Bacterial Degradation of Folic Acid*

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SUMMARY

Two *Pseudomonas* sp. able to grow with folic acid as sole source of carbon and nitrogen were isolated from soil. Strain FO1 converts folic acid to pterioic acid which accumulates in the culture medium. Strain FO8 grows with various pteridine compounds. From the culture medium of this strain the following compounds were isolated: deaminated folic acid, pterioic acid, deaminated pterioic acid, and lumazine-6-carboxylic acid. Extracts deaminated various pteridines with high activity. A pathway of folic acid degradation is proposed.

INTRODUCTION

A number of bacteria utilize the glutamate moiety of folate (Lemon *et al.* 1948; Levy & Goldman, 1967; Pratt, Crawford & Friedkin, 1968) but little is known about the process of degradation beyond the level of pteroate. Folate-degrading micro-organisms deserve attention as possible sources of enzymes which may be of some value in the treatment of malignant tumours. Folate depletion of the culture medium by a carboxypeptidase converting folate to pteroate was shown to inhibit the growth of human and murine leukaemia cells *in vitro* (Bertino, O'Brien & McCullough, 1971). Folate-degrading enzymes might be effective against cell lines resistant to the folate analogue methotrexate.

We report the isolation of two bacteria, tentatively identified as *Pseudomonas* sp., which grow on folic acid as sole source of carbon and nitrogen. Four degradation products of folic acid have been identified by chromatography and spectrophotometry, and a pathway of folic acid degradation is proposed.

METHODS

Identification tests. These were performed according to the *Manual of Microbiological Methods* (1957). Some tests were carried out as described by Stanier, Palleroni & Doudoroff (1966).

Media. Medium A contained (g/l deionized water): K_2HPO_4 , 1.0; KH_2PO_4 , 0.5; $MgSO_4$, 0.05; NaCl, 0.05; pH 7. Folic acid or pteridine compounds (0.1 %, w/v) were added as required. Medium B consisted of medium A, plus (mg/l): H_3BO_3 , 0.5; $CuSO_4$, 0.04; KI, 0.1; $FeCl_3$, 0.2; $MnSO_4$, 0.4; $(NH_4)_2MoO_4$, 0.2; $ZnSO_4$, 0.4; biotin, 0.1; pH 7. Medium C contained 8 g nutrient broth (Difco)/l deionized water; pH 6.5. Medium D contained (g/l deionized water): nutrient broth (Difco), 6.5; yeast extract (Oxoid), 1.5; glucose, 1.0; NaCl, 3.5; K_2HPO_4 , 3.7; KH_2PO_4 , 1.5; pH 6.8. Medium E contained (g/l deionized water): yeast extract (Oxoid), 10; peptone (Oxoid), 10; glucose, 20; pH 7.2.

For the preparation of plates, agar was added to a final concentration of 2.1 % (w/v).

Chemicals. Pterioic acid and neopterin were gifts from Dr R. B. Angier, and 6,7-di-

* In memory of Dr Otto Oltmanns.

methylpterin from Professor W. Pfeleiderer. The following compounds were prepared according to published procedures (Wolf *et al.* 1947; Angier *et al.* 1952; Weygand & Schaefer, 1952; Baugh & Shaw, 1964): 2-amino-4-hydroxypteridine (pterin), 6-hydroxymethylpterin, pterin-6-carboxylic acid, rhizopterin, deaminated folic acid, deaminated pteric acid, lumazine-6-carboxylic acid. Xanthopterin was obtained from C. Roth OHG, Karlsruhe, Germany, aminopterin from Serva AG, Heidelberg, Germany, and folic acid from Merck AG, Darmstadt, Germany. Dowex 50 WX 8 (200 to 400 mesh) and DEAE-cellulose SS were purchased from Serva AG, Heidelberg, MN-cellulose 300 from Macherey und Nagel, Düren, Germany, and Multodisks S1 from Oxoid.

