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Thymidylate Synthetase in Mouse Erythrocytes Infected with *Plasmodium berghei*

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SUMMARY

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Thymidylate synthetase in mouse erythrocytes infected with *Plasmodium berghei* increases markedly during cellular parasitization. The malaria enzyme has been compared with enzymes from normal mouse reticulocytes and *Escherichia coli* by kinetic and physical means. When folic acid analogues were used as cofactors, distinct kinetic differences were apparent when thymidylate synthetases from *P. berghei* and *E. coli* were compared. However, no significant kinetic dissimilarities were revealed in a comparison of enzymes from *P. berghei* and the host mouse reticulocytes. Upon gel filtration the *P. berghei* thymidylate synthetase exhibits an apparent molecular weight in excess of 100,000 whereas the normal mouse reticulocyte enzyme shows an apparent molecular weight of 68,000, similar to the enzyme from *E. coli*.

INTRODUCTION

The emergence of drug-resistant strains of *Plasmodium falciparum* in South America, Southeast Asia, and other tropical areas is an unpleasant reminder that malaria is still a worldwide problem. Increasing reports of drug-refractory malaria have spurred efforts to develop new antimalarials effective against chloroquine-resistant parasites.

As part of a study aimed at elucidating the role of folic acid in malaria (1), a folate-requiring enzyme, thymidylate synthetase, was investigated in mouse erythrocytes parasitized with *Plasmodium berghei*.

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Thymidylate synthetase catalyzes the following reaction:

$$\text{Deoxyuridine 5'-phosphate} + N^5,N^{10}\text{-methylenetetrahydrofolate} \xrightarrow{Mg^{++}} \text{deoxythymidine 5'-phosphate} + \text{dihydrofolate}$$

Our rationale has been that specific information about this key enzyme in nucleic acid metabolism could lead to the design of more effective antimalarials. This paper describes various kinetic and physical properties of thymidylate synthetase characteristic of mouse malaria.

METHODS

1. *Preparation of Extracts from Parasitized Erythrocytes*

The strain of *P. berghei* (NK658) used for this study was kindly supplied by Drs. H. Most and M. Yoeli of New York University, Bellevue Medical Center, New York. The strain was carried primarily in DBA/2J

male mice obtained from Jackson Laboratories, Bar Harbor, Maine. During interim periods when infected blood was not required, *P. berghei* was carried in male Syrian hamsters also supplied by Jackson Laboratories. DBA/2J mice received an injection of blood containing 2×10^7 infected red blood cells. In approximately 5–7 days the mice were bled by cardiac puncture. At term, 25–45% of the red cells were infected. Ether anesthesia was used to facilitate intracardial sampling of blood.

After collection of blood, the erythrocytes were centrifuged, washed twice by resuspension in 0.15 M KCl for removal of plasma, and then lysed in buffer C. The lysate was poured into a French pressure cell, and 10,000–13,000 psi were applied, after which it was centrifuged for 10 min to remove red cell ghosts. The supernatant fluid was used as the source of crude enzyme. All steps were carried out at 0–4°.

2. *Assay of Thymidylate Synthetase*

The method for determining thymidylate synthetase activity was a modification of procedures described by Smith-Lomax and Greenberg (2) and Roberts (3). The assay depends on the labilization of tritium attached to carbon 5 of deoxyuridine 5′-phosphate.

The components of the thymidylate synthetase assay system consisted of [^{3}H]deoxyuridylate, 0.1 mM (4.2×10^7 dpm/μmole); *dl*-tetrahydrofolate, 1.6 mM; formaldehyde, 3.0 mM; $MgCl_2$, 7.8 mM; and Tris buffer, pH 7.4, 0.25 M. The total volume, including the enzyme, was 116 μl. After 30 min of incubation at 37°, 0.5 ml of 0.8 mM dUMP and 0.5 ml of charcoal suspension (200 mg/ml) in 8% trichloracetic acid were added. The mixture was then centrifuged, and to a 700-μl sample of the supernatant fluid were added a further 0.4 μmole of dUMP and 0.5 ml of the charcoal suspension. The suspension was centrifuged, and 1 ml of supernatant fluid was taken for assay of radioactivity by addition to 15 ml of DAM 611 liquid scintillation fluid (4).

