Plasmodium berghei Dihydrofolate Reductase

Isolation, Properties, and Inhibition by Antifolates

R. FERONE, J. J. BURCHALL, AND G. H. HITCHINGS

The Wellcome Research Laboratories, Burroughs Wellcome & Co. (U. S. A.), Inc., Tuckahoe, New York 10707

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SUMMARY

Dihydrofolate reductase has been isolated from cells of the rodent malarial organism *Plasmodium berghei* by separation from the host cells with saponin and extraction by rupture in a French pressure cell. The enzyme exhibits certain distinctive properties which clearly distinguish it from the dihydrofolate (H_2-folate) reductase isolated from the host cells (mouse erythrocytes) and from H_2-folate reductases obtained from other sources, as reported in the literature. The molecular weight determined by gel filtration (190,000 ± 10%) is 9–10-fold higher than reported for most other H_2-folate reductases. This value was not significantly decreased when the enzyme was passed over Sephadex G-100 columns in the presence of KCl, urea + 2-mercaptoethanol, or the substrate, H_2-folate. The plasmodial H_2-folate reductase is unlike bacterial enzymes in its stimulation by high concentrations of KCl and urea, and its approximately 10-fold lower K_m value for NADPH (1.3 μM).

The enzyme from *P. berghei* differs markedly from other dihydrofolate reductases in its sensitivity to several diaminoheterocyclic inhibitors. Most striking is the inhibition by pyrimethamine; it exhibits a 50% inhibitory concentration of approximately 0.5 nM compared with 1 μM for the mouse erythrocyte enzyme. The binding of pyrimethamine to *P. berghei* dihydrofolate reductase is stoichiometric when enzyme and drug have been incubated for 5–10 min prior to the addition of H_2-folate, and reversible in the absence of prior incubation. A positive correlation was observed between the binding of pyrimethamine and three dihydrotriazines by the enzyme and the activity of these compounds *in vivo* against *P. berghei* infections. These data establish that the selective action of pyrimethamine in malaria is due to the greater sensitivity to this drug of the plasmodial enzyme as compared to the host enzyme and reflect the unique, potent binding of pyrimethamine to plasmodial H_2-folate reductase.

INTRODUCTION

The enzyme dihydrofolate reductase catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (1, 2). This enzyme is of considerable chemotherapeutic interest, because of its key position in the cellular biosynthesis of purines, pyrimidines, and certain amino acids (1). Close structural analogues of the substrate, such as aminopterin and amethopterin, have been found useful in the treatment of some neoplastic diseases, and the mechanism of action of these drugs has been established as inhibition of this enzyme (3, 4). Hitchings and co-workers have synthesized a number of antifolates less similar in structure to the substrate (see ref. 1). Compounds of this type ["small molecule" antifolates (1)] have been designed which selectively bind the parasite enzymes with greater affinities than the host enzymes, forming the basis

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for successful chemotherapeutic agents (1, 5, 6). We (1, 5, 7) have documented the close correlation between antibacterial activity *in vivo* and the degree of binding of a diverse group of these compounds to enzymes isolated from several bacteria.

One member of this group is the antimalarial agent pyrimethamine (8). It has been shown to be an antifolate in various studies in growing cells (1, 8), and it is moderately inhibitory against dihydrofolate reductases isolated from mammals and bacteria (6, 7). However, the site of action of pyrimethamine against plasmodia has not yet been fully documented.

The growth of plasmodia *in vivo* is stimulated by the folate precursor, *p*-aminobenzoic acid (9, 10), and inhibited by antimetabolites of this precursor, the sulfonamides (11–13). This inhibition is reversed more effectively by *p*-aminobenzoic acid than by folic acid (11–13). These data suggest that plasmodia synthesize folate-containing cofactors *de novo*, instead of utilizing preformed folates. In the pathway of folate biosynthesis *de novo* established in various microorganisms, H_2-folate is formed and subsequently reduced to the tetrahydro level by dihydrofolate reductase (1). In plasmodia, the presence of H_2-folate reductase is suggested by studies *in vivo*, and could be inferred from the demonstration (14) that the rodent malarial organism *Plasmodium berghei* carries out the conversion of H_2-folate to citrovorum factor. However, the presence of dihydrofolate reductase in plasmodia had not been demonstrated by direct enzymatic assay. In fact, the only protozoal dihydrofolate reductases isolated to date have been from certain trypanosomid flagellates (15–17). This paper presents evidence that *P. berghei* possesses a unique dihydrofolate reductase and that the potent inhibition of this enzyme by pyrimethamine is the basis of the chemotherapeutic action of this antimalarial agent.

