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DIHYDROFOLATE REDUCTASE FROM GUINEA PIG LIVER AND SMALL INTESTINE*

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Abstract—Dihydrofolate reductase has been purified 2000-fold from guinea pig liver, and 450-fold from guinea pig small intestine. The chromatographic properties, pH optima, and substrate and cofactor requirements of the two enzymes have been compared; the susceptibility of the enzymes to a series of activators and inhibitors also has been determined. No differences were noted between dihydrofolate reductases from the two sources; it is concluded that the unusually high resistance of the guinea pig to folate antagonists cannot be attributed to differences in the properties of small intestine and liver dihydrofolate reductase, or to differences between guinea pig dihydrofolate reductase and the analogous enzyme from other mammalian species.

AMONG laboratory animals, the guinea pig is much more resistant to the folate analog aminopterin§ (AM) than are the rat, mouse, and dog.[1–3] In addition, the guinea pig differs from these species in that a state of folate deficiency can readily be produced by dietary means.[4] Previous studies from this laboratory have demonstrated that the AM- and methotrexate¶ (MTX)-sensitive enzyme, dihydrofolate reductase, is present in liver and kidney of the guinea pig in high activity; small intestine, spleen, and bone marrow had moderate activity, while low levels were found in skeletal muscle and brain.[5] Fourteen days after administration of MTX to guinea pigs, bioassay showed that the inhibitor was present in liver and kidney; in contrast, the inhibitor was not detectable in bone marrow or small intestine even 24 hr after MTX.

Since a possible explanation for the relative refractoriness of the guinea pig to MTX might be that intestinal dihydrofolate reductase is less sensitive to this inhibitor than the liver enzyme, the present study was undertaken to purify and characterize the enzyme from guinea pig liver and small intestine, and to compare the inhibition of enzyme from these two sources by MTX. In addition, since species differences between dihydrofolate reductases have been shown to exist,[6, 7] it was of interest to compare a number of properties of dihydrofolate reductase obtained from two different organs from the same species.

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§ 4-Amino-4-deoxypteroylglutamate.

¶ 4-Amino-4-deoxy-10-methylpteroylglutamate.

EXPERIMENTAL

Chemicals. The commercial sources of chemicals and the methods for preparation of substrates were detailed in a previous publication.[8] A series of 2,4-diaminopteridines was kindly supplied by Dr. A. Maass of Smith, Kline & French Laboratories; the 2,4-diaminopyrimidines studied were a gift of Dr. G. Hitchings of the Burroughs-Wellcome Laboratories.

Methods. All spectrophotometric measurements were made in a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder. Protein was determined by the biuret method in crude extracts or, after the ammonium sulfate stage of purification, by absorbance at 280 mμ with bovine serum albumin as a standard. Specific activity of dihydrofolate reductase is expressed as micromoles substrate reduced per hour per milligram protein. The assay used has been described elsewhere;[8, 9] details of the individual experiments are given in the legends for the figures and tables.

Purification of guinea pig liver and small intestine dihydrofolate reductase. Four female guinea pigs (400–500 g) were anesthetized with ether and killed by exsanguination. The livers from two animals were pooled; 40 g liver was added to 120 ml cold saline and subsequently used for enzyme purification. The entire duodenum and jejunum of the four animals were excised by stripping them free of peritoneum. After removing the intestinal contents by gentle pressure with a spatula, 40 g of the pooled specimens was added to 120 ml cold saline. In all further steps, the preparations from liver and small intestine were treated identically; all steps were carried out at 4°. Homogenization was performed with a Waring Blendor for 2 min. The pH of the homogenate was adjusted to 5·1 by careful addition of 1 N HCl, and the homogenate was immediately centrifuged at 30,000 *g* for 15 min. The supernatant solution was then fractionated with ammonium sulfate by slowly adding the crystalline solid to raise the concentration to 55% of saturation; the pH was adjusted to 6·0 with 1 N KOH. After allowing 10 min for mixing, the precipitate that formed was discarded after centrifugation, and additional ammonium sulfate was added to the supernatant solution to achieve 70% saturation; once again the solution was adjusted to pH 6·0 and stirred for an additional 10 min. The precipitate was recovered by centrifugation and suspended in 8 ml of 0·01 M K_2HPO_4–KH_2PO_4 buffer, pH 7·0, containing 0·1 M KCl. This fraction was then applied to a column of Sephadex G-100 (80 × 3 cm), previously washed with 0·01 M phosphate buffer, pH 7·0, 0·2 M with respect to KCl; phosphate–KCl solution of the same molarity was used to elute the enzyme from the column; fractions of 5 ml each were collected. The enzyme was eluted from the column in tubes no. 48–55. In each instance the peak of enzyme activity appeared shortly after the hemoglobin peak (tubes 38–39). The final step in the purification was chromatography on calcium phosphate, prepared according to the modification by Mathews *et al.*[10] of the method of Main and Cole.[11] A column of 9 × 2·5 cm was prepared and washed with 100 ml of 0·01 M K_2HPO_4–KH_2PO_4 buffer, pH 7·0, containing 0·1 M KCl; 40 ml of the eluate from the Sephadex G-100 column (tubes 48–55) was then applied to the calcium phosphate column. Elution was carried out with successive 60-ml portions of 0·01 M, 0·025 M, and 0·05 M K_2HPO_4–KH_2PO_4, buffer, pH 7·0, all containing 0·1 M KCl; 10-ml fractions were collected. The greater

part of the enzyme activity was eluted when the buffer concentration was raised to 0·05 M (tube 15 for liver; tubes 15 and 16 for small intestine).

RESULTS

Enzyme purification

Table 1 summarizes the individual steps of the enzyme preparation. By this relatively simple procedure about 2000-fold purification of liver enzyme and 450-fold

TABLE 1. PURIFICATION OF DIHYDROFOLATE REDUCTASE FROM GUINEA PIG LIVER AND SMALL INTESTINE

Purification step	Volume (ml) L.	Volume (ml) S.I.	Total protein (mg) L.	Total protein (mg) S.I.	Specific activity (μmoles/hr mg/) L.	Specific activity (μmoles/hr mg/) S.I.	Total activity (μmoles/hr) L.	Total activity (μmoles/hr) S.I.	Recovery (%) L.	Recovery (%) S.I.
1. pH 5·1 Extract	120	120	3240	1720	0·11	0·06	360	103		
2. Ammonium sulfate (55–70%)	8	8	260	200	1·2	0·2	312	100	87	97
3. Sephadex (G–100) Tubes 48–55	40	40	15·6	20·1	13·9	1·5	217	31	60	30
4. Calcium phosphate Tube 15*	10	20	0·6	0·5	217	26·8	130	13·4	36	13

Liver, L.; small intestine, S.I.

* Tubes 15 and 16 for S.I.

purification of intestinal dihydrofolate reductase were accomplished. The lower initial activity of the small intestine previously noted, as well as the poorer recovery of dihydrofolate reductase activity during purification, might explain the lower specific activity obtained for the purified intestinal enzyme, as compared to the purified liver enzyme. Both enzymes migrated on Sephadex G-100 and calcium phosphate in an identical fashion.

Purified preparations from both liver and intestine were stable for at least 2 months when stored at 4°.

pH optima

When the effect of pH on the activities of the two enzymes was determined with saturating amounts of substrate and cofactors, no obvious differences were observed when the intestine and liver enzyme were compared (Fig. 1). For both enzymes, the reaction velocity was greater with acetate buffer than with citrate buffer.

Substrate and cofactor

When dihydrofolate was used as a substrate, NADPH was a more effective cofactor than was NADH for both enzymes when tested at a concentration of 8×10^{-5} M (Table 2). At pH 5·0, the activity with NADH was less than 10% of the activity

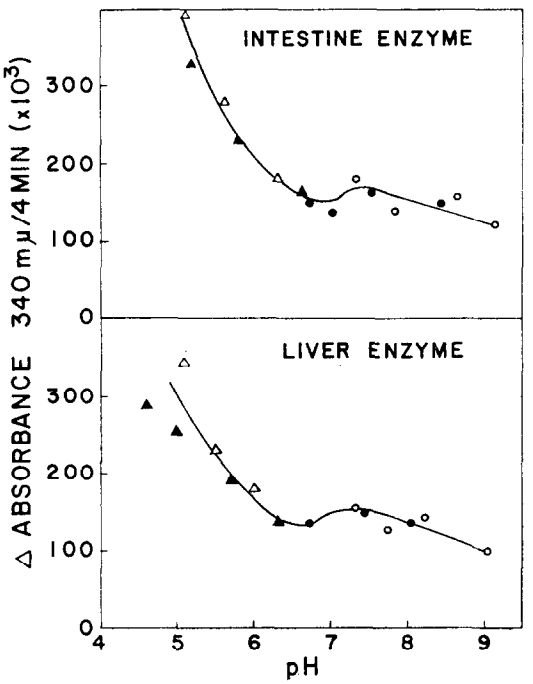


FIG. 1. The effect of pH on the dihydrofolate reductase activity of guinea pig liver and small intestine. The complete reaction mixture contained, in a final volume of 1 ml: buffer, 100 μmoles; KCl, 150 μmoles; NADPH, 0·08 μmole; purified enzyme, 0·1 ml; and 0·04 μmole dihydrofolate containing 10 μmoles 2-mercaptoethanol. The decrease in absorbance at 340 mμ was measured at 15-sec intervals. The results have been corrected for the oxidation of NADPH that occurs in the absence of substrate. The pH values plotted are measured values obtained immediately on completion of the reaction. ▲: sodium citrate buffer; △: sodium acetate buffer; ○: Tris-HCl buffer; ●: potassium phosphate buffer.

TABLE 2. COFACTOR AND SUBSTRATE SPECIFICITY FOR DIHYDROFOLATE REDUCTASE OF GUINEA PIG LIVER AND SMALL INTESTINE

pH	Cofactor	Substrate	Activity	
			L.	S.I.
	(8 × 10^{-5} M)	(4 × 10^{-5} M)	(ΔOD 340 mμ/6 min)	
1. 5·0	NADPH	Folic	0·52	0·13
	NADH	Folic	0·00	0·00
	NADPH	Dihydrofolate	3·50	1·80
	NADH	Dihydrofolate	0·80	0·40
2. 7·5	NADPH	Folic	0·05	0·03
	NADH	Folic	0·06	0·04
	NADPH	Dihydrofolate	1·40	0·45
	NADH	Dihydrofolate	0·14	0·04

Assay conditions were similar to those described in the legend of Fig. 2. Acetate buffer was used at pH 5·0; Tris buffer at pH 7·5.

obtained with NADPH; essentially no activity was noted with NADH at pH 7·5. Folic acid was reduced only at an acid pH, and much less rapidly than was dihydrofolate; in this instance, an absolute requirement for NADPH existed, and no activity was noted with NADH.

Two analogues of dihydrofolate also were tested as substrates for the purified enzymes, 3′,5′-dichlorodihydrofolate and N^{10}-methyldihydrofolate. The activity of

TABLE 3. SUBSTRATE ACTIVITY OF 3′,5′-DICHLORODIHYDROFOLATE (Cl_2FH_2) AND N^{10}-METHYLDIHYDROFOLATE ($N^{10}MeFH_2$) AS COMPARED TO DIHYDROFOLATE (FH_2) FOR DIHYDROFOLATE REDUCTASE OF GUINEA PIG LIVER AND SMALL INTESTINE

Source	Enzyme activity (Δ absorbance at 340 mμ/4 min)					
	FH_2		Cl_2FH_2		$N^{10}MeFH_2$	
	−KCl	+KCl	−KCl	+KCl	−KCl	+KCl
Liver	0·125	0·210	0·240	0·265	0·190	0·210
Small intestine	0·100	0·160	0·260	0·270	0·160	0·200

The complete system contained: Tris-HCl buffer, pH 7·0, 100 μmoles; NADPH, 0·08 μmole; enzyme, 0·1 ml; and the indicated substrate, 0·04 μmole. The substrate was added last to initiate the reaction. When KCl was present, the concentration was 0·15 M.

these substrates, as compared with dihydrofolate, is given in Table 3. The maximal velocity attained with these compounds was greater than that achieved with dihydrofolate; however, in the presence of 0·15 M KCl, no further increase in activity was observed with these substrates, while a twofold increase in activity was noted with dihydrofolate. Both enzymes reduced these substrates at the same rates.

The effect of activators on enzyme activity

Protein denaturants such as urea and guanidine-HCl have been reported to stimulate dihydrofolate reductase from chicken liver[12] and also from Ehrlich ascites cell extracts.[13] It has also been observed that organic mercurials stimulate dihydrofolate reductase from these sources; but not from *Escherichia coli* dihydrofolate reductases.[14] It was of interest, therefore, to compare the effects of these substances on the enzymes obtained from the guinea pig. The organic mercurial, *p*-chloromercuribenzoate (PMB), stimulated both enzyme activities, but only to a slight extent. Changing the pH or buffer, or adding mercaptoethanol[13] did not result in any marked differences in the stimulation observed (Table 4). EDTA, added before PMB, prevented the stimulation in rate. In contrast to results obtained with the chicken liver enzyme, but similar to those obtained with the Ehrlich ascites enzyme, CH_3HgBr did not stimulate either the guinea pig intestine or liver enzyme. Marked stimulation was observed for the enzyme from these two sources when urea or guanidine-HCl was added to the reaction mix, but again the enzyme from both sources was activated to the same extent.

Inhibition by methotrexate

Because of the resistance of the guinea pig to 4-amino analogs of folate, it was of interest to examine in some detail inhibition by MTX of the liver enzyme, as well as of

the intestinal enzyme. The inhibition of dihydrofolate reductase activity with increasing amounts of MTX is plotted in Fig. 2. This study was carried out in reaction mixtures with and without added KCl, and at two pH values. A pH of 5·9 was used, since "stoichiometric" inhibition is produced at this pH;[15, 16] this study may be compared to the titration carried out at pH 8·2, where less inhibition is observed.[16] The results of studies with the liver enzyme are shown in this figure; identical results were obtained with the purified enzyme from small intestine.

TABLE 4. ACTIVATION OF DIHYDROFOLATE REDUCTASE FROM GUINEA PIG LIVER AND SMALL INTESTINE BY ORGANIC MERCURIALS

Conditions	Organic mercurial	Concentration (M)	Stimulation L.	Stimulation S.I.
			(%)	
1. Complete system	PMB	5×10^{-4}	35	45
2. Omit mercaptoethanol	PMB	5×10^{-4}	32	42
3. Phosphate buffer (50 μmoles)	PMB	5×10^{-4}	43	40
4. Phosphate buffer, omit mercaptoethanol	PMB	5×10^{-4}	42	45
5. Phosphate buffer, omit mercaptoethanol, plus EDTA (5×10^{-3} M) added before PMB	PMB	5×10^{-4}	5	12
6. Complete system	CH_3HgBr	1×10^{-4}	0	0
7. Omit mercaptoethanol	CH_3HgBr	1×10^{-4}	5	5

The complete system contained, in a final volume of 1 ml: Tris-HCl buffer, 50 μmoles; KCl, 50 μmoles; NADPH, 0·08 μmole; purified liver or intestinal enzyme, 0·1 ml; organic mercurial, as indicated. Dihydrofolate, 0·03 μmole, and 2 μmoles 2-mercaptoethanol were added last to initiate the reaction. Substitutions or omissions in the complete system are indicated in the table.

Inhibitors of the two enzymes by substituted 2,4-*diaminopyrimidines and* 2,4-*diaminopteridines*

The studies of Burchall and Hitchings,[6] which demonstrate that dihydrofolate reductase purified from several bacterial sources can be differentiated on the basis of sensitivity to certain dihydrofolate reductase inhibitors, suggested to us the possibility that differences in the enzymes from the guinea pig liver and intestine might be obtained by using a series of closely related inhibitors. Three 2,4-diaminopyrimidines, as well as several substituted 2,4-diaminopteridines, were tested; the results are shown in Tables 5 and 6 respectively. No appreciable differences between the enzymes obtained from the two sources could be demonstrated. Trimethoprim was a poor inhibitor for both enzymes, in contrast to the potent inhibition produced in certain bacterial species,[6] while pyrimethamine, and in particular DDMP (see Table 5), were potent inhibitors of the guinea pig enzymes. Certain 2,4,7-triaminopteridines, substituted in the 6-position with aromatic constituents, 2′-thienyl, phenyl, *o*-tolyl, and 2′-furyl, had moderate inhibitory activity, while the 6-substituted 3′-pyridyl and *p*-aminophenyl-substituted compounds had less inhibitory activity. When the 2,4,6-triamino-7-phenylpteridine is compared with 2,4,7-triamino-6-phenylpteridine, it may be seen that the former compound is a much weaker inhibitor of the guinea pig

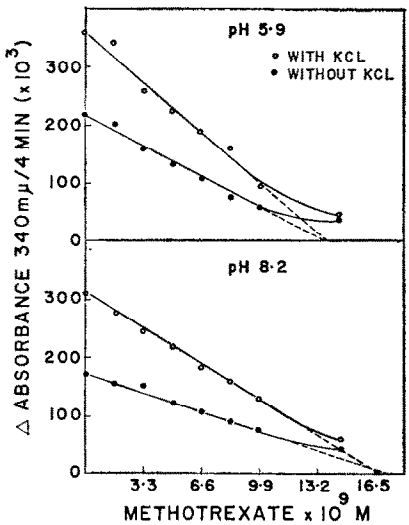


FIG. 2. Inhibition of dihydrofolate reductase guinea pig liver by methotrexate measured at pH 5·9 (upper panel) and at pH 8·2 (lower). The complete reaction mixture contained, in a final volume of 1 ml: buffer, 100 μmoles (sodium citrate buffer, upper; Tris-HCl buffer, lower); KCl, when present, 150 μmoles; NADPH 0·08 μmole; purified liver enzyme, 0·1 ml; and dihydrofolate, 0·04 μmole, containing 10 μmoles 2-mercaptoethanol. The dihydrofolate was added last to initiate the reaction.

TABLE 5. INHIBITION BY SUBSTITUTED 2,4-DIAMINOPYRIMIDINES OF DIHYDROFOLATE REDUCTASE OF GUINEA PIG LIVER AND SMALL INTESTINE

Compound	50% inhibition (M)	
	L.	S. I.
2,4-Diamino-5-(3′,4′,5′-trimethoxy benzyl)pyrimidine (trimethoprim)	3×10^{-5}	2×10^{-5}
2,4-Diamino-5-*p*-chlorophenly-6-ethyl-pyrimidine (pyrimethamine)	5×10^{-8}	5×10^{-8}
2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP)	8×10^{-9}	6×10^{-9}

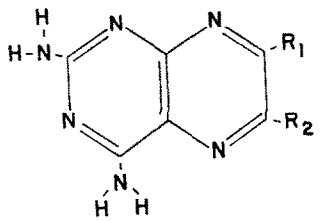
The complete system contained in a final volume of 1 ml: Tris-HCl buffer, pH 7·4, 100 μmoles; KCl, 150 μmoles; purified liver or intestinal enzyme, 0·1 ml; NADPH, 0·08 μmole; and inhibitor. After mixing and allowing the mixture to stand at room temperature for 2 min, dihydrofolate (0·03 μmole) was added to initiate the reaction. The dihydrofolate solution contained 20 μmoles 2-mercapto-ethanol per μmole dihydrofolate. Several inhibitor concentrations were studied for each compound in order to determine the concentration necessary to obtain 50% inhibition.

enzyme.[8] Substitution of a methyl group for a hydrogen atom at the 4-amino position of 2,4,7-triamino-6-phenylpteridine also resulted in a marked decrease in affinity of the inhibitor for the enzyme.

DISCUSSION

Although the explanation for the increased natural resistance of the guinea pig to challenge by methotrexate has not been provided by these studies, several possibilities

TABLE 6. INHIBITION OF THE DIHYDROFOLATE REDUCTASE OF GUINEA PIG LIVER AND SMALL INTESTINE BY SUBSTITUTED 2,4-DIAMINOPTERIDINES



Compound	R_1	R_2	Concentration necessary for 50% inhibition (M) S.I.	L.
8542	NH_2	Phenyl	$2{\cdot}6 \times 10^{-7}$	$2{\cdot}6 \times 10^{-7}$
9107	NH_2	*o*-Chlorophenyl	$6{\cdot}0 \times 10^{-7}$	$7{\cdot}0 \times 10^{-7}$
9123	NH_2	*o*-Tolyl	$2{\cdot}1 \times 10^{-7}$	$1{\cdot}9 \times 10^{-7}$
11,812	NH_2	3′-Pyridyl	$4{\cdot}0 \times 10^{-6}$	$4{\cdot}0 \times 10^{-6}$
11,976	NH_2	2′-Thienyl	$8{\cdot}0 \times 10^{-8}$	$8{\cdot}0 \times 10^{-8}$
12,344	NH_2	*p*-Aminophenyl	$1{\cdot}2 \times 10^{-5}$	$1{\cdot}0 \times 10^{-5}$
12,375	NH_2	2′-Furyl	$2{\cdot}6 \times 10^{-7}$	$2{\cdot}4 \times 10^{-7}$
13,287	Phenyl	NH_2	$3{\cdot}0 \times 10^{-5}$	$3{\cdot}0 \times 10^{-5}$
2,7-Diamino-4-methyl-amino-6-phenylpteridine			$3{\cdot}8 \times 10^{-5}$	$3{\cdot}8 \times 10^{-5}$

Inhibition studies were performed as described in the legend of Table 5.

have been eliminated. Probably of most importance is the finding that purified dihydrofolate reductase not only from liver but also from intestine, an organ vulnerable to methotrexate toxicity, has equal sensitivity to inhibition with this drug *in vitro*. Three additional conclusions can be drawn from these studies. (1) The percentage inhibition produced by a given amount of methotrexate is similar either in the presence or absence of KCl. (2) Less inhibition is produced at pH 8·2 than at pH 5·9 with equimolar amounts of methotrexate and with the same amounts of enzyme present; furthermore, the amount of enzyme "titrated" (the extrapolation to zero enzyme activity) is not similar at the two pH values studied. (3) Dihydrofolate reductase from these sources is as sensitive to methotrexate inhibition as is the enzyme from livers of sensitive species such as the mouse[17] and rat.[15] Furthermore, no differences between the enzyme from the two sources, or unusual binding to cofactors or substrates, were noted. The molecular weights of the enzymes, as estimated by chromatography on Sephadex G-100, were similar, as were some kinetic properties of the enzyme, including activation by organic mercurials, urea and guanidine, or inhibition by several folic acid antagonists. The enzyme of the guinea pig may be differentiated from those of chicken liver, Ehrlich ascites cells, and *E. coli* by virtue of its moderate stimulation by PMB, in contrast to (a) the marked stimulation that

this compound produced with the enzyme of Ehrlich ascites cells and with that of chicken liver, and (b) the lack of stimulation that was produced with the enzyme of *E. coli*. Additional differences exist between the enzymes of chicken liver and the guinea pig in that the enzyme of the former tissue is markedly stimulated by CH_3HgBr.[18] The *E. coli* enzyme differs from the mammalian enzymes in several respects; namely, that dichlorodihydrofolate is not a substrate and that the enzyme is not activated by either mercurials or guanidine and only slightly by urea.

An alternative explanation is now being sought for the refractoriness of the guinea pig toward the folic acid antagonists; a possible cause for this refractoriness may be enzymic inactivation of these agents. Studies of the oxidation of MTX and AM by hepatic aldehyde oxidase from guinea pig and other species are presented in the accompanying paper.[19]

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