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PRESENCE AND PROPERTIES OF DIHYDROFOLATE REDUCTASES WITHIN THE GENUS CRITHIDIA

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SUMMARY

1. The properties of dihydrofolate reductases in the non-pathogenic easily cultured trypanosomatid flagellates *Crithidia oncopelti* and *Crithidia fasciculata* have been investigated. Similar amounts of reductase activity/mg protein were found in high speed centrifugal supernatant fractions of these organisms after disruption in the Mickle disintegrator. The enzyme in *C. oncopelti* was highly active between pH values of 5.6 and 7.0 and that in *C. fasciculata* between pH values of 5.6 and 8.0. Michaelis' constants for the two enzymes were about $3 \cdot 10^{-6}$ M for dihydrofolate and $1 \cdot 10^{-5}$ M for NADPH. Folate, biopterin and dihydrobiopterin (all at concentrations of 10^{-4} M) were ineffective as substrates for either enzyme; however, folate inhibited both enzymes at this concentration by about 50% while dihydrobiopterin and biopterin were without effect. The enzymes were also inhibited about 50% by methotrexate ($6 \cdot 10^{-9}$–$8 \cdot 10^{-9}$ M), pyrimethamine ($5 \cdot 10^{-6}$ M) and trimethoprim ($2 \cdot 10^{-5}$–$2 \cdot 10^{-4}$ M).

2. The dihydrofolate reductases in these two flagellates are thus similar in general properties to all such enzymes that have so far been described.

3. Some evidence is presented which suggests that *C. oncopelti* preparations may contain more than one dihydrofolate reductase.

INTRODUCTION

Recent comparative studies of trypanosomal dihydrofolate reductases (EC 1.5.1.4) (ref. 1) have been hampered by the lack of available material. Since this enzyme had been described in freeze/thawed preparations of the easily cultured trypanosomatid flagellates, *Crithidia fasciculata* and *Crithidia oncopelti*[2] we were prompted to investigate the properties of this enzyme in these organisms, to determine if either would serve as a model trypanosomal dihydrofolate reductase. In addition, we wished to ascertain if the nutritional requirements of *C. fasciculata* for both a conjugated pteridine (folate) and an unconjugated pteridine (biopterin)[3] were an indication that the one-carbon

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metabolism of the organism involved features not found in other folate-requiring organisms.

MATERIALS AND METHODS

Crithidia fasciculata (Anopheles strain, ATCC 11745) was obtained from Prof. G. W. Kidder, Amherst College, Amherst, Mass. (U.S.A.). It was grown at 25° in an undefined medium containing 2% (w/v) Difco proteose peptone, 1% (w/v) Difco liver infusion and 1% (w/v) glucose, together with supplements of adenine (50 mg/l), folic acid (5 mg/l) and haemin (25 mg/l). Medium (initial pH value 8.0) was distributed 5 ml/boiling tube or 100 ml/l Roux bottle and the containers were closed with cotton wool plugs. *Crithidia oncopelti* (obtained from Dr. B. A. Newton, Department of Biochemistry, University of Cambridge) was grown in the undefined, peptone-containing medium described by NEWTON AND HORNE[4]. Organisms were always harvested while in the exponential phase of growth ($1 \cdot 10^7$–$2 \cdot 10^7$ organisms/ml).

Harvesting was carried out at 0° by centrifugation (2500 × *g* for 10 min). Organisms were then washed twice in cold buffered salts (NaCl, 9 g; KCl, 0.4 g; Na_2HPO_4, 3 g; KH_2PO_4, 0.4 g; glass distilled water to 1 l, pH value to 7.2) and then resuspended at 10^9 organisms/ml in buffered salts supplemented with *N*-acetylcysteine (0.56%, w/v). These washed cell suspensions were then disrupted in the MICKLE[5] disintegrator and the supernatant fluids, after centrifugation at 100 000 × *g* for 60 min were used in the dihydrofolate reductase assay. Their protein content was estimated by the modification of LOWRY *et al.*[6] of the Folin reagent. The method of assay has previously been described[7] and is based on a decrease in absorbance at 340 mμ as NADPH is oxidised and dihydrofolic acid is reduced. The reaction was carried out, except where indicated, in 0.1 M phosphate buffer, pH 7.0.

