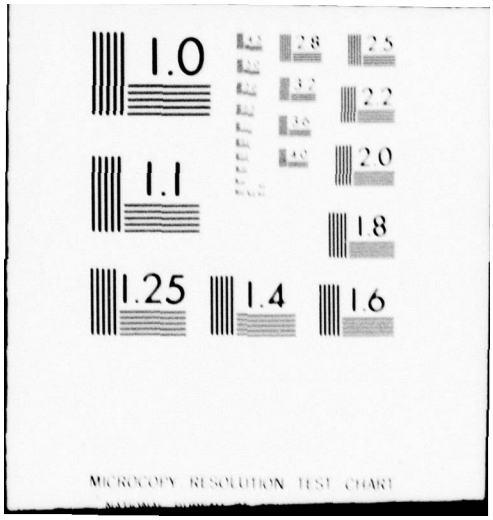
AD-A060 461 MASSACHUSETTS COLL OF PHARMACY BOSTON F/G 6/15
MECHANISM OF SULFONAMIDE RESISTANCE AND SYNERGISM IN PATHOGENS.(U)
JUN 78 R I HO, L CORMAN DAMD17-74-C-4039
UNCLASSIFIED NL

1 OF 1
AD A060 461

END
DATE FILMED
1-79
DDC


1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8
MICROCOPY RESOLUTION TEST CHART

LEVEL II

12

AD A060461

MECHANISM OF SULFONAMIDE RESISTANCE AND SYNERGISM IN PATHOGENS

JUNE, 1978

CONTRACT NO. DAMD17-74-C-4039

REPORT NUMBER 5

TITLE

(6) MECHANISM OF SULFONAMIDE RESISTANCE AND SYNERGISM IN PATHOGENS.

(9) FINAL REPORT. Sep 71 — Aug 77,

by

(10) Richard I. Ho, ~~Richard I. Corman~~, Leonard Corman

(11) JUNE 1978 (12) 44 p.

Supported by (16) 3M161102A (17) 00

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

(15) Contract No. DAMD17-74-C-4039

Massachusetts College of Pharmacy

Boston, Massachusetts 02115

DDC AVAILABILITY STATEMENT

APPROVED FOR PUBLIC RELEASE DISTRIBUTION UNLIMITED

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: Five	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): Mechanism of Sulfonamide Resistance and Synergism in Pathogens		5. TYPE OF REPORT & PERIOD COVERED: Final Report Sept. 1971-August 1977
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Ho, Richard I. Corman, Leonard		8. CONTRACT OR GRANT NUMBER(s): DAMD17-74-C-4039
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Massachusetts College of Pharmacy Boston, Massachusetts 02115		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A 3M161102BS01.00.002
11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medical Research & Development Command, Fort Detrick, Frederick, MD 21701		12. REPORT DATE: June, 1978
		13. NUMBER OF PAGES: 44 pages
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report): Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Dihydropteroate Synthetase, Dihydrofolate Synthetase, Dihydrofolate Reductase, Sulfonamides, Sulfones, Sulfides, Synergism, Gonorrhea, Leukemia, Trimethoprim, Methotrexate, Folic Acid, Sulfonamide-Trimethoprim Resistance.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

There are growing concerns regarding the frequent occurrences of antibiotic resistant microorganisms and its direct impetus on the clinical treatment of infectious diseases. This is particularly true of sulfonamide where sulfonamide-resistant strains of micro-organisms have been encountered (e.g. Neisseria gonorrhoeae and Neisseria meningitidis). The sulfonamide-resistant strains of

DD FORM 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Neisseria gonorrhoeae are most efficiently treated by the combinative applications of a sulfonamide (i.e. sulfamethoxazole) with the anti-folate drug of trimethoprim. However, whether such a combination will be equally effective in the treatment of sulfonamide-resistant Neisseria meningitidis still remains to be tested in the future.

Therefore, detailed understanding of the intimate nature of sulfonamide resistance and the effectiveness of chemotherapy are clearly in great demand. It is the primary objective of our proposed research to explore the mechanism of sulfonamide resistance and synergism at the molecular level.

Since the enzyme dihydropteroate synthetase of bacterial folic acid biosynthesis is the target enzyme of sulfonamide chemotherapy and its sequential enzyme dihydrofolate reductase is inhibited by the anti-folate drug of trimethoprim, a detailed biochemical study of such double blockage enzymes are obviously vitally important in fulfilling our research objectives. Furthermore, the results of such understandings will in turn provide us with important leads in future drug design on sulfonamides and more specifically, it will offer logical considerations for synthesizing antigonococal agents.

Our experimental results both from cell-free and whole cell bacterial studies with naturally occurrring clinical isolates of N. meningitidis and N. gonorrhoeae revealed that drug resistance to sulfonamide was achieved through an alteration in dihydropteroate synthetase structure expressed as a reducing binding capacity for the inhibitor, sulfonamide, rather than a change in affinity for the substrate, paraaminobenzoate (pABA). The enzyme dihydropteroate synthetases from Neisseria meningococci were found to possess much better chromatographic behaviors toward gel-filtrations as well as ion-exchangers. However, great difficulties have been encountered in attempting to purify the same enzyme from Neisseria gonorrhoeae by employing the same techniques. So far, only ammonium sulfate fractionated enzyme extract is obtainable for enzyme properties and kinetic studies. Gel-filtration studies indicated the molecular weight of the enzyme from gonococcal extract is unusually large and complexed and the stability of such a molecule is relatively fragile. Our preliminary kinetic analysis of the relatively crude ammonium sulfate fractionated enzyme extract showed that dihydropteroate synthetase from sulfonamide sensitive strain (CDC-9) is an allosteric enzyme with negative cooperativity. This result may offer us a biochemical mechanism for the development of sulfonamide resistance in Neisseria gonorrhoeae. The enzyme dihydropteroate synthetase occurring in the sulfonamide resistant strains must have been altered in such a manner that in order to develop a reducing binding capacity for the inhibitor, sulfonamide, the structure of the enzyme has to also change accordingly and consequently makes the enzyme behave with a much more profoundly negative allosteric property toward its natural substrate, pABA. As a consequence of this, resistant strains were found to excrete much more pABA into the growth medium. This has been repeatedly confirmed by our experimental data.

over

In order to obtain a final proof for the above mentioned statements, we are currently investigating the possibility of purifying the enzyme by affinity column chromatography, and hope by using the purified enzymes, a much more negative cooperativity property of the enzyme dihydropteroate synthetase from the sulfonamide resistant strains of N. gonorrhoeae can be confirmed.

