Inhibition by Sulfonamides of the Biosynthesis of Folic Acid[1]

Gene M. Brown[2]

Woods ([22]) laid the groundwork for the elucidation of a biologic function for p-aminobenzoic acid (p-AB) with the observation that this compound antagonizes the bacteriostatic properties of sulfonamides. Later work established that p-AB is a structural unit of a more complex compound, folic acid ([1, 9]). No evidence exists to indicate that p-AB is needed for any purpose other than as a precursor of folic acid. Work with intact bacterial cells had established that sulfonamides interfere with the cellular synthesis of folic acid ([10, 12, 23]), and that p-AB reverses this inhibition in a competitive manner. The elucidation of the enzymatic reactions that result in the biosynthesis of folic acid by Shiota and coworkers ([15–18]) and by Brown and his collaborators ([5, 6, 20]) provided an opportunity to study the effects of sulfonamides on the enzymatic utilization of p-AB for the formation of folic acid and related compounds.

This paper will be presented in the form of a short review in which the biosynthesis of folic acid will be discussed briefly, followed by a somewhat more detailed description of the work with sulfonamides.

Enzymatic synthesis of folic acid and related compounds. Folic acid contains three structural units: a pteridine moiety, p-AB, and glutamic acid. Most of the significant work that has led to an understanding of the enzyme system that catalyzes the synthesis of the vitamin from these precursors has come from the laboratories of Shiota ([15–18]) and of Brown ([5, 6, 20]). These workers used, respectively, cell-free extracts of *Lactobacillus plantarum* and extracts of *Escherichia coli* as sources of enzymes. It has been established that, in both systems ([6, 16]), the pteridine used as substrate is 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine (Fig. 1). This compound can be converted enzymatically to either dihydropteroic acid (in the presence of p-AB as cosubstrate) or dihydrofolic acid (in the presence of p-aminobenzoylglutamic acid) only when ATP and magnesium ions are also present in the reaction mixtures. Weisman and Brown ([20]) have fractionated extracts of *E. coli* and obtained two enzyme fractions (fractions A and B), both of which are needed for the formation of dihydropteroic acid from p-AB, ATP and the dihydropteridine substrate. One of these enzyme fractions (fraction A) is needed to catalyze the formation from the dihydropteridine and ATP of a substance that can be converted by the second enzyme fraction (fraction B) to dihydropteroate in the presence of p-AB. Shiota *et al.* ([17, 18]) have synthesized the monophospho and pyrophospho esters of 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine and showed that in the *L. plantarum* system the pyrophosphate ester, but not the monophosphate, can be converted to dihydropteroate in the presence of p-AB and in the absence of ATP. Weisman and Brown ([20]) then found that their fraction B, prepared from *E. coli* extracts, could catalyze the synthesis of dihydropteroate from p-AB and the pyrophosphate ester, but the monophosphate ester was not utilized as substrate by either fraction B alone, or in the presence of ATP and both fractions A and B. These results suggested that the pyrophosphate ester is an intermediate in the pathway and that its

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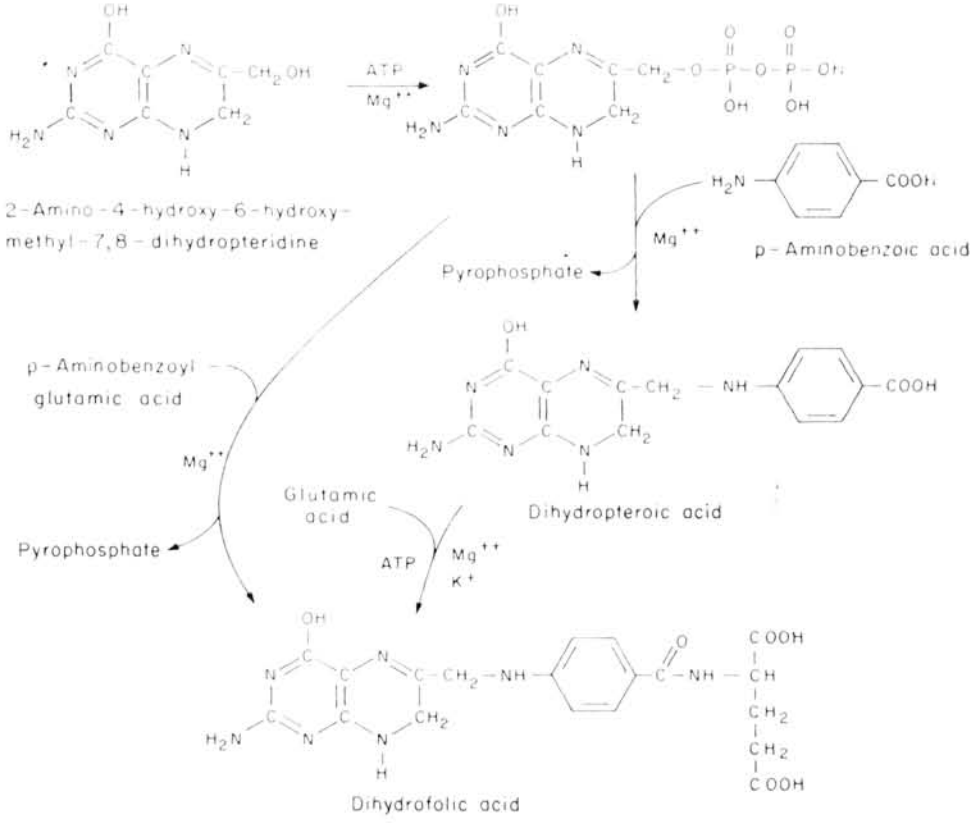

