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p-Cresol and 3,5-Xylenol Methylhydroxylases in *Pseudomonas putida* N.C.I.B. 9869

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Pseudomonas putida N.C.I.B. 9869, when grown on 3,5-xylenol, hydroxylates the methyl groups on 3,5-xylenol and on *p*-cresol by two different enzymes. 3,5-Xylenol methylhydroxylase, studied only in relatively crude extracts, requires NADH, is not active with *p*-cresol and is inhibited by cyanide, but not by CO. The *p*-cresol methylhydroxylase requires an electron acceptor and will act under anaerobic conditions. It was purified and is a flavocytochrome *c* of mol.wt. approx. 114000 consisting of two subunits of equal size. The enzyme catalyses the hydroxylation of *p*-cresol (K_m 16 μM) and the further oxidation of product, *p*-hydroxybenzyl alcohol (K_m 27 μM) to *p*-hydroxybenzaldehyde. A different *p*-cresol methylhydroxylase of the flavocytochrome *c* type is induced by growth on *p*-cresol. It too was purified and has mol.wt. approx. 100000, and again consisted of two equal-size subunits. The K_m for *p*-cresol is 3.6 μM and for *p*-hydroxybenzyl alcohol, 15 μM.

Although the bacterial oxidation of methyl-substituted aromatic compounds may proceed with retention of the methyl group intact until after ring fission, often the initial attack involves oxidation of the methyl group to carboxyl (Chapman, 1972). The first step of hydroxylation to yield an alcohol is followed by oxidation to an aldehyde and then an acid, in a manner analogous to the conversion of alkanes into carboxylic acids or the ω-oxidation of fatty acids (Gholson *et al.*, 1963). Detailed studies have been made on the enzymes used by some bacteria for alkane hydroxylation (McKenna & Coon, 1970; Jurtshuk & Cardini, 1971; Ruettinger *et al.*, 1977). These use molecular oxygen with a requirement for reduced nicotinamide nucleotide and are multi-component systems. Less is known about the enzymes that oxidize methyl groups attached to the benzene ring, although the *p*-xylene hydroxylase from *Pseudomonas aeruginosa* appears to be a mixed-function oxygenase and has been resolved into two components by DEAE-cellulose chromatography (Nozaka & Kusunose, 1968). An enzyme that hydroxylates the methyl group of *p*-cresol has been purified from a strain of *Pseudomonas putida* (Hopper, 1976; Hopper & Taylor, 1977). It is a flavocytochrome and will act under anaerobic conditions with a requirement for an electron acceptor.

The degradation of both *p*-cresol and 3,5-xylenol by *Ps. putida* N.C.I.B. 9869 is initiated by oxidation of a methyl group (Hopper & Chapman, 1971; Hopper & Taylor, 1973), and the results of a study of the hydroxylases involved are presented in the present paper.

Materials and Methods

Maintenance and growth of organism

The organism, *Ps. putida* N.C.I.B. 9869, was maintained by growth on nutrient-agar slants for 24h at 30°C, followed by storage at 4°C. It was subcultured monthly.

Cells were grown at 30°C in medium containing (per litre): Na_2HPO_4, 4.33g; KH_2PO_4, 2.65g; NH_4Cl, 2.0g; nitrilotriacetic acid, 0.1g; salts solution (Rosenberger & Elsden, 1960), 4.0ml; 3,5-xylenol or *p*-cresol, 0.3g. For whole-cell studies cells were grown in 1 litre of medium in 2-litre Erlenmeyer flasks at 30°C in an orbital shaker (Gallenkamp and Co., Widnes, Lancs., U.K.). Larger quantities of cells for preparation of extracts and for enzyme purification were grown in a Microferm MF-114 fermenter (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) which was aerated at a rate of 4 litres/min and stirred at 150rev./min. Growth was followed by measuring turbidity at 540nm in a Unicam SP.500 spectrophotometer. Further additions of substrate were made when necessary to give higher yields. Cells were harvested in an Alfa Laval LAB 102B continuous-flow centrifuge (Alfa Laval Co., Brentford, Middx., U.K.) and stored at −20°C.

Enzyme assays

The procedures for assaying *p*-cresol methylhydroxylases A and B were those described by Hopper & Taylor (1977) modified by using 50mM-glycine/NaOH buffer, pH9.6, the pH optimum for both enzymes.

Preparation of extracts

Extracts were prepared by suspending each 1g of wet cells in 2ml of ice-cold 50mM-KH_2PO_4/NaOH buffer, pH7.0, cooling the suspension in an ice/water bath and disrupting the bacteria with a Dawe Soniprobe, type 7530 A, fitted with a micro-tip for volumes up to 20ml or in a 500W MSE ultrasonic disintegrator fitted with a 3cm probe for larger volumes. The smaller volumes were disrupted for a total of 4min in $\frac{1}{2}$min periods and the larger volumes for 8min also in $\frac{1}{2}$min periods. The extracts were centrifuged at 2°C for 10min at 40000*g* and the supernatants, referred to as crude extracts, retained. Such extracts were red–brown in colour and contained 35–45mg of protein/ml. Ice-cold saturated $(NH_4)_2SO_4$ solution was added slowly, with stirring, to crude extract to adjust it to 70% $(NH_4)_2SO_4$ saturation. After 1h at 0°C the precipitate was removed by centrifuging at 25000*g* for 20min. The pellet was dissolved in a volume of 50mM-phosphate buffer, pH7.0, equal to that of the original crude extract and dialysed with stirring for 3h against two changes of 100vol. of the same buffer. This extract is referred to as $(NH_4)_2SO_4$-treated extract.