The results of MIC studies indicated that many strains of N. gonorrhoeae exhibiting sulfonamide resistance are found to be also relatively insensitive to trimethoprim. However, the results of our parallel cell-free studies showed that such biological phenomena of coresistance can not be explained by the mechanism involving different trimethoprim susceptibility of dihydrofolate reductases, even though the reductases as isolated from different resistant strains (i.e. CDC-9 and 7134) do show some small but subtle structural differences. Resistance may be suggested, therefore due to decreased permeability of the cell envelope or increased production of dihydrofolate reductase.

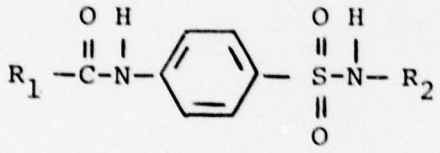
The dihydrofolate reductase of a sulfonamide sensitive strain (CDC-9) is further purified to a homogeneous state by a methotrexate affinity chromatography. A molecular weight of 20,300 is estimated for the gonococcal reductase by analytical gel-filtration. Studies on the structural properties of this enzyme indicate that the enzyme contains -SH groups which are not in the active site pocket of tne enzyme and may be spatially oriented quite close to each other in the enzyme's tertiary structure; formation of intramolecular -S-S- link from such SH groups results in complete inactivation of the enzyme. The SH groups and -S-S- bonds are exhangeable under effects of air oxidation and disulfide reducing agents by which enzyme activity could be regenerated.

Enzymes of dihydrofolate reductases from resistant strains (they are also trimethoprim resistant) behave nicely toward purification procedures. All the data collected so far on the dihydrofolate reductase from sensitive strains are highly purified (homogenous state) and currently we are attempting to purify the resistant dihydrofolate reductase by affinity column chromatographic technique. Furthermore, we are collecting more resistant strains to confirm our present experimental findings.

Due to the fact that very low activities can be detected for the enzyme dihydrofolate synthetase from Neisseria gonorrhoeae, all the experimental data collected so far is based on rather crude enzyme extract. Such data showed that this enzyme behaves in most parts similarly to the enzyme from E. coli. However, the sigmoidal response to Mg^{+2} of this enzyme is quite unique and worth future efforts to investigate. Additionally whether the enzyme dihydrofolate synthetase will play any role in the sulfonamide chemotherapy (i.e. sulfa drugs resistance and synergistic effects of a combinative drug chemotherapy) is unknown to date.

Therefore, based on our present experimental data about the three enzymes, we have no doubt established certain criteria in regards to the development of sulfonamide resistance and its direct influence on the development of trimethoprim resistance particularly for the clinical isolates of Neisseria gonorrhoeae. A final proof depends on the homogenous purification of all the concerned enzymes, and a delicate reconstruction of the enzyme complex. Nevertheless, the preliminary information on the three enzymes suggests logical developments of modified therapeutic agents that probably will be most effective in the treatment of gonococcal diseases or infectious diseases in general.

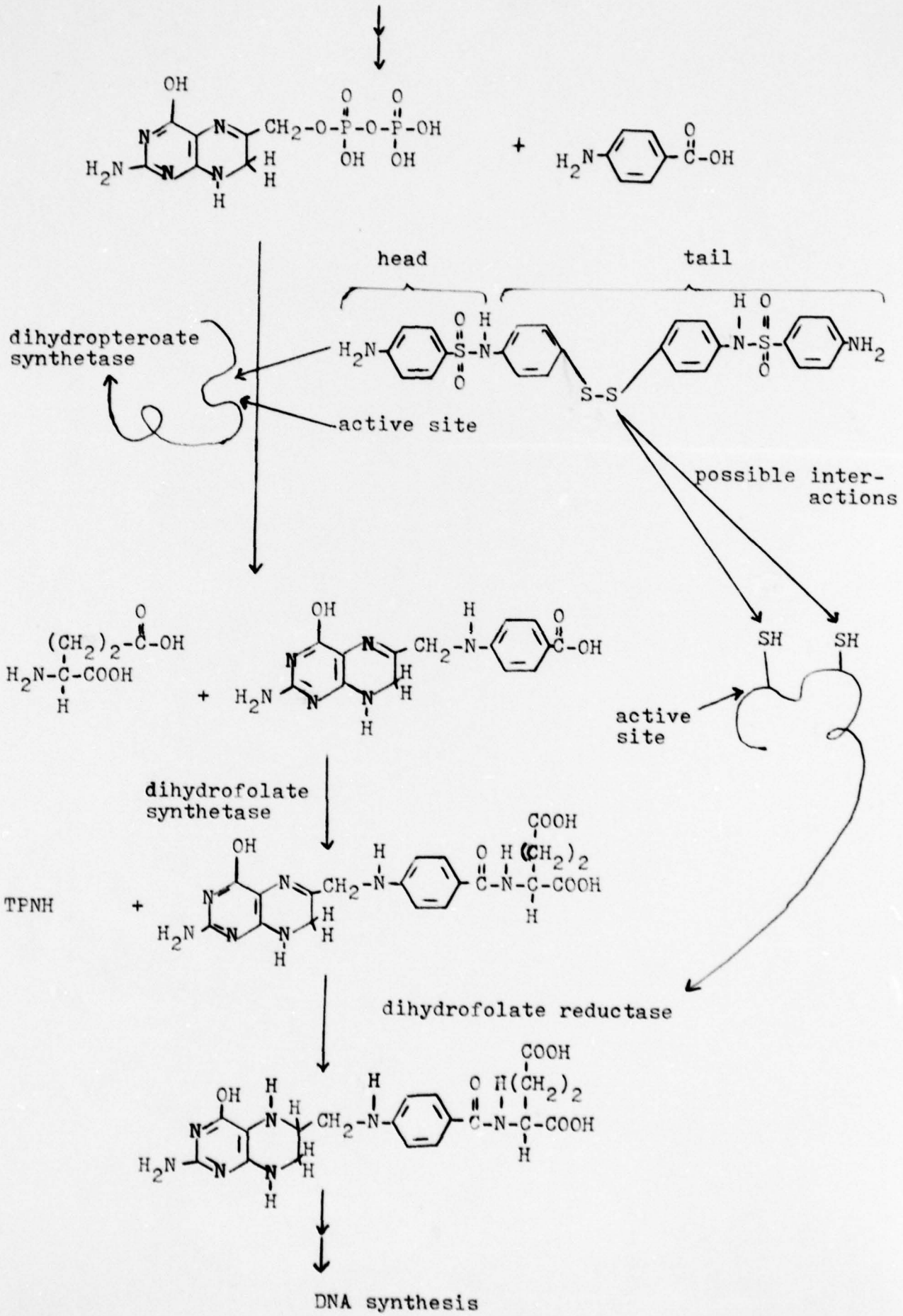
Based on our specific biochemical findings on the enzymes of dihydropteroate synthetase and dihydrofoalte reductase, we feel that a future potential irreversible sulfonamide enzyme drug should possess the following specific structural features:



R_1 = any alkyl group (or groups) that are most likely to be cleaved at physiological pH

R_2 = any chemical group (or groups) that contains a moiety which is reactive toward the adjacent -SH groups

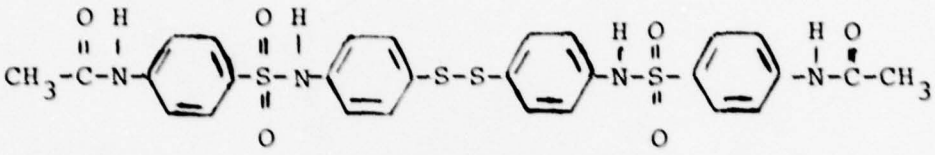
The biochemical reasoning for such a molecular design is: 1) acyl groups of the amide bonds are usually easily cleaved at the physiological pH of both the mammalian and bacterial cells; (2) p-aminobenzene-sulfonamido group will serve as the head of the molecule to be directed into the active site of the enzyme dihydropteroate synthetase of both sensitive and resistant strains of Neisseria gonorrhoeae (or any other bacteria); (3) the disulfide group, through its general ability to undergo mixed disulfide formation with the thiol groups. The proposed drug will therefore interact with the essential mercapto groups of the enzyme dihydrofolate reductases. The whole concept can be schemed below:


head
tail
dihydropteroate synthetase
active site
possible interactions
SH
SH
active site
dihydrofolate synthetase
TPNH
dihydrofolate reductase
DNA synthesis

MECHANISM OF SULFONAMIDE RESISTANCE AND SYNERGISM IN PATHOGENS

SUMMARY

One of the drugs proposed as described in the abstract was synthesized by Dr. William O. Foye, possessing the following structure.

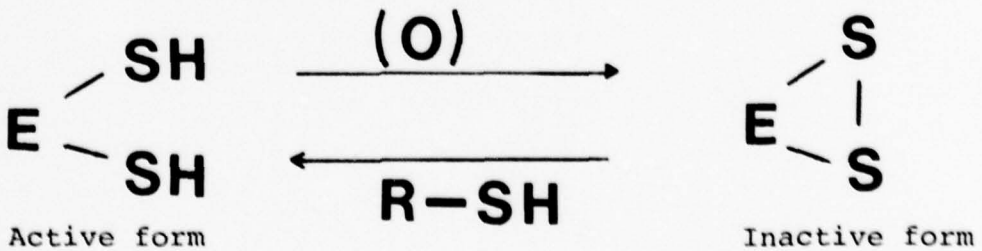


Its in Vitro antigonococcal activity has been thoroughly investigated by our group both to sulfonamide sensitive and resistant strains. The activities are observed to be better than the sulfonamide of sulfamethoxazole (the sulfa drug that is combined with trimethoprim in Septra). Therefore, it is a drug worth further investigation, particularly on the cell free enzymes of dihydropteroate synthetase, dihydrofolate synthetase and dihydrofolate reductase and their possible combinative complexes. Because of lack of time due to the termination of our contract, this work has not been carried out yet. However, based on the in Vitro antigonococcal properties so far studied, more of these types of drugs should be synthesized and biologically evaluated in the future.

Two things are observed experimentally to be extremely interesting and worth further investigation for the enzyme dihydropteroate synthetase: 1) No matter what purification techniques have been used, this enzyme can not be purified beyond the step of ammonium sulfate fractionation without significant loss of enzyme activity. Anion exchanger step of various technique, for some unknown reason, which remains to be resolved, has been found detrimental to its activity. Affinity column chromatography technique can only be considered as a theoretical proposal.

However, since it has been successfully used in the purification of the same enzyme from E. coli, it should also be tried for the enzyme dihydropteroate synthetase from Neisseria gonorrhoeae. The rapid elutions of this enzyme from Sephadex G-100, G-200 without any purification indicate to us that the molecular weight (or molecular size) of this enzyme may be extremely large and complex (allosteric enzyme ?) which may be responsible for its easy inactivation. 2) A sigmoidal activity/substrate pABA curve has always been observed with the crude enzyme extract as well as the partially ammonium sulfate fractioned enzyme extract. These kinetic behaviors show that the enzyme probably is allosteric in nature. More detailed studies indeed revealed that dihydropteroate synthetase from sulfonamide sensitive strain (CDC-9) is an allosteric enzyme with negative cooperativity. Further biochemical studies will not only probe into the actual shape of the active site of this enzyme but more importantly the biological functions due to such a confirmational analysis will certainly provide vital information for future drug design.

The implication of our most recent experimental findings that the enzyme dihydrofolate reductase from either sulfonamide sensitive or resistant strains of Neisseria gonorrhoeae are interconvertible among its active and inactive forms are extremely important. The SH group (s) in dihydrofolate reductase has been so far found as essential group (s) that will decide the activities of that enzyme via ring closure (or ring opening) mechanism.



Active form Inactive form

This interconvertible relationship between active and inactive forms are thus far observed only among bacteria of E. coli and N.gonorrhoeae . Whether this is an unique enzyme property only for bacterial enzyme or it holds true also for the same enzyme of mammalian origin remains to be clarified experimentally in the future (the preliminary data show however this unique enzymatic property of dihydrofolate reductase does not exist in mammalian cells). This interconvertible relation as discovered by our group has only been described for the first time. Consequently there are lots of work to be done before the information of SH group (s) can be utilized advantageously in terms of future drug design. The well known fact that N. gonorrhoeaecan only be grown anaerobically may reflect the essentiality of free mercapto group (s) for its activity and therefore the enzyme will be inactivated by the oxygen from air, the microorganism will die out eventually. If such a relationship does not exist in mammalian enzyme, the medical consequences will be significant enough that a new specific selectivity has been found that may be utilized as a new basis for new drug design either in the antibacterial or antileukemia fields (the elevation of the enzyme dihydrofolate reductase in leukemia patients is a common observation).