FIG. 1. The enzymatic reactions that lead to the biosynthesis of dihydrofolic acid.

formation and subsequent utilization for the enzymatic synthesis of dihydropteroate is as shown in Figure 1.

Also shown in Figure 1 is the reaction whereby dihydropteroic acid and glutamic acid are utilized for the formation of dihydrofolic acid. The enzyme that catalyzes this reaction has been found in extracts of a large number of microorganisms ([7]), including such diverse varieties as yeast, *Neurospora*, *Mycobacterium avium*, and various species of enterobacteria. The enzyme has been purified some 35-fold from extracts of *E. coli* and some of its properties described ([7]). As shown in Figure 1, ATP, Mg^{++} and monovalent cation (either K^+ or NH_4^+) are all needed for this enzyme to be active. Neither pteroic acid nor tetrahydropteroic acid can be used in place of dihydropteroic acid as substrate.

Although p-aminobenzoylglutamic acid p-ABG) can be utilized in place of p-AB for the direct formation of dihydrofolic acid ([6, 15]), it is felt that p-ABG is probably not of importance as an intermediate in the biosynthesis of folic acid compounds, since p-ABG is not utilized as effectively as p-AB in most systems and no evidence could be obtained that p-ABG is a product formed enzymatically from p-AB and glutamate in *E. coli* ([6]).

The general importance of the pathway given in Figure 1 is evident in the fact that the reactions for the synthesis of dihydropteroic acid are known to occur in a wide variety of organisms, including (in addition to *L. plantarum* and *E. coli*) pneumococci ([13, 21]), micrococci ([2]), yeast ([8]), *Pekinensis brassica* ([11]), *Neurospora* ([3]), *Mycobacterium avium* ([3]), *Bacillus subtilis* ([3]), *Corynebacterium sp.* ([3]), *enterobacteria* ([3]) such as *Proteus morgani*, *Aerobacter aerogenes*, and *Salmonella typhimurium*, and *Micrococcus lysodeikticus* ([3]).

Inhibition by sulfonamides. The effects of sulfonamides on the enzymatic synthesis of