METHODS

Preparation of P. berghei dihydrofolate reductase. The strain of *P. berghei* used was obtained from the Laboratory of Parasite Chemotherapy, National Institute of Allergy and Infectious Diseases, National Institutes of Health. It was maintained in 20–25-g male CD-1 mice (Charles River Mouse Farms, Wilmington, Mass.) by weekly transfer of sterile, citrated heart blood obtained from infected mice. The mice were inoculated interperitoneally with 0.1 ml of infected blood, diluted 1:4–1:7 (inoculum of $20–40 \times 10^6$ parasitized erythrocytes). Blood for enzyme extraction was withdrawn on the fifth day of infection, when the parasitemia was 45–65% (average survival time of the infected mice was 7 days). For preparation of extracts, blood from 100–150 infected mice was obtained by decapitation and collected into 5% sodium citrate solution (one-tenth the final volume).

The plasma and buffy coat were removed by centrifugation at $2200 \times g$ for 5 min at room temperature. The cells were washed twice in cold 0.85% NaCl, and then resuspended in half their packed cell volume of 1.5 mg of saponin per milliliter (in 0.85% NaCl). The suspension was shaken vigorously at 5-min intervals during a 20-min incubation at 37°, diluted 2–3-fold with cold 0.85% NaCl, and centrifuged at $3800 \times g$ for 20 min. The dark red supernatant fluid was drawn off, and the brownish pellets of freed parasites were washed four times with cold 0.85% NaCl. After the last centrifugation, the brown pellets (consisting of free parasites, some leukocytes, and fibrous matter) were resuspended in an equal volume of 1 mM EDTA (alone or in 0.01 M Tris-HCl, pH 7.3). This suspension was passed twice through a French pressure cell (Aminco) at 15,000–20,000 psi and centrifuged at $41,300 \times g$ for 20 min at 4° in a Lourdes model A-2 Betafuge. The reddish-brown, turbid supernatant was stored frozen for at least 1 night and centrifuged again upon thawing. The final supernatant was not turbid and was used as the crude extract.

The extracts were passed, at 4°, over a Sephadex G-100 column (2.95×35 cm) which had been equilibrated with 1 mM EDTA (alone or in 0.01 M Tris-HCl, pH 7.3) and eluted with the same buffer, and

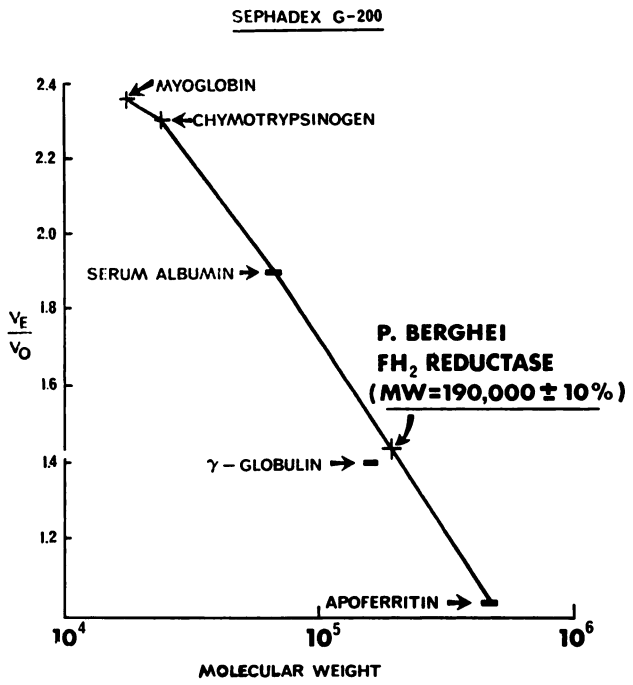

FIG. 2. *Estimation of molecular weight of P. berghei dihydrofolate (FH_2) reductase by gel filtration on Sephadex G-200*

See METHODS for experimental details.

