# The Enzymatic Synthesis of Dihydrofolate and Dihydropteroate in Cell-Free Preparations from Wild-Type and Sulfonamide-Resistant Pneumococcus*

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ABSTRACT: In the presence of adenosine triphosphate and $Mg^{2+}$, cell-free enzyme preparations of wild-type pneumococcus couple 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and *p*-aminobenzoate to form dihydropteroate. In the absence of adenosine triphosphate, 2-amino-4-hydroxy-6-pteridinylmethyl pyrophosphate in its dihydro form serves as substrate; the corresponding monophosphorylated pteridine is not utilized.

Dihydrofolate is the product formed when *p*-aminobenzoylglutamate replaces *p*-aminobenzoic acid. Compared to enzyme preparations derived from the wild-type strain, those of a sulfanilamide resistant mutant, F*d*, display a markedly reduced capacity for the utilization of *p*-aminobenzoylglutamate and a slightly higher $K_m$ for this substrate. Sulfanilamide competitively inhibits the synthesis of both dihydropteroate and dihydrofolate; the $K_i$ values for the mutant enzyme preparations are significantly higher than those for the wild-type preparations. These findings are consistent with the hypothesis that the genetic alteration, phenotypically expressed as sulfanilamide resistance, has produced characteristic differences in the enzyme which catalyzes the condensation of 2-amino-4-hydroxy-6-dihydropteridinylmethyl pyrophosphate with *p*-aminobenzoic acid or *p*-aminobenzoylglutamate.

Cell-free extracts of several different microorganisms catalyze the condensation of 2-amino-4-hydroxy-6-dihydropteridinylmethyl pyrophosphate[1] with *p*-aminobenzoate (PAB) or *p*-aminobenzoylglutamate (PABG) to form dihydropteroate and dihydrofolate, respectively (Shiota *et al.*, 1964; Weisman and Brown, 1964). With partially purified preparations of *Veillonella*, Shiota *et al.* (1964) have demonstrated the following reactions

$$\text{hydroxymethyldihydropteridine-PP} + \text{PAB} \longrightarrow \text{dihydropteroate} + PP_i \quad (1)$$

$$\text{hydroxymethyldihydropteridine-PP} + \text{PABG} \longrightarrow \text{dihydrofolate} + PP_i \quad (2)$$

The participation of a phosphorylated pteridine in the synthesis of folate-like compounds had been suggested earlier by the observation that ATP is required for synthesis by bacterial extracts when hydroxymethyldihydropteridine is the cosubstrate with PAB or PABG (Bocchieri and Koft, 1965; Brown *et al.*, 1961; Shiota and Disraely, 1961; Jaenicke and Chan, 1960). The last-mentioned workers further concluded that for yeast a pyrophosphorylated pteridine was the active intermediate.

Wolf and Hotchkiss (1963) have described the synthesis of folic acid compounds by cell-free extracts of wild-type pneumococcus and by preparations of several sulfanilamide-resistant mutant strains. The synthesis, dependent upon endogenous pteridine precursor and an exogenous supply of PAB or PABG, was subject to competitive inhibition by specific PAB analogs and exhibited resistance patterns characteristic of the mutant strain used. Analysis of the kinetics of synthesis by some of the mutant strains indicated that they have characteristic $K_m$ and $V_{max}$ values for PAB and widely varying $K_i$ values for the various inhibitors. These and earlier experiments with whole cells (Hotchkiss and Evans, 1960) suggest that these differences are attributable to genetically controlled modifications of the binding groups of the enzyme system which utilizes PAB for the synthesis of folic acid compounds.

The present study is concerned with further characterization of the specific enzyme present in extracts of pneumococcus which catalyzes the synthesis of folic acid compounds. The properties of the *in vitro* synthesis are compared for enzyme preparations derived from the wild-type and those derived from the sulfonamide-resistant mutant strain F*d*, with particular emphasis on the substrate and cofactor requirements for maxi-

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[1] The abbreviations used are: ATP, adenosine triphosphate; PAB, *p*-aminobenzoic acid; PABG, *p*-aminobenzoylglutamate; hydroxymethylpteridine and hydroxymethyldihydropteridine for 2-amino-4-hydroxy-6-hydroxymethylpteridine and its dihydro form; hydroxymethyldihydropteridine-P, -PP, and -PPP for 2-amino-4-hydroxy-6-dihydropteridinylmethyl monophosphate, pyrophosphate, and triphosphate, respectively.

mum synthesis. The response of the reactions catalyzed by wild-type and mutant enzyme preparations to sulfanilamide and *p*-nitrobenzoate has also been examined.

