THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 237, No. 2, February 1962
Printed in U.S.A.

The Biosynthesis of Folic Acid

II. INHIBITION BY SULFONAMIDES*

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(Received for publication, July 26, 1961)

The biological importance of *p*-aminobenzoic acid was recognized only in 1940 when Woods showed that the compound antagonized the bacteriostatic effects of sulfonamides (2). Later, *p*-aminobenzoic acid was found to be an essential part of the folic acid molecule (3, 4), and it now seems quite likely that the sole metabolic function of *p*-aminobenzoate is to serve as a precursor for the biosynthesis of folic acid compounds. Experiments with a variety of bacterial species have established that sulfonamides inhibit synthesis *in vivo* of compounds with folic acid activity and that *p*-aminobenzoate competitively reverses the inhibition (5–7). These results, along with other observations that folate-utilizing bacteria are not affected by sulfonamides (7–10), have led to the conclusion that sulfonamides inhibit the growth of bacteria by competitively inhibiting the utilization of *p*-aminobenzoate for the biosynthesis of folic acid.

Recent observations that bacterial cell-free extracts can form folate compounds from pteridine compounds, *p*-aminobenzoate, and glutamate (11–16) have provided the opportunity to study the effects of sulfonamides on the enzymatic synthesis of folate. It has already been reported in brief notes that sulfonamides are effective inhibitors of enzymatic synthesis of folic acid compounds (12–15). The results to be presented in the present paper show that various sulfonamides inhibit folate synthesis to varying degrees by specifically inhibiting the enzymatic step whereby *p*-aminobenzoate and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine react to yield dihydropteroate. Also, evidence will be presented which indicates that pteridine compounds can react with sulfonamides in place of *p*-aminobenzoate to yield products thought to be sulfonamide analogues of dihydro- and tetrahydropteroic acid.

EXPERIMENTAL PROCEDURE

Materials—Crystalline ATP and TPN were purchased from the Pabst Laboratories; *p*-aminobenzoic acid, from Fisher Scientific Company; folic acid and sodium ascorbate, from the California Corporation for Biochemical Research; *p*-aminosalicylic acid and various sulfonamides, from Nutritional Biochemicals Corporation; and sulfanilic acid, from Eastman Organic Chemicals. *p*-Aminobenzoyl-L-glutamic acid was a gift from Lederle Laboratories Division of the American Cyanamid Company. 2-Amino-4-hydroxy-6-hydroxymethylpteridine was prepared by the procedure of Waller *et al.* (17) and reduced to the tetrahydro compound, hydroxymethyltetrahydropteridine,[1] as described previously (1). S^{35}-sulfanilic acid containing 48 mc per mmole was purchased from Nuclear-Chicago Corporation.

Determination of Folic Acid Compounds—Enzymatically produced folic acid compounds were determined by microbiological assay with *Streptococcus faecalis* (ATCC 8043) (1). Results will be presented as "folate equivalents produced," although it should be noted that compounds such as dihydropteroate, tetrahydropteroate, dihydrofolate, and tetrahydrofolate, all of which are active in replacing folate as a growth factor for *Streptococcus faecalis*, constitute the products of enzymatic action.

Preparation of Enzymes—A strain of *Escherichia coli* B resistant to T1 bacteriophage was used as a source of enzymes. Large scale growth of cells and preparation of cell-free extracts were as described previously (1). Protein was precipitated from the extracts by the addition of a volume of a saturated ammonium sulfate solution adjusted to pH 7.0 with NH_4OH sufficient to give a 90% saturated solution. The precipitated protein was dissolved in 0.05 M phosphate buffer (K salts), pH 7.0, and the solution was dialyzed at 4° for approximately 16 hours against about 200 volumes of the same buffer. Solutions prepared in this way were used as a source of enzymes in all of the work to be reported in this paper.

