Dihydrofolate and Dihydropteroate Synthesis by Partially Purified Enzymes from Wild-Type and Sulfonamide-Resistant Pneumococcus*

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ABSTRACT: The enzyme which catalyzes the condensation of 2-amino-4-hydroxy-6-dihydropteridinylmethyl pyrophosphate with *p*-aminobenzoate or *p*-aminobenzoylglutamate to form dihydropteroate and dihydrofolate, respectively, has been purified approximately 30-fold from wild-type pneumococcus and from two sulfonamide-resistant strains. In all cases the K_m values for *p*-aminobenzoylglutamate were higher than those for *p*-aminobenzoate, and the enzymes from drug-resistant strains displayed slightly higher K_m values for *p*-aminobenzoylglutamate than wild-type preparations. The K_m values for *p*-aminobenzoate for the wild-type and drug-resistant strains were essentially the same. The K_i values for sulfanilamide were identical with both substrates; enzymes from drug-resistant strains displayed a reduced binding capacity for the drug.

Both dihydrofolate and dihydropteroate synthesis was inhibited by *p*-hydroxybenzoate. Dihydrofolate synthesis showed greater sensitivity to inhibition by this compound than dihydropteroate synthesis. Enzyme activity is stimulated by Mn^{2+} and Mg^{2+}. Although in all instances a greater stimulatory effect was observed with Mn^{2+}, only 3–4-fold increases in activity were observed with enzymes from the wild-type strain. One drug-resistant strain responded similarly, however, the enzyme from the other drug-resistant strain showed a 10–13-fold increase in activity in the presence of Mn^{2+}. This strain also displayed a very low rate of dihydrofolate synthesis relative to both wild-type or the other drug-resistant strain examined. The estimated molecular weight of the partially purified enzyme preparation from the wild-type strain is 75,000–95,000.

The enzymatic condensation of DHP-PP[1] with *p*-aminobenzoate or *p*-aminobenzoylglutamate to form H_2-pteroate or H_2-folate, respectively, has been demonstrated in cell-free extracts derived from several different microorganisms (Shiota *et al.*, 1964; Richey and Brown, 1969).

Wolf and Hotchkiss (1963) described the synthesis of folic acid compounds by cell-free extracts of pneumococcus in a system dependent upon endogenous pteridine precursor and an exogenous supply of *p*-aminobenzoic acid or *p*-aminobenzoylglutamate. Competitive inhibition by specific *p*-aminobenzoic acid analogs was observed, and enzyme preparations from sulfonamide-resistant strains exhibited characteristic K_m and V_{max} values for *p*-aminobenzoic acid, and distinctive, widely varying K_i values for various inhibitors.

Further characterization of the pneumococcal enzymes derived from wild-type and sulfonamide-resistant strain F*d* demonstrated the requirement for DHP-PP as cosubstrate, and the identity of the products of the reaction with *p*-aminobenzoic acid or *p*-aminobenzoylglutamate as cosubstrate (Ortiz and Hotchkiss, 1966). Several specific differences in the properties of the enzyme systems derived from the drug-resistant strain and those prepared from the wild-type strain were also described.

The present study describes the partial purification of the enzyme from wild-type and two sulfonamide-resistant strains, F*d* and F*a*. The properties of the enzymic reaction catalyzed by these preparations and their response to sulfanilamide or *p*-hydroxybenzoate has also been examined.

Materials and Methods

Materials. DEAE-Sephadex A-50 (3.5 mequiv/g, particle size 40–120 mμ) was washed with 0.60 M NaCl in 0.01 M potassium phosphate buffer (pH 7.5), by mixing 10 g with 500 ml of buffer for 1 hr. After decantation of the supernatant, washing was repeated with H_2O until Cl^- was removed. For storage the washed material was suspended in 0.01 M potassium phosphate buffer (pH 7.5). DEAE-cellulose (0.90 mequiv/g) was washed according to the method of Shiota *et al.* (1964). 2-Amino-4-hydroxy-6-hydroxymethylpteridine was prepared by the method of Waller *et al.* (1950). 2-Amino-4-hydroxy-6-pteridinylmethyl pyrophosphate was chemically prepared, purified by DEAE-cellulose column chromatography, and immediately prior to use was reduced to the dihydro level with sodium borohydride, according to the method of Shiota *et al.* (1964). Beef liver catalase, an aqueous ammonium sulfate suspension (Worthington), was a gift of Dr. Irene Schulze.

Strains of Pneumococcus. Three strains of pneumococcus were used for the present study; R6, the wild type, which will not grow (in neopeptone broth) in the presence of sulfanilamide concentrations greater than 5 μg/ml, strain F*a*, which is resistant to about 20 μg/ml of sulfanilamide, and strain F*d*, which is resistant to about 80 μg/ml of sulfanilamide. The latter two strains were obtained *via* transformation with DNA of a highly resistant mutant (Hotchkiss and Evans, 1958).

