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TITLE

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DATE

September 1976 - August 1977  
CONTRACT NO. DAMD17-74-C-4039

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GONORRHOEAE

REPORT NUMBER 4

TITLE

6 COMPARISON OF DIHYDROFOLATE REDUCTASES OBTAINED FROM TRI-METHOPRIM AND SULFONAMIDE RESISTANCE OF NEISSERIA GONORRHOEAE

TYPE OF REPORT

9 Annual Summary Report. no. 4, Sep 76-Aug 77

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DATE

11 Sep 1977

12 16P.

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Washington, D.C. 20314

Contract No. 15 DAMD17-74-C-4039

Massachusetts College of Pharmacy  
Boston, Massachusetts 02115

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Four	2. GOVT ACCESSION NO. A	3. RECIPIENT'S CATALOG NUMBER
4. U.L.R. (and Subtitle) Comparison of Dihydrofolate Reductase from Trimethoprim and Sulfonamide Resistance of <u>Neisseria Gonorrhoeae</u>		5. TYPE OF REPORT & PERIOD COVERED Annual Summary Report Sept. 1976-August 1977
7. AUTHOR(s) Ho, Richard I. Corman, Leonard		6. PERFORMING ORG. REPORT NUMBER A
9. PERFORMING ORGANIZATION NAME AND ADDRESS Massachusetts College of Pharmacy Boston, Massachusetts 02115		8. CONTRACT OR GRANT NUMBER(s) DAMD17-74-C-4039
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command, Washington, D.C. 20314		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A 3M161102BS01.00.002
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE September, 1977
		13. NUMBER OF PAGES 16 pages
		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, Sulfonamides, Sulfones, Gonorrhoea, Meningitis, Folic Acid		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Dihydrofolate reductases from two strains (CDC 9 and 7134) of <u>Neisseria gonorrhoeae</u> with different sensitivity to sulfamethoxazole (60 fold) and trimethoprim (2 fold) have been isolated and purified. The reductases are strikingly similar in most respects. Purification steps produced essentially similar results with both enzymes. The pH profiles of both reductases were virtually identical; and the effect of increasing salt concentration was the same. There were no		

significant differences in any of the kinetic parameters. The enzymes do differ with respect to thermostability, substrate protection against heat inactivation and their response to inhibition by methotrexate.

The observation that the less sensitivity of the organism to trimethoprim is accompanied by the greater resistance of the organism to sulfamethoxazole can not be explained by the mechanism involving different trimethoprim susceptibility of dihydrofolate reductase.

Dihydrofolate reductase of the strain CDC 9 is further purified to homogeneous state, the purification procedure involves the use of 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 sulfhydryl groups which are not in the active site pocket of the 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 are exchangeable under effects of air oxidation and disulfide reducing agents by which enzyme activity could be regenerated.

Inhibition of dihydrofolate reductase by dihydrofolate above the concentrations that possibly pertain within the cell is found to occur with the gonococcal enzyme. The kinetic data indicate that the substrate inhibition is uncompetitive.

- A. A Comparison of Dihydrofolate Reductase from Trimethoprim- and Sulfonamide-Resistant Strains of Neisseria Gonorrhoeae.
- B. Purification and Kinetic Characteristics of Dihydrofolate Reductase from Neisseria Gonorrhoeae.
- C. Interconvertible Active and Inactive Forms of Dihydrofolate Reductase from Neisseria Gonorrhoeae.

## SUMMARY

The purpose of this work is to provide a rational chemotherapeutic approach to diseases of bacterial origin. It focuses on gonorrhoea but has wider applicability. The methodology involves molecular considerations and therefore requires the purification of the particular enzymes responsible for folic acid biosynthesis.

The enzymes particularly concerned in our work are dihydropteroate synthetase, dihydrofolate synthetase and dihydrofolate reductase. However, due to the basic properties of the enzymes we have found that dihydrofolate reductase from gonococcal extract is most easily purified. This particular enzyme can be purified into an homogeneous state by a series of steps of purification and the step of affinity column chromatography was found most efficient.

A synergistic effect is usually observed both in vitro and clinically with the combinative uses of various sulfonamides and trimethoprim. This biological phenomenon is usually explained by the biochemical knowledge that there is a sequential blockage of the enzyme dihydropteroate synthetase by sulfonamide and dihydrofolate reductase by trimethoprim. Therefore the fact that many strains of Neisseria gonorrhoeae exhibiting sulfonamide resistance are found to be also relatively insensitive to trimethoprim indicate to us that there must be a different biochemical interrelationship between the above mentioned two enzymes when they are existing in the different sulfonamide resistant gonococcal strains. The difference may be that the concerned enzyme is structurally altered or their enzymatic complexes are constructed quite differently.

In order to be able to offer meaningful biochemical explanations for the above mentioned synergistic effects, states of highly purified enzymes are desirable. Due to the above mentioned excellent chromatographic properties of the enzyme dihydrofolate reductase, we have indeed made great progress in the studies of this enzyme during the past year. Because the nature of these studies is exploratory, we have been simultaneously pursuing several parallel investigations. As a result, three areas of activity are reaching varying stages of completion and they are reported under the following topics.

A. A Comparison of Dihydrofolate Reductase from Trimethoprim- and Sulfonamide-Resistant Strains of Neisseria Gonorrhoeae.

In vitro; strains CDC 9 and 7134 exhibited a two-fold difference in the MIC of trimethoprim (TMP) and methotrexate (MTX) and a sixty-fold difference in the MIC of sulfamethoxazole (SMZ). Although it was previously shown that the affinity of dihydropteroate synthetase for sulfonamide in sulfonamide-sensitive (strain CDC 9) and -resistant (strain 7134) strains was markedly different, no conclusive difference can be demonstrated in the binding of TMP by dihydrofolate reductase from these organisms. It is possible that a two-fold difference in the affinity of the enzyme for TMP could not be measured accurately under our conditions but the striking similarities in all other parameters examined persist. Two-fold differences in MIC values are not often considered significant when obtained by serial two-fold dilutions of the drug. However, in our experiments the MIC of TMP and MTX was obtained by using 10 ug/ml increments of the drug. Thus, the two-fold difference in the MIC observed with TMP and MTX represent significant differences in the sensitivity of these strains.

Purification by DEAE Bio-Gel A column chromatography suggested a similarity in the two enzyme proteins as they eluted at approximately the same NaCl concentration. The pH activity profiles are superimposable indicating similarity in the amino acids participating at the active site(s) and /or structural elements essential to activity. Although many other reductases from avian and mammalian sources also showed two pH optima, E. coli and gonococcal reductases are the only known bacterial enzymes exhibited two pH optima. The effect of increasing salt concentration on the activity of these enzymes further supports their similarity.

The kinetic parameters ( $K_m$ ,  $V_{max}$ ) revealed no significant differences between the two dihydrofolate reductases. The apparent  $K_m$  for dihydrofolate (DHF) and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and the calculated  $V_{max}$  were for all practical purposes, indistinguishable. The  $K_i$  ( $3.4 \times 10^{-8}$  M) of TMP for gonococcal dihydrofolate reductases is very similar to the average  $K_i$  value ( $5 \times 10^{-8}$  M) obtained for enzymes from E. coli, P. vulgaris and S. aureus. In contrast, the average value for the

same enzyme from a variety of mammalian sources was about  $3 \times 10^{-3}$  M. Thus, TMP is about 10,000 times more potent as an inhibitor of gonococcal reductase than the mammalian enzyme. The inhibition of the gonococcal dihydrofolate reductase by high DHF concentration is of special interest as DHF substrate inhibition of dihydrofolate reductase has been reported recently, so far, only with bovine liver and L. casei reductases.

Examination of the heat stability of dihydrofolate reductase indicated a difference between enzyme from strain CDC 9 and 7134. When the dihydrofolate reductase was incubated in the presence of its substrate, DHF, it was not only protected to a significant extent against heat inactivation, but interestingly enough, was substantially activated at room temperature. This phenomenon was seen most dramatically with the enzyme from the more sensitive strain (CDC 9) but also occurred at room temperature, with the enzyme from the more resistant strain (7134). At higher temperatures (40, 55 C), the protective effect of DHF was more apparent with the enzyme from CDC 9. Additional experiments have indicated that these enzymes are essentially identical with respect to the other parameters measured. Therefore, we must assume that the difference in enzyme structure revealed by substrate protection against thermal inactivation is small or subtle in its nature. It appears unlikely to be a matter of molecular size since that should have been revealed as a difference in elution during purification. Significant charge differences should also have been observed during purification on DEAE Bio-Gel A. The active site(s) of these enzymes cannot be very different in view of the similarity of the kinetic constants, but if the difference is slight it seems reasonable to assume that it exists at or near the active site(s). This reasoning led us to try MTX, a potent competitive inhibitor of dihydrofolate reductase, in an attempt to distinguish between the two enzymes. The results indicated that the enzymes from strains CDC 9 and 7134 respond differently to MTX. Titration experiments with MTX suggest that this partially reversible inhibitor was bound more tightly by the reductase of the more resistant strain 7134. This observation directly conflicts with our MIC determinations. The difference between MTX effects on whole bacterial cells (MIC studies) and

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partially purified enzyme preparations may be attributable to permeability characteristics of the cell envelope with respect to MTX. Other possibilities should also be considered. Mutation to aminopterin (a compound related to MTX) has been reported for some species of microorganisms. Two types of resistant mutants were described: the first produced as much as 100 times more dihydrofolate reductase than was formed in the sensitive strains and a second type involved an alteration in the dihydrofolate reductase protein. The most important feature of this altered enzyme is that aminopterin was much less effective as an inhibitor and its action could be overcome by an intracellular accumulation of the natural competitor DHF.

Thus, although there is a minor structural difference between the two enzymes at or near the active site(s), it may not be related to the increased resistance of strain 7134 to TMP and MTX. In this strain, resistance may be due to decreased permeability of the cell envelope or increased production of dihydrofolate reductase. Additional studies are required to determine whether other naturally occurring TMP-resistant strains are similar.

#### B. Purification and Kinetic Characteristics of Dihydrofolate Reductase from Neisseria Gonorrhoeae.

##### Enzyme Purification

In order to reduce the volume of crude extracts, concentration with sucrose has been found more beneficial than ultrafiltration or ammonium sulfate precipitation.

The modified purification procedure on the step of DEAE Bio-gel A column chromatography will result in better recovering and resolution of the enzyme reductase. Subsequent ultrafiltration with Amicon PM-10 instead of concentration with ammonium sulfate also doubled the yield of enzyme activity. A change of the DEAE Bio-Gel A column eluting buffer pH from 7.0 to 6.5 increased the enzyme retardation on this column, so that a contaminating protein eluted prior to the major enzyme peak could be achieved.

At least a 40-fold purification has been achieved by the MTX affinity chromatography of the gonococcal reductase. MTX is unstable

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at a higher pH, and it was found necessary to immediately rewash the affinity gel back to lower pH (6.5) after enzyme elution at the higher pH (8.5) buffer to save the life of the affinity gel.

The application of affinity chromatography to the purification of dihydrofolate reductase has become part of the routine purification procedures. It is usually used earlier in the purification profile of the reductase. The MTX affinity chromatography experience obtained in this study revealed that, except for large scale preparation of the enzyme, the use of MTX affinity chromatography with partially purified enzyme preparation is more beneficial for three reasons: 1). a small affinity column could be used, 2). it is easier to elute the enzyme from the column, 3). the column could be easily recycled for subsequent use.

The MTX affinity chromatography is particularly important in this study, since in addition to its powerful ability to purify the reductase, the MTX affinity column can also be used as a differentiating tool to distinguish the inactive form from the active form of the enzyme. The inactive enzyme was copurified with the active enzyme throughout the previous purification procedures. This study presents the first application of affinity chromatography to the separation of structural enzyme isomers.

The non-retardation of the inactive enzyme in the affinity column indicates that the changed conformation of the enzyme during inactivation is unfavorable for the binding of MTX.

## Purity and Molecular Weight

The enzyme purified from the affinity column has a high purity (about 5500-fold purification) and is homogeneous electrophoretically; only one single protein band could be detected with disc gel electrophoresis. Since the enzyme activity could not be shown by staining the protein with MTT tetrazolium salt, this protein band shown as stained with amido black is the inactive enzyme form. Electrophoresis of the autooxidized inactive enzyme gave the same  $R_f$  value.

Early in this study, it was found that the enzyme is labile at a pH higher than 8.0 and, in the absence of EDTA, lost considerable activity during purification and storage. The probable factors causing this inactivation during electrophoresis are the higher pH(8.3) and the absence of EDTA in the tank buffer used in discontinuous gel electrophoresis. The addition of folate or NADPH did not change the

mobility of the single protein band, which again indicates that this single protein band is the inactive enzyme. Addition of folate or NADPH which results in a different electrophoretic mobility from that of the apoenzyme has been reported.

The molecular weight (20,300) estimated for the gonococcal reductase falls into the range of molecular weight of the known dihydrofolate reductase.

#### Dihydrofolate Substrate Inhibition

Since the gonococcal reductase is inhibited by its substrate, DHF, the  $K_m$  value for DHF is reexamined. A  $K_m$  value of 5.9  $\mu\text{M}$  is obtained from 14 data points on the curve by the cleland method described for substrate inhibition. The results of the present study also show that the DHF inhibitory effect on enzyme activity is nonallosteric (the low molecular weight of this enzyme also suggests a hypothesis without involving subunit structure). Substrate inhibition by DHF becomes significant above 13  $\mu\text{M}$ , and the calculated  $K_i$  for DHF is 29  $\mu\text{M}$  which is only 5-fold higher than the  $K_m$  for DHF.

Although dihydrofolate reductase has been the subject of intensive investigation, its DHF substrate inhibition has so far been observed only with the gonococcal reductase, bovine liver and L. casei enzymes. Rowe and Rusel (1) with bovine liver reductase observed also that the substrate inhibition by NADPH, and DHF became inhibitory above a concentration of 70  $\mu\text{M}$  which is very close to the value found with the gonococcal reductase (13 $\mu\text{M}$ ). Other reports that have noted inhibition of the reductase at high concentrations of DHF are Kaufman and Kameron (2) with bovine liver reductase, Jackson et al. (3) with the reductases from four cultured mammalian cell lines (the inhibition was interpreted as caused by a degradation product of commercial DHF), and Blakley et al. (4) with a bacterial reductase (which DHF become inhibitory above  $10^{-4}$  M). Since the DHF used was freshly prepared from the purified folate, the observed substrate inhibition is not caused by an artifact or by impure substrate. Since folate would be a competitive inhibitor, the possibility that unreduced folate acts as alternative substrate resulting in substrate inhibition can also be excluded. According to Dalziel (5), only a noncompetitive inhibitor existing in the

substrate preparation at a constant ratio will show an inhibition pattern resembling that of substrate inhibition

It is possible that substrate inhibition by DHF is a general property of all dihydrofolate reductases and, its observation requires adequate assay conditions. The favored assay conditions for demonstrating this inhibition are an enzyme preparation which is free from non-specific NADPH oxidase activity contamination; an adequately low concentration of enzyme which otherwise will result in a too fast velocity to give comparable rates of reaction at short observation intervals (e.g., 15 sec or 30 sec intervals); a high enough concentration of substrate to obtain a higher ratio of (S) to  $K_m$  (the (s) commonly used is only ten times higher than the value of  $K_m$  and is too low to show appreciable inhibitory effects).

C. Interconvertible Active and Inactive Forms of Dihydrofolate Reductase from Neisseria Gonorrhoeae.

Participation of SH Groups in the Gonococcal Dihydrofolate Reductase Polymorphism

Previously, multiple forms of dihydrofolate reductase from chicken liver, the insect, Galleria mellonella, and the bacterium, Diplococcus pneumoniae have been identified. These multiple forms of the chicken liver enzyme can mutually transform under the influence of mercaptoethanol. It was also observed that activity of the reductases from chicken liver and the insect could be effected by sulfhydryl reagents.

One general property of the multiple forms of the reductases from these three sources is that they all showed the enzyme activity (active forms), besides, activation of both chicken liver and the insect reductases by sulfhydryl reagents could be prevented by thiols.

The gonococcal reductase is the first case describing the regeneration of the reductase activity from an inactive form of the enzyme by thiols and the interconversion of the active and inactive forms of the reductase which are the unique differences from the known structural isomers of dihydrofolate reductase.

Since the sulfhydryl reagents used in this study, namely,

PCMB and IAA are capable of reacting with amino acid residues other than cysteine, inhibition of reductase activity by these reagents could not be readily attributed to a reaction between cysteine residue(s) and such reagents. However the observation that the enzyme activity could be regenerated from the inactive enzyme solution by mercaptoethanol and DTT which are widely used as disulfide-reducing agents in protein chemistry does speak for the presence of SH group(S) in the gonococcal reductase molecule. DTT and 2-mercaptoethanol showed different mechanisms of enzyme activity activation which can be explained by the ability of DTT to form a stable 6-membered ring when it is oxidized and indicates that this is a thiol-disulfide exchange.

The inhibition of the enzyme activity by PCMB and IAA under the described conditions (pH 7.6 and a very short reaction time) seems very likely to be due to a reaction between SH group(S) and the SH reagents. The inability of NADPH or DHF to protect the enzyme from inhibition by SH reagents suggests that the SH group(S) involved is not at the binding sites of both substrates. That the SH group(S) is not involved in catalysis is quite obvious since the reductases from L. casei and S. faecium contain no SH group. The observation that SH group(S) is not involved in catalysis has been reported with the SH containing reductases from chicken liver, sheep liver and bovine liver.

On one hand the gonococcal reductase is similar to those from calf thymus, sarcoma 180, E. coli and bovine liver in that they are inhibited by SH reagents, on the other hand, it is different from those of Ehrlich ascites cell, chicken liver and L1210 lymphoma cells which are reported to be activated by SH reagents.

The appearance of one enzyme activity peak suggests that the SH groups involved may be spatially oriented closely to each other in the enzyme's tertiary structure. Disulfide formation may also cause conformational changes near or at the active site of the reductase such that the enzyme-substrate complex(es) is not formed or reduction of dihydrofolate can not be catalyzed at all. Since the inactive enzyme is not retarded on the MTX affinity column, it seems likely that inactivation is due to a loss of substrate

binding ability of the oxidized enzyme.

Formation of intramolecular disulfide link has also been reported for the E. coli reductase, whether its oxidized form shows reductase activity is not known.

An aggregate containing at least 28 monomers of the reductase due possibly to disulfide bonding were noted for an E. coli enzyme. Since the reported assay mixture contained 12 mM 2-mercaptoethanol, a disulfide-reducing agent, it is doubtful that such an aggregate would show activity.

Argument has been made that lyophilized E. coli reductase might contain a cysteine residue which was oxidized to a higher oxidation state, i.e., sulfenate, than a disulfide. Sulfenates are generally unstable and would undergo further oxidation to an even higher oxidation state which would not be reduced by thiols. Iodine oxidation of the SH groups of creatine kinase to higher oxidation state than the disulfide has been known to result only in a partial recovery of the enzyme activity by thiol reducing agents. (It can be seen that a constant recovery of enzyme activity was observed when the enzyme preparation was incubated with DTT). It is unlikely that the inactivation of the gonococcal reductase is due to oxidation of SH groups to a higher oxidation state than disulfide under mild conditions.

#### SH Group(S) in Dihydrofolate Reductases

At the present moment, the role of SH group(S) in the reductase molecule remains unknown. However most of the known dihydrofolate reductases contain cysteine residue(s). The only known exceptions to this generalization are the two reductases from L. casei and S. faecium which have to depend on the foreign folates for growth; moreover, all the known vertebrate, i.e., chicken liver, L1210 cells, and bovine liver dihydrofolate reductases contain only one cysteine residue. (The number of cysteinyl residues in the bovine liver reductase reported by Peterson et al. (6) and Baumann and Wilson (7) who showed two and three residues respectively is somewhat uncertain. The value of one cysteinyl residue reported by Kaufman and Kermerer (2) is more reliable since this value represents the results from experimentations using four different amino acids as references for the performic acid oxidation product of cysteine in the enzyme molecule). Several strains of E. coli

have two cysteine residues in the reductase molecule. The existence of cysteine in E. coli, in gonococcal reductases and in vertebrate enzymes may indicate a physiological role for these residues which L. casei and S. faecium reductases do not possess. Furthermore, these facts may suggest some implications on enzyme evolution or physiological regulation at the molecular level.

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