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LEVELS OF DIHYDROFOLATE REDUCTASE AND THE FORMATE-ACTIVATING ENZYME ACTIVITIES IN GUINEA PIG TISSUES BEFORE AND AFTER AMETHOPTERIN ADMINISTRATION*

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Abstract—Formate-activating enzyme activity and dihydrofolate reductase activity have been measured in the tissues of the guinea pig; the highest levels of these enzyme activities occur in the liver and kidney. After the administration of amethopterin, the liver and kidney retained this drug for at least 14 days, while bone marrow, small intestine, and spleen contained amounts not detectable by bioassay methods (less than 1×10^{-7} μmoles/mg protein in lysates). Dihydrofolate reductase activity in liver, kidney, spleen, bone marrow, and small intestine did not increase after amethopterin administration (5 mg/kg body weight given subcutaneously); however, a two- to four-fold increase was observed in circulating erythrocytes 7 to 21 days after the administration of the drug. Dietary deficiency of folate did not produce significant changes in the dihydrofolate reductase activity of liver, bone marrow, erythrocytes, or leukocytes of guinea pigs. The erythrocytes and leukocytes of various animal species have measurable levels of dihydrofolate reductase activity, except the erythrocytes of the dog and the red and white cells of man.

DIHYDROFOLATE reductase (also called folic acid reductase) has been studied in bacteria,[1] mouse leukemia cells,[2–4] calf thymus,[5] chicken liver,[6–8] and normal and leukemic leukocytes from humans.[9, 10] This enzyme has been of particular interest because of the key role the coenzyme formed (tetrahydrofolate) plays in biosynthesis of purine, thymidylate, histidine, and methionine.[11, 12] In particular, the function of dihydrofolate reductase in the synthesis of thymidylate may be the key metabolic pathway inhibited by the folic acid antagonists.[13, 14]

The recent finding that this enzyme activity is 'induced' in leukocytes and erythrocytes of humans treated with amethopterin[10] prompted the study of this phenomenon in various tissues of an experimental animal. The present study was undertaken to determine the level of dihydrofolate reductase activity, as well as formate-activating enzyme activity, in guinea pig tissues and subsequently to examine the effects of amethopterin administration or folic acid deficiency upon these enzyme levels. The guinea pig was chosen as the experimental animal, since dietary folate deficiency can be produced readily in this species.[15, 16] In addition, previous studies[17, 18] have indicated that the guinea pig is remarkably refractory to the toxic effects of amethopterin.

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It was therefore of interest also to relate these findings to the natural resistance of these animals to this substance. A preliminary report of a portion of this work has appeared.[19]

EXPERIMENTAL

Materials

NADP, NAD, NADPH, NADH, and ATP were obtained from the Sigma Chemical Co. and folic acid from Nutritional Biochemicals Corp. Dihydrofolate was prepared by the method of Futterman[20] as modified by Blakley.[21] Tetrahydrofolate was obtained from Nutritional Biochemicals Corp. or was prepared according to Hatefi *et al.*[22] Amethopterin was kindly supplied by Dr. J. M. Ruegsegger of the American Cyanamid Co. (Lederle Division). DEAE cellulose, purchased from Eastman Co., was washed prior to use, according to the method of Sober *et al.*[23]

Methods

Except for the studies with special diets, adult female guinea pigs, weighing between 450 and 550 g, were used. The animals were etherized and killed by exsanguination.

Erythrocytes and leukocytes were separated from heparinized whole blood and lysed in the manner previously described.[24, 25] Bone marrow was procured by removing both femurs, cracking open both ends of the bones, and washing out the marrow cavity with 3 ml of 0·15 M $KHCO_3$. Small intestine was obtained by removing a 25-cm segment distal to the pylorus; gentle pressure with a spatula sufficed to empty the intestinal contents. The entire segment was then homogenized in five volumes of 0·15 M $KHCO_3$.

Liver, spleen, kidneys, and the remainder of the organs assayed were homogenized in approximately 5 volumes of 0·15 M $KHCO_3$. All tissues and extracts were kept at 4°.

