Inhibitor Binding Analysis of Dihydrofolate Reductases from Various Species[1]

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SUMMARY

Dihydrofolate reductases have been purified approximately 100-fold from *Escherichia coli, Staphylococcus aureus,* and *Proteus vulgaris* and to a lesser degree from rat, rabbit, guinea pig, and human liver. Average Michaelis constants of 2.3×10^{-5} M for dihydrofolic acid and 1.8×10^{-5} M for NADPH have been obtained for the three bacterial enzymes. NADPH is the preferred reductant but could be replaced by NADH with about 25% efficiency. pH-activity curves, in the case of the bacterial enzymes, show a single peak with maximum activity between pH 6.8 and 7.2.

Strong correlations have been found between the binding of a drug by a particular dihydrofolate reductase and the capacity of that drug to inhibit the source organism *in vitro*. In addition, each dihydrofolate reductase was shown to possess a pattern of inhibition that is distinct for the species under investigation. These differences between and among bacterial and mammalian reductases may be due to changes in amino acid composition at the active sites.

INTRODUCTION

Antagonism to the utilization of folates was recognized a number of years ago as a general property of 2,4-diaminopyrimidine derivatives (1). The synthesis and testing of a variety of such substances revealed that considerable selectivity could be achieved through molecular modification of an original active molecule, and a number of these substances have found chemotherapeutic applications (2). The view that their selective toxicities depended on a "differential affinity of the antimetabolite for the receptors of the parasite" (3) was supported in the main by considerations of structure:activity relationships in studies *in vitro* and in chemotherapeutic trials. For example, it could be shown that among the 5-benzylpyrimidines, substituents which increased activity against bacteria diminished that against plasmodia and vice versa (4). Since comparative trials could be carried out in the same host (the mouse) factors such as absorption, elimination, metabolic disposal, and cellular transport were regarded as similar, and the selective effects of the drugs were attributed to their relative affinities to the cell receptors of the parasites and the host.

Meanwhile a more precise definition of the cell receptor has become available. Early work has shown the small-molecule

[1] Preliminary reports of this work were presented at the 48th Annual Meeting of the Federation of Societies for Experimental Biology, Chicago, Illinois, 1964, and at the VIth International Congress of Biochemistry, New York, 1964.

Abbreviations used: NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NADH, reduced form of nicotinamide adenine dinucleotide; pABA, *para*-aminobenzoic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; K_m, Michaelis constant; K_i, inhibition constant.

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type of "antifolate" to interfere with the conversion of folic acid to citrovorum factor (5). The reduction of the pteridine moiety of folate (or dihydrofolate) has been recognized as an essential step in the process, and the *locus* of action of the 4-amino analogs of folate was identified as the reductase which carries it out (6). The data presented in this paper similarly identify dihydrofolate reductases as the sites of action of the small molecule type of antifolate, and they document the large differences in binding that may occur when similar reductases from bacterial and mammalian species are exposed to the same inhibitor. Recently, Baker (7) has noted that the *E. coli* reductase binds inhibitors differently than does the similar enzyme from mouse and pigeon liver; however, no large differences were observed between the latter sources.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms used in this study were: *Escherichia coli* M48-34, a pABA-requiring mutant (originally from Dr. B. D. Davis), *Staphylococcus aureus,* strain 209, and *Proteus vulgaris,* ATTC 9920. *E. coli* was grown initially at 38° for 18 hr in the synthetic medium of Lascelles and Woods (8) supplemented with pABA (5 μg per liter). In later experiments the organism was grown on the medium of Brown (9) in order to obtain better cell yields. Both media were aerated vigorously. *S. aureus* and *P. vulgaris* were grown for 18 hr at 38° in Brain Heart Infusion Broth (Difco) without aeration. Slants of the organisms were maintained on Nutrient Agar (Difco).

Enzyme assay. The assay for dihydrofolate reductase activity was based on the decrease in absorbancy at 340 mμ observed in the presence of dihydrofolate, NADPH, and enzyme. The standard reaction system contained 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 dihydrofolic acid; 0.24 μmole NADPH, and 33 μmole 2-mercaptoethanol, in a final volume of 3.0 ml. Control reactions consisted of either the complete reaction system without dihydrofolate or without NADPH, or the complete reaction system with 10^{-5} M amethopterin. One unit of enzyme is defined as that quantity of protein which catalyzes the reduction of 1 mμmole of dihydrofolate per minute under assay conditions and was calculated according to the method of Blakley (10). Specific activity is defined as the number of enzyme units per milligram of protein.