When the microorganism of N.gonorrhoeae develops sulfonamide resistance, it also shows relative resistance to trimethoprim. In order to prove whether the enzyme dihydrofolate reductase is playing any biologically significant role in the synergistic phenomenon, dihydrofolate reductases have been isolated and purified from two strains of Neisseria gonorrhoeae . The strains (clinical isolates, strains of CDC-9 and 7134) are selected for study on the basis of the difference in their sensitivity to sulfamethoxazole (60 fold) and trimethoprim (2 fold). The reductases are strikingly similar in most respects. The two fold

in Vitro difference in trimethoprim sensitivity can not be demonstrated conclusively in the purified enzymes. Purification steps produce essentially similar results with both extracts. The pH profiles are also virtually identical. The effect of increasing salt concentration is the same. There are no significant differences in any of the kinetic parameters. The enzymes do differ with respect to substrate protection against thermal inactivation and their response to inhibition by methotrexate. Titration experiments with methotrexate indicate that this partially reversible inhibitor is bound more readily by the reductase of the more resistant strain 7134. The implication is that there is a structural difference between the two enzymes at or near the active site(s), possibly a slight difference in hydrophobicity attributed to a change even in one amino acid. The activation that is seen in the presence of substrate may be attributable to its effect upon the structure of the protein molecule at the active site (s).

The small structural differences on the enzyme dihydrofolate reductases from the above-mentioned different sulfonamide-trimethoprim resistant strains indicate to us that the enzyme dihydrofolate reductases seem not to play any important biological role in the phenomenon of synergism. However, when four strains of clinical isolates of Neisseria gonorrhoeae with varying susceptibility to sulfonamides have been quantitatively investigated, the most resistant strain (whole bacterial cells) responds most synergistically to sulfonamide-trimethoprim combination as compared to the sensitive strain. Therefore, the synergistic effect, as we interpret it, is due to probably the difference on the enzyme structure of dihydropteroate synthetase (as mentioned previously this enzyme from resistant strain has not been studied yet due to our time schedule). Of course, other enzyme(s) (such as dihydrofolate synthetase) or other factor (s) can never be excluded before any experimentations are implemented.

The exact biological functions of the enzyme dihydrofolate synthetase is still unknown due to the lack of biochemical understanding. Dihydrofolate synthetase from Neisseria gonorrhoeae has been isolated, purified and characterized for the first time by our group. The summary of the enzyme purification is shown in Table 1. The properties of this enzyme from N. gonorrhoeae are also studied. The results of such studies can be represented by the following statements.

1. Linear relations of activity with increasing protein concentration (up to 0.8 mg/ml protein used) and with increasing time (up to 2.5 hrs.) have been observed under our standard assay conditions.

2. Maximum activity is observed when the pH of the reaction mixture is fixed at 9.3 (pH of 0.2 M Tris-HCl buffer is used in this study).

3. The molecular weight of dihydrofolate synthetase has been found to be 58,000 ± 3000 by using Sephadex G-100 as the filtration Gel.

4. The enzyme is unstable when stored at 4 C (about 50% of the activity was lost within 6 weeks). It is relatively stable at -60 C with only 5% activity loss in about two months.

5. The summary of the effects of monovalent cations are shown in Table 2. NH_4Cl showed activation at $1x10^{-2}M$ concentration, while the higher concentration shows inhibition.

6. The effects of divalent cations are summarized in Table 3. The results indicate that at 2mM Mn^{+2} concentration, 93% activity is observed and the activity is decreased at higher concentrations (at 5mM, only 60% activity remained). Other divalent cations such as Fe^{+2} and Cu^{+2} give very low activity while Hg^{+2}, Ba^{+2}, Ca^{+2}, and Ni^{+2} give no activity.

7. Different anions of ammonium salts are used, the results are summarized in Table 4.

8. The effects of adenosine nucleotides on dihydrofolate synthetase have been studied. Adenosine diphosphate is shown to be only half as effective as adenosine triphosphate. Adenosine monophosphate is not effective at all.

The effects of the substrates dihydropteroate, L-glutamate, and cofactor ATP on the dihydrofolate synthetase from N. gonorrhoeae has been studied and the K_m values are determined by the use of computer program. The following are the summary of the results.

1. Only dihydropteroate can be used as the substrate. No activity is observed when pteroic acid is used as its substrate. The enzyme shows substrate inhibition at high concentrations. The double reciprocal plot of 1/v versus 1/S was constructed by using computer program and consequently K_m value of dihydropteroate has been found to be 3.57×10^{-5} M.

2. It has been found that L-glutamate has a K_m value of 6.5×10^{-4} M. K_m for ATP is 6.7×10^{-4} M.

Inhibition studies on this particular enzyme have also been studied in great detail. The summary of the enzyme inhibition by dihydropteroic acid derivatives is shown in Table 5, in which K_i and the concentration required for 50% inhibition (I_{50}) values are indicated. The inhibition of dihydrofolate and its derivatives is summarized in Table 6.

Dihydro-10-oxo-pteroic acid is a most potent inhibitor among the dihydropteroic acid derivatives, while dihydro-10-oxo-folic acid is the best among the derivatives of dihydrofolate. The results seem to suggest that 10-carbonyl moiety is important for its inhibitory activity in both types of compounds. Such compounds are not known to be synthesized. If the in Vitro activities are found to be good (unfortunately we are lacking in time to pursue this), these types of compounds should be designed and synthesized in the near future.

The most interesting experimental observations that we have found recently concerning the properties of the enzyme dihydrofolate synthetase is the effect of mercaptoethanol on this enzyme's activity. As completely different from the enzyme of dihydrofolate reductase, the enzyme dihydrofolate synthetase behaves quite differently in the presence of mercaptoethanol. The enzyme activity has been destroyed almost completely within 2 weeks at 4 C. At 0 C and -60 C the activity is destroyed more rapidly than without 2-mercaptoethanol. This indicates to us that the enzyme is sensitive to the effects of SH group (s). The biochemical reasoning for such behavior is probably that there is (or are) extremely sensitive mercapto groups located at the active site in such a manner that slight distortion on its geometrical position will completely destroy the enzyme activity. This is possible because a disulfide bridge is usually formed in the presence of excess mercaptoethanol and this may cause the distortion of the active site mercapto groups. However, the enzyme of dihydrofolate reductase possesses entirely different chemical properties as described previously.

