Dihydrofolate Reductase from *Trypanosoma equiperdum*

I. Isolation, Partial Purification, and Properties

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SUMMARY

Dihydrofolate reductase was isolated from acetone powder extracts of *T. equiperdum*. Folic acid was not an effective substrate; indeed, it was a weak inhibitor, the concentration required for 50% inhibition being 3×10^{-5} M. NADPH was a much more efficient hydrogen donor than NADH. Michaelis constants of 4.3×10^{-6} M for dihydrofolate and 1.2×10^{-5} M for NADPH were obtained. Trypanosomal dihydrofolate reductase showed a broad peak of maximal activity between pH 6.0 and 7.7, with activity sharply decreasing above and below those values. Activity increased with increasing temperature within the range of 20°–40°, but fell off sharply above 40°. 4-Amino analogs of folic acid are potent inhibitors of trypanosomal dihydrofolate reductase, the concentration for 50% inhibition by methotrexate, for example, being 2×10^{-10} M. The trypanosomal reductase differs from those of mammalian and bacterial origin with respect to susceptibility to inhibition by a number of 2,4-diamino-heterocyclic compounds.

A substance (or substances) was isolated from homogenates of *T. equiperdum* which possessed spectrophotofluorometric properties closely resembling those of 2-amino-4-hydroxypteridines and which effectively replaced folic acid as a growth-promoting factor for *Lactobacillus casei*.

INTRODUCTION

Dihydrofolate reductases, which catalyze the conversion of dihydrofolic acid to tetrahydrofolic acid, have previously been isolated and partially purified from bacterial (1–4), avian (5–7), and mammalian (2, 8–11) sources, but not, to our knowledge, from a protozoal source. Yet there is evidence to indicate that certain protozoa can synthesize dihydrofolic acid *de novo* (12, 13) and can convert this substance to tetrahydro derivatives (12, 14–17).

A requirement for pteridine cofactors such as biopterin (2-amino-4-hydroxyl-(1-*threo*)-6-1′,2-hydroxypropylpteridine) has been established for certain trypanosomatids (18). However, the metabolic functions of pteridine cofactors in trypanosomes has not been elucidated, although the operation of some sort of one-carbon transfer system in *Trypanosoma equiperdum* can be inferred (19).

This paper will describe the isolation and partial purification of dihydrofolate reductase from *T. equiperdum* and will compare some of the properties of this enzyme with those of the analogous enzyme from different sources. We also shall report the isolation of a substance (or substances) from cell-free extracts of *T. equiperdum* which exhibits spectrophotofluorometric properties closely resembling those of folic acid or related 2-amino-4-hydroxypteridines, and which can replace folic acid as a growth-promoting factor for *L. casei*.

MATERIALS AND METHODS

Materials. NADPH and NADH were purchased from P-L Biochemicals, Inc.,

Milwaukee, Wisconsin; folic acid from Calbiochem, Los Angeles, California; EDTA from Eastman Organic Chemicals, Rochester, New York. ECTEOLA Type 20 ion exchange cellulose is manufactured by Carl Schleicher and Schuell Co., Keene, New Hampshire. Methotrexate (amethopterin) is a product of Lederle Laboratories, Pearl River, New York, and its purity was confirmed by paper chromatography and ultraviolet spectroscopy before use. Dihydrofolic acid was prepared by the method of Futterman (5). Dr. G. H. Hitchings, Wellcome Research Laboratories, Burroughs Wellcome and Co., Tuckahoe, New York, kindly provided the following 2,4-diaminoheterocyclic compounds: BW 50-63: 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (pyrimethamine); BW 56-72: 2,4-diamino-5 (3′,4′,5′-trimethoxybenzyl)-pyrimidine (trimethoprim); BW 60-212A: 2,4-diamino-6-butylpyrido[2,3-*d*]pyrimidine isethionate; BW 58-283: 2,4-diamino-5-methyl-6-*sec*-butylpyrido [2,3-*d*] pyrimidine; and BW 57-43: 2,4-diamino-1-(4′-butylphenyl)-6,6-dimethyl-1, 6-dihydro-1,3,5-triazine.