Polyacrylamide-gel electrophoresis

Electrophoresis of enzymes was carried out on 6.4% (w/v) polyacrylamide gels with an average pore radius of 1.5nm (Gordon, 1969) by the method of Ornstein & Davis (1964) at pH8.9. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969). Disaggregation of the enzymes required incubation for 2h at 37°C in 10mM-phosphate buffer, pH7.0, containing 1% (v/v) sodium dodecyl sulphate and 1% (w/v) mercaptoethanol. The incubation mixture was then dialysed overnight against 10mM-phosphate buffer, pH7.0, containing 0.1% (w/v) sodium dodecyl sulphate and 0.1% (w/v) mercaptoethanol. Gels were stained for protein with either Amido Black 10B (Colour Index 20470) or Coomassie Brilliant Blue G-250 (Colour Index 42655) and cleared by washing with 7% (v/v) acetic acid in water or acetic acid/ethanol/water (2:9:9, by vol.) respectively. Gels were stained for enzyme activity by placing them in reaction mixtures containing, in 1.5ml of 50mM-glycine/NaOH buffer, pH9.6, 2μmol of either *p*-cresol or *p*-hydroxybenzyl alcohol, 2μmol of phenazine methosulphate and 1mg of either Nitro Blue Tetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride. These were incubated at 20°C in the dark. The Nitro Blue Tetrazolium gave a blue colour with the enzyme and the latter compound gave a red colour. Gels, stained for protein, were scanned at 600nm in a Gilford 2400S spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.) fitted with a 2410S gel-transport attachment.

Ultracentrifuge studies

These were carried out with a Beckman Spinco model E ultracentrifuge equipped with Rayleigh interference optics. Samples were previously dialysed for 24h against 16.7mM-phosphate buffer, pH7.0, at 2°C. Sedimentation-equilibrium experiments were conducted by using the short-column meniscus-depletion method of Yphantis (1964) and were performed at 20410rev./min for 24h at 20°C for hydroxylase A and at 17890rev./min for 24h at 9°C for hydroxylase B. The initial protein concentration was 0.5mg/ml. In these runs the solution compartment in the double-sector cells, equipped with sapphire windows, contained 100μl of enzyme solution on 50μl of fluorocarbon oil (Beckman, Palo Alto, CA, U.S.A.); the reference compartment contained 120μl of diffusate on 40μl of fluorocarbon oil. The attainment of equilibrium was determined experimentally by measuring fringe displacements of given radial positions. A value of 0.725cm^3/g was assumed for the partial specific volume, $\bar{v}$, of the proteins.

Aerobic incubations

Oxidation of *p*-cresol by crude extracts was followed at 30°C in Warburg respirometers containing, in a 2.8ml reaction volume, 33mg of protein, 125μmol of KH_2PO_4/NaOH buffer, pH7.0, 2μmol of phenazine methosulphate and 6μmol of substrate, with 0.2ml of 20% (w/v) KOH in the centre well. For oxidation of *p*-cresol or *p*-hydroxybenzyl alcohol by partially purified hydroxylase A flasks contained, in 2.8ml of 50mM-glycine/NaOH buffer, pH9.6, 2μmol of phenazine methosulphate, 3.2mg of protein and 6μmol of substrate, with 0.2ml of 20% (w/v) KOH in the centre well.

Anaerobic incubations

Reaction mixtures in Thunberg tubes contained 5ml of purified enzyme (1.2mg and 0.8mg of protein/ml for hydroxylase A and B respectively), 5ml of 50mM-glycine/NaOH buffer, pH9.6, 50μmol of phenazine methosulphate, 60μmol of 2,6-dichlorophenol-indophenol and 30μmol of either *p*-cresol or *p*-hydroxybenzyl alcohol in the side arms. The tubes were alternately evacuated and flushed with N_2 five times and, after tipping of substrate, were incubated at 30°C for 3h. The reaction was stopped by addition of 5ml of 20% (w/v) orthophosphoric acid.

Isolation of products

Protein from acidified reaction mixtures was removed by centrifuging at 10000*g* for 10min and

the supernatants were extracted twice with an equal volume of diethyl ether. The pooled extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness.

Chromatography

T.l.c. was carried out on Kieselgel GF_{254} (E. Merck A.-G., Darmstadt, Germany) coated on glass plates to a thickness of 0.25mm and activated at 110°C for 30min. Phenolic compounds were separated by using solvent A [benzene/dioxan/acetic acid (90:25:4, by vol.) (Pastuska, 1961)]. For 2,4-dinitrophenylhydrazones solvent B [benzene/tetrahydrofuran (19:1, v/v) (Byrne, 1965)] was used. Descending paper chromatography of phenolic compounds was carried out on Whatman no. 1 paper with solvent C [benzene/acetic acid/water (125:72:3, by vol.) (Smith, 1960)] and solvent D [5% (w/v) sodium formate/formic acid (200:1, v/v) (Smith, 1960)].