## Materials and Methods

*Materials.* ATP was purchased from Pabst Laboratories, PAB from Eastman Kodak Co., PABG from Mann Research Laboratories, crystalline folic acid from Nutritional Biochemicals, and alkaline phosphatase (*E. coli*) from Worthington Biochemical Co. 2-Amino-4-hydroxy-6-hydroxymethylpteridine was prepared as described by Waller *et al.* (1950), recrystallized several times, and reduced to the dihydro form by the method of Futterman (1957). Phosphate esters of 2-amino-4-hydroxy-6-hydroxymethylpteridine were chemically prepared, chromatographically separated on DEAE-cellulose columns, and reduced to the dihydro level with sodium borohydride according to the method of Shiota *et al.* (1964). The total phosphorus content of the products was determined by measuring the phosphate released after treatment with alkaline phosphatase. For this assay, 0.05–0.10 μmole of phosphorylated pteridine was incubated for 60 min at 37° with 0.05 mg of alkaline phosphatase in a total volume of 1 ml of 0.10 M Tris buffer, pH 8.0. Phosphate was measured according to the method of Chen *et al.* (1956). The ultraviolet absorption spectrum was utilized to confirm the reduction; the concentration of the reduced compounds was derived from the absorbance in 0.1 N NaOH ($E_{253m\mu}$ 23,500) (Shiota *et al.*, 1964).

*Strains of Pneumococcus.* Two strains of pneumococcus were used for the preparation of cell-free extracts: R6, the wild type, which will not grow (in neopeptone broth) in the presence of sulfanilamide concentrations greater than 5 μg/ml, and RF6-7 (or simply, F*d*), a mutant obtained *via* transformation (Hotchkiss and Evans, 1958), which is resistant to about 80 μg of sulfanilamide/ml.

*Preparation of Cell-Free Extracts.* Pneumococci were grown, harvested, and washed as previously described (Wolf and Hotchkiss, 1963), and acetone powders of the bacterial cells were prepared by the general procedure outlined by Gunsalus (1955). When stored at −10°, the powders retained their activity for several months.

The preparation of extracts and all subsequent treatments were conducted at 3°. For extraction, about 10 mg of powder was suspended, with frequent stirring, for 60 min in 1 ml of 0.01 M potassium phosphate buffer, pH 8.0, containing 0.01 M sodium thiomalate. The supernatant fluid obtained after centrifugation for 10 min at 10,000 rpm was used directly for ammonium sulfate fractionation; in all other cases it was adsorbed for 10 min with acid-washed Norit A (2 mg/mg of protein). The Norit was removed by centrifugation and the supernatant fluid, where indicated, was used as enzyme source.

*Ammonium Sulfate Precipitation.* Extracts, prepared as described above and containing 2 mg of protein/ml, were brought to 55% saturation with solid ammonium sulfate. After stirring for 10 min at 3° the precipitate was collected by centrifugation, and the pellet was dissolved in Tris buffer, 0.1 M, pH 8.6. Before use the fraction was dialyzed overnight against $10^{-3}$ M Tris, pH 8.6. The dialyzed preparations contained about 80% of the total protein and 85% of the total enzyme activity, assayed with either PAB or PABG as substrate. Stored at 3°, the enzyme preparations maintained full activity for about 1 week. Extracts of both the wild type and the mutant F*d* strains responded similarly to this fractionation treatment with ammonium sulfate, and the distribution of activity and protein was identical for both.

*Microbiological Assay.* Enzyme activity was measured as the growth response of *Streptococcus faecalis* (ATCC 8043) to the reaction products. After dilution of reaction mixtures to halt the enzymic reaction, samples for assay were withdrawn and added to Difco folic acid assay medium containing 0.5 mg of sodium ascorbate/ml. Growth of the organism was assayed as turbidity measurements with a Coleman Model 9 nephelometer. Folic acid standards were used in each assay; all results are reported as folate equivalents and represent the mean values obtained from duplicate or triplicate analyses. Although neither dihydropteroate nor dihydrofolate are as active for *S. faecalis* as folate (Brown *et al.*, 1961; Shiota *et al.*, 1964), no factors were applied to the values reported here to correct for this discrepancy. Protein was determined by the method of Oyama and Eagle (1956) using crystalline bovine plasma albumin as standard.

*Bioautography.* The method used was essentially that

TABLE I: Requirements for Dihydropteroate Synthesis.[a]

| Additions | | Folate Equivalents (mμg/mg protein/hr) |
|---|---|---|
| Complete | | 19.0 |
| Complete | −$MgCl_2$ | 1.9 |
| Complete | −ATP | 1.9 |
| Complete | −Hydroxymethyl-dihydropteridine | 0.5 |
| Complete | −PAB | 2.0 |
| Complete | −Enzyme | 0.9 |

[a] The complete reaction mixture (1.0 ml) contained: 1 μmole of $MgCl_2$, 2 μmoles of ATP, 1.5 μmoles of PAB, 44 mμmoles of hydroxymethyldihydropteridine, 60 μmoles of Tris, pH 8.0, and Norit-adsorbed extract of wild-type pneumococcus containing 0.21 mg of protein. After incubation for 60 min at 37° under nitrogen gas the reaction mixtures were diluted, and the amount of product formed was determined by microbiological assay.