Purification of [5-^{3}H]deoxyuridine 5′-monophosphate. Radiochemical destruction of ^{3}H-labeled dUMP leads to increasing blanks in the assay. A simple procedure involving application of ^{3}H-labeled dUMP to a small column of DEAE-cellulose, followed by washing with 1 mM NH_4HCO_3, elution of the radioactive nucleotide with 250 mM NH_4HCO_3, and removal of NH_4HCO_3 by evaporation under vacuum (water added several times), yields material with low blanks.[3]

3. *Catalytic Hydrogenation of Folate Analogues*

Analogues of folic acid were reduced as follows. Approximately 10 mg of platinum oxide were suspended in 1.5 ml of potassium phosphate buffer, pH 7.1, and hydrogenated. This reduction took about 15–20 min. Then approximately 2 mg of *N*-pteroyl-DL-α-aminopimelic acid, N^{α}-pteroyl-N^{ϵ}-(*tert*-butyloxycarbonyl)-L(+)-lysine, or N^{α}-pteroyl-L(+)-lysine (5) were added to the reaction flask through a serum cap after being dissolved in 0.3 ml of water with the addition of 1 NaOH. Aliquots of the mixture were removed periodically for spectrophotometric assay in order to follow the progress of the reduction. After reduction was complete, enough mercaptoethanol was added to make the final concentration 1 M.

4. *Preparation of Extracts of Reticulocytes*

A 2.5% phenylhydrazine hydrochloride solution was prepared and neutralized with sodium hydroxide. The solution was stored at 15° in the presence of 1 mM reduced glutathione (6). To produce reticulocytosis, 0.1 ml of the phenylhydrazine solution was injected intraperitoneally into mice daily for 4 days. No injections were given on the fifth day, to allow the phenylhydrazine to be metabolized. The blood withdrawn from the mice by cardiac puncture was washed twice with 0.15 M KCl and stored frozen. An extract was prepared from these cells by suspension in buffer C and passage through the French pressure cell as described above.

5. *Buffers*

Buffer A was 50 mM Tris-Cl (pH 8.4)–10 mM mercaptoethanol–1 mM ethylenediaminetetraacetate; buffer B, same as buffer A but adjusted to pH 7.4 instead of 8.4; buffer C, same as buffer A except that the

[3] L. VandeVenter and M. Friedkin, unpublished method.

concentration of Tris-Cl was 5 mM instead of 50 mM.

RESULTS

Infection of Mouse Erythrocytes by P. berghei (NK658)

The differential infection of mouse erythrocytes and *not reticulocytes* by the strain of *P. berghei* (NK658) used in our study made possible a comparison of thymidylate synthetases from *P. berghei* and from mouse reticulocytes.

Infected and noninfected mature erythrocytes as well as infected and noninfected reticulocytes could be distinguished easily by use of the following staining procedure.

A small amount of infected blood was mixed with an equal volume of new methylene blue and allowed to react for 3 min (usually in a micropipette to reduce evaporation). Then a thin blood smear was made. Wright's stain was applied for 2.5 min, followed by rinsing with water and counterstaining with Giemsa. After 25 min the slide was rinsed with water and air-dried. With this technique reticulocytes, infected reticulocytes, and infected mature erythrocytes could be distinguished. Erythrocytes were stained a buff color and reticulocytes exhibited blue-stained granules superimposed on the buff background. Infected reticulocytes were easily distinguishable by the presence of the stained plasmodium in a reticulocyte. *P. berghei* is characterized by a blue cytoplasm with red nuclei. The cytoplasm of the parasite was uniformly dense. An infected mature erythrocyte was distinguishable by exhibiting the stained plasmodium on a buff background without granules.

Nine DBA/2J male mice received an injection of the *P. berghei* (NK658) strain, and a similar group of animals were treated a strain of *P. berghei* known to infect reticulocytes (kindly provided by Dr. King, ITT Research Institute, Chicago). Blood smears from both groups of infected animals were prepared as described above during the first 7 days of infection.