Isolation and partial purification of trypanosomal dihydrofolate reductase. At 48 hr after intraperitoneal inoculation of male Wistar rats, 300–500 g, with 8×10^7 trypanosomes, parasitemia levels averaged 2×10^9 per milliliter. After exsanguination of such heavily parasitized rats under ether anesthesia, the trypanosomes were separated from other blood elements by differential centrifugation, as described elsewhere (19). Contamination with formed blood cells was generally less than 1%. Routinely, 3 to 6×10^{10} trypanosomes (equivalent to 200–400 mg dry weight) served as the source for each new batch of enzyme.

An acetone powder was prepared by first adding a thick suspension of the trypanosomes, in calcium-free 0.116 M phosphate buffer, pH 7.6, containing 0.01 M glucose (20), dropwise to 20 volumes of acetone at −12°, with constant stirring. The flocculated material was then rapidly deposited on Whatman No. 2 filter paper by suction filtration under nitrogen. After through drying, the filter paper coated with the acetone powder was cut into small strips which were placed in a 20-ml beaker on ice and immersed in 7 ml of 0.01 M phosphate buffer, pH 5.5, containing 1 mM EDTA and 0.25 M sucrose.

When the filter paper was thoroughly macerated, the mixture of crude extract and filter paper pulp was centrifuged in a Beckman Model L ultracentrifuge at 10^5 *g* for 40 min. Before centrifugation, the specific activity of the crude extract was generally around 13 (line 1, Table 1). The

TABLE 1
Partial purification of Trypanosoma equiperdum dihydrofolate reductase

Fraction	Total units	Percent recovery	Specific activity
Before centrifugation	425	—	13
After centrifugation	240	56	31
After dialysis	81	19	94

crude extract also possessed high NADH oxidase, but no NADPH oxidase, activity. After centrifugation, the specific activity of the particle-free supernate was generally around 30 (line 2, Table 1), and high NADH oxidase activity was retained.

The supernatant was then dialyzed at 5° for 20 hr against 100 volumes of 0.01 M phosphate buffer, pH 5.5, containing 1 mM EDTA. Under these conditions, a fraction of the nondialyzable protein was precipitated. This fraction was collected by centrifugation at 20,000 *g* for 60 min and was then dissolved in ice-cold 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA. The specific activity of the extract after dialysis was generally around 100 (line 3, Table 1), but NADH oxidase activity was completely absent at this stage of purification. The preparation was stable for at least 4 days when stored at 5°.

Enzyme assay. The method used by Burchall and Hitchings (2), based on the decrease in absorbancy at 340 mμ in the presence of dihydrofolate, NADPH, and enzyme, was used to assay dihydrofolate reductase activity. The composition of our standard reaction system was the same as

theirs, namely, 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4 in a molar ratio of 6:4), pH 7.0; 0.16 μmole of dihydrofolic acid; 0.24 μmole of NADPH; 33 μmoles of 2-mercaptoethanol; and the partially purified enzyme (usually a 0.2 ml aliquot), in a final volume of 3.0 ml.

The assay was carried out in matched 1-cm silica cells using a Beckman DU spectrophotometer equipped with thermospacers to maintain the cell compartment at 37°. Phosphate buffer (0.1 M; pH 7.0) served as the reference solvent. The temperature of the reaction mixtures was allowed to equilibrate with that of the cell compartment before addition of NADPH.

Protein was measured according to the method of Lowry *et al.* (21). One unit of enzyme is defined as that quantity of protein which catalyzes the reduction of 1 mμmole of dihydrofolate per minute and was estimated by the method of Blakley and McDougall (1). Specific activity is defined as the number of enzyme units per milligram of protein.

Concentration of drug required for 50% inhibition of trypanosomal dihydrofolate reductase. At least 4 levels of drug were tested in the standard reaction system after 10 min preincubation with the enzyme, 2-mercaptoethanol, and buffer. All reaction systems contained approximately the same number of enzyme units.

Extraction of folic acid and/or related pteridines from trypanosomal homogenates. A suspension of 3 to 6×10^{10} trypanosomes in 2 ml of 0.05 M phosphate buffer (pH 7.5) was subjected to one freeze-thaw cycle, using a dry ice-alcohol freezing mixture. Eight milliliters of the same buffer was added to the suspension of disrupted trypanosomes, which was then homogenized with a Tri-R Stirr-R equipped with a glass-embedded Teflon pestle. The homogenate was centrifuged in a Beckman Model L ultracentrifuge at 20,000 *g* for 60 min, and the precipitate was discarded.