TABLE II: Phosphorylated Hydroxymethyldihydropteridines as Substrates for the Synthesis of Dihydropteroate.[a]

| Substrate | Folate Equivalents (mμg/mg protein/hr) |
|---|---|
| Hydroxymethyldihydropteridine | 2–5 |
| Hydroxymethyldihydropteridine + ATP | 20–50 |
| Hydroxymethyldihydropteridine-P | <3 |
| Hydroxymethyldihydropteridine-P + ATP | <3 |
| Hydroxymethyldihydropteridine-PP | 675 |
| Hydroxymethyldihydropteridine-PP + ATP | 640 |
| Hydroxymethyldihydropteridine-PP (oxidized) | <2 |
| Hydroxymethyldihydropteridine-PPP | 199 |
| Hydroxymethyldihydropteridine-PPP + ATP | 192 |

[a] Reaction mixtures (0.50 ml) contained: 0.5 μmole of $MgCl_2$, 25 μmoles of Tris, pH 8.0, 0.5 μmole of PAB, 20 mμmoles of hydroxymethyldihydropteridine or 10 mμmoles of the respective phosphorylated pteridines, 0.17 mg of Norit-adsorbed extract protein, and where indicated 1 μmole of ATP. Other conditions were as described in Table I.

described by Brown (1962), with the exception that no ascorbate was incorporated into the buffer used as solvent for chromatography, and the chromatography paper was not washed with ascorbate before use. The solvent was 0.1 M potassium phosphate buffer, pH 7.0, and the test organism for bioautography was *S. faecalis* (8043).

## Results

Table I illustrates the requirements for synthesis of dihydropteroate by extracts of wild-type pneumococcus which have been treated with Norit to remove endogenous substrates. With the complete system, linear rates of synthesis are observed for about 60 min, in contrast to the short-lived synthesis observed with extracts not supplemented with pteridine (Wolf and Hotchkiss, 1963). Incubation of reaction mixtures in air results both in lower rates of synthesis and earlier curtailment of linearity. The addition of reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide or 2-mercaptoethanol to reaction mixtures did not alter the rates of synthesis in air or in a nitrogen atmosphere. The same requirements for maximal synthesis are observed when PAB is replaced by PABG as cosubstrate.

The demonstration of an ATP requirement for synthesis when hydroxymethyldihydropteridine is cosubstrate with PAB and PABG suggested the participation of a phosphorylated intermediate. Phosphate esters of hydroxymethylpteridine were chemically synthesized, and separated according to the method of Shiota *et al.* (1961, 1964). The phosphate:pteridine ratios of the first three column peaks were 1.1, indicating the monophosphorylated compound, 2.3 for the pyrophosphorylated derivative, and 2.7 for the triphosphorylated compound. Following reduction to the dihydro form, the ability of these compounds to function as substrate was measured. With the monophosphate ester, no synthesis occurs in the absence or presence of ATP (Table II). However, with the pyrophosphorylated pteridine maximal rates of synthesis occur which are independent of the presence of ATP and linear for about 60 min. The marked increase in rates of synthesis observed when the pyrophosphorylated ester of hydroxymethyldihydropteridine replaces the nonphosphorylated pteridine as substrate may reflect the limited capacity of the extracts to produce the phosphorylated intermediate enzymatically, under the conditions employed.

The limited activity observed with the triphosphorylated compound probably results from phosphatase activity in the extracts employed. No reaction occurs if the pyrophosphorylated pteridine is not reduced to the dihydro level.

*Products of the Reaction.* Since synthesis occurs when either PAB or PABG are cosubstrate with the pyrophosphate ester of hydroxymethyldihydropteridine, the identification of the products of the reaction with each of these substrates was undertaken. From the schematic representation of a typical bioautogram (Figure 1) it is seen that, when PAB is substrate, one area of growth is observed, which corresponds to the pteroic acid marker. With PABG as substrate, two areas are visible: the major one corresponding in $R_F$ to folic acid, and a minor zone of growth, corresponding in $R_F$ to the pteroic acid marker. It is not known whether the pteroic acid formed when PABG is substrate represents some cleavage of glutamate from PABG during the incubation or whether small amounts of PAB contaminate the PABG preparations used. Because no precautions were taken against oxidation, the reduced forms of the products were not observed. The control containing all the components except the enzyme showed no activity.

*Properties of the Reactions Catalyzed by Ammonium Sulfate Fractions of Wild-Type and Mutant Enzymes.*