Examination of the slides indicated that *P. berghei* strain NK658 was a mature erythrocyte infection as carried in DBA/2J male mice. No reticulocytosis was produced during the course of the infection. In contrast, the strain of *P. berghei* obtained from Dr. King was shown to infect reticulocytes. During the course of infection by the King strain no infected erythrocytes could be detected.

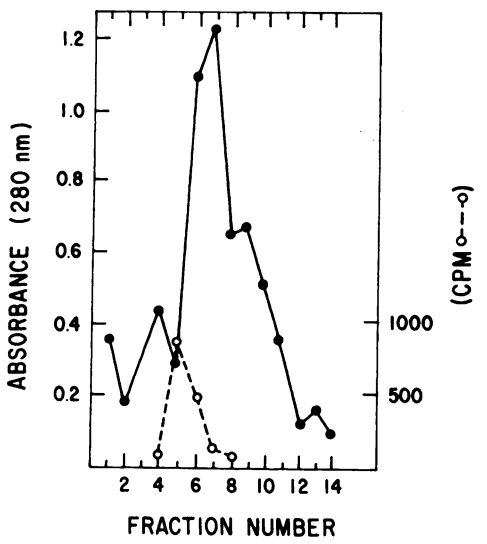

FIG. 1. *Separation of thymidylate synthetase from hemoglobin on DEAE-cellulose columns*

See the text for details of the procedure. The solid line represents elution of protein, measured by absorbance at 280 nm. The dashed line indicates thymidylate synthetase activity.

Separation of Thymidylate Synthetase from Hemoglobin by DEAE-cellulose Chromatography

DEAE-cellulose columns were prepared in two sizes, 1.0 × 10 cm for 100 mg of protein and 3.0 × 10 cm for 500 mg of protein. Each column was equilibrated with buffer A, and then an enzyme extract of parasitized erythrocytes (see METHODS) was applied, followed by washing with buffer B. At this point hemoglobin was eluted from the column and the enzyme remained bound to the DEAE-cellulose. Thymidylate synthetase was eluted with 0.4 M $(NH_4)_2SO_4$ in buffer B. A typical elution pattern is shown in Fig. 1. With the removal of hemoglobin there was a 50-fold purification of thymidylate synthetase.

Increase of Thymidylate Synthetase Activity during P. berghei Infection of Erythrocytes

Preparations of whole blood from infected and noninfected DBA/2J mice were diluted so that all samples contained 2.6×10^9 erythrocytes. Cells were lysed with buffer C. After centrifugation the supernatant fluid, containing 60 mg of protein, was subjected to chromatography on a DEAE-cellulose column (1×10 cm) for removal of hemoglobin (see previous section); 1.1-ml fractions were collected and samples were taken for determination of thymidylate synthetase activity.

After removal of hemoglobin by chromatography on DEAE-cellulose the thymidylate synthetase activity in extracts of erythrocytes was proportional to the time of incubation and the amount of extract used (Fig. 2). This validation of the assay procedure allowed us to estimate changes of thymidylate synthetase activity during the course of parasitemia. As shown in Fig. 2, the enzyme activity in erythrocytes increased approximately 7-fold during the infection with *P. berghei*.

The source of thymidylate synthetase in blood samples from *noninfected* mice was then investigated. Red cells were separated from white cells by the following procedure. Red and white cells were counted in samples of whole blood from normal uninfected mice. Rouleaux formation was induced by the addition of 1 volume of fibrinogen, 2.7% in 0.15 M KCl, to 2 volumes of whole blood. The erythrocytes were allowed to settle and the supernatant fluid was removed. The erythrocytes were diluted to the original volume, and a fraction of blood equivalent to 2×10^{10} erythrocytes was taken. Fibrinogen in KCl was added to induce rouleaux formation, and the procedure was repeated three times. After the final sedimentation of red cells, the blood was centrifuged and the buffy coat was removed.

Despite the fact that over 73% of the white blood cells were removed by this method of differential centrifugation, the amount of thymidylate synthetase per red cell remained constant. We conclude that the basal level of thymidylate activity in mouse blood samples is due to the intrinsic activity of normal mouse erythrocytes. Although very low, this activity was a constant finding in all preparations of noninfected mouse blood samples tested.