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[1] The abbreviations used are: DHP-PP, 2-amino-4-hydroxy-6-dihydropteridinylmethyl pyrophosphate; H_2-pteroate, 7,8-dihydropteroic acid; H_2-folate, 7,8-dihydrofolic acid.

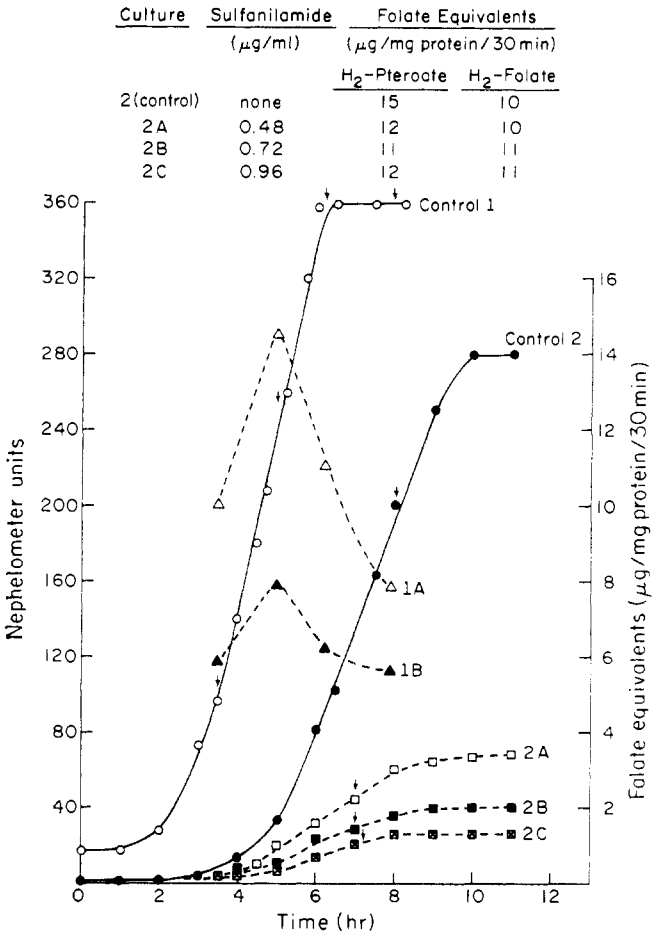

FIGURE 1: Cell growth and enzyme activity. Control 1: growth curve in meat infusion broth. Curve 1A: H_2-pteroate activity. Curve 1B: H_2-folate activity. Control 2: growth curve for expt 2. Curves 2A, B, C: growth curves in the presence of 0.48, 0.72, and 0.96 μg of sulfanilamide per ml, respectively. Samples were withdrawn at times indicated by arrows, lysed, and assayed for enzymic activity as described in Materials and Methods. Results for expt 2 are recorded at the top of figure.

Methods

Enzyme Assay. The complete reaction mixture (0.25 ml) in 1 × 7 cm test tubes contained: 40 μmoles of Tris-Cl buffer (pH 8.0), 50 mμmoles of DHP-PP, 10 mμmoles of $MnCl_2$, 3 mμmoles of *p*-aminobenzoic acid or 50 mμmoles of *p*-aminobenzoylglutamate, 1 mg of ascorbic acid (pH 6.0), and 0.10–1.00 μg of protein. For *Fd* enzyme assays reaction mixtures were altered to contain 20 mμmoles of $MnCl_2$ and 100 mμmoles of *p*-aminobenzoylglutamate. After the tubes were stoppered and incubated 30 min at 37°, samples of the reaction mixtures were diluted immediately into cold water. The amount of product formed was determined in samples of the dilutions by microbiological assay. Because the time of incubation was reduced from that previously reported (Ortiz and Hotchkiss, 1966), the necessity for running the reaction under nitrogen was eliminated.

Because reaction mixtures containing all the components of the system *except* enzyme elicit a significant and not completely predictable growth response by the test organism, such controls are routinely included in each assay. This blank, which is larger when *p*-aminobenzoylglutamate is substrate than when *p*-aminobenzoic acid is substrate, is also dependent upon the presence and concentration of the cosubstrate DHP-PP. Each value reported has been corrected accordingly.

Microbiological Assay. Enzyme activity was measured as the growth response of *Streptococcus faecalis* (ATCC 8043) to the reaction products, as previously described (Ortiz and Hotchkiss, 1966). Since folic acid was used as standard in all assays, results are reported as folate equivalents and represent the mean values obtained from triplicate analyses.

Protein Assay. The protein concentration of all enzyme fractions was determined by the modification of the Lowry method suggested by Oyama and Eagle (1956), using crystalline bovine plasma albumin as standard.