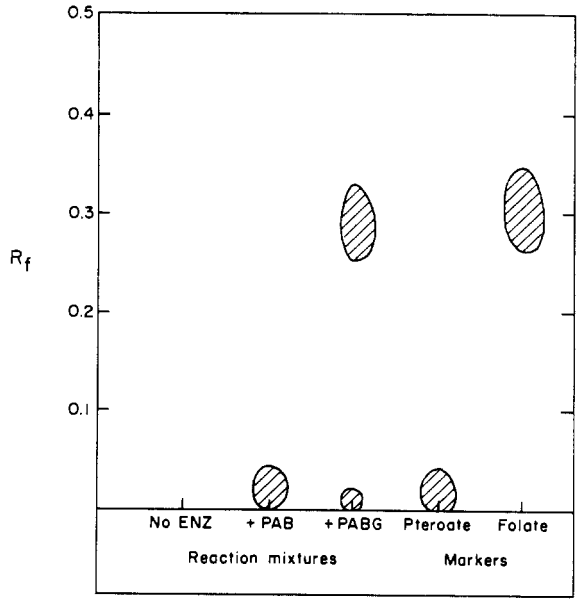


FIGURE 1: Bioautogram of reaction products. Reaction mixtures were identical with those described for Table I except that they contained 40 $\mu$g of an ammonium sulfate fraction of wild-type extract. In one case PABG (2.14 $\mu$moles) replaced PAB. After incubation at 37° for 2 hr samples were removed for microbiological assay as described for Table I. Samples of 0.04 ml, containing 2.6 $\mu$g of dihydropteroate and 5.6 $\mu$g of dihydrofolate, and reference samples (5.0 $\mu$g each) were applied to Whatman No. 1 paper. After ascending chromatography for 3 hr at 29° the chromatogram was dried and placed in contact for 10 min with a plate of solidified Difco folic acid assay medium seeded with *S. faecalis* (8043). After incubation of the plate overnight at 37° the zones of growth indicated were observed.

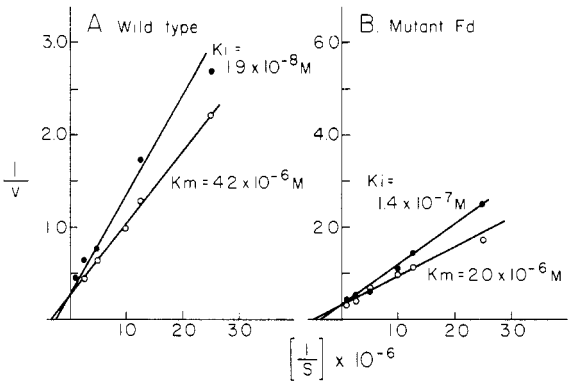


FIGURE 2: Effect of PAB and sulfanilamide on dihydropteroate synthesis. Experimental conditions were as described for Table III. PAB concentration was varied between 0.1 and 10.0 $\times$ $10^{-6}$ M. The concentration of sulfanilamide was 0.5 $\times$ $10^{-8}$ M for the wild-type extract and 5.0 $\times$ $10^{-8}$ M for the F*d* extract. Velocity is given in $\mu$g of product formed/mg of protein per hour. Each reaction mixture contained ammonium sulfate enzyme fraction equivalent to 120 $\mu$g of protein.

TABLE III: Dihydropteroate and Dihydrofolate Synthesis in the Presence of $PP_i$ and $P_i$.[a]

| Additions | Folate Equivalents ($\mu$g/mg protein/hr) Dihydropteroate | Dihydrofolate |
|---|---|---|
| None | 4.9 | 2.4 |
| $PP_i$: 4 $\times$ $10^{-5}$ M | 2.4 | 1.2 |
| 2 $\times$ $10^{-4}$ M | <0.5 | <0.3 |
| $P_i$: 2 $\times$ $10^{-4}$ M | 4.4 | 2.4 |
| 2 $\times$ $10^{-3}$ M | 3.9 | 1.9 |

[a] Reaction mixtures (0.50 ml) contained: 100 $\mu$moles of Tris, pH 8.6, 5 m$\mu$moles of PAB or 50 m$\mu$moles of PABG, 1 mg of sodium ascorbate, pH 6.0, 50 m$\mu$moles of hydroxymethyldihydropteridine-PP, and ammonium sulfate enzyme fraction (wild-type) equivalent to 20 $\mu$g of protein. The values for $P_i$ and $PP_i$ are final concentrations. Other conditions were as described in Table I.

Dialyzed ammonium sulfate fractions (see Methods) were used in all subsequent experiments because they provide more stable enzyme preparations and give lower blanks. Rates of synthesis of both dihydrofolate and dihydropteroate are optimal for both enzyme preparations between pH 8.5 and 8.7 (Tris buffers). At pH 8.4 and 8.8, respectively, 40 and 60% of the maximum rates are observed.

Essentially complete inhibition of the synthesis of both dihydrofolate and dihydropteroate by wild-type and mutant enzyme preparations is observed when $PP_i$, at final concentrations of 2 $\times$ $10^{-4}$ M or higher, is added (Table III). The presence of $P_i$ at equivalent concentrations is without effect, and only about 20% inhibition of synthesis of both products is observed at a final $P_i$ concentration of 2 $\times$ $10^{-3}$ M.

While the synthesis of both dihydropteroate and dihydrofolate by wild-type preparations is inhibited by $MgCl_2$ and $MnCl_2$ (2 $\times$ $10^{-3}$ M), both these compounds stimulate the synthesis of dihydropteroate and dihydrofolate catalyzed by the mutant enzyme preparations (Table IV). The addition of $CoCl_2$ is inhibitory to both enzymes, while $NH_4Cl$ and KCl are without effect on either enzyme preparation.