of the *P. berghei* enzyme prompted us to consider the possibility that the enzyme might exist as an aggregation of smaller units. To test this possibility, gel chromatography of the enzyme was carried out under conditions commonly used to dissociate aggregated subunit proteins (41). In the first experiment, *P. berghei* dihydrofolate reductase was precipitated with ammonium sulfate, and the active fraction (35–75% ammonium sulfate saturation) was dissolved in 0.2 M KCl in 0.01 M Tris-HCl, pH 7.3, containing 1mM EDTA. This was then passed over a Sephadex G-100 column equilibrated and eluted with the same medium. The V_e/V_0 ratio was found to be 1.06, unchanged from the values found in the absence of KCl. Next, a *P. berghei* crude extract was adjusted to contain 4 M urea, 0.1 M 2-mercaptoethanol, 0.01 M Tris (pH 7.3), and 1 mM EDTA, dialyzed for 20 hr against the same medium, and then passed over a Sephadex G-100 column equilibrated and eluted with the same solution.[2] A V_e/V_0 ratio of 1.05 was obtained, again indicating no change in the molecular weight. A third experiment was carried out in which the enzyme was diluted in a solution containing 50 μM H_2-folate + 0.04 M 2-mercaptoethanol (approximately the same concentrations as used in the assays), and the column was equilibrated and eluted with the same solution. The position of exit of the enzyme from the column was not affected by the presence of the substrate; again the V_e/V_0 ratio was 1.05.

Effects of dihydrofolate reductase inhibitors. Pyrimethamine is known to be an inhibitor of H_2-folate reductase obtained from mammalian and microbial sources,

[2] This experiment was possible because of the unusual stability of the *P. berghei* enzyme to urea. A half-life of 20 hr was observed when the enzyme was allowed to stand at 4° in a urea solution prepared as above (in the presence or absence of the 2-mercaptoethanol). The loss of activity did not follow first-order kinetics, for 38% of the activity was still present at 75 hr.

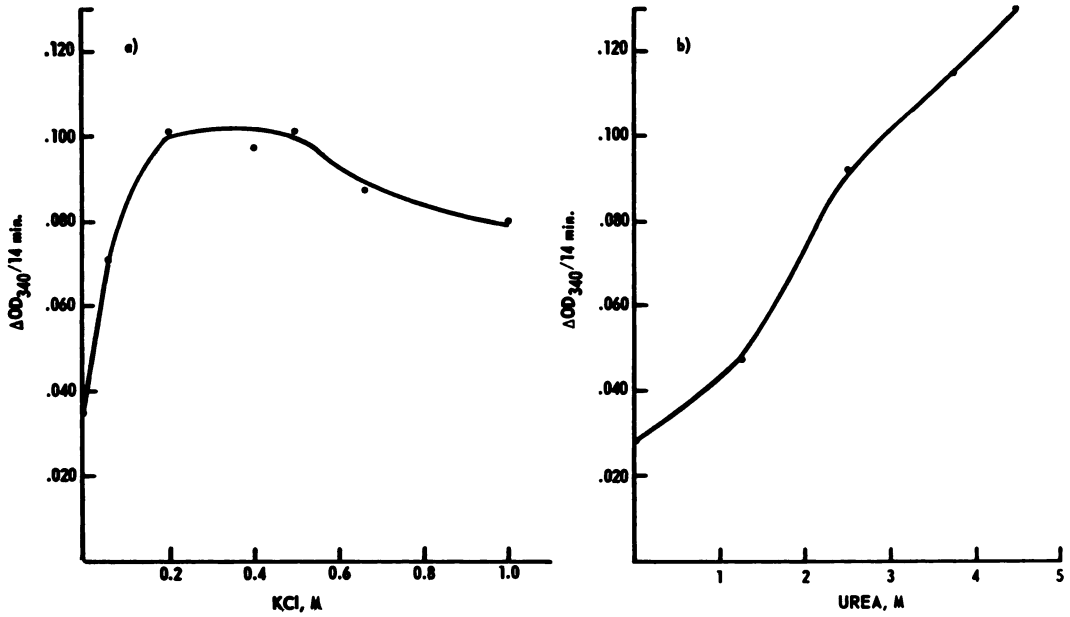

FIG. 1. *Effects of KCl and urea on the activity of P. berghei dihydrofolate reductase*

The reaction systems were prepared as described in METHODS, except that 0.0133 M Tris-HCl buffer, pH 7.3, was used, and KCl (a) or urea (b) was added as indicated.

ever, since the enzyme is not fully soluble below this pH value.

P. berghei dihydrofolate reductase is inhibited 50% by 20 μM folic acid at pH 7.2. No reduction of folate was noted at this pH value, and it was not possible to test the enzyme at the low pH values usually used for this substrate (2, 27, 28, 38) because of the lack of enzyme solubility below pH 6, as noted above. The preparations are stable for months when stored at —20° but lose 50% of their activity in 10 days when kept at 4°. A half-life of 35 min was observed at 45°. The enzyme was inhibited 50% by 0.3 mM *p*-chloromercuribenzoate when only 0.33 mM 2-mercaptoethanol was present in the cuvettes. The inhibition was decreased to 36% in the presence of 14.3 mM 2-mercaptoethanol, and the inhibition was completely reversed at the usual concentration of 2-mercaptoethanol in the assay (38.3 mM). The activity of the enzyme in 0.33 mM 2-mercaptoethanol was only about 50% of that found at the higher concentration.