Spectroscopic methods. Ultraviolet spectra were determined with a DMR 21 spectrophotometer (Zeiss). Infrared spectra were recorded with a spectrometer equipped with a beam condenser permitting measurements of small samples (Ernst Leitz Optical Works, Wetzlar, Germany). Mass spectra were recorded on an MS 902/DS 30 spectrometer (AEI; courtesy of Dr Alberti, AEI, Manchester).

Thin-layer chromatography. Thin-layer chromatography was performed with plates covered with MN-cellulose 300. The following solvent systems were used: 3 % NH_4Cl ; methanol-conc. NH_4OH -water (60:10:30, by vol.); isopropanol-conc. NH_4OH -water (60:5:35, by vol.); and *n*-butanol-acetic acid-water (60:15:25, by vol.).

Growth tests. Growth tests were performed in 200 ml Erlenmeyer flasks at 30 °C on a rotary shaker for three days. Strain Fo1 was incubated in medium A, strain Fo8 in medium B. Growth was measured by plating diluted samples on medium E plates.

Enzymic tests. Bacteria were grown in medium D for two days at 30 °C on a rotary shaker. Crude extracts were prepared by sonication. Folate amidase activity was determined by the procedure of Pratt *et al.* (1968). Solutions for the assay of pteridine deaminase activity contained the compounds tested (3×10^{-5} to 1×10^{-4} M) in 0.1 M-tris buffer pH 8. The reaction was followed by measuring the absorbance with a recording spectrophotometer at 37 °C. The wavelengths used were selected for the maximum difference of absorbance of the respective substrate and product.

Isolation of folate metabolites. Strains Fo1 and Fo8 were incubated in their respective media A supplemented with folic acid on a rotary shaker at 30 °C. The accumulation of metabolites was followed by thin-layer chromatography of the culture medium. Incubation times used for the isolation of the different metabolites from Fo8 are shown in Table 1. The incubation and isolation procedures were performed in the dark or in dim light to prevent photochemical degradation.

Compound 1 (ptericoic acid): ptericoic acid was isolated from the culture fluid and purified as described by Lemon *et al.* (1948).

Compound 3 (deaminated ptericoic acid): culture fluid of strain Fo8 (1 l) was concentrated to a small volume under reduced pressure. The solution was applied to a column of DEAE-cellulose (Cl^- form, 5×30 cm). Deaminated ptericoic acid was eluted with deionized water and the eluate evaporated to dryness. The residue was dissolved in 1 N- NH_4OH and adjusted to pH 4 by the addition of 2 N-acetic acid. The resulting precipitate was collected by centrifugation and treated with 5 ml conc. HCl at room temperature. The precipitate was collected on a filter and subsequently dissolved in 3 ml conc. HCl on a steam bath. Deionized water (50 ml) was added to the solution and the resulting yellow crystals collected and dried.

Compound 2 (deaminated folic acid) and compound 4 (lumazine-6-carboxylic acid): Culture fluid of strain Fo8 (1 l) was evaporated to dryness under reduced pressure. The residue was dissolved in 2 N-HCl and applied to a column of Dowex 50 WX 8 (200 to

Fig. 1. Electron micrographs of (a) strain FO1 and (b) strain FO8.

400 mesh, H^+ form, 1.5×30 cm). The column was developed with 1 N-HCl and fractions (10 ml) collected. Lumazine-6-carboxylic acid was eluted in fractions 3 to 12, and deaminated folic acid in fractions 16 to 40.

Fractions containing deaminated folic acid were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 3 ml conc. HCl and the solution poured into 50 ml hot water. Deaminated folic acid crystallized in the cold.

Table 1. *Yields of compounds isolated from 1 l medium B (supplemented with folic acid) incubated with strain FO8*

Compound	Incubation time (days)	Yield (mg)
Compound 1 (pteroic acid)	7	10
Compound 2 (deaminated folic acid)	3	7
Compound 3 (deaminated pterioic acid)	3	14
Compound 4 (lumazine-6-carboxylic acid)	15	1.8

Fractions containing lumazine-6-carboxylic acid were combined and evaporated to dryness. The residue was dissolved in 2 ml 1 N-HCl and applied to a column of Dowex 50 WX 8 (200 to 400 mesh, H⁺ form, 0.5 × 40 cm). The column was developed with 1 N-HCl. Fractions containing lumazine-6-carboxylic acid were combined and evaporated to dryness. This compound was crystallized from water.

