Inhibition of Dihydropteroate Synthetase from
Escherichia coli by Sulfones and Sulfonamides

JERRY L. McCULLOUGH1 AND THOMAS H. MAREN
Department of Pharmacology and Therapeutics, University of Florida College of Medicine,
Gainesville, Florida 32601

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The inhibitory action of various diphenylsulfones and sulfonamides on
dihydropteroate synthetase partially purified from Escherichia coli was
examined. 4,4'-Diaminodiphenylsulfone (DDS; \( I_{50} = 2 \times 10^{-8} \) M) and the
monosubstituted derivatives 4-amino-4'-formamidodiphenylsulfone (\( I_{50} = 5.8 \times 10^{-6} \) M) and 4-amino-4'-acetamidodiphenylsulfone (\( I_{50} = 5.2 \times 10^{-6} \) M) were
effective inhibitors of dihydropteroate synthetase activity. Disubstitution of the
arylamine groups of DDS (4,4'-diformamidodiphenylsulfone and 4,4'-
diacetamidodiphenylsulfone) resulted in complete loss of inhibitory activity.
Both DDS (\( K_{i} = 5.9 \times 10^{-6} \) M) and sulfadiazine (\( K_{i} = 2.5 \times 10^{-4} \) M) were found
to be competitive inhibitors of dihydropteroate synthetase. These findings are
discussed in regard to the Bell and Robin theory of structure-activity relation-
ships for \( p \)-aminobenzoic acid antagonists.

Diphenylsulfones such as 4,4'-diaminodiphenylsulfone (DDS) are currently used in the
chemotherapy of leprosy (19) and malaria (9, 11). Both the antibacterial and antimarial
activities of DDS (3, 8, 13), like those of the sulfonamides (14, 15, 25), can be reversed by
\( p \)-aminobenzoic acid (PABA). Dihydropteroate
synthetase catalyzes the synthesis of dihydropteroate from 2-amino-4-hydroxy-6-hydroxy-
methylidihydropyridine pyrophosphate and PABA. Although it is generally assumed that,
as with the sulfonamides (6), the target enzyme of the sulfones is the synthetase, definitive
studies on the action of sulfones on the isolated enzyme have not yet been reported. We have
now examined the inhibitory action of several diphenylsulfones on the in vitro enzymatic
synthesis of dihydropteroate, using a partially purified enzyme from Escherichia coli. A comparison
is made between the inhibitory activities of various sulfones and sulfonamides and the
reversal of this activity by PABA.

MATERIALS AND METHODS

Ethylendiaminetetraacetate (EDTA), tris(hy-
droxymethyl)aminomethane (Tris), and bovine
serum albumin were purchased from Sigma Chemical Co.; ribonuclease and deoxyribonuclease, from Calbi-
ochem; Sephadex G-25, from Pharmacia Fine Chemi-
cals, Inc.; diethylaminoethyl (DEAE)-cellulose, from Whatman; 2-mercaptoethanol, from Eastman Or-
ganic Chemicals; pyrophosphoric acid, from Fluka AG, Chemische Fabrik; and \( p \)-aminobenzoic-7,14C
acid (specific activity, 5.85 mCi/mmol), from New
England Nuclear Corp. 1,3-Dihydroxyacetone, 6-
hydroxy-2,4, 5-triaminopyrimidine sulfate, sulfanila-
mide, and sulfanilic acid were purchased from Aldrich Chemical Co. DDS, 4-amino-4'-formamidodiphenyl-
sulfone, 4-acetamido-4'-aminodiphenylsulfone, 4,4'-
diformamidodiphenylsulfone, 4,4'-diacetamidodiphenyl-
sulfone, 2-amino-5-sulfanilthiazole, and homo-
sulfanilamide were obtained from Parke, Davis & Co.
through the courtesy of Edward F. Elslager. Sulfadiaz-
ine was obtained from Lederle Laboratories. Frozen
cells of E. coli B harvested from the late log phase
were purchased from General Biochemicals Corp.
Stock solutions of the sulfone and sulfonamide
compounds were made by dissolving the compound in
1% dimethylsulfoxide.

Protein was determined by the biuret method (12),
with crystallized bovine albumin as the standard.

