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ENZYMATIC SYNTHESIS OF POLYGLUTAMATE DERIVATIVES OF 7-HYDROXYMETHOTREXATE

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Abstract—7-Hydroxymethotrexate, an important metabolite of methotrexate, is a substrate for folylpolyglutamate synthetase (FPGS) isolated from rat liver and several human leukemia cell lines. The substrate activity it displays over a wide range of concentrations (0–200 μM) is nearly equivalent to that of methotrexate. The 7-hydroxy derivative of dichloromethotrexate is also a substrate for FPGS. The pattern of polyglutamate products synthesized by rat liver FPGS was nearly identical with both 7-hydroxymethotrexate and methotrexate. In addition, conversion of MTX polyglutamates to the corresponding 7-hydroxy compounds was demonstrated using partially purified rabbit liver aldehyde oxidase. The rate of conversion was concentration dependent, and the relative rate decreased as the MTX polyglutamate chain length increased. We propose that 7-hydroxymethotrexate polyglutamates may be formed by initial hydroxylation of methotrexate and subsequent polyglutamate formation or by direct hydroxylation of methotrexate polyglutamates. It was further shown that the relative substrate activity of folate analogs for folylpolyglutamate synthetase is dependent on the source of the enzyme.

Methotrexate (MTX)† is one of the most useful drugs for cancer chemotherapy. It is used extensively in treatment of acute lymphocytic leukemia, choriocarcinoma, breast cancer, and head and neck cancer [1, 2]. Initial studies with this drug indicated that at conventional doses it was not significantly metabolized [3, 4]. Recent studies, however, employing more sensitive analytical methods have demonstrated that there are three routes of MTX metabolism and the degree to which each is utilized is dose dependent. Removal of the glutamate from MTX, presumably by the action of enteric bacteria [5], yields 4-amino-10-methyl-pteroic acid. This metabolite has been detected in urine and plasma of patients receiving high-dose MTX therapy [6] and may account for up to 6% of the excreted drug. MTX may also be metabolized by hydroxylation of C-7 [7]. 7-Hydroxymethotrexate (7-OH-MTX) is a much less potent inhibitor of dihydrofolate reductase (DHFR) than is MTX and therefore this reaction represents an inactivation of MTX [7]. In addition, 7-OH-MTX is much less soluble than MTX, and precipitation of 7-OH-MTX may contribute to the nephrotoxicity encountered in high-dose MTX therapy [8]. Formation of 7-OH-MTX is thought to be principally mediated by hepatic aldehyde oxidase [8], although it may be formed in many other tissues [9]. Methotrexate is also metabolized by the intracellular formation of poly (γ-glutamyl) derivatives analogous to those formed by natural folates [10–12]. These methotrexate polyglutamates are equipotent with MTX as DHFR inhibitors [13] but are more inhibitory toward other folate-dependent enzymes such as thymidylate synthase [14]. These polyglutamyl derivatives are also retained intracellularly to a greater extent than is MTX [12, 15, 16], thus allowing high intracellular concentrations of drug to be attained.

We have determined that two of these pathways of MTX metabolism—the hydroxylation and polyglutamylation reactions — may occur sequentially, yielding new metabolites, the 7-hydroxymethotrexate polyglutamates.

MATERIALS AND METHODS

Methotrexate (4-amino-10-CH_3-PteGlu) was obtained from the Division of Cancer Treatment, National Cancer Institute. The derivative containing one additional glutamate (4-amino-10-CH_3-$PteGlu_2$) was the gift of Dr. S. Jacobs, University of Pittsburgh School of Medicine. The derivatives containing two or three additional glutamates (4-amino-10-CH_3-$PteGlu_{3,4}$) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Purity of each compound was assayed by u.v. spectroscopy and HPLC analysis (see below). (*dl*)-Tetrahydrofolate was synthesized and purified as previously described [17].

7-Hydroxymethotrexate was synthesized from MTX [18] using partially purified (46-fold) aldehyde oxidase from rabbit liver [19]. The 100-ml reaction mixture contained 50 mM NH_4HCO_3, pH 8.0,

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† Abbreviations: MTX (4-NH_2-10-CH_3-PteGlu), methotrexate (4-amino-10-methyl-pteroylglutamate); 7-OH-MTX, 7-hydroxymethotrexate; DCM and 7-OH-DCM, 3′,5′-dichloromethotrexate and its 7-hydroxylated derivative respectively. Length of the poly (γ-glutamyl) derivatives of these compounds is indicated by the use of a subscript in the chemical formula, e.g. 4-NH_2-10-CH_3-$PteGlu_2$ is the methotrexate derivative containing one additional glutamate in γ-linkage. DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; and HPLC, high pressure liquid chromatography.

250 mM $(NH_4)_2SO_4$, 0.005% Na_2-EDTA, 100 μmoles MTX, catalase (10,000 units; Worthington Biochemicals), and 5 ml of aldehyde oxidase preparation which was capable of causing ΔA_{341}/min/ml = 50 when assayed under standard reaction conditions. After the reaction proceeded to completion at 37° (3 hr), the solution was deproteinized by boiling for 5 min. The supernatant fraction obtained after centrifugation (15 min; 12,000 *g*) was diluted with 5 vol. of water and applied to a 1.5 × 26 cm column of DEAE-cellulose (DE-23; Whatman, Inc.) equilibrated (22°) with 0.1 M NH_4HCO_3, pH 8.0 [8]. Fractions of 6.2 ml were collected at 2 ml/min. After loading, the column was washed with 2 column volumes of equilibration buffer and eluted with a linear gradient (900 ml total volume) from 0.1 to 0.4 M NH_4HCO_3, pH 8.0. At the end of the gradient, a wash of 0.4 M NH_4HCO_3, pH 8.0, was initiated to complete elution of the compound. Absorption spectra of fractions were obtained and those with spectra identical to 7-OH-MTX [20] were pooled, lyophilized to dryness, dissolved in H_2O, and relyophilized to a fluffy yellow solid. Absorption spectra at pH 1, 7, and 13 were identical to those in the literature [20]. This purification scheme easily separates 7-OH-MTX from trace residual MTX [8], a finding verified by HPLC analysis of the final product. The compound was further characterized by its inhibition of homogeneous dihydrofolate reductases from MTX-resistant L1210 cells and human leukemic spleen (both were gifts of Dr. C. A. Lindquist of this laboratory). The L1210 DHFR had I_{50} values of 1×10^{-8} M and 1.3×10^{-6} M, while human leukemic spleen DHFR had I_{50} values of 1.8×10^{-9} M and 8×10^{-7} M for MTX and 7-OH-MTX respectively. These differences in inhibitory potency between MTX and 7-OH-MTX against DHFR compare favorably with those previously reported [7, 8, 18]. These differences are also consistent with those obtained with a rat liver DHFR and chemically synthesized 7-OH-MTX which would not be contaminated by MTX [20].

Products of the reaction between MTX polyglutamates and aldehyde oxidase were also purified on DEAE-cellulose columns (0.4 × 10.5 cm) except that the NH_4HCO_3, pH 8.0, gradient (200 ml) extended from 0.02 to 0.4 M.

Enzyme assays. All assays were performed under conditions of time and enzyme linearity. Aldehyde oxidase was assayed by measuring the change in absorbance at 341 nm as MTX or one of its polyglutamates was converted to a 7-hydroxy derivative according to the procedure of Johns *et al.* [7] as modified to include the activator $(NH_4)_2SO_4$ [19]. Although 70 μM MTX was routinely used in assays, it was later determined that its K_m was 45 μM; thus, assays were run at subsaturation. DHFR activity was assayed spectrophotometrically according to Osborn and Huennekens [21]. I_{50} values (concentration required for 50% inhibition) for DHFR inhibitors were estimated visually from graphs of log drug concentration versus activity. For measurement of inhibition, standard DHFR activity of 0.02 ΔA_{340}/min (L1210) or 0.036 ΔA_{340}/min (leukemic spleen) was used in assays. Folylpolyglutamate synthetase was assayed by measuring the incorporation of [^{3}H]glutamate into the polyglutamates of different substrates [17]. Control experiments demonstrated that polyglutamates of 7-OH-MTX would be quantitatively measured under routine assay conditions. Pteroylpolyglutamate hydrolase (conjugase) was measured with the assay of Krumdieck and Baugh [22] using $PteGlu_2$-γ-[^{14}C]Glu substrate supplied by Dr. C. M. Baugh.

Enzymes. Rat liver folylpolyglutamate synthetase (FPGS) was partially purified according to the procedure of McGuire *et al.* [17]. The folylpolyglutamate synthetases of K562 and CCRF-CEM human leukemia cell lines were purified by ammonium sulfate fractionation and Sephadex G-150 chromatography (J. J. McGuire, P. Hsieh and J. R. Bertino, unpublished method). Crude extracts of HL-60 and MOLT-3 human leukemia cell lines were also used as FPGS sources.

Cell culture. Cells were cultured using standard techniques. The K562 and MOLT-3 cell lines were grown in RPMI 1640, containing 10% fetal bovine serum. The CCRF-CEM cells were cultured in RPMI-1640 containing 10% horse serum. The HL-60 cells grew in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and garamycin (40 μg/ml). To measure EC_{50} values (drug concentration inhibiting growth by 50%), logarithmically growing cells were seeded at 1×10^4 cells/ml in the absence or presence of various concentrations of drug. At either 48 or 72 hr, the cell count was performed using a Coulter counter. The EC_{50} values were estimated visually from plots of log drug concentration versus percent of control growth.

High pressure liquid chromatography (HPLC). HPLC was performed on a microparticulate anion exchange column (Whatman Partisil SAX; 10 μm) by a modification [23] of the procedure of McGuire *et al.* [17]. To assay purity of 7-OH-MTX, the buffer used was 20 mM sodium-phosphate, pH 3.3. MTX and 7-OH-MTX were separated isocratically with baseline resolution. When 7-OH-MTX or MTX polyglutamates were examined, the initial buffer was 60 mM sodium-phosphate, pH 3.3, and the gradient was as described [23].

Miscellaneous. All substrate concentrations were determined from absorption spectra and published extinction coefficients [20, 24].

RESULTS

Substrate activity of 7-hydroxymethotrexate with mammalian folylpolyglutamate synthetases. Substrate activity of 7-OH-MTX was comparable to that of MTX using partially purified FPGS from either rat liver (Fig. 1) or K562 human leukemia cells (Fig. 2). Activity similar to that of the K562 cell FPGS was also observed with the FPGS of CCRF-CEM cells, a human lymphoid leukemia cell line, and two other human leukemia cell lines, HL-60 and MOLT-3 (data not shown). In the normal pharmacologically achievable concentration range (≥ 50 μM), 7-OH-MTX was equivalent to MTX as a substrate for the leukemia cell FPGS, while it was slightly better than MTX for the liver FPGS.

Analysis of products synthesized from 7-OH-MTX by rat liver FPGS. The radiolabeled products synthe-

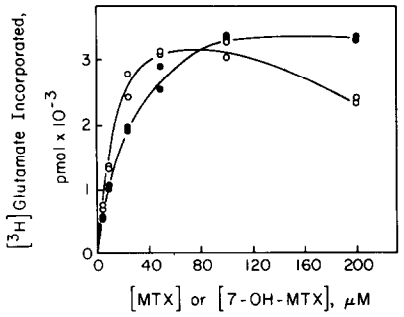

Fig. 1. Substrate activity of methotrexate and 7-hydroxymethotrexate for rat liver folylpolyglutamate synthetase. Standard folylpolyglutamate synthetase reaction mixtures contained either MTX (●) or 7-OH-MTX (○) at the indicated concentrations.

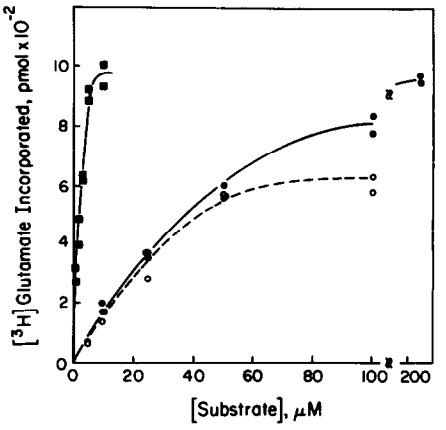

Fig. 2. Substrate activity of methotrexate, 7-hydroxymethotrexate, and tetrahydrofolate for K562 leukemia cell folylpolyglutamate synthetase. Standard folylpolyglutamate synthetase reaction mixtures contained either MTX (●), 7-OH-MTX (○), or (*dl*)-tetrahydrofolate (■) at the indicated concentrations.

sized from 7-OH-MTX and MTX by rat liver FPGS were analyzed by HPLC. Authentic 7-OH-MTX polyglutamates were not available as standards, so identification was based on two facts: (a) the retention time of each of the polyglutamate derivatives of any single type of folate is a defined function of its total number of glutamates; two different types of folates may have different absolute retention times but derivatives with the same number of glutamates will elute in the same position relative to one another [25], and (b) 7-OH-MTX elutes just after MTX. Thus, a 7-OH-MTX polylgutamate would elute just after the MTX polyglutamate of corresponding length. The analysis showed that rat liver FPGS synthesized 7-OH-MTX polyglutamates containing one and possibly two additional glutamates (Fig. 3, bottom). The predominant product, containing one additional glutamate, was present at high enough concentration to give a substantial absorbance peak immediately after the corresponding MTX polyglutamate standard peak. The analysis of an identical reaction mixture containing MTX showed a very similar distribution of product lengths (Fig. 3, top) which co-eluted with the MTX polyglutamate standards. No absorbance peak corresponding to the first 7-OH-MTX product (Fig. 3, top) was detected in MTX-containing reaction mixtures or reaction mixtures lacking any pteridine substrate. The assignment of these products as polyglutamates of 7-OH-MTX is further supported by the known absolute specificity of mammalian FPGS for intact pteridine-like structures [26, 27] and the fact that [^{3}H]glutamate incorporation is followed in these experiments [17].

Substrate activity of MTX and MTX polyglutamates for mammalian hepatic aldehyde oxidase. Polyglutamates of MTX (4-NH_2-10-CH_3-$PteGlu_n$) containing up to four total glutamates were substrates for a partially purified rabbit liver aldehyde oxidase (Table 1). Activity was concentration dependent. There was a marked decrease in activity as the chain length increased at both concentrations tested.

Several experiments verified that the products were 7-OH-MTX polyglutamates. Separate reaction mixtures for each MTX derivative were prepared and absorption spectra were obtained before the addition of aldehyde oxidase and again after the reaction had run to completion. All spectra taken before reaction (pH 7.8) were typical of MTX and those taken at completion (at pH 7.8 and pH 1) were identical [20] to those of 7-OH-MTX (data not shown). Samples of each reaction mixture at completion were chromatographed on mini-columns of DEAE-cellulose. Products having spectra identical to 7-OH-MTX eluted at progressively later positions in the salt gradient as the glutamate chain length of the initial MTX substrate was increased. This indicated that the polyglutamate chains were still intact. This finding was corroborated by showing that the aldehyde oxidase preparation contained no pteroylpolyglutamate hydrolase (conjugase) activity when hydrolase was measured under the same reaction conditions as aldehyde oxidase but at a 20-fold higher enzyme concentration for a 30-fold longer incubation period than was used for aldehyde oxidase. Pteroylpolyglutamate hydrolase is an activity which specifically hydrolyzes γ-glutamyl bonds in polyglutamates of folates and folate analogs [22]. Finally, if removal of the polyglutamate chain were required before aldehyde oxidase activity would be detectable, a lag might be expected in the reaction rate; none was detected.

Cytotoxicity of MTX and 7-OH-MTX for human leukemia cell lines. Five human leukemia cell lines were tested for sensitivity to 7-OH-MTX with MTX as a standard (Table 2). Longer exposure times (72 hr) were used for cell lines with doubling times greater than 24 hr. In all cases, 7-OH-MTX was about two orders of magnitude less toxic than MTX.

Substrate activity of dichloromethotrexate and 7-hydroxydichloromethotrexate with rat liver FPGS. Dichloromethotrexate (DCM), an MTX analog, and its 7-hydroxy derivative were also tested as FPGS substrates (Fig. 4). The substrate activity of these two was nearly equivalent. Since MTX and DCM are also nearly equivalent as substate for this FPGS (data not shown), 7-OH-DCM is as good an FPGS substrate as is 7-OH-MTX.

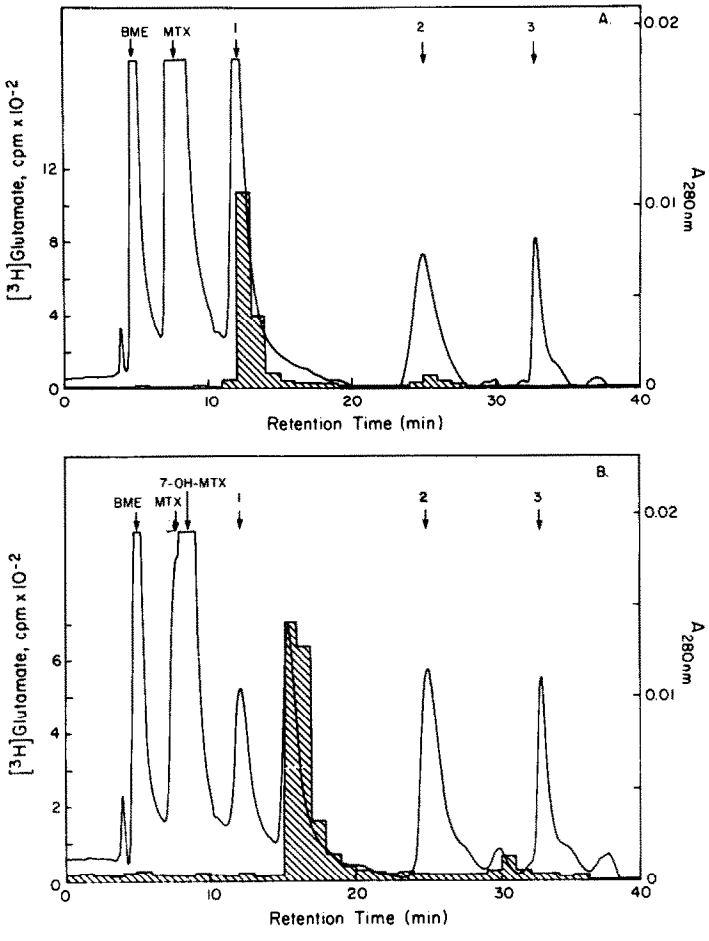

Fig. 3. Analysis of products synthesized from methotrexate and 7-hydroxymethotrexate by rat liver folylpolyglutamate synthetase. Products were analyzed by HPLC as described in Materials and Methods. Sufficient chemically synthesized MTX polyglutamates were co-injected with each sample to provide internal standards measured by absorbance at 280 nm. The elution positions of 2-mercaptoethanol (BME), MTX, and 7-OH-MTX are indicated. MTX polyglutamate peaks 1, 2, and 3 correspond to 4-NH_2-10-CH_3-$PteGlu_2$, 4-NH_2-10-CH_3-$PteGlu_3$, and 4-NH_2-10-CH_3-$PteGlu_4$, respectively. See text for details of other peak assignments. (A) Products synthesized from 50 μM MTX; (B) products synthesized from 50 μM 7-OH-MTX.

DISCUSSION

Methotrexate is now known to form three different types of metabolites in humans. Two of these metabolites, the 4-amino-10-methyl-pteroic acid produced by intestinal flora and 7-hydroxymethotrexate, are much less potent inhibitors of DHFR [5, 7]. The third route of metabolism, the formation of polyglutamyl derivatives, gives products which are at least as potent inhibitors of DHFR as is MTX [13] and which are often more potent inhibitors of other folate-dependent enzymes [14]. In addition, these polyglutamyl derivatives are better retained by cells, the degree of retention increasing as the number of glutamates increases [12, 15, 16]. The data presented here suggest that two of these routes of MTX metabolism may operate sequentially to yield a new series of metabolites, 7-hydroxymethotrexate polyglutamates (Fig. 5). These derivatives may be formed either by direct polyglutamylation of 7-OH-MTX after the action of aldehyde oxidase or by prior formation of MTX polyglutamates by folylpolyglutamate synthetase and their subsequent hydroxylation.

Table 1. Substrate activity of methotrexate polyglutamates (4-NH_2-10-CH_3-$PteGlu_n$) for rabbit liver aldehyde oxidase*

Conc. (μM)	4-NH_2-10-CH_3-$PteGlu_n$ (ΔA_{341}/min)			
	N = 1	2	3	4
10	9	2.5	0.8	0.8
50	34	7	3	2

* Substrate activity of each compound was measured under standard conditions (see Materials and Methods).

Dichloromethotrexate is even more extensively metabolized to its 7-hydroxy derivative than is MTX [7, 28]. Since clinical interest in DCM has revived [29], we tested the FPGS substrate activity of DCM and 7-OH-DCM (Fig. 4). Their excellent substrate

Table 2. Cytotoxicity of methotrexate and 7-hydroxymethotrexate (7-OH-MTX) for human leukemia cell lines*

	MTX EC_{50}		7-OH-MTX EC_{50}	
	Concn (M)	Exposure (hr)	Concn (M)	Exposure (hr)
K562	1.3×10^{-8}	72	1.8×10^{-6}	72
HL-60	5.2×10^{-9}	72	1.6×10^{-6}	72
CCRF-CEM	7×10^{-9}	48	7×10^{-7}	48
MOLT-3	2.6×10^{-8}	48	5×10^{-6}	48
SKL-7	3.8×10^{-8}	72	3.3×10^{-6}	72

* Cytotoxicity was measured by outgrowth techniques using continuous exposure to drug (see Materials and Methods).

activity indicates that 7-OH-DCM polyglutamates may be formed by the same pathways as described for MTX (Fig. 5).

It is difficult to assess which of the pathways to 7-OH-MTX polyglutamates would be quantitatively the most significant in humans. The absolute concentrations of both intracellular MTX polyglutamates [15, 16] and urinary or plasma 7-OH-MTX [8, 30] are dependent on the dose of MTX administered; thus, the activity of each pathway would also be dependent on dose. The apparent affinity of 7-OH-MTX for FPGS is as high as that of MTX, but this enzyme is present in very low amounts in all tissues including liver [26]. The affinity of MTX [31] and presumably MTX polyglutamates (Table 1) for human aldehyde oxidase is low but high activity is present in liver. However, even tissues which do not synthesize 7-OH-MTX could still contain the derivative because the extremely high (250 μM) plasma levels [30] achievable in some patients could allow its influx by passive diffusion. It should be noted that 7-OH-MTX is detectable in plasma during conventional doses such as might be used in maintenance therapy of acute lymphocytic leukemia and under these conditions synthesis of 7-OH-MTX polyglutamates by one or both routes would still be possible.

The consequences of formation of 7-OH-MTX polyglutamates are at present unknown. Since 7-OH-MTX and its polyglutamates are less potent inhibitors of DHFR, their formation from MTX or MTX polyglutamates would clearly lessen acute cytotoxicity. Further, 7-OH-MTX might reduce MTX cytotoxicity by preventing the synthesis of MTX polyglutamates, thus reducing its duration of tissue retention. Since the liver has relatively high levels of aldehyde oxidase, efficient conversion to 7-OH-MTX polyglutamates may account for the low incidence of hepatotoxicity in MTX therapy [1]. The 7-OH-MTX metabolite is clearly cytotoxic at high concentrations (Table 2), however, and the formation of 7-OH-MTX polyglutamates in particular tissues might contribute to cytotoxicity by allowing high intracellular levels to be maintained for long time periods. Also, the slow release of 7-OH-MTX from polyglutamates and its subsequent efflux might contribute, along with low renal clearance, to the long plasma half-life of 7-OH-MTX [6, 8, 30]. Since it has been proposed [8] that precipitation of 7-OH-MTX in kidney may account for the occasional nephrotoxicity seen with MTX therapy, sustained slow release of 7-OH-MTX might increase the possibility of this complication. Finally, formation of MTX polyglutamates has been suggested to lower cellular folate stores [32]. If 7-OH-MTX polyglutamates have a similar effect, they could increase toxicity via this mechanism.

There are several interesting features of MTX and 7-OH-MTX substrate activity for folylpolyglutamate synthetases. The 7-hydroxylation did not diminish activity with either the rat liver or human leukemia cell FPGS and, in fact, increased activity at low concentration with the rat liver FPGS. These results

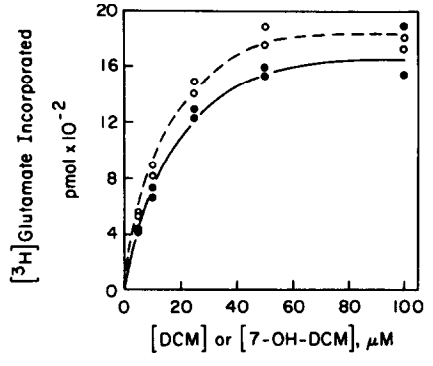

Fig. 4. Substrate activity of dichloromethotrexate and 7-hydroxydichloromethotrexate with rat liver folylpolyglutamate synthetase. Standard folylpolyglutamate synthetase reaction mixtures contained either DCM (●) or 7-OH-DCM (○) at the indicated concentrations.

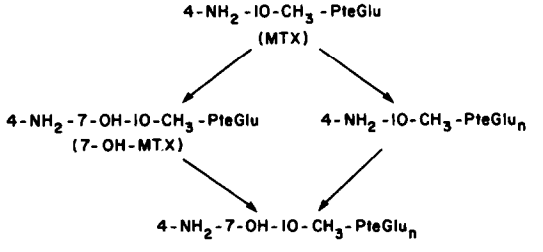

Fig. 5. Proposed pathways of formation of 7-hydroxymethotrexate polyglutamates.

indicate that an unsubstituted 7-position is not essential for folate-like substrate activity. This is consistent with, and extends, results from our laboratory and others demonstrating that extensive modification of the pteridine moiety is possible with retention of substrate activity [26, 27]. Analysis of the products of the FPGS reaction demonstrated that 7-OH-MTX was converted qualitatively to the same products as MTX (Fig. 3). The majority of the product contained one additional glutamate with the product containing two additional glutamates being the only other detectable. The relative product length distribution from 7-OH-MTX and MTX was also the same as in Fig. 3 at a lower substrate concentration (10 μM; data not shown) indicating again that MTX and 7-OH-MTX were similar in substrate activity and also that they both differed from tetrahydrofolate. When tetrahydrofolate is used as a substrate, longer products, up to pentaglutamate, are preferentially synthesized at lower substrate concentrations [17]. Dehydroxylation of the 7-OH-MTX substrate before reaction with FPGS appeared to be ruled out because the products did not co-elute with the authentic MTX polyglutamate standards which were co-injected (Fig. 3).

One cautionary note on the relative activity of 7-OH-MTX, MTX, and tetrahydrofolate with FPGS. With the rat liver FPGS, MTX (and thus 7-OH-MTX; Fig. 1) is only a slightly poorer substrate than tetrahydrofolate [23]. However, with the human leukemia cell FPGS (Fig. 2), both were much poorer substrates than tetrahydrofolate. Thus, it is not possible to make strict generalizations about relative substrate activity of folate analogs as this activity may depend upon the enzyme source.

Michaelis contants (K_m) based on the data of Figs. 1 and 2 have not been presented. Such values would be meaningless because Michaelis–Menten kinetics are not maintained [17] in that multiple products are formed (Fig. 3) and the products of each reaction are competing substrates of unknown affinity.

MTX polyglutamates served as substrates for hepatic aldehyde oxidase in a concentration-dependent manner. Only two concentrations were tested because of the limited availability of these compounds. Activity decreased with each increase in chain length at both concentrations (Table 1). This result extends the findings of Valerino *et al.* [5] who showed that addition of the first glutamate to 4-amino-10-methyl-pteroic acid to form MTX resulted in a decrease in V_{max} in the aldehyde oxidase reaction. The decrease in activity with increasing chain length is probably a charge, rather than steric, effect since bulky di-esters of MTX are much better substrates than MTX for aldehyde oxidase [33].

Although the intracellular metabolites of MTX have been examined in rat liver [10], hepatoma cells [11], and hepatocytes [34, 35], only polyglutamates of MTX have been detected. There are several possible reasons for not finding 7-OH-MTX polyglutamates. In cultured cells systems, the 7-OH-MTX may efflux before it is polyglutamylated or cultured cells may lack aldehyde oxidase. A more important reason may be in the labeling pattern of [^{3}H]MTX [36]. Greater than 50% of the label in [^{3}H]MTX is at C-7 and this is released as [^{3}H]H_2O during hydroxylation. This would decrease the sensitivity of detection of 7-OH-MTX by at least a factor of 2. If, in addition, the aldehyde oxidase displays an isotope effect at this position, the labeled material would be hydroxylated more slowly, again causing a decrease in the sensitivity of detection [36]. Finally, since these metabolites had not been suggested before, it is not clear that the analytical methods used to resolve MTX polyglutamates would resolve 7-OH-MTX polyglutamates as well. Our demonstration of the cell-free enzymatic synthesis of 7-OH-MTX polyglutamates suggests that they should be looked for in model systems and in the clinical situation, and a determination made of their significance. In this regard, we are currently studying the effect of the presence of 7-OH-MTX on the cytotoxicity of MTX in cell culture.

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