Catalase Assay. Catalase activity was assayed by following the decrease in absorbancy at 240 mμ, under the conditions described by Martin and Ames (1961).

Growth of Bacteria and Cell Lysis. Pneumococcus was grown in a meat infusion broth containing 1% neopeptone, 0.03% glucose, and 0.005 M K_2HPO_4. Cultures were harvested in log phase, and the cells were collected by centrifugation and washed twice with 0.01 of the original culture volume of 0.002 M potassium phosphate buffer (pH 8.0), containing 0.002 M sodium thiomalate.

For lysis, the cells were resuspended in 0.01 of the original culture volume in the same buffer and incubated at 37° for 2 min. Sodium deoxycholate (5%, pH 8–9) was added to a final concentration of 0.28%, and the cells were incubated at 37° for an additional 6 min. Cell debris and unlysed cells were removed by centrifugation of the lysates for 20 min at 12,000 rpm (3°).

Results

Cell Growth and Enzyme Activity. Maximum enzyme activity for both substrates is observed in the logarithmic phase of growth of wild-type pneumococcus in either supplemented meat infusion broth (see Materials and Methods), or in a chemically defined medium (Tomasz, 1964) (Figure 1, curves 1, 1A,1B). The absolute activities observed in the latter case were slightly higher than in the former. Since a greater variation in activity during the growth of the culture was observed with *p*-aminobenzoic acid as substrate (Figure 1, curve 1A), the ratio of the two activities displayed an apparent change of as much as 1.4 to 1.9.

When wild-type cells are grown in the meat infusion broth containing moderate concentrations of sulfanilamide (Figure 1, curves 2A,B,C), inhibition of growth is observed but no significant differences in enzyme activity are found in lysates prepared from cells grown in the presence of the drug from those grown in its absence. Similar results were obtained in a comparison of the enzymatic activity of lysates of cells grown in the presence of 0, 60, and 224 μg per ml of *p*-hydroxybenzoic acid.

Enzyme Purification. Unless otherwise noted, all of the following steps were performed below 5°.

Ammonium Sulfate Fractionation. The lysate, containing about 2 mg of protein/ml, was brought to 25% saturation with solid $(NH_4)_2SO_4$. After the solution had been stirred for 10 min, the precipitate was collected by centrifugation and the supernatant solution was brought to 65% saturation by the

TABLE I: Enzyme Purification.

Strain	Fraction	Total Protein (mg)	Folate Equivalents (μg/mg of Protein/30 min) H_2-Pteroate	H_2-Folate	H_2-Pteroate: H_2-Folate
R6	Lysate	465	14	9	1.56
	AS 1	186	19	12	1.59
	AS 2	13	102	50	2.04
	AS 3	2	429	200	2.14
F*d*	Lysate	205	7	0.4	17.1
	AS 1	87	15	2.5	27.7
	AS 2	8	89	3.4	25.7
	AS 3	1	230	10.5	21.8
F*a*	Lysate	426	8	5	1.60
	AS 1	143	17	11	1.55
	AS 2	21	90	39	2.30
	AS 3	1	238	157	1.52

addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 0.01 M Tris-Cl buffer (pH 8.5), and dialyzed overnight against 2×10^{-3} M Tris-Cl buffer (pH 8.5) (AS #1).

DEAE-cellulose Chromatography. The dialyzed solution was chromatographed on a DEAE-cellulose column (2.5 × 30 cm), previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). The column was developed stepwise with the same buffer containing 0.10, 0.20, and 0.35 M NaCl, respectively. Fractions of 8 ml were collected at a flow rate of 60 ml/hr. The enzyme activity eluted with 0.35 M NaCl. Fractions of highest specific activity were pooled and concentrated by precipitation with $(NH_4)_2SO_4$. The fraction precipitating between 30 and 60% saturation was collected by centrifugation, dissolved in 0.01 M Tris-Cl buffer (pH 8.5), and dialyzed overnight against 2×10^{-3} M Tris-Cl buffer (pH 8.5) (AS #2).

DEAE-Sephadex Chromatography. The dialyzed solution was applied to a column of DEAE-Sephadex (1.0 × 34 cm) which was previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). The column was developed stepwise with solutions of the same buffer containing 0.10, 0.20, 0.30, and 0.35 M NaCl, respectively. Fractions of 5 ml were collected at a flow rate of 30 ml/hr. The enzyme activity eluted with 0.35 M NaCl. Fractions with the highest specific activity were pooled and concentrated by precipitation with $(NH_4)_2SO_4$ (0–65% saturation). The precipitate was collected and treated as described for step 2 (AS #3). At protein concentrations of about 1 mg/ml this fraction when stored at −30° is stable for several months, and unless otherwise noted was used in the experiments reported here.