After its pH was adjusted to 2.5 with 2–3 drops of concentrated H_3PO_4, the supernatant was extracted twice with 5 volumes of diethyl ether to remove lipid-soluble materials. The ether phases were discarded, and the aqueous phase, after its pH was adjusted to 7.4 with NaOH, was centrifuged at 20,000 *g* for 15 min.

The precipitate was discarded, and the supernatant was subjected to ECTEOLA-cellulose ion-exchange column (8 × 0.8 cm; pH 2.7) chromatography, using double-distilled water as the eluant. Aliquots of the effluent were analyzed using an Aminco-Bowman spectrophotofluorometer with its activating wavelength set at 365 mμ. The first 10 ml of effluent showed the strongest emission at 450 mμ, indicating that material possessing spectral properties closely resembling those of 2-amino-4-hydroxypteridines (22) was concentrated in this fraction. The volume was reduced to 2 ml by evaporation under reduced pressure.

The ability of this presumptive "pteridine fraction" to replace folic acid as a growth-promoting factor for *L. casei* was assayed by Dr. J. J. Burchall, Wellcome Research Laboratories, Burroughs Wellcome & Co., Tuckahoe, New York, using the method of Herbert (23) except that Difco Laboratory's folic acid *L. casei* medium was substituted for that provided by the Baltimore Biological Laboratory.

RESULTS AND DISCUSSION

Substrate and Cofactor Requirements for Trypanosomal Dihydrofolate Reductase

The rates of the enzymic reduction of dihydrofolate were determined with various concentrations of dihydrofolate in the reaction system. The results were plotted by the method of Lineweaver and Burk (24), and yielded a Michaelis constant (K_m) of 4.3×10^{-6} for dihydrofolate (Fig. 1). This value is reasonably close to those obtained for dihydrofolate using reductases from various bacteria—*Streptococcus faecalis* (4×10^{-6} M) and *Diplococcus pneumoniae*, (3×10^{-6} M) (25); *Lactobacillus leichmannii* (6×10^{-6} M) (4); and a thymine-requiring mutant of *Escherichia coli*, (7×10^{-6} M) (26)—as well as from certain cells of neoplastic origin: Ehrlich ascites carcinoma cells (1.5×10^{-6} M) (27); and a methotrexate-resistant subline of

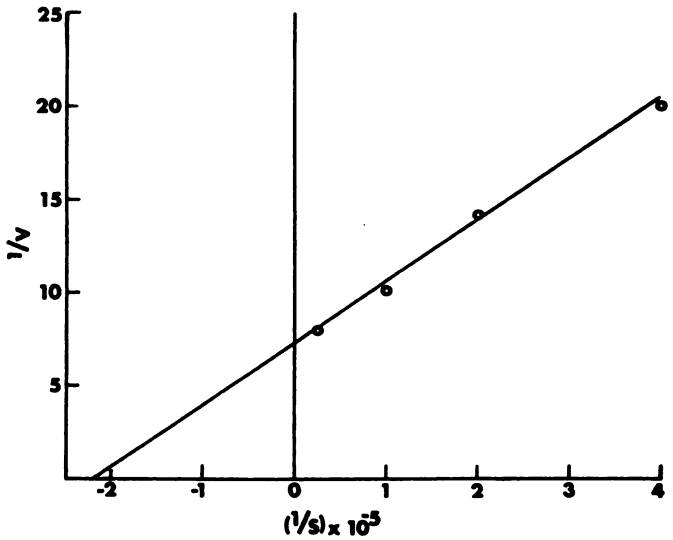

FIG. 1. *Reciprocal of the reaction velocity versus the reciprocal of the concentration of dihydrofolate at pH 7.0 and 37°*

The reaction system was identical with that described in Materials and Methods, except for the concentration of dihydrofolate. *V* represents the decrease in absorbancy at 340 mμ over a period of 10 min.