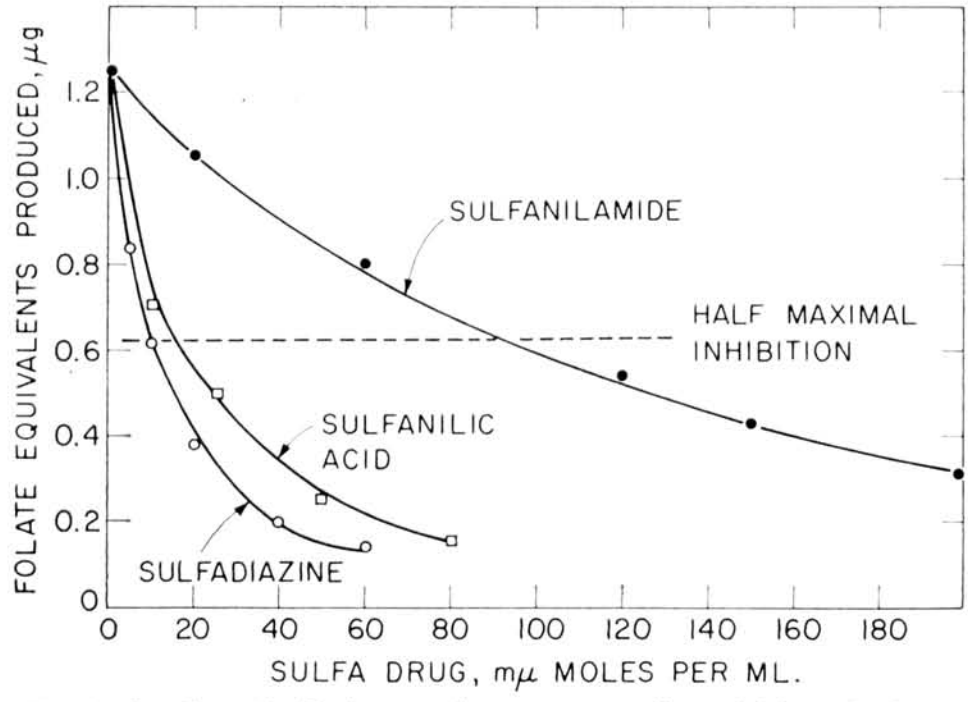

FIG. 2. The effects of sulfa drugs on the enzymatic synthesis of folic acid. The contents of the reaction mixtures are given in the text. Details of this experiment can be found in a paper by Brown (4).

dihydropteroic acid (expressed as "folate equivalents" produced) by an enzyme system from *E. coli* are shown in Figure 2. Each reaction mixture contained: 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine, ATP, Mg^{++}, the enzyme preparation, p-AB (5 mμmoles per ml.), and sulfa drug as shown in the figure. The three sulfa drugs tested were all effective as inhibitors of product formation, but the three were not equally effective. Sulfadiazine was most effective and sulfanilamide the least. Sulfanilic acid is, of course, not a sulfonamide, but nevertheless is a potent inhibitor. The dotted line shown in Figure 2 represents half-maximal synthesis of product under the experimental conditions. A useful term that has been used to characterize the effects of competitive inhibitors in biologic systems is the "inhibition index." This refers to the ratio of the concentration of the inhibitor to the concentration of the substrate (with which it competes) that results in half-maximal synthesis of product where maximal synthesis of product is measured under the same experimental conditions in the absence of inhibitor. For example, from the data of Figure 2 it is possible to calculate inhibition indices of approximately 2.0, 3.0 and 20 for sulfadiazine, sulfanilic acid and sulfanilamide, respectively.

In an experiment where the effect of a competitive inhibitor on bacterial growth is measured, the inhibition index is defined in the same way except that growth is the process measured quantitatively rather than the formation of a product. A number of sulfonamides have been analyzed for their abilities to compete with p-AB and thus to inhibit the growth of *E. coli*, and also for their properties as inhibitors of the utilization of p-AB for the enzymatic production of dihydropteroic and by an enzyme system prepared from *E. coli* (4). A listing of the calculated inhibition indices determined both in growth and enzymatic experiment is given in Table 1. For the details of this experiment, the reader is referred to a paper by Brown (4). It is evident from the data of Table 1 that a particular sulfonamide is a much better

TABLE 1. *Effectiveness of sulfonamides as inhibitors of the growth of* E. coli *and as inhibitors of the 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

inhibitor at the enzyme level than it is as an inhibitor of bacterial growth.

A comparison of the inhibitory properties of the sulfonamides indicates that, in general, if a sulfonamide is a good growth inhibitor it is also a relatively good inhibitor of the enzymatic reaction, although some exceptions are evident (*cf.* sulfabenzamide, sulfaguanidine, sulfasuxidine). Two compounds that were tested, sulfanilic acid and p-amino-salicylic acid, are not sulfonamides but were included because of their similarities in structure to p-AB. Sulfanilic acid was completely ineffective in inhibiting bacterial growth, but was quite effective as an inhibitor in the enzymatic experiments. This suggests that the cells are not permeable to the highly negatively charged sulfanilic acid. The fact that all of the sulfonamides were far better inhibitors of the enzymatic synthesis of dihydropteroate than they were of the growth of *E. coli* suggests that these bacteria are far less permeable to these compounds than they are to p-AB.

