGS as judged by the apparent first order rate constants of these compounds (Fig. 2A and Table 1). The major factor contributing to this difference was the concentration at which each compound saturated the reaction (Table 1). Similarly, DCM was a significantly better substrate for FPGS than was MTX (Fig. 2A and Table 1). Notably, there were only minor differences among the maximal velocities measured at saturating concentrations of MTX, DCM, and aminopterin (Fig. 2A).

7-OH-MTX has been shown to accumulate to substantial levels in patients being treated with high dose MTX (19, 23). As shown by the data of Fig. 2B, 7-OH-MTX was a moderately good substrate for mouse liver FPGS. The FPGS reaction proceeded half-maximally at equivalent concentrations of 7-OH-MTX or MTX (Fig. 2B and Table 1). However, the maximal velocity of the reaction observed for 7-OH-MTX was half that of MTX.

Effect of structural modification of the amino acid side chain on the substrate activity of 4-amino antifolates. Mouse liver FPGS was found to have a stringent requirement for the L-glutamic acid side chain in the 4-amino antifolate substrate (Tables 2 and 3). The enzyme was stereospecific for the L configuration in the glutamic acid side chain, i.e., D-MTX did not react at even 500 μM. Likewise, the γ-monobenzylamide and γ-*t*-butyl ester of MTX did not have detectable substrate activity. Low substrate activity was detected with the γ-monohydroxamate derivative of MTX, but this was judged to be probably due to a small amount of hydrolysis of this derivative to free MTX at the assay pH (8.3 at 37°). Each of these γ-substituted MTX derivatives inhibited the formation of product to a low extent when PteGlu was added at equimolar concentrations (500 μM).

FPGS activity was dependent upon the presence of a free α-carboxyl group (Table 2). Thus, neither the α-*n*-butyl nor the α-*t*-butyl esters of MTX was active as a substrate. In addition, the analogue of MTX that lacked the α-carboxyl altogether (i.e., MeAPA-Gaba) was found to be without activity as a substrate for FPGS. Each of these compounds was a poor inhibitor of the FPGS reaction using PteGlu as substrate (Table 2). Deoxoaminopterin, the aminopterin analogue in which the amide carboxyl is replaced with a methylene group (14), was also inactive as a substrate for mouse liver FPGS (Table 2).



SCHEME 1

The substrate activities of a series of 4-amino antifolates differing by the length of the hydrocarbon chain between the asymmetric α-carbon and the ω-carboxyl were compared (see Scheme 1). These studies indicated a strict requirement of FPGS for the length of this chain (Table 3). Thus, whereas aminopterin was a substantially better substrate than PteGlu (Fig. 1B), the formation of product utilizing the L-aspartate analogue of aminopterin (APA-Asp) as substrate could not be detected under conditions in which PteGlu reacted extensively (Table 3). Likewise, the addition of one methylene group to the glutamate residue of MTX resulted in a compound, i.e., MeAPA-Adi, for which product formation was barely detectable (Table 3). The next higher homologue, MeAPA-Pim, was found to be a substrate for this enzyme albeit a poorer one than MTX. This surprising partial recovery of substrate activity with the addition of a second methylene group to the glutamate residue of MTX was confirmed on duplicate batches of these compounds. As shown in Table 1, the estimated first order rate constant for MeAPA-Pim was only 18% of that of the PteGlu control, and both V_{max} and the apparent K_m were less favorable to reaction. Interestingly, when one more methylene group was introduced into the amino acid side chain of MTX, i.e., in MeAPA-Sub, substrate activity once again diminished markedly. The analogue of MTX in which the amino acid was 3-aminoglutarate rather than 2-aminoglutarate (i.e., glutamate) was completely inactive as a substrate for this enzyme (Table 3).

Substrate activity of compounds altered at the 10-position. Modification at the 10-position affects substrate activity for FPGS. As shown by the data of Table 1, the analogue of aminopterin in which N^{10} was replaced by a

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TABLE 3

Activity of 4-amino antifolates with altered side chain lengths as substrates and inhibitors of FPGS

The listed compounds were incubated with FPGS under standard conditions for 1 hr at 37° and the product of the reaction was isolated by adsorption onto charcoal. For details, see text. The FPGS activity of each enzyme preparation for these compounds was compared with that of 500 μM PteGlu as control except where noted. The ability of these compounds to inhibit FPGS was tested by incubating enzyme with 500 μM PteGlu (combined activity). The structural features of these compounds denoted by *R*, *n*, and *m* are shown in Scheme 1.

