Pteridines Produced by *Methylococcus capsulatus*

ISOLATION AND IDENTIFICATION OF A NEOPTERIN 2':3'-PHOSPHATE

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Three pteridines have been isolated from the methane- or methanol-oxidizing bacterium *Methylococcus capsulatus*. Two of these are known compounds, 2-amino-6-carboxy-4-hydroxypteridine and 2-amino-4-hydroxy-6-methylpteridine. The third is shown by degradative and synthetic experiments to be *L-threo*-neopterin 2':3'-phosphate. Labelling experiments show that both the pteridine moiety and phosphate residue are derived from a single GTP molecule. The possible metabolic significance of these compounds in methanol oxidation is discussed.

Pseudomonas sp. M27 can grow on methanol as a sole source of carbon and energy. Methanol is oxidized by a non-specific primary alcohol dehydrogenase. The enzyme has been purified and was reported to contain a tightly bound pteridine derivative as its prosthetic group (Anthony & Zatman, 1967). Similar alcohol dehydrogenases have been reported in other methanol-oxidizing bacteria (Johnson & Quayle, 1964) and in some methane-oxidizing bacteria either directly (Patel & Hoare, 1971) or by implication (Whittenbury, Phillips & Wilkinson, 1970). These findings prompted us to examine some of these species to identify the pteridines in them. The present paper reports the results of structural analyses on the pteridines from *Methylococcus capsulatus*, an organism that grows only on methane or methanol.

Three major pteridines have been isolated. Two of these, 2-amino-6-carboxy-4-hydroxypteridine and 2-amino-4-hydroxy-6-methylpteridine, are known compounds, the latter having been identified as occurring naturally in a variety of methane- or methanol-utilizing micro-organisms (Urushibara & Forrest, 1970). The third is a new compound; evidence is presented that it is *L-threo*-neopterin 2':3'-phosphate. [Throughout this paper the trivial name neopterin is used for 2-amino-4-hydroxy-6-(1',2',3'-trihydroxypropyl)pteridine, with no implication as to the stereochemistry of the hydroxyl groups on the side chain.] Some results have been presented (Cone & Guroff, 1971) on the structure of a pteridine isolated from the *Comamonas* sp. A.T.C.C. 11299a having an inducible phenylalanine

hydroxylase; these authors tentatively identify this pteridine as a neopterin cyclic phosphate.

EXPERIMENTAL

Growth of M. capsulatus. Cultures were grown on the basal medium of Foster & Davis (1966) with methane as sole carbon and energy source. Carboys (14-litre capacity) containing 6 litres of basal medium were inoculated with 600 ml of exponential-phase cells. Cultures were gassed and sealed under an atmosphere of methane+air (50:50) and were grown for 3 days at 37°C on a reciprocal shaker.

Cells were harvested by centrifuging at 12000g for 15 min in a Sorvall RC-2 centrifuge, and were washed twice in 50 mM-potassium phosphate buffer, pH 7.

Separation and purification of pteridines. A 2 g (wet wt.) portion of cells was suspended in water (400 ml) and the suspension, acidified with acetic acid (2 ml), was treated with MnO₂ (1 g). After being stirred for 2 h, the cloudy suspension was filtered, the filtrate treated with charcoal (Norit A; 1 g) and the whole shaken for 30 min. The charcoal was collected by centrifugation and washed with water, and fluorescent materials were eluted from it with ethanol-aq. 4% (w/v) ammonia (1:1, v/v) (three elutions, 50 ml each). The combined eluates, after concentration *in vacuo* to 1 ml, were streaked on Whatman no. 3MM paper, which was then irrigated with the propanol-ammonia solvent (solvent II; Table 1). Fluorescent bands were eluted from the dried chromatogram with water, and the compounds were further purified by chromatography on Whatman no. 3MM paper with solvent I (Table 1). After elution of the bands from this chromatogram with water, the eluates were evaporated to small bulk *in vacuo*.

Permanganate oxidations. A mixture of sample solution and KMnO₄ solution (saturated in 0.1 M-NaOH) was

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heated at 98°C for 15 min. Excess of permanganate was destroyed by addition of ethanol, and the MnO_2 was removed by centrifugation. The supernatant was treated with solid CO_2 and concentrated to dryness. The residue was dissolved in aq. 0.1% (w/v) ammonia.

Acid hydrolysis of pteridines. Hydrolysis of pteridines was accomplished in 2M-HCl at 98°C for 2 h. The reaction mixture was concentrated to dryness *in vacuo*, and the residue was dissolved in water and evaporated to dryness. This procedure was repeated a few times to remove acid. Finally the residue was dissolved in aq. 0.1% (w/v) ammonia.