The mean $K_m$ value at 37° for PAB, obtained from the reciprocal plots of the velocity *vs.* substrate concentration (Lineweaver and Burk, 1934), for the wild-type enzyme is 2.7 $\times$ $10^{-6}$ M, with a range of 1.1–4.2 $\times$ $10^{-6}$ M (Figure 2A). For the F*d* mutant enzyme, a mean $K_m$ value for PAB of 1.4 $\times$ $10^{-6}$ M was obtained, with a range of 0.8–2.0 $\times$ $10^{-6}$ M (Figure 2B).

The $K_m$ for PABG for the wild-type enzyme was

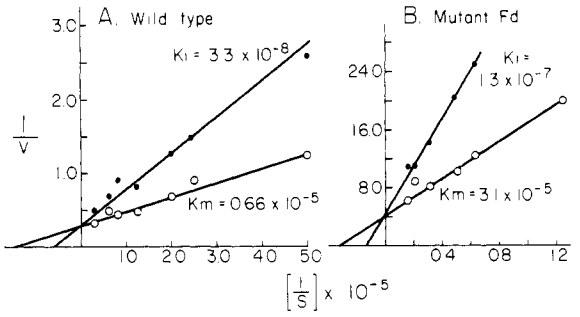


FIGURE 3: Effect of PABG and sulfanilamide on dihydrofolate synthesis. Experimental conditions were as described for Table III. PABG concentrations were varied between 3.2 and 10.0 $\times$ $10^{-5}$ M for the wild-type extract and between 0.8 and 10.0 $\times$ $10^{-5}$ M for the mutant extract. The concentration of sulfanilamide was 5.0 $\times$ $10^{-8}$ M for (A) and 2.0 $\times$ $10^{-7}$ M for (B). Reaction mixtures contained ammonium sulfate enzyme fractions equivalent to 100 $\mu$g of protein.

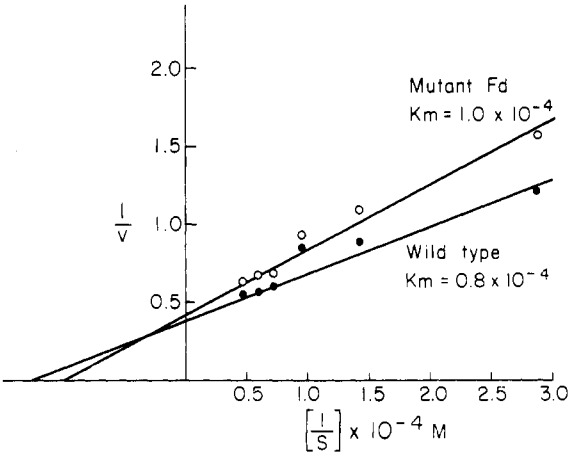


FIGURE 4: Effect of concentration of hydroxymethyldihydropteridine-PP on the synthesis of dihydropteroate. Reaction mixtures were as described for Table III. Each contained ammonium sulfate enzyme fraction equivalent to 75 $\mu$g of protein. Hydroxymethyldihydropteridine-PP concentrations varied from 0.4 to 2.1 $\times$ $10^{-4}$ M. Other conditions were as described for Table I.

TABLE IV: Effect of Metals on Dihydrofolate and Dihydropteroate Synthesis.[a]

| Enzyme | Salt Added | Folate Equivalents ($\mu$g/mg protein/hr) Dihydropterotate | Dihydrofolate |
|---|---|---|---|
| Wild type | None | 3.80 | 2.24 |
| | $MgCl_2$ | 2.80 | 1.92 |
| | $MnCl_2$ | 1.88 | 1.20 |
| | $CoCl_2$ | 0.94 | *b* |
| Mutant F*d* | None | 0.49 | 0.16 |
| | $MgCl_2$ | 1.65 | 0.46 |
| | $MnCl_2$ | 0.79 | 0.25 |
| | $CoCl_2$ | <0.20 | <0.08 |

[a] Reaction mixtures were as described for Table III. For the experiment with wild-type enzyme ammonium sulfate fraction equivalent to 25 $\mu$g of protein was used; for the experiments with mutant enzyme 27 $\mu$g of protein and 10 m$\mu$moles of PAB or 150 m$\mu$moles of PABG were used. Final concentration of metal salts was 2 $\times$ $10^{-3}$ M. Other conditions were as described for Table I. [b] Not tested.

found to range between 0.7 and 2.3 $\times$ $10^{-5}$ M (mean = 1.3 $\times$ $10^{-5}$ M), while that for the mutant enzyme was significantly higher, ranging from 3.0 to 9.4 $\times$ $10^{-5}$ M, with a mean of 6.2 $\times$ $10^{-5}$ M (Figure 3). Each of the $K_m$ values reported above was obtained from three separate experiments.

Sulfanilamide competitively inhibits the synthesis of dihydrofolate and dihydropteroate. The $K_i$ for the synthesis of the latter compound by the wild-type enzyme preparation was 1.9 $\times$ $10^{-8}$ M (Figure 2A), and for the former the $K_i$ was found to be about 3.3 $\times$ $10^{-8}$ M (Figure 3A). For the mutant enzyme the $K_i$ for synthesis of dihydropteroate was 1.4 $\times$ $10^{-7}$ M (Figure 2B), while that for dihydrofolate synthesis was essentially the same, 1.3 $\times$ $10^{-7}$ M (Figure 3B).