Growth Experiments with E. coli—The growth inhibition indices of the various sulfonamides, *i.e.* the ratio of concentration of sulfonamide to *p*-aminobenzoate which will just permit half-maximal growth, were determined with the *E. coli* B strain resistant to T1 and the medium which was used to grow this bacterium previously (1). Operationally, a series of tubes (25 × 200 mm) was prepared to contain medium plus varying amounts of *p*-aminobenzoate and sulfonamide in a final volume of 10 ml. Each tube was inoculated with 1 drop of a washed suspension (absorbancy of approximately 0.3) of cells taken from a 24-hour glucose-yeast extract slant culture. The inoculated liquid cultures were incubated for 20 hours at 37° in a slanted position to give the maximal amount of surface area. Growth was estimated turbidimetrically with a Coleman junior spectrophotometer at 660 mμ.

RESULTS

Inhibition of "Folate" Synthesis by Sulfonamides—The inhibitory effects of sulfanilamide and sulfathiazole of "folate" synthesis by cell-free extracts of *E. coli* are shown in Fig. 1. From

* Supported by Research Grants G-4580 from the National Science Foundation and A-3442 from the National Institutes of Health of the United States Public Health Service. See (1) for Paper I of this series.

[1] The abbreviation used is: hydroxymethyltetrahydropteridine, 2-amino-4-hydroxy-6-hydroxymethyltetrahydropteridine.

the data, the ratios of concentrations of sulfonamide to *p*-aminobenzoate required to inhibit synthesis to the half-maximal level, which will hereafter be referred to as the "enzymatic inhibition index," were calculated to be 18 and 0.77 for sulfanilamide and sulfathiazole, respectively.

It was also found (see Fig. 1) that sulfathiazole effectively antagonized the utilization of *p*-aminobenzoylglutamate, which can be used as substrate in place of *p*-aminobenzoate in the enzyme system, although less effectively (1). The calculated enzymatic inhibition index is 0.1, a value which is approximately one-eighth of that obtained when *p*-aminobenzoate was used as substrate. When one considers that *p*-aminobenzoylglutamate is utilized as substrate approximately 10 times less effectively than *p*-aminobenzoate (1), it would appear that sulfathiazole antagonizes *p*-aminobenzoate and *p*-aminobenzoylglutamate to an approximately equal extent. The fact that *p*-aminobenzoylglutamate does not reverse sulfonamide inhibition indicates that the mechanism of action of sulfonamides can probably be explained as an inhibition of the enzymatic reaction whereby *p*-aminobenzoate or *p*-aminobenzoylglutamate becomes attached to a pteridine compound.

The products of enzymatic action in the experiments described above and in the experiments to be described below were found by paper chromatographic and bioautographic techniques (1) to be dihydro- and tetrahydropteroic acid from *p*-aminobenzoate and dihydro- and tetrahydrofolic acid and derivatives of tetrahydrofolate (probably containing 1-carbon units) from *p*-aminobenzoylglutamate. The inclusion of sulfonamide in reaction mixtures in amounts which caused approximately half-maximal formation of products did not qualitatively alter the nature of the products.

Comparison of Sulfonamides as Inhibitors of Bacterial Growth and Inhibitors of Enzymatic Production of "Folate"—A number of sulfonamides as well as sulfanilic acid and *p*-aminosalicylic acid were tested as inhibitors of the enzymatic synthesis of "folate." Each compound yielded an inhibition curve with the same shape as those shown in Fig. 1 for sulfathiazole and sulfanilamide. However, the amounts needed for inhibition varied, depending on the inhibitor tested. Each compound was also tested as an inhibitor of growth of *E. coli* B, the strain from which the enzyme system was extracted, according to the conditions described in "Experimental Procedure." For each compound, the growth and enzymatic inhibition indices were determined (see Table I). In general, those compounds which were relatively effective inhibitors of enzymatic synthesis of "folate" were also relatively effective growth inhibitors. The striking exception is sulfanilic acid, which was quite effective in inhibiting "folate" synthesis but did not have any effect on growth, even when present in very high concentrations. Also, sulfabenzamide and sulfaguanidine were relatively better inhibitors of "folate" synthesis than might have been predicted from their properties as growth inhibitors. The fact that much higher values were obtained when growth was used as a measure of the inhibition index than when enzymatic synthesis of "folate" was used suggests that the sulfonamides may have some difficulties in getting to the intracellular site of enzymatic synthesis of "folate." This might be due to limited permeability of the cells to these compounds. Indeed, it would appear that sulfanilate does not permeate the cells to any extent, since it had no effect on growth but was a good inhibitor of "folate" synthesis *in vitro*.

