Phosphanilic acid (PA) is a structural analog of p-aminobenzoic acid (pABA) as well as sulfanilic acid and the sulfonamides. Chemically, PA is p-aminobenzene phosphonate. Thus, it has a phosphonate group in the position occupied by a sulfonate group in the sulfa drugs and by a carboxy group in pABA.

The antimicrobial activity of PA, as that of the sulfonamides, has been reported to be antagonized by pABA in intact cells (4-6). The antimicrobial spectrum of PA resembled that of the sulfonamide sulphanmethoxazole, but PA had significantly greater activity against Pseudomonas aeruginosa (6). Thus, while PA was effective in treating P. aeruginosa in intraperitoneally inoculated mice, the therapeutic effectiveness of PA against other organisms when given by this parenteral route was unimpressive (6). However, PA was found to be effective as a topical treatment in the burned rat model for both P. aeruginosa and Proteus mirabilis (unpublished observations). PA therefore may have value in topical treatment in the burned rat model for both P. aeruginosa and Proteus mirabilis.

The sulfonamides, being structural analogs of pABA, compete with pABA for the same binding site on dihydropteroate synthase. In a total volume of 200 µl, the following reagents, in final concentrations, were added to the reaction mixture: Tris hydrochloride buffer (pH 7), 18.3 µM; dihydropteroate, 40 mM; 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-PP; Mg²⁺, 1 mM; dithiothreitol, 5 mM; 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-PP; 20 µM; [ring-UL-¹⁴C]pABA, 20 µM (2.72 Ci/mmol); cell extract, 0.5 mg protein per ml; and potassium phosphanilate and sodium phosphate buffer (pH 7) to 55% saturation with protein per ml; and potassium phosphanilate and sodium phosphate buffer (pH 7) to 55% saturation with

Since the antimicrobial activity of PA is antagonized by pABA in intact bacterial cells, PA would be expected to inhibit organisms at the dihydropteroate synthase level. However, no published experimental evidence on the mode of action of PA at the molecular level could be found. Thus, the experiments described herein were undertaken to determine at the enzyme level whether PA acts against dihydropteroate synthesis.

MATERIALS AND METHODS

Organisms. The two experimental organisms used in these studies were Escherichia coli, a clinical isolate from a urine specimen; and P. aeruginosa ATCC 27317.

Medium. Unless otherwise indicated, the organisms were grown in a chemically defined basal salts medium (3) supplemented, in final concentration, with 20 mM glucose (BSG).

MIC determinations. The tube serial dilution technique was used to determine MICs with BSG as the test medium.

Preparation of cell extracts. Cells of P. aeruginosa and E. coli were grown overnight at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) on a reciprocating shaker. The cells were harvested by centrifugation, washed once with 10 mM phosphate buffer (pH 7), and then suspended in 2 volumes of the same buffer. The cells were ruptured by two passages through a French pressure cell at 18,000 lb/in². DNase and RNase were added (100 µg/ml, final concentration) to the broken-cell suspension. Intact cells were removed by centrifugation at 36,000 × g for 30 min at 4°C. The supernatant was then centrifuged at 100,000 × g for 1 h at 4°C.

The supernatant from the latter centrifugation was brought to 55% saturation with (NH₄)₂SO₄ by dropwise addition of a saturated (NH₄)₂SO₄ solution at 0 to 4°C. The precipitated proteins were sedimented by centrifugation at 36,000 × g for 45 min at 4°C. The sediments were then dissolved in 10 mM phosphate buffer (pH 7) and dialyzed with gentle agitation for 24 h against the same buffer at 0 to 4°C. The extracts were stored in 0.5-ml samples at −70°C until needed.

Protein concentrations were determined by the Coomassie blue technique (1) with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used as the standard.