Potassium periodate oxidation of pteridines. Periodate oxidation of pteridines was carried out in aqueous solution at room temperature for 2 days.

Micro determination of phosphorus. Phosphorus was determined by a slight modification of the method of Chen, Toribara & Warner (1956). Pteridine concentration was measured spectrophotometrically in neutral solution at 345 nm. The same solution was placed in a Pyrex test tube (15 mm $\times$ 87 mm) and solvent was removed by flame. Conc. HCl (1 ml) was added to the test tube and the mixture was heated at 110°C for 1 h. After removal of the acid, the residue was treated with 1 ml of molybdic acid reagent (3M- H_2SO_4 -water-10% ascorbic acid-2.5% ammonium molybdate, 1:7:1:1, by vol.; prepared just before use); the mixture was incubated at 37°C for 2 h. The extinction at 820 nm was measured after the mixture had cooled to room temperature.

Enzymic hydrolysis of pteridines. A mixture of 1 vol. of sample solution, 2 vol. of 0.1M- NH_4Cl , pH 9.5, 1 vol. of 10 mM-magnesium acetate and 1 vol. of a saturated solution of alkaline phosphatase (*Escherichia coli*; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) in 0.1M- NH_4Cl was incubated at 37°C for 1 h. Acetone was added and the precipitate was removed by centrifugation. The supernatant was evaporated to dryness *in vacuo* and the residue dissolved in aq. ammonia.

Incorporation of radioactivity from [^{14}C]GTP. A 2 g (wet wt.) portion of *M. capsulatus* cells, grown as described above, was thoroughly washed with 50 mM-tris-HCl buffer, pH 8.0, and suspended in 7 ml of the same buffer. The suspension was sonicated in a Branson Sonifier (7 kHz for five 1 min intervals with 30 s rest between each). The resulting suspension was centrifuged (12000g for 15 min).

The orange supernatant (3 ml) was treated with 2 μCi of [^{14}C]GTP (tetrasodium salt) (389 mCi/m mol; New England Nuclear Corp., Boston, Mass., U.S.A.), or with 5 μCi of [α - ^{32}P]GTP (original specific radioactivity 290 mCi/mmol), or with a mixture of 5 μCi of [^{14}C]GTP and 10 μCi of [α - ^{32}P]GTP. After 3.5 h at 37°C, the solution was adjusted to pH 3 with acetic acid and heated at 100°C for 3 min with 50 mg of MnO_2 . The precipitate was removed by centrifugation and pteridines in the supernatant were purified by chromatography on Whatman 3MM paper with solvent system II followed by system I (Table 1). Appropriate areas were cut out from the paper, and fluorescent material was eluted from them by adding 1 ml of aq. 0.1% ammonia. After 3-4 h the paper was removed, and the eluate evaporated to dryness *in vacuo*. Scintillation fluid [10 ml; toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazol-2-yl)benzene] was added and radioactivity was determined

in an Ansitron Scintillation Counter (counting efficiency for ^{14}C , 92%).

Other procedures. Absorption spectra were determined with a Cary 14 spectrophotometer or a Beckman DB-G spectrophotometer. Fluorescence and excitation spectra were measured with a Farrand Mk 1 spectrofluorimeter. Identification of pteridines by paper chromatography was achieved on Whatman no. 1 papers with the solvent systems shown in Table 1.

Electrophoretic identification of pteridines was carried out on Whatman 3MM papers in a Beckman Duostate paper-electrophoresis apparatus. Fluorescent spots were detected with a Mineralite Black-Ray UVL-22 lamp.

Synthesis of L-threo-neopterin. L-threo-Neopterin was made by the method described by Rembold & Metzger (1963) and Rembold & Buschmann (1963).

Synthesis of L-threo-neopterin cyclic phosphate. L-threo-Neopterin cyclic phosphate was made from L-threo-neopterin by treatment with POCl_3 , or from L-threo-neopterin phosphate by using trifluoroacetic anhydride (Baddiley, Buchanan, Carss & Mathias, 1956).

RESULTS AND DISCUSSION

Paper-chromatographic separation of fluorescent materials gave three blue fluorescent bands. For convenience they were named A-1, A-2 and A-3 in order of increasing R_F in solvent I. Some minor fluorescent materials were eliminated during purification; they were not present in sufficient amount to be identified.

Compound A-2 was not attacked by potassium periodate or by alkaline potassium permanganate. Direct comparison showed it to be identical with authentic 2-amino-6-carboxy-4-hydroxypteridine. It may occur only as a degradation product from compound A-1 or compound A-3 during isolation and purification. Compound A-1, for example, slowly decomposed to 2-amino-6-carboxy-4-hydroxypteridine during purification.