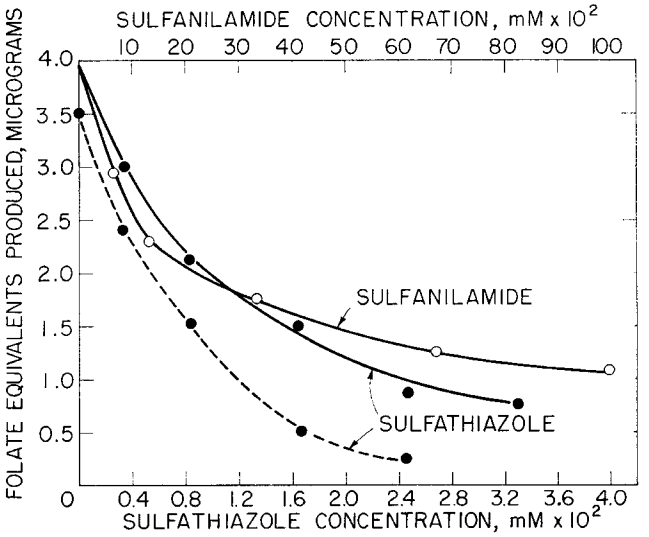

FIG. 1. The inhibition of enzymatic synthesis of "folate" by sulfathiazole and sulfanilamide. Reaction mixtures contained either 0.013 mM *p*-aminobenzoate (*solid lines*) or 0.067 mM *p*-aminobenzoylglutamate (*dashed line*), 0.033 mM hydroxymethyltetrahydropteridine, 3.3 mM ATP, 6.7 mM $MgCl_2$, 0.067 mM TPN, 67 mM mercaptoethanol, 0.07 M phosphate buffer, pH 7.0, 0.1 ml (4 mg of protein) of dialyzed enzyme preparation, and either sulfanilamide or sulfathiazole as shown; final volume was 1.5 ml. Incubation was for 2 hours at 37° under anaerobic conditions (1). After incubation, 1 mmole of mercaptoethanol was added to each reaction mixture, and "folate" synthesis was estimated by microbiological assay with *S. faecalis*.

TABLE I

Effectiveness of sulfonamides as inhibitors of growth of E. coli and as inhibitors of enzymatic formation of "folate"

Inhibitor	Inhibition index	
	Enzymatic*	Growth†
Sulfathiazole	0.70	250
Sulfabenzamide	0.90	1,100
Sulfamerazine	1.2	270
Sulfadiazine	1.9	270
Sulfanilic acid	2.5	
Sulfamethazine	2.9	625
Sulfapyridine	3.8	1,400
Sulfaguanidine	5.0	16,300
Sulfacetamide	7.0	2,900
Sulfanilamide	20.0	21,500
Sulfasuxidine	23.0	6,800
p-Aminosalicylic acid	24.0	64,000

* Each value is an average of at least three separate determinations. Reaction mixtures, incubation conditions, and analytical procedure were as described in Fig. 1. *p*-Aminobenzoate was added as substrate.

† *p*-Aminobenzoic acid was added to the growth medium at a concentration of 10^{-4} mM. Sulfanilic acid was added at concentrations as high as 24 mM with no apparent growth inhibitory effects.

