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Alterations in Dihydropteroate Synthetase in Cell-Free Extracts of Sulfanilamide-Resistant *Neisseria meningitidis* and *Neisseria gonorrhoeae*

RICHARD I. HO,[1] LEONARD CORMAN, STEPHEN A. MORSE, AND MALCOLM S. ARTENSTEIN

Harvard School of Dental Medicine and Department of Microbiology, Harvard School of Public Health, Boston, Massachusetts 02115, and Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

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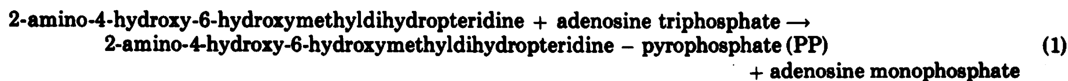
Extracts from *Neisseria meningitidis* and *N. gonorrhoeae* with varying susceptibility to sulfanilamide have been investigated for dihydropteroate synthetase activity. Sulfanilamide was a competitive inhibitor of dihydropteroate synthetase with respect to *p*-aminobenzoate in extracts from both species. Though the K_m for *p*-aminobenzoate was unaffected, the K_i for sulfanilamide increased and the V_{max} decreased as the strains' resistance to sulfanilamide increased. Temperature studies have revealed differences in the dihydropteroate synthetase from *N. meningitidis* and *N. gonorrhoeae*. A direct relationship was observed between the minimal inhibitory concentration of sulfanilamide determined in vitro and the ratio of K_i/K_m. This ratio may be a molecular explanation of sulfanilamide resistance for both *N. meningitidis* and *N. gonorrhoeae*.

Woods (20) showed that sulfanilamide owed its bacteriostatic activity to its competition with *p*-aminobenzoic acid (PABA). Later, Lascelles and Woods (10) demonstrated that sulfanilamide blocked the sequence of reactions leading to the biosynthesis of folic acid. Since that time, the inhibition of in vivo folic acid biosynthesis by sulfonamides has been established (21) as well as the enzymatic sequence involved in folic acid biosynthesis by cell-free preparations derived from both bacteria and plants (14).

The biosynthetic pathway for dihydropteroate proceeds via the two reactions:



2-amino-4-hydroxy-6-hydroxymethyldihydropteridine + adenosine triphosphate ⟶ 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine − pyrophosphate (PP) + adenosine monophosphate (1)

2-amino-4-hydroxy-6-hydroxymethyldihydropteridine − PP + PABA → dihydropteroate + PP (2)

Recently, Ortiz (15) has shown that K_m values for PABA in wild-type and drug-resistant strains of *Diplococcus pneumoniae* were essentially identical. Enzymes from the drug-resistant strains displayed a decreased affinity for sulfanilamide when compared with those from susceptible strains. Wolf and Hotchkiss (19) had suggested that the variation in sulfonamide resistance in mutant strains of *D. pneumoniae* was attributable to alterations in the PABA and sulfonamide binding groups of one enzyme (dihydropteroate synthetase). They were able to partially purify this enzyme and indicated that both the sulfa resistance and susceptibility observed in intact cells was correlated with dihydropteroate synthetase activity in cell-free extracts. Since then several laboratories (1, 8, 16; G. M. Brown, Fed. Proc. 18:19) have attempted to define the nature of this alteration.

The present study evaluates alterations in the enzyme dihydropteroic acid synthetase, which may explain variations in sulfonamide sensitivity observed in clinical isolates of *Neisseria meningitidis* and *N. gonorrhoeae*.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: hydroxymethylpteridine for 2-amino-4-hydroxy-6-hydroxymethylpteridine; hydroxymethyldihydropteridine for 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine; and hydroxymethyldihydropteridine-PP for 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine-PP.

[1] Present address: Massachusetts College of Pharmacy, Boston, Mass. 02115.