All determinations of radioactivity were made in a
Beckman model 1650 scintillation spectrometer with the use of Bray's (4) scintillation fluid.

Synthesis of pteridines. 2-Amino-4-hydroxy-6-
hydroxymethylpteridine (hydroxymethylpteridine) was prepared by the condensation of 6-hydroxy-
2,4, 5-triaminopyrimidine sulfate with 1,3-dihydroxyacetone according to the method of Baugh and Shaw
(1). The pyrophosphate ester of hydroxymethylpteridine (hydroxymethylpteridine pyrophosphate) was
prepared and purified by column chromatography on
DEAE-cellulose, according to the method of Shiota et
al. (22). Hydroxymethylpteridine pyrophosphate was reduced to 2-amino-4-hydroxy-6-pyrophosphorylmethyl-7,8-dihydropteridine (hydroxymethylidihydropteridine pyrophosphate) with dithionite as described by Shiota et al. (21). This material was prepared fresh prior to each experiment. Formation of the dihydro compound was assessed by examination of the ultraviolet absorption spectrum (21, 22). The concentration of dihydropteridine compounds was determined at 330 nm (pH 7.1), with the use of a molar extinction coefficient of 6,200 (21).

Purification of dihydropterate synthetase. Unless otherwise noted, all of the following steps were performed at 4 C.

Cell-free extracts were prepared by modifications of the procedures of Weisman and Brown (24) and Shiota (21). A 3g-g amount of frozen cells (E. coli B) was suspended in 5 volumes of cold 0.05 M Tris-hydrochloride buffer, pH 8.0. This suspension was passed twice through a French press cell (Amino) at 15,000 to 20,000 psi. The disrupted cells were centrifuged for 20 min at 91,000 × g. The turbid supernatant solution was stored frozen for 16 h and centrifuged as above after thawing.

The supernatant material was treated with deoxyribonuclease (5 mg/100 ml) to decrease the viscosity. This solution was stirred for 30 min and centrifuged for 1 h at 118,000 × g.

Ribonuclease (1 μg of protein/ml) was added to the deoxyribonuclease-treated supernatant fluid. This solution was incubated in a bath at 37 C for 1 h with occasional mixing. The cloudy solution was centrifuged at 91,000 × g for 20 min to remove insoluble material.

The supernatant solution was then fractionated with ammonium sulfate by slowly adding solid ammonium sulfate (11.4 g/100 ml) to achieve a 20% saturation at 4 C. The mixture was stirred for 1 h and centrifuged for 1 h at 27,000 × g. Ammonium sulfate (34 g/100 ml) was added to the supernatant solution to achieve a 70% saturation at 4 C. The mixture was stirred for 1 h and centrifuged for 1 h at 27,000 × g.

The supernatant was then dialyzed in 20 ml of 0.05 M Tris-hydrochloride buffer, pH 8.0, and was dialyzed for 36 h against the same buffer (three changes of 6 liters of buffer).

The dialyzed material was then centrifuged for 1 h at 118,000 × g. The clear extract was applied to a column of Sephadex G-25 (2.5 by 60 cm) which had been equilibrated with 0.05 M Tris-hydrochloride buffer, pH 8.0. The same buffer was used to elute the enzyme from the column. The initial protein eluates were combined and used as the dihydropterate synthesizing extract.

Assay of dihydropterate synthetase activity. Dihydropterate synthetase activity was measured by a modification of the radioactive assay of Richey and Brown (16), based on the incorporation of 14C-PABA into dihydropterate. Each reaction mixture was prepared to contain in a volume of 0.4 ml Tris-hydrochloride buffer, pH 8.5, 100 mM; MgCl2, 10 mM; 2-mercaptoethanol, 50 mM; hydroxymethylidihydropteridine pyrophosphate, 0.12 mM; 14C-PABA, 0.01 mM; inhibitor, as indicated; and partially purified dihydropterate synthetase extract, 0.2 mg. Blank determinations contained all components except enzyme. The reaction mixtures, contained in test tubes (1 by 7 cm), were stoppered and incubated for 1 h at 37 C. The reactions were stopped immediately by the addition of 25 μmol of EDTA (pH 8.3) to each reaction mixture. Each reaction mixture was evaporated to dryness under reduced pressure and was redissolved in 0.075 ml of 0.05 M Tris, pH 8.0; 0.05 ml was then applied (each in an area of 1.0 by 4.0 cm) to Whatman 3MM chromatography paper. The chromatograms were developed by descending chromatography with 0.1 M potassium phosphate buffer, pH 7.0, for 4 h at 25 C. Under these conditions, pterate (dihydropterate is oxidized to pterate during the evaporation step) remains at the origin, whereas unreacted 14C-PABA migrates with an Rf value of 0.78. Areas corresponding to the origin of the developed chromatograms were cut out and counted in a liquid scintillation counter. All enzyme assays were done in triplicate. Enzyme activity is expressed as picomoles of dihydropterate produced per hour.

The concentration of various sulphones and sulfonamides required for 50% inhibition (I50) of dihydropterate synthetase activity was determined by titration of at least five levels of drug in the standard reaction system.

Analysis of kinetic data. The values of the inhibition constants (Ki) together with their standard errors were determined by fitting the primary data for each experiment to the overall rate equation for linear competitive inhibition through the use of the Fortran programs of Cleland (7) and a digital computer. The points drawn in the kinetic figures showing double reciprocal plots are an average of three experimentally determined values. In general, the error of the individually determined values was less than 10% of the average value reported.

RESULTS

Comparative effects of sulphones and sulfonamides on dihydropterate synthetase. Table 1 shows the I50 values of several sulphones and sulfonamides determined against dihydropterate synthetase. Of a number of sulphones that were tested, DDS (I50 = 2 × 10−5 M) was the most effective inhibitor. The two monosubstituted derivatives, 4-amino-4'-acetamidodiphenylsulfone (I50 = 5.2 × 10−5 M) and 4-amino-4'-formamidodiphenylsulfone (I50 = 5.8 × 10−5 M), were of similar inhibitory activity. Monosubstitution of the alyamine group of DDS by acetylation or formylation resulted in approximately a twofold decrease in inhibitory activity as compared with the parent compound DDS.

No inhibitory activity was observed with the diacetyl derivative of DDS (4,4'-diacetamidodiphenylsulfone) or with the diformyl derivative (4,4'-diformamidodiphenylsulfone) at concentrations as high as 10−4 M. The low solubility of these disubstituted compounds...
Table 1. Effectiveness of sulfones and sulphanilamides as inhibitors of dihydropteroate synthetase

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{i}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4, 4'-Diaminodiphenylsulfone</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>4-Amino-4'-formamidodiphenylsulfone</td>
<td>$5.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>4-Acetamido-4'-aminodiphenylsulfone</td>
<td>$5.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>4, 4'-Diformamidodiphenylsulfone</td>
<td>No inhibition at $10^{-4}$</td>
</tr>
<tr>
<td>4, 4'-Diacetamidodiphenylsulfone</td>
<td>No inhibition at $10^{-4}$</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>$3.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>2-Amino-5-sulfanylthiazole</td>
<td>$7.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Sulfolanilamide</td>
<td>$3.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Homosulfolanilamide</td>
<td>No inhibition at $10^{-2}$</td>
</tr>
</tbody>
</table>

* Concentration of compound required to effect a 50% decrease in enzyme activity.

prevented us from testing concentrations greater than $10^{-4}$ M. These results would indicate that only those diphenylsulfone congeners having a free arylamine group are effective inhibitors of dihydropteroate synthetase.

Of a number of sulfonamides that have been tested, sulfadiazine ($K_{i} = 10^{-4}$ M) was the most potent inhibitor, whereas sulfanilamide ($K_{i} = 3.2 \times 10^{-4}$ M) was a relatively poor inhibitor. These results are in agreement with those previously reported by Brown (6), who also determined the $K_{m}$ for PABA ($2.5 \times 10^{-6}$ M) for this enzyme (16). A comparison of the inhibitory properties of the sulfones and the sulphanilamides shows the most active sulfone, DDS, to be half as active as sulfadiazine.

It has been reported that substitution of the 4-amino group of sulphanilamide by an aminomethyl group (NH$_2$—CH$_2$—) results in a compound, homosulphanilamide, having anti-bacterial action which is not antagonized by PABA (5). It was of interest, therefore, to test the effect of this compound on dihydropteroate synthetase activity in vitro. No inhibition was observed with concentrations of homosulphanilamide as high as $10^{-2}$ M (Table 1).

Reversal by PABA of DDS and sulfadiazine inhibition. A reciprocal plot of dihydropteroate synthetase activity versus substrate concentration at two levels of DDS is shown in Fig. 1A. A pattern of competitive inhibition was obtained by DDS with respect to PABA. These findings are in accord with previous reports on the reversal by PABA of the antibacterial activity of DDS (13). As shown in Fig. 1B sulfadiazine also acts as a competitive inhibitor of dihydropteroate synthetase. The reversibility of sulphanilamide inhibition of E. coli dihydropteroate synthetase by PABA is in agreement with that previously reported by Brown (6). Apparent $K_{i}$ values of $5.92 \pm 0.85$ μM and $2.55 \pm 0.32$ μM for DDS and sulfadiazine, respectively, were obtained by fitting these data to the equation for linear competitive inhibition (7). The inhibitory activity of DDS and sulfadiazine against the enzyme is comparable to their activity against bacterial growth in vitro (17).

**DISCUSSION**

The data show that the diaminodiphenylsulfonyl, like the sulphanilamides, are effective inhibitors of dihydropteroate synthetase in vitro. Of particular importance is the demonstration that a free arylamine group of the parent sulfone is required for inhibitory activity. This substantiates previous conclusions that the antimalarial efficacy of 4, 4'-diformamidodiphenylsulfone in *P. berhei* (23) and the antimalarial

![Fig. 1](http://aac.asm.org/)

**Fig. 1.** (A) Inhibition of dihydropteroate synthetase by 4, 4'-diaminodiphenylsulfone (DDS) with p-aminobenzoic acid (PABA) as variable substrate. The concentrations of DDS were 0 (●), $10^{-4}$ (▲), and $5 \times 10^{-4}$ M (■). (B) Inhibition of dihydropteroate synthetase by sulfadiazine with PABA as variable substrate. The concentrations of sulfadiazine were 0 (●), $10^{-4}$ (▲), and $2.5 \times 10^{-4}$ M (■).
and antileprotic action of 4,4'-diacetamidodiphenylsulfone in vivo (20, 23) are due to the conversion to DDS.

The competitive inhibition of dihydropteroteate synthetase by the sulfones with respect to PABA suggests that, like the sulfonamides (6), these compounds are acting as a structural analogue of PABA and are thereby competing for the active site of the enzyme. Bell and Robin (2) in 1942 pointed out the structural similarity between the SO₃⁻ group of the sulfones and sulfonamides and the CO₂⁻ ion of PABA. They postulated that the antibacterial activity of the PABA antagonists depended upon the negativity of the SO₃⁻ group. They used the acid dissociation constant (pKₐ) as an indirect measure of the negative character of the SO₃⁻ group and showed that the activity of a group of N₁-substituted sulfonamides was related to the acid dissociation constants of the sulfonamide groups. Plotting the minimal inhibitory concentration (MIC) for E. coli against the acid dissociation constant (pKₐ) values of the sulfonamides, Bell and Robin found a bell-shaped curve with the lowest MIC at pKₐ 6.7. Although this relation between pKₐ and activity has been widely used as a prediction and reflection of potency in this series, it must be pointed out that the fundamental point of Bell and Robin concerned negativity of the SO₃⁻ group. This property, through an inductive effect, influences both the basicity of the aromatic amine and (in the case of sulfonamides) the acidity of the sulfonamide proton. In DDS, the latter is lacking; however, its basic pKₐ of 2 is similar to the basic pKₐ of the active sulfonamides (18, 26). These basic pKₐ values have too narrow a range (about 0.3 unit) to account for the wide (about 100-fold) variation in activity between, for example, sulfanilamide and DDS ≅ sulfadiazine. Thus, it appears that neither acid nor basic properties alone can explain the activity in this group of drugs. The high potency of DDS as a PABA competitor probably is based on steric factors that remain to be explained.

The lack of inhibition of dihydropteroteate synthetase by homosulfanilamide is in agreement with previous observations that the antibacterial action of this compound is not antagonized by PABA (10).

ACKNOWLEDGMENTS

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