Sarcoma 180 cells (6.1×10^{-6} M) (11). On the other hand, the K_m value for dihydrofolate in the trypanosomal reductase system is lower, by a factor of 6, than those reported for reductases from *E. coli M 48-34* (2.6×10^{-5} M) and *Proteus vulgaris* (2.4×10^{-5} M) (2); and it is markedly higher than those reported for reductases

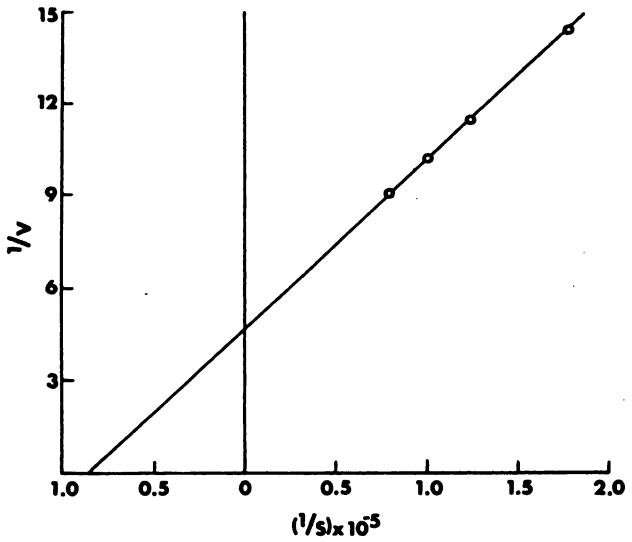

FIG. 2. *Reciprocal of the reaction velocity versus the reciprocal of the concentration of NADPH at pH 7.0 and 37°*

The reaction system was identical with that described in Materials and Methods, except for the concentration of NADPH. *V* represents the decrease in absorbancy at 340 mμ over a period of 10 min.

from chicken liver (5×10^{-7} M) (28) and (1.7×10^{-7} M) (7) and rat liver (2×10^{-7} M) (29).

When an equimolar amount of folic acid was substituted for dihydrofolic acid in the standard reaction system, very little decrease in absorbancy at 340 mμ was observed (Table 2), indicating that folate

TABLE 2
Substrate and cofactor specificity for Trypanosoma equiperdum dihydrofolate reductase

Conditions	$A_{340\ m\mu}$/10 min	Percent of control value
Complete system[a]	0.126	—
Omit DHF; add F	0.002	2
Omit NADPH; add NADH	0.011	9

[a] The composition of the complete system is described in Materials and Methods. Where indicated, equimolar amounts of folic acid (F) or NADH were substituted.

is an extremely poor substrate for the trypanosomal reductase. This finding is consistent with those of others (1, 2, 11) regarding the relative efficacy of dihydrofolate and folate as substrates for reductases from various sources.

The Michaelis constant for NADPH was also obtained using the trypanosomal reductase reaction system, and, as can be calculated from the data in Fig. 2, was 1.2×10^{-5} M. This value is essentially the same as those reported for reductases from *E. coli* (1.0×10^{-5} M) and *Staphylococcus aureus* (1.02×10^{-5} M) (2). Michaelis constants for NADPH reported by others using reductases from a variety of sources are included here for purposes of comparison: *S. faecalis* (4.6×10^{-5} M) (1); *L. leichmannii* (3.8×10^{-5} M) (4); *P. vulgaris* (2.78×10^{-5} M) (2); *D. pneumoniae* (2.0×10^{-5} M) (25); mouse leukemia L1210 cells (1.95×10^{-5} M) (30); Ehrlich ascites carcinoma cells (5.6×10^{-6} M) (9); and sheep liver (1.7×10^{-6} M) (8).

When an equimolar amount of NADH was substituted for NADPH in the standard reaction system, the rate of decrease in absorbancy at 340 mμ fell to about 10% of control values (Table 2). This finding that NADH is a relatively inefficient cofactor for trypanosomal dihydrofolate reductase is similar to those reported by others (1–3, 30) using reductases from a variety of sources.

Effect of pH

As is shown in Fig. 3, trypanosomal dihydrofolate reductase maintained essentially the same degree of activity between pH 6.0 and 7.7, such activity falling off sharply above and below these values. In this respect, the trypanosomal reductase differs from those obtained from various bacterial species (1, 2, 4), which exhibit well-defined single pH optima within this range, and also differs from reductases of avian and mammalian origin, which were found to exhibit two pH optima (7, 9, 27).