From the data presented in Table I, it is evident that sulfathiazole is the most effective of all the sulfonamides as an antagonist of the utilization of p-AB. In order to determine whether the observed inhibition of the formation of product was competitive, a number of reaction mixtures were prepared to contain varying concentrations of both p-AB and sulfathiazole. In one series, a constant amount of sulfathiazole was added to each mixture and the concentration of p-AB was varied. Three other such series of reaction mixtures were prepared, each series containing a different amount of sulfathiazole. The data are presented in Figure 3 as double reciprocal plots (the so-called Lineweaver-Burke plot) that are traditionally used to determine whether the inhibition is competitive. The resulting family of straight lines, each line with a different slope but the same intercept, indicates that the inhibition by sulfathiazole was competitive with p-AB.

In order to confirm that competitive inhibition kinetics applied to the system described above, experiments were designed in which reaction mixtures were prepared to contain sulfathiazole and every component (including the enzyme system) needed for the enzymatic production of dihydropteroate, except for p-AB. After a preliminary incubation period (37° C) of two hours, p-AB was then added to the reaction mixture and incubation was continued for another two hours. Control experiments indicated that the amount of p-AB added was great enough so that if it had been added at the same time as the sulfathiazole, no inhibition by the drug would have been evident. It was expected that if strictly competitive inhibition kinetics were followed, the preliminary incubation in the presence of the sulfathiazole and the absence of p-AB would have no effect on the production of dihydropteroate during the second incubation period (after the addition of the p-AB). However, the results shown in Table 2 indicate that the preliminary incubation with sulfathiazole resulted in inhibition that was not reversed by subsequent addition of p-AB (Reaction Mixture 4, Table 2). Reaction Mixture 2 of Table 2 shows that when both p-AB and sulfathiazole were added together, the amount of p-AB added was sufficient so that very little inhibition occurred (compare with Reaction Mixture 1). The effect of the length of the first incubation period

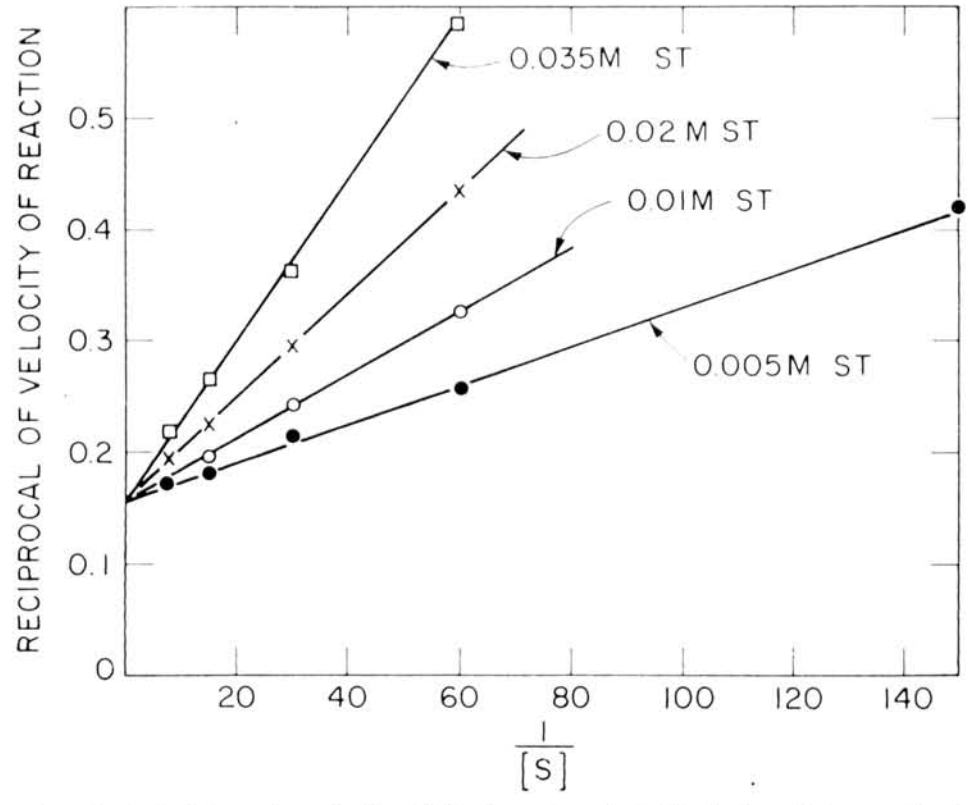

FIG. 3. A double reciprocal plot of 1/substrate against 1/velocity of the reaction in the presence of various concentrations of sulfathiazole. [S] = The concentrating of p-AB; ST = sulfathiazole. The other components of the reaction mixtures were: 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine, ATP, $MgCl_2$, and enzyme preparation. The velocity of the enzymatic reaction was determined by measuring the amount of product, dihydropteroic acid, that was formed. Experimental details were similar to those reported by Brown (1).