Preparation of 2,4-dinitrophenylhydrazones

Reaction mixtures or 5ml of solutions of standards were incubated at 30°C for 30min with 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl. The precipitated 2,4-dinitrophenylhydrazones were collected by centrifuging at 10000*g* for 10min. The pellet was washed with 2M-HCl and dissolved in 3ml of ethyl acetate. The solution was dried over anhydrous Na_2SO_4.

Absorption spectra

Visible-absorption spectra were measured in a Unicam SP.1800 double-beam recording spectrophotometer. For difference spectra of the *p*-cresol methylhydroxylases 2ml of enzyme solution containing 0.5–1.0mg of protein/ml were first oxidized with 2μl of 50% (w/w) H_2O_2, and the baseline spectrum recorded with oxidized enzyme in both sample and reference cuvettes (1cm light-path). The enzyme in the sample cuvette was then reduced by addition of a few crystals of sodium dithionite or 0.1ml of 20mM substrate, and the difference spectrum recorded.

Spectrophotofluorimetric studies

Fluorimetric measurements were made in 1cm light-path cuvettes in an Aminco–Bowman spectrophotofluorimeter (American Instrument Co., Silver Spring, MD, U.S.A.) fitted with a xenon light-source and with slit arrangement no. 3 (Aminco instruction manual).

NADH- and NADPH-generating systems

NADH was generated in Warburg flasks by including 0.1ml of 10mM-NAD^+, 0.1ml of 0.2M-ethanol and 0.1ml of alcohol dehydrogenase (1mg/ml, 300 units/mg) in the reaction mixture. NADPH was generated by inclusion of 0.1ml of 10mM-$NADP^+$, 0.1ml of 0.2M-glucose 6-phosphate and 0.1ml of glucose 6-phosphate dehydrogenase (30 units/ml). One unit is defined as the conversion of 1μmol of substrate/min under the manufacturers' recommended conditions.

Protein determinations

The biuret method of Gornall *et al.* (1949) was used to determine protein, except where very low concentrations were present, when the method of Lowry *et al.* (1951) was used. Standard curves were prepared by using bovine serum albumin. The elution of protein from columns was followed by measuring A_{280} of the fractions.

Purification of p-cresol methylhydroxylase A

Crude extract from 3,5-xylenol-grown cells was heated rapidly, with continuous stirring, to 70°C, kept at this temperature for 5min and then quickly cooled in ice/water. The precipitated protein was removed by centrifuging at 18000*g* for 10min. The supernatant was loaded on a DEAE-cellulose column (18.5cm×2.5cm), which was washed with 200ml of 16.7mM-KH_2PO_4/NaOH buffer, pH7.0. The enzyme was then eluted with a linear gradient from 0 to 0.3M-KCl in 1 litre of the buffer and 10ml fractions were collected. Fractions 52–58 inclusive were pooled and dialysed for 4h against 2 litres of 16.7mM-KH_2PO_4/NaOH buffer, pH7.0, with three changes of buffer. The enzyme was concentrated by loading on a small DEAE-cellulose column (3cm×2cm) and eluting with the phosphate buffer containing 0.5M-KCl, and then loaded on a Sephadex G-200 column (45cm×2.5cm). It was eluted with 50mM-KH_2PO_4/NaOH buffer, pH7.0. Fractions of 5ml were collected and fractions 33–38 were pooled. A summary of the purification is shown in Table 1.

Purification of p-cresol methylhydroxylase B

Crude extract from *p*-cresol-grown cells was heat-treated as described for *p*-cresol methylhydroxylase A. The extract was then loaded on a DEAE-cellulose column (12cm×2.5cm) and the enzyme washed straight through with 16.7mM-KH_2PO_4/NaOH buffer, pH7.0. The enzyme was concentrated by the gradual addition at 2°C of 0.48g of $(NH_4)_2SO_4$ per ml of extract followed by centrifuging at 25000*g* for 20min. The pellet of precipitated protein was redissolved in a small volume of 50mM-KH_2PO_4/NaOH buffer, pH7.0, and applied to a Sephadex G-200 column (45cm×2.5cm). The enzyme was eluted with the same buffer and 5ml fractions were collected.

Table 1. *Summary of the purification of p-cresol methylhydroxylase from Ps. putida N.C.I.B. 9869 grown on 3,5-xylenol (hydroxylase A)*

The starting material was 90g wet wt. of cells. One unit of enzyme activity is the amount that catalyses the uptake of ½μmol of O_2/min at 30°C in the O_2-monitor assay.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Crude extract	240	7900	1529	0.19	100	1
(2) Heat-treatment	210	1125	446	0.40	29.2	2.1
(3) DEAE-cellulose chromatography	70	77	407	5.28	26.6	27.7
(4) Sephadex G-200 gel filtration	30	19	189	9.95	12.4	52.3

Table 2. *Summary of the purification of p-cresol methylhydroxylase from Ps. putida N.C.I.B. 9869 grown on p-cresol (hydroxylase B)*