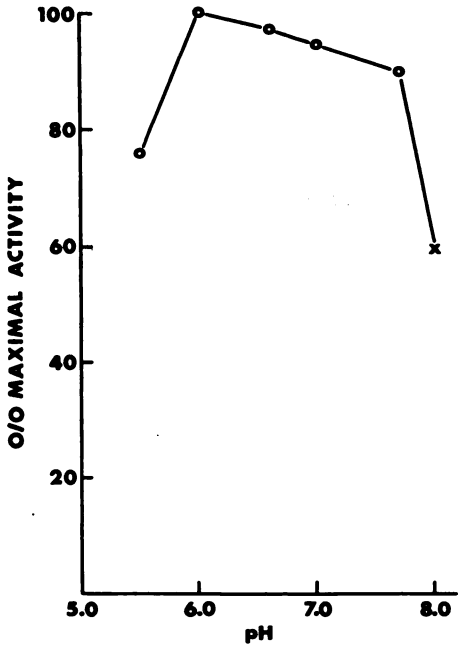

FIG. 3. *The effect of pH on the activity of Trypanosoma equiperdum dihydrofolate reductase at 37°.*

The reaction system was identical with that described in Materials and Methods, except for the buffer systems as indicated: ○ = 0.1 M phosphate; × = 0.1 M borate.

Effects of Temperature

The velocity of the enzymic reduction of dihydrofolic acid was determined at 20°, 25°, 30°, 35°, and 40°. A plot of the logarithm of the reaction velocity against the reciprocal of the absolute temperature gave a straight line (Fig. 4). The enthalpy of activation was calculated from the slope of this line, and a value of approximately 8000 cal was obtained. This value is much lower than the value of 14,700 cal obtained by Zakrzewski *et al.* (11) for the NADPH-mediated reduction of dihydrofolate by the reductase isolated from a methotrexate-resistant subline of Sarcoma 180 cells; it is closer, however, to the value of 5700 calories they obtained when folic acid was substituted for dihydrofolic acid in the same system.

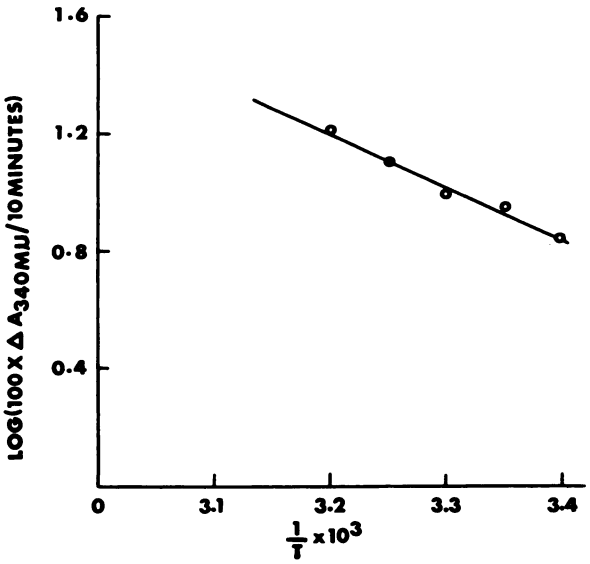

FIG. 4. *Relationship between the reciprocal of the absolute temperature and the log of the reaction velocity*

The temperature (T) at each setting was controlled by means of a Techne Tempunit, which circulated water at the appropriate temperature through the supply system of the cell compartment of the spectrophotometer.

The heat stability of the trypanosomal reductase was also determined. When incubated at 40° for 15 min, the enzyme lost less than 10% of its activity; at 50° for 15 min, 50% of original activity was lost; and at 60° for 15 min, essentially all activity was lost. Similar findings concerning the instability of reductases from different sources at temperatures above 40° have been reported (3, 25). Kaufman and Gardiner (7) found that NADPH, but not dihydrofolate or 2-mercaptoethanol, was able to protect reductase from chicken liver from heat inactivation, but we were unable to detect such protection of the trypanosomal reductase under similar experimental conditions. It is of interest that Hakala and Suolinna (31) recently reported that substrates, cofactors, and a number of competitive inhibitors protected dihydrofolate reductase obtained from a methotrexate-resistant subline of Sarcoma 180 cells from chemical and enzymic inactivation.

