The Characteristics and Significance of Sulfonamides as Substrates for *Escherichia coli* Dihydropteroate Synthase*

(Received for publication, May 29, 1979)

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Sulfonamides are known to compete with *p*-aminobenzoic acid for dihydropteroate synthase. Others have reported that some sulfonamides are alternate substrates, but the significance of these observations to the antimicrobial action of sulfonamides has not been studied. We have shown that sulfanilamide, sulfathiazole, and sulfathymoxazole are efficient alternate substrates for this reaction, with apparent *Km* values equivalent to their *Kp* values as competitive inhibitors.

The products synthesized from the sulfonamides in *vitro* were chromatographically similar to chemically prepared dihydropterin-sulfonamides. A culture of *Escherichia coli* B converted 28% of 0.625 μM [35S]sulfamethoxazole to a product which was identified as dihydropterin-sulfamethoxazole. Greater than 99% of the product was found in the medium and the cellular concentration of radiolabel was 2 μM. This lack of accumulation was consistent with our finding that sulfonamides diffuse into *E. coli* and that the active transport of [35S]sulfanilamide could not be demonstrated.

The growth rate of *E. coli* B was not inhibited by 2 μM of chemically synthesized dihydropterin-sulfamethoxazole. No significant inhibition of thymidylate synthase, N6,N10-methylenetetrahydrofolate dehydrogenase, N6,N10-methylene-tetrahydrofolate cyclohydrolase, or dihydrofolate reductase was found with the aromatic and dihydropterin-sulfonamides. High concentrations (50 to 150 μM) of some of the compounds were inhibitory to GTP cyclohydrolase, hydroxymethyl-dihydropterin pyrophosphokinase, and serine hydroxymethyltransferase. The dihydropterin-sulfonamides were product inhibitors of dihydropteroate synthase, and were inhibitors of dihydrofolate synthetase. However, to obtain substantial inhibition of these enzymes by the dihydropterin-sulfonamides in *vivo*, higher concentrations of these compounds are required than those which are attainable intracellularly.

These data show that sulfonamides are effective alternate substrates for *E. coli* dihydropteroate synthase, but that the dihydropterin-sulfonamide products formed do not contribute significantly to the growth inhibition by sulfonamides.

Sulfonamides have been used for many years in clinical practice as effective chemotherapeutic agents. It has been documented that the most sensitive locus of sulfonamide inhibition is competition with *p*-AB for Hpteroate synthase.

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* The abbreviations used are: p-AB, *p*-aminobenzoic acid; H2- as a prefix before pt, pteroate, or folute, indicates the 7,8-dihydro forms; H4folate, 5,6,7,8-tetrahydrofolate; ptCH2SMX, 2-amino-6-(*N*-[(5-methyl-3-oxazolyl)sulfanoyl]anilinomethyl)pteridin-4(3H)-one; ptCH2SA, 2-amino-6-((sulfamoylanilino)methyl)pteridin-4(3H)-one; ptCH2STZ, 2-amino-6-[(2-thiazolylsulfamoyl)anilinomethyl] pteridin-4(3H)-one; ptCH2OMP, 2-amino-4-oxo-6-hydroxymethylpteridine pyrophosphate; ptCH2OH, 2-amino-4-oxo-6-hydroxymethylpteridine; SMX, sulfamethoxazole; STZ, sulfathiazole; SA, sulfanilamide.
Sulfonamides as Substrates for Dihydropteroate Synthase

Attempts to couple either STZ or SMX by the method of Taylor (21) were unsuccessful, and the pterin-sulfonamides were prepared by sodium dithionite reduction (22). Evidences for successful reduction of the pterin-sulfonamides were a UV spectral shift characteristic of reduced pterin and folates (21), the visual change under UV light (254 nm) from UV-absorbing material (aromatic pterins) to UV blue fluorescing material (7,8-dihydropterins), and the fact that 7,8-dihydropterin-sulfonamides were substrates for rat liver PTF reductase, whereas STZ, 20 ml of anhydrous dimethyl sulfoxide, and 2.0 g of 3 Å molecular sieves stood at room temperature under nitrogen for 2 h.

The pyrazine ring is fully oxidized in the aromatic species.

Radiochemicals

[carboxy-14C]p-AB (10 and 55 Ci/mol) was purchased from ICN Pharmaceuticals, Cleveland, O. [U-14C]Glutamic acid (260 Ci/mol) was purchased from Schwartz/Mann, Orangeburg, N. Y. [1-14C]HDL (1 mCi/g), [14C]leucine (1.25 mCi/mg), and [U-14C]leucine (60 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. L-[3-14C]Serine (54 Ci/mol), [3-15N]sulfamethizole (sulfamethoxazole) (9.1 Ci/mol) were purchased from New England Nuclear, Boston, Mass. L-[U-15N]Glutamic acid (260 Ci/mol)

Preparation of Pteridines

2-Amino-6-[4-[(5-methyl-3-isoxazoyl)sulfamoyl]anilinomethyl]pteridin-4(3H)-one (PTCH-SA) (see Fig. 1)—Under a nitrogen atmosphere, a mixture of 0.70 g (3.0 mmol) of 2-amino-6-[4-(2-thiazoylsulfamoyl)]anilinomethyl]pteridin-4(3H)-one (14) and 140 ml of 0.2 N NaOH was heated at reflux for 2 h to give a greenish yellow solution which was then adjusted to pH 5 with glacial acetic acid. The yellow precipitate was collected by filtration, washed with water and methanol, and dried under vacuum (70°C); yield 0.46 g of the crude pteridine. A 54-mg sample of this material was subjected to DEAE-cellulose chromatography to separate the PtCH-STZ from highly fluorescent contamination (2). The yellow precipitate was isolated by centrifugation, washed with water, and dried under vacuum (70°C); yield 21 mg; m.p. >300°C; NMR (MeSO-4-d6) δ 4.46 (d, J = 4.5 Hz, 1H), 6.85 (br s, 2H), 7.08 (br t, J = 6 Hz, 1H), 7.16 (d, J = 4.5 Hz, 1H), 7.50 (d, J = 8.7 Hz, 2H), 8.63 (s, 1H), 11.3 (br s, 1H), 12.1 (br s, 1H); UV λmax (0.1 N NaOH) 597 nm (ε 37,840), 971 sh (32,700), 360 (9,600); mass spectrum, m/e 430 (M).