Compound	*R*	*n*	*m*	Exp.	FPGS activity				
					Control	Compound	% Control	Combined activity	% Inhibition
					nmol/hr			*nmol/hr*	
APA-Asp	H	1	0	1	0.65 ± 0.03	<0.005[a]	<0.5	0.26 ± 0.001	60.1
				2	0.71 ± 0.02[b]	<0.01[a,c]	<1.0		
MeAPA-Adi	CH_3	3	0	1	0.79 ± 0.06	0.001 ± 0.014	1.4	0.59 ± 0.01	25.8
				2	1.18 ± 0.004	0.023 ± 0.004		0.87 ± 0.005	
MeAPA-Pim	CH_3	4	0	1	0.79 ± 0.06	0.16 ± 0.003	21.2	0.57 ± 0.03	27.6
				2	1.18 ± 0.004	0.26 ± 0.008		0.86 ± 0.02	
MeAPA-Sub	CH_3	5	0	1	1.18 ± 0.022	0.016 ± 0.001	1.0	0.88 ± 0.004	32.6
				2	1.41 ± 0.001	0.008 ± 0.001		0.85 ± 0.004	
MeAPA-3-NH_2-Glu	CH_3	1	1	1	0.78 ± 0.006	<0.005	<1.0	0.64 ± 0.01	23
				2	1.96 ± 0.06	<0.01	<0.5	1.43 ± 0.03	

[a] Product was not detectable.
[b] 500 μM MTX was used as a control in this experiment.
[c] 340 μM compound was used in this experiment.

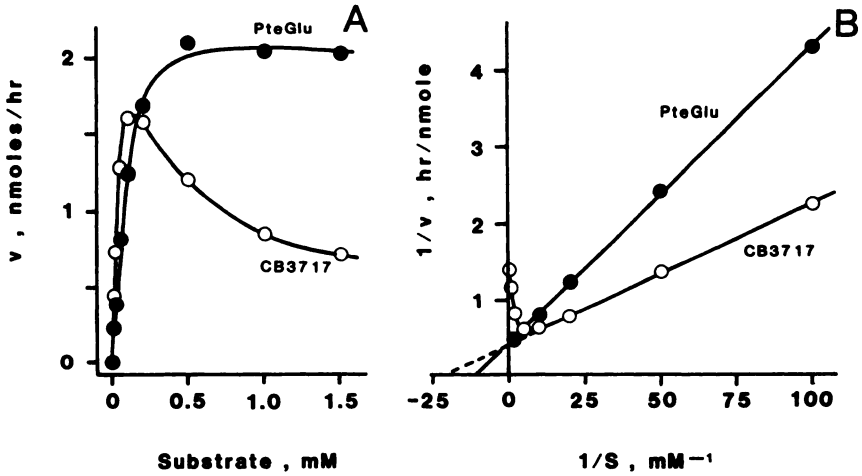

FIG. 3. *Substrate concentration-reaction velocity (A) and double reciprocal (B) plots for the activity of CB 3717 as a substrate for mouse liver FPGS*

TABLE 4

The activity of folate analogues with other ring systems as substrates and inhibitors of FPGS

For details, see Table 2. The structural features of these compounds denoted by *R* and *n* are shown in Scheme 2.

Compound			Exp.	FPGS activity				
				Control	Compound		Control + compound	
R	*n*	Side chain			nmol/hr	% Control (mean)	nmol/hr	% Inhibition (mean)
CH	0	L-Aspartic acid	1	0.65 ± 0.03	<0.01[a]	<0.8	0.15 ± 0.008	78
			2	1.33 ± 0.01	<0.0003[a]		0.28 ± 0.008	
N	1	DL-3-Aminoglutaric acid	1	0.65 ± 0.03	<0.01[a]	<0.6	0.24 ± 0.01	63
			2	1.33 ± 0.01	<0.002[a]		0.49 ± 0.002	
CH	1	DL-3-Aminoglutaric acid	1	0.65 ± 0.03	<0.01[a]	<0.8	0.18 ± 0.009	74
			2	1.33 ± 0.01	<0.002[a]		0.33 ± 0.01	

[a] Product not detectable. See Table 2.