Table I summarizes the steps in the partial purification of enzymes derived from wild-type R6 and F*d* and F*a* strains of pneumococcus. In all cases, about 30-fold purification was obtained with an average yield of 10%. With a given strain no significant variations in the ratios of activities of the various fractions for H_2-folate and H_2-pteroate synthesis were observed at the different stages of purification. Only slight differences between the specific and relative activities of enzyme F*a* and those from wild-type R6 are obtained. The extremely low activity of F*d* fractions for H_2-folate synthesis, which has been reported earlier (Wolf and Hotchkiss, 1963; Ortiz and Hotchkiss, 1966), is observed throughout the purification procedure. No inhibition of H_2-folate synthesis by wild-type enzyme fractions occurred when mixed with either crude or partially purified fractions from F*d*.

TABLE II: K_m Values for *p*-Aminobenzoic Acid and *p*-Aminobenzoylglutamate.[a]

Enzyme	K_m (*p*-Aminobenzoic Acid) M × 10^5 (Range)	(Mean)	K_m (*p*-Aminobenzoylglutamate) M × 10^5 (Range)	(Mean)
R6	0.15–0.50	0.33	1.0–2.5	1.8
F*a*	0.05–0.25	0.15	5.0–12.0	8.5
F*d*	0.56–0.90	0.73	4.0–9.0	6.5

[a] Components of reaction mixtures, incubation conditions, and details of biological assay are as described in Materials and Methods.

Properties of the Reactions Catalyzed by Partially Purified Enzymes. The pH optimum for H_2-folate or H_2-pteroate synthesis was 8.0–8.2 for enzyme preparations from R6, F*a*, and F*d*.

The range and mean of K_m values for *p*-aminobenzoic acid and *p*-aminobenzylglutamate, obtained from reciprocal plots of the velocity *vs.* substrate concentration (Lineweaver and Burk, 1934) for the wild-type and mutant enzymes are given in Table II. Because of the limitations of the assay method, the range of values obtained is broad. Nevertheless, it is clear that the K_m values for *p*-aminobenzoylglutamate are in all cases significantly higher than that for *p*-aminobenzoic acid, and that somewhat higher values are obtained with F*a* and F*d* enzymes than with wild type. However there seem to be no striking differences in the K_m values for *p*-aminobenzoic acid for the three enzyme preparations.

The inhibition by sulfanilamide of H_2-folate and H_2-pteroate synthesis with crude R6 and F*d* enzyme preparations has been reported (Ortiz and Hotchkiss, 1966). The K_i values now obtained with partially purified enzymes of the two strains are essentially the same for both reactions catalyzed: 2.0×10^{-8} for R6 and 1.5×10^{-7} for F*d*. These are almost identical with values obtained with crude enzyme preparations (Ortiz and Hotchkiss, 1966). The K_i values for F*a* for H_2-folate and H_2-pteroate are 5.4×10^{-7} and 7.6×10^{-7}, respectively.

Because the growth of strain F*d* displays greater sensitivity to inhibition by *p*-hydroxybenzoate than wild-type strains (Hotchkiss and Evans, 1960), it was of interest to determine the effect of this *p*-aminobenzoic acid analog on the *in vitro* synthesis of H_2-folate and H_2-pteroate (Tables III and IV). *p*-Hydroxybenzoate inhibits the synthesis of both compounds when tested with the enzyme from either R6 or F*d*. The inhibition is slightly greater with F*d* than with R6

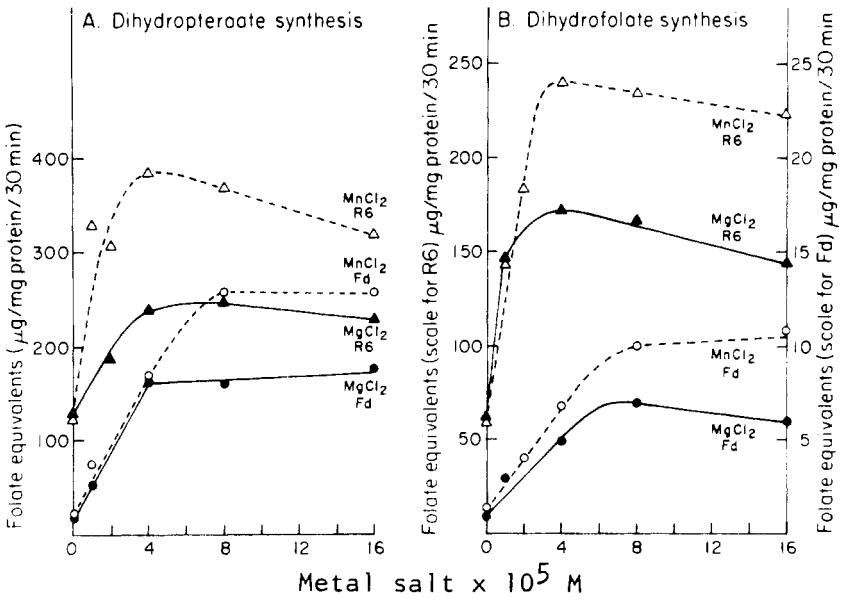

FIGURE 2: The effect of divalent cations on enzymic activity; 2A: H_2-pteroate activity, 2B: H_2-folate activity. Reaction mixtures and assays were as described in Methods, except that metal salt concentration was varied as indicated.