Relationship between Protein Content of Extract and Enzyme Activity

Figure 5 depicts the relationship between the rate of reduction of dihydrofolate and the amount of protein in aliquots of the partially purified (specific activity of around 100) trypanosomal reductase. Such a linear relationship is predicted by con-

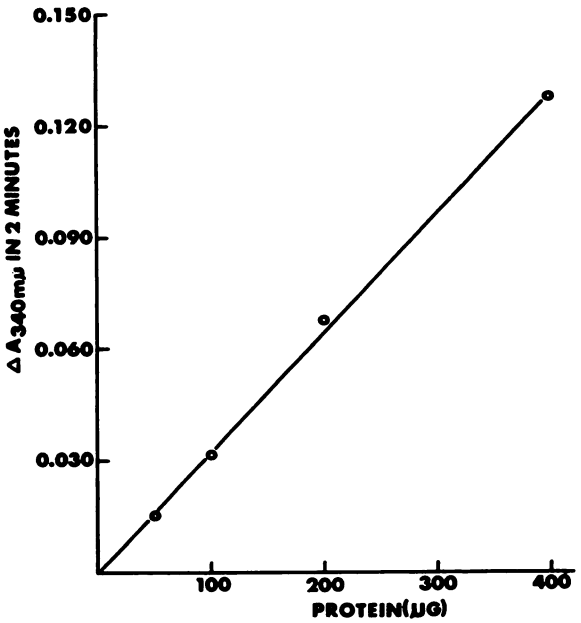

FIG. 5. *Relationship between protein content in aliquots of extract and dihydrofolate reductase activity*

The reaction system was identical with that described in Materials and Methods, except that as the volume of added enzyme extract was increased, the volume of buffer in the reaction system was reduced to maintain a final total volume of 3.0 ml.

sideration of the kinetic equation for the enzymic transformation of a single substrate under conditions in which the concentration of the enzyme is very low compared with the concentration of the substrate (32).

Chemical Inhibitors of Trypanosomal Reductase

4-Amino analogs of folic acid are strong inhibitors of dihydrofolate reductases from a variety of sources. Indeed, dihydrofolate reductase has been termed "the cellular receptor of the antileukemic drug, amethopterin, and other diamino antagonists of folic acid" (11). Trypanosomal dihydrofolate reductase is also extremely sensitive to the action of 4-amino analogs of folic acid. The activity of the enzyme in the standard reaction system was reduced to 50% of the control value in the presence of 2×10^{-10} M methotrexate (amethopterin). Thus, the sensitivity of the trypanosomal reductase to inhibition by methotrexate is as high as that of the reductase from *S. aureus*, which Burchall and Hitchings (2) found to be the most sensitive in this regard of the various mammalian and bacterial reductases they studied (Table 3).

Aminopterin was found to be a somewhat less potent inhibitor of trypanosomal reductase than was methotrexate, a concentration of 3×10^{-9} M of the former being required for 50% inhibition of the enzyme. This concentration is similar to that found to produce 50% inhibition of dihydrofolate reductase from *E. coli* B infected with T6 phage (4×10^{-9} M), but is somewhat lower than that producing 50% inhibition of the reductase from uninfected *E. coli* B (8×10^{-9} M) (3). The ID_{50} of aminopterin for the trypanosomal reductase is also quite similar to that found for the reductase from mouse leukemia L1210 cells (5×10^{-9} M) (33).

The diuretic agent 2,4,7-triamino-6-phenylpteridine (triamterene) was found to produce 50% inhibition of dihydrofolate reductases from guinea pig liver and small

intestine at a concentration of 2.6×10^{-7} M (10). Bertino *et al.* (9) also found that triamterene was a potent inhibitor of dihydrofolate reductase from Ehrlich ascites cells ($K_I = 1.3 \times 10^{-8}$ M). This pteridine derivative was a much weaker inhibitor of the trypanosomal reductase, a concentration of 2.5×10^{-6} M being required for 50% inhibition.

inhibit the reduction of dihydrofolate by reductases from a number of sources. Folic acid was also found to inhibit the reduction of dihydrofolate by the trypanosomal reductase, the concentration required for 50% inhibition being 3×10^{-5} M. Blakley and McDougall (1) have observed that folate produced 60–80% inhibition of dihydrofolate reductase from *S. faecalis* at a

TABLE 3

Comparative sensitivity of mammalian, bacterial, and Trypanosoma equiperdum dihydrofolate reductases to the action of diaminoheterocycles