Assay of inhibitors in vitro. The activities of the inhibitors used in this study were determined in the following manner: 50 ml of phenol coefficient agar was poured into 150 × 15 mm petri dishes and allowed to solidify. An inoculum of 10^{-3} ml of an 18-hr culture of the appropriate organism was mixed thoroughly with 10 ml of the warm agar and poured in a smooth layer over the hardened agar base. Paper disks, 10 mm in diameter, were saturated with a solution containing 1 mg/ml of the test compound (approximately 20 μg per disk) and placed on the surface of the agar. After incubation overnight the diameter of any clear zone around the disk was measured. The molecular weights of the compounds, and hence the molar concentration of drugs, were approximately equal within each series.

Determination of inhibitor constants (K_i values). The inhibition constants of the various antimetabolites were determined at pH 7.0 in the standard assay system except that the concentration of dihydrofolate was varied over approximately a 5- or 10-fold range. Two concentrations of each drug were preincubated with the enzyme and buffer for 10 min prior to the addition of either substrate or cofactor. The K_i values were calculated from reciprocal plots of velocity vs. substrate concentration (11).

The concentration of drug required for 50% inhibition of the mammalian reductases was determined by titration of at least 5 levels of drug in the standard reaction system after 10 min preincubation with the enzyme and buffer. In the case of amethopterin all reaction systems contained approximately the same number of enzyme units.

Purification of bacterial enzymes. The procedure below describes the purification of *E. coli* and *S. aureus* dihydrofolate re-

ductases. For the purification of the *P. vulgaris* enzyme the procedure was modified as noted.

Cells were harvested in a Lourdes SL centrifuge at 5900 *g* for 20 min, washed once with 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA, and resuspended in sufficient buffer to make a thick suspension. The addition of the chelating agent was found to be necessary to preserve the activity of the enzyme during purification. The cells were broken by 2 passages through a French pressure cell, and cellular debris and unbroken cells were removed by centrifugation in a Servall SS-1 centrifuge at 14,500 *g* for 10 min. Crude extracts of *E. coli* were found to contain an NADPH oxidase of high activity. The specific activity of this extract (obtained from 2 liters of medium) was 1.05 (line 1, Table 1). A

TABLE 1
Purification of E. coli dihydrofolate reductase

	Fraction	Total units	Per cent recovery	Specific activity
1)	Crude extract	240	—	1.05
2)	1% streptomycin	205	85	1.24
3)	55%–95% $(NH_4)_2SO_4$	203	75	4.60
	Sephadex G-75 Eluates:			
4)	Tube No. 22	56	23	103.0
5)	Tube No. 23	60	23	102.0

10% solution of streptomycin sulfate was then added to the extract in an amount equal to one-tenth of the volume of the extract. The brown precipitate which formed was removed by centrifugation at 14,500 *g* and discarded. Solid ammonium sulfate was now added to the supernatant solution, with stirring, to a final concentration of 55%. The gray precipitate which formed was removed by centrifugation as before and discarded. Additional ammonium sulfate was now added to the supernatant solution, raising the salt concentration to 90%. The second white precipitate was removed by centrifugation, redissolved in a minimum volume of cold 0.001 M phosphate buffer, pH 7.0, containing 1 mM EDTA, and dialyzed for several hours against 100 vol of the same buffer. The specific activity at this point had risen to 4.6 (line 3, Table 1). The contents of the dialysis sac were applied to G-75 Sephadex column (2 × 48 cm) previously equilibrated with 1 mM EDTA. Dihydrofolate reductase was eluted from the column with 1 mM EDTA. The enzyme activity appeared on the trailing edge of a single protein peak at between one and two void volumes of eluate. The fractions in tubes 22 and 23 (approximately 3 ml of eluate each) contained 48% of the original activity with a specific activity of 103 (lines 4 and 5, Table 1). NADPH oxidase was completely absent at this stage of purification, and the preparation was stable for several months when stored at —20°. The ability of Sephadex G-75 to resolve this enzyme is particularly fortunate since the *E. coli* dihydrofolate reductase, unlike the *S. faecalis* reductase isolated by Blakley (10), rapidly loses activity on contact with DEAE-cellulose. For the purification of *P. vulgaris* dihydrofolate reductase, the concentration of ammonium sulfate was first raised to 35% (precipitate discarded) and then raised to 65% during the second precipitation.