enzyme for *p*-aminobenzoic acid as substrate (Table III). The opposite is true when *p*-aminobenzoylglutamate is substrate (Table IV). The synthesis of H_2-folate is much more sensitive to *p*-hydroxybenzoate inhibition. Thus, with a ratio of *p*-hydroxybenzoate:*p*-aminobenzoic acid of 16, negligible inhibition of H_2-pteroate synthesis occurs, but the same ratio of *p*-hydroxybenzoate:*p*-aminobenzoylglutamate (for R6) resulted in 89% inhibition of H_2-folate synthesis (Tables III and IV). Qualitatively similar results are obtained if lysates are the source of enzyme rather than the partially purified enzyme preparations employed in these experiments. No inhibition of the growth of *Streptococcus faecalis* (used as test organism in the microbiological assay) by *p*-hydroxybenzoate was observed at concentrations comparable with those transferred into the microbiological assay tubes in diluted reactions mixtures. In no instance was synthesis observed in the presence of *p*-hydroxybenzoate and the absence of *p*-aminobenzoic acid or *p*-aminobenzoylglutamate.

In agreement with earlier findings with more crude enzyme preparations (Ortiz and Hotchkiss, 1966), *p*-nitrobenzoate does not inhibit either H_2-folate or H_2-pteroate synthesis with partially purified enzymes derived from either wild type or *Fd*.

Figure 2A,B shows the stimulation by divalent cations of the synthesis of H_2-pteroate and H_2-folate with R6 and *Fd* enzyme preparations. In all cases, including enzyme *Fa* (not shown), Mn^{2+} is more effective than Mg^{2+}, and Ca^{2+} and Zn^{2+} are less effective than Mg^{2+}. Monovalent cations (Na, K) are without effect when substituted for divalent cations or when added to reaction mixtures supplemented with cations. Maximal rates of synthesis with R6 enzyme are obtained at $MnCl_2$ concentrations of 4×10^{-5} M; *Fd* requires twice this concentration to obtain maximum rates of synthesis. Dialysis of enzyme preparations first against EDTA, then against buffer, resulted in somewhat lower activity measured in the absence of added cation, but the highest rates again were obtained with Mn^{2+} and at the same concentrations. Rates of H_2-pteroate and H_2-folate synthesis are increased 10–13-fold when reaction mixtures containing *Fd* enzyme are supplemented with Mn^{2+} at optimal concentrations, while increases of 3–4-fold are noted with *Fa* or R6 enzymes.

TABLE III: The Effect of *p*-Hydroxybenzoate on Dihydropteroate Synthesis.[a]

p-Hydroxybenzoate ($M \times 10^4$)	*p*-Hydroxybenzoate:*p*-Aminobenzoic Acid (Molar)	% Inhibition R6	% Inhibition *Fd*
2	16.6	11	4
4	33.2	25	19
8	66.4	19	37
16	132.8	36	61
28	233.3	44	73

[a] Components of reaction mixtures, incubation conditions and details of biological assay are as described in Materials and Methods.

TABLE IV: The Effect of *p*-Hydroxybenzoate on Dihydrofolate Synthesis.

p-Hydroxybenzoate ($M \times 10^4$)	*p*-Hydroxybenzoate:*p*-Aminobenzoylglutamate (Molar) R6	*p*-Hydroxybenzoate:*p*-Aminobenzoylglutamate (Molar) *Fd*	% Inhibition R6	% Inhibition *Fd*
0.8	0.4	0.2	9	0
2	1.0	0.5	51	26
4	2.0	1.0	74	15
8	4.0	2.0	68	46
20	10.0	5.0	83	50
32	16.0	8.0	89	62

