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THE ENZYMATIC HYDROLYSIS OF FOLATE ANALOGUES

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Abstract—The enrichment culture technique has been used to isolate bacteria which contain enzymes for the hydrolysis of the terminal amino acid in various folate analogues. Each of the three purified enzymes is specific for the hydrolysis of only one of the following compounds: 4-amino-4-deoxypteroylalanine, 4-amino-4-deoxypteroylaspartate and 4-amino-4-deoxypteroylglutamate. The hydrolysis in each reaction is in accord with the equation:

4-amino-4-deoxypteroyl-N-(amino acid) + H_2O → 4-amino-4-deoxypteroate + amino acid.

A SOIL pseudomonad which can grow with methotrexate as its only source of carbon and nitrogen contains an enzyme capable of catalyzing reaction 1:[1]

$$\text{Methotrexate} + H_2O \rightarrow \text{4-amino-4-deoxy-10-methylpteroate} + \text{glutamate} \quad (1)$$

In addition to catalyzing this reaction, the enzyme carboxypeptidase G, hydrolyzes many compounds with an *N*-acyl linkage to glutamate represented by the general formula RCO-N,L-glutamate, where RCOOH represents not only various pteroic acid analogues but also a variety of organic acids, including all the amino acids tested.[2] Contrasting with the lack of specificity for the R-group is the complete specificity of the enzyme for the carboxyl terminal glutamate. Specificity such as this for a single amino acid with a variety of *N*-acyl substituents seems to be unique among peptidases.

Aside from making available a peptidase with interesting new properties, the study with methotrexate illustrates the usefulness of microorganisms in elucidating biochemical reactions on compounds with potential interest as drugs. In order to explore further this method of studying the biological degradation of such compounds, enrichment culture[3] was used to select microorganisms that could degrade other folate analogues.

Since carboxypeptidase G can probably degrade all folate analogues in which the alteration occurs in the pteroyl portion of the molecule, organisms were sought which could degrade folate analogues in which another amino acid is substituted for glutamate.

It is the purpose of this paper to report the finding of new enzymes which can degrade 4-amino-4-deoxypteroylaspartate according to reaction 2 and 4-amino-4-deoxypteroylalanine according to reaction 3. These reactions are comparable to

the action of carboxypeptidase G on 4-amino-4-deoxypteroylglutamate (aminopterin), reaction 4.

4-amino-4-deoxypteroylaspartate + H_2O → 4-amino-4-deoxypteroate + asparatate (2)

4-amino-4-deoxypteroylalanine + H_2O → 4-amino-4-deoxypteroate + alanine (3)

4-Amino-4-deoxypteroylglutamate + H_2O → 4-amino-4-deoxypteroate + glutamate (4)

MATERIALS AND METHODS

Materials. 4-Amino-4-deoxypteroylaspartate, 4-amino-4-deoxypteroylalanine and 4-amino-4-deoxypteroate were synthesized by the method of Seeger *et al.*[4] 4-Amino-4-deoxypteroylglutamate (aminopterin) was a gift from Lederle Laboratories, Pearl River, N.Y. Benzoyl-aspartate and benzoyl-alanine were synthesized by a Shotten–Baumann reaction.[5]

Isolation and cultivation of bacteria. The conditions for the preparation of carboxypeptidase G from an organism which can utilize methotrexate as a source of carbon and nitrogen have been described.[1,2] A pseudomonad containing an enzyme which catalyzes reaction 3 was isolated by the enrichment culture technique with benzoyl-aspartate as a source of carbon and nitrogen.[6] An enzyme for reaction 4 has been found in an unclassified gram-negative coccobacillus isolated from Rock Creek mud by the enrichment culture technique with benzoyl-alanine as a source of carbon and nitrogen.

Each organism was isolated and allowed to grow at 25–30° on a medium with the following composition per liter: HK_2PO_4, 0·5g; K_2HPO_4, 1·2 g; Salts A, 5 ml, and Salts B, 1·5 ml.[6]

For the pseudomonad, 2 g benzoyl-aspartate was added, growth was in 15-l. carboys with heavy aeration, and harvest was by centrifugation (Sharples) in late logarithmic growth. For the unclassified coccobacillus, 2 g benzoyl-alanine was added and the organism was allowed to grow in 2-l. Erlenmeyer flasks on a rotary shaker (120 rpm). This organism was harvested in late logarithmic growth by centrifugation at 10,000 *g*.

The yield of the pseudomonad cells was about 2 g wet wt./1 l. of medium.

The coccobacillus contained a mucoid capsular material which prevented tight packing of the cells even after centrifugation at 70,000 *g*. The yield of these cells was 6 g wet wt/l. of medium.

Enzyme purification. Procedures for the 100-fold purification of the enzyme which catalyzes reaction 2 have been described.[6] To obtain the 10-fold purified enzyme for catalyzing reaction 3, the following operations were performed at 0–5°. The bacterial paste was suspended in three times its weight of 0·05 M Tris-HCl buffer at pH 7·6 and the cells were disrupted at 9000 lb/in^2 in a French pressure cell (Aminco). To the extract, which had been clarified by centrifugation at 75,000 *g* for 30 min, a protamine sulfate solution (2% in water adjusted to pH 7·3 with 1 N NaOH) was added slowly with stirring; in this mixture the final ratio of protein to protamine was 3:1. The extract was again clarified by centrifugation and solid ammonium sulfate (31 g/100 ml) was added slowly with stirring to the protein solution. After 1 hr of stirring, the clear supernatant solution was collected by centrifugation and additional

ammonium sulfate (14 g/100 ml) was added as above. The precipitate obtained after centrifugation was dissolved in a minimal volume of 0·05 M Tris-HCl buffer at pH 7·3 and dialyzed for 2 hr against 0·05 M Tris-HCl at pH 7·3. This enzyme solution was stable for several weeks at — 20° and was used in the experiments described.

A typical enzyme preparation by this purification scheme is summarized in Table 1. Like carboxypeptidase G[2] and other enzymes, the yield increases during purification.

TABLE 1. SUMMARY OF ENZYME PURIFICATION

Step	Volume (ml)	Protein (mg)	Enzyme activity (units)	Sp. act. (units/mg)
Crude extract	20	460	17	0·037
Protamine supernatant	24	264	20	0·076
Ammonium sulfate	5	40	15	0·37

Assay of the enzyme hydrolyzing 4-*amino*-4-*deoxypteroylalanine*. The hydrolysis of 4-amino-4-deoxypteroylalalanine according to equation 3 is accompanied by the spectral changes shown in Fig. 1. These spectral alterations are comparable to those accompanying the hydrolysis of 4-amino-4-deoxypteroylglutamate and 4-amino-4-deoxypteroylaspartate.[4] The spectral change for reaction 3 is maximal at approximately 300 mμ (Fig. 1) and accordingly the extent of hydrolysis was measured at

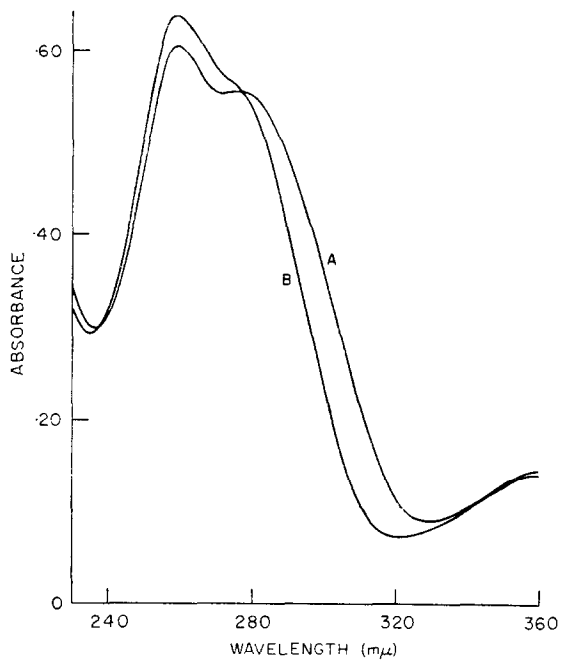

FIG. 1. Spectral characteristics of the enzymatic reaction with 4-amino-4-deoxypteroylalanine. The reaction mixture contained 70 mμmoles 4-amino-4-deoxypteroylalanine, 150 μmole Tris-HCl buffer at pH 7·3 and 0·6 μmoles $ZnCl_2$. Total volume was 3 ml in a cuvette (1 cm light path) held at 30°. After curve A was recorded, 0·1 unit of enzyme (0·03 ml) was added and when the change in optical density at 300 mμ indicated that the reaction had gone to completion (1 min), curve B was recorded.

this wavelength. At pH 7·3, the extinction coefficient for the reaction is given by the difference between ϵ_{300} (4-amino-4-deoxypteroylalanine) = 20,400 and ϵ_{300} (4-amino-4-deoxypteroate) = 12,300. An enzyme unit is defined as the activity required to hydrolyze 1 μmole of 4-amino-4-deoxypteroylalanine per minute under the conditions stated in Fig. 1.

Isolation of pteridine products of the reactions. For the identification of the pteridine product of the hydrolytic reaction on the folate analogues, large-scale incubations of substrate with enzyme were conducted. The reaction mixture contained 110 mg 4-amino-4-deoxypteroylalanine, 1·75 m-moles Tris-HCl at pH 7·3 and 4 units of the enzyme catalyzing reaction 3 in a volume of 60 ml. After incubation at 30° for 8 hr, the reaction had gone to completion as indicated by the absence of further spectral changes, and the reaction mixture was brought to 100°. The solution was clarified by centrifugation and its volume reduced to about 10 ml on a rotary evaporator at 50°. The product was then precipitated by bringing the solution to pH 3 with 1 N HCl. After 3 hr at 0°, the precipitate was collected on a sintered glass filter and redissolved in 10 ml of 0·1 N NaOH. This solution was heated with charcoal and filtered; solid CO_2 was added to the clear filtrate and the product was collected as the sodium salt; yield 41 mg (43 per cent of theoretical).

Comparable incubations of 4-amino-4-deoxypteroylglutamate and 4-amino-4-deoxypteroylaspartate with their respective hydrolyzing enzymes yielded similar amounts of product when isolated in this manner.

Characterization of the reaction products. The amino acids released in the hydrolytic reactions were isolated from the reaction mixtures by elution from a column of Dowex 50 (H^+)[7] and quantified by a photometric ninhydrin method.[8] The amino acids eluted from the column were chromatographed on Eastman silica gel chromagram sheets in order to establish their identity with authentic compounds. The following systems were used: 60% ethanol in water; *n*-propanol–NH_4OH (2:1); and ethanol–NH_4OH (3:1).

Ascending paper chromatography on Whatman No. 1 paper was used to substantiate the identity of isolated 4-amino-4-deoxypteroate relative to its authentic standard in two systems: 5% acetic acid in H_2O and 3% NH_4Cl in H_2O. Protein was measured by the method of Gornall *et al.*[9] or of Warburg and Christian.[10]

RESULTS

Identification of the products and stoichiometry of the hydrolytic reactions with the folate analogues. Infrared spectroscopy (Fig. 2) and the extinction coefficients (Table 2) of the isolated and authentic compounds indicate that the reactions of the various 4-amino-4-deoxypteroyl analogues yielded 4-amino-4-deoxypteroate as expected from eqns (2–4).

The stoichiometry of the hydrolytic reactions proposed in eqs. (2–4) are confirmed by the data of Table 3. Studies with each of these enzymes have indicated that a divalent cation is an absolute requirement of the hydrolytic reactions shown. This requirement is satisfied by Zn^{2+} for carboxypeptidase G[2] and by either Co^{2+} or Zn^{2+} for the aspartate-splitting enzyme.[6] Studies on the alanine-splitting enzyme indicate a requirement for Zn^{2+}. Accordingly, the reaction conditions for each enzyme include the appropriate divalent cation as specified in the legend of Table 3. In other experiments, each enzyme was tested under these incubation conditions for reactivity against each of the other two folate analogues and no hydrolysis of the bond to the terminal

amino acid was detected. This finding is of particular interest since it indicates that the specificity of these enzymes is toward the carboxyl terminal amino acid rather than toward the pteroyl group in compounds of this type.

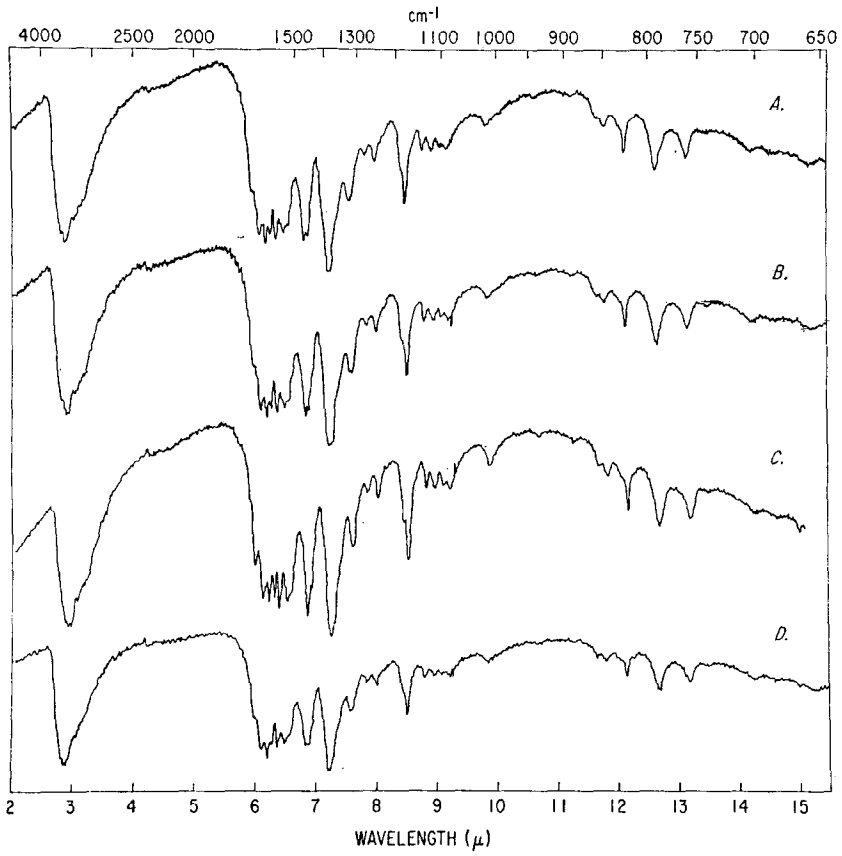

FIG. 2. Infrared spectra of authentic and isolated 4-amino-4-deoxypteroate. Curve A is the synthetic compound. Curves B, C and D are of the 4-amino-4-deoxypteroate recovered from the hydrolysis of 4-amino-4-deoxypteroylalanine, 4-amino-4-deoxypteroylglutamate and 4-amino-4-deoxypteroylaspartate respectively. The spectra were taken in KBr pellet on a Perkin–Elmer, model 21.

TABLE 2. EXTINCTION COEFFICIENTS OF ISOLATED AND AUTHENTIC 4-AMINO-4-DEOXYPTEROATE*

Source of compound	λ_{max} (mμ)	ε ($M^{-1}cm^{-1}$)	λ_{min} (mμ)	ε ($M^{-1}cm^{-1}$)	λ_{max} (mμ)	ε ($M^{-1}cm^{-1}$)
Authentic	370	7600	321	3200	261	29,000
4-Amino-4-deoxypteroylglutamate	372	7800	321	3000	261	32,000
4-Amino-4-deoxypteroylaspartate	370	7500	321	3100	261	31,000
4-Amino-4-deoxypteroyllalanine	370	7900	321	3300	261	31,000

* All spectra were recorded on samples in 0·05 M Tris-HCl buffer at pH 7·3 on a Beckman DK-2A ratio-recording spectrophotometer. Extinction coefficients were calculated from the weights of the isolated product, on the assumption that they had the molecular weight of the sodium salt of 4-amino-4-deoxypteroic acid.

TABLE 3. STOICHIOMETRY OF FOLATE ANALOGUE HYDROLYSIS*

Starting compound		Products	
Identity	Amount (μmoles)	4-Amino-4-deoxypteroate (μmoles)	Amino acid (μmoles)
4-Amino-4-deoxypteroylglutamate	2·2	2·1	1·9
4-Amino-4-deoxypteroylaspartate	3·1	2·9	2·4
4-Amino-4-deoxypteroylalanine	2·7	2·6	2·3

* Reaction mixtures contained the amount of starting compound shown and 125 μmole Tris-HCl buffer in a total volume of 4·0 ml. For the incubation with 4-amino-4-deoxypteroylglutamate, the reaction mixture also contained 0·4 unit of carboxypeptidase G (30-fold purified). For the incubation with 4-amino-4-deoxypteroylaspartate, 0·2 unit of the aspartate-removing enzyme was added with 0·8 μmole $CoCl_2$. For the incubation with 4-amino-4-deoxypteroylalanine, 0·8 μmole $ZnCl_2$ and 0·2 unit of the alanine-removing enzyme were added. Incubation was conducted for 30 min at 30°. 4-Amino-4-deoxypteroate was calculated from its extinction coefficient at 300 mμ. The amino acids were determined quantitatively as described in Methods.

DISCUSSION

The degradation of this series of folate analogues illustrates how microorganisms may be selected to give information on biochemical reactions involving various compounds of pharmacological interest. The metabolism of the folate analogues in this study seems to depend not on their common 4-aminopteroyl structure, but rather on the differences in their respective amino acid substituents. Each of the enzymes seems to be a peptidase or deacylase specific for its particular carboxyl terminal amino acid.[2,6]

Thus, it is not surprising that each enzyme fails to hydrolyze folate analogues which differ in amino acid substituent. A distinction between the various analogues on the basis of their amino acids rather than a similarity due to the common pteroyl group might be correlated with the limited effectiveness of compounds such as 4-amino-4-deoxypteroylasparatate *in vivo*.[11]

Enzymes isolated from organisms which can degrade compounds of pharmacological interest may serve another useful function by providing the basis for a specific assay of that compound. This is illustrated in the present study where the spectral changes at 300 mμ accompanying each of the three reactions would be specific for the compound reactive with that enzyme.

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