Nature of Inhibition—When the *p*-aminobenzoate concentration of reaction mixtures was varied, the amount of sulfonamide required for inhibition of enzymatic synthesis of "folate" also varied, so that the enzymatic inhibition index remained rela-

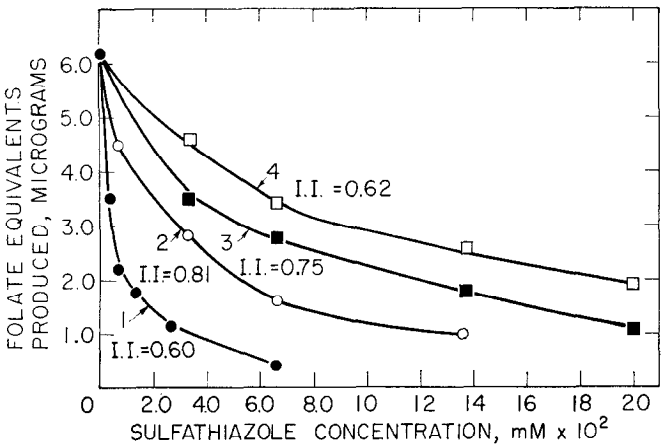

FIG. 2. The relation between concentration of *p*-aminobenzoate and the amount of sulfathiazole required for inhibition of "folate" synthesis. Reaction mixtures were as described in Fig. 1, except that an amount of enzyme preparation equal to 6 mg of protein was added, and the concentrations of *p*-aminobenzoate were: *curve 1*, 0.0067 mM; *curve 2*, 0.033 mM; *curve 3*, 0.067 mM; and *curve 4*, 0.133 mM. Incubated reaction mixtures were analyzed as described in Fig. 1. The calculated enzymatic inhibition index (*I.I.*) is listed for each concentration of *p*-aminobenzoate used.

TABLE II

Irreversible inhibition by sulfathiazole of "folate" synthesis

The complete reaction mixture contained *p*-aminobenzoate, 0.067 mM; hydroxymethyltetrahydropteridine, 0.033 mM; ATP, 3.3 mM; TPN, 0.067 mM; $MgCl_2$, 6.7 mM; sulfathiazole, 0.017 mM; phosphate buffer, 0.07 M; mercaptoethanol, 67 mM; and enzyme preparation equivalent to 6 mg of protein; the total volume of each reaction mixture was 1.5 ml. The first incubation was for 2 hours anaerobically at 37° in special tubes (1). Missing components were then added to the reaction mixtures (except for Reaction Mixtures 5 and 6; see footnote) by means of a syringe equipped with a hypodermic needle in order not to disturb anaerobic conditions, and the reaction mixtures were reincubated for 2 hours at 37°. Analysis of reaction mixtures was conducted as described in Fig. 1.

Reaction mixture No.	Component omitted in first incubation period	Component added between first and second incubation periods	Folate equivalents produced
			μg
1	Sulfathiazole	None	5.8
2	None	None	5.2
3	Sulfathiazole, *p*-aminobenzoate	*p*-Aminobenzoate	6.4
4	*p*-Aminobenzoate	*p*-Aminobenzoate	1.1
5*	Sulfathiazole, *p*-aminobenzoate	All components, except enzyme and sulfathiazole	6.5
6*	*p*-Aminobenzoate	All components, except enzyme and sulfathiazole	5.5

* After the first incubation period, these reaction mixtures were dialyzed for 16 hours against 2000 volumes of 0.1 M phosphate buffer. New substrates and cofactors were then added to the dialyzed solutions, and the reaction mixtures were incubated a second time.