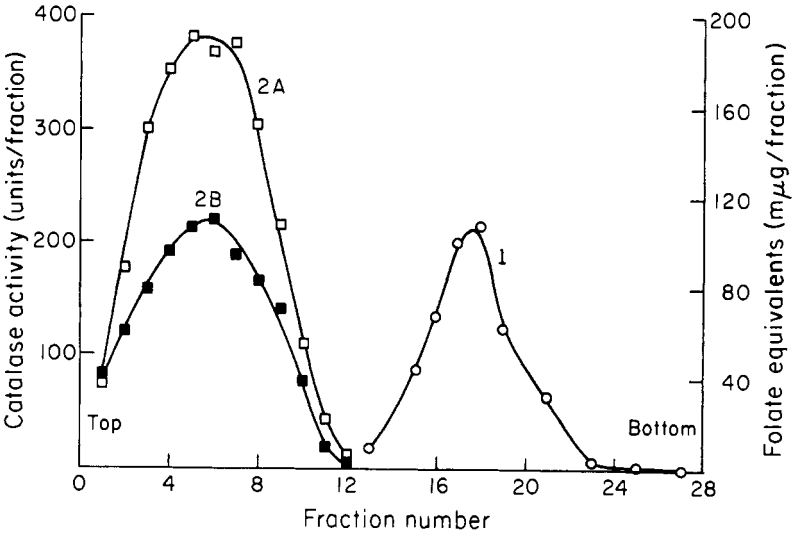

FIGURE 3: Sedimentation of enzyme preparation R6 (AS 3) and catalase in a sucrose gradient (5–20%). Centrifugation was performed with a SW-39 rotor in the Spinco Model L preparative ultracentrifuge at 38,000 rpm for 10.5 hr at 3°. Fractions of 0.10 ml were collected and assayed as described in Methods. Curve 1: catalase. Curve 2A: H_2-pteroate activity. Curve 2B: H_2-folate activity.

The earlier finding that $MgCl_2$ and $MnCl_2$ inhibited both H_2-folate and H_2-pteroate synthesis by crude wild-type preparations and stimulated synthesis by F*d* preparations was a consequence of the high (2×10^{-3} M) concentration of metal salts employed (Ortiz and Hotchkiss, 1966). Qualitatively identical results are obtained at this metal concentration with the partially purified preparations employed in the present experiments.

The approximate molecular weight of the wild-type enzyme was determined by sucrose gradient centrifugation as described by Martin and Ames (1961), using beef liver catalase as a standard. One such gradient is represented in Figure 3. As can be seen, peak activity for H_2-pteroate and H_2-folate synthesis is coincident, and the ratio of the activities over the major portion of the peak are constant. When fractions were assayed without added $MnCl_2$ lower absolute activity was found, but the distribution pattern was identical with that found in the presence of added cation. From the two sharpest gradients molecular weights of 75,000 and 90,000 were calculated.

Because both *p*-aminobenzoic acid and *p*-aminobenzoylglutamate are substrates for crude and partially purified enzyme preparations, the question arises whether two separate enzymes are involved, one for each of these substrates. To gain some insight into this question, competition experiments were performed with the partially purified preparation (AS #3) from the wild-type strain as enzyme source. (Figure 4). Here the concentration of one substrate (*p*-aminobenzoylglutamate) is held constant (1×10^{-4} M), while the other is varied from limiting to saturation levels. If two independent proteins were involved, an additive response in the total synthesis measured should be observed. Long before the saturation level with *p*-aminobenzoic acid was reached, the values obtained in the presence of both *p*-aminobenzoic acid and *p*-aminobenzoylglutamate were significantly lower than the sum of the values for the separate syntheses. However, a slight increase in synthesis in the presence of both substrates above that found with either substrate separately has been consistently observed. That no limitation of other components necessary for maximal enzyme action existed was indicated by permitting portions of the reaction mixtures to continue incubation for an additional 30-min period. The synthesis observed after 60 min was approximately twice that reported for the 30-min incubation time.

Discussion

The variation in enzyme activity during the bacterial culture growth reported here for pneumococcus adds to the list of those enzymes in the folic acid pathway which display the same behavior. Osamu and Silber (1969) have shown for *Lactobacillus casei* that dihydrofolate reductase, 10-formyltetrahydrofolate synthetase, and 5,10-methylentetrahydrofolate dehydrogenase activity increases during the period of logarithmic growth of the cultures and decreases during the stationary phase.

If sulfanilamide not only inhibited the enzyme but also altered the control of synthesis of the enzyme, lysates of cells grown in the presence of the drug (assayed with sufficient *p*-aminobenzoic acid or *p*-aminobenzoylglutamate to overcome possible inhibition of enzyme-bound drug) should display differences in specific enzyme activity from lysates of cells grown in the absence of drug. This was not the case, and seems contrary to a finding by Woods (1954), confirmed by Sevag and Ishii (1958), that washed cell suspensions of a *p*-aminobenzoic acid requiring, sulfonamide-sensitive strain of *Escherichia coli*, when grown in subinhibitory concentrations of sulfonamide synthesized five- to tenfold greater amounts of folic acid than when the cells were grown in the absence of the drug. Both these and the present observations can be reconciled if one considers that in the earlier work folic synthesis was assayed in a system containing only buffer, glucose and *p*-aminobenzoic acid. Thus in cells grown in the drug, pteridine cosubstrate (DHP-PP) might accumulate and result in a rapid burst of synthesis by cell suspensions when supplemented with *p*-aminobenzoic acid, without any alteration in enzyme activity or enzyme synthesis. In the case of the cells grown in the absence of the drug, where normal turnover occurs, pteridine does not accumulate and a low but normal rate of synthesis is observed.

With only 30-fold purification of the pneumococcal enzymes, additional information has been gained concerning

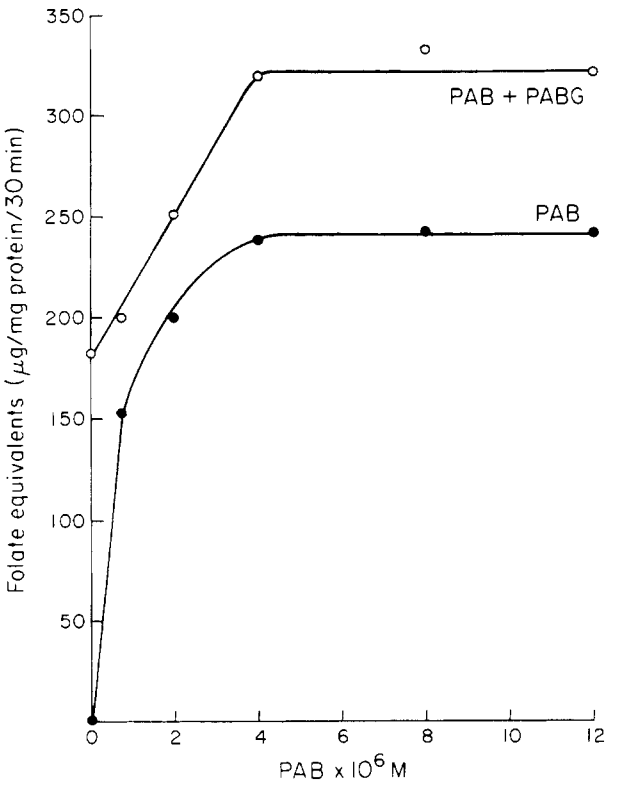

FIGURE 4: Enzyme activity with mixtures of *p*-aminobenzoic acid and *p*-aminobenzoylglutamate. *p*-Aminobenzoylglutamate concentration = 1×10^{-4} M; *p*-aminobenzoic acid concentration varied as indicated.

the properties of the system which were not apparent with crude extracts. Thus the stimulation of activity by divalent cations, particularly Mn^{2+} and Mg^{2+} is of special interest since one sulfonamide-resistant enzyme (*Fd*) shows a much greater stimulation by these cations than does either enzyme *Fa* or wild-type enzyme R6. Richey and Brown (1969) have reported that *E. coli* B enzyme preparations require Mg^{2+} for maximal activity with *p*-aminobenzoic acid, but the magnitude of the response to the cation was not given. As indicated by these authors, a requirement for divalent cation for the condensation of *p*-aminobenzoic acid or *p*-aminobenzoylglutamate with DHP-PP is expected in analogy to other reactions of this type. The response of enzyme *Fd* may reflect a type of alteration which distinguishes it from both wild type and *Fa*. Whether this is related to the low activity of this enzyme with *p*-aminobenzoylglutamate is not known.

Although the K_m values for *p*-aminobenzoylglutamate are in all cases higher than those for *p*-aminobenzoic acid, the K_i values for sulfanilamide are in all cases almost identical for both substrates, and the enzymes from drug-resistant strains display a reduced binding capacity for sulfanilamide. Thus while strains *Fa* and *Fd* differ quantitatively in their levels of resistance to sulfanilamide, the measured affinities of their enzyme systems for substrate and drug are similar although quite distinct from wild type.

No inhibition of crude or partially purified R6 or *Fd* enzymes by NOB has been observed despite the fact that *Fd* and all strains carrying this genetic subunit show greater growth inhibition in the presence of this compound than do wild-type strains (Hotchkiss and Evans, 1960). Although the same growth response is observed with *p*-hydroxybenzoate as with *p*-nitrobenzoate, *in vitro* inhibition studies with this compound indicated that both H_2-folate and H_2-pteroate synthesis are inhibited by this compound. Of particular interest is the finding that H_2-folate synthesis is appreciably more sensitive to such inhibition. This leads one to question the possibility that the *in vivo* effect of *p*-nitrobenzoate may be attributed to conversion by the cells of *p*-nitrobenzoate into *p*-hydroxybenzoate, which then inhibits growth by inhibition of folate biosynthesis. No direct evidence for this specific conversion is available.