Mammalian liver extracts. Livers of rat, rabbit, and guinea pig were removed immediately after the animals were killed. The organs were washed in 0.002 M Tris buffer, pH 7.8, containing 0.25 M sucrose and 1 mM EDTA. The tissue was minced with scissors into fresh buffer of the same composition; the final concentration was between 10 and 50% (w/v). The resulting preparation was homogenized for 60 sec in a cold Potter-Elvehjem homogenizer and then spun for 40 min at 92,000 *g* in a Spinco model L centrifuge. Human liver was removed at autopsy performed within 4 hours after accidental death of the individual. The tissue was sliced into sections approximately 1 cm thick, quick-frozen, and delivered within 12 hr. Since human liver dihydrofolate reductase is extremely unstable in the frozen state, extracts were prepared as soon after receipt as possible. Extracts of the enzymes were stable in the frozen state and were usually stored in that form until further purification was performed.

Purification of mammalian dihydrofolate reductases. The dihydrofolate reductases obtained from rat, rabbit, guinea pig, and human were prepared by a modification (R. Ferone, unpublished results) of the method of Werkheiser (12). The final purity of these enzymes, as well as their stability during purification, was considerably more variable than that of the bacterial enzymes. Rat, rabbit, and guinea pig enzymes varied between 25- and 60-fold purification with an average yield of 25%. Human liver enzyme was obtained in about 25% yield with an average purification of 10- to 25-fold and was the most unstable of the enzyme preparations studied.

The extract, as described in Methods, was adjusted to a pH value of 4.5 with dilute HCl and recentrifuged in an International refrigerated centrifuge for 15 min at 1030 *g*. The pH value of the supernatant solution was adjusted to 7.0 by the addition of dilute NaOH. (The enzymes were considerably more stable at this point than in the frozen tissue and could be stored at −20° for at least several months.) Solid ammonium sulfate now was slowly added with mechanical stirring to the clarified extract to a final concentration of 65%. The suspension was centrifuged in an Servall SS-1 centrifuge, and the precipitate was discarded. The ammonium sulfate concentration now was raised to 90%, the suspension was centrifuged as before, and the precipitate was dissolved in a minimal volume of 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA. Originally, preparations at this stage of purification were dialyzed against 100 vol of 0.01 M phosphate buffer containing 1 mM EDTA, as were the bacterial enzymes, but subsequent experiments showed that the enzyme solutions could be directly applied to the Sephadex G-75 column. As with the bacterial extracts, dihydrofolate reductase activity appeared at the trailing edge of the single protein peak.

Materials. NADPH, folic acid, EDTA, and streptomycin sulfate were all purchased from Calbiochem, Los Angeles, California. Sephadex G-75 was manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden. Enzyme grade ammonium sulfate was a product of General Biochemicals, Chagrin Falls, Ohio. Amethopterin (methotrexate) was a product of Lederle Laboratories, Pearl River, New York. All other dihydrofolate reductase inhibitors used in this study were synthesized previously in the Wellcome Research Laboratories (13). Dihydrofolic acid was reduced according to the method of Futterman (14) as modified by Blakley (15) and stored as a suspension of 0.005 M HCl. Protein was measured according to the method of Lowry *et al.* (16).

RESULTS

Bacterial Dihydrofolate Reductases

Michaelis constants for dihydrofolate and NADPH. Table 2 lists the Michaelis constants obtained for dihydrofolate and NADPH in the presence of the appropriate enzyme. The results were calculated by the method of Lineweaver and Burk (11). The Michaelis constants obtained for dihydrofolate are 2.6, 2.0, and 2.35×10^{-5} M for *E. coli*, *S. aureus*, and *P. vulgaris*, respectively. These enzymes cannot be distinguished from each other by their ability to bind dihydrofolic acid. It is of interest to note that when folic acid at 10^{-4} M was substituted for dihydrofolic acid in the standard assay system in the presence of the *E. coli* enzyme no decrease in absorbancy at 340 mμ was observed. Moreover, no diazotizable amine was detected by the Bratton-Marshall reaction after 1 hr of incubation at 38° in the presence of the above levels of folic acid and a NADPH regenerating system. It has not been possible to decide whether the enzyme cannot catalyze the reduction of folic acid or merely does so at an extremely slow rate.