The $K_m$ value for hydroxymethyldihydropteridine-PP for both the wild-type and the mutant enzyme preparations is about 1.0 $\times$ $10^{-4}$ M, measured in the presence of PAB (Figure 4).

Although earlier observations indicated that the synthesis of dihydropteroate catalyzed by crude extracts of the mutant F*d* is more heat sensitive than the reaction catalyzed by wild-type extracts (Wolf and Hotchkiss, 1963), under the experimental conditions presently employed this differential heat sensitivity is not observed. This is the case both when the pteridine supplied as cosubstrate for the reaction is hydroxymethyldihydropteridine-PP (Figure 5) and when hydroxymethyldihydropteridine (in the presence of ATP and $Mg^{2+}$) is provided (Figure 6).

The apparent heat sensitivity previously observed may have been the consequence of limiting PAB concentration. The concentrations of PAB employed in the present experiments are in excess of the $K_m$ value determined at 37°, while considerably lower concentrations, based on $K_m$ determinations obtained at 0°, were used in the earlier experiments. This explanation was offered by Wolf and Hotchkiss (1963) and supported by their preliminary finding that some synthesis did occur at 50° with F*d* extracts when the PAB concentration was increased.

In addition, and in confirmation of the previous findings, no differences between the heat sensitivities of

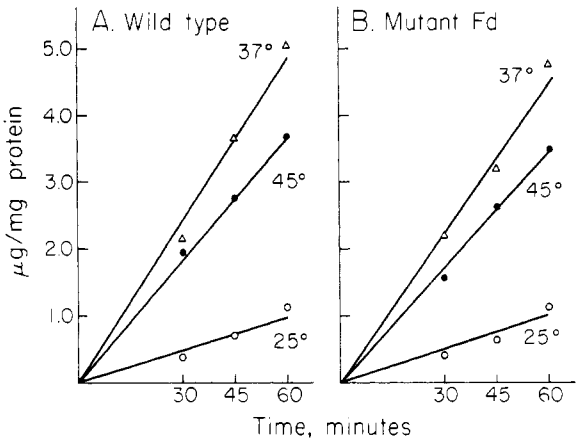


FIGURE 5: The synthesis of dihydropteroate at different temperatures with hydroxymethyldihydropteridine-PP as substrate. Reaction mixtures were as described for Table III. Ammonium sulfate enzyme fraction was used in the amounts of protein (A) 28 μg (wild type) and (B) 25 μg (mutant F*d*), and 1 μmole $MgCl_2$ was included in the mutant reaction mixtures. After addition of all of the components of the reaction the solutions were divided into approximately three equal portions, one incubated at each temperature indicated. Samples were withdrawn at indicated time intervals, diluted, and assayed.

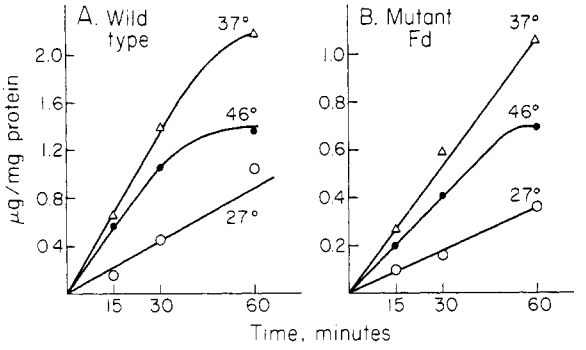


FIGURE 6: The synthesis of dihydropteroate at different temperatures with hydroxymethyldihydropteridine as substrate. Reaction mixtures (0.50 ml) contained: 100 μmoles of Tris, pH 8.6, 1 μmole of ATP, 81 mμmoles of hydroxymethyldihydropteridine, 1 μmole of $MgCl_2$, 1 mg of sodium ascorbate, pH 6.0, 5 mμmoles of PAB and (A) 80 μg of protein (wild type) or (B) 65 μg of protein (mutant F*d*). Other conditions were as described for Figure 5.

the wild-type and mutant enzyme preparations are observed when they are heated to about 46°, then assayed for enzymatic activity at 37°.

Because the inhibition of the growth of pneumococci by *p*-nitrobenzoate is reversed competitively by PAB (Hotchkiss and Evans, 1960), it was of interest to determine the effect of this PAB analog on the *in vitro* synthesis of dihydropteroate and dihydrofolate. For greater convenience in testing high concentrations of *p*-nitrobenzoate relative to PAB and PABG concentrations, the levels of PAB or PABG were reduced in these experiments below $K_m$ concentrations. Under these conditions, no inhibition of synthesis was observed even at concentrations of *p*-nitrobenzoate several thousand times greater than that of the PAB or PABG employed (Table V).

Although the growth of the mutant F*d* and all strains containing this genetic subunit displays considerably greater sensitivity to *p*-nitrobenzoate than do wild-type strains (Hotchkiss and Evans, 1960), the synthesis of dihydropteroate or dihydrofolate catalyzed by extracts of the mutant F*d* is equally as resistant to inhibition by *p*-nitrobenzoate as that catalyzed by extracts prepared from wild-type strains.