tively constant over a wide range of concentrations (see Fig. 2 for a typical example). This behavior suggested that sulfonamides competitively antagonize the utilization of *p*-aminobenzoate by the enzyme system. Efforts to determine whether or not competitive inhibition kinetics were followed have not been very successful, primarily because the microbiological assay values are not sufficiently accurate for the precision required in the Lineweaver-Burk method, in which the reciprocal of reaction velocity is plotted against the reciprocal of the substrate concentration. An alternate procedure which proved useful in determining whether or not the inhibition was of the competitive type is as follows. A reaction mixture was prepared to contain a sulfonamide and all of the components required for "folate" production except *p*-aminobenzoate. After a preliminary incubation period, *p*-aminobenzoate was added in an amount which was known to reverse sulfonamide inhibition when both compounds are added at the same time, and the reaction mixture was reincubated. It was reasoned that if the inhibition were solely of the competitive type, there should be no inhibition of "folate" synthesis whether *p*-aminobenzoate was added at the same time as the sulfonamide or after the preliminary incubation period. The results of such an experiment, summarized in Table II, show that *p*-aminobenzoate did not reverse sulfathiazole inhibition in the reaction mixture from which *p*-aminobenzoate had been omitted in the first incubation period (Reaction Mixture 4). On the other hand, when *p*-aminobenzoate and sulfathiazole were added to the reaction mixture at the same time (Reaction Mixture 2), little inhibition occurred. The same results were obtained when sulfathiazole was replaced by either sulfanilate or any of the other sulfonamides listed in Table I.

The results described above indicated that the sulfonamide inhibition could not be described solely as the competitive type. The possibility that the preliminary incubation with sulfathiazole may render the enzyme irreversibly inactive was checked by the procedure described for Reaction Mixtures 5 and 6, Table II. These experiments revealed that almost complete enzymatic activity was recovered by dialyzing the enzyme preparation which had been exposed to sulfathiazole during the first incubation period, a fact which indicates that an enzyme was not inactivated irreversibly by reaction with sulfathiazole.

A summary of the correlation of the "irreversible inhibition," *i.e.* inhibition produced by a preliminary incubation and not reversed by the subsequent addition of *p*-aminobenzoate, with time of incubation is given in Table III. These data established that the phenomenon was relatively slow, with considerable in-

TABLE III

Relation of incubation time to irreversible inhibition by sulfathiazole of "folate" synthesis

Reaction mixtures were prepared to contain substrates and sulfathiazole (see Table II for concentrations), and two successive incubations were conducted as described in Table II, except the length of the first incubation period was varied as shown.

Length of first incubation period	Component omitted from first incubation and added before second	Folate equivalents produced
min		μg
120	None	6.6
1	*p*-Aminobenzoate	6.6
10	*p*-Aminobenzoate	5.6
30	*p*-Aminobenzoate	3.0
60	*p*-Aminobenzoate	1.7
120	*p*-Aminobenzoate	0.9

hibition occurring only after approximately 30 to 60 minutes. This rate of "irreversible inhibition" is reminiscent of the rate of "folate" synthesis in the absence of sulfonamides (1) and suggested that perhaps during the preliminary incubation period the sulfonamide may have reacted with the pteridine to give a product which is inactive as folate in the assay. Thus, the "irreversible inhibition" effect might then be due merely to depletion of the pteridine substrate. The formation of a product would be expected to be dependent on the presence of pteridine substrate and, perhaps, of Mg^{++}. Both of these were absolute requirements for "folate" synthesis by the enzyme preparation. This hypothesis was tested in the experiment summarized in Table IV. The results obtained show that both the pteridine and Mg^{++} were necessary for the "irreversible inhibition" to occur; *i.e.* no inhibition resulted when either of these was omitted during the first incubation period. These facts support the hypothesis that a product was formed from the pteridine and sulfathiazole.

If the "irreversible inhibition" is to be explained as an exhaustion of pteridine substrate, it should be possible to reduce or eliminate this inhibition by increasing the amount of the pteridine compound added to a reaction mixture. In Table V it can be seen that increasing the concentration of the pteridine substrate resulted in a reduction of the degree of "irreversible inhibition" but did not eliminate the inhibition entirely. Thus, it would appear that the "irreversible inhibition" phenomenon involves more than the mere depletion of pteridine substrate. Possibly, the postulated product formed enzymatically might itself be an effective inhibitor of *p*-aminobenzoate utilization.