TABLE 2
Michaelis constants of several microbial dihydrofolate reductases

Drug	Michaelis constants (M × 10^5)		
	E. coli	*S. aureus*	*P. vulgaris*
Dihydrofolate	2.6	2.0	2.4
NADPH	1.0	1.8	2.8

The K_m values obtained for NADPH are 1.0, 1.02, and 2.78×10^{-5} M for *E. coli*, *S. aureus*, and *P. vulgaris*, respectively. Again it does not appear possible to distinguish the enzymes from each other on this basis, although the *S. aureus* dihydrofolate reductase may possess a maximum velocity somewhat lower than that of the other two enzymes. When NADH was substituted for NADPH the reduction of dihydrofolate proceeded at about 25% of the maximum rate.

pH optimum. The pH optimum for the reduction of dihydrofolic acid was determined for each enzyme; the results are shown in Fig. 1. The enzymes exhibit a strong similarity in this respect also. Each enzyme has a single peak of maximum activity at approximately pH 7.0. These patterns are similar to those reported for the *S. faecalis* dihydrofolate reductase (10) and differ markedly from the bimodal activation curves reported for the mammalian dihydrofolate reductases (17).

Relation of Inhibitor Binding to Antibacterial Activity

Table 3 shows the inhibition constants (K_i values) of several diaminopyrimidine inhibitors determined against the bacterial dihydrofolate reductases and compares these values to the antibacterial activity of the compounds *in vitro*. Each compound possesses approximately the same activity against each species of microorganism. Thus, compounds BW 48-210 and BW 48-224 show no activity against any of the three organisms. These inhibition constants range from 95 to 690×10^{-8} M. Trimethoprim, on the other hand, possesses high antibacterial activity and binds very strongly to the purified enzymes as indi-

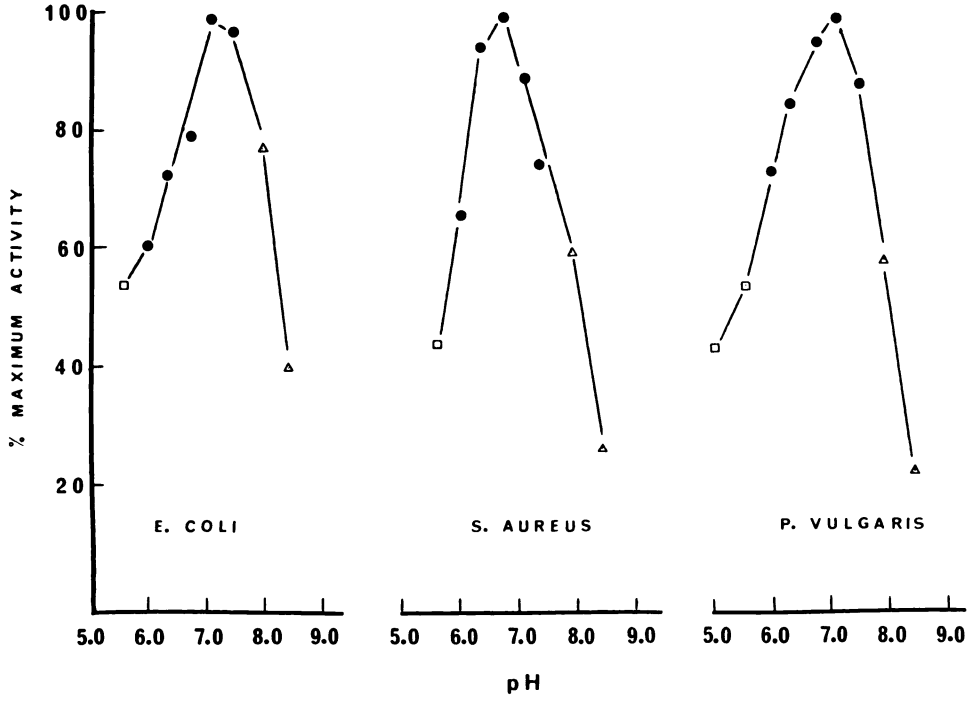

FIG. 1. *The effect of pH on the activity of the bacterial dihydrofolate reductases*

The reaction mixtures contained the components of the complete system described in Materials and Methods except for the buffer systems as indicated: ●, 0.1 M phosphate; ⊡, 0.1 M acetate; △, 0.1 M borate buffer.