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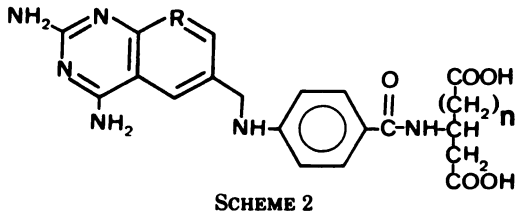
methylene group, i.e., 10-deazaaminopterin, was a poorer substrate than aminopterin, as evidenced by a substantially higher apparent K_m. In fact, this near-isosteric replacement reduced the substrate activity of the compound to that of MTX. On the other hand, the first order rate constants of 10-thiofolate and folate were equivalent. The substrate activity of homofolic acid, which has an additional methylene group between the pteridine ring and the *p*-aminobenzoyl nitrogen, was 2-fold less than that of folic acid (Table 1). It was again noted that the major difference between the activities of these compounds was their relative apparent K_m values.

Substrate activity of folate analogues with other ring systems. CB3717 is a quinazoline analogue of folic acid with an N^{10}-propargyl substituent, and is a potent inhibitor of thymidylate synthase (24). As shown in Fig. 3, CB3717 was a substrate for mouse liver FPGS but moderate substrate inhibition was observed at concentrations >100 μM. Extrapolation of the linear portion of the double reciprocal plot indicated an apparent K_m of about 40 μM (Table 1). The L-aspartic and DL-3-aminoglutaric acid derivatives of the 2,4-diaminoquinazoline equivalent of aminopterin and the DL-3-aminoglutaric acid derivative of the 2,4-diaminopyridopyrimidine equivalent of aminopterin were devoid of substrate activity for mouse liver FPGS but showed significant inhibition of the utilization of PteGlu by this enzyme (Table 4; see Scheme 2). This inhibition by the L-aspartic acid derivative of the 2,4-diaminoquinazoline was similar to the effect of the corresponding aminopterin analogue (Table 3).



SCHEME 2

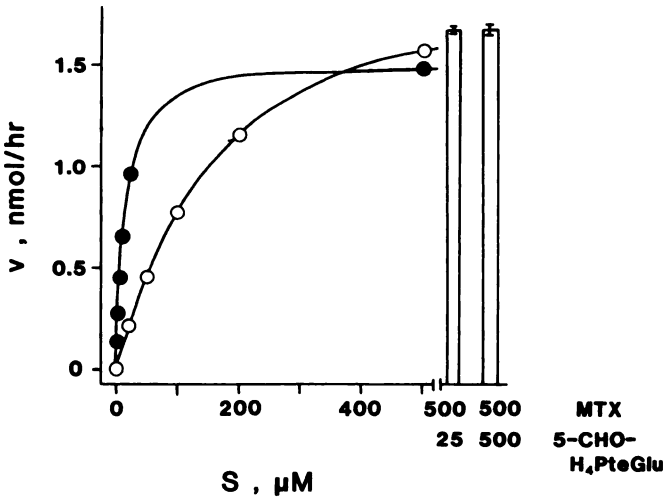


FIG. 4. *Lack of additivity of the rate of reaction of MTX (○) and 6-(R,S)-5-CHO-H_4PteGlu (●) with mouse liver FPGS*

The symbols in the body of the graph represent the mean rate of reaction of two determinations observed at various concentrations of each compound alone. The bar graph on the right represents the reaction rate (± standard deviation) observed in the presence of 500 μM MTX *and* either 25 or 500 μM 6-(*R,S*)-5-CHO-H_4PteGlu.

Reaction velocity in the presence of multiple substrates. As an approach to the question of whether the reactions we have studied were being catalyzed by one or by multiple enzymes, we measured the rate of the mouse liver FPGS reaction in the presence of high concentrations of several folate analogues taken two at a time. As shown by the data of Fig. 4, the rates of reaction with saturating concentrations of MTX and 6-(*R,S*)-5-CHO-H_4PteGlu were intermediate between the maximal velocities observed with a saturating concentration of either compound. The reaction rates observed in the simultaneous presence of 500 μM MTX and 25 or 500 μM 6-(*R,S*)-5-CHO-H_4PteGlu were 1.69 ± 0.01 and 1.68 ± 0.02 nmol/hr, respectively. The maximal velocities for MTX and 6-(*R,S*)-5-CHO-H_4PteGlu taken one at a time (extrapolated by computer from the data of this same experiment) were 2.13 ± 0.01 and 1.51 ± 0.02 nmol/hr, respectively (Fig. 4). If two independent enzyme activities in this protein preparation were acting on these two substrates, a reaction rate of 2.54 and 3.05 nmol/hr would be predicted for these combinations of concentrations of MTX and 6-(*R,S*)-5-CHO-H_4PteGlu, respectively. If a single enzyme were involved, the predicted rates would be estimated[2] to be 1.55 and 1.52 nmol/hr, respectively (see Discussion). Similarly, the rates of reaction observed in the simultaneous presence of several K_m values for MTX and PteGlu, 7-OH-MTX, or 6-(*R,S*)-5-CH_3-H_4PteGlu did not indicate any additivity of reaction rates (Table 5).

Characteristics of FPGS partially purified from other tissues. It was of interest to determine whether the structure-activity relationships found for mouse liver FPGS could be extrapolated to enzyme from other sources. We determined the relative substrate activity of PteGlu, MTX, and aminopterin using enzyme of similar purity from a second normal organ of the mouse (kidney), from a neoplastic tissue of the mouse (L1210 leukemia), and from the homologous organ from another species (rat liver). Folate-independent enzyme reactions that interfered with our FPGS assay were undetectable in 30% $(NH_4)_2SO_4$ preparations from these tissues (21). A similar pattern of relative activities of these substrates was observed using enzyme from all three mouse tissues: the apparent K_m for aminopterin was substantially less than those of MTX and PteGlu and the V_{max} for aminopterin was higher than those seen with MTX and PteGlu, which were similar. Interestingly, a distinct substrate inhibition was observed using PteGlu as a substrate with the L1210 enzyme. PteGlu also inhibited reaction at high concentrations using mouse kidney FPGS, albeit to a lesser degree than with the L1210 enzyme. This phenomenon has not been observed in about 50 experiments with the mouse liver enzyme using PteGlu as a substrate at con-

[2] The maximal velocity to be expected for two compounds if only one enzyme species were involved can be estimated by the equation:

$$v_{combined} = \frac{V_{max,1}}{1 + \frac{K_{m,1}}{S_1}\left(1 + \frac{S_2}{K_{i,2}}\right)} + \frac{V_{max,2}}{1 + \frac{K_{m,2}}{S_2}\left(1 + \frac{S_1}{K_{i,1}}\right)}$$

if one makes the assumption that each substrate acts as a strict competitive inhibitor of the reaction of the other substrate and that the K_i of each compound as an inhibitor is equal to the K_m of that compound as a substrate.

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TABLE 5

The rate of addition of [^{3}H]glutamate to two substrates simultaneously incubated with FPGS

Compound(s)	Concentration(s)	FPGS activity		
		Observed	Predicted if additive[a]	Predicted if mutually exclusive[b]
	μM		*nmol/hr*	
MTX	500	1.53 ± 0.03		
PteGlu	500	1.69 ± 0.02		
6-(*R,S*)-5-CH_3-H_4PteGlu	275	2.21 ± 0.001		
7-OH-MTX	500	0.73 ± 0.02		
MTX + PteGlu	Both 500	1.66 ± 0.04	3.22	1.82
MTX + 6-(*R,S*)-5-CH_3-H_4PteGlu	500 & 275	1.94 ± 0.06	3.74	2.12
MTX + 7-OH-MTX	Both 500	0.95 ± 0.006	2.26	1.25

[a] Calculated by adding the reaction rates observed with each compound separately.

[b] Calculated by assuming that each compound acted as a competitive inhibitor of the reaction of the other and that $K_m = K_i$ (see Discussion). The K_m values assumed for this calculation were as in Table 1 and, for 6-(*R,S*)-5-CH_3-H_4PteGlu, 87 μM (10).

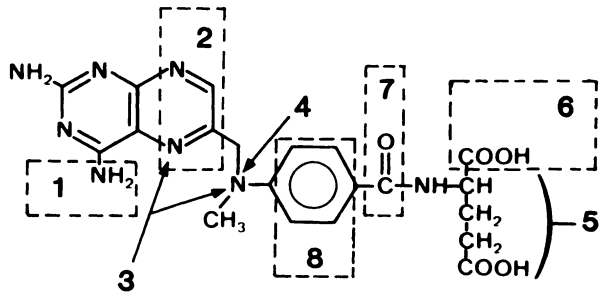
centrations up to 1.0 mM. The relative substrate activities with the rat liver enzyme were aminopterin ≅ MTX ≫ PteGlu, whereas the pattern seen with enzyme from the mouse tissues was aminopterin ≫ MTX ≅ PteGlu.

The apparent K_m values of aminopterin for FPGS from rat liver, mouse liver, mouse kidney, and L1210 cells were not significantly different [15.0 ± 2.1 (n = 2), 17.6 ± 5.8 (n = 4), 19.0 ± 1.6 (n = 2), and 18.6 ± 1.9 (n = 2) μM, respectively]. MTX was a better substrate for rat liver FPGS than for any of the mouse preparations studied with respect to both V_{max} and apparent K_m. This was reflected in the apparent first order rate constants for MTX (relative to that of PteGlu in the same experiment): rat liver, 6.5 ± 0.3 (n = 2); mouse liver, 0.90 ± 0.05 (n = 3); mouse kidney, 0.65 ± 0.07 (n = 2); and mouse L1210 leukemia, 0.53 ± 0.03 (n = 3). Using rat liver FPGS, reaction velocities passed through a maximum at ~200 μM for all three substrates studies, although the subsequent substrate inhibition was not pronounced (data not shown).

DISCUSSION

Our studies indicate that structural modification at any of several loci will alter the reactivity of a folate and/or folate analogue with mouse liver FPGS.

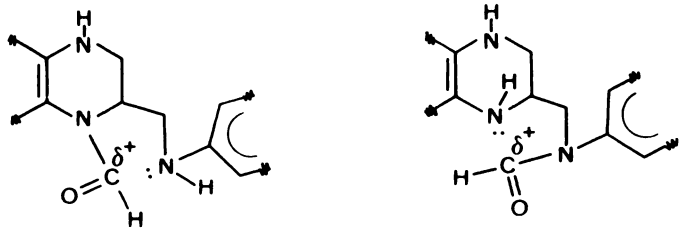
1) Substitution of an amino function for the 4-oxo group will enhance activity as a substrate primarily by decreasing K_m, cf. aminopterin and PteGlu or MTX and 10-CH_3-PteGlu (Table 1 and Fig. 1). Presumably, this reflects a tighter binding of 4-amino compounds to the enzyme (Scheme 3).



SCHEME 3. *Positions in the 4-amino folate structure important for reactivity with FPGS*

2) Reduction of the pyrazine ring to the dihydro- or tetrahydro-oxidation state has been previously shown to substantially enhance the substrate activity of 4-oxo compounds with mouse liver FPGS[3] (10), again by an effect on K_m.

3) Methylation of either N^{10} or N^5 diminished substrate activity by increasing K_m. Thus, 10-CH_3-PteGlu was a poor substrate relative to the activity of PteGlu (Fig. 1); MTX was a proportionally worse substrate than aminopterin for mouse liver enzyme (Fig. 2 and Table 1). 5-CH_3-H_4PteGlu has been shown to be a poor substrate relative to H_4PteGlu (10).[3] However, 5,10-CH_2-H_4PteGlu, 10-CHO-H_4PteGlu and 5-CHO-H_4PteGlu are all excellent substrates for this enzyme and are at least as active as H_4PteGlu (10).[3] This suggests to us that folates bind to FPGS in a conformation in which the N-5, C-6, C-9, N-10 system approximates the shape of 5,10-CH_2-H_4PteGlu. Thus, 5-CHO- or 10-CHO-H_4PteGlu might adopt such a conformation as a result of a nonbonded dipole interaction between the formyl group and the opposite nitrogen. N^5- or N^{10}-methyl substituents would not favor such a conformation (Scheme 4).



SCHEME 4

4) Replacement of N^{10} of the 4-aminopteroyl structure with a methylene group appeared to diminish binding to FPGS, as judged by the difference in apparent K_m between aminopterin and 10-deazaaminopterin. However, 10-thiofolate was found to be approximately as active as a substrate as PteGlu (Table 1).

[3] The relative first order rate constants for PteGlu, H_2PteGlu, H_4PteGlu, 5,10-CH_2-H_4PteGlu, 10-CHO-H_4PteGlu, 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu were estimated to be 1.0, 28, 30, 26, 44, 16, and 2.0, respectively. As reported elsewhere (10), these differences were mainly due to variation in apparent K_m among these compounds.

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5) Any major change in the length of the amino acid side chain examined to date resulted in a major decrease of substrate activity.

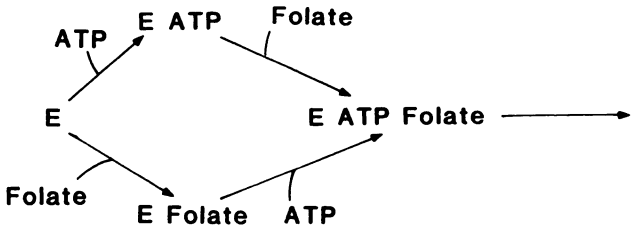
6) The presence of the α-carboxyl is an important structural requirement for the FPGS reaction. These data suggest to us that the α-carboxyl contributes to binding of the glutamic acid moiety of the substrate in a catalytically active orientation within the active site. This is consistent with the strict requirement for the length of the amino acid side chain (Table 3). It is interesting to note that FPGS shares with dihydrofolate reductase and thymidylate synthase the requirement of an α-carboxyl for substrate or inhibitor binding (25, 26).

7) Replacement of the amide carboxyl of the side chain with a methylene group eliminated substrate activity.

8) Finally, the activity of DCM (Table 1) suggests that mouse liver FPGS can tolerate bulky substituents in the phenyl ring.

It is not known, at present, whether modification of a substrate at several of these positions would allow additive increments in binding affinity for FPGS. If this were true, then a tetrahydro derivative of aminopterin, for instance, would be expected to be highly retained within cells as would 3′,5′-dichloroaminopterin. In addition, it seems reasonable that inhibitors of FPGS are likely to be more potent if they incorporate the structural factors outlined above to maximize the strength of initial binding.

We surmise that the substrate inhibition observed with CB3717 indicates that mouse liver FPGS either has an ordered sequential kinetic mechanism (27), as previously documented for FPGS from two bacterial sources (8, 9) or can bind at least some folates in two configurations. This could arise either 1) through a "preferred pathway" kinetic mechanism (28), e.g., whereby saturation of a kinetically slower path at higher folate concentration slows the overall reaction (Scheme 5) or 2) by simultaneous binding of a second mole of folate at a second site. If this latter possibility were the case, CB 3717 might be a poor analogue of other naturally occurring folates that bind much more tightly to this second site. McGuire *et al.* (29) have suggested that folylpentaglutamates fulfill a regulatory function that controls FPGS activity *in vivo*.



SCHEME 5

It has been previously stated by others that aminopterin was nearly as good a substrate as MTX for the rat liver FPGS while PteGlu and 10-CH_3-H_4PteGlu were poor substrates for this enzyme (29). In light of the data of Fig. 1, this might imply that the relative substrate activity of different folates is a function of the enzyme source or that results obtained using the charcoal adsorption technique differ from those obtained using other (29) assays. The data of Fig. 5 clearly confirm the results reported by McGuire *et al.* (29) with our assay and also indicate that FPGS from different sources obey different structure-activity relationships. It remains to be seen how different various enzymes are. However, the data of Fig. 5 suggest that it is not that aminopterin is a poorer substrate for rat liver FPGS than for mouse liver enzyme, but rather that MTX is a better substrate for FPGS from rat liver than from mouse liver. In addition, whereas the rat and mouse liver enzymes were distinctly different with respect to the behavior of MTX as a substrate, the K_m for aminopterin and the relative V_{max}/K_m values for PteGlu and aminopterin were similar with both enzymes. Finally, it should be noted that the kinetic behavior of enzyme from all the murine tissues was very similar and

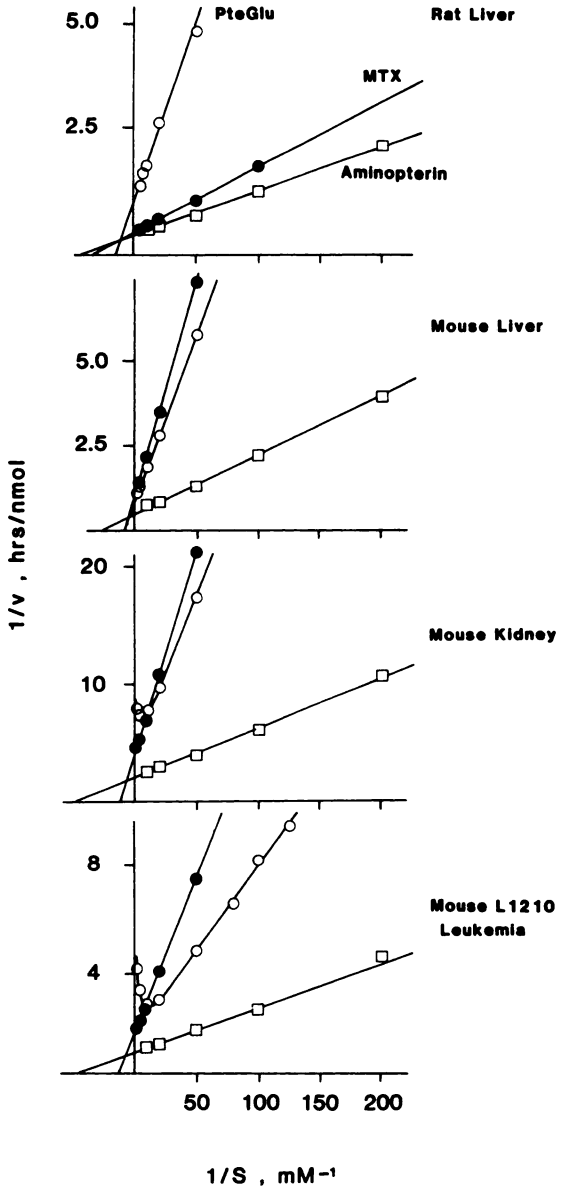


FIG. 5. *Relative substrate activity of PteGlu (○), MTX (●), and aminopterin (□) for FPGS preparations from mouse and rat tissues*

For details, see text. Each symbol represents the mean of duplicate assays from a representative experiment. The data of each panel were obtained from the same experiment.

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differed only in the degree of substrate inhibition seen with PteGlu.

The comparisons that we have made between the activity of various substrates for mouse liver FPGS have been possible only because we have chosen to obtain full saturation curves for each compound and to analyze quantitatively the resultant data. The apparent Michaelis constants that we have determined must be interpreted in the light of the limitations imposed by our conditions: 1) the enzyme used in these studies was minimally purified (~12-fold, specific activity ~1.2 nmol/hr/mg of protein) and 2) FPGS has been shown to use diglutamates produced in such assays as substrates for the synthesis of longer chain polyglutamates (10, 29). Under saturation conditions or under conditions of low conversion of substrate to product, however, this latter effect does not interfere with proper determination of the rate of formation of a folate diglutamate product (10, 29). In addition, we have limited our incubation time to 1 hr since we (10) and others (29) have shown low to negligible formation of higher folyloligoglutamates under these conditions even at relatively low substrate concentrations. It should be noted that the information available to date on the substrate specificity of mammalian FPGS from other sources (Ref. 1 and references quoted therein) is based on incubation with a single or a few substrate concentrations and enzyme of a similar degree of purity to that used in our experiments.

A question of considerable importance to the interpretation of our results is whether the reactions observed herein have been catalyzed by one or multiple enzymes. It would be predicted that simultaneous saturation of two enzymes in our relatively crude preparation with two pteridines would result in the addition of [^{3}H]glutamate at a rate equal to the sum of the maximal velocities observed with each substrate alone. This clearly was not the case (Fig. 4 and Table 5). If both reactions were catalyzed by a single enzyme, then the reactions should be mutually exclusive and the maximal velocity observed in the presence of both substrates should be intermediate between the V_{max} values observed with both substrates.[2] We observed this to be the case for MTX and either PteGlu, 6-(*R,S*)-5-CHO-H_4PteGlu, 6-(*R,S*)-5-CH_3-H_4PteGlu, or 7-OH-MTX. The combined reaction velocities of MTX and 6-(*R,S*)-5-CHO-H_4PteGlu agree with this prediction within 10% (Fig. 4). Two *independent* enzymes can be excluded by the data of Fig. 4 and Table 5. This direct competition between tetrahydrofolate compounds and MTX at the level of FPGS provides an explanation for the inhibition of the formation of polyglutamates of MTX by 6-(*R,S*)-5-CHO-H_4PteGlu (30, 31) and 6-(*R,S*)-5-CH_3-H_4PteGlu (31). However, it remains to be established whether this effect plays a role in the competitive (32) phenomenon of "leucovorin rescue."

The formation of polyglutamates of 7-OH-MTX in these experiments raises the question of whether such metabolites are formed *in vivo* and whether they interfere with or participate in the therapeutic effects of MTX. This is particularly relevant because 1) the formation of 7-OH-MTX varies considerably among patients, 2) urinary 7-OH-MTX can account for up to 11% of the administered dose of MTX (23), and 3) this metabolite is cleared by the body significantly more slowly than is MTX so that serum 7-OH-MTX is often >10-fold higher than serum MTX at the time of leucovorin rescue. In addition, 7-OH-MTX polyglutamates could be formed in kidney and liver and could be involved in the renal and hepatic toxicity of MTX. Finally, it is conceivable that some of the MTX polyglutamate fractions that have been observed *in vivo* might be contaminated by 7-OH-MTX derivatives.

The role of FPGS in the selective retention of MTX in tumor cells *vis à vis* normal tissues is currently being examined, as is the role of MTX polyglutamates as a factor in the renal and hepatic toxicity of long term or high dose MTX therapy. The high levels of FPGS in liver and kidney (21) suggest that these toxicities may be averted by the use of dihydrofolate reductase inhibitors that are not substrates for FPGS, such as MeAPA-Adi or MeAPA-Sub (Table 3). On the other hand, the activity of aminopterin for FPGS was sufficiently greater than that of MTX for FPGS from murine tissues (Figs. 2, 3, and 5; Table 1) to lead to the expectation that aminopterin would be much more extensively polyglutamated than MTX in mice. Preliminary reports document that this is the case in mouse L1210 cells (33) and in other mouse tumors (34). The similar level of polyglutamation of aminopterin and MTX with rat liver FPGS and the remarkably more facile polyglutamation of aminopterin than MTX with mouse liver FPGS may explain the similar lethal potencies of MTX and aminopterin in rats (LD_{50} = 4.5 and 6–25 mg/kg, respectively) and their dissimilar toxicities in mice (LD_{50} = 90–110 and 2–5 mg/kg, respectively) (35–37). Extensive formation of aminopterin polyglutamates might explain the delayed kinetics of cellular loss of aminopterin previously observed in mouse tumors and intestine (reviewed in Ref. 7) and the lower therapeutic ratio of aminopterin than MTX for treatment of an experimental tumor (37). However, because a treatment schedule appropriate for MTX might be suboptimal for an agent as highly polyglutamated as aminopterin, the desired schedule for antifolates that are excellent FPGS substrates might be radically different, e.g., high dose treatments spaced several weeks apart. Caution seems advised in attempts to develop more extensively polyglutamated antifolates without a more thorough understanding of the role of FPGS as a determinant of the *selective* toxicity of antifolates.

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