Biochemicals

Folate was purchased from Calbiochem, LaJolla, Calif. Pterostre was a gift from Dr. Charles M. Baugh, University of South Alabama, Mobile, Ala. All other biochemicals were from Sigma Chemical Co., St. Louis, Mo. unless otherwise specified. All chemicals were of reagent grade.

Preparation of Pteridines

2-Amino-6-[4-sulfamoylanilinomethyl]pteridin-4(3H)-one (PTCH-SA) (see Fig. 1)—Under a nitrogen atmosphere, a mixture of 0.55 g (1.6 mmol) of 2,4-diamino-6-(4-sulfamoylanilinomethyl)pteridine (14) and 140 ml of 0.2 N NaOH was heated at reflux for 2 h to give a greenish yellow solution which was then adjusted to pH 5 with glacial acetic acid. The yellow precipitate was collected by filtration, washed with water and methanol, and dried under vacuum (70°C); yield 0.46 g (88%); m.p. >300°C, NMR (MeSO-4-d6) δ 4.49 (d, J = 4.5 Hz, 1H), 6.48 (br s, 2H), 6.68 (d, J = 9 Hz, 2H), 6.90 (br s, 4H), 7.09 (t, J = 6 Hz, 1H), 7.50 (d, J = 9 Hz, 2H), 8.63 (s, 1H), 11.51 (br s, 1H); UV λmax (0.1 N NaOH) 257 nm (ε 36,200), 364 (ε 60,000); mass spectrum, m/e 430 (M).
analyzed for radioactivity as described previously (5). The apparent $K_v$ value for Hpt$\text{CH}_2\text{OH}$ was 0.7 $\mu\text{M}$.

3. H.pertroate synthase (EC 2.5.1.15) was assayed with [carboxy-$^{14}$C]P-AB (10 $\mu$Ci/ml) (26). The enzyme was a partially purified E. coli B preparation eluted from a Sephadex G-100 column (27) with a specific activity of 1 to 2 nmol/min/mg of protein.

4. H.pertroate synthase (EC 2.5.1.15) was assayed with $\text{L-[1-^{14}C]}$glutamic acid (1.25 $\mu$l/ml) according to the procedure of Webb and Ferone (28). The enzyme source was the same as for H.pertroate synthase.

5. H.pertroate reductase (EC 1.5.1.3) was assayed spectrophotometrically according to the procedure of Baccanari et al. (29) with the use of enzyme from E. coli RT2000 purified to homogeneity by methotrexate affinity chromatography. Rat liver dihydrofolic acid reductase was purified until the Arjoo 5 $0.6$.

6. Serine hydroxymethyltransferase (EC 2.1.2.1) was assayed with $\text{L-[3-^{3}H]}$serine (0.2 $\mu$l/ml) according to the method of Taylor and Weissbach (31). E. coli B cells (stored at $-70^\circ\text{C}$), purchased from the Gram Processing Corp., Muscataine, Ia., were suspended in 0.05 $\mu$l Tris-HCl buffer, pH 8.3 (25$^\circ\text{C}$), and ruptured by passage twice through a French pressure cell (Aminco) at 11,000 p.s.i. The 200,000 $\times g$ (1 h) supernatant fluid was used as the enzyme source.

7. Thymidyline synthetase (EC 2.1.1.45) was assayed spectrophotometrically by the method of Friedkin (22) with a 200,000 $\times g$ (1 h) supernatant fluid made partially pure by centrifugation (Sorvall GSA column) (32) and the enzyme from E. coli B cells harvested from glucose minimal medium (32) as the source of enzyme.

8. N$^6,N^{\text{methyl}}$methylene-H.pertroate dehydrogenase (EC 1.5.1.5) was assayed by the method of Siringeour and Hueneakens (33) except that $N^6,N^{\text{methyl}}$methylene-H.pertroate was purified prior to assay, and 0.03 $\mu$l Tris/malente, pH 8.3, buffer was used. The enzyme source was a partially purified E. coli B enzyme eluted from a DEAE-cellulose column (94).

9. N$^6,N^{\text{methyl}}$methylene-H.pertroate cyclohydrolase (EC 3.5.4.9) was assayed according to the method of Greenberg (35). The enzyme source was the same as for serine hydroxymethyltransferase.

E. coli Growth Cultures for Permeability Studies

E. coli ML30 cultures ($A_{600} = 1.3$ to 1.5) were harvested and the cells were washed with 30 $\mu$l phosphate buffer, pH 7.0, before being used for diffusion studies. Cultures of cells (100 ml) prepared for active transport studies (E. coli ML30 or E. coli B SS206) were harvested when the $A_{600}$ reached 0.4. These cells were washed and resuspended in 50 $\mu$l phosphate, pH 7.0, buffer to an $A_{600}$ of 8 to 14.

E. coli Growth Inhibition Studies

Aerobic cultures containing 40 ml of glucose minimal medium and 0.025 to 4 mM SA, or 0.5 to 100 $\mu$l STZ, or 5 to 34 $\mu$l $\text{ptCH}_2\text{SMX}$, or no drug were inoculated with an overnight growth culture at an $A_{600}$ of 0.3, grown aerobically in Tris medium, was added to 100 ml of Tris medium containing 0.625 $\mu$l (5$^{3}$H)SMX (28 Ci/ml), and was incubated at 30$^\circ\text{C}$ with shaking.