Compound A-3 was not attacked by potassium periodate; however, it was attacked by alkaline potassium permanganate to give 2-amino-6-carboxy-4-hydroxypteridine. Direct comparison showed compound A-3 to be identical with authentic 2-amino-4-hydroxy-6-methylpteridine.

Compound A-1 could be distinguished from a large number of authentic known pteridines. Its absorption spectrum showed maxima (relative absorption in parentheses) in 0.1M-hydrochloric acid at 256 (0.499) and 318 nm (0.105); in water at 260 (0.637) and 344 nm (0.100); and in 0.1M-sodium hydroxide at 257 (0.725) and 359 nm (0.108).

Fig. 1 shows a comparison of the excitation and fluorescence spectra of compound A-1 in water with those of authentic D-erythro-neopterin made from D(+)-ribose and 2,4,5-triamino-6-hydroxypyrimidine.

These results suggest that compound A-1 is a

Fig. 1. Excitation (left) and fluorescence (right) spectra of *D-erythro*-neopterin (—) and compound A-1 (---) in water, pH 7.0. For recording fluorescence an activating wavelength of 360 nm was used, and for excitation a wavelength of 450 nm was used.

6-substituted 2-amino-4-hydroxypteridine derivative, and its chromatographic behaviour indicated that it might be a phosphate ester.

Compound A-1 was not attacked by potassium periodate, whereas, as expected, authentic neopterin phosphate was oxidized by such treatment.

However, compound A-1 was readily oxidized by alkaline potassium permanganate to give 2-amino-6-carboxy-4-hydroxypteridine. Acid hydrolysis of compound A-1 gave a blue fluorescent derivative (compound B-1). Compound B-1 was almost identical with the authentic *D-erythro*-neopterin phosphate isolated from *E. coli* (Goto & Forrest, 1961; Goto, Ohno, Forrest & Lagowski, 1965). However, the R_F values of compound B-1 were slightly lower than those of *D-erythro*-neopterin phosphate in all solvents.

Compound B-1 was attacked by potassium periodate or by alkaline potassium permanganate to give 2-amino-6-carboxy-4-hydroxypteridine. Alkaline phosphatase treatment of compound B-1 gave a blue-fluorescent derivative (compound B-2), whereas the original compound A-1 was not attacked by this enzyme. Compound B-2 was almost identical with authentic *D-erythro*-neopterin; however, they were markedly different in R_F values in solvent system VI. Since such a difference is

indicative of stereo isomers (Rembold & Metzger, 1963; Rembold & Buschmann, 1963), a comparison with *threo* isomers was made. The results showed compound B-2 to be identical with *L-threo*-neopterin [from *L*(-)-xylose and 2,4,5-triamino-6-hydroxypyrimidine]. Paper-chromatographic and electrophoretic comparisons are summarized in Table 1.

Micro determination of phosphorus in compound A-1 suggested that the ratio of phosphorus to pteridine nucleus was 1:1.

We conclude, therefore, that compound A-1 is a 2':3'-cyclic phosphate derived from *L-threo*-neopterin. A 1':3'-cyclic phosphate is ruled out because of the ease of acid hydrolysis of the compound and because the natural product is readily synthesized from the 3'-phosphate by using trifluoroacetic anhydride (Baddiley *et al.* 1956). The *L-threo* configuration of the side chain is supported by microbiological assays on compound B-2 and related compounds with *Criethidia fasciculata*. Over a wide range of concentrations compound B-2 had the same growth-factor activity as synthetic *L-threo*-neopterin and, at a concentration of 3 ng/ml, about twice the activity of synthetic *D-threo*-neopterin. These results are in accord with previous observations (Kidder & Dewey, 1968). We are grateful to

Table 1. *Chromatographic and electrophoretic comparison of compounds from bacteria with authentic compounds*

Chromatographic solvents were as follows: I, butan-1-ol-acetic acid-water (4:1:1, by vol.); II, propan-1-ol-aq. 1% (w/v) ammonia (2:1, v/v); III, propan-1-ol-2% (w/v) ammonium acetate (1:1, v/v); IV, propan-2-ol-5% (w/v) boric acid (4:1, v/v); V, 3% (w/v) NH_4Cl . Electrophoresis was carried out at pH 4.70 in acetate buffer at 20 V/cm for 1 h. Further experimental details are given in the text.

Compound	Solvent ...	R_F value					Electrophoretic mobility to anode (mm)
		I	II	III	IV	V	
Compound A-1		0.02	0.15	0.40	0.04	0.68	33
L-threo-Neopterin-2':3'-cyclic phosphate		0.02	0.15	0.40	0.04	0.68	33
Compound B-1		0.01	0.02	0.29	0.01	0.72	34
D-erythro-Neopterin-3'-phosphate		0.01	0.03	0.30	0.02	0.74	34
Compound B-2		0.14	0.24	0.50	0.11	0.64	-3
L-threo-Neopterin		0.14	0.24	0.50	0.11	0.64	-3
D-erythro-Neopterin		0.15	0.26	0.52	0.16	0.66	-3
Compound A-1, KMnO_4 oxidation product		0.14	0.15	0.31	0.02	0.59	35
Compound B-1, KMnO_4 oxidation product		0.14	0.15	0.31	0.02	0.59	35
Compound B-2, KMnO_4 oxidation product		0.14	0.15	0.31	0.02	0.59	35
Compound B-1, KIO_4 oxidation product		0.14	0.15	0.31	0.02	0.59	35
Compound B-2, KIO_4 oxidation product		0.14	0.15	0.31	0.02	0.59	35
Compound A-2		0.14	0.15	0.31	0.02	0.59	35
2-Amino-6-carboxy-4-hydroxypteridine		0.14	0.15	0.31	0.02	0.59	35
Compound A-3		0.40	0.41	0.64	0.35	0.57	-3
2-Amino-4-hydroxy-6-methylpteridine		0.40	0.41	0.64	0.35	0.57	-3
Compound A-3, KMnO_4		0.14	0.15	0.31	0.02	0.59	35

Scheme 1. Flow sheet for degradative reactions on compound A-1 described in the text.

Table 2. Incorporation of radioactivity from labelled GTP into pteridines

Experimental details are given in the text. The ratio for the starting material was determined with a portion from original solution at the same time as the isolated materials.

Expt. no.	Isotope in GTP	Radioactivity incorporated (c.p.m./ μ mol)			$^{32}\text{P}/^{14}\text{C}$ ratio
		6-Methyl compound	Cyclic phosphate		
			^{14}C	^{14}C	
I	^{14}C	1260000	1630000	—	—
II	^{32}P	—	—	34996*	—
III	^{14}C and ^{32}P	1530	1975†	21987*	11.1
	Starting mixture	—	—	—	10.2

* Only a part of the reaction mixture was worked up to obtain these values, since only the ratio of ^{32}P to ^{14}C in the cyclic phosphate is important.

† Corrected for loss of one carbon atom from the GTP on transformation into a pteridine.

Fig. 2. Excitation (left) and fluorescence (right) spectra of the alcohol dehydrogenase from *M. capsulatus* in water, pH 7.0. For recording fluorescence an activating wavelength of 360 nm was used, and for excitation a wavelength of 450 nm was used.

Dr Ian MacLean, Dalhousie University, for carrying out these assays.

A flow sheet for the degradative reactions described above is shown in Scheme 1.

The synthesis of both 2-amino-4-hydroxy-6-methylpteridine and L-threo-neopterin 2':3'-phosphate from $[\text{U-}^{14}\text{C}]\text{GTP}$ has been achieved in low yield with a cell-free extract from *M. capsulatus*. The ratio of radioactivities found in the two com-

pounds (2-amino-6-carboxy-4-hydroxypteridine was not observed in working up the reaction mixture) corresponds closely to the theoretical ratio for a compound with 7 radioactive carbon atoms and one with 9 (Found, 0.773; theoretical, 0.778) (Table 2), indicating that the two compounds have a common pathway of biosynthesis. In a second experiment with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, radioactivity was incorporated into the cyclic phosphate. Finally, with a mixture of $[\text{U-}^{14}\text{C}]\text{GTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ the $^{14}\text{C}/^{32}\text{P}$ radioactivity ratios in the starting solution and in the final product were identical (Table 2). This provides convincing evidence that the cyclic phosphate is derived directly from GTP.

We have also examined the activation and fluorescence spectra of a partially purified preparation of the alcohol dehydrogenase from *M. capsulatus*. This preparation has activation and fluorescence peaks at the wavelengths expected for an aromatic pteridine (Fig. 2). The significance of this is difficult to judge at this time, since the molar ratio of pteridine to enzyme is not known and, in any case, one would expect a reduced non-fluorescent pteridine to be present as coenzyme.

The methanol dehydrogenases from a number of methane- and methanol-oxidizing bacteria, including the enzyme isolated by Anthony & Zatman (1967), appear to be quite similar by various criteria (D. S. Hoare & R. N. Patel, unpublished work). The cyclic phosphate described above has been tentatively identified after isolation from a highly purified preparation of the methanol dehydrogenase from a *Pseudomonas* sp. ('pink organism'). The implication is that this pteridine is closely related to or derived from a cofactor common to them all.

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