TABLE 2. *Irreversible inhibition of folate synthesis by sulfathiazole.*

Reaction mixture[a]	Component omitted during first incubation period	Component added after first and before the second incubation period	Folate equivalents produced
			μg
1	Sulfathiazole	None	5.8
2	None	None	5.2
3	Sulfathiazole, p-AB	p-AB	6.4
4	p-AB	p-AB	1.1

[a] The preparation of the reaction mixtures and the incubation conditions were as described in the text. Other details of this experiment were as described by Brown (1).

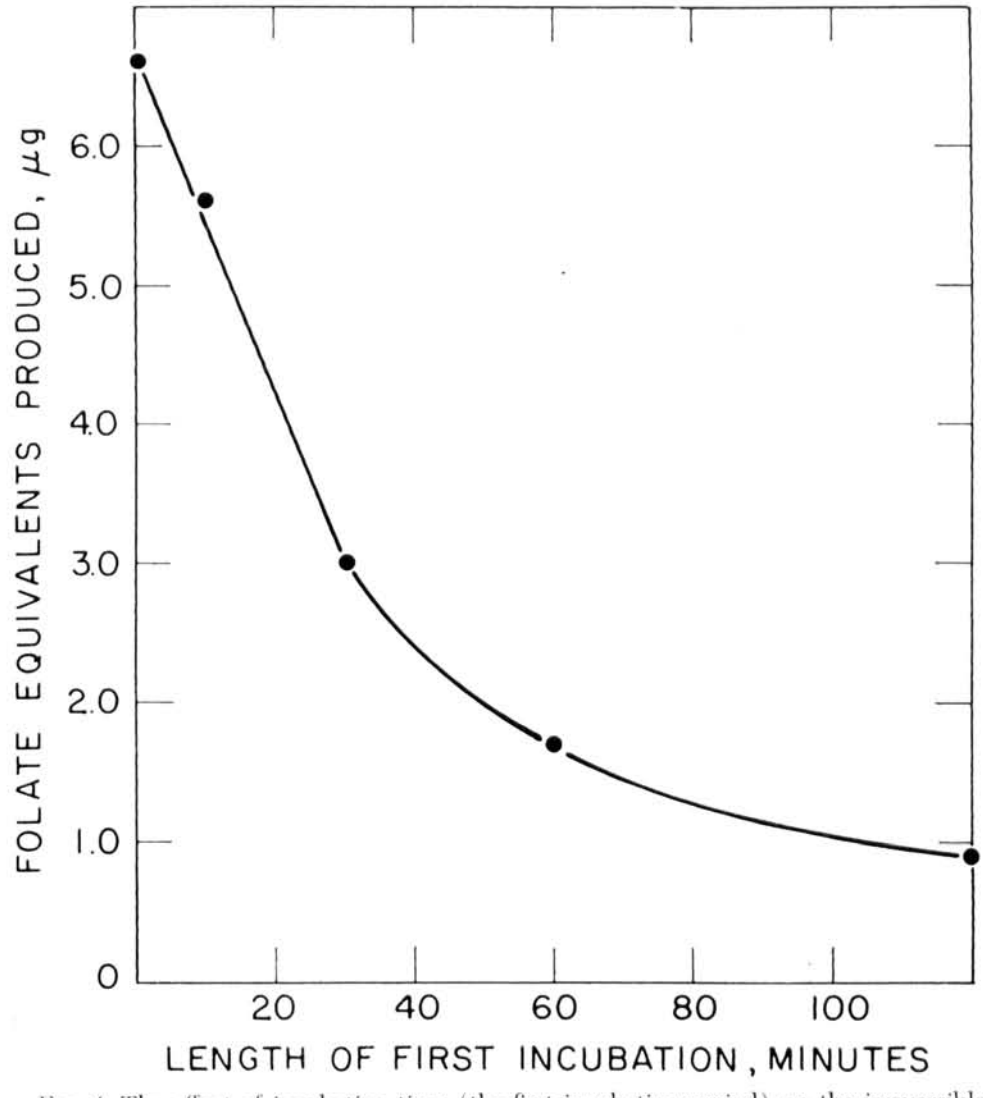

FIG. 4. The effect of incubation time (the first incubation period) on the irreversible inhibition by sulfathiazole. During the first incubation period, reaction mixtures contained sulfathiazole, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine, ATP, $MgCl_2$, and enzyme preparation. After incubation at 37° for the times shown, p-AB was then added to each reaction mixture and all were then reincubated for 2 hours. Enough p-AB was added so that if the p-AB and sulfathiazole had been added at the same time no inhibition by sulfathiazole would have been observed.

on this "irreversible inhibition" is given in Figure 4. As the incubation time is increased the degree of irreversible inhibition is also increased. This suggested that an enzymatic reaction involving sulfathiazole as a substrate might be occurring during the first incubation and that the net effect of this reaction is to either use up other substrate or to form a product that affects the formation of dihydropteroate from p-AB. Further experiments indicated that in order for the irreversible inhibition to occur, Mg ++, ATP and the dihydropteridine substrate had to be present in the reaction mixture during the first incubation period. This supported the view that a

product was formed and suggested that the product was an analog of dihydropteroate in which the p-AB portion of this product was replaced by sulfathiazole. One of the effects of such a phenomenon should be that the dihydropteridine substrate would be consumed by reaction with sulfathiazole and thus not be available for reaction with p-AB after the latter compound was added to the reaction mixture. If this were the case, then by increasing the concentration of the dihydropteridine, one should observe a reversal of the inhibition. Such an experiment was done and it was, indeed, found that high concentrations of the dihydropteridine in reaction mixtures did tend to reverse the inhibition ([4]).