The extracellular concentration of products was measured when 2 ml samples of the growth culture were removed at 30-min intervals and passed through a 0.25-mm HA Millipore filter that had previously been washed with 3 ml of Tris medium. Before the entire sample had filtered, the filtrate was removed and samples (100 $\mu$l) were analyzed chromatographically. The amounts of the intracellular concentration of products. At 30-min intervals, medium (3 ml, 30$^\circ\text{C}$) was applied to a prewashed filter. A 2-ml sample of the growth culture was added and the total 5 ml was filtered almost to dryness. An additional 3 ml of medium (30$^\circ\text{C}$) was added to wash the cells and just before the filters became dry, the vacuum was removed. The filters were dried and counts removed were assayed as described by Black and Gerhardt (38).

The calculation of the cellular concentrations assumes that 1 ml of a culture at an $A_{600}$ of 1.0 contains 0.05 $\mu$l of intracellular water (96).

Chromatography

Paper Chromatography—Chromatograms ($6 \times 20$ cm, Whatman No. 1) were developed ascendingly with 0.1 $\mu$l Sorenson's PO, pH 7.0 buffer, to a length of 18 cm from the origin. The dried chromatograms were cut into 1-cm sections which were analyzed for radioactivity in vials containing 10 ml of a mix of 15.2 $\mu$l 2.5-bis-(5-4-butybenzoxicarbonil)-thiophene/gal, toluen.

Thin Layer Chromatography—Thin layer chromatography was performed on cellulose ($20 \times 20$ cm) (0.26 mm) plates (Machery and Nagel, Germany). Chromatograms were developed for 12 cm in Na$_2$HPO$_4$ (5%, w/v), LiCl (0.21%, w/v), or NH$_4$Cl (3%, w/v). Chromatograms of reaction mixtures were scanned with a thin layer scanner (model LB 2760, Roehrd, West Germany).

High Performance Liquid Chromatography Techniques

Reverse phase high performance liquid chromatography was performed on a Bondapak C$_8$ column ($0.78 \times 30$ cm) in a model ALC-GPC 244 unit equipped with model 6000A pumps and a model 660 solvent programmer (the Waters Associates, Milford, Mass.). Peaks were detected by a Waters Associates model 440 UV detector ($A = 254$ nm) and a model FS-970 continuous flow fluorescence monitor (Scheel Instrument Co., Westwood, N. J.) with an excitation wavelength set at 350 nm and an emission filter (KV 418) with a cutoff below 410 nm. The fluorescent monitor was used in a 1 $\mu$l range with a sensitivity setting of approximately 51 and a time constant at 6 s. The column was developed with a linear gradient of 0 to 60% methanol in water at an approximate flow rate of 1 ml/min. Fractions (0.5 $\mu$l) were collected and aliquots (50 $\mu$l) were analyzed for radioactivity in 10 ml of Bray’s scintillation fluid. The volume of samples injected ranged from 2.5 to 55 $\mu$l.

In Vivo Concentration of $\text{ptCH}_2\text{SMX}$

A 20-ml culture of E. coli B SS206 ($A_{600} = 0.3$), grown aerobically in Tris medium, was added to 100 ml of Tris medium containing 0.625 $\mu$l (5$^{3}$H)SMX (28 Ci/ml), and was incubated at 30$^\circ\text{C}$ with shaking.

In Vivo Synthesis of $\text{ptCH}_2\text{SMX}$

The radioactivity in the supernatant (785 ml) was adjusted to pH 7.0 and pumped at 40 ml/h onto a column (2.5 x 30 cm) of DE52 (Whatman, Clifton, N. J.) at 5$^\circ\text{C}$, equilibrated with 0.01 M Tris-Cl, pH 8.0 at 5$^\circ\text{C}$. A linear gradient (800 ml of buffer, 0 to 1.0 M KCl) was applied to the column, followed by 1500 ml of buffer with 1.0 M KCl. Fractions (12 to 15 ml) were collected and samples of each were assayed for radioactivity and were chromatographed to evaluate the contents of the peaks. The fractions of interest were pooled and applied at 60 ml/h to a column (0.7 x 30 cm) containing 5 mg of acid-washed Norit A (activated charcoal) layered over 10 mg of CF11 cellulose (Whatman). The column was washed with 50 ml of H$_2$O. The bound material was eluted with 10 ml of NH$_4$H$_2$O$_2$:H$_2$O (1:4) and the void volume was provided as an energy source. Reaction mixtures (100 $\mu$l) were added to 100 $\mu$l Tris medium, was added to 100 ml of Tris medium containing 0.625 $\mu$l (5$^{3}$H)SMX (28 Ci/ml), and was incubated at 30$^\circ\text{C}$ with shaking. A linear gradient of 0 to 60% methanol in water at an approximate flow rate of 1 ml/min. Fractions (0.5 $\mu$l) were collected and aliquots (50 $\mu$l) were analyzed for radioactivity in 10 ml of Bray’s scintillation fluid. The volume of samples injected ranged from 2.5 to 55 $\mu$l.

Transport Studies

Active transport functions were assayed according to the method of Kaback (37) except that whole cells were used and 10 nm glucose was provided as an energy source. Reaction mixtures (100 $\mu$l) were incubated at 3$^\circ\text{C}$ in 0.05 M 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer, pH 7.2, over a 3-minute time course.
where \( v_0 \) is the known volume of the solution added to packed cells, 0.5 ml in this study; \( v_p \) is the known volume of packed cells; \( C_0 \) is the solute concentration of \( v_0 \); \( C_f \) is the final concentration of solute in the presence of cells; and \( S' \) is per cent of cell pack penetrated. This method is based on the dilution of radiolabel of the solute by the intracellular water of a packed cell pellet compared to the dilution of \([\text{H}]\text{H}_2\text{O}\) by these cells. When \( S' \) is determined with \([\text{H}]\text{H}_2\text{O}\) as solute, it is a measure of the extracellular and the intracellular space. When \( S' \) is determined with \([\text{C}]\text{dextran} (M_r = 20,000)\) as solute, it represents the total extracellular space. The difference between these values is the intracellular water space.