Compound[a]	Concentration (X 10^8 M) for 50% inhibition						
	Human[b]	Guinea pig[b]	Rat[b]	*E. coli*[b]	*S. aureus*[b]	*P. vulgaris*[b]	*T. equiperdum*
Methotrexate	0.2	0.35	0.21	0.1	0.02	0.1	0.02
Pyrimethamine	180	3	70	250	300	150	20
Trimethoprim	30,000	2,000	26,000	0.5	1.5	0.5	100
BW 57-43	55	4	14	65,000	50,000	10,000	2,000
BW 60-212	95	4	46	50	4	50	40

[a] Methotrexate: 2,4-diamino-N^{10}-methylpteroyl glutamate.
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.

[b] From J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.* 1, 126 (1965).

Although 2,6-diaminopurine was found to be a fairly strong inhibitor of dihydrofolate reductases from chicken liver (34) and Ehrlich ascites cells (9), it was inactive against the trypanosomal reductase even at concentrations as high as 10^{-3} M. It is unlikely that this striking difference in sensitivity to 2,6-diaminopurine between the trypanosomal reductase and the reductase from Ehrlich ascites cells can be attributed to differences in the state of ionization of the purine analog, since the pH of the reaction systems in both cases (pH 7.0 and pH 7.5) was well above the pK_a (5.09) determined for the formation of the monocation of 2,6-diaminopurine from the neutral species (35). We were unable to detect spectrophotometric evidence for degradation of 2,6-diaminopurine when it was incubated in the standard reaction system.

A number of investigators (1, 3, 4, 6, 9) have found that folic acid can significantly

level approximately 10,000-fold greater than the level of aminopterin required to produce equivalent degrees of inhibition; in this regard it is noteworthy that the concentration of folate (3×10^{-5} M) required to effect a 50% inhibition of the trypanosomal dihydrofolate reductase is 10,000 times higher than the concentration of aminopterin (3×10^{-9} M) required to achieve the same degree of inhibition.

Burchall and Hitchings (2) have recently compared the ability of a number of 2,4-diaminopyrimidines and related heterocyclic compounds to inhibit dihydrofolate reductases from mammalian and bacterial sources. The susceptibility of the trypanosomal reductase to inhibition by several of these compounds has been determined, and the results are summarized in Table 3, which includes the data of Burchall and Hitchings for purposes of comparison. It can be seen that the overall pattern of response of the trypanosomal reductase to

various diaminoheterocycles is distinctly different from that of the mammalian or bacterial reductases. Thus, for example, the trypanosomal reductase more closely resembles the bacterial than the mammalian reductases in its sensitivity to 1-(*p*-butylphenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine (BW 57-43). On the other hand, the trypanosomal reductase is intermediate in its sensitivity to trimethoprim, between the extreme insensitivity of the mammalian reductases and the high sensitivity of the bacterial reductases. The sensitivity of the trypanosomal reductase to pyrimethamine more closely resembles that of reductases from guinea pig and rat than that of the bacterial or human reductases. Yet, the sensitivity of the trypanosomal reductase to the broad-spectrum dihydrofolate reductase inhibitor 2,4-diamino-6-butylpyrido [2,3-*d*] pyrimidine (BW 60-212) is within the range of that found for the other reductases. It is extremely interesting that 2,4-diamino-6-*sec*-butyl-5-methylpyrido[2,3-*d*]pyrimidine (BW 58-283), a closely related structural analog of BW 60-212, was 100 times more potent ($ID_{50} = 4 \times 10^{-9}$ M) than the latter as an inhibitor of trypanosomal reductase. This pyridopyrimidine thus is only 20 times less potent than methotrexate as an inhibitor of the trypanosomal reductase. This finding is consistent with those of Burchall and Hitchings (2), who reported the K_I values for BW 58-283 against reductases from *E. coli* and *P. vulgaris* to be 50 and 72 times lower, respectively, than those for BW 60-212. On the other hand, these investigators found the K_I value of BW 58-283 to be almost twice as high as the K_I value for BW 60-212 against the reductase from *S. aureus*.