RESULTS

Isolation of folate-degrading bacteria

Erlenmeyer flasks containing 100 ml medium A supplemented with folic acid were inoculated with samples of garden soil and incubated on a rotary shaker at 30 °C. Portions (10 ml) were transferred to fresh medium at weekly intervals. After five serial transfers, portions were plated on medium B agar supplemented with folic acid. Two bacteria (strains FO1 and FO8) able to grow on folic acid as sole source of carbon and nitrogen were obtained. Colonies of strain FO1 growing on medium A or medium B agar supplemented with folic acid accumulated at their surface a yellow-orange substance which was assumed to be pterioic acid. Strain FO8 growing on medium B agar supplemented with folic acid showed colourless spots in the yellow agar plate around the bacterial colonies. The colourless zones showed a bright-blue fluorescence under u.v. light, resulting from the accumulation of lumazine-6-carboxylic acid as shown below.

Characterization of bacteria

Strain FO1. Morphological features: straight rods, 2 to 3 µm by 0.7 to 1 µm occurring singly, motile in every medium used, polarly flagellated with additional fimbriae (Fig. 1a). No endospores; Gram-negative. Colonies on medium E agar incubated two days at 30 °C: brownish; diameter of single colonies 2 to 4 mm; colony form circular or slightly irregular because of undulate margin, flat. Blue fluorescence under u.v. light on Paton medium (Paton, 1959); no pigment. No pigment on King A medium, yellow-green fluorescence on King B medium (King, Ward & Raney, 1954).

Strain FO8. Morphological features: small rods, straight or slightly curved, 1 to 2 µm by 0.5 to 0.8 µm, highly motile by means of one terminal flagellum with subpolar insertion (Fig. 1b). Colonies on medium E agar incubated three days at 30 °C: brownish; punctiform; diameter of single colonies about 0.2 mm. No endospores; Gram-negative. No fluorescence on Paton medium; no pigment. No pigment on King A medium, yellow-green fluorescence on King B medium.

Table 2. Fragments in the high-resolution mass spectrum of compound 2 (deaminated folic acid)

Peak at m/e	Fragment	Relative intensity
313	C ₁₄ H ₁₁ O ₄ N ₅ (deaminated pteronic acid)	8.5
178	C ₇ H ₆ O ₂ N ₄ (6-methyl-lumazine)	9.7
164	C ₆ H ₄ O ₂ N ₄ (lumazine)	0.5
137	C ₇ H ₇ O ₂ N ₁ (4-aminobenzoate)	8.4
84	C ₄ H ₆ O ₁ N ₁ (pyrrolidone-2)	100

Fig. 2. (a) Growth test with strain Fo1 on medium D (x), or on medium A supplemented with folic acid (O) or glutamic acid (Δ). (b) Growth test with strain Fo8 on medium D (x), or on medium B supplemented with folic acid (O), pteronic acid, deaminated folic acid or deaminated pteronic acid (□), or glutamic acid (Δ).

Classification was performed according to standard procedures (*Manual of Microbiological Methods*, 1957; Stanier, Palleroni & Doudoroff, 1966). The biochemical characteristics and the antibiotic sensitivity of both strains were in agreement with the characteristics described for the genus *Pseudomonas*.

Metabolites of folic acid

Strain Fo1 was grown in medium A supplemented with folic acid on a rotary shaker at 30 °C until a bulky precipitate was formed (5 to 10 days). The precipitate was harvested by filtration and purified as described in Methods. The isolated compound and an authentic sample of pteronic acid had similar u.v. and i.r. spectra and similar mobilities on t.l.c. In a typical experiment 40 mg pteronic acid were obtained from 100 mg folic acid.