Evidence for Enzymatic Formation of Sulfonamide-containing Product—In an attempt to provide evidence that sulfonamides can be utilized by the enzyme system in place of *p*-aminobenzoate to yield a product containing pteridine and sulfonamide moieties, S^{35}-sulfanilic acid was incubated in reaction mixtures along with hydroxymethyltetrahydropteridine and other components, as described in Fig. 3. As shown in the figure, two new radioactive compounds were produced in the complete reaction mixture. When either Mg^{++} or the enzyme preparation was omitted from the reaction mixture, neither of these products was formed, and only a trace of one product was formed when hydroxymethyltetrahydropteridine was omitted. The formation of the small amount of compound of R_F 0.27 in the absence of added pteridine compound probably resulted from the presence in the enzyme preparation of a small amount of pteridine substrate. The presence of pteridine was established by other experiments. These results provide convincing evidence for the utilization of sulfanilate for the formation of products of enzymatic action. The exact identities of the two products remain unknown. The most reasonable explanation for the detection of two products is that they differ only in the state of oxidation of the pteridine ring (tetrahydro, dihydro, or perhaps even fully oxidized). From what is known about the sequence of reactions for the biosynthesis of folate compounds (1), the primary product probably should be a dihydro compound; how-

TABLE IV

Pteridine and Mg^{++} requirements for irreversible inhibition by sulfathiazole of "folate" synthesis

Reaction mixtures were prepared and incubation procedures were followed as described in Table II.

Component omitted from first and added before second incubation	Folate equivalents produced
	μg
None	5.6
p-Aminobenzoate	1.2
p-Aminobenzoate, hydroxymethyltetrahydropteridine	6.0
Hydroxymethyltetrahydropteridine	5.0
p-Aminobenzoate, $MgCl_2$	5.1
$MgCl_2$	5.1

TABLE V

Effect of concentration of pteridine substrate on irreversible inhibition by sulfathiazole of "folate" synthesis

Reaction mixtures were prepared, and incubations were conducted, as described in Table II except that hydroxymethyltetrahydropteridine was added in the amounts shown. In all of the reaction mixtures, *p*-aminobenzoate was omitted during the first incubation period and was added before the second incubation period.

Hydroxymethyltetrahydropteridine added	Folate equivalents produced	
	Absence of sulfathiazole	Presence of sulfathiazole
mM	μg	μg
0.033	4.1	0.5
0.067	8.0	1.2
0.100	9.1	3.6
0.133	9.0	4.8

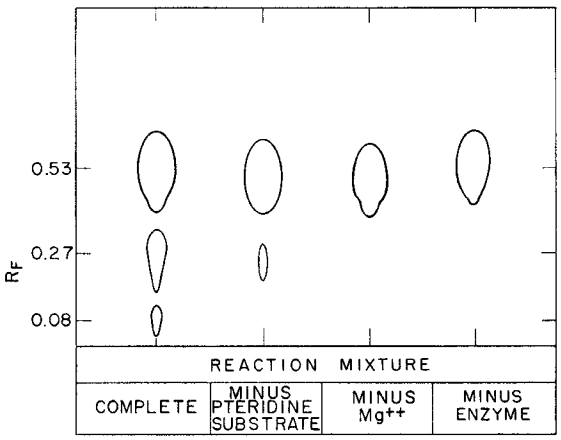

FIG. 3. A drawing of an autoradiogram which shows the enzymatic formation of radioactive products from S^{35}-sulfanilic acid. The complete reaction mixture contained 0.67 mM neutralized S^{35}-sulfanilic acid (4.8 mc per mmole), 0.17 mM hydroxymethyltetrahydropteridine, 3.3 mM ATP, 0.67 mM TPN, 6.7 mM $MgCl_2$, 67 mM mercaptoethanol, and an amount of dialyzed enzyme preparation equivalent to 10 mg of protein; the total volume of the reaction mixtures was 1.5 ml. Incubation anaerobically was for 3 hours at 37°. After incubation, 1 mmole of mercaptoethanol was added to each reaction mixture, and a total of approximately 0.05 ml of each reaction mixture was spotted in 0.005-ml amounts on a sheet of Whatman No. 1 paper. The chromatogram was developed (ascending technique) with 0.1 M phosphate (K salts) buffer, pH 7.0, which also was 0.03 M with respect to sodium ascorbate. The developed chromatogram was allowed to remain in contact with Ansco Non-Screen X-ray Safety Film for 7 days, after which the film was developed. The *spots* shown in the figure appeared on the developed film as dark areas against a clear background. The compound of R_F 0.53 is unchanged S^{35}-sulfanilic acid.