Organisms and preparation of cell-free extracts. The properties of the organisms used in this study are shown in Table 1. Agar-grown cultures of *N. meningitidis* were harvested after 18 to 24 h of growth at 37 C on Mueller-Hinton agar (Difco) plates. Cells were removed by gentle scraping, suspended in a minimal volume (usually 5 ml for five to six plates) of 0.1 M phosphate-buffered saline (pH 7.2), and ruptured by use of a Ribi cell fractionator (Ivan Sorvall Inc. Norwalk, Conn.). Extracts were centrifuged (45,000 × *g* for 30 min) to remove particulate material. Agar-grown cultures of *N. gonorrhoeae* were harvested after 18 to 24 h growth on GC agar (Difco) plates at 37 C in a CO_2 incubator containing 5% CO_2. Cells were removed by gentle scraping, suspended in a minimal volume of 0.1 M phosphate-buffered saline (pH 7.2), and disrupted in a cell homogenizer (Braun, model MSK). The resulting suspension was centrifuged at low speed (12,000 × *g* for 15 min) to remove the glass beads followed by centrifugation at high speed (45,000 × *g* for 30 min) to remove the remaining particulate material. All cell-free extracts were stored at −30 C until assayed.

Treatment of cell-free extracts. All extracts contained small amounts of folic acid, or some other substance(s), which stimulated the growth of *Streptococcus faecium* (ATCC 8043). Therefore, it became necessary to subject the extracts to ammonium sulfate fractionation and extensive dialysis to minimize the effect of this contaminant. Extracts were brought to 30% saturation by the slow addition of solid ammonium sulfate and gently stirred for 60 min at 4 C. If no precipitate was observed at this point, the solution was brought to 65% saturation and stirred for an additional hour. After centrifugation, the precipitate was dissolved in a minimal volume of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.1 to 8.2) and dialyzed against 9 liters of this buffer for a period of 9 h.

Preparation and reduction of substrates. Hydroxymethylpteridine was prepared by the method of Forrest and Walker (4) and purified by repeated recrystallization from water. The purity of the recrystallized product was demonstrated by paper chromatography (14). The absorption spectra and R_f values were identical with those of authentic samples (14). Hydroxymethyldihydropteridine-PP was chemically prepared (17) and purified on a Dowex-1 anion exchange column as suggested in a personal communication from T. Shiota (Department of Microbiology, University of Alabama). Immediately before use the above compound, as well as pteroic acid, was reduced to the dihydro-form with sodium hydrosulfite by the method of Futterman (5).

Enzyme assay. Dihydropteroate synthetase was assayed by a direct assay system. The reaction mixture consisted of: 50 mM Tris-hydrochloride buffer (pH 8.1 to 8.2); 0.025 mM hydroxymethyldihydropteridine-PP; 1.8 to 15 mM PABA; and 24 mM 2-mercaptoethanol in a total volume of 6.42 ml. The complete enzyme reactions were carried out under anaerobic conditions (argon gas). The constituents were mixed well, and the reaction was started by the addition of partially purified extract. Reaction mixtures were incubated at 25 C and, at specific intervals, 1-ml samples were transferred to tubes containing 0.1 ml of potassium ascorbate solution (3 mg of potassium ascorbate per ml) and heated in a boiling water bath for 4 min. The amount of product formed was assayed for folate activity by microbiological assay.

Microbiological assay. The product of enzyme activity, dihydropteroic acid, was measured by the growth response of *S. faecium* (ATCC 8043). The assay organism was grown in the medium of Flynn et al. (3), and growth was quantified turbidimetrically at 640 nm. A standard control containing known amounts of folic acid was used with each assay, and all results are reported as folate equivalents.

Chemicals. ATP was purchased from Sigma Chemical Co. (St. Louis, Mo.), PABA was from Eastman Kodak Co. (Rochester, N.Y.), and crystalline folic acid was from Nutritional Biochemicals (Cleveland, Ohio). Pteroic acid was a gift from R. Kisliuk (Department of Biochemistry, Tufts University). All other reagents were of analytical grade.

Miscellaneous measurements. Protein was determined by a modification of the method of Lowry et al. (11), with crystalline bovine serum albumin as a standard.

TABLE 1. *Properties of clinical isolates used in this study*

Organism[a]	Strain	Serogroup	MIC[b] (μg/ml)
N. meningitidis	M-60	C	0.8
	M-166	B	2.0
	M-126	C	12.5–25
N. gonorrhoeae	A-12	—	0.8
	Kohn	—	8.0
	7134	—	12.5–25

[a] All strains were obtained from the Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

[b] Minimal inhibitory concentration of sulfanilamide.

RESULTS

Enzyme activity was demonstrated in dialyzed extracts of all strains of *N. meningitidis* and *N. gonorrhoeae* examined when assayed at 25 C in the presence of required substrates. No activity was observed in the absence of added PABA. The reaction rate was proportional to protein concentration, and the rate of dihydropteroate formation (measured as "folate equivalents") was linear during a 1-h incubation period.

The activities of dihydropteroate synthetase in extracts of strains of *N. meningitidis* with varying degrees of sulfanilamide susceptibility were compared. Values (K_m, K_i, and V_{max})

calculated from Lineweaver-Burk analysis of the data (Fig. 1) are summarized in Table 2. The value for K_m was essentially the same for all three strains. The V_{max} of the enzyme reaction decreased as the sulfanilamide resistance of the strain increased. The competitive nature of the inhibition of dihydropteroate synthetase by sulfanilamide is shown in Fig. 1. The increase in the K_i values (Table 2) indicates that dihydropteroate synthetase has less affinity for sulfanilamide as the resistance of the strain to the drug increases. On the other hand, the relative constancy of the K_m values suggests that the affinity of the enzyme for its substrate PABA remains unchanged in increasingly resistant strains.

For comparison, activities of dihydropteroate synthetase in extracts of strains of *N. gonorrhoeae* with varying degrees of sulfanilamide resistance were examined. The results (Table 3) were similar to those observed with *N. meninigitidis*. The values for K_m were essentially the same for both species. The V_{max} of the enzyme reaction also decreased as the sulfanilamide resistance of the strain increased, but the magnitude of these values was 3.5- to 4.0-fold less than the values observed with *N. meningitidis*. Figure 2 demonstrates the competitive nature of the inhibition of dihydropteroate synthetase by sulfanilamide; the increase in the K_i values (Table 3) indicated a decreased affinity for sulfanilamide as the resistance of the strain to the drug increased.

The effect of assay temperature on the activities of dihydropteroate synthetase from *N. meningitidis* and *N. gonorrhoeae* is shown in Table 4. Maximal activity was observed at 37 C for all three strains of *N. meningitidis*; the temperature range broadened as the resistance of the strain to sulfanilamide increased. No activity was observed at 55 C. In contrast, appreciable activity was observed at 55 C in extracts from all three strains of *N. gonorrhoeae*. Of particular interest was the observation that the optimal temperature, as well as the heat stability of the enzyme, increased as the resistance to sulfanilamide increased.

The effect of temperature on dihydropteroate

TABLE 2. *Rate constants for dihydropteroate synthetase in cell-free extracts of sulfanilamide-resistant N. meningitidis*[a]

Strain	MIC (μg/ml)	K_i (M × 10^6)	K_m (M × 10^3)	K_i/K_m[b] (× 10^3)	V_{max}[c]
M-60	0.8	1.5	5	0.44	250
M-166	2.0	3.0	1.4	0.88	111
M-126	12.5–24	24	4	7.05	25

[a] Values represent the average of 25 assays with each strain.

[b] 3.4×10^{-3} M is used as the mean K_m for calculation of ratio.

[c] Expressed as nanograms of folate equivalents per milligram of protein per 30 min.

TABLE 3. *Rate constants for dihydropteroate synthetase in cell-free extracts of sulfanilamide-resistant N. gonorrhoeae*[a]

Strain	MIC (μg/ml)	K_i (M × 10^6)	K_m[b] (M × 10^3)	K_i/K_m (×10^4)	V_{max}[c]
A-12	0.8	0.19	1.8	1.0	71.4
Kohn	8.0	1.9	2.3	10.0	27.7
7134	12.5–25	3.1	1.6	16.0	6.6

[a] Values represent the average of 25 assays with each strain.

[b] 1.9×10^{-3} M is used as the mean K_m for calculation of ratio.

[c] Expressed as in Table 2.

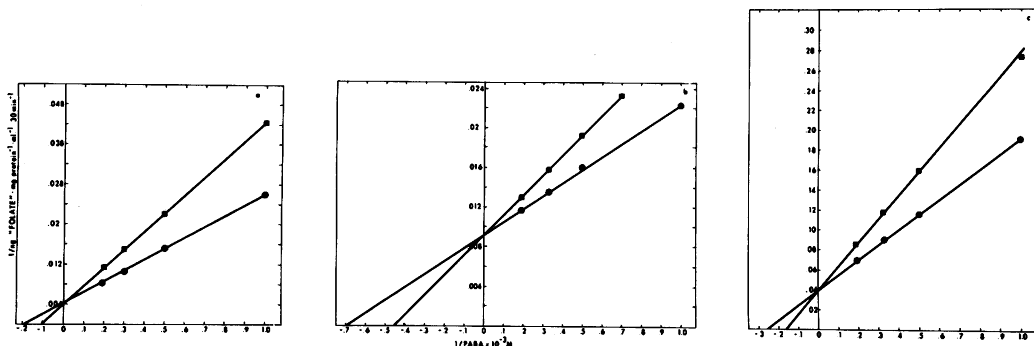


FIG. 1. *Competitive inhibition of dihydropteroate synthetase by sulfanilamide in cell-free extracts of N. meningitidis. Enzyme activity was measured as described in Materials and Methods. (a) Strain M-60; (b) strain M-166; (c) strain M-126. Symbols: ●, without sulfanilamide; ■, with 1.55 × 10^{-6} M sulfanilamide. Values represent the average of 25 assays with each strain.*

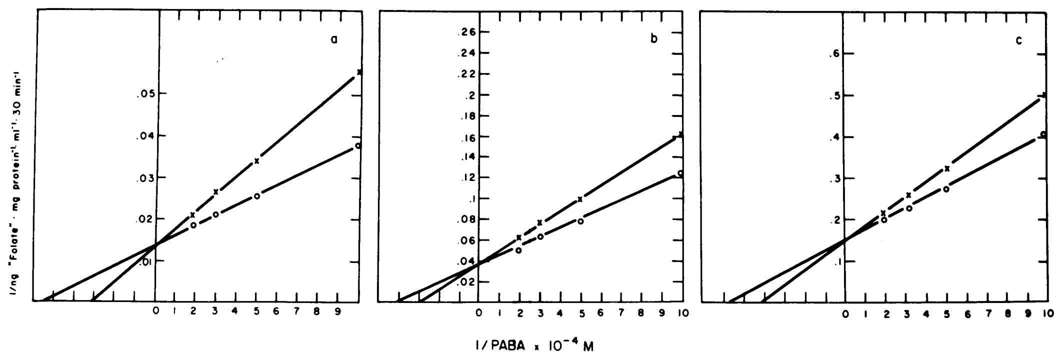


FIG. 2. *Competitive inhibition of dihydropteroate synthetase by sulfanilamide in cell-free extracts of N. gonorrhoeae. Enzyme activity was measured as described in Materials and Methods. (a) Strain A-12; (b) strain Kohn; (c) strain 7134. Symbols: O, without sulfanilamide; ×, with 1.55 × 10^{-6} M sulfanilamide. Values represent the average of 25 assays with each strain.*

TABLE 4. *Effect of assay temperature on dihydropteroate synthetase activity in sulfanilamide-resistant strains of N. meningitidis and N. gonorrhoeae*

Organism	Strain	Percent of maximal activity at				
		25 C	30 C	37 C	45 C	55 C
N. meningitidis	M-60	30	50	100	40	0
	M-166	30	50	100	73	0
	M-126	40	65	100	60	0
N. gonorrhoeae	A-12	100	80	75	60	40
	Kohn	65	81	100	72	54
	7134	50	66	75	100	70

TABLE 5. *Effect of preincubation temperature on the loss of dihydropteroate synthetase activity in extracts of sulfanilamide-resistant N. meningitidis*

Temper-ature (C)	Activity (%) lost[a]					
	Strain M-60		Strain M-166		Strain M-126	
	30 min	60 min	30 min	60 min	30 min	60 min
25	30	44	12	28	8	22
30	40	60	15	35	10	26
37	45	65	23	40	20	35
45	75	100	40	90	35	75

[a] Enzyme extracts assayed at 25 C as described in Materials and Methods.

synthetase activity in cell-free extracts of *N. meningitidis* was investigated further by preincubating extracts for 30 or 60 min at the temperatures indicated in Table 5. After this preincubation period, extracts were chilled in an ice bath and then assayed for dihydropteroate synthetase activity. The results (Table 5) showed that the heat stability of this enzyme also increased with increasing resistance to sulfanilamide.

DISCUSSION

Hotchkiss and Evans (6) studied the effect of sulfanilamide on dihydropteroate synthetase activity in genetically transformed mutants of *D. pneumoniae*. These investigators concluded that drug resistance was achieved through an alteration in enzyme structure expressed as a reduced binding capacity for the inhibitor, sulfanilamide, rather than a change in affinity for the substrate, PABA. Our results with naturally occurring clinical isolates of *N. meningitidis* and *N. gonorrhoeae* support this conclusion. Kinetic studies showed that dihydropteroate synthetase from both species had a reduced affinity for sulfanilamide which paralleled the increased resistance of the organism to the drug. To the best of our knowledge, this is the first report confirming that the mechanism of sulfanilamide resistance in clinical isolates is the same as in genetically manipulated organisms.

Temperature stability studies demonstrated differences in the enzyme in extracts of *N. meningitidis* and *N. gonorrhoeae*. The heat stability of dihydropteroate synthetase in extracts of either organism increased with increasing resistance. However, although the optimal temperature for activity was the same for all strains of *N. meningitidis*, it increased as the sulfanilamide resistance of the strain of *N. gonorrhoeae* increased. These differences substantiate in part the work of Catlin (2), who showed that sulfanilamide resistance in *N. meningitidis* and *N. gonorrhoeae* resulted from a species-specific genetic alteration.

TABLE 6. *Relationship between K_i/K_m and the MIC of sulfanilamide*

Organism	Strain	(MIC)/ (K_i/K_m)
N. meningitidis	M-60	1.82
	M-160	2.27
	M-126[a]	2.27
N. gonorrhoeae	A-12	1.25
	Kohn	1.25
	7134[b]	1.23

[a] On basis of K_i value, MIC estimated to be 16 µg/ml.

[b] On basis of K_i value, MIC estimated to be 13 µg/ml.

Landy and Gerstung (9) reported that sulfanilamide-resistant strains of *N. gonorrhoeae* excreted two to five times more PABA than sulfanilamide-susceptible strains during growth in the absence of sulfanilamide. Ivler et al. (7) observed the same phenomenon in a sulfadiazine-resistant strain *N. meningitidis*. If the rate of PABA synthesis is similar in both sensitive and resistant strains, these observations can be explained by our finding of a decreased V_{max} with increased sulfanilamide resistance.

It is interesting to note that the "drug resistance ratio" (K_i/K_m) reflects the relative drug resistance of the strain as represented by its minimal inhibitory concentration (MIC) value. A direct relationship between MIC and the ratio K_i/K_m is observed for the strains of *N. meningitidis* and *N. gonorrhoeae* used in this study (Table 6). This ratio may be a molecular explanation of the MIC of sulfanilamide for these particular organisms. Studies are in progress to test the application of this ratio with other strains of these pathogens and with other sulfa drugs as McCullough and Maren (12) and Thijssen (18) have done with *Escherichia coli*.

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