TABLE 3
Growth vs. inhibition of dihydrofolate reductase

Compound	Substituents 5	Substituents 6	*E. coli* Zone[a] (mm)	*E. coli* $K_i \times 10^9$	*S. aureus* Zone (mm)	*S. aureus* $K_i \times 10^9$	*P. vulgaris* Zone (mm)	*P. vulgaris* $K_i \times 10^9$
2,4-Diaminopyrimidines								
BW 48-210	OC_6H_5Cl	CH_3	10	5100	10	6900	10	4900
BW 48-224	$CH_2C_6H_5$	H	10	3400	10	2310	10	950
BW 56-72	$CH_2C_6H_5(OCH_3)_3$	H	34	2.6	26	3.9	28	1.5
2,4-Diaminoquinazolines								
BW 57-192	5,6-trimethylene		36	4.4	16	144	33	2.2
BW 55-200	H	CH_2CH_3	32	68	27	16	26	27.9
2,4-Diaminopyrido(2,3-d)pyrimidines								
BW 58-283	CH_3	*sec.* C_4H_9	34	1.05	23	30	26	1
BW 58-265	CH_3	*n* C_4H_9	31	3.8	20	34.5	22	2
BW 60-205	CH_3	$CH_2C_6H_5$	31	6.7	24	30	25	1
BW 60-385	H	$CH_2C_6H_5$	24	17.4	32	1.65	17	10
BW 60-212	H	*n* C_4H_9	22	50.0	26	18.3	11	72

[a] Diameter of zone of inhibition produced by a 10-mm disk containing *ca.* 20 μg of compound on agar plate.

cated by its low inhibition constant.

Similarly, two diaminoquinazolines showed excellent antibacterial activity in the plating test and extremely low inhibition constants. It is particularly pertinent to note the low antibacterial activity of compound BW 57-192 against the purified *S. aureus* reductase. This inactivity is parallel to the weak binding of the inhibitor to this reductase as shown by the inhibition constant which is 32- and 65-fold higher than those of the *E. coli* and *P. vulgaris* dihydrofolate reductases, respectively. This observation strongly suggests that the *S. aureus* enzyme differs from the other two enzymes.

In certain circumstances the quinazolines showed higher *in vitro* activity than would have been predicted from a knowledge of their inhibition constants. Thus the quinazoline BW 55-200 produces an inhibition zone against *P. vulgaris* that is equal to that of the pyridopyrimidine BW 58-283 (26 mm) although the inhibition constant of the quinazoline is almost 30 times higher than that of the pyridopyrimidine. The behavior of the quinazolines in this regard suggests the possibility of unique properties or *loci* of action which may be worth further investigation.

Further evidence of the dissimilarities among the bacterial enzymes is seen in the series of 2,4-diaminopyrido(2,3-*d*)pyrimidines listed in decreasing order of activity *in vitro*. A remarkably precise correlation can be seen between these activities and the binding of the compounds by the particular dihydrofolate reductases. Equally important is the pattern of the binding revealed in this series which projects an "inhibitor profile" characteristic of each particular dihydrofolate reductase. The 6-benzyl derivative is most active against *S. aureus* while its analog, the 5-methyl-6-benzyl derivative, is more effective against *E. coli* and *P. vulgaris* and less active against *S. aureus*. The 6-*n*-butyl derivative is most effective against *S. aureus* whereas its 5-methyl analog is most effective against *E. coli* and *P. vulgaris*.

Each of the diaminoheterocycles used in the above study was observed to be a competitive inhibitor of the microbial dihydrofolate reductases. Reciprocal plots of enzyme activity *versus* dihydrofolate concentration are shown in Fig. 2 for the *P.*