Gel filtration studies. In order to obtain *P. berghei* dihydrofolate reductase free of hemoglobin, and to purify the enzyme partially, a crude extract was passed over a Sephadex G-75 column. Since this enzyme from a variety of sources (26–28, 31, 34, 37–40) has a molecular weight of approximately 20,000–22,000, it was expected that the enzyme activity would appear after the hemoglobin had been eluted from the column. Instead, the enzyme was not retarded at all; it was found in the same volume as was the blue dextran. Further experiments revealed that the enzyme was only slightly retarded on Sephadex G-100 columns; V_e/V_0 ratios of 1.04–1.10 were observed in numerous experiments. To determine the molecular weight, the enzyme was passed over a calibrated Sephadex G-200 column (Fig. 2), and an average molecular weight of 190,000 ± 10% was calculated. The mouse erythrocyte dihydrofolate reductase was eluted from Sephadex G-100 at the expected position for a mammalian dihydrofolate reductase, and an average molecular weight of 21,000 ± 10% was estimated by passing this enzyme over a calibrated Sephadex G-100 column.

The unexpectedly high molecular weight

were obtained from Pharmacia Fine Chemicals, Inc. Protein markers for the calibrated Sephadex columns were purchased from Mann Research Laboratories, Inc. Tris, ammonium sulfate, and EDTA were products of General Biochemicals. Amethopterin was purchased from Lederle Laboratories; 2-mercaptoethanol, from Eastman Organic Chemicals; and urea, from Fisher Scientific Company. Folic acid, saponin, and *p*-chloromercuribenzoate were obtained from Calbiochem; NADPH and NADH were products of Sigma Chemical Company. Dihydrofolic acid was prepared by the method of Futterman (20) as modified by Blakley (21), and stored frozen as a suspension in 0.005 N HCl containing 0.05 M 2-mercaptoethanol. The diamino heterocyclic antifolates were synthesized previously in these laboratories.[1] Hemoglobin was determined as cyanmethemoglobin with Hycel reagent and standard, purchased from Hycel, Inc. Protein was determined by the method of Lowry *et al.* (25).

RESULTS

Enzyme extraction. In initial experiments to detect the presence of dihydrofolate reductase in *P. berghei*, free parasites were ruptured by sonic oscillation, freeze-thaw cycling, osmotic shock, or combinations of these techniques. The resultant extracts contained barely sufficient dihydrofolate reductase activity to be detected by the spectrophotometric assay (specific activities, approximately 0.1–0.2 mμmole/min/mg of protein). We then found, however, that adequate extracts could be obtained when the parasites were ruptured by passage through a French pressure cell, as described in METHODS. Specific activities of 1.2–2.5 mμmoles/min/mg of protein were observed, which are within the range of values reported for this enzyme in various other crude extracts, such as *Escherichia coli* (7), *Diplococcus pneumoniae* (26), chicken liver (27), Erlich ascites cells (28), and others.

General properties. The Michaelis constants for H_2-folate and NADPH for the *P. berghei* and mouse erythrocyte enzymes are listed in Table 1. The values observed

TABLE 1
Michaelis constants for H_2-folate and NADPH for P. berghei and mouse liver dihydrofolate reductases

	Michaelis constants	
	P. berghei	Mouse erythrocyte
	μM	μM
H_2-folate	2.6	~4
NADPH	1.3	5.9

are similar to those reported for dihydrofolate reductases isolated from various sources (1). It is noteworthy that (a) the K_m value determined for NADPH for the *P. berghei* enzyme is 4.5-fold lower than that observed for the mouse erythrocyte enzyme, and (b) the K_m value for NADPH for the *P. berghei* enzyme is 5–10-fold lower than the values reported for other microbial dihydrofolate reductases (7, 15, 29–31). The reaction proceeded at 30% of the maximum rate when NADPH was replaced by NADH.