Miscellaneous Findings

The labilization of tritium from [^{3}H]dUMP was completely dependent on the presence of tetrahydrofolate and active enzyme. The pH optimum was found to be quite broad, pH 7.0–8.8, in Tris-Cl buffer. The enzyme activity in whole blood extracts was rapidly inactivated by heating at 55° for 2 min.

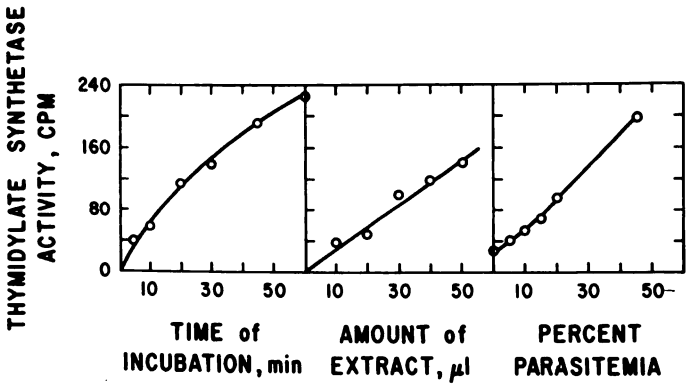

FIG. 2. *Thymidylate synthetase activity as a function of time of incubation, amount of extract, and percentage of parasitemia*

See METHODS for experimental details of assays.

Fluorodeoxyuridylate at a concentration of 0.1 μM produced 50% inhibition.

Localization of Thymidylate Synthetase

All extracts of parasitized erythrocytes were prepared from lysed cells followed by passage through the French pressure cell (see METHODS), a procedure which disrupted the parasites. However, the pressure treatment was not necessary for release of thymidylate synthetase; gentle lysis of parasitized erythrocytes in buffer C sufficed to release 85% of the enzyme in a soluble form.

When parasites were first separated from lysates by centrifugation and then disrupted in the French pressure cell, less than 10% of the thymidylate synthetase activity of the original lysates was present. The enzymatic activity in the supernatant fluid from centrifuged parasites was not diminished by the pressure cell treatment, indicating that this procedure did not denature the enzyme. We are forced to the conclusion that the plasmodium thymidylate synthetase is probably released from the parasite during lysis of the erythrocytes. A much less likely, but not excluded, possibility is that the enzyme can diffuse through the plasmodium cell membrane during infection of the erythrocyte.

Comparison of Thymidylate Synthetases from Parasitized Erythrocytes and Normal Mouse Reticulocytes

Kinetic studies with analogues of tetrahydrofolic acid. Thymidylate synthetases from *P. berghei*-infected erythrocytes and from host reticulocytes were examined for possible differences in kinetic properties by using a variety of tetrahydrofolate analogues as cofactors. This method is a very useful and well-known means of revealing species differences. For example, the pattern of activities with various cofactor analogues is strikingly dissimilar when thymidylate synthetases from *Escherichia coli* and mouse reticulocytes are compared (Table 1). Thymidylate synthetases from parasitized erythrocytes and from reticulocytes yielded a more similar pattern; however, some differences were apparent when tetrahydrohomofolate and tetrahydropteroyl(*tert*-butyloxycarbonyl)lysine were used as cofactors.

TABLE 1

Thymidylate synthetase activities with various tetrahydrofolate analogues

Tetrahydrofolate analogues were prepared as described under METHODS, section 3, and in each case were used in place of tetrahydropteroylglutamate in the incubation mixture (METHODS, section 3). *P. berghei* and reticulocyte enzymes were fractions obtained by DEAE-cellulose chromatography of lysed cells by the method described in Fig. 1. The thymidylate synthetase of *E. coli* was prepared by a modification of previously described procedures (7).