Homogenization of leukocytes and bone marrow cells was accomplished by using a Virtis '45' high-speed homogenizer (45,000 rpm for 90 sec). All other tissues were homogenized in a Waring Blendor at high speed for 2 min. In some experiments liver and small intestine were homogenized with a glass Potter-Elvehjem homogenizer in 0·25 M sucrose; in other experiments 0·015 M acetic acid was employed (instead of 0·15 M $KHCO_3$).

After homogenization of the various tissues, the supernatant fluid obtained by centrifuging at 10,000 $\times$ *g* for 30 min (high-speed attachment, refrigerated International centrifuge, model PR-2) was used for assays.

Assay procedures

The formate-activating enzyme was assayed as previously described.[24]

Dihydrofolate reductase activity was determined at pH 7·5 either by a coupled assay, measuring the amount of terahydrofolate formed with the aid of an excess of formate-activating enzyme,[9] or by following the conversion of NADPH and dihydrofolate at 340 mμ in a Beckman DU spectrophotometer;[2, 6] the former assay was used routinely. With this assay low levels of dihydrofolate reductase activity could be detected, since the product of the reaction, N^{10}-formyl tetrahydrofolate, may be conveniently estimated after conversion of this compound to N^5,N^{10}-methenyl

tetrahydrofolate after deproteinization with acid. In order to insure that dihydrofolate reductase activity was rate limiting, a partially purified formate-activating enzyme from guinea pig liver was added in excess. ATPase activity was measured by determining phosphate liberated according to Fiske and Subbarow.[26]

Protein was measured by the biuret reaction or by light absorption at 280 mμ with bovine serum albumin as the standard. Hemoglobin was estimated by light absorption at 540 mμ in 0·04% NH_4OH. Enzyme activity is expressed as micromoles of product formed per hour per milligram protein.

The amount of amethopterin in the tissues was measured in neutralized trichloroacetic acid (TCA) extracts of lysates of tissues prepared as described. The amethopterin present in the extract was estimated by determination of the amount of inhibition produced, in comparison with that caused by known amounts of amethopterin, when the extract was added to a purified guinea pig dihydrofolate reductase preparation. This assay has been described in detail by Werkheiser, *et al.*, using folate as a substrate,[27] and by Bertino and Fischer, using dihydrofolate as a substrate.[28]

Folic acid-deficient guinea pigs

Guinea pigs, aged 5 to 8 days and weighing 90 to 130 g, were placed on a folate-free test diet, prepared according to Reid and Briggs.[16] The animals of a control group were treated subcutaneously with folate, 5·0 mg three times weekly. After 3 weeks on this diet, animals from both groups were sacrificed and the tissues removed and prepared as described previously. Six of the twelve animals on the folate-free diet, which developed low levels of folate-derived substances in the serum (Table 1),

TABLE 1. LEVEL OF FOLATES IN SERA OF GUINEA PIGS FED A DIET DEFICIENT IN FOLATE, AS COMPARED WITH THAT OF CONTROL ANIMALS

Group	No. of animals	Serum folate levels* (mμg/ml serum)
I Folate-deficient diet	4	2·3 (2–2·7)
II Folate-supplemented diets	4	37 (13–51)

* We are indebted to Dr. V. Herbert for performing these determinations, for which *Lactobacillus casei* was utilized as the test organism.[38] In this and other tables the numbers within parentheses indicate range. A. Iannotti in this laboratory has recently measured the serum folate level in 4 guinea pigs on a regular laboratory diet; the average serum folate was 9·0 mμg/ml (range 3·5 to 15·0).

demonstrated hair loss, diarrhea, poor weight gain, moderate anemia, and leukopenia, and these were used for the enzyme studies. The group of animals on a similar diet, but supplemented with folate, were used for the control studies.