The *P. berghei* enzyme is similar to dihydrofolate reductases from avian and mammalian sources in its responses to high levels of salts and urea (32–37). Figure 1a and b shows the response of this enzyme to KCl and urea; approximately 3–4-fold stimulation could be observed in the presence of 0.2–0.5 M KCl or 4.5 M urea. The activation by KCl presumably was due to the chloride ion, since similar stimulation was observed at comparable levels of Tris-HCl, but not Tris-maleate. Thus the activity observed in the presence of 0.067 M Tris-HCl buffer is approximately twice that found in 0.067 M phosphate buffer (sodium or potassium salt) or in 0.067 M Tris-maleate buffer. A pH-activity maximum is observed at pH 7.0–7.2 in Tris-HCl or phosphate buffer. Accurate enzyme assays below pH 6.0 were not possible, how-

[1] Pyrimethamine was synthesized by the method of Russell and Hitchings (22); trimethoprim, Roth *et al.* (23); dihydrotriazines, B. Roth (to be published); and pyrido[2,3-*d*]pyrimidine, Hurlbert and Valenti (24). Systematic names are given in a footnote to Table 2.

the active fractions were pooled. Most experiments were carried out with Sephadex G-100 enzyme prepared in this manner, although in some cases Sephadex G-75 or G-200 was used instead.

Preparation of mouse erythrocyte H_2-folate reductase. Blood, obtained by decapitation of uninfected mice and collected into one-tenth the final volume of 5% sodium citrate, was centrifuged, and the red cells were washed twice in 0.85% NaCl as described above for infected blood. The packed cells were lysed by the addition of twice their volume of 0.01 M sodium phosphate buffer, pH 6.5, containing 1 mM EDTA, and the lysate was adjusted to pH 6.5 by dropwise addition of dilute HCl. The lysate was stirred magnetically on an ice bath, and an equal volume of carboxymethyl Sephadex gel was added (prepared by suspending 5 g of carboxymethyl Sephadex in 300 ml of 0.5 M sodium phosphate buffer, pH 6.1, overnight, and then washing the gel three or four times with about 300 ml of 0.01 M phosphate buffer, pH 6.5, containing 1 mM EDTA). The stirring was continued for 30 min, and the mixture was centrifuged at 41,300 $\times$ *g* for 20 min at 4°. The supernatant fluid was carefully decanted, and if the amount of hemoglobin remaining was still too high (as judged from the intensity of the red color), a second treatment with carboxymethyl Sephadex gel was performed. Most of the dihydrofolate reductase was found in the supernatants after this procedure, but an additional 10–20% could be obtained by extracting the gel obtained from the first treatment with 0.01 M sodium phosphate, pH 6.5, containing 1 mM EDTA (15 min of stirring, with centrifugation as above). The combined supernatants had only 1–5% of the hemoglobin present in the lysate, with 50–90% recovery of enzyme. The supernatants were lyophilized and redissolved in a minimum amount of buffer. The specific activity of such preparations was 0.5–1.0 mμmole/min/mg of protein.

Enzyme assay. The assay was based on the decrease in optical density at 340 mμ due to the conversion of dihydrofolate and NADPH to tetrahydrofolate and NADP (1, 2). The enzyme was incubated for 5 min in 0.067 M Tris-HCl, pH 7.3, in the presence of 30 mM 2-mercaptoethanol, in a 37° cell chamber of a Beckman DU spectrophotometer equipped with a Gilford model 220 optical density converter. Then NADPH (67 μM, final concentration) was added, and the reaction was started by the addition of H_2-folate (50 μM, containing 8.3 mM 2-mercaptoethanol). The control consisted of the complete system plus 10 μM amethopterin. One unit of enzyme is defined as the amount of protein which catalyzes the reduction of 1 mμmole of dihydrofolate per minute, calculated from a molar absorbance change of 12,300 M^{-1} cm^{-1} (18). Specific activity is defined as units per milligram of protein. All additions to the reaction systems were first incubated with enzyme for 5 min unless indicated otherwise. For the determination of the 50% inhibitory concentration of inhibitors, at least four levels of compound were tested and the percentage of inhibition was plotted on semilog paper.

Molecular weight estimation. Sephadex G-200 was allowed to swell for 5 days at room temperature in 0.01 M Tris-HCl, pH 7.3, containing 1 mM EDTA, poured (at 4°) into a 2.95 $\times$ 40 cm column to a height of 26 cm, and washed with 5 void volumes of buffer. *P. berghei* crude extract (3 ml) was applied and eluted with buffer at a flow rate of 3.5–4 ml/hr/cm^2. The position of exit of the enzyme was determined by assay of the fractions (4 ml) collected. The column was calibrated by passing over 3 ml of a solution containing 5.2 mg each of blue dextran, myoglobin, bovine serum albumin, human γ-globulin, and horse apoferritin, and monitoring the position of exit (extrapolated to the nearest milliliter) by the absorption maxima for each (19). The plot of V_e (elution volume)/V_0 (void volume as determined by elution of blue dextran) vs. the log molecular weight of the known proteins was essentially linear [including human γ-globulin, which behaves on Sephadex G-200 as if it were a protein of molecular weight 205,000 (19)].