Cofactor	Relative rates		
	E. coli	Reticulocytes	Parasitized erythrocytes
	%	%	%
H_4-pteroyl-L(+)-glutamate	100	100	100
H_4-pteroyl-DL-α-aminopimelate	63	26	21
H_4-pteroyl-N^{α}-L(+)-lysine	40	11	11
H_4-pteroyl-N^{ϵ}-(*tert*-butyloxycarbonyl)-N^{α}-L(+)-lysine	50	124	91
H_4-homopteroyl-L(+)-glutamate	0	13	3

It is interesting in regard to the low but significant activity of tetrahydrohomofolate with reticulocyte enzyme that this analogue can act as a cofactor of thymidylate synthetase in *Streptococcus faecium* and *Lactobacillus casei* (8) but is a powerful inhibitor of the enzyme from *E. coli* (5).

Gel filtration of thymidylate synthetases from parasitized erythrocytes and uninfected reticulocytes. Thymidylate synthetase from lysed erythrocytes parasitized by *P. berghei* appeared at the void volume upon filtration through Sephadex G-100, whereas the enzyme in uninfected reticulocytes was detected just before the elution of hemoglobin (Fig. 3). The reticulocyte enzyme showed the same elution pattern as bovine serum albumin and *E. coli* thymidylate synthetase, with an apparent molecular weight of 68,000 (7).

In order to rule out the possibility that the *P. berghei* enzyme formed a high molecular weight complex with other proteins, the

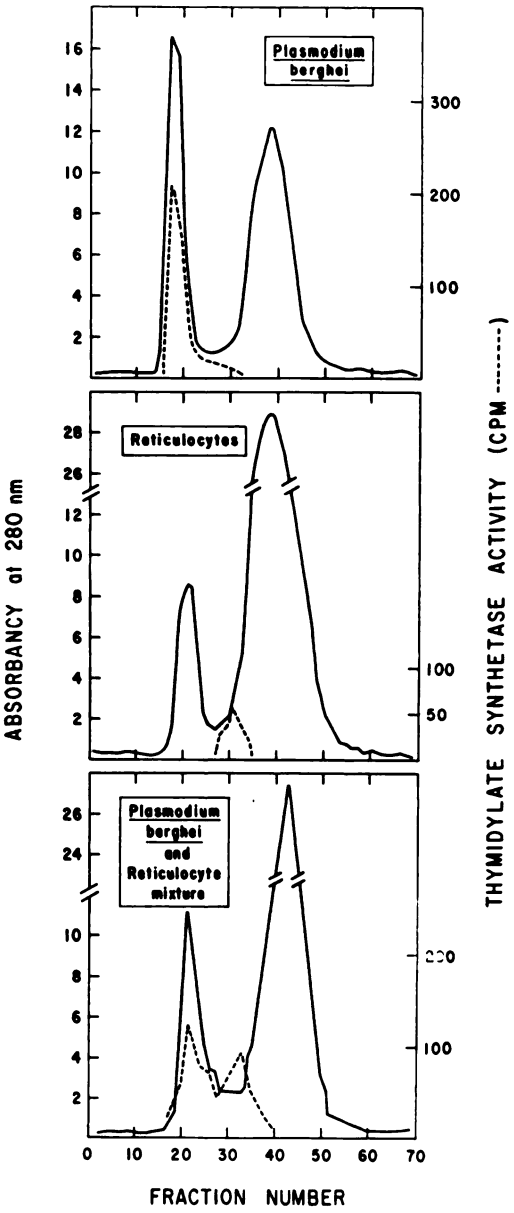

FIG. 3. *Gel filtration of thymidylate synthetases from P. berghei-infected erythrocytes and from reticulocytes*

Lysates (see METHODS, sections 1 and 4) of infected erythrocytes (200 mg of protein) and/or of noninfected reticulocytes (138 mg of protein) were layered onto a column of Sephadex G-100 (2.2 × 64.5 cm) that had been equilibrated with buffer A, and then eluted with buffer A. Fractions 1–15 contained 4.5 ml, and each succeeding fraction, 2 ml. Top, extract of enzyme from infected cells; center, extract of reticulocytes; bottom combined extracts from infected cells and from reticulocytes.

thymidylate synthetases from infected erythrocytes and normal reticulocytes were mixed and then subjected to gel filtration. As shown in the bottom panel of Fig. 3, two distinct peaks of enzyme activity were seen, one appearing at the void volume and the other at the ascending foot of the hemoglobin peak.