The major achievements that we have made during the period of our contract with the Army can be represented by the following articles. These articles have been adapted for publication in the books of Method in Enzymology. Their effects on the progress of

biochemistry of pteridine as well as on the therapeutic drug design and synthesis against infections and cancerous diseases will take time to prove. However, from what we know about this particular field today, we have felt that we have definitely made a breakthrough, and only time will substantiate what we believe today.

TABLE 1

SUMMARY OF PURIFICATION OF DIHYDROFOLATE SYNTHETASE FROM N. GONORRHOEAE

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification ratio	% Yield
1. Crude extract	2430	1215	0.5	1	100
2. 30-50% Saturated ammonium sulfate fraction	1015	1160	1.1	2.2	95
3. Sephadex G-200 column	113	1001	8.9	18	82
4. DEAE Biogel-A column	14.4	758	52.6	105	62

* 1 Unit activity = 1 mole dihydrofolate formed per 90 min. under standard assay condition

Table 2

Effect of monovalent cations on dihydrofolate synthetase. The standard assay condition was used except that the monovalent cations indicated were added. Percent of dihydrofolate formed compared to 100% dihydrofolate formed under standard assay condition. Monovalent cations were used in the form of chloride.

Monovalent cations	Concentration 1×10^{-2}M	Dihydrofolate formed (%)
K^+	2	53
	5	90
	8	100
	10	100
NH_4^+	2	110
	5	106
	8	93
	10	88
Na^+	2-10	0
Li^+	2-10	0

Table 3

Effect of divalent cations on dihydrofolate synthetase from N. gonorrhoeae. Percent dihydrofolate formed compared to the standard assay condition which was equivalent to 100%. Divalent cations were used in the form of chloride, except Fe^{+2} was sulfate. The standard assay was used except that the divalent cations indicated were added.

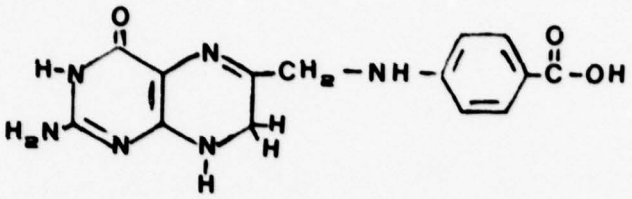
Divalent cations	Concentrations 1×10^{-3}M	Dihydrofolate formed (%)
Mg^{+2}	1	10
	2	31
	3	70
	4	96
	5	100
Mn^{+2}	1	80
	2	93
	3	86
	4	75
	5	60
Fe^{+2}	1-3	0
	4	3
	5	5
Cu^{+2}	1	6
	4-5	0
Hg^{+2}	1-5	0
Ba^{+2}	1-5	0
Ca^{+2}	1-5	0
Ni^{+2}	1-5	0

Table 4

Effect of anions on dihydrofolate synthetase. Percent dihydrofolate formed compared to 100% dihydrofolate formed under standard assay condition. Anions were used in the form of ammonium salts.

Anions	Concentrations 1×10^{-2}M	Dihydrofolate formed (%)
$SO_4^{=}$	1	137
	5	107
	10	86
Cl^-	1	131
	5	107
	10	80
$HPO_4^{=}$	1	156
	5	18
	10	0
NO_3^-	1	142
	5	81
	10	48
CH_3COO^-	1	131
	5	128
	10	102

Table 5



Dihydropteroic Acid

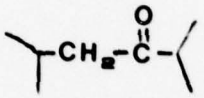
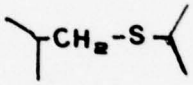
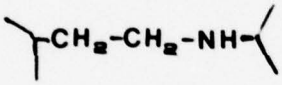
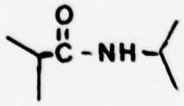
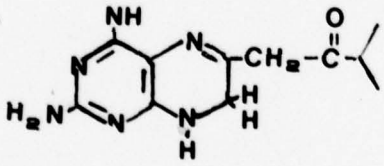
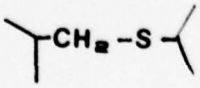
Compounds		K_i (M)	I_{50} (M)
Dihydro-10-pteroic Acid		6.9×10^{-5}	5×10^{-5}
Dihdydro-10-Thiopteroic Acid		1.5×10^{-4}	1.6×10^{-4}
Dihydrohomopteroic Acid		3.4×10^{-4}	3.2×10^{-4}
Dihydroisopteroic Acid		17.5×10^{-4}	21×10^{-4}
Dihydro-9-oxo-pteroic Acid			Inactive
4-Amino-4-Deoxy-Dihydro-10-Oxo-Pteroic Acid			Inactive
4-Amino-4-Deoxy-Dihydro-10-Thiopteroic Acid			Inactive

Table 6

Compounds	K_i (M)	I_{50} (M)
Dihydrofolic Acid	5.7×10^{-4}	5.3×10^{-4}
Dihydro-10-Oxo-Folic Acid	2.9×10^{-4}	2.2×10^{-4}
Dihydro-10-Thio-Folic Acid	4.9×10^{-4}	3.8×10^{-4}
Dihydro-10-Oxo-Aminopterin	Inactive	
Dihydro-10-Thio-aminopterin	Inactive	

Synthesis and Biological Evaluation of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate

Running Head: 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate

Address Correspondence to:

Dr. Richard I. Ho
Massachusetts College of Pharmacy
179 Longwood Avenue
Boston, Massachusetts 02115

ACKNOWLEGEMENT

This work was supported by Research Grant DAMD 17-74-C-4039 AFEB from the Armed Forces Epidemiological Board, Department of Defense.

Synthesis and Biological Evaluation of 2-Amino-4 hydroxy-6-hydroxymethylpteridine Pyrophosphate

By Richard I. Ho

Principle

The enzymatic synthesis of dihydropteroate and dihydrofolate involves the coupling of hydroxymethylpteridine pyrophosphate[1] with p-AB[1] or p-ABG.[1,2-4] The first synthesis of hydroxymethylpteridine pyrophosphate was described by Shiota et al.[5,6], and this method has been employed extensively for studies related to the biosynthesis of pteroate and analogs. This method of synthesis, however, is tedious and results in preparations that are invariably contaminated with simple pteridines, particularly pteridine monophosphate. Purification of these products is difficult, and yields are quite low[6]. A similar synthesis of hydroxymethylpteridine pyrophosphate was reported, but it is also unsatisfactory since it yields a variety of phosphorylated pteridines, requiring extensive purification.[7]

[1] Abbreviations used are as follows: hydroxymethylpteridine pyrophosphate for 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate; and p-AB for p-aminobenzoic acid; p-ABG for p-aminobezoylglutamate.

[2] T. Shiota, Arch. Biochem. Biophys. 80,155(1959).

[3] L. Jaenicke, and P.C. Chan, Angrew. Chem. 72, 752(1960).

[4] G.M. Brown, R.A. Weisman, and S.A. Molnar, J. Biol. Chem. 236, 2534(1961).

This article describes an improved synthesis of hydroxymethylpteridine pyrophosphate, which is obtained free of contaminating impure materials. Furthermore, this procedure requires only one step, and no further purification is needed beyond recrystallization.

Procedure

Guidelines for Synthesis. In this procedure, an excess of crushed pyrophosphoric acid is melted slowly, and the 6-hydroxymethylpteridine, prepared by the method of Forrest and Walker is added slowly to avoid polymerization.[8] The product is adsorbed on acid-washed active charcoal and eluted rapidly with a carefully controlled amount of ammonium hydroxide solution. The eluate is checked for impurities by paper chromatography, using water as the solvent. The desired compound moves with the solvent front; if another spot appears, the extraction is stopped. The compound is obtained as the diammonium salt, and its purity was substantiated by passage of the eluate through a column of Sephadex G-10; a single peak resulted.

Synthesis of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate. Pyrophosphoric acid was crushed and dried under vacuum for 4 days, and 25 g was melted slowly in a glass-stoppered flask at 60-65° C. 2-Amino-4-hydroxy-6-hydroxymethylpteridine

[5] T. Shiota, M.N. Disraely, and M.P. McCann, Biochem. Biophys. Res. Commun. 7,194(1962).

[6] T. Shiota, M.N. Disraely and M.P. McCann, J. Biol. Chem.239,2259 (1964).

[7] L. Jaenicke and M. Silverman, Deut. Med. Forsch.1,24(1963).

[8] H.S. Forrest, and J. Walker, J. Chem. Soc.2077(1949).

(300 mg, 0.0008 mole) was added slowly, and the flask was protected from light by aluminum foil. The mixture was stirred and heated at 60-65°C for 2 hr. After addition of 80 ml of distilled water, the contents were transferred to a beaker, and an aqueous charcoal suspension (Darco 60, 3 g in 20 ml) was added. The mixture was stirred for 30 min and was filtered through a 0.45-um filter (millipore). The charcoal pad was carefully removed from the filter and washed with 500 ml of distilled water to remove excess pyrophosphoric acid. The pteridine adsorbed on the charcoal pad was eluted by suspending the pad in 75 ml of 3 N ammonium hydroxide, and the mixture was stirred for 15 min. The resulting film suspension was filtered through a fresh millipore filter paper.

This procedure was repeated three times with the same quantity of 3 N ammonium hydroxide as eluant. The combined filtrate was evaporated under reduced pressure at room temperature until no odor of ammonia could be detected. The solution was lyophilized to yield about 300 mg (80%) of greenish-yellow amorphous powder. An analytical sample was obtained by recrystallization from aqueous ethanol, mp 166-175°C; UV $_{max}$(0.1 N NaOH): 255 and 362 nm; IR$_{\nu\ max}$(mineral oil): 3300, 1675 (NH_2), 1240 (P=O), 1040 (P-OH), and 820 (pteridine) cm^{-1}; NMR: 5.2 (m, 2H, CH_2) and 7.0 (d,1H, aromatic H); R_f (water) 0.98. Anal.-Calc. for $C_7H_{15}N_7O_8P_2$: C,21.71; H, 3.90, N, 25.30, P, 15.99. Found: C,21.86; H, 4.10; N, 24.50; P, 15.95.

[9] S. Futterman, J. Biol. Chem. 228,1031(1957).

Biological Evaluation

Reduction of the hydroxymethylpteridine pyrophosphate to its dihydro form using sodium dithionite[9] gave a product that showed full enzymatic activity with the dihydropteroate synthetase extracted from both Neisseria meningitidis and Neisseria gonorrhoeae. By using the enzyme from one strain (Strain M-60 EUR; obtained from the Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C., through the courtesy of Dr. M. Artenstein) of N. meningitidis, rates of formation of dihydropteroate for different concentrations of the hydroxymethyldihydropteridine pyrophosphate were measured. The dihydropteroate, formed using p-aminobenzoate-^{14}C, was eluted from the origin and identified by its characteristic UV spectrum. Total dihydropteroate was radioassayed; this procedure was found to be highly reproducible. The rate of dihydropteroate synthesis was dependent on hydroxymethyldihydropteridine pyrophosphate concentration in the presence of a constant concentration of p-aminobenzoate. A Lineweaver-Burke plot (of the reciprocal of rate of dihydropteroate synthesis versus the reciprocal of substrate concentration) gave a K_m value for the hydroxymethylpyrophosphate of 2×10^{-4} M, obtained from the average of three duplicate experiments.

The optimal substrate concentration of the pyrophosphate for the dihydropteroate synthetase employed was 0.26 mM. The pyrophosphate becomes inhibitory beyond that concentration.

Inhibition Studies

Inhibition studies using the hydroxymethylpteridine pyrophosphate as substrate for the dihydropteroate synthetase of N. meningitidis were carried out using sulfanilamide, sulfadiazine, dapsone, and bis(4-aminophenyl) disulfide. Dapsone(4,4'-diaminodiphenylsulfone) competitively inhibited the synthesis of dihydropteroate, which is in accord with a previous finding that the antibacterial action of dapsone is reversed by p-aminobenzoate.[10] This has been further substantiated by recent reports that dapsone and the sulfonamides were competitively inhibitory to the same enzyme from a cell-free extract of Escherichia coli.[11,12] The other compounds observed also produced competitive inhibitions at concentrations of 1.7×10^{-5}M, in the following order of decreasing activity: sulfadiazine dapsone sulfanilamide bis(4-aminophenyl)disulfide. The disulfide has been postulated to function analogously to dapsone in malaria chemotherapy.[13,14]

10 C. Levaditi, and R. Perault, C.R. Soc. Biol. 135, 1043(1941).

11 J.L. McCullough, and T.H. Maren, Antimicrob. Ag. Chemother. 3, 665 (1973).

12 J.K. Seydel, J. Pharm. Sci. 57, 1455 (1968).

13 W.O. Foye, and J.P. Speranza, Ibid. 59,259 (1970).

14 R.I. Ho, L. Corman, S.A. Morse, and M.S. Artenstein, Antimicrob. Ag. Chemother. 5, 388 (1974).

A Simple Radioassay for Dihydrofolate Synthetase Activity in *Escherichia coli* and Its Application to an Inhibition Study of New Pteroate Analogs

Running Head: Dihydrofolate Synthetase

Address Correspondence to:

Dr. Richard I. Ho
Massachusetts College of Pharmacy
179 Longwood Avenue
Boston, Massachusetts 02115

ACKNOWLEGEMENT

This work was supported by Research Grant DAMD 17-74-C-4039 AFEB from the Armed Forces Epidemiological Board, Department of Defense.

A Simple Radioassay for Dihydrofolate Synthetase Activity in Escherichia coli and Its Application to an Inhibition Study of New Pteroate Analogs

By Richard I. Ho

Dihydropteroate + glutamate → dihydrofolate

Assay Method

Principle. The method involves measurement of the enzymatic formation of dihydrofolate. Very little is known about this enzyme beyond its presence in Escherichia coli and other microorganisms reported briefly by G. Brown.[1] In that investigation some of the characteristics of the enzyme were examined by the microbiological assay with Lactobacillus casei (ATCC No. 7469) as described by Herbert and Griffin.[2] This microbiological method is cumbersome and inaccurate. Moreover, the L. casei assay is not suitable for determining the effects of inhibitors and metabolites on dihydrofolate synthetase because the assay microorganism itself may be inhibited by such inhibitors. A simple, rapid and accurate radioassay method, using L-(^{14}C) glutamic acid as substrate, has now been established for a quantitative determination of dihydrofolate synthetase activity. Pteroic acid was synthesized according to the method of L.T. Plante and further purified by the method of Houlihan.[3,4] And its dihydro form used as substrate was prepared by reduction with sodium dithionite as described by Futterman.[5]

Reagents

Dihydropteroic acid, 0.038-0.12 mM

L-(^{14}C)glutamic acid uniformly labeled and the specific activity was 237 mCi/millimoles, 1.1mM

ATP, 3.4 mM

$MgCl_2$, 1.1 mM

KCl, 44.4 mM

Tris-HCl buffer, 10 mM, pH 8.2

2-Mercaptoethanol, 11.1 mM

Enzyme, 0.6 mg

Inhibitor as indicated

Procedure. The reaction mixtures were prepared to contain in a total volume of 360 ul: the above reagents. The reaction mixtures were incubated in 10 x 75 mm-test tubes under argon gas for 30 min at 37°C. Reaction mixtures without either enzyme, dihydropteroic acid, ATP, or Mg^{2+} served as blanks. After incubation, the reactions were terminated by the addition of 20 ul of concentrated mercaptoethanol (final concentration 0.75 M). Aliqots from each reaction tube, mixed with 20 ul of carrier dihydrofolic acid (12 mg/3 ml of 1 M mercaptoethanol) were applied to Whatman 3 MM paper. The descending chromatograms were developed in 0.1 M potassium phosphate buffer, pH 7.0 , containing 6 mg/ml of ascorbic acid. The applied spots on the chromatogram were dried under nitrogen gas. L-(^{14}C)dihydrofolic acid, traveled with an R_f of 0.12. Carrier dihydrofolic acid aided in visualizing, under ultraviolet light, the position of the product on the chromatogram. The area of fluorescence corresponding to the quantitative formation of enzymatic product was carefully excised and put into counting vials containing 10 ml of Bray's

solution.[6] The vials were counted in a Packard Tri-Carb liquid scintillation counter Model 3380.

Detections of the Enzymatic Product Dihydrofolic Acid.

Chromatograms of reaction mixtures were analyzed for their ability to support the growth of L. casei. They were also carefully examined by serial sections and radioactive scans and revealed no enzymatic product at R_f 0.12 when either enzyme, dihydropteroic acid, ATP, or Mg^{2+} was omitted from the reaction mixture. The amount of product (dihydrofolic acid) formed was observed to increase linearly with time up to 50 min of incubation at 37°C and to be directly proportional to the amount of protein added up to 2 mg/ml. Concentrations of 4 mM ATP and 1.1 mM Mg^{2+} were found to be optimal for activity; higher concentrations of these cofactors were slightly inhibitory. Commercially available radioactive folic acid was used to confirm our enzymatic product chromatographically in different solvent systems.[7] The radioactive dihydrofolic acid formed enzymatically from dihydropteroic acid was isolated from the paper chromatogram by extraction with 0.1M Tris-HCl buffer, pH 8.2. Folic acid was obtained easily by passing oxygen gas through the extracted reaction mixture and was chromatographically identical to the known radioactive (monoglutamyl) folic acid in nuetral phosphate buffer (0.1 M, pH 6.2-7.7), R_f 0.39, or acetate buffer (0.1M, pH 6.3), R_f 0.43.

Enzyme Preparation

Crude extracts are known to contain intrinsic dihydropteridines, Mg^{2+}, and adenosine triphosphatase.[8] Therefore, the following

procedure was employed for the removal of these folate compounds, metal ions, and undesireable proteins from the crude extract. Approximately 30 g of frozen cells of E. coli (harvested from the late log-phase, 3/4 log) were thawed and suspended in 30 ml of cold 0.05 M Tris-HCl buffer, pH 8.2. The cells were disrupted by a Bronwill homogenizer for 5 min. An additional 70 ml of cold 0.05 M Tris-HCl buffer, pH 8.2, was added to the suspension which was then centrifuged for 20 min at 12,100 g to remove glass beads. The supernatant was recentrifuged at 90,000 g for an additional 20 min to remove suspended particles. The clear supernatant material was treated with deoxyribonuclease (5 mg/100 ml) to decrease the viscosity. This solution was stirred for 30 min at 25°C and centrifuged for 1 hr at 120,000 g. Ribonuclease (0.1 mg) was added to the above-mentioned supernatant fluid and incubated at 37°C for 40 min. The cloudy solution was centrifuged at 120,000 g for 20 min to remove insoluble materials. An amount of charcoal, equal in weight to the amount of protein present (80 ml, equivalent to 960 mg of protein) was added slowly to the solution and the suspension was stirred slowly at 4°C for 15 min and then centrifuged at 18,000 g to remove the charcoal. The resulting protein solution was then dialyzed overnight against 0.05 M phosphate buffer, pH 7.6, with three changes (3 liters each) of the same buffer. The enzyme preparation was free from folate compounds active in promoting the growth of L. casei, and dihydrofolate production by this preparation was dependent on the addition to the reaction mixture of dihydropteroic acid, L-glutamic acid, Mg^{2+}, and ATP.

Definition of a Unit. A unit of enzyme is that amount that is required for the formation of 1 mug of dihydrofolate under the conditions described above. Protein is determined by the method of Lowry et al.[9] with bovine serum albumin as the standard.

Inhibition Studies

The concentration of various inhibitors required for 50% inhibition (I_{50}) of dihydrofolate synthetase activity was determined by titration of at least five levels of drug in the standard reaction system. The inhibitors studied in this work have been chosen primarily because of their analogy to pteroate. They represent selective modifications of molecular size and of the substituents at the 4- and 10-positions. Changes in substituents at these positions have been shown in the case of dihydrofolate reductase to affect inhibitory activity profoundly; for example, the 4-amino group of methotrexate or trimethoprim and the added methylene group of homopteroic acid or thiol substitution in 10-thiofolic acid.[10] Eight analogs have been tested for inhibition of dihydrofolate synthetase, and their relative effectiveness is shown in Table 1. Compound 8 is inactive as an inhibitor and, in our opinion, this lack of activity can be attributed to the presence of the very polar carbonyl group at position 9. The inactivity of compounds 6 and 7 may be ascribed to the amino group in the 4-position. Comparison with compound 3 substantiates this view. We have found that trimethoprim, which may be considered a 4-amino analog as well, is also inactive as an

inhibitor of dihydrofolate synthetase. It is noteworthy that the 4-amino group, so essential for the inhibition of dihydrofolate reductase (e.g., methotrexate, aminopterin, and trimethoprim) should be ineffective with dihydrofolate synthetase, since the substrate in each case carries a hydroxyl group in the 4-position. The hydroxyl substituent at position 4 of pteroic acid analogs does allow for some measure of electronic interaction at the active site of the enzyme while the 4-amino group, as the protonated form under physiological conditions, presumably exerts electronic repulsion. It is not easy to offer an explanation for the lack of inhibition revealed by compound 5 since it bears a distinct resemblance to compounds 2 and 3, which are both significantly inhibitory. It is conceivable that the substitution of a sulfur instead of nitrogen in the 10-position, in addition to the glutamate moiety, extends the molecule beyond the tolerance of the enzyme's stereospecificity. It is apparent from Table 1 that dihydrohomopteroic acid, compound 1, is the most effective of the inhibitors examined. The affinity of the enzyme for this analog as expressed by the apparent K_i value is substantially greater than the affinity of the enzyme for its substrate, assuming that the apparent $K_m = 2.5 \times 10^{-4}$ for dihydropteroic acid is a reasonable binding constant. Along with compound 2,10-thiopteroic acid, the homopteroic acid inhibition indicates that efficacy can be improved by the addition or substitution of nonpolar species in the 10-position of dihydropteroic acid. Compound 3, dihydrofolic acid, is the product of the reaction catalyzed by dihydrofolate synthetase,

so it is not surprising that it should be a competitive inhibitor and may represent a reversal of the reaction. Nair and Baugh[11] demonstrated inhibition in vitro of Streptococcus faecium by isopteroic acid. This compound failed to inhibit the growth of L. casei. Inasmuch as L. casei cannot grow on pteroic acid and can grow on folic acid, it appears the organism lacks the enzyme dihydrofolate synthetase. S. faecium, on the other hand, can grow on pteroic acid, and presumably, must have dihydrofolate synthetase. In both instances the assumption must be made that the bacteria have the capacity to reduce these compounds to the dihydro state. The effectiveness in our cell-free system of compound 2, dihydrolo-thiopteroic acid (Table 1), confirms the recent observations[7] from one of our laboratories with whole cells of L. casei and S. faecium and suggests that its site of activity in the latter organism is dihydrofolate synthetase. The eight compounds in Table 1 and the substrate itself were all tested for inhibitory activity in their oxidized states. In no instance was inhibition observed. It is clearly mandatory and the 7- and 8-positions of the pteridine moiety be reduced to achieve binding of substrate and inhibitor to the enzyme. In order to confirm the kinetic behavior of the enzyme toward these newly synthesized pteroate analogs, independent experiments were carried out with a constant concentration of each drug and at least five levels of substrate concnetration (i.e., dihydropteroic acid). From such velocity data, Lineweaver-Burk kinetic plots were obtained for each of the active compounds. The inhibition exerted by compounds 1 through 4 of Table 1 are found to be competitive in nature. The apparent K_i value for each compound

is the result of at least five experiments, and it was observed experimentally that the kinetic data were reproducible to within 1%.

TABLE 1

COMPOUND	I_{50} (M)	K_i (M)
1. Dihydrohomopteroic acid	5.0×10^{-6}	1.4×10^{-5}
2. Dihydro-10-thiopteroic acid	1.3×10^{-5}	4.0×10^{-5}
3. Dihydrofolic acid	2.5×10^{-5}	1.1×10^{-4}
4. Dihydroisopteroic acid	6.0×10^{-5}	1.7×10^{-4}
5. Dihydro-10-thiofolic acid	no activity	
6. 4-Amino-deoxy-7,8-dihydro-10-thiopteroic acid	no activity	
7. Dihydro-10-thioaminofolic acid	no activity	
8. 9-oxo-Pteroic acid	no activity	

REFERENCES

1. G.M. Brown, R.A. Weisman, and D.A. Molnar, J. Biol. Chem. 236, 2534 (1961).

2. V. Herbert, J. Clin. Invest. 40, 81 (1961).

3. L.T. Plante, J. Org. Chem. 36, 860 (1971).

4. C.E. Houlihan, P.H. Boyle, and J.M. Scott, Anal. Biochem. 46, 1 (1972).

5. S. Futterman, J. Biol. Chem. 228, 1031 (1957).

6. G.A. Bray, Anal. Biochem. 1, 279 (1960).

7. M.G. Nair, P.T. Campbell, and C.M. Baugh, J. Org. Chem. 239, 310 (1964).

8. M.J. Griffin, and G.M. Brown, J. Biol. Chem. 239,310 (1964).

9. O.H. Lowry, M.J. Rosebrough, A.L. Farr, and R.J. Randell, J. Biol. Chem. 193, 265(1951).

10. M.G. Nair, and C.M. Baugh, V International Symposium on Pteridines, Konstanz, West Germany, 1975.

11. J.R. Bertino, ed. Folate Antagonists as Chemotherapeutic Agents. Ann. N.Y. Acad. Sci. 186, 475 (1971).