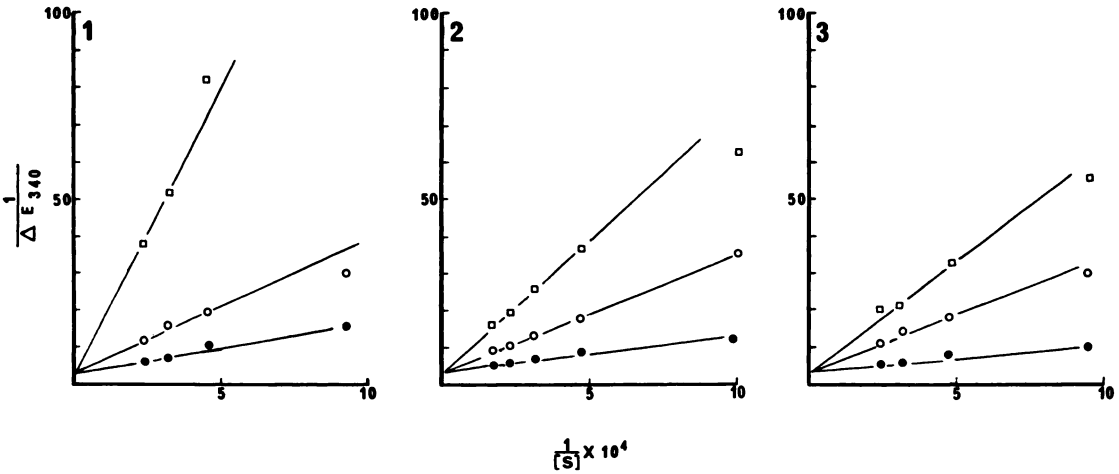

FIG. 2. *Reciprocal of the reaction velocity versus the reciprocal of the concentration of dihydrofolic acid*

The reaction systems were identical with those described in Materials and Methods except for the concentration of dihydrofolic acid and the addition of the inhibitor as explained below. Source of enzyme: *P. vulgaris* dihydrofolate reductase. Graph *1:* BW 58-265 (2,4-diamino-5-methyl-6-butylpyridopyrimidine), ○ = 3×10^{-8} M; □ = 3×10^{-9} M; ● = no drug. Graph *2:* BW 57-192 (2,4-diamino-5,6-trimethylenequinazoline), ○ = 1×10^{-8} M; □ = 5×10^{-9} M; ● = no drug. Graph *3:* BW 56-72 [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine], ○ = 1×10^{-8} M; □ = 5×10^{-9} M; ● = no drug.

vulgaris enzyme in the presence of a diaminopyrimidine, quinazoline, pyridopyrimidine or without drug. In no case does the presence of drug lower the maximum reaction velocity. All three lines in each plot have a common intercept on the ordinate.

Amethopterin also appears to act as a competitive inhibitor. Reciprocal plots of *E. coli* reductase activity vs. substrate concentration at two levels of amethopterin are shown in Fig. 3 (upper figure). The inhibition curves pass through a common intercept on the ordinate axis, suggesting that amethopterin is a competitive rather than a noncompetitive inhibitor. Calculation of the inhibition constant yields a K_i value of 4.1×10^{-9} M. Evidence that amethopterin is a reversible inhibitor of *E. coli* dihydrofolate reductase is also obtained when the results of a titration of enzyme vs. activity are plotted according to the method of Ackermann and Potter (18) (Fig. 3, lower portion). Plots of both the inhibited and uninhibited reaction systems present straight lines passing through the origin. Irreversible inhibition results, on the other hand, in a line with a slope the same as that of the control but an intercept on the x-axis which is displaced by an amount proportional to the concentration of the inhibitor.

Binding of Inhibitors to Mammalian Dihydrofolate Reductases

The dissimilarity of the bacterial inhibitor profiles suggested that the inhibitor profile analysis might also distinguish among mammalian dihydrofolate reductases. Partially purified enzymes were prepared from rat, rabbit, guinea pig, and human liver (see Materials and Methods). Five inhibitors were tested against each enzyme: amethopterin (the 2,4-diamino-N^{10}-methyl analog of folic acid), 2,4-diamino-5-methyl-6-benzylpyrido(2,3-*d*)-pyrimidine, 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine, 2,4-diamino-6-butylpyrido (2,3-*d*)-pyrimidine, and 1-(*p*-butylphenyl)-1, 2-dihydro-2, 2-dimethyl-4, 6-di-

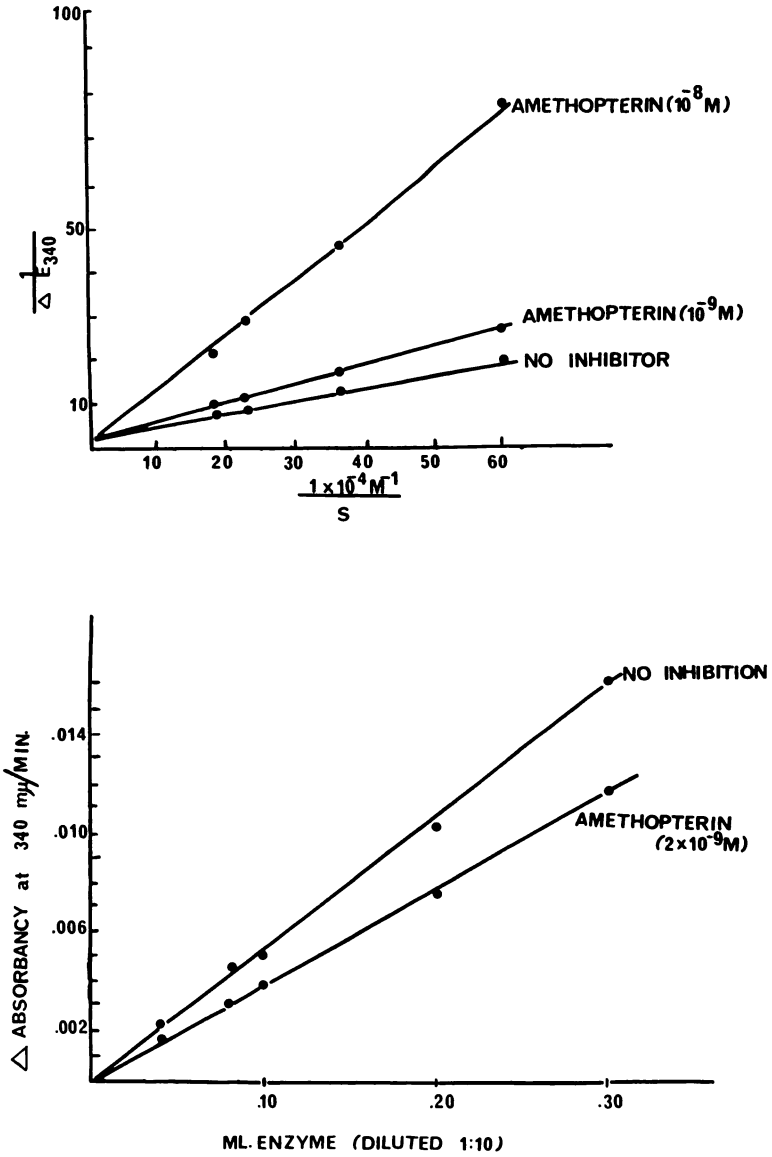

FIG. 3. *Inhibition of E. coli dihydrofolate reductase by amethopterin* (upper figure)

Reciprocal of the reaction velocity *versus* the reciprocal of the concentration of dihydrofolic acid in the presence of amethopterin at 10^{-8} M, 10^{-9} M, and without drug. The reaction systems contained the components of the complete system described in Materials and Methods except for the concentration of dihydrofolic acid.

(Lower figure) *Ackermann-Potter plot of the reaction velocity versus amount of purified E. coli dihydrofolate reductase (ml of 1:10 dilution) in the presence of amethopterin at* 2×10^{-9} M *and without drug*

amino-*s*-triazine. The Michaelis constants for dihydrofolate were extremely low (e.g., human enzyme: $<10^{-6}$ M) for the enzymes studied and could not be determined with accuracy by the optical assay employed. Therefore, the results are presented as the molar concentrations of the compounds required to reduce the activity to 50% of the

TABLE 4
Comparative binding of diaminoheterocycles by bacterial and mammalian dihydrofolate reductases

Compound[a]	Concentration ($\times 10^8$ M) for 50% inhibition						
	Human	Guinea pig	Rat	Rabbit	*E. coli*	*S. aureus*	*P. vulgaris*
BW 50–63	180	3	70	50	2,500	300	1,500
Amethopterin	9	1	9	6	0.6	0.1	0.5
BW 60–212	95	4	46	40	50	4	50
BW 58–265	24	0.7	26	9	2	7	1
BW 57–43	55	4	14	16	10,000	30,000	3,000
BW 56–72	30,000	—	26,000	37,000	0.5	0.5	0.4

[a] BW 50–63: 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (Daraprim, pyrimethamine).
Amethopterin: 2,4-diamino-N^{10}-methylpteroylglutamate (methotrexate).
BW 60–212: 2,4-diamino-6-butylpyrido(2,3-*d*)pyrimidine.
BW 58–265: 2,4-diamino-5-methyl-6-butylpyrido(2,3-*d*)pyrimidine.
BW 57–43: 1-(*p*-butylphenyl)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine.
BW 56–72: 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (trimethoprim).

control value in the standard assay (Table 4). Of all the enzymes investigated that of the guinea pig is the most sensitive to the inhibitors listed. Amethopterin is the most potent inhibitor of all species except the guinea pig, where the 2,4-diamino-5-methyl-6-butylpyrido(2,3-*d*)-pyrimidine is the most active. The 2,4-diamino-6-butylpyridopyrimidine also is active for all the species studied but is either equally or less inhibitory than its 5-methyl analog in all species except *S. aureus*. The dihydrofolate reductases of all the mammalian species tested proved extremely insensitive to trimethoprim (average concentration of 50% inhibition = 3×10^{-4} M) while the same compound produced 50% inhibition of the isolated bacterial enzymes at 5×10^{-9} M. Exactly the opposite specificity can be seen in the action of the dihydrotriazine, BW 57-43, which inhibits the mammalian reductases at approximately the same concentrations as do the pyridopyrimidines but shows extremely poor binding to the bacterial enzymes. The other antimalarial compound, BW 50-63, pyrimethamine, exhibits a pattern of selective binding similar to that of the dihydrotriazine.

DISCUSSION

The present paper demonstrates that differences in the binding sites of enzymes from different sources can be detected through the effects of a spectrum of inhibitors. The resultant "inhibition profile" is a phenotypic characteristic of the species that is independent of the stage of purification of the enzyme and the particular individual that served as its source. The magnitude of the differences in binding may be exceedingly large. For example trimethoprim produces 50% inhibition of the *P. vulgaris* dihydrofolate reductase at a concentration of 4×10^{-9} M while a similar inhibition of the rat liver reductase occurs only when the concentration is raised to 3.6×10^{-4} M. In contrast the dihydrotriazine, BW 57-43, is bound more tightly by a factor of almost 2000 to the rat liver enzyme. These, and other binding values, correlate extremely well with the biological data. Trimethoprim is an active antibacterial both *in vitro* and *in vivo* (19) while the dihydrotriazine is toxic to mice and ineffective against bacteria, although it possesses activity against plasmodial infections in chicks and mice. The correlation between enzyme binding and activity in bacterial plating tests is especially remarkable when one considers the multiplicity of factors that might obscure this relationship. Variations in the intracellular levels of folate cofactors, drug transport, and degradation might be expected to exert modifying roles.

The finding that amethopterin competitively inhibits the bacterial reductases was unexpected since Blakley and McDougall (10) reported that this compound inhibited the same enzyme from *S. faecalis* noncompetitively. It is difficult to compare these observations since the *S. faecalis* study was performed at pH 6.3 and the *E. coli* study was at pH 7.0. In mammalian systems (17), amethopterin is more tightly bound at pH 6.3 than at the higher pH. In addition, the Michaelis constant for dihydrofolate is five times lower for the *S. faecalis* enzyme than for the *E. coli* reductase.

Experiments with whole cells (20) have shown that whereas *S. faecalis* requires in the order of several mμg/ml of amethopterin for inhibition, quantities in the order of μg/ml are necessary to inhibit *E. coli*. It appears likely that this effect is due to differential permeability of these two organisms to amethopterin; however, the possibility cannot be excluded that the qualitative nature of amethopterin binding to dihydrofolic reductase influences the degree of its inhibition.

The pyrimidine reductase inhibitors differ strikingly from the structural analogs of folic acid in several respects. They appear to pass cell membranes without obvious hindrance, where the structural analogs require active transport similar to that of folate, and therefore in general inhibit only folate-requiring species (20). The structural analogs possess the full complement of binding sites of the parent metabolite molecule, and thus exhibit very little difference in binding to enzymes from different sources, which above all must be capable of binding the natural substrate. The small molecule, on the other hand, is capable of seeking out and exploiting differences between enzymes in intermediate regions that may be less important to the binding of the substrate.

The precise nature of the species differences in dihydrofolate reductases has not yet been established, but it is plausible that these are due to differences in primary (amino acid) structure in regions at or near the active center. Variations in the primary structure of proteins and enzymes from various species now have been recorded for hemoglobin (21), cytochromes (22), aldolases (23), and lactic acid dehydrogenases (24). Wolf and Hotchkiss (25) and Pato and Brown (26) have related resistance to sulfonamides to mutational changes in folate-synthesizing enzymes. Recently Sirotnak *et al.* (27) have reported differences in binding of methotrexate to the dihydrofolate reductases of sensitive and resistant strains of *Diplococcus pneumoniae*. It is a matter of considerable importance to establish the nature of such differences on a more precise basis.

Note added in proof:

An error has been found in the experiments with methotrexate reported herein. Decomposition (photolysis?) of dilutions of the stock solutions of methotrexate appears to have been progressively greater as the dilution increased. Both the type of inhibition and the inhibitory concentrations are subject to revision on the basis of experiments now in progress.

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