Conversion of *p*-nitrobenzoate to *p*-aminobenzoate did not occur under the conditions employed; no synthesis was observed with either enzyme preparation when PAB and PABG were absent from reaction mixtures containing the highest *p*-nitrobenzoate concentrations tested.

TABLE V: Dihydropteroate and Dihydrofolate Synthesis in the Presence of *p*-Nitrobenzoate.[a]

| *p*-Nitrobenzoate (μg/ml) | Folate Equivalents (μg/mg protein/hr) | | | |
|---|---|---|---|---|
| | Wild type | | Mutant F*d* | |
| | with PAB | with PABG | with PAB | with PABG |
| None | 3.83 | 0.40 | 1.57 | 0.10 |
| 167 | 3.71 | 0.45 | 1.52 | 0.09 |
| 334 | 3.87 | 0.45 | 1.52 | 0.11 |
| 835 | 3.55 | 0.37 | 1.63 | 0.10 |
| 1970 | 3.71[b] | | 1.54[b] | |
| 6680 | | 0.33[c] | | 0.08[d] |

[a] Reaction mixtures (0.50 ml) were as for Table III, except: for experiments with PAB they contained ammonium sulfate enzyme preparations equivalent to 26 μg of protein and 0.50 mμmole of PAB. For PABG experiments they contained 2.5 mμmoles of PABG and ammonium sulfate enzyme preparations equivalent to 65 μg of protein (wild-type enzyme preparations) or 4.0 mμmoles of PABG and 60 μg of protein (mutant enzyme). Reaction mixtures with mutant enzyme preparations also contained 1 μmole of $MgCl_2$. Other conditions were as described in Table I. [b] Molar ratio NOB/PAB = 14,380. [c] NOB/PABG = 5172. [d] NOB/PABG = 3140.

## Discussion

As with other bacterial systems studied, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine is required as substrate for the synthesis of dihydropteroate and

dihydrofolate by cell-free preparations of pneumococcus. A prerequisite for the condensation with PAB or PABG is the phosphorylation of this pteridine, presumably through the action of a pyrophosphokinase, resulting in the formation of hydroxymethyldihydropteridine pyrophosphate. Although direct evidence for this enzymatic conversion has not been presented, the requirement for ATP when nonphosphorylated pteridine is provided as substrate and the preferential utilization of the chemically synthesized pyrophosphorylated derivative in the absence of ATP suggest that this is the case. The pyrophosphate ester of the pteridine is apparently not formed from the monophosphate, for no utilization of the monophosphorylated pteridine was observed in the absence or presence of ATP. This is in contrast to the results of Jaenicke and Chan (1960) which indicated the utilization of the monophosphorylated pteridine in the presence of ATP.

The products of synthesis by extracts of wild-type pneumococcus were shown to be pteroic acid when PAB is substrate and mainly folic acid when PABG is substrate. Since the reduced form of hydroxymethylpteridine pyrophosphate has been shown to be required for synthesis, and because the products may have become oxidized during the bioautographic assay, it is postulated that dihydropteroate and dihydrofolate are the primary products of the reaction.

Although the release of $PP_i$ concomitant with the condensation of either PAB or PABG with hydroxymethyldihydropteridine-PP has not been demonstrated, the inhibitory effect of $PP_i$ and the absence of inhibition by $P_i$ suggest that $PP_i$ is a product of the reaction. The stoichiometric release of $PP_i$ has been directly demonstrated for the *in vitro* synthesis of dihydrofolate by partially purified preparations of *Veillonella* (Shiota *et al.*, 1964).

Although the synthesis of dihydropteroate and dihydrofolate may be the result of the activity of two separate enzymes, one which utilizes PABG as substrate, the other, PAB, no conclusive evidence concerning this question has resulted from the present studies. It should be noted, however, that the $K_i$ for sulfanilamide with respect to both activities is increased severalfold in the mutant F*d*.

Enzyme preparations derived from the sulfonamide-resistant mutant strain F*d* exhibit several distinctive properties. Most significant are an approximately 12-fold lower maximal rate of PABG utilization, the somewhat higher $K_m$ for this substrate, and the relatively reduced binding capacity for sulfanilamide. The correlation between the resistance of this strain to sulfanilamide and the efficiency with which enzyme preparations derived from it bind the drug is obvious. Less apparent is the relationship between drug resistance and PABG utilization. The importance of the reaction involving PABG as substrate to the major pathway of folate biosynthesis has been questioned; indeed, it has been suggested that dihydropteroate is an obligatory intermediate in the pathway for *E. coli* (Brown *et al.*, 1961). While no conclusive evidence is available relative to this question, the present data indicate that the major differences in behavior of enzyme preparations derived from the mutant and those of the wild-type strain particularly affect PABG and not PAB utilization. It is also pertinent that, in experiments with growing cultures, those of the F*d* strain produced and accumulated smaller amounts of folic acid than did parallel wild-type cultures (Hotchkiss and Evans, 1960). These observations suggest that the condensation of PABG with hydroxymethyldihydropteridine-PP may be the biosynthetically significant reaction responsible for dihydrofolate synthesis in the living cell. The opposite effect of $Mg^{2+}$ and $Mn^{2+}$ salts in inhibiting the wild-type and stimulating the mutant enzyme reactions may be an indication of other interesting differences between the strains.