**Miscellaneous Methods**

Radioactivity was determined by the use of a Beckman LS-230 liquid scintillation spectrometer. Ultraviolet spectra were recorded by either a Cary 118 or Gilford 240 spectrophotometer. NMR spectra were determined on a Varian XL-100 spectrometer by the Fourier transform method. Field desorption mass spectral data were obtained with a Varian MAT 731 spectrometer. Elemental microanalyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. Specific growth rates and ED_{50} values were calculated as described previously (39). Chromatograms of standards (5 to 10 nmol) were viewed under UV light (254 nm) to locate the spots.

Enzyme kinetic data were evaluated with computer programs for the analysis of competitive and noncompetitive inhibitors (40). Evaluations were made at two inhibitor concentrations compared to the control. Apparent \( K_m \) values were calculated from the slope (\( K_m/V_{max} \)) of plots of \( 1/[V] \) versus \( 1/[S] \). Apparent \( K_m \) values were calculated from the intercept of the \( 1/[V] \) axis of double reciprocal plots. Kinetic studies were conducted with assay procedures adequate to insure the measurement of initial velocities (25).

Protein was determined by the method of Lowry et al. (41), with bovine serum albumin as standard.

**RESULTS**

**Enzymatic Product Formation from Radioactive Sulfonamides**—The enzymatic synthesis of a product containing a radioactive sulfur moiety was demonstrated when H\(_9\)pteroate synthase was incubated with \([3-\text{S}]\text{SMX} (9 \text{ Ci/mol})\) as substrate in place of p-AB. When an aliquot (100 \mu l) of each reaction mixture was chromatographed on paper, a peak of radioactivity at an \( R_F \) value of 0.53 was evident (Fig. 2A). This peak represents 64% of the total radioactivity and it is not present in a control reaction mixture lacking H\(_9\)pteroate synthase. The radioactive material migrated to the same position as authentic H\(_9\)pteroate SMX (Fig. 2D). The small peak at an \( R_F \) value of 0.45 represents pCH\(_2\)SMX. No precautions were taken to limit oxidation during chromatography, although the reaction mixtures were incubated in the presence of 5 \mu M dithiothreitol.

To demonstrate that the product contained a pteridine moiety, radioactive dihydropteridine substrate, \([2-\text{C}]\text{H}_9\)pteroate, was enzymatically synthesized in situ from \([2-\text{C}]\text{H}_9\)ptCH\(_2\)OH (10 Ci/mol); H\(_9\)pteroate synthase and SMX were then added, and the reaction mixtures were incubated an additional period. The chromatogram of the complete reaction indicates a peak at \( R_F \) value of 0.24 (54% of the total radioactivity) that is identical with the \( R_F \) value for the radioactive peak demonstrated from the reaction mixtures incubated with \([3-\text{S}]\text{SMX}\) and for authentic H\(_9\)ptCH\(_2\)SMX. The chromatogram of the control reaction mixture, lacking H\(_9\)pteroate synthase, shows two peaks and a shoulder (Fig. 2B). The peak at \( R_F \) value of 0.58 corresponded to \([2-\text{C}]\)ptCH\(_2\)OH and remained unchanged throughout the enzymatic incubation and chromatography. The peak at an \( R_F \) value of 0.73 co-chromatographs with H\(_9\)ptCH\(_2\)OPP and represents the product of H\(_9\)ptCH\(_2\)OH pyrophosphokinase (the substrate for H\(_9\)pteroate synthase). Since no precautions were taken to avoid oxidation during chromatography, the shoulder at an \( R_F \) value of 0.84 probably represents \([2-\text{C}]\)ptCH\(_2\)OPP.

Further evidence that the product at \( R_F \) value of 0.24 contained both a pteridine moiety and sulfamethoxazole was obtained from a double label experiment with \([2-\text{C}]\)H\(_9\)ptCH\(_2\)OPP and \([3-\text{S}]\)SMX. As seen in Fig. 2C, the complete reaction mixture shows a new peak at an \( R_F \) value of 0.24 which accounts for 70% more radioactivity than the product synthesized from either radioactive substrate alone.
The radioactive compound synthesized in the complete reaction mixture also migrates to the same position as authentic HptCH2SMX in several other solvents tested (see Table I). The enzymatic product fluoresced under UV light which is consistent with the behavior of HptCH2SMX. As observed by Dock et al. (12), the protein content of the reaction mixture probably alters the RF value of SMX compared to the RF value of SMX when chromatographed alone.

Other sulfonamides were tested as substrates in assays similar to those described above. The product synthesized from HptCH2OPP and [35S]SA migrates to an RF value of 0.06, whereas [35S]SA migrates to an RF value of 0.76. When STZ and [2-14C]HptCH2OPP were the substrates, the radioactive product had an RF value of 0.07, whereas [2-35S]STZ migrated to an RF value of 0.70. The RF values of these products correspond to the RF values of authentic HptCH2SA and HptCH2STZ, respectively.

**Kinetics of Hpteroate Synthase in the Presence of Sulfonamides**—[carboxy-14C]p-AB incorporation into Hpteroate was inhibited competitively by the sulfonamides tested and, a replot of the slopes versus the inhibitor concentrations indicated linear competitive inhibitions (11). The kinetic constants are shown in Table II. SMX was noncompetitive with HptCH2OPP with an apparent KI value of 8.19 ± 1.36 μM when the concentration of p-AB was 20 μM. Replots of both the slopes and the intercepts versus inhibitor concentration indicate linear noncompetitive inhibition.