RESULTS

The initial experiments were directed toward obtaining reproducible levels of activity of the formate-activating enzyme and dihydrofolate reductase in the guinea pig tissues. As shown in Fig. 1, dihydrofolate reductase activity is linear with increasing concentration of extract, but only with a restricted range of levels of extract; larger amounts of homogenate resulted in inhibition of enzyme activity. This inhibition was most pronounced in small intestine, liver, and muscle—tissues in which a significant amount of

ATPase activity is present. If these tissues were frozen overnight and assayed the next day, increased specific activity resulted and less inhibition was found; this finding paralleled the diminution in ATPase activity. Addition of fluoride, or removal of inert material by treatment with protamine sulfate, also decreased the degree of inhibition observed. When tissues were homogenized in sucrose with a Potter-

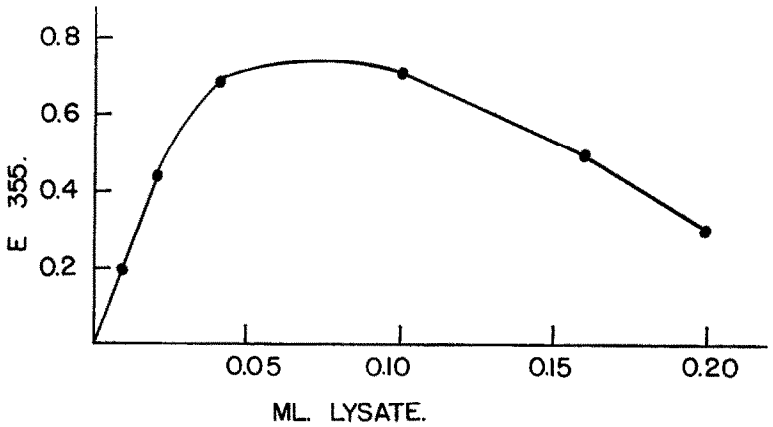

FIG. 1. The effect of increasing enzyme concentration on dihydrofolate reductase activity. The complete system contained in a 1-ml volume: 100 μmoles Tris (pH 7·5), 10 μmoles $MgCl_2$, 5 μmoles ATP, 50 μmoles NH_4Cl, 0·2 μmole NADPH, 0·2 μmole dihydrofolate, and 50 μmoles sodium formate. The enzyme concentration (supernatant solution from centrifuging a homogenate of guinea pig liver) was varied as indicated. The assay mixture was incubated at 37° for 30 min ,and 10% trichloracetic acid was added to stop the reaction. The product was estimated by light absorption at 355 mμ; a reading blank (less formate) was treated in a similar manner.

Elvehjem homogenizer and a supernatant solution 37,000 × *g* was used as the enzyme source, linearity over a wider range was noted. The use of 0·015 M acetic acid in the extraction resulted in clear homogenates that could be used in the direct assay for dihydrofolate reductase, but this procedure resulted in a marked reduction of formate-activating enzyme activity. As a result of these difficulties, and in order to obtain a value in a range where dihydrofolate reductase activity was linearly dependent on the amount of homogenate added, the enzyme activity of each tissue was assayed in three concentrations of enzyme. The greater activity of the formate-activating enzyme found in the tissues assayed allowed for the use of smaller aliquots of homogenates and consequently little difficulty was observed in obtaining activity proportional to enzyme concentration.

The activities of these two enzymes in guinea pig tissues are listed in Table 2. Of interest is the high level of both these enzymes in liver and kidney. The level of the formate-activating enzyme is an order of magnitude greater than that of dihydrofolate reductase activity in every tissue examined. Formate-activating enzyme activity was present in appreciable amount in every tissue assayed, as contrasted to the low levels of dihydrofolate reductase found in skeletal muscle and brain.

The distribution of administered amethopterin in guinea pig tissues

A single dose of 5 mg amethopterin/kg body weight was administered subcutaneously to five guinea pigs. At days 1, 3, 5, 7, and 14 after the injection of drug, a treated and

untreated animal were sacrificed and the amethopterin in liver, kidney, spleen, bone marrow, and small intestine was determined in the manner described. Kidney and liver contained measurable amounts of the inhibitor on each of these days, whereas little or no activity was found in the spleen, bone marrow, and small intestine (Table 3). Lysates of the tissues of control animals, sacrificed at the same time, showed no inhibitor activity.

TABLE 2. ACTIVITY OF DIHYDROFOLATE REDUCTASE AND FORMATE-ACTIVATING ENZYME IN GUINEA PIG TISSUES

Source	No. of samples	Formate-activating enzyme (μmoles/hr/mg protein)	Dihydrofolate reductase (μmoles/hr/mg protein)
Liver	10	14·2 (10·7 –20·1)	0·28 (0·18–0·39)
Kidney	10	2·4 (1·2 – 4·3)	0·13 (0·08–0·16)
Small intestine	10	2·5 (1·8 – 3·1)	0·06 (0·04–0·09)
Bone marrow	10	0·80 (0·38– 1·2)	0·07 (0·03–0·10)
Spleen	10	0·70 (0·42– 0·91)	0·05 (0·04–0·09)
Leukocytes	6	0·60 (0·50– 0·70)	0·08 (0·07–0·11)
Erythrocytes*	12	360 (190–492)	6·2 (5·0 –8·6)
Ovary	3	0·60 (0·47– 0·70)	0·03 (0·02–0·06)
Cerebrum	3	0·40 (0·26– 0·50)	0·02 (0·01–0·03)
Skeletal muscle	3	0·40 (0·18– 0·52)	0·01 (0 –0·01)

* Micromoles per hour per gram hemoglobin.

TABLE 3. AMETHOPTERIN CONTENT OF TISSUES OF GUINEA PIGS SACRIFICED AT VARIOUS INTERVALS AFTER ADMINISTRATION OF AMETHOPTERIN

Days after amethopterin	Liver	Kidney	Spleen	Bone marrow	Small intestine
	(10^{-6} μmole amethopterin/mg protein)*				
0	<0·1	<0·1	<0·1	<0·1	<0·1
1	4·4	4·9	<0·1	<0·1	<0·1
3	4·6	0·3	<0·1	<0·1	<0·1
5	4·6	1·7	0·4	<0·1	<0·1
7	10·0	1·9	<0·1	<0·1	<0·1
14	9·3	0·1	<0·1	<0·1	<0·1

* Under the conditions of the assay, 1×10^{-7} μmole amethopterin/mg protein was detectable in the lysates.

The effect of amethopterin on dihydrofolate reductase activity in guinea pig tissues

The level of dihydrofolate reductase activity was determined in tissue extracts of liver, spleen, bone marrow, small intestine, and erythrocytes in animals sacrificed 1, 3, 5, 7, 14, and 21 days after the subcutaneous administration of amethopterin (5 mg/kg body weight). As shown in Table 4, except for a possible decrease in dihydrofolate reductase activity produced in the kidney, spleen, and bone marrow 24 hr after the injection of amethopterin, appreciable inhibition of enzyme activity was not observed. On the other hand, a *rise* in erythrocyte enzyme activity was seen at days 7, 14, and 21, and this observation has been confirmed repeatedly in other studies. Although spleen and bone marrow may have exhibited a transient increase in activity, the range of values observed in tissues of untreated animals precludes any valid

conclusions concerning this observation, in view of the small number of animals used in this experiment. Leukocyte activity was not measured, since the small amount of blood obtained from single animals did not yield enough white blood cells to permit a determination of their dihydrofolate reductase activity at three levels of enzyme.

TABLE 4. LEVELS OF DIHYDROFOLATE REDUCTASE ACTIVITY IN GUINEA PIG TISSUES AFTER ADMINISTRATION OF 5 MG AMETHOPTERIN/KG BODY WEIGHT

Days after amethopterin	Liver	Kidney	Dihydrofolate reductase (μmoles/hr/mg) Spleen	Bone marrow	Small intestine	RBC†
0*	0·28	0·13	0·05	0·07	0·06	6·2
1	0·24	0·07	0·02	0·04	0·08	7·0
3	0·28	0·11			0·08	7·0
5	0·31	0·14	0·09	0·05	0·05	
7	0·31	0·07	0·12	0·10	0·07	13·0
14	0·26	0·12	0·04	0·09	0·06	11·0
21	0·32	0·15	0·04		0·05	13·0

* Average of ten animals (see Table 2 for range).
† Micromoles per hour per gram hemoglobin.