It is evident from the data presented that *p*-nitrobenzoate is not an inhibitor of the *in vitro* synthesis of dihydropteroate or dihydrofolate catalyzed by cell-free enzyme preparations of wild-type or mutant F*d* pneumococcal strains. It is possible that a preliminary conversion of *p*-nitrobenzoate to an effective inhibitor of this reaction occurs in whole cells, but not under the conditions of the present experiments. Alternatively, *p*-nitrobenzoate may exert an inhibitory action on another essential biosynthetic pathway. In support of this hypothesis are preliminary experiments which indicate that *p*-nitrobenzoate inhibits the growth of *S. faecalis* although this organism, which requires folate or pteroate for growth, lacks the enzyme which catalyzes the synthesis of these compounds from PAB.

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# The Biosynthesis of Hadacidin*

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ABSTRACT: Glycine, formate, and the β-carbon of serine were all found to be very rapidly incorporated into hadacidin during its synthesis by *Penicillium aurantio-violaceum*. Degradation of hadacidin showed that C-1 of glycine was found almost exclusively in the glycyl portion of hadacidin while formate, the β-carbon of serine, and the α-carbon of glycine were incorporated into both the glycyl and formyl portions of the hydroxamate. *N*-Hydroxyglycine was incorporated into hadacidin at a rate equal to that for glycine in 3-hr periods and to a much greater extent in longer time periods. *N*-Hydroxyglycine, but not glycine, brought about a net stimulation of hydroxamate production. Nitroacetic acid, glyoxylic acid oxime, and formylglycine were not rapidly incorporated into hadacidin. Experiments with $^{18}O$ showed that the hydroxylamino oxygen atom of hadacidin is derived from oxygen gas rather than water. The experimental results are consistent with the hypothesis that the biosynthesis of hadacidin occurs by *N*-oxygenation of glycine to yield *N*-hydroxyglycine followed by *N*-formylation to yield the hydroxamate.

Nitrogen at the oxidation state of −1 is found in biological systems as the hydroxylamino group of hydroxamic acids of fungal origin. The hydroxylamino group is frequently donated by a hydroxylamino acid (*N*-hydroxyamino acid), such as δ-*N*-hydroxyornithine found in the siderochromes (Keller-Schierlein *et al.*, 1964). Tracer studies using labeled amino acids have been carried out with aspergillic acid (MacDonald, 1961), mycelianamide (Birch and Smith, 1958), and ferrichrome (T. F. Emery, unpublished). In every case it was found that the carbon skeleton of the amino acid is incorporated into the hydroxylamino acid portion of the hydroxamic acid. Nothing is known, however, about the mechanism of formation of the hydroxamate group itself.

Hadacidin, or *N*-formyl-*N*-hydroxyglycine, is the simplest known naturally occurring hydroxamic acid. This compound, isolated and characterized by Kaczka *et al.* (1962), seemed well suited for a study of the route of hydroxamic acid biosynthesis. The hydroxamate bond may be considered to be a peptide bond with an oxygen atom on the amide nitrogen, but there is no *a priori* reason to decide whether the oxygen atom is introduced before or after the formation of the amide bond. In the latter case, formylglycine would be an intermediate in hadacidin biosynthesis. *N*-Hydroxylation of an amide bond was reported by Cramer *et al.* (1960), who found that *N*-hydroxy-2-acetylaminofluorene was formed in the intact rat upon administration of 2-acetylaminofluorene. Nevertheless, this finding cannot be considered direct proof of *N*-hydroxylation of an amide bond because, as the authors point out, the acetyl group is labile in their experiments, and hydroxylation of the amino group might have occurred.

Formation of the hydroxylamino group prior to amide bond formation, that is, at the level of the free amino acid, might occur by several routes. (A) DIRECT OXIDATION OF AN AMINO GROUP. No such reaction has been described in biological systems, although Baker and Chaykin (1960) observed a reduced triphosphopyridine nucleotide dependent oxidation of trimethylamine to trimethylamine *N*-oxide, and the *N*-oxide of *N,N*-dimethyltryptamine can be formed by mouse liver homogenates (Fish *et al.*, 1955). Oxidation of aniline to nitrosobenzene has also been reported (Böttcher and Kiese, 1960). However, hydroxylamino acids have not been found free even in hydroxamate synthesizing organisms.

(B) REDUCTION OF A NITRO GROUP. Nitro groups in compounds of biological origin are very rare. Enzymic reduction of an aromatic nitro group yields the amine (Zucker and Nason, 1955), although in one instance 4-hydroxylamino-2,6-dinitrotoluene was found as the product of enzymic reduction of trinitrotoluene (Bueding and Jolliffe, 1946). 1-Amino-5-nitropentane stimu-

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