Materials. Sephadex G-75, G-100, G-200, carboxymethyl Sephadex, and blue dextran

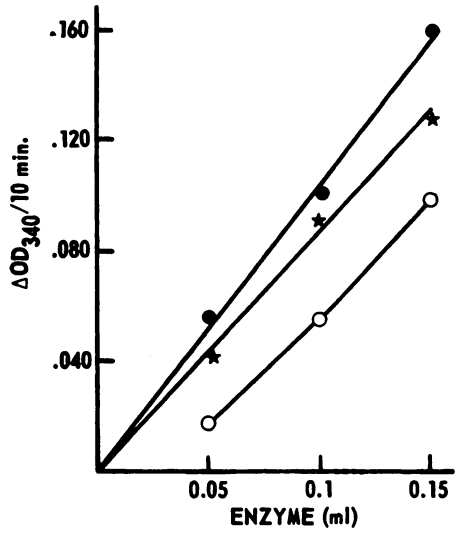

FIG. 3. *Ackermann-Potter plot (42) of P. berghei dihydrofolate reductase and pyrimethamine, with and without prior incubation*

Reaction mixtures were prepared as described in METHODS, with various amounts of enzyme as indicated. ●, Enzyme initially incubated for 10 min without drug; ★, enzyme initially incubated for 10 min, with 1 nM pyrimethamine added immediately before the reaction was started; ○, enzyme plus 1 nM pyrimethamine incubated for 10 min before the reaction was started by the addition of NADPH.

for which 50% inhibitory concentrations of 10^{-7}–10^{-6} M have been reported (see Table 2). Since this drug is a potent antimalarial agent, it was anticipated that it would be bound much more tightly to the plasmodial enzyme than to the host enzyme. Indeed, the *P. berghei* dihydrofolate reductase was inhibited 50% by 0.5 nM pyrimethamine when drug and enzyme were incubated for 5 min prior to the addition of H_2-folate. The 50% inhibitory concentration of pyrimethamine for the mouse erythrocyte enzyme is 1 μM, a 2000-fold higher concentration than for the plasmodial enzyme. A comparison of the 50% inhibitory concentrations of five antifolates against the *P. berghei* enzyme with dihydrofolate reductases from mouse erythrocytes, rat liver, *Trypanosoma equiperdum*, and *E. coli* is presented in Table 2. The *P. berghei* enzyme has a unique inhibitor profile (1, 7), demonstrated clearly by its sensitivity to pyrimethamine and BW 57-43 [1-(*p*-butylphenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine], both of which have significant antimalarial activity. It can be seen that all of the enzymes bound amethopterin in a like manner, but large differences may be observed in the binding of the small-molecule antifolates to the enzyme from different sources. A comparison between enzyme inhibition and antimalarial activity *in vivo* for pyrimethamine and three dihydrotriazines is shown in Table 3. The results indicate a positive correlation between these two parameters, for increased enzyme binding is reflected in increased activity *in vivo*.

TABLE 2

Comparative binding of diamino heterocyclics by protozoal, mammalian, and bacterial dihydrofolate reductases

Compound[a]	Concentration (× 10^{-8} M) for 50% inhibition				
	P. berghei	Mouse erythrocyte	Rat liver[b]	*T. equiperdum*[c]	*E. coli*[b]
Pyrimethamine	*ca.* 0.05	100	70	20	250
Trimethoprim	7.0	>100,000	26,000	100	0.5
BW 57-43	0.8	60	14	2000	65,000
BW 60-212	1.7	ND[d]	46	40	50
Amethopterin	0.07	ND[d]	0.21	0.02	0.1

[a] Pyrimethamine, 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine; trimethoprim, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine; BW 57-43, 1-(*p*-butylphenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine; BW 60-212, 2,4-diamino-6-butylpyrido[2,3-*d*]pyrimidine; amethopterin, 2,4-diamino-N^{10}-methylpteroylglutamic acid.

[b] From Burchall and Hitchings (7).

[c] From Jaffe and McCormack (15).

[d] Not done.