The starting material was 45g wet wt. of cells. One unit of enzyme activity is the amount that catalyses the uptake of ½μmol of O_2/min at 30°C in the O_2-monitor assay.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Crude extract	130	5410	1929	0.36	100	1
(2) Heat-treatment	110	908	470	0.52	24.4	1.4
(3) DEAE-cellulose chromatography	120	540	432	0.80	22.4	2.2
(4) Sephadex G-200 gel filtration	55	106	190	1.79	9.9	5.0
(5) Hydroxyapatite chromatography	40	5.6	38	6.78	2.0	18.8

Fractions 27–37 were pooled and dialysed against 1 litre of 40mM-KH_2PO_4/NaOH buffer, pH6.8, at 2°C for 4h with three changes of buffer. The extract was loaded on a hydroxyapatite column (13cm × 2.5cm) and the enzyme eluted with a linear gradient constructed from 350ml of 40mM-KH_2PO_4/NaOH buffer, pH6.8, and 350ml of 220mM-KH_2PO_4/NaOH buffer, pH6.8. Fractions of 5ml were collected and the protein in pooled fractions 75–80 was concentrated by addition of $(NH_4)_2SO_4$ as described before. The protein pellet was redissolved in 2ml of 50mM-KH_2PO_4/NaOH buffer, pH7.0, and dialysed for 3h at 2°C against 1 litre of the same buffer, which was changed twice. A summary of the purification is shown in Table 2.

Chemicals

CO was prepared by the controlled addition of formic acid to conc. H_2SO_4 (Vogel, 1956).

o-Cresol, *m*-cresol, *p*-cresol, 2,3-xylenol, 2,5-xylenol, 2,6-xylenol, 3,4-xylenol, *o*-hydroxybenzyl alcohol, DL-1-phenylethanol, Nitro Blue Tetrazolium, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride and phenazine methosulphate were from BDH, Poole, Dorset, U.K. 3,5-Xylenol, *p*-methoxyphenol, *p*-ethylphenol and 2,4-xylenol (redistilled before use) were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Amido Black 10B and 2,6-dichlorophenol-indophenol were from Hopkin and Williams, Chadwell Heath, Essex, U.K. Coomassie Brilliant Blue G-250 was from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany. 4-Hydroxybenzyl alcohol was from K & K Laboratories, Plainview, NY, U.S.A. *p*-Methylbenzyl alcohol was from NIPA Laboratories, Pontypridd, Glam., U.K. Bovine serum albumin (Cohn fraction V) was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Preswollen micro-granular DEAE-cellulose DE-52 was from W. R. Balston, Maidstone, Kent, U.K. Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Fumarase, glyceraldehyde 3-phosphate dehydrogenase, phosphorylase *a*, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, NAD^+, $NADP^+$ and glucose 6-phosphate were from Boehringer Corp., London W.5, U.K.

Results

Oxidation of compounds by whole cells

The oxidation of a number of compounds by washed-cell suspensions of *Ps. putida* N.C.I.B. 9869 grown on either 3,5-xylenol or *p*-cresol was followed in Warburg respirometers (Fig. 1). Although 3,5-

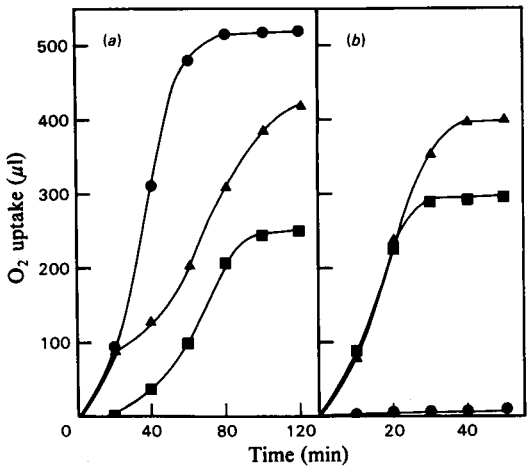

Fig. 1. *Oxidation of compounds by washed cells grown with (a) 3,5-xylenol and (b) p-cresol*

Each flask contained 1.5ml of bacterial suspension (5mg dry wt.) in 50mM-KH_2PO_4/NaOH buffer, pH7.0, 0.3ml of 10mM-substrate, tipped from the side arm and 0.2ml of 20% (w/v) KOH in the centre well. Substrates were: ●, 3,5-xylenol; ▲, *p*-cresol; ■, *p*-hydroxybenzoic acid. The temperature was 30°C. The gas phase was air. Oxygen uptakes in the absence of substrate [74μl/h for (*a*) and 54μl/h for (*b*)] have been subtracted.

xylenol-grown cells showed initial rapid oxidation of *p*-cresol as well as of growth substrate, oxidation of 3,5-xylenol by *p*-cresol-grown cells was very slow. Neither of these compounds was oxidized by succinate-grown cells.

p-Cresol methylhydroxylase activity in cell extracts

Crude cell-free extracts of the organism grown on 3,5-xylenol or *p*-cresol were centrifuged at 150000*g* for 60min at 4°C and tested in the Warburg apparatus for ability to oxidize *p*-cresol. There was only very slow oxidation of *p*-cresol above endogenous and the rate was not enhanced by inclusion of 6μmol of NAD^+, 6μmol of $NADP^+$, an NADH-generating system or an NADPH-generating system. Inclusion of 2μmol of phenazine methosulphate, however, resulted in very rapid oxidation of substrate. This phenazine methosulphate-stimulated oxidation of *p*-cresol, similar to that described by Hopper (1976), could be followed in the O_2 monitor and formed the basis of the routine enzyme assay. Similar activity was also found in the extract from *p*-cresol-grown cells, but not in that from succinate-grown cells.