Confirmation was obtained for the formation of a sulfa drug-containing product by the incubation of sulfanilic acid-S^{35} with the enzyme preparation, the dihydropteridine, ATP, and Mg++. Analysis of the incubated reaction mixture by means of paper chromatography followed by radioautography indicated that a radioactive product has been formed ([4]). The formation of this product was shown to be dependent on the presence in the reaction mixture of the dihydropteridine, ATP and Mg++. This provides strong support for the view that sulfa drugs are able to replace p-AB as substrate for the enzyme. Thus the observed competitive inhibition can properly be described as a case of competition of sulfonamides with p-AB as substrates for the enzyme. Once the sulfa has competed successfully and becomes bound to the enzyme, it can then react in the way that p-AB reacts to yield a product.

Resistance to sulfonamides. Pato and Brown ([11]) have isolated a number of mutants of *E. coli* that will grow in the presence of a higher concentration of sulfonamide than the parent wild-type strain will tolerate. An examination of these mutants revealed that they can be divided into two classes. The enzyme system extracted from cells from one group of these mutants was identical with that obtained from the parent strain as regards sensitivity to sulfonamides and the Michaelis constant for p-AB. These observations suggest that these mutants are less sensitive to the growth-inhibitory properties of sulfonamides by being relatively impermeable to the drugs. The second class of mutants contained an enzyme system with a Michaelis constant for p-AB identical to that of the enzyme from the wild-type strain, but was less sensitive to the inhibitory properties of the sulfonamides. Thus, these mutants appeared to contain enzymes altered to the extent that the combination of the enzyme with sulfonamide is affected. One such mutant from this group contained an enzyme so altered that it was not stable to heating at 46° C, although the enzyme from the wild-type strain was completely stable to this treatment.

Since these mutants were all isolated by the multistep technic, it is likely that the mutants that were examined contained more than mutation and that the observed phenotypic characteristics were therefore the result of more than one mutational event. The chances seem good, for example, that those mutants that were observed to contain altered enzymes as a result of a mutation or mutations probably also might have super-imposed on these mutations others that might cause these organisms to be relatively impermeable to sulfonamides.

Wolf and Hotchkiss ([21]) have analyzed a number of mutants of pneumococci that are resistant to sulfanilamide and have reported that the enzymes from these mutants have a lower affinity for the drug than the enzyme extracted from the wild-type sensitive strain. They could find no evidence that altered permeability played any part in imparting resistance to pneumococci.