TABLE 3
Comparison of dihydrofolate reductase inhibition and antimalarial activity in vivo of pyrimethamine and several dihydrotriazines

Compound[a]	H_2-folate reductase, 50% inhibitory concentration		ED_{50}[b]
	P. berghei	Mouse erythrocyte	
			mg/kg
Pyrimethamine	$\sim 0.5 \times 10^{-10}$ M	1.0×10^{-6} M	0.03
BW 52-81	3.6×10^{-9} M	1.6×10^{-6} M	0.27
BW 57-43	8.0×10^{-9} M	6.0×10^{-7} M	2.0
BW 63-49	1.4×10^{-7} M	1.4×10^{-5} M	5.0

[a] BW 52-81, 1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine; BW 63-49, 1-(*p*-butoxyphenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine.

[b] Compounds were administered intraperitoneally to infected mice (groups of 10), starting on the afternoon of the day the mice were inoculated and continuing twice a day for 3 days. On the morning of the fourth day smears were made from tail blood of the individual mice, stained with Giemsa, and the average percentage of parasitemia was calculated for each group. The ED_{50} is defined as the dose of drug that reduced the parasitemia of treated animals to 50% of that of control mice.

The nature of the binding of pyrimethamine to the plasmodial dihydrofolate reductase depends on the manner in which the assay is carried out. If the drug is added immediately before the reaction is started (by the addition of H_2-folate), or if H_2-folate is included during the initial incubation of enzyme plus drug, the 50% inhibitory concentration is increased 6–10-fold. The drug is bound stoichiometrically when incubated first with enzyme in the absence of H_2-folate, but reversible binding is observed when the drug is added immediately before the reaction is started (Fig. 3). NADPH had no effect on the inhibition by pyrimethamine. Prior incubation was without effect on several other antifolates tested, including amethopterin. Dialysis of a mixture of *P. berghei* dihydrofolate reductase and pyrimethamine for 20 hr against 200 volumes of 1 mM EDTA did not dissociate the drug-enzyme complex, but at the end of the dialysis complete activity could be regenerated by incubation of an aliquot of the dialyzed enzyme-drug solution with H_2-folate for 10 min before the assay was started by the addition of NADPH.

DISCUSSION

The dihydrofolate reductase activity present in extracts of *P. berghei* exhibits characteristics which differ markedly from the properties of dihydrofolate reductases isolated from various other sources, including the mouse erythrocyte, the host cell of this parasite. The differences (K_m value for NADPH, sensitivity to antifolates, and molecular weight) between the enzyme from the parasite and the host cell are so striking that one can only conclude that the enzymatic activity observed in the *P. berghei* extracts resides in a protein native to the parasite itself. Contaminating dihydrofolate reductases from host blood cells were eliminated by gel chromatography, since the dihydrofolate reductase activity associated with the parasite was eluted from Sephadex G-100 columns almost a full void volume before the host enzyme would be expected to be eluted.

In its possession of an effective dihydrofolate reductase [and apparent lack of a folate reductase (14)], *P. berghei* resembles the microorganisms that synthesize dihydrofolate *de novo* and convert it to tetrahydrofolate-containing cofactors through reduction and reaction with 1-carbon fragments (1). The earlier demonstration that *P. berghei* could convert dihydrofolate to citrovorum factor (substances capable of supporting the growth of *Pedicoccus cerevisiae*) (14) suggested the presence of one or more of the 1-carbon addition enzymes. Direct demonstration of these and, indeed, direct evidence for synthesis of di-

hydrofolate *de novo* from *p*-aminobenzoic acid remain for further studies, although both are strongly suggested by several lines of evidence.

P. berghei dihydrofolate reductase exhibits certain properties previously reported only for this enzyme isolated from mammalian and avian sources. The K_m value for NADPH (1.3 μM) is substantially lower than the range of values (10–46 μM) reported for microbial dihydrofolate reductases (7, 15, 29–31) but is similar to the values reported for several mammalian (43–45) and avian (27) enzymes. Also, the strong stimulation of activity by urea and KCl (Fig. 1a and b) have been reported only for dihydrofolate reductases from mammalian and avian origins (32–37), and not for bacterial dihydrofolate reductases (31, 40). However, the *P. berghei* enzyme shows outstanding differences from the mammalian and avian enzymes in certain respects: (a) the 20-hr half-life in the presence of urea for the malarial enzyme, in contrast to half-lives in the order of minutes reported for the other enzymes (33, 35); (b) the striking differences in binding to the small-molecule antifolates, such as trimethoprim, pyrimethamine, and the dihydrotriazines (Tables 2 and 3, ref. 7); and (c) the 9–10-fold differences in molecular weight (Fig. 2 and refs. 27, 28, 34, 37, 38), as determined by gel filtration. It is noteworthy that the *P. berghei* and *T. equiperdum* dihydrofolate reductases differ in the two properties by which they may be compared [affinity for NADPH and binding of small-molecule antifolates (15)], indicating that the properties of the plasmodial enzyme are not common to all protozoa.