Purification of p-cresol methylhydroxylase from 3,5-xylenol- and p-cresol-grown cells

Schemes for the purification of *p*-cresol methylhydroxylase from 3,5-xylenol-grown cells, referred to as hydroxylase A, and from *p*-cresol-grown cells, referred to as hydroxylase B, were developed and are described in the Materials and Methods section. Both procedures yielded red-coloured proteins active with both *p*-cresol and *p*-hydroxybenzyl alcohol.

Enzyme activity was stimulated approx. 2-fold in each case by the particles obtained from centrifuging crude extract at 150000*g* for 60min at 4°C or, in the case of *p*-cresol methylhydroxylase A, by the early fractions from DEAE-cellulose chromatography of crude extract. Heat treatment of the stimulatory material at 50°C for 1min resulted in complete loss of its activity, which accounts for the apparent high loss of enzyme units on heat treatment of crude extracts in the purifications.

Polyacrylamide-gel electrophoresis of p-cresol methylhydroxylases

After electrophoresis on polyacrylamide gels, hydroxylase B (100μg) gave a single band when stained for protein (Fig. 2) or when stained for enzyme activity with either *p*-cresol or *p*-hydroxybenzyl alcohol as substrate. Hydroxylase A (100μg), however, gave one major band followed by two other bands of decreasing intensity when gels were stained for protein (Fig. 2). None of these corresponded to hydroxylase B and all three bands appeared, with the same pattern of intensities, when gels were stained for enzyme activity, again with either *p*-cresol or *p*-hydroxybenzyl alcohol as substrates. A red-coloured band could be seen on electrophoresis of hydroxylase A corresponding to the major protein band. When this band was excised from a number of gels and extracted with 16.7mM-phosphate buffer, pH7.0, electrophoresis of the preparation on fresh gels again gave three bands. Similar treatment of the portion of gel 0.5cm behind the red band gave the same pattern. A mixture of hydroxylase A and B preparations gave a pattern equivalent to the sum of the separate proteins. A similar mixed pattern was seen when 10μl of heat-treated extract (5.4mg of protein/ml) from cells grown on a mixture of 3,5-xylenol and *p*-cresol (0.015%, w/v, of each) was subjected to electrophoresis and gels stained for enzyme activity.

Substrate specificity of p-cresol methylhydroxylases

Hydroxylases A and B showed similar substrate specificities and were active with a number of compounds (Table 3). Neither enzyme was active under these conditions with *m*-cresol, *o*-cresol, 2,3-xylenol,

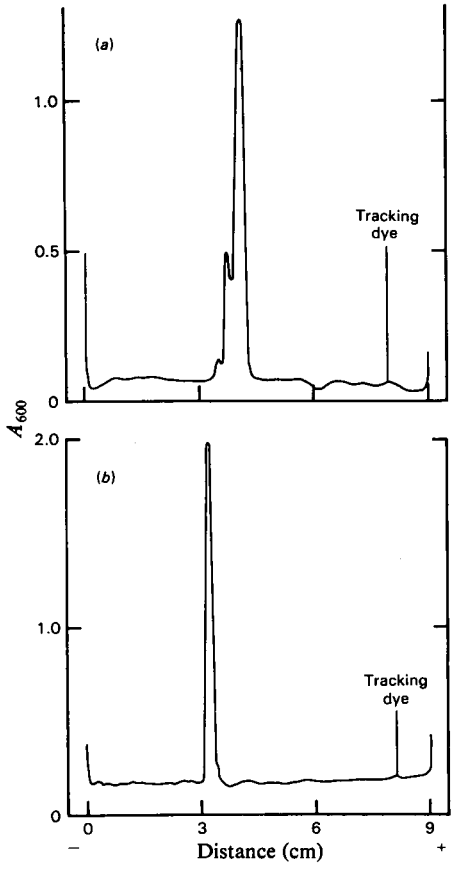

Fig. 2. *Scans of polyacrylamide gels after electrophoresis of purified p-cresol methylhydroxylases*
Gels were loaded with 100 μg of purified enzyme, hydroxylase A (*a*) and hydroxylase B (*b*), and after electrophoresis were stained for protein with Amido Black. They were then scanned at 600 nm.

2,5-xylenol, 2,6-xylenol, 3,5-xylenol, *m*-hydroxybenzyl alcohol, *o*-hydroxybenzyl alcohol, *p*-methoxyphenol, DL-1-phenylethanol, *p*-xylene, *p*-hydroxyacetophenone, *o*-hydroxyacetophenone or *p*-methylbenzyl alcohol.