ever, the enzyme preparation used was relatively crude, and it might contain enzymes and cofactors that could reduce the dihydro compound to the corresponding tetrahydro compound.

DISCUSSION

The data of the present paper establish conclusively that sulfonamides inhibit the synthesis of folic acid compounds by inhibiting the specific enzymatic reaction whereby what is believed to be hydroxymethyldihydropteridine and *p*-aminobenzoate react in the presence of ATP and Mg^{++} to yield dihydropteroic acid. The nature of the inhibition appears to involve a competition between the sulfonamide and *p*-aminobenzoate for the active area on the enzyme; however, once the sulfonamide competes favorably for the active site, it apparently can react with the pteridine compound to yield products which are inactive as folic acid in the microbiological assay system. The inactivity of these compounds has been established by bioautographic experiments. Thus, the inhibition probably can most properly be described as one involving the competition of two substrates, *p*-aminobenzoate and the inhibitor being tested, for the active region of the enzyme. There is some indirect evidence that the inactive products formed from the sulfonamides may exert some inhibitory effects on the utilization of *p*-aminobenzoate as substrate; however, proof must await the testing of the pure compounds. These compounds should also be tested as possible inhibitors of the formation of dihydrofolate from dihydropteroate and glutamate.

Although the experiments reported here provided direct evidence only for the interaction of the pteridine and sulfanilic acid, other workers have reported that the *p*-aminobenzoate analogue, *p*-aminosalicylate, which is an effective inhibitor of growth of some bacteria, can be used as substrate to form folic acid-like compounds. Thus, Wacker, Kolm, and Ebert (18) found that C^{14}-*p*-aminosalicylate was converted by certain enterococci into a compound which could be recovered in the folic acid fraction of the cells, and Hotchkiss and Evans (19) observed that *p*-aminosalicylate is used by pneumococci to form a compound with folic acid activity for pneumococci but not for *S. faecalis*.

SUMMARY

Sulfonamides were effective inhibitors of the biosynthesis of folic acid compounds by cell-free extracts of *Escherichia coli*. The inhibited enzymatic reaction was established as the one whereby dihydropteroic acid is formed from *p*-aminobenzoic acid and what is believed to be 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine in the presence of adenosine triphosphate and Mg^{++}.

A comparison of the inhibitory effects of various sulfonamides on the growth of *Escherichia coli* and on the enzymatic synthesis of folate compounds revealed that the potencies of sulfonamides as inhibitors varied with the specific compound tested; sulfathiazole was the most effective. However, all the sulfonamides are several times more potent as inhibitors at the enzyme level than they are as inhibitors of growth, and sulfanilic acid was ineffective as a growth inhibitor but was quite active as an inhibitor of enzymatic synthesis of folic acid compounds. These facts indicate that these compounds may not easily pass from the medium into the bacterial cells.

Sulfonamide inhibition of the synthesis of folic acid compounds was readily reversed by increasing the concentration of *p*-aminobenzoic acid supplied in the reaction mixtures. The data obtained indicated that sulfonamides can be used as substrates in the enzyme system to form products which are probably analogues of reduced forms of pteroic acid and which are inactive as pteroic acid or folic acid in microbiological assays. Thus, it appears that sulfonamides inhibit *p*-aminobenzoic acid utilization by competing with the latter compound as a substrate in the enzyme system.

Acknowledgment—It is a pleasure to acknowledge the very skillful technical assistance of Mrs. Chai Ho Lo.

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