The finding that *P. berghei* dihydrofolate reductase has an average molecular weight of 190,000 ± 10% sharply differentiates this enzyme from most dihydrofolate reductases reported to date. Molecular weights of approximately 20,000–22,000 have been reported for dihydrofolate reductases from mammalian (28, 34, 37, 38), avian (27), and bacterial (26, 31, 39, 40) sources. Molecular weights of 31,000 and 33,500 were found for the enzyme from T6 bacteriophage-infected *E. coli* (40) and calf thymus (46), respectively. The only previously reported example of a high molecular weight dihydrofolate reductase is from an amethopterin-resistant strain of *D. pneumoniae* (26). A component of that enzyme was completely excluded from Sephadex G-100 and G-200 and Bio-Gel P-300 columns; the authors suggested that "the excluded component is an association of reductase molecules" (26). In contrast, the data on the *P. berghei* enzyme are consistent with the hypothesis that the protein exists as a molecule of average molecular weight 190,000 ± 10%. The position of exit of the enzyme from Sephadex G-100 columns was not changed when the chromatography was carried out in the presence of 0.2 M KCl, 4 M urea, and 0.1 M 2-mercaptoethanol or 50 μM H_2-folate + 0.04 M 2-mercaptoethanol. Thus, neither agents which break hydrogen bonds or disulfide linkages nor the substrate was able to decrease significantly the molecular weight of the malarial enzyme. While this evidence is not as rigorous as one might obtain from physical studies of a pure protein, it strongly suggests that the enzyme exists naturally as a molecule of 190,000 ± 10% average molecular weight.

The data in Table 2 suggest that *P. berghei* dihydrofolate reductase binds pyrimethamine even more tightly than it binds methotrexate. In fact, the *P. berghei* enzyme–pyrimethamine binding is greater than any other small-molecule–dihydrofolate reductase combination tested in this laboratory to date. We estimated (6) that the serum level of pyrimethamine in man after a minimum effective dose would not exceed 60 nM and predicted that "under natural conditions the effective concentration might be one or two orders of magnitude lower than this." This estimate is remarkably close to the 50% inhibitory concentration of pyrimethamine found for the *P. berghei* enzyme: 0.5 nM when enzyme and drug are incubated for 5 min prior to the addition of H_2-folate, and 3 nM without prior incubation. Figure 3 shows that the binding is stoichiometric when enzyme and drug are incubated prior

to the reaction, and reversible when they are not. The type of binding occurring *in vivo* remains unknown at this time. If the enzyme *in vivo* were exposed to a level of H_2-folate close to that used in these studies (50 μM), reversible binding of pyrimethamine might be expected. Although blood levels of H_2-folate have not been reported, they are probably much lower than the concentration used in these assays. Whole blood folates in the order of 1.5 μM and 0.14–0.25 μM for the rat (47) and man (48), respectively, have been reported, and in both these cases the bulk of the folates were in the form of N^5-methyltetrahydrofolate. The issue is confused, however, since it would be the concentration of dihydrofolate within the plasmodium that would be definitive, and presumably this level would be higher than that in the *milieu exterieur* as a consequence of synthesis *de novo*. Indeed the observation of Trager (49) that duck cells infected with *Plasmodium lophurae* contain more tetrahydrofolates than uninfected cells would be consistent with this view. Nevertheless, the remarkable potency and persistent action of pyrimethamine (8) suggest that stoichiometric binding of the plasmodial enzyme might occur *in vivo*.

The correlation between enzyme binding and antimalarial activity *in vivo* (Table 3) is reminiscent of our data (5, 7) for the action of small-molecule antifolates on several species of bacteria. An excellent correlation between enzyme binding and antibacterial activity was demonstrated in that case, including several crossovers, in which compounds ranked differently for different bacteria. Although only four compounds are included in the series of antimalarials reported in this paper, it is gratifying to observe that binding to the enzyme reflects chemotherapeutic activity in the whole animal. This correlation, and the over 1000-fold greater binding of pyrimethamine to the parasite than to the host enzyme, suggest that the basis for the chemotherapeutic action of pyrimethamine is the difference in sensitivity of plasmodial and host enzyme to this drug. This difference reflects the unique, potent binding of pyrimethamine to the plasmodial dihydrofolate reductase.

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