Identification of products from aerobic and anaerobic incubations

p-Cresol and *p*-hydroxybenzyl alcohol were incubated aerobically in Warburg flasks with hydroxylase A in the presence of phenazine methosulphate. After incubation for 50 min at 30°C the contents of the flasks were acidified to pH 1.0 with 5 M-HCl, and the products were isolated. The products were chromatographed by t.l.c. on silica gel in solvent A and on paper in solvents C and D. In all systems the product from both *p*-cresol and *p*-hydroxybenzyl alcohol corresponded to authentic *p*-hydroxybenzaldehyde, with no trace of starting material.

Table 3. *Relative activities of p-cresol methylhydroxylases A and B with various substrates*
Substrates were tested by the O_2-monitor assay with 100 μg of hydroxylase A or 80 μg of hydroxylase B and 1.0 μmol of substrate. The values are given as a percentage of the rate with *p*-cresol.

Substrate	Hydroxylase activity A	B
p-Cresol	100	100
2,4-Xylenol	71	74
3,4-Xylenol	70	78
p-Hydroxybenzyl alcohol	69	81
p-Ethylphenol	66	55

Both hydroxylases A and B were active under anaerobic conditions. Addition of *p*-cresol or *p*-hydroxybenzyl alcohol from the side arm of a Thunberg cuvette, made anaerobic by alternate evacuation and filling with N_2 four times, to the reaction mixture for spectrophotometric assay resulted in a rapid decrease in A_{600} due to reduction of 2,6-dichlorophenol-indophenol. The presence of both enzyme and phenazine methosulphate were essential for this activity.

The anaerobic reaction mixtures were scaled up to obtain sufficient products for identification. Chromatography by t.l.c. and on paper in the same systems as before showed *p*-hydroxybenzaldehyde as the major product from *p*-cresol and *p*-hydroxybenzyl alcohol with both hydroxylases. A minor product from *p*-cresol corresponded to *p*-hydroxybenzyl alcohol. Further confirmation of *p*-hydroxybenzaldehyde as a product came from t.l.c. of the 2,4-dinitrophenylhydrazone of the product in solvent B, where it corresponded to the derivative from authentic material.

Kinetic studies

Spectrophotometric assays were performed with 14 μg of pure hydroxylase A or 17 μg of pure hydroxylase B per assay and a range of substrate concentrations from 3.3 to 13.3 μM. Double-reciprocal plots of the rate obtained and the substrate concentrations gave straight lines from which the K_m values for *p*-cresol and *p*-hydroxybenzyl alcohol were calculated. Hydroxylase A gave a K_m of 16 μM for *p*-cresol and 27 μM for *p*-hydroxybenzyl alcohol. The corresponding values for hydroxylase B were 3.6 and 15 μM.

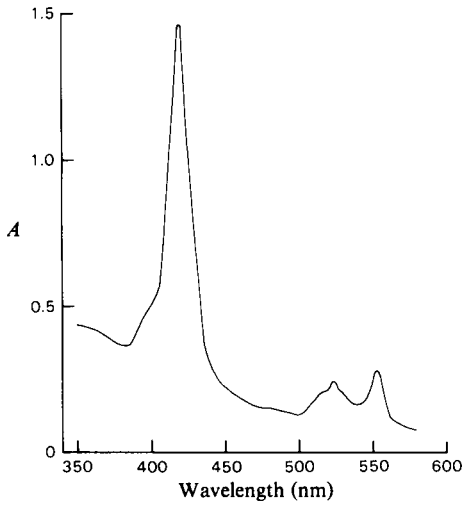

Fig. 3. *Absorption spectrum of hydroxylase A*
The spectrum of purified *p*-cresol methylhydroxylase A (1.3mg/ml) was recorded against a blank of 50mM-KH_2PO_4/NaOH buffer, pH7.0.

Molecular-weight determinations of p-cresol methylhydroxylases

A value of 114000 was obtained for the molecular weight of hydroxylase A by the sedimentation-equilibrium (meniscus-depletion) method. The value for hydroxylase B, from a similar experiment, but run at a lower tempe rature,was 99000. The respective values for hydroxylase A and B from their relative elution volumes (V_e/V_o) from a Sephadex G-200 column calibrated by plotting relative elution volumes of known proteins against log(molecular weight) (Andrews, 1965) were 108000 and 100000.

Electrophoresis of the enzymes, after incubation with sodium dodecyl sulphate and mercaptoethanol, on polyacrylamide gels containing sodium dodecyl sulphate gave single bands when stained for protein with Coomassie Brilliant Blue. Comparison of their mobilities relative to Bromophenol Blue with the mobilities of standard proteins gave mol.wts. of approx. 58000 for hydroxylase A and 56000 for hydroxylase B. The standard proteins were glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 37000), fumarase (mol.wt. 48500), bovine serum albumin (mol.wt. 68000) and phosphorylase *a* (subunit mol.wt. 94000).

Absorption spectra of p-cresol methylhydroxylases

The visible-absorption spectra of the two enzymes are very similar and typical of haemoproteins. The spectrum of hydroxylase A, shown in Fig. 3, has $\lambda_{max.}$ for α-, β- and δ-peaks of 553, 523 and 418nm respectively. The equivalent peaks for hydroxylase B are at 551, 523 and 418nm. These peaks also appeared in difference spectra of reduced against oxidized enzymes and the same spectra were produced by reducing oxidized enzyme with sodium dithionite or by adding the substrates *p*-cresol or *p*-hydroxybenzyl alcohol. No reduction occurred with 3,5-xylenol.