Assay for effect of various agents on dihydropteroate synthase. In a total volume of 200 µl, the following reagents, in final concentration, were used to measure the activity of dihydropteroate synthase: Tris hydrochloride buffer (pH 8.3), 40 mM; MgCl₂, 5 mM; dithiothreitol, 5 mM; 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-PP, 20 µM; [ring-UL-¹⁴C]pABA, 20 µM (2.72 µCi); cell extract, 0.5 mg of protein per ml; and potassium phosphanilate and sodium phosphate buffer (pH 7) to 55% saturation with protein per ml; and potassium phosphanilate and sodium phosphate buffer (pH 7) to 55% saturation with protein per ml; and potassium phosphanilate and sodium phosphate buffer (pH 7) to 55% saturation with.
TABLE 1. MICs of PA and sulfanilamide in the absence and presence of pABA and the MIC of pABA alone for *P. aeruginosa* and *E. coli* in BSG medium

<table>
<thead>
<tr>
<th>Agent</th>
<th>MIC (mM) for:</th>
<th>*P. aeruginosab</th>
<th>*E. coli&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfanilamide</td>
<td>1.161 (1.483)</td>
<td>1.161</td>
<td></td>
</tr>
<tr>
<td>Sulfanilamide + 1.142 mM pABA</td>
<td>&gt;5.805</td>
<td>2.322</td>
<td></td>
</tr>
<tr>
<td>Phosphanilate</td>
<td>0.117 (0.585)</td>
<td>&gt;0.585</td>
<td></td>
</tr>
<tr>
<td>Phosphanilate + 1.142 mM pABA</td>
<td>&lt;0.059 (0.176)</td>
<td>&gt;5.85</td>
<td></td>
</tr>
<tr>
<td>pABA</td>
<td>2.855 (2.855)</td>
<td>1.142</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The tube serial dilution technique was used in which the concentrations of the agents were increased by increments of 0.585 mM from 0.585 to 5.85 mM, or by increments of 0.059 mM from 0.059 to 0.585 mM. (These values are for PA. Since the molecular weights of all three agents used are nearly equal, these values closely approximate those of PA and sulfanilamide.)

<sup>b</sup> The nonparenthetical numbers were results after 24 h of incubation, while the parenthetical numbers were results after 48 h of incubation.

<sup>c</sup> E. coli grew slowly on BSG and turbidity did not occur until in excess of 24 h of incubation. Thus, the results shown here represent 48 to 72 h of incubation.

sulfadiazine in final concentrations as appropriate as indicated in Table 2.

The reactions were incubated for 5 min in 1.5-ml microcentrifuge tubes in a 37°C water bath. The reactions were started by the addition of the cell extracts and were stopped by placing the incubation tubes on ice.

A 100-μl sample from each reaction mixture was added to an area of 2 by 3 cm on Whatman 3MM chromatography paper. Ascending chromatography was run in 0.1 M phosphate buffer (pH 7). After developing, the origins (i.e., the 2-by-3-cm areas) were cut out and placed in minivials with 6 ml of Opti-fluor scintillation fluid (Packard Instrument Co., Inc., Downers Grove, Ill.), and their radioactivities were determined. In this procedure, the [14C]dihydropteroate remained at the origin while the unreacted [14C]pABA migrated with an Rf = 0.8.

Reagents, DNase, RNase, and [ring-UL-14C]pABA (specific activity, 6.8 mCi/mmol) were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Phosphanilic acid was purchased from Raylo Chemicals (Edmonton, Alberta, Canada). All other reagents were purchased from commercial sources in their highest state of purity.

RESULTS

MICs of PA, sulfanilamide, and pABA for *P. aeruginosa* and *E. coli* in BSG medium. The MIC procedure used in these experiments should not be confused with the standardized procedures with enriched Mueller-Hinton medium. Instead, the minimal medium BSG was used to avoid the presence of intermediates and end products of folic acid metabolism which might mask the inhibitory effects of weak inhibitors of dihydropteroate synthase. Using this procedure, the results in Table 1 indicate that *P. aeruginosa* was more susceptible to inhibition by PA than *E. coli*.