Pteronic acid from cultures of strain Fo8 was isolated in the same way, except that the medium (B supplemented with folic acid) was adjusted to pH 6 to prevent further degradation of pteronic acid.

Compounds 2 to 4 were isolated from cultures of strain Fo8 grown in medium B which was supplemented with folic acid and adjusted to pH 7 as described in Methods. Yields are shown in Table 1.

Table 3. *Substrate specificity of pteridine deaminase from strain Fo8*

Substrate	Specific activity (nmol/mg protein/min)	Wavelength (nm)
Pterin	316	340
6-Hydroxymethylpterin	219	360
Pterin-6-carboxylic acid	143	264
Neopterin	19.2	320
Pteric acid	13.7	285
Folic acid	11.4	295
Aminopterin	8.8	300
6,7-Dimethylpterin	< 1	350
Xanthopterin	< 1	360

Permanganate oxidation of compound 2 yielded lumazine-6-carboxylic acid which was identified by thin-layer chromatography. High-resolution mass spectra showed the fragments presented in Table 2. From these data the compound was assigned the structure of deaminated folic acid. This was confirmed by comparison with a synthetic sample by means of i.r. and u.v. spectrometry, t.l.c., and thin-layer chromatographic detection of glutamic acid after hydrolysis of compound 2 with 6 N-HCl.

Oxidation of compound 3 with KMnO_4 yielded lumazine-6-carboxylic acid. Compound 3 was indistinguishable by i.r. and u.v. spectrometry and by t.l.c. from a synthetic sample of deaminated pteric acid.

Compound 4 was identified as lumazine-6-carboxylic acid by comparison with a synthetic sample by means of i.r. and u.v. spectra and by t.l.c.

Growth tests

The results of growth tests are shown in Fig. 2. All added compounds were tested as sole source of carbon and nitrogen.

Strain Fo1 grew well in medium D. Growth in medium A with folic acid or glutamic acid as sole source of carbon and nitrogen was slightly delayed. No growth occurred with 4-aminobenzoate and 4-aminobenzoylglutamic acid.

Strain Fo8 grew well in medium D. Folate, deaminated folate, pterate, and deaminated pterate as supplements of medium B gave equal growth characteristics. Lumazine-6-carboxylic acid was not a good substrate for growth. No growth occurred with 4-aminobenzoate.

Enzymic assays

Extracts of strain Fo1 converted folate to pterate with a specific activity of 1.5 nmol pterate formed/mg protein/minute.

Extracts of strain Fo8 deaminated various pteridine compounds (Table 3). Unsubstituted pterin was deaminated most efficiently. 6-Hydroxymethylpterin and pterin-6-carboxylic acid were also deaminated at a high rate.

DISCUSSION

From morphological and biochemical studies, the folate-degrading strains Fo1 and Fo8 are tentatively classified as *Pseudomonas* sp. Both organisms are incapable of liquefying gelatin and seem to belong to the 'fluorescent group'. Definite assignment to one of the species considered by Stanier *et al.* (1966) was not possible.

Fig. 3. Proposed pathway of folate degradation by strain Fo8. Gln, glutamic acid.

Four degradation products of folic acid were isolated from the culture fluid of strain Fo8. On this basis we propose the scheme of folic acid degradation shown in Fig. 3.

Strain Fo1 contains an enzyme hydrolysing the amide bond of folic acid which is similar to the folate amidases from *Flavobacterium* sp. (Pratt *et al.* 1968) and *Pseudomonas* sp. (Levy & Goldman, 1967). Enzymic deamination of folic acid and pteric acid by crude extracts from *Alcaligenes faecalis* was described by McNutt (1963). However, this organism was unable to grow with folic acid as sole source of carbon and nitrogen. Another pteridine deaminase was partially purified by Levenberg & Hayaishi (1959) from *A. metalcaligenes* isolated with pterin-6-carboxylic acid as sole source of carbon and nitrogen. The enzyme deaminates various pteridines, but not aminopterin. The specific activity in crude extracts is comparable with strain Fo8.

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