The enzymes behaved differently when attempts were made to prepare their alkaline pyridine haemochromagens. A solution of hydroxylase A (1ml containing 0.83mg of protein) was mixed with 1ml of 0.2M-NaOH/pyridine (3:1, v/v), and a few crystals of sodium dithionite were added. The α-, β- and δ-peaks appeared at 550, 520 and 414nm respectively with an A_{550} of 0.12. The spectrum is characteristic of a *c*-type cytochrome (Bartsch, 1971) and from the millimolar absorption coefficient of 31.18 litre·mmol^{-1}·cm^{-1} for the α-peak of the pyridine haemochrome of cytochrome *c* and a mol.wt. of 114000, the ratio of haem to protein for hydroxylase A was calculated as 1.06:1. When the same treatment was applied to hydroxylase B, reoxidation of the ferrohaemochromagen was too rapid for the spectrum to be recorded. However, a spectrum with an α-peak at 550nm was obtained when 1.5ml of purified enzyme (0.8mg of protein/ml) was mixed with 1.5ml of the NaOH/pyridine mixture and placed, with a few crystals of sodium dithionite, in a Thunberg cuvette which was alternately evacuated and flushed with argon three times.

Flavin content of p-cresol methylhydroxylase

A solution of hydroxylase A (6ml containing 0.71mg of protein/ml) was acidified to pH2.0 with 2M-HCl at 4°C and 3vol. of ice-cold acetone was added. The solution was centrifuged at 18000*g* for 15min at 4°C, yielding a yellow pellet that contained approx. half of the protein. The pellet did not dissolve in 1.0ml of 50mM-phosphate buffer, pH7.0, but, after acidification of the suspension to pH2.0 with 2M-HCl and incubation in the dark at 100°C for 3min followed by centrifugation at 18000*g* for 15min, some yellow colour remained in the supernatant. The spectrum (Fig. 4) suggests the presence of flavin and some haem. Addition of sodium dithionite resulted in the disappearance of the peak at 450nm, which was then restored by oxidation by bubbling air through the solution.

Attempts to dissociate flavin from pure enzyme, by incubating at 100°C for 5min or by adding $(NH_4)_2SO_4$ to 90% saturation followed by acidification to pH3, were unsuccessful. However, further evidence for flavin came from fluorescence studies on pure enzyme or on a tryptic digest of the yellow protein precipitated by acid/acetone. The latter was prepared by resuspending the pellet in 2ml of 50mM-phosphate buffer, pH7.0, and incubating at 38°C for

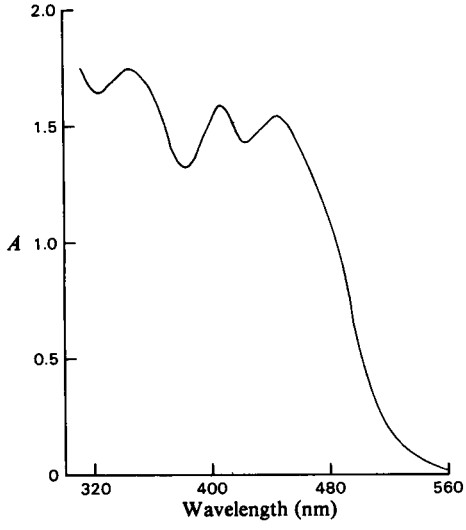

Fig. 4. *Absorption spectrum of yellow component of hydroxylase A after acetone/HCl extraction*
The spectrum of the material described in the text was recorded against a blank of 50mM-KH_2PO_4/NaOH buffer, pH7.0.

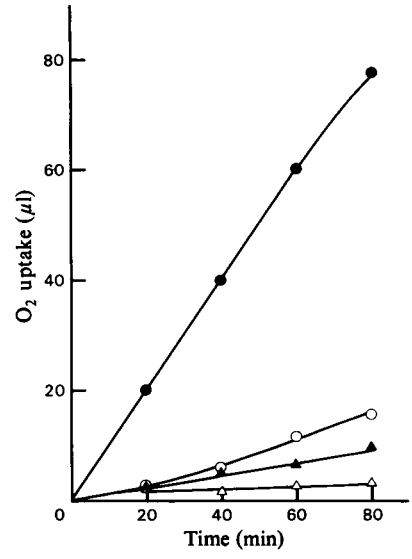

Fig. 5. *Oxidation of 3,5-xylenol by $(NH_4)_2SO_4$-treated extracts*
Warburg flasks contained $(NH_4)_2SO_4$-treated extract (40mg of protein) from 3,5-xylenol-grown cells and 6μmol of 3,5-xylenol in 2.8ml of 50mM-phosphate buffer, pH7.0. The centre well contained 0.2ml of 20% (w/v) KOH. Flasks also contained an NADH-generating system (●), NAD^+ (3μmol) (○), an NADPH-generating system (▲) or $NADP^+$ (3μmol) (△).