All bacterial extracts or partially purified preparations tested have been found to utilize both *p*-aminobenzoic acid and *p*-aminobenzoylglutamate as substrates (Shiota *et al.*, 1964; Brown *et al.*, 1961; Wolf and Hotchkiss, 1963; Ortiz and Hotchkiss, 1966). While in all cases cited the K_m values for *p*-aminobenzoylglutamate are higher than those for *p*-aminobenzoic acid, the rates of synthesis of H_2-folate and H_2-pteroate do not vary by more than a factor of two; the higher rate being that of H_2-pteroate synthesis. The only two exceptions are *E. coli* (Brown *et al.*, 1961), and the pneumococcal enzyme *Fd* reported here. In these cases rates of H_2-folate synthesis are very much lower than found elsewhere. This being the case, it does not seem appropriate at this time to apply a name (Richey and Brown, 1969) to this enzyme(s) which perhaps reflects properties of an enzyme preparation which differ from those most frequently described.

No definitive evidence is available to indicate that two independent proteins are responsible for the utilization of the two substrates. However, the coincidence of activity qualitatively and quantitatively in sucrose gradient fractions, and the lack of significant distortion of the ratios of the two activities during partial purification suggest that only one enzyme is involved.

However, the rates of synthesis obtained in the presence of mixtures of *p*-aminobenzoic acid and *p*-aminobenzoylglutamate would suggest that if two separate enzymes are involved, they do not act independently when confronted simultaneously with both substrates.

Alternatively these results may be interpreted as the result of partial competition by the two substrates for a single enzyme site.

Further studies with more purified enzyme preparations are necessary to extend these observations and provide evidence in support of these possibilities.

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Amino Acid Transfer Factors from Yeast. III. Relationships between Transfer Factors and Functionally Similar Protein Fractions*

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ABSTRACT: The functions of the factors FI and FII from yeast, complementary in peptide chain elongation, have been further characterized. Factor FI resembles the translocase of bacterial and mammalian systems. Factor FII seems to be a functional analog to the bacterial T factor and to the binding enzyme from mammalian sources. Fusidic acid, as well as diphtheria toxin and nicotinamide–adenine dinucleotide, inhibits the functions of FI but not of FII. Both yeast factors exist in more than one protein fraction. An FI-related form, FI_G, has been isolated; its properties closely resemble those of FI except that FI_G shows a significant ribosome-dependent guanosine triphosphatase activity. Specific treatments selectively eliminate this guanosine triphosphatase activity of FI_G, leaving its transfer activity intact. Besides the binding enzyme FII, two fractions, T_1 and T_2, can be obtained with functions similar to FII.

However, T_1 and T_2 combined with the translocase FI fail to stimulate peptide chain elongation. A relationship between the T fractions and translocase FI can be demonstrated by studying the effect of FI on the guanosine triphosphate–T_1 complex without ribosomes. In the presence of phenylalanyl transfer ribonucleic acid and T_2, factor FI removed the γ-phosphate and the guanine moiety from the guanosine triphosphate–protein complex.

Studies in polypeptide chain elongation so far have led to the finding of separable factors involved in distinct reactions. In microbial systems, two factors (T_u and T_s) have been shown to be involved in the binding of aminoacyl-tRNA to ribosomes (Lucas-Lenard and Lipmann, 1966; Lucas-Lenard and Haenni, 1968; Ravel, 1967), and a third (G) in translocation of peptidyl-tRNA between ribosomal sites (Haenni and Lucas-Lenard, 1968; Erbe and Leder, 1968; Lucas-Lenard and Haenni, 1969). Partial reactions associated with these factors have been (1) the formation of an intermediate aminoacyl-tRNA–T_u–GTP complex (Ravel *et al.*, 1967; Ertel *et al.*, 1968; Gordon, 1968; Skoultchi *et al.*, 1968), and (2) a G factor and ribosome-dependent GTP hydrolysis (Conway and Lipmann, 1964). A GTP hydrolysis has also been associated with transfer of aminoacyl-tRNA from the aminoacyl-tRNA–T_u–GTP complex to ribosomes (Ono *et al.*, 1969; Shorey *et al.*, 1969; Gordon, 1969).

In mammalian systems two factors have been recognized with properties partially analogous to the microbial factors. One catalyzes aminoacyl-tRNA binding to ribosomes (Rao and Moldave, 1967; Arlinghaus *et al.*, 1964; McKeehan *et al.*, 1969) and a concomitant GTP hydrolysis (Arlinghaus *et al.*, 1964; McKeehan *et al.*, 1969), and the other one catalyzes translocation as well as ribosome-dependent GTP cleavage (Skogerson and Moldave, 1968a,b; Felicetti and Lipmann, 1968; Klink *et al.*, 1967).

We have been attempting to find parallels between the yeast system and microbial or mammalian systems. Ayuso and Heredia (1968) described a yeast factor which catalyzed aminoacyl-tRNA binding to ribosomes, and we have isolated two fractions T_1 and T_2 which resemble T_u and T_s from bacteria in that they are complementary in aminoacyl-tRNA-dependent GTP hydrolysis (Richter *et al.*, 1968). They fail to function in chain elongation, however.

Here, we described two factors FI and FII which have been

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