Other possible mechanisms that could theoretically be responsible for resistance to sulfonamides are: over-production by the resistant cells of p-AB or over-production of the p-AB-utilizing enzyme. The only report that has appeared that supports either of these alternatives in one by Landy *et al.* ([9]) in which they claim that strains of *Staphylococcus aureus* resistant to sulfonamides over-produce p-AB.

SUMMARY

The enzymatic reactions that lead to the biosynthesis of tetrahydrofolic acid are as follows:

(1) 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine + p-AB $\xrightarrow{\text{ATP}}$ dihydropteroic acid

(2) dihydropteroic acid + glutamic acid $\xrightarrow{\text{ATP}}$ dihydrofolic acid

(3) dihydrofolic acid $\xrightarrow{\text{TPNH}}$ tetrahydrofolic acid.

Two enzymes are involved in the formation of dihydropteroic acid (reaction 1). These two have been separated by chromatographic technic and it has been found that one of these enzymes (Enzyme A) catalyzes the formation of an intermediate from the pteridine substrate and ATP. The second enzyme (Enzyme B) catalyzes the conversion of this intermediate and p-AB to dihydropteroic acid. The enzymatic formation of dihydropteroic acid from p-AB and the pteridine intermediate is inhibited by sulfonamides. Of a series of sulfonamides tested, sulfathiazole was the most effective inhibitor and sulfanilamide the least effective. Experimental evidence has been obtained that the enzyme actually uses the sulfonamides as substrates with the formation of sulfonamide-containing products that would be analogs of dihydropteroic acid. Thus, the inhibitory properties of sulfonamides can be ascribed to these compounds competing with p-AB as substrates for the enzyme. This differs from the traditional view that sulfonamides are classic competitive inhibitors of the utilization of p-AB.

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DISCUSSION

Dr. Prabhakaran. In studies of this type it is common practice to do Lineweaver-Burke plots of the results. Your data did not show any. Have you done so?

Dr. Brown. We have done so on the effect of the inhibitors on the initial velocity of the enzyme.

Dr. Prabhakaran. The pathways of biosynthesis of reduced forms of folic acid have been well worked out in other microorganisms and in animal tissues. Do you find anything new or different in *E. coli*?

Dr. Brown. The pathways were the same.

Dr. Morrison. I wonder if you have any kinetic information on the Michaelis constant for p-AB as compared with sulfathiazole when the latter functions as a substrate for Enzyme B, the condensing enzyme.

Dr. Brown. The K_s for p-AB has been determined to be approximately 2.0×10^{-5}M. No exact value for the K_s for sulfathiazole has been determined, although we do know that the latter compound is utilized more effectively than p-AB as substrate.

Dr. Bushby. Dr. Brown has shown us the stage at which sulfonamides act in the synthesis of the co-enzyme, tetrahydrofolic acid, and we shall later refer to the potentiating effect of pyrimethamine and trimethoprim on the antibacterial and antimalarial activity of dapsone. I should like to take this opportunity to point out the stage in this synthesis in which pyrimethamine and trimethoprim act. They interfere with the conversion of dihydrofolate to its tetrahydro form through combining with tetrahydrofolate reductase. The potentiation is therefore due to the sulfonamides and these pyrimidines acting in the same biochemical pathway but at different stages.

Dr. Prabhakaran. Is it not true that the sulfonamides differ from the sulfones in that they do not cause anemia and are not effective in leprosy?

Dr. Brown. I would have to ask Dr. Shepard to answer that question.

Dr. Shepard. Initially the impression was gained that DDS was much more toxic than the sulfonamides with respect to the production of methemoglobinemia and anemia. Later it was learned that the difference lay in the much slower excretion of DDS. As a consequence, when DDS was given in the same daily dosage as sulfonamides a very high blood level was built up, with the production of severe hemolytic reactions. However, when the dosage was decreased to one that produced the same blood levels of drug, DDS was found to be no more hemolytic than sulfonamides.

Dr. Rees. What experience have you had on the emergence of resistance to DDS, and other sulfonamides, to strains of bacteria other than mycobacteria? What was the pattern of resistance, and, in particular, were there examples of "single-step mutants"?

Dr. Brown. We have done no work with DDS as an inhibitor. The mutants that we have examined that are resistant to sulfonamides have all been derived from *E. coli*, and were obtained as multi-step mutants. The resistance patterns that we have observed are described in the text.