To prepare a particle fraction from Mickle-disrupted preparations of *C. oncopelti*, the homogenate was first centrifuged at 2500 × *g* for 10 min to remove large cell debris. The supernatant fluids were then further centrifuged at 10 000 × *g* for 10 min, the resulting pellet being termed the particle fraction. This fraction was washed 3 times in cold buffered salts and was then disrupted in the Hugh's Press. The supernatant fluids, after a high speed centrifugation (100 000 × *g* for 60 min) were then assayed directly for dihydrofolate reductase activity.

The sources of the inhibitors used in this study have previously been mentioned[1]. Biopterin was obtained from Dr. Alfred Maass, of Smith, Kline & French Inc., Philadelphia, Pa. (U.S.A.). 7,8-Dihydrobiopterin was synthesized by the method of KAUFMAN[8].

RESULTS

Similar amounts of dihydrofolate reductase activity were detected in preparations of both *C. fasciculata* and *C. oncopelti* (about $1 \cdot 5$ μmoles dihydrofolate reduced/min per mg protein). The relationship between the rate of the reaction and the concentration of dihydrofolate for the enzyme in *C. oncopelti* is shown in Fig. 1. The apparent Michaelis' constants (K_m) with respect to dihydrofolate were the same for both enzymes, the values obtained of $3 \cdot 10^{-6}$–$4 \cdot 10^{-6}$ M being well within the extremes ($1 \cdot 10^{-7}$ and $2 \cdot 10^{-5}$ M) for this parameter which appear in the literature[7]. Folic acid,

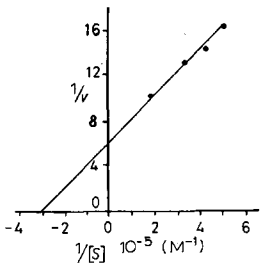

Fig. 1. Reciprocal of reaction velocity *versus* reciprocal of concentration of dihydrofolate of enzyme of *C. oncopelti* in 0.1 M potassium phosphate buffer (pH 7.0) containing NADPH ($8 \cdot 10^{-5}$ M) and measured at 37°. v is expressed as decrease in absorbance at 340 mμ over a period of 5 min, or, at lower substrate concentrations, where the linear part of the reaction is completed within this time, the calculated rate assuming the reaction had continued to be linear.

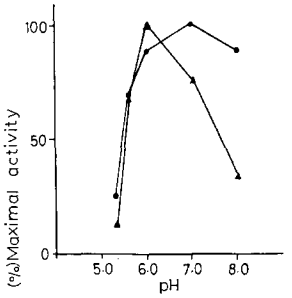

Fig. 2. Effect of pH on the activity of dihydrofolate reductases from *C. fasciculata* and *C. oncopelti*. The incubations (enzyme in 0.1 M potassium phosphate buffer with NADPH and dihydrofolate both to $8 \cdot 10^{-5}$ M) were identical in all cases except for the pH value of the incubation medium. ●—●, *C. fasciculata*; ▲—▲, *C. oncopelti*.

however, was not an effective substrate and nor were biopterin or 7,8-dihydrobiopterin. Folic acid (10^{-4} M) in fact inhibited the activity of the enzyme by about 50% but dihydrobiopterin and biopterin at the same concentrations were without significant effect. NADH did not replace NADPH as hydrogen donor; the apparent K_m values of the 2 enzymes with respect to cofactor were essentially the same ($1 \cdot 10^{-5}$ M). The enzyme in *C. oncopelti* was highly active between pH values of 5.6 and 7.0 and that in *C. fasciculata* between pH values of 5.6 and 8.0. pH optima were between pH 6 and 7

TABLE I

COMPARATIVE SENSITIVITIES OF DIHYDROFOLATE REDUCTASES TO INHIBITION BY DIAMINOHETEROCYCLIC ANTIMETABOLITES

The incubations were carried out in 0.1 M potassium phosphate buffer (pH 7.0) containing NADPH and dihydrofolate (both $8 \cdot 10^{-5}$ M). The figures represent the concentration of drug (expressed in 10^{-8} M) required to inhibit the activity of the enzyme 50%.