Growth-Promoting Factor for L. casei Isolated from Trypanosomal Homogenates

The requirements for pteridine cofactors such as biopterin [2-amino-4-hydroxy-(1-*threo*) - 6 - 1′,2 - dihydroxypropylpteridine] have been established for certain trypanosomatids (*Crithidia* spp.) (18). However, the metabolic requirements for pteridine derivatives such as biopterin or folic acid are not well defined for most trypanosomatids, and, until this report, there was no evidence suggesting the presence of folic acid or other pteridines in *Trypanosoma* spp.

A substance (or substances) has now been isolated from homogenates of *T. equiperdum* which exhibits spectrophotofluorometric properties closely resembling those of folic acid and related 2-amino-4-hydroxypteridines and which possesses a rather marked ability to replace folic acid as a growth-promoting factor for *L. casei* (Table 4). At present, the exact chemical nature of this substance is not known.

TABLE 4

Ability of an extract of Trypanosoma equiperdum to promote the growth of folate-requiring L. casei

System[a]	0.1 N NaOH (ml)		
	0	LFA	SFA
Control	0.9	5.0	10.7
Extract, 0.05 ml	11.6	12.0	12.3

[a] Aliquots (0.05 ml) of the extract, sterilized by Millipore filtration, were added to the medium in the presence of limiting (0.05 mμg/ml; LFA) or saturating (0.625 mμg/ml; SFA) concentrations of folic acid or when folic acid was absent. Assay was kindly performed by J. J. Burchall, Wellcome Research Laboratories, Burroughs Wellcome & Co., Tuckahoe, New York.

General Considerations

This paper reports the isolation and partial purification of a dihydrofolate reductase from *Trypanosoma equiperdum* which possesses properties that are shared by dihydrofolate reductases from a variety of sources: (a) dihydrofolate is a much better substrate than folate, the latter, indeed, being able to act as a weak inhibitor of the enzyme; (b) NADPH is a much more efficient hydrogen donor than NADH; and (c) 4-amino analogs of folic acid are very strong inhibitors of the enzyme.

On the other hand, the trypanosomal reductase exhibits an overall pattern of sensitivity to a number of pyrimidine reductase inhibitors which is distinctly dif-

ferent from that of analogous reductases from mammalian or bacterial sources. This distinguishing characteristic is not surprising in the light of the findings of Hitchings and Burchall (2, 12, 36) concerning species differences among dihydrofolate reductases. A similar difference has been demonstrated in susceptibility of schistosomal and mammalian phosphofructokinases to trivalent organic antimonials (37). Kinetic and immunologic differences between analogous glycolytic enzymes of *Schistosoma mansoni* and its mammalian host have been found in the cases of hexokinases and phosphoglucose isomerases (38, 39), and of lactic dehydrogenases (40–42). One can wholeheartedly agree with Saz and Bueding (43) that "such differences suggest the availability of opportunities for interfering with the functional integrity of enzymes of the parasite without affecting those catalyzing the same reactions in the host."

Now that the presence of dihydrofolate reductase has been demonstrated in at least one species of *Trypanosoma,* as well as its presumptive substrate, an opportunity exists for chemotherapeutic exploitation of the differences between the parasite and its mammalian host with regard to this enzyme and the metabolic pathways it subserves. Unfortunately, we have found so far that neither the intermediary metabolism of *T. equiperdum in vitro* nor its rate of reproduction *in vivo* is significantly affected by 4-amino analogs of folic acid or by the 2,4-diaminopyrimidines available to us, alone or in combination with sulfadiazine. The failure of the structural analogs of folic acid to inhibit the trypanosomes might possibly be explained by the finding (44) that such compounds require active transport for their assimilation by cells, and that they, therefore, will inhibit only folate-requiring species, among which most protozoa are not included (12). On the other hand, Wood *et al.* (44) have also demonstrated that the pyrimidine reductase inhibitors (e.g., pyrimethamine) appear to enter and leave cells by passive diffusion without hindrance. The failure of these diaminoheterocyclic compounds to inhibit *T. equiperdum in vitro* or *in vivo,* despite the fact that some are strong inhibitors of the dihydrofolate reductase isolated from these parasites, could mean that these compounds are not assimilated by *T. equiperdum* (which seems unlikely), or that this species synthesizes greater amounts of dihydrofolate reductase than can be inactivated by the amounts of such compounds that do penetrate. The reasons for the inefficacy of dihydrofolate reductase inhibitors against the trypanosomes remain to be elucidated.

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