The effect of folic acid deficiency on the formate-activating enzyme activity and dihydrofolate reductase activity in guinea pig tissues

Table 5 summarizes the results of experiments performed on the two groups of animals fed folate-free diets, with and without folate supplementation. No significant differences in enzyme activities were found in the tissues examined.

TABLE 5. FORMATE-ACTIVATING ENZYME ACTIVITY AND DIHYDROFOLATE REDUCTASE ACTIVITY IN TISSUES OF FOLATE-DEFICIENT AND FOLATE-SUPPLEMENTED GUINEA PIGS

Tissue	Formate-activating enzyme* Control	Deficient	Dihydrofolate reductase* Control	Deficient
Liver			0·18 (0·14–0·23)	0·15 (0·10–0·20)
Bone marrow	0·60 (0·32–1·0)†	0·79 (0·45–1·3)	0·07 (0·06–0·08)	0·07 (0·04–0·15)
Erythrocytes	228 (140–356)‡	180 (104–264)	10·1 (8·0–13·9)	9·4 (6·8–18·8)
Leukocytes	0·91 (0·81–1·6)	1·5 (0·4–2·3)	0·07 (0·05–0·11)	0·10 (0·05–0·14)

* Micromoles per hour per milligram protein.
† Average of 6 animals for each tissue.
‡ Micromoles per hour per gram hemoglobin.

Formate-activating enzyme activity and dihydrofolate reductase activity in the blood cells of various animals

The finding that the erythrocytes and leukocytes of guinea pigs, unlike those of man, contained easily measurable levels of dihydrofolate reductase activity prompted a survey of the level of this enzyme activity in the leukocytes and erythrocytes of various animal species. Measurable levels of dihydrofolate reductase activity were present in the erythrocytes of the chicken and of the rodent species studied; in contrast, the pig had relatively low enzyme activity, and the erythrocytes of both dog and man

had virtually no measurable enzyme activity (Table 6). The relative activity of this enzyme in erythrocytes of various species is similar to that observed for formate-activating enzyme activity.[24] Leukocytes of all animal species, including those of the dog, had measurable dihydrofolate reductase enzyme activity, in contrast to the extremely low levels present in leukocytes from normal human subjects. The presence

TABLE 6. DIHYDROFOLATE REDUCTASE ACTIVITY IN ERYTHROCYTES AND LEUKOCYTES OF VARIOUS ANIMAL SPECIES

Species	No. of samples	Dihydrofolate reductase activity RBC (μmoles/hr/g Hb)	WBC (μmoles/hr/mg)
Chicken	1	6·3	
Mouse	3	18·1 (14·5 –24·1)	
Rat	3	1·3 (0·92– 1·6)	0·02 (0·01–0·3)
Guinea pig	12	8·5 (5·0 –12·4)	0·08 (0·5 –0·11)
Rabbit	3	2·5 (2·0 – 3·5)	0·16 (0·10–0·21)
Pig	1	0·53	0·03
Dog	5	<0·1	0·01 (0–0·03)
Man	20	<0·1	0·005 (0–0·005)

of this enzyme activity in animal leukocytes may be related to the number of dividing cells, or those capable of mitosis. The rat, guinea pig, rabbit, and pig all have a higher percentage of mononuclear cells in the blood, as compared to the percentage of granulocytes.

DISCUSSION

Except for the studies of Werkheiser,[29] who measured the levels of folic reductase activity in the liver, small intestine, and kidney of the rat, guinea pig, and mouse, to our knowledge there have been no studies of the distribution of this enzyme activity in the tissues.* The results reported here are in general agreement with those of Werkheiser in that the relative amounts of enzyme activity in liver, kidney, and small intestine are similar; however, the enzyme activities were measured by different methods and are expressed differently, and for these reasons it is difficult to make quantitative comparisons. Whiteley[31] measured the levels of certain tetrahydrofolate-dependent enzymes, including the formate-activating enzyme activity, in a number of organisms including the rabbit and chicken. The relative activities of the formate-activating enzyme in rabbit tissues were comparable to those found in the guinea pig in the present study.

In view of the importance of dihydrofolate reductase in the synthesis of precursors of nucleic acids, in particular thymidylate,[13,14,32] it was of interest to find that this enzyme activity can be correlated in a general way with the amount of mitotic activity; however, the liver and kidney appear to offer important exceptions. These organs not only contain the highest levels of folate compounds,[33] but also, as has been shown

* Since the completion of this work, Roberts and Hall[30] have reported the folate reductase activity in a number of tissues from various animal species. Although levels were not reported in this preliminary communication, it is of interest that these authors were not able to detect appreciable enzyme activity in muscle or lung tissue.

previously[34–36] and in the present study, they retain the greatest amount of administered amethopterin. These findings support the concept that the liver and kidney represent the organs in which synthesis and storage of 5-methyl tetrahydrofolate, probably the major storage form of the folates, occurs. Thus, in man, an early response to a folate-deficient diet[37] as well as to amethopterin administration is a decrease in the amount of circulating 5-methyl tetrahydrofolate.[38]

The finding that peripheral leukocytes and erythrocytes of various animal species have measurable dihydrofolate reductase activity, as compared to that in human leukocytes and erythrocytes, is of interest. Studies of the formate-activating enzyme in the erythrocytes of animal species have shown that the amount of this enzyme activity is inversely proportional to the life span of these cells. The increased levels of dihydrofolate reductase activity may be accounted for in part by the presence in the peripheral blood of these animals of an increased number of immature cells or cells capable of reproduction, since bone marrow leukocytes have higher levels of this enzyme activity than have mature granulocytes.[39] Other enzymes involved in the synthesis of nucleic acids, such as thymidylate synthetase,[40] pyrimidine synthesizing enzymes,[41] and thymidine kinase,[42] also are decreased in normal circulating leukocytes, as compared to those in bone marrow cells or in the cells obtained from patients with acute or chronic myelocytic leukemia.

The increase in dihydrofolate reductase activity observed in the erythrocytes of guinea pigs after the administration of amethopterin is of interest, since similar results have been observed in man.[10] Unlike human erythrocytes, however, the untreated cells of the guinea pig have a relatively high level of activity, and the rise observed after single, nonlethal doses of amethopterin has been at most fourfold. It was not possible to obtain valid data on possible 'induction' of dihydrofolate reductase in guinea pig leukocytes because cells were not obtainable in sufficient amounts. The other tissues studied did *not* show any significant increase of dihydrofolate reductase activity after the administration of amethopterin.

The natural resistance of the guinea pig to relatively high doses of amethopterin is not well understood. In these studies, large single doses of amethopterin did not result in an accumulation of the drug in the two important tissues (bone marrow and intestine) probably involved especially in the lethal effects of the drug. This finding may be important in understanding the natural resistance to amethopterin exhibited by this species. The lack of significant enzyme inhibition produced by the doses of amethopterin used, when enzyme activity is measured at pH 7·5 with dihydrofolate as the substrate, also is of interest; however, it may not accurately reflect conditions *in vivo* because of the conditions of the assay.[39] Nevertheless, the lack of either significant amethopterin uptake or retention by small intestine and bone marrow would indicate that little or no inhibition was produced in these tissues. The lack of uptake of amethopterin by bone marrow and small intestine is being investigated further. Although the most likely explanation for this finding is lack of transport of the drug into the cells, other possibilities must be considered—e.g. poor binding of inhibitor to enzyme, inactivation of amethopterin by these tissues, and the capacity of these tissues to utilize either tetrahydro derivatives of folates or preformed purines and thymidylate.

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