Species	*Methotrexate**	*Pyrimethamine***	*Trimethoprim****	*60–212*†	*57–43*††
E. coli[1]	0.1	250	0.5	50	65 000
T. rhodesiense[2]	0.1	20	25	60	700
T. lewisi[2]	0.2	750	2 000	80	6 000
Man[1]	0.2	180	30 000	95	55
C. fasciculata	0.8	300	2 000	110	4 000
C. oncopelti	0.6	500	25 000	100	6 600

[1] Data of BURCHALL AND HITCHINGS[9].
[2] Blood stream forms. Data of JAFFE *et al.*[1].
* 2, 4-Diamino-N^{10}-methylpteroyl glutamate.
** 2, 4-Diamino-5-*p*-chlorophenyl-6-ethylpyrimidine.
*** 2, 4-Diamino-5-(3′,4′,5′-trimethoxybenzyl) pyrimidine.
† 60–212 (B W–60–212): 2,4-diamino-6-butylpyrido[2,3-d]pyrimidine.
†† 57–43 (BW–57–43): 2, 4-diamino-1-(4′-butylphenyl)-1,6-dihydro-6,6-dimethyl-1,3,5-triazine.

for both enzymes (see Fig. 2). The enzymes were extremely sensitive to inhibition by methotrexate, a 4-amino-analogue of folic acid. Concentrations of only $6 \cdot 10^{-9}$ M for the enzyme in *C. oncopelti* and $8 \cdot 10^{-9}$ M for the enzyme in *C. fasciculata* were required to effect inhibitions of 50% (Table I).

In these general properties, the dihydrofolate reductases in these two flagellates are very similar to all the enzymes of this type that have been described, whether from trypanosomal, bacterial, avian or mammalian sources. However, BURCHALL AND HITCHINGS[9] were able to differentiate between mammalian and bacterial dihydrofolate reductases by measuring the concentration of certain 2,4-diaminopyrimidines and related heterocycles (particularly trimethoprim and B.W. 57-43) required to effect a 50% decrease in enzyme activity. Such measurements give an approximate indication of the sensitivity of the reductase systems to inhibition. Using this approach, two distinct patterns of sensitivity were found for mammalian and bacterial dihydrofolate reductases, Man being a typical mammal and *Escherichia coli* a typical bacterium in this respect (Table I). A third pattern of sensitivity to these agents has now been described for the reductases of trypanosomal origin[1], their sensitivities lying between the bacterial and mammalian extremes. The pattern of sensitivity of the reductases from *C. fasciculata* and *C. oncopelti* also falls between the two extremes, being closely similar to the pattern shown by trypanosomes of the section Stercoraria (genus Trypanosoma) (*e.g. T. lewisi*) and rather different from the pattern shown by trypanosomes of the section Salivaria (*e.g. T. rhodesiense*) (Table I).

The dihydrofolate reductase in *C. oncopelti* was only one tenth as sensitive to inhibition by trimethoprim, as was the analogous enzyme in *C. fasciculata*. Indeed the relative insensitivity of the reductase of *C. oncopelti* to this inhibitor stands in sharp contrast to the marked sensitivity of bacterial reductases to it (see Table I). It seemed possible that this enormous difference in sensitivity might be used to resolve the controversy concerning the nature of the bipolar bodies which occur in this organism (see NEWTON[10] for review). If these unusual intracellular structures represent endosymbiotic bacteria as suggested by GILL AND VOGEL[11,12] they would be expected to contain a dihydrofolate reductase which could be distinguished from the endogenous reductase of the organism by its extreme sensitivity to trimethoprim. The high speed centrifugal supernatant fluids of a disrupted preparation of the particle fraction of the organism (which contained bipolar bodies and kinetoplasts) had some dihydrofolate reductase activity (about 1% of the total amount in the organism) but the concentration of trimethoprim required to inhibit this activity 50% was as high as $4 \cdot 10^{-5}$ M. Thus, although it is possible that there might be more than one dihydrofolate reductase in *C. oncopelti*, it is unlikely that either of them are of bacterial origin. In agreement with this conclusion was our observation that organisms incubated for 16 h in a growth inhibiting concentration of trimethoprim (10^{-3} M) contained one nucleus and one kinetoplast but mostly two and sometimes three rather than the normal one bipolar body, indicating that the reproduction of the bipolar body is not particularly sensitive to inhibition by trimethoprim.

DISCUSSION

We have shown that in *C. fasciculata* there is an enzyme which can reduce dihydrofolate (but not folate, biopterin and dihydrobiopterin) efficiently in the pre-

sence of NADPH (but not NADH). The relatively low K_m value of the enzyme with respect to dihydrofolate suggests that this compound is in fact the natural substrate of the enzyme. The enzyme activity was extremely sensitive to inhibition by methotrexate. It can be concluded therefore that the organism contains a classical dihydrofolate reductase with the same basic properties as those of the analogous enzyme in trypanosomes, bacteria, birds and mammals. Since it has already been shown that thymidine and methionine (in the presence of biopterin) can spare the folic acid requirements of *C. fasciculata*[3] it now seems likely that the one-carbon metabolism in the organism is also entirely conventional. This conclusion raises the question as to how the organism synthesises dihydrofolate. Studies with bacterial and mammalian cells suggest that dihydrofolate is synthesised either from preformed folate or by a biosynthetic pathway *de novo*. The observation of KIDDER AND DUTTA[3] that *C. fasciculata* showed a requirement for folate as well as for biopterin would suggest that the former pathway of synthesis is utilised. However, we were unable to detect any evidence of folate utilisation by our preparations, either in the presence of NADPH or NADH. The conclusion reached above also raises the question as to why the organism has a nutritional requirement for biopterin if its folic acid metabolism is conventional. The recent studies of DEWEY AND KIDDER[13] which showed that the biopterin requirement of the organism could be spared by unsaturated fatty acids may be the clue which will give the answer to this question, but further investigations are clearly required.

The relative similarity of the pattern of sensitivity of crithidial reductases and those of stercorarian section trypanosomes to inhibition by 2,4-diaminopyrimidine inhibitors is of interest since such a similarity may indicate a close phylogenetic relationship. Our data indicate, however, that the crithidial reductases cannot be used as model systems to study the reductases of salivarian section trypanosomes (*e.g. T. rhodesiense*) and, unfortunately, it is these species which are most difficult to grow *in vitro*.

Our data further indicate that there might be two dihydrofolate reductases in *C. oncopelti*. However, as was indicated above, our analyses give only an approximate indication of the true sensitivities of the dihydrofolate reductases to inhibitors, so that a 10-fold difference between the two crithidial enzymes may not be significant. HILLCOAT AND BLAKLEY[14] have demonstrated the presence of more than one species of dihydrofolate reductase in a mutant strain of *Streptococcus faecalis* and multiple forms of chicken liver dihydrofolate reductase have recently been described[15] but in neither instance has the intracellular location of these forms been ascertained. The existence and location of this possible second reductase in *C. oncopelti* is at present under investigation. Preliminary results suggest that if it exists, it is not located in the bipolar bodies since their reproduction is not particularly sensitive to inhibition by trimethoprim.

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