As demonstrated above, SMX can replace p-AB as a substrate for Hpteroate synthase. The product synthesized from [3-35S]SMX was chromatographed on paper and quantitated by analysing for radioactivity a section of the chromatogram that was shown to include all the radioactivity associated with the product and none of the unreacted [3-35S]SMX. The kinetic constants for SMX are included in Table II. [3-35S]SMX incorporation into product was competitively inhibited by p-AD with an apparent KI value of 0.53 ± 0.07 μM. Hpteroate synthase in the presence of [35S]SA (18.6 Ci/mol) or [35S]STZ (2.1 Ci/mol) was also studied. Since the products formed from these sulfonamides migrated only slightly when chromatographed on paper they were quantitated as described for Hpteroate (5). Although the specific activity of the [35S]STZ was too low for accurate kinetic determinations, the apparent KI value appeared to be <1 μM. Each sulfonamide was evaluated as a growth inhibitor of E. coli B and these results are included in Table II. The values reported here are consistent with previously published values (1).

**Penetration Studies**—The permeability of the cells to p-AB and sulfonamides was determined by measuring the cellular and extracellular concentrations of radioactive compounds. The results presented in Table III indicate that p-AB was concentrated no more than 3.4-fold when diffusion was studied for 30 min at 4°C. This type of experiment does not eliminate active transport as a means of uptake, nor does it necessarily mean that the cellular concentration indicated is actually the intracellular concentration.

Studies conducted with each of the sulfonamides indicated that the concentration factor (cellular concentration/Ci) was never greater than 2.7 and generally was approximately 2.0 (Table III). The inclusion of 0.1% tolenue to increase the cellular permeability did not lower the results with sulfanilamide, thus suggesting that the radiolabel was bound to cell components (42). [3-35S]SMX could be washed away from the cells with nonradioactive SMX. The diffusion of STZ, with a KI of 7.1 (43), was studied at pH 6.0, pH 7.0, and pH 8.0, and no differences were seen in the concentration factor, indicating that the degree of ionization of this sulfonamide extracellularly was not a factor in its uptake. Incubating SA and SMX at 37°C for 5 min or 4°C for 30 min resulted in similar concen-

### Table I

<table>
<thead>
<tr>
<th></th>
<th>Migration on TLC plates of substrates and products from reaction mixtures containing [3-35S]SMX</th>
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<tbody>
<tr>
<td></td>
<td>5% Na2HPO4</td>
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<tr>
<td>SMX (20 nmol)</td>
<td>0.86 (A)</td>
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<tr>
<td>Control reaction</td>
<td>0.70 (A)</td>
</tr>
<tr>
<td>Complete reaction</td>
<td>0.23 (F)</td>
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<tr>
<td>HptCH2SMX (6 nmol)</td>
<td>0.28 (F)</td>
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* A = ultraviolet absorbant.
* F = ultraviolet fluorescent.

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substrate activity apparent KI, μM</th>
<th>Substrate Vmax as % Vmaxp-AB</th>
<th>Inhibitor activity apparent KI, μM</th>
<th>Growth inhibition ED50, μM</th>
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<tr>
<td>p-AB</td>
<td>0.57 ± 0.05</td>
<td>(100)</td>
<td>0.53 ± 0.07</td>
<td>3.8 ± 0.3</td>
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<td>Sulfathiazole</td>
<td>&lt;1.0</td>
<td>~75</td>
<td>0.035 ± 0.003</td>
<td>1.5 ± 0.36</td>
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<td>Sulfamethoxazole</td>
<td>0.12 ± 0.02</td>
<td>75</td>
<td>0.18 ± 0.017</td>
<td>11560 ± 68</td>
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<tr>
<td>Sulfanilamide</td>
<td>4.8 ± 1.4</td>
<td>30</td>
<td>5.7 ± 0.5</td>
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</tbody>
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* KI ± S.E.
* Inhibition constant determined from slopes of 1/[v] versus 1/[s] plots ± S.E.
* Ref. 25.
* Value determined using [carboxy-14C]p-AB as substrate.
* Values determined using [carboxy-14C]p-AB as substrate.
* Concentration required to inhibit the growth rate by 50% ± S.E.
Summary of diffusion studies on p-AB and the sulfonamides

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>A</th>
<th>B</th>
<th>Average of the range of concentration factor, R/A</th>
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</thead>
<tbody>
<tr>
<td>p-AB</td>
<td>(0.026-3.89)</td>
<td>(0.065-13.3)</td>
<td>2.8</td>
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<td>SMX</td>
<td>(0.302-6.38)</td>
<td>(0.47-1.75)</td>
<td>1.49</td>
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<tr>
<td>SMX</td>
<td>(0.317-6.47)</td>
<td>(0.40-7.43)</td>
<td>1.16</td>
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<tr>
<td>STZ</td>
<td>(2.5-49)</td>
<td>(5.3-104)</td>
<td>2.12</td>
</tr>
<tr>
<td>SA</td>
<td>(6.88)</td>
<td>(10-1300)</td>
<td>2.2</td>
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<td>SA</td>
<td>(300)</td>
<td>(597)</td>
<td>1.88</td>
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<tr>
<td>SA</td>
<td>(5.9-609)</td>
<td>(16-1253)</td>
<td>2.25</td>
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\(a\) The final concentration of solute in the presence of cells.

\(b\) Measured in the presence of 0.1% toluene.

The identification of the [\(^{35}\)S]ptCH\(_2\)SMX was confirmed by employing reverse phase high pressure liquid chromatography techniques. After initiation of the solvent gradient authentic ptCH\(_2\)SMX was eluted at 54 min and [\(^{35}\)S]ptCH\(_2\)SMX at 40 min. The elution profiles of authentic ptCH\(_2\)SMX and biosynthesized [\(^{35}\)S]ptCH\(_2\)SMX are identical (Fig. 4). The radiolabel (94%) was recovered in a peak that directly overlays the ultraviolet absorbing peak of authentic ptCH\(_2\)SMX at 54-min elution time. The remaining \(^{35}\)S label elutes at 40 min which probably represents slight breakdown of the [\(^{35}\)S]ptCH\(_2\)SMX to free [\(^{35}\)S]SMX.

In Vivo Concentration of H\(_2\)ptCH\(_2\)SMX—Since we confirmed the in vivo synthesis of H\(_2\)ptCH\(_2\)SMX by E. coli B, it was necessary to determine the cellular concentration of the product in order to evaluate the physiological significance of the pterin-sulfonamides. The cellular concentration of \(^{35}\)S label was measured during the aerobic growth of E. coli B because of the relatively short duration of the experiment and the ease of sampling every 30 min. Since the amount of \(^{35}\)S concentration was observed to be 50 to 70 cpm. If 100% of 0.5 \(\mu\)M of [\(^{35}\)S]SMX was transported, an additional 1000 cpm would be retained. There were no additional counts associated with the cells over the range of 0.5 to 1000 \(\mu\)M of [\(^{35}\)S]SMX incubated at 31°C for up to 3 min.

In order to determine whether sulfonamides are actively transported, the uptake of [\(^{35}\)S]SA (9.1 Ci/mmol) by E. coli ML30 was studied. The radioactivity retained by the filter in the presence or absence of the cells at zero incubation time was 50 to 70 cpm. If 100% of 0.5 \(\mu\)M [\(^{35}\)S]SA was transported, an additional 1000 cpm would be retained. There were no additional counts associated with the cells over the range of 0.5 to 1000 \(\mu\)M of [\(^{35}\)S]SA incubated at 31°C for up to 3 min. As a control for this methodology, the energy-dependent uptake of \(L\)-\([\text{\(^{14}\)C}]\)leucine (308 Ci/mol) was demonstrated. E. coli ML30 cells transported 65% of 0.5 \(\mu\)M radioactive leucine added to the cells over a 2- min time course of incubation at 25°C.

In Vivo Synthesis of ptCH\(_2\)SMX—Bock et al. (12) found materials chromatographically similar to ptCH\(_2\)SMX and H\(_2\)ptCH\(_2\)SMX in the extracellular medium of E. coli grown with SMX, but no attempts were made to isolate, identify, or quantify these materials. In our study, a large culture of E. coli was grown with [\(^{35}\)S]SMX anaerobically (to prevent oxidative cleavage of the product) in Tris medium. Glucose minimal medium was unacceptable because the product breaks down to a compound that absorbs at 420 nm. Following incubation, the cells were collected and a chromatogram of a 400-\(\mu\)l of the culture supernatant fluid was taken. The radiolabel concentration of [\(^{35}\)S]SA was determined in separate experiments. The solid line is the \(A_{260}\) tracing and the radioactivity is denoted by \(x\).

![Fig. 3. Ultraviolet absorption spectra of authentic ptCH\(_2\)SMX (○—○) and of [\(^{35}\)S]ptCH\(_2\)SMX (□—□) isolated from E. coli B. Spectra were recorded in 0.1 \(\mu\)l NaOH at 20 \(\mu\)M (est. 32,400) pterin.](http://www.jbc.org/)

![Fig. 4. High performance liquid chromatography elution profile of authentic ptCH\(_2\)SMX and of [\(^{35}\)S]ptCH\(_2\)SMX. A sample (55 \(\mu\)l) containing 5.3 nmol of [\(^{35}\)S]ptCH\(_2\)SMX (by radiolabel) was combined with 10 \(\mu\)l containing 29 nmol of ptCH\(_2\)SMX and then separated by the use of high performance liquid chromatography. The arrows denote the positions of authentic ptCH\(_2\)SMX and of [\(^{35}\)S]ptCH\(_2\)SMX as determined in separate experiments. The solid line is the \(A_{264}\) tracing and the radioactivity is denoted by \(x\).](http://www.jbc.org/)
found associated with the cells was \( \pm 5 \) pmol, this experiment was repeated several times with the highest specific activity \([3-^{35}S]\)SMX available \((28 \text{ Ci/mol})\). Although this procedure measures both the product and the unreacted \([3-^{35}S]\)SMX found intracellularly, several separate experiments indicated that the intracellular concentration of total \( ^{35}S \) label was \( \pm 2 \) \( \mu \text{M} \). In the experiment presented in Fig. 5, the concentration was \( 1.06 \pm 0.6 \) \( \mu \text{M} \), where the large standard error reflects the inherent inaccuracy of these small numbers.

The rate of the appearance of the products extracellularly was monitored from identical cultures. The concentration of the extracellular products presented in Fig. 5 represents both oxidized and reduced ptCHSMX, separated from \([3-^{35}S]\)SMX available \((28 \text{ Ci/mol})\). Although this procedure was not concentrated in the cell. The culture supernatant had remained inside the cell and did not diffuse out, the steady state intracellular concentration would have been \( 911 \pm 26 \) \( \mu \text{M} \). This value is calculated from the regression analysis of a plot of the total volume of intracellular water calculated from the optical density versus the total nanomoles of the product found outside the cell. These experiments show that \( >99\% \) of the ptCHSMX biosynthesized by \( E. \ coli \) B diffused into the extracellular media and was not concentrated in the cell.

**Growth Inhibition Studies**—Because HptCHSMX is freely diffusible, monitoring the growth rate of \( E. \ coli \) B in the presence of \( 2 \mu \text{M} \) HptCHSMX extracellularly should elucidate its effects on growth. The anaerobic growth rate \((k = 0.422 \pm 0.003)\) of \( E. \ coli \) B \((k = 0.946 \pm 0.009)\) was inhibited by \( 12\% \) at \( 34 \mu \text{M} \) ptCHSMX \((k = 0.833 \pm 0.005)\) was inhibited by \( 40\% \) at \( 2 \mu \text{M} \) HptCHSMX. In both cases, the inhibition could be accounted for by the slight contamination of the pterin-sulfamethoxazoles by free SMX.

In order to investigate the inherent effects of the pterin-sulfonamides, the authentic aromatic and reduced compounds were tested as inhibitors of various folate biosynthetic and cofactor-utilizing enzymes and the results are presented in Table IV. The substrate concentrations were within 4-fold higher than their apparent \( K_{i} \) values. The variation in the highest concentration of the compounds tested is directly related to the poor solubility of these compounds at the pH of the assay described.

HptCHSMX and HptCHSTZ are moderately inhibitory to the folate biosynthetic enzymes tested, with \( I_{50} \) values ranging from 2 to 150 \( \mu \text{M} \). These compounds are less inhibitory than Hpterote (\( I_{50} \) value of 55 \( \mu \text{M} \)) against GTP cyclohydrolase \((44)\). They were most potent as product inhibitors of Hpterote synthase. The inhibition by HptCHSTZ was competitive with HptCHOPP with an apparent \( K_{i} \) of \( 1.33 \pm 0.11 \mu \text{M} \); a value similar to the apparent \( K_{i} \) value (0.6 \( \mu \text{M} \)) for Hpterote. HptCHSTZ was noncompetitive with p AB, with an apparent \( K_{i} \) of \( 12.5 \pm 3.8 \mu \text{M} \) (Hpterote apparent \( K_{i} \) of \( 9.5 \pm 1.7 \mu \text{M} \)). Since the \( I_{50} \) values are similar for HptCHSTZ and HptCHSMX against Hpterote synthase, it would be consistent for their \( K_{i} \) values to be much the same also. HptCHSTZ and HptCHSMX also inhibit Hfolate synthetase by \( 50\% \) at 18 \( \mu \text{M} \) and 9.1 \( \mu \text{M} \), respectively. The free sulfonamides at millimolar concentrations have no effect on this enzyme.

The aromatic and 7,8-dihydropterin-sulfonamides were inactive or only weakly inhibitory against the activity of the following Hfolate cofactor-utilizing enzymes: serine hydroxymethyltransferase (EC 2.1.2.1), thymidylate synthetase (EC 2.1.1.45), \( N^{5},N^{10} \)-methyl-Hfolate cyclohydrolase (EC 3.5.4.9), and \( N^{5},N^{10} \)-methylene-Hfolate dehydrogenase (EC 1.5.1.5).

**DISCUSSION**

Although previous reporters have indicated that sulfonamides are metabolized in the presence of Hpterote synthase,

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**Table IV**

**Inhibition by pterin-sulfonamide adducts of folate enzymes isolated from \( E. \ coli \) B**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( \text{ptCHSA} )</th>
<th>( \text{HptCHSA} )</th>
<th>( \text{ptCHST} )</th>
<th>( \text{HptCHSTZ} )</th>
<th>( \text{ptCHSMX} )</th>
<th>( \text{HptCHSMX} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP cyclohydrolase</td>
<td>( %_{i} \mu \text{M} )</td>
<td>( %_{i} \mu \text{M} )</td>
<td>( %_{i} \mu \text{M} )</td>
<td>( %_{i} \mu \text{M} )</td>
<td>( %_{i} \mu \text{M} )</td>
<td>( %_{i} \mu \text{M} )</td>
</tr>
<tr>
<td>HptCHOH pyrophosphokinase</td>
<td>ND(^{a})</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hpterote synthase</td>
<td>50</td>
<td>200</td>
<td>50</td>
<td>148</td>
<td>50</td>
<td>158</td>
</tr>
<tr>
<td>Hfolate synthetase</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>148</td>
<td>50</td>
<td>18</td>
</tr>
<tr>
<td>Hfolate reductase</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>35</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>Serine hydroxymethyltransferase</td>
<td>61</td>
<td>140</td>
<td>41</td>
<td>140</td>
<td>118</td>
<td>16</td>
</tr>
<tr>
<td>Thymidylate synthetase</td>
<td>0</td>
<td>112</td>
<td>0</td>
<td>120</td>
<td>18</td>
<td>74</td>
</tr>
<tr>
<td>( N^{5},N^{10} )-methylene-Hfolate dehydrogenase</td>
<td>0</td>
<td>56</td>
<td>65</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>( N^{5},N^{10} )-methylene-Hfolate cyclohydrolase</td>
<td>0</td>
<td>24</td>
<td>24</td>
<td>28</td>
<td>0</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^{a}\) ND, not determined.

\(^{b}\) These values determined at 20 \( \mu \text{M} \) HptCHOPP.
neither the detailed kinetics of the reaction, nor its role in growth inhibition by sulfonamides have been investigated. We found that the apparent \( K_m \) values for \( p \)-AB and for each sulfonamide as a substrate for \( H_2 \)pterate synthase are equivalent to their apparent \( K_v \) values as competitive inhibitors, a characteristic of competitive inhibitors which are alternate substrates of the physiological substrate (11). The kinetic values presented in this paper show that SMX and probably sulfonamide as a substrate for \( H_2 \)pterate synthase are equivalent to competitive inhibitors which are alternate substrates of the physiological substrate (11). The kinetic values presented in this paper show that SMX and probably sulfonamide as a substrate for \( H_2 \)pterate synthase are equivalent to competitive inhibitors which are alternate substrates of the physiological substrate (11).

As a result, the rate of \( H_2 \)pterCH OPP utilization will be similar in the presence and absence of these sulfonamides. Accumulation of the pterin substrate within the cell will thus not occur, as it most probably would if sulfonamides were not alternate substrates. Although SMX has been shown to be a noncompetitive inhibitor with respect to \( H_2 \)pterCH OPP, under certain conditions accumulation of the pterin substrate could nevertheless decrease the inhibition by SMX. Therefore, by preventing the accumulation of \( H_2 \)pterCH OPP, the ability of sulfonamides to act as alternate substrates of \( H_2 \)pterate synthase contribute to their effectiveness as inhibitors of folate biosynthesis, and thus, of growth.

There have been several previous reports on the uptake of sulfonamides by various microorganisms. Although Reddy et al. (45) has reported that sulfadiazine is actively transported by \( E. coli \), Büttner and Büttner (42) have shown sulfadiazine to be concentrated no more than 1.5-fold by \( E. coli \). \( H_2 \)pt was not concentrated by various sensitive and sulfonamide resistant Staphylococci strains (46). In the latter two studies, the sulfonamide could easily be washed out of the cells.

The results presented in this paper (Table III) show that SA, SMX, and STZ diffuse into \( E. coli \) B. The results of the uptake studies are supported by the experiment determining the steady state intracellular concentration of \(^{35}S\) radiolabel to be \( \pm 2 \mu M \). This finding is totally consistent with the fact that SMX diffuses into \( E. coli \) at micromolar concentrations, and, in addition, establishes the upper limit for accumulation of the \( H_2 \)ptCH-SMX product.

Based on the kinetic data, the intracellular concentrations of SMX predicted by the penetration studies are sufficient to inhibit \( H_2 \)pterate synthase to the extent demanded by the observed inhibition of growth, if the intracellular concentration of \( p \)-AB is \( \pm 5 \mu M \) \((K_m = 0.5 \mu M)\).

Among the enzymes tested, the most potent inhibition by the \( H_2 \)pterin-sulfonamides was against \( H_2 \)pterate synthase. Product inhibition patterns with \( H_2 \)pterate synthase indicate an ordered reaction sequence where \( H_2 \)pterCH OPP is the first substrate to bind and \( H_2 \)pterate is the second product released (22). The \( H_2 \)pterin-sulfonamides are competitive inhibitors with \( H_2 \)pterCH OPP, and therefore they are acting as \( H_2 \)pterate analogs and are binding to the same form of the enzyme. The \( H_2 \)pterin-sulfonamides were also found to be inhibitors of the next enzyme of the folate biosynthesis sequence, dihydrofolate synthetase. To evaluate the effects of these actions on the overall inhibition of folate biosynthesis, a rate expression for the operation of the sequence was derived, similar to and based on that for a two-step product-inhibited sequence (47). \( H_2 \)pterin-sulfonamide was assumed to diffuse from the cell, in accordance with the data presented in this paper, with the steady state concentration determined by the choice of value of the first order rate constant for diffusion.

Steady state reaction velocities of the sequence were calculated using the kinetic parameters for substrates and inhibitors reported in this paper and in Refs. 25 and 28. It was assumed that \( p \)-AB and \( H_2 \)pterCH OPP were present at concentrations equal to their \( K_m \) values. \( H_2 \)pterin-sulfonamides were assumed to be either competitive or noncompetitive with \( H_2 \)pterate in the inhibition of dihydrofolate synthetase. None of these assumptions had any substantial effect upon the conclusions. The calculations showed that inhibition of \( H_2 \)pterate synthase and \( H_2 \)folate synthetase by \( H_2 \)pterin-sulfonamides could be significant only when the product accumulated in the cell to a considerably higher level than that observed experimentally. For example, with SMX present at a concentration sufficient to inhibit the pathway velocity by 40% due to competition with \( p \)-AB, a further 2-fold reduction in pathway velocity due to inhibition of \( H_2 \)pterate synthase and \( H_2 \)folate synthetase by \( H_2 \)pterCH OPP occurred only when the product accumulated to a concentration of \( 8 \mu M \). When accumulation was limited to \( 2 \mu M \), product inhibition by \( H_2 \)pterCH OPP increased the calculated inhibition of pathway velocity from 40 to 49%. Even this increase is less than expected from the effect on the enzyme in isolation, because of the associated reduction in product inhibition by \( H_2 \)pterate.

The negligible contribution of this inhibition results from the fact that it is readily overcome by a slight increase in \( H_2 \)pterate concentration, balanced by a similarly small decrease in \( H_2 \)pterCH OPP. Calculations were made assuming a wide range of intracellular concentrations of SMX, and the results described above are typical of all. The conclusions drawn from the calculations are supported by the observed lack of inhibition of growth of \( E. coli \) by exogenously supplied \( H_2 \)pterCH OPP. We therefore conclude that inhibition by \( H_2 \)pterin-sulfonamides of \( H_2 \)pterate synthase, or of the other folate enzymes tested, is not physiologically significant. The use of sulfonamides as alternate substrates by \( H_2 \)pterate synthase contributes to growth inhibition only through preventing the accumulation of \( H_2 \)pterCH OPP which would otherwise occur. Competition with \( p \)-AB is thus the primary mode of action of sulfonamides. The consequent reduction in the rate of \( H_2 \)pterate synthesis decreases the concentration of \( H_2 \)folate cofactors in the cell, reducing the rate of synthesis of the products of the pathway, thus reducing the cell growth rate.

Acknowledgments—We are grateful to Dr. Paul H. Ray for his advice and instruction in the cellular permeability studies. We would like to thank Dr. Jon C. Nixon for his assistance in performing the high performance liquid chromatography techniques and helpful discussions. We express our appreciation to Inderjit K. Dev, Par’Novak, and Alice L. Warkew for their technical assistance.

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