Sulfanilamide was used as a control because it is known to be a weak inhibitor of dihydropteroate synthesis (i.e., sulfanilamide is weakly competitive with pABA). A comparison of the inhibition of *P. aeruginosa* by PA with that by sulfanilamide showed that the amount of PA required to inhibit this organism was less than 1/10th that of sulfanilamide (Table 1). PA was less effective against *E. coli* than against *P. aeruginosa*, whereas sulfanilamide was about equally effective against *E. coli* and *P. aeruginosa*.

At the concentration used, pABA was effective in overcoming the inhibitory effect of sulfanilamide but not that of PA for *P. aeruginosa* (Table 1). In fact, pABA appeared to enhance the activity of PA against *P. aeruginosa*. These results can be explained, at least in part, by the observation that pABA itself was inhibitory under these experimental conditions (Table 1).

**Effect of PA on dihydropteroate synthase.** PA inhibited the synthesis of dihydropteroate by dihydropteroate synthase in extracts of both *P. aeruginosa* and *E. coli* (Table 2). As a control, sulfadiazine, which is a well-documented inhibitor of dihydropteroate synthesis, was used. Thus, at a mol/mol ratio of 10:1 of sulfadiazine-pABA, sulfadiazine completely inhibited the synthesis of dihydropteroate by extracts of both *P. aeruginosa* and *E. coli*. PA, on the other hand, was a weak inhibitor of dihydropteroate synthase. At a mol/mol ratio of 100:1 with pABA, PA inhibited dihydropteroate synthase activity of *P. aeruginosa* and of *E. coli* only 22 and 28%, respectively. A mol/mol ratio with pABA of 1,000:1 was required to achieve a high level of inhibition.

**DISCUSSION**

At the intact-cell level, our data showed (i) that *P. aeruginosa* was more susceptible to the inhibitory effects of PA than *E. coli*, thus confirming observations by others (6); (ii) that pABA prevented inhibition by PA of *E. coli* in BSG medium but not of *P. aeruginosa*; and (iii) that pABA itself exhibited an inhibitory effect.

At the enzyme level, our experimental results strongly suggested that PA inhibited dihydropteroate synthase by acting as a competitor of pABA. Thus, PA appeared to exert its inhibitory effect in the same manner as the sulfonamides with which PA is structurally similar. PA, however, was a weak inhibitor of dihydropteroate synthase. Curiously, patterns of susceptibility and resistance of bacteria to PA did not always parallel those of the sulfonamides (unpublished observations).

It is interesting that the dihydropteroate synthases of *P. aeruginosa* and *E. coli* were about equally affected by PA (actually, the dihydropteroate synthase of *E. coli* appeared to be somewhat more sensitive to PA than that of *P. aeruginosa*), whereas intact cells of *P. aeruginosa* were much more susceptible to PA than intact cells of *E. coli*. A probable explanation is that intact cells of *P. aeruginosa* were more permeable to PA than intact cells of *E. coli*. Thus, since *P. aeruginosa* can utilize a variety of aromatic substances such as benzoate, catechol, or toluate that are structurally related to PA, it is likely that PA is taken into the cells of *P. aeruginosa* by diffusion and permeation mecha-
nisms physiologically designed for the uptake of aromatic compounds.

Finally, the fact that intact cells, especially those of \textit{P. aeruginosa}, were more susceptible to PA than the dihydropteroate synthase in extracts was surprising. Equally surprising was the inhibitory effect of pABA on intact cells, with \textit{E. coli} being the more sensitive. Our data did not permit us to determine the reason for this anomaly. We speculate, however, that PA has a second mode of action other than inhibition of dihydropteroate synthase. Moreover, this second mode of action also appeared to be shared by pABA.

**ACKNOWLEDGMENTS**

This work was done in the Laboratories of the U.S. Army Institute of Surgical Research where R.G.E. was a participant in the U.S. Army Summer Faculty Research and Engineering Program.

We thank Robert Ferone, Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, N.C., for the protocol for the measurement of dihydropteroate synthase activity. We are also especially grateful to Carmen Allegra, National Cancer Institute, Bethesda, Md., who very graciously supplied us with a sample of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-PP, without which this work would not have been possible.

**LITERATURE CITED**