These findings support the view that thymidylate synthetase in parasitized erythrocytes is distinctly different from normal host enzyme obtained from reticulocytes. Although the exact molecular weight of the plasmodium enzyme was not determined in this study, it must be 100,000 or greater in view of its exclusion from Sephadex G-100.

DISCUSSION

Pyrimidine synthesis in parasites has been an obvious target for chemotherapeutic attack, generating a large number of biochemical studies. The central role of folic acid in nucleic acid metabolism and the known action of the sulfonamides and antifolates, such as pyrimethamine and chloroguanide (9), have prompted investigations of folic metabolism in plasmodia by Trager in 1961 (10), by Ferone and Hitchings in 1966 (11), and by Platzer in a very recent work (12).

Of special interest to us has been the enzymatic synthesis of thymidylate *de novo* from deoxyuridylate via a tetrahydrofolate-dependent 1-carbon transfer reaction. Thymidylate synthetase has been detected in a number of protozoa—in the avian malaria parasite *Plasmodium lophurae* (13), in *Plasmodium chabaudi*, *Trypanosoma gambiense*, and *Trypanosoma lewisi* (14), and now, as reported in this paper, in *Plasmodium berghei*.

In the present study we have shown that upon infection of mouse erythrocytes by *P. berghei* a marked increase of thymidylate synthetase occurs in the red cell, a result very similar to what happens when nucleated avian erythrocytes become infected with *P. lophurae* (13). In view of the fact that the salvage pathway for utilization of thymidine is apparently inoperative in *P. berghei* (15), we concur with Walsh and Sherman (13) that the synthesis of thymidylate is probably the point in the pathway of nucleic acid

synthesis most sensitive to the action of folate analogues. There is strong evidence that folate-dependent purine synthesis *de novo* is *not* required by the parasite, since endogenous host purines can be utilized (13, 15).

Our interest in comparing the plasmodial and host thymidylate synthetases was aroused by the strikingly selective action of pyrimethamine on plasmodial and erythrocyte dihydrofolate reductases. As shown by Ferone, Burchall, and Hitchings (16), pyrimethamine is 50% inhibitory at 0.5 nM with the plasmodial enzyme, compared to the much higher value of 1 μM required to affect the host dihydrofolate reductase.

Although our kinetic data with tetrahydrofolate analogues did not reveal sharp differences between the plasmodial and host thymidylate synthetases (Table 1), the finding of a much higher molecular weight for the *P. berghei* enzyme, 100,000 or greater, compared to a molecular weight of approximately 68,000 for the host enzyme (Fig. 3), convinced us that the two enzymes are indeed dissimilar.

During the early stages of our investigation the similarity of activities with the tetrahydrofolate analogues suggested that the plasmodial infection might result in the production of more *host* thymidylate synthetase. Another possibility was that parasitized erythrocyte preparations also contained reticulocytes, known to be a source of thymidylate synthetase. We believe this to be highly unlikely, since during the period of infection in which blood was sampled there was no increase in reticulocytes.

The exclusion of plasmodial enzyme on Sephadex G-100, in contrast to the retardation of the reticulocyte enzyme (Fig. 3), strongly suggests that *P. berghei* thymidylate synthetase is indeed a protein with different properties from those of the host enzyme. It is a curious coincidence that *P. berghei* dihydrofolate reductase also has a very high molecular weight, 190,000 (16), in contrast to a host erythrocyte enzyme molecular weight of 20,000. These unusually high molecular weights of two plasmodial enzymes that are so closely linked in function, dihydrofolate reductase and thymidylate synthetase, may be a reflection of unknown regulatory mechanisms unique for plasmodia.

In any event, our finding of a major physical difference between plasmodial and host thymidylate synthetase holds out the possibility that specific antimalarials can be designed that will cut off plasmodial thymidylate synthesis without harm to the host. Certainly the experience with dihydrofolate reductases from different species (17) serves as a promising model for screening compounds that may act as specific inhibitors of the protozoal thymidylate synthetase.

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