3h with 3mg of trypsin. Undigested protein was removed by heating at 100°C for 3min followed by centrifuging. The preparations gave a fluorescence emission peak at 523nm with maximum fluorescence when excited at 450nm and for the tryptic digest the excitation spectrum for emission at 520nm gave peaks at 465, 450, 380 and 295nm.

The peak at 465nm was much larger in the spectrum obtained with pure enzyme and is probably due to the presence of the haem group.

The acetone from the supernatant of the original extraction was evaporated with a stream of N_2, and the pyridine haemochromagen was prepared, as before, with the residue. This gave a spectrum with peaks at 550, 523 and 418nm.

A similar acid/acetone extraction of hydroxylase B again resulted in a yellow precipitate. Attempts to dissociate flavin were again unsuccessful, although the pure enzyme fluoresced at 525nm with a maximum when excited at 450nm and the excitation spectrum gave peaks at 465, 450, 380 and 295nm. Although the supernatant from acid/acetone extraction was red–brown in colour, attempts to prepare the alkaline pyridine haemochrome failed.

Oxidation of 3,5-*xylenol by cell extracts*

Measurements of the oxidation of 3,5-xylenol by extracts of cells grown on 3,5-xylenol were made in a Warburg apparatus. There was no increase in the rate of oxygen uptake above the endogenous rate when 6μmol of 3,5-xylenol was added to a reaction mixture (40mg of crude-extract protein in 2.8ml of 50mM-phosphate buffer, pH7.0) unless NAD^+ (3μmol) was included, when a rate of 600μl of O_2/h was measured compared with 260μl of O_2/h for a flask without substrate. This stimulation was not given by $NADP^+$ or an NADPH-generating system. When an NADH-generating system was used there was no significant oxidation above endogenous until after a lag of around 70min. However, when $(NH_4)_2SO_4$-treated extracts were used, an NADH-generating system was required for oxidation of 3,5-xylenol, and NAD^+ alone was not effective (Fig. 5). The rate after such a treatment was much less than that with crude extract. Attempts to assay the 3,5-xylenol hydroxylase in the oxygen monitor were unsuccessful.

Substrate specificity of 3,5-*xylenol hydroxylase*

The relative rates of oxygen uptake when four phenols were tested as substrates are shown in Table 4.

Table 4. *Relative rates of oxidation of phenols by $(NH_4)_2SO_4$-treated extracts*

Oxidation of substrates by $(NH_4)_2SO_4$-treated extracts from 3,5-xylenol-grown cells was followed in a Warburg apparatus. Flasks contained approx. 40mg of protein in 50mM-phosphate buffer, pH7.0, an NADH-generating system and 6μmol of substrate in a total volume of 2.8ml with 0.2ml of 20% (w/v) KOH in the centre well. The endogenous rate was subtracted from each and the rates relative to that for 3,5-xylenol are given.

Substrate	Relative rate
3,5-Xylenol	100
2,5-Xylenol	100
m-Cresol	18
p-Cresol	0

Inhibition of 3,5-*xylenol hydroxylase*

The oxidation of 3,5-xylenol by crude extracts supplemented with NAD^+ was completely inhibited by NaCN at a concentration of 0.5mM. No inhibition was observed when the atmosphere in the flask consisted of approx. 50% CO in air.

Discussion

The oxidation of both 3,5-xylenol and *p*-cresol by cells grown on 3,5-xylenol (Fig. 1) suggested the presence of a methylhydroxylase capable of attacking groups both *meta* and *para* to the hydroxy group. However, the more detailed study of these activities has shown that the initial hydroxylations are due to two very different enzymes.

The requirement of the 3,5-xylenol hydroxylase for NADH, as shown by experiments with $(NH_4)_2SO_4$-treated extracts, is typical of an enzyme of the monooxygenase type, and inhibition by cyanide but not CO suggests a system more like the octane hydroxylase of McKenna & Coon (1970) than the cytochrome *P*-450 system described by Jurtshuk & Cardini (1971). The apparent lag in 3,5-xylenol oxidation by crude extracts supplied with NADH may be an artefact and reflect high activities of alternative NADH-oxidizing systems. A similar observation was reported for a bacterial methane-oxidizing system and it was shown that addition of methane to extracts caused a redirection of electron flow to O_2 rather than any overall stimulation of the rate of O_2 consumption (Tonge *et al.*, 1974).

Extracts also oxidized other *m*-methyl-substituted phenols (Table 4), which is in accord with the partial oxidation of 2,5-xylenol to 3-hydroxy-4-methylbenzoate by this organism and the oxidation of *m*-cresol by 3,5-xylenol-grown cells (Hopper & Chapman, 1971). However, there was no activity with *p*-cresol and clearly this enzyme does not account for its oxidation by 3,5-xylenol-grown cells (Fig. 1).

Oxidation of the methyl group of *p*-cresol is initiated by a second hydroxylase with very different properties, closely resembling the enzyme described by Hopper & Taylor (1977). The requirement for an electron acceptor and the activity under anaerobic conditions suggests hydroxylation by dehydrogenation followed by hydration, by the mechanism proposed by Hopper (1976) rather than by an oxygenase enzyme. