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THE PHARMACOLOGICAL AND BIOCHEMICAL ACTIVITY OF 4-AMINO-4-DEOXY-10-METHYL-PTEROYLASPARTIC ACID

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Abstract—4-Amino-4-deoxy-10-methylpteroylaspartic acid (AspMTX), the aspartate analog of methotrexate (MTX), was synthesized, and its pharmacological and biochemical effects were studied. AspMTX, even at comparatively large doses, was less effective against mouse leukemia L1210 than MTX. Pretreatment with MTX, but not with AspMTX, abolished the protection afforded by folate against a lethal dose of aminopterin in mice. Dihydrofolic reductase activity in the liver of normal mice, as well as incorporation of formate-^{14}C into acid-soluble adenine in the spleen of leukemic mice, was inhibited much less after administration of AspMTX than of MTX. AspMTX was a competitive inhibitor ($K_i/K_m = 1{\cdot}8 \times 10^{-3}$ at pH 7·4) of dihydrofolic reductase prepared from an antifolic-resistant subline of leukemia L1210. The findings are explained by a decreased affinity of the inhibitor for the enzyme after substitution of aspartate for glutamate.

JACOBSON and Cathie[1, 2] have put forward the hypothesis that 4-amino-4-deoxypteroylglutamic acid (aminopterin) could be inactivated *in vivo* by cyclization of the terminal glutamic acid moiety and noted that the aspartate analog, 4-amino-4-deoxypteroylaspartic acid (amino-an-fol), was not subject to such ring closure. Comparison of the effectiveness of the two compounds against advanced mouse leukemia L1210 showed[3] that both produced similar increases in median survival time but that the optimal daily dose was much higher in the case of amino-an-fol, suggesting that substitution of aspartate for glutamate markedly reduced the toxicity for both tumor and host.

Since methotrexate (MTX), which is the *N*-10-methyl derivative of aminopterin, produces a greater increase, at optimal doses, in the survival time of leukemic mice than the parent compound,[3,4] the synthesis and investigation of 4-amino-4-deoxy-10-methylpteroylaspartic acid (AspMTX) was undertaken. The present report deals with a comparison of MTX and AspMTX with respect to some of their pharmacological and biochemical effects.

MATERIALS AND METHODS

Hybrid male CDBA mice [(BALB/cAn × DBA/2J)F_1], 11 to 14 weeks old and weighing 24 to 28 g, were maintained on Purina laboratory chow pellets and water *ad libitum*. In studies of the antileukemic effectiveness of the drugs, the mice were inoculated subcutaneously, in the right inguinal region, with the lymphoid leukemia L1210 or its antifolic-resistant subline, L1210/FR-8,[5] as previously described.[4]

Aminopterin and methotrexate were products of the Lederle Laboratories Division, American Cyanamid Co. Folic acid, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and ethylenediaminetetraacetic acid (EDTA) were obtained from Calbiochem; 2-mercaptoethanol from Eastman Organic Chemicals; and sodium formate-^{14}C from the New England Nuclear Corp. Dihydrofolic acid was prepared by the method of Futterman[6] and stored frozen as a suspension in 0·001 N hydrochloric acid containing 0·01 M mercaptoethanol.[5, 7]

4-Amino-4-deoxy-10-methylpteroylaspartic acid was synthesized by a procedure analogous to the preparation of MTX.[8] *p*-Methylaminobenzoylaspartic acid was obtained from *p*-iodobenzoylaspartic acid by the method reported for *p*-methylaminobenzoylglutamic acid.[9] A mixture of 13·4 g (89% pure, 0·05 mole) of 2,4,5,6-tetraaminopyrimidine sulfate and 12·2 g (0·05 mole) of barium chloride dihydrate in 250 ml of water was stirred at 60° for 10 min, then cooled to 45°. Disodium *N*-methyl-*p*-aminobenzoylaspartate (7·75 g, 0·025 mole) was added, and the pH adjusted to 4. At 40°–45° were added 10·8 g (0·05 mole) of 2,3-dibromopropionaldehyde in 10 ml of acetic acid, a solution of 6·4 g of iodine and 12·5 g of potassium iodide in 50 ml of water, and sodium hydroxide solution as necessary to maintain pH 4. The resulting mixture was cooled well and filtered. The crude product was stirred at 60° for half an hour in 1,600 ml water containing 8 g lime. The insoluble material was filtered and washed with 800 ml hot water. The filtrate was adjusted to pH 10·8 with aqueous zinc chloride, clarified, acidified to pH 4, and filtered. This cake was slurried at 60° with 1,350 ml water and enough sodium hydroxide to give pH 11–12. The pH was then adjusted to 7 while cooling to 20°. After clarification the filtrate was acidified to pH 4, chilled, and filtered. The precipitate was slurried with 800 ml water and just enough lime to give pH 9·0–9·4 at 60°. The mixture was treated with Norite, clarified, and the filtrate acidified to pH 4. The product (2 g) was estimated to be 87% pure by its u.v. spectrum. This is the material that was used in the present investigation. A 50-mg sample of the product was place on a Celite column and developed with a butanol:methanol:0·5% aqueous ammonium carbonate mixture (15:3:10). The major fraction was evaporated to dryness under reduced pressure, and the product was reprecipitated from aqueous alkaline solution (10 ml) by acidification to pH 4. The yield was 33 mg. Microanalysis was correct for a monohydrate. This agrees well with the estimated purity (by u.v. spectrum) of 95% as anhydrous product.

Anal. Calcd. for $C_{19}H_{20}N_8O_5 \cdot H_2O$: C, 49·8; H, 4·82; N, 24·4. Found: C, 50·0; H, 5·02; N, 24·1.

Ultraviolet spectra: in 0·1 N sodium hydroxide λ_{max} 223 mμ (log ε 4·34), 258 mμ (log ε 4·39), 304 mμ (log ε 4·39), 370 mμ (log ε 3·91), λ_{min} 240 mμ, 271 mμ, 340 mμ; in 0·1 N hydrochloric acid λ_{max} 242 mμ (log ε 4·26), 307 mμ (log ε 4·36), 333 mμ (inflection) (log ε 4·14), 344 mμ (inflection) (log ε 4·02), λ_{min} 233 mμ, 261 mμ.

For the studies *in vivo*, MTX, AspMTX, folic acid, and aminopterin were dissolved in 2% sodium bicarbonate and injected s.c. into the axillary region (0·01 ml/g body weight). Sodium formate-^{14}C (1 μc/μmole) was dissolved in saline and administered i.p. (0·2μc/g body weight). The animals were killed by cervical dislocation. Incorporation of formate-^{14}C into acid-soluble adenine was determined by the method previously described.[10]

Acetone-dried powders were prepared at −60° to −20° from the local tumors of the L1210/FR-8 subline[5] and stored at −20°. Extracts were obtained from the powders

with 20 volumes of the appropriate buffer at 3° and centrifuged at 35,000 *g*. In other experiments, livers of freshly killed mice were homogenized with 0·25 M sucrose containing 0·01 M mercaptoethanol and 0·001 M EDTA (preadjusted to pH 7·4), and the supernatant fractions obtained by centrifugation at 100,000 *g*. Dihydrofolic reductase activity was determined according to published procedure.[7, 11, 12, 13] The reaction was carried out in cuvets containing the enzyme (extract of acetone-dried powder or supernatant fraction), 0·24 μmole NADPH, and 0·08 μmole dihydrofolate in 0·05 M potassium phosphate buffer (pH 7·4) with 0·01 M mercaptoethanol and 0·001 M EDTA (total volume, 3 ml). Absorbance readings at 340 mμ were made at 1-min intervals at 28° against a reference cuvet from which dihydrofolate was omitted. Protein was determined by the method of Lowry *et al.*[14] Specific enzyme activity (mμmoles dihydrofolate reduced per hr/mg protein) was based on the combined decrease in absorbance[13] for NADPH ($\Delta\epsilon = 6{,}200$) and dihydrofolate ($\Delta\epsilon = 5{,}800$) at 340 mμ. In the *in vitro* inhibition studies, the enzyme, antimetabolite, and buffer were preincubated at 36° for 5 min before the addition of NADPH and dihydrofolate.

RESULTS

The possibility, suggested by Jacobson and Cathie,[2] that resistance of leukemia to therapy with aminopterin and MTX could be related to inactivation of the antimetabolites made it desirable to study the effects of AspMTX against both leukemia L1210 and an antifolic-resistant subline. As shown in Table 1, AspMTX was less

TABLE 1. EFFECTS OF METHOTREXATE AND ITS ASPARTATE ANALOG AGAINST MOUSE LEUKEMIA L1210 AND AN ANTIFOLIC-RESISTANT SUBLINE

Tumor	Drug	Dose (mg/kg/day)	Median survival time (days)
L1210	None		11·0
	MTX	0·28	21·5
	MTX	0·47	26·5
	MTX	0·78	>50*
	MTX	1·30	41·0
	MTX	2·16	26·0
	AspMTX	0·5	10·5
	AspMTX	5·0	11·5
	AspMTX	50·0	16·0
L1210/FR-8	None		12·0
	MTX	0·78	13·0
	MTX	1·30	12·0
	MTX	2·16	12·0
	AspMTX	0·5	12·0
	AspMTX	5·0	12·5
	AspMTX	50·0	12·5

Each experimental group consisted of 6 animals, except that 10 mice were used in the untreated control groups. Treatment was initiated on day 3 after s.c. implantation of the tumor and continued daily until the death of the animals. Methotrexate (MTX) and 4-amino-4-deoxy-10-methylpteroylaspartic acid (AspMTX) were administered s.c. in 2% $NaHCO_3$. The antifolic-resistant subline L1210/FR-8[5] exhibited about 50-fold increased dihydrofolic reductase activity as compared to the parent leukemia. Another MTX-resistant subline (L1210/M46R), in which dihydrofolic reductase was increased 6-fold, was tested in the same experiment and also failed to respond to treatment with AspMTX.

* The experiment was discontinued on day 50, when 3 of the 6 animals were still alive.

effective, even at the maximal dose used, than MTX in prolonging the life span of mice bearing the sensitive leukemia L1210. The relatively small quantity of AspMTX available for our studies prevented testing higher doses which might have yielded increased survival times. In previous studies,[3] the optimal daily dose of amino-an-fol (28 mg/kg) was 170 times the optimal dose of aminopterin (0·17 mg/kg). If the same dose ratio were to hold for the *N*-10-methyl derivatives, the dose of 50 mg AspMTX/kg (median survival times 16 days) would be equivalent to the dose of 0·28 mg MTX/kg (median survival time 21·5 days). The L1210/FR-8 subline[5] was fully resistant to both MTX and its aspartate analog. It was to be expected that an analog of MTX which was less effective against the parent tumor would be ineffective against this subline, since the latter apparently differs from the parent strain only by its greatly increased dihydrofolic reductase level.[5]

The effect of AspMTX on the normal host was tested indirectly. Goldin *et al.*[15] have shown that, in nonleukemic mice, the protective action of folate administered 1 hr before a lethal dose of aminopterin was abolished by pretreatment (24 hr earlier) with sublethal levels of aminopterin. This finding is most readily explained by the assumption that the protective action of folate is caused by accumulation in the tissues of tetrahydrofolate or its derivatives, and that the reduction of folate is blocked when the previously administered aminopterin is bound to the dihydrofolic reductase* present in the cells.[16, 17] In the experiment summarized in Table 2, administration of 25 mg folate/kg protected mice from the lethal effect of 50 mg aminopterin/kg injected 1 hr later. Treatment with nonlethal doses of MTX 24 hr before the administration of folate nullified its protective effect, but treatment with AspMTX did not. In fact, even 10 mg of the aspartate analog/kg failed to reproduce the action of 0·1 mg MTX/kg. This finding suggests that, 24 hr after administration of AspMTX, sufficient free dihydrofolic reductase was available for the reduction of adequate amounts of folate or that folate was capable of reversing the inhibitory effect of AspMTX.

Werkheiser[17] has demonstrated that, 24 hr after injection of mice with 1 mg MTX/kg, essentially all the dihydrofolic reductase in the liver was bound by the drug. Table 3 shows that, with the same treatment, reduction of dihydrofolate at pH 7·4 by the supernatant fraction of mouse liver was inhibited 84%.† Inhibition was not increased significantly when the dose of MTX was raised to 10 mg/kg and when repeated injections were given, in confirmation of previous findings[17] that after 24 hr no free drug was present in the animals. When AspMTX was substituted for MTX, reduction of dihydrofolate was inhibited to a much lesser extent, again suggesting that most of the enzyme remained free or that dihydrofolate reversed the inhibition produced by the drug.

The inhibition of dihydrofolic reductase by the folic acid antagonists is reflected in an inhibition of metabolic pathways involving one-carbon metabolism, including biosynthesis *de novo* of purine nucleotides.[18] Previous studies [10, 20, 21] have provided evidence for a correlation between the relative effectiveness against leukemia L1210 of

* The enzyme that reduces both folate and dihydrofolate has been called "dihydrofolic reductase" in this paper for reasons discussed by Huennekens.[18]

† The residual dihydrofolic reductase activity of 16% is consistent with the activity measured *in vitro* when equivalent amounts of enzyme and MTX were preincubated.[7] Werkheiser's findings of complete inhibition [17] can be explained by the different assay conditions (folate instead of dihydrofolate as a substrate and pH 6 instead of pH 7·4). [7, 19]

TABLE 2. EFFECT OF PRETREATMENT WITH METHOTREXATE OR ITS ASPARTATE ANALOG ON THE PROTECTION PROVIDED BY FOLATE AGAINST AMINOPTERIN TOXICITY IN NONLEUKEMIC MICE

Preatreatment*		Folate after 24 hr (mg/kg)	Aminopterin after 25 hr (mg/kg)	Survivors†
Drug	Dose (mg/kg)			
			50	1/6
		25	50	6/6
MTX	0·1	25	50	2/6
MTX	1·0	25	50	1/6
MTX	10·0	25	50	0/6
MTX	10·0			6/6
AspMTX	0·1	25	50	5/6
AspMTX	1·0	25	50	6/6
AspMTX	10·0	25	50	6/6
AspMTX	10·0			6/6

* Male CDBA mice (6 per group) were injected s.c. with MTX or AspMTX in 2% $NaHCO_3$. Folic acid (25 mg/kg) was administered similarly 24 hr later, and aminopterin (50 mg/kg) after another hour.

† Observations of mortalities were carried out for 20 days. None of the mice died before day 4 or after day 7 following administration of aminopterin, and most of the deaths occurred on day 5.

TABLE 3. INHIBITION OF MOUSE LIVER DIHYDROFOLIC REDUCTASE AFTER ADMINISTRATION *IN VIVO* OF METHOTREXATE AND ITS ASPARTATE ANALOG

Drug	Dose (mg/kg)	Number of treatments	Specific activity (mμmoles/hr/mg protein)
2% $NaHCO_3$		1	94
2% $NaHCO_3$		4	88
MTX	1	1	15
MTX	10	1	16
MTX	10	4	13
AspMTX	1	1	90
AspMTX	10	1	93
AspMTX	10	4	75

Male CDBA mice (3 per group) were injected s.c. with MTX or AspMTX in 2% $NaHCO_3$, Controls received an equivalent volume of 2% $NaHCO_3$. Treatments were given at 24-hr intervals, and the animals were killed 24 hr after the last injection. The pooled livers were homogenized at 3° with 4 ml of 0·25 M sucrose containing 0·01 M mercaptoethanol and 0·001 M EDTA (pH 7·4) per g (wet weight) in a Potter-Elvehjem homogenizer, and the homogenates were centrifuged at 40,000 rpm in the Spinco preparative ultracentrifuge for 45 min. Dihydrofolic reductase activity of the supernatant fractions, after removal of the lipid layer, was determined at pH 7·4 (see Materials and Methods). The control livers had a reductase activity of 6–7 μmoles per hr/g wet weight.

folic acid antagonists administered under various conditions and the inhibition of purine biosynthesis *in vivo*, as measured by incorporation of formate-^{14}C into acid-soluble adenine of mouse spleen infiltrated with leukemia. Figure 1 shows that, in this system, 40 to 50 times the amount of AspMTX was required to produce the inhibition obtained with a given dose of MTX when the drugs were administered s.c. 20 min before the injection of formate-^{14}C. Moreover, at the higher dose levels, MTX caused more complete inhibition than the aspartate analog.

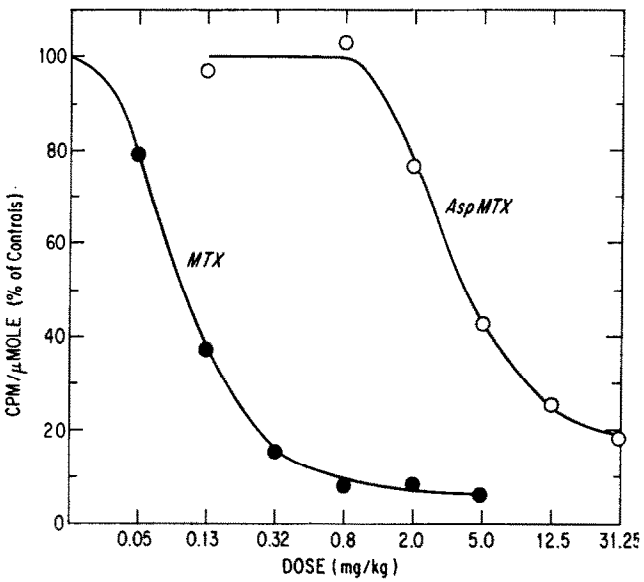

FIG. 1. Effect of methotrexate (MTX,●) and of 4-amino-4-deoxy-10-methylpteroylaspartic acid (AspMTX, ○) on the incorporation of formate-^{14}C into acid-soluble adenine of mouse spleen infiltrated with leukemia L1210. The experiment was performed on day 8 after leukemic inoculation. The mice (3 per group) received a subcutaneous injection of MTX or AspMTX in 2% $NaHCO_3$, then after 20 min an intraperitoneal injection of sodium formate-^{14}C (200 μc/kg), and were killed another 20 min later. Acid-soluble adenine (from the soluble portion of a cold trichloroacetic acid homogenate) was isolated from the pooled spleens of each group, and its specific radioactivity (counts per min/μmole) determined as described previously.[10] Results are expressed as per cent of the specific radioactivity measured in the spleens of control mice, which received 2% $NaHCO_3$ instead of the drugs.

The kinetics of dihydrofolic reductase inhibition *in vitro* were studied with an enzyme preparation (specific activity = 4·2 μmoles per hr/mg protein) from the anti-folic-resistant FR-8 subline[5] of leukemia L1210. Figure 2 shows that, at pH 7·4, AspMTX was much less inhibitory than MTX in equimolar concentrations. In addition, under the conditions of the experiment, the aspartate analog was a competitive rather than a "stiochiometric" inhibitor. As reported previously,[7] titration of dihydrofolic reductase with MTX gave a straight line, characteristic of stiochiometric inhibition,[22] when there was an excess of enzyme over MTX such that essentially all of the latter was bound,[23] although with increased amounts of MTX reversible

inhibition was observed.[7, 19] This behavior is consistent[19] with the inhibition kinetics in "mutal depletion systems" discussed by Webb.[24] Moreover, the initial reaction rate following preincubation of the enzyme with MTX was the same with the two dihydrofolate concentrations used (Fig. 2), both of which gave the maximal rate in the absence of inhibitor. In contrast, inhibition of the enzyme by AspMTX was less pronounced with increased substrate concentrations. When the ratio V/V_i was plotted against

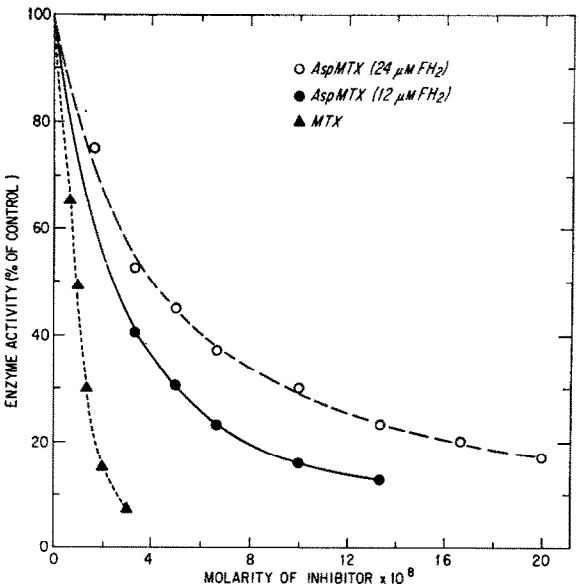

FIG. 2. Inhibition of L1210/FR-8 dihydrofolic reductase by variable amounts of MTX and its aspartate analog (AspMTX) at a constant enzyme level. Acetone powder of antifolic-resistant tumor[5] was extracted with 0·05 M potassium phosphate buffer, pH 7·4, containing 0·01 M mercaptoethanol and 0·001 M EDTA. The extract (0·20 mg protein, specific activity = 4·2 μmoles per hr/mg), inhibitor, and buffer mixture were preincubated 5 min at 36° before addition of NADPH and dihydrofolate (FH_2). Enzyme activity (initial rate at 28°) is expressed as per cent of a control in which extract was preincubated in absence of inhibitor. ○, AspMTX and 24 μM FH_2; ●, AspMTX and 12μM FH_2; ▲, MTX and 24 or 12 μM FH_2.

the AspMTX concentration I (Fig. 3), straight lines resulted with unit intercept and with the slope dependent on the dihydrofolate concentration, showing[25] that the aspartate analog behaved as a competitive inhibitor of dihydrofolic reductase.* The Michaelis constant for dihydrofolate at pH 7·5 was found to be $K_m = 0{\cdot}5 \times 10^{-6}$ in the case of chicken liver dihydrofolic reductase[12] and $1{\cdot}3 \times 10^{-6}$ with the enzyme from Ehrlich ascites carcinoma.[19] Assuming a value of 10^{-6} for the L1210/FR-8 tumor enzyme, the K_i for AspMTX, as calculated from the slopes of the plots in Fig. 3 or from the equation, $IV_i/(V-V_i) = K_iS/K_m$,[26] was approximately 2×10^{-9}.

* The plot corresponds to the equation[25] for a competitive inhibitor: $V/V_i = 1 + K_mI/K_i(K_m + S)$ where V and V_i are the reaction rates in the absence and presence of inhibitor, K_m the Michaelis constant for the substrate, K_i the dissociation constant of the enzyme-inhibitor (EI) complex, and I and S the concentrations of inhibitor and substrate. With a noncompetitive inhibitor, where $V/V_i = 1 + I/K_i$, the slope is independent of S.[25] A "stoichiometric" inhibitor does not yield a straight line.[24]

DISCUSSION

AspMTX, in spite of its very low K_i (about 2×10^{-9}), was found to be a competitive inhibitor of dihydrofolic reductase under conditions where MTX produced inhibition characteristic of kinetics in a "mutual depletion" system.[24] It would, therefore, appear that the K_i of MTX is even lower, in support of Werkheiser's[22] assumption.* On the other hand, the K_i of several 2,4-diaminopteridines, which also inhibited dihydrofolic reductase competitively, but which lacked the *p*-aminobenzoylamino acid portion,

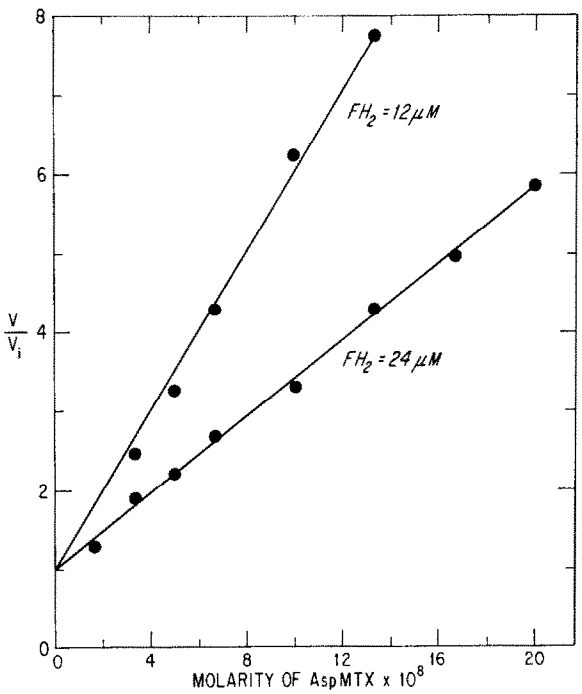

FIG. 3. Plot of V/V_i against I for the inhibition of L1210/FR-8 dihydrofolic reductase by AspMTX at two different molarities of dihydrofolate (FH_2). Experimental conditions as in Fig. 2. V and V_i are the initial reaction rates in the absence and presence of inhibitor; I is the molarity of AspMTX. A similar plot for methotrexate curved upward with increasing I.

was higher by several magnitudes.[27, 28] Thus, the presence of the *p*-aminobenzoylglutamate residue is required for the very tight binding to dihydrofolic reductase characteristic of MTX and aminopterin. Even substitution of aspartate for glutamate renders the *EI* complex more dissociable. The spatial dimensions of the molecule, in addition to the required functional groups, therefore influence the affinity of the inhibitor for the enzyme.

* Werkheiser has calculated a maximal value of $K_i = 3 \times 10^{-11}$ for MTX, whereas Osborn *et al.*[12] have reported $K_i = 2{\cdot}3 \times 10^{-9}$. The latter value, however, was calculated from the Lineweaver-Burk plot of $1/V$ against $1/S$, which appeared to represent noncompetitive inhibition. Werkheiser[22] and Webb[24] have pointed out that the Lineweaver-Burk plot cannot distinguish between a classical noncompetitive and a "stoichiometric" inhibitor, and that calculation of K_i by the equation valid for noncompetitive inhibition would give an incorrect value in a mutual depletion system. Similar considerations may apply to the maximal value of $K_i = 6{\cdot}7 \times 10^{-10}$, reported by Bertino *et al.*[19]

The finding that dihydrofolic reductase is inhibited competitively by AspMTX may be relevant to an understanding of the interaction of the enzyme with MTX. It is difficult to distinguish between competitive and noncompetitive inhibition in a mutual depletion system on the basis of kinetic data.[17, 22, 24] However, because MTX differs from AspMTX only by the terminal amino acid and because the latter is identical in MTX and dihydrofolate, there exist no functional groups in MTX other than those present in either AspMTX or dihydrofolate capable of reacting with the enzyme. Since a noncompetitive inhibitor reacts with a different site on the enzyme than the substrate, Werkheiser's suggestion,[22] supported by recent data of Wang and Werkheiser,[29] that the inhibition of dihydrofolic reductase by MTX is competitive appears reasonable.

The much lower toxicity and antileukemic effectiveness of AspMTX, when compared with those of MTX, are consistent with the weaker inhibitory action on dihydrofolic reductase *in vitro*. However, additional factors may be involved. Thus, it is possible that AspMTX enters cells less readily than MTX. Moreover, MTX persists in tissues for prolonged periods because of its tight binding to dihydrofolic reductase.[16, 17] The aspartate analog, is less likely to remain localized inside cells once the drug has disappeared from the circulation because the enzyme–AspMTX complex is dissociated more readily. Comparison of the data would, indeed, seem to suggest that the difference between equally effective or inhibitory doses of MTX and AspMTX is much larger *in vivo* than *in vitro*.

The present results neither confirm nor invalidate Jacobson and Cathie's suggestion[1, 2] that MTX and aminopterin may suffer inactivation *in vivo* by cyclization of the terminal glutamate residue. They indicate, however, that the presence of this amino acid is required for the very tight binding to dihydrofolic reductase characteristic of the 4-amino analogs of folic acid. This finding may be important for the design of antifolic drugs with improved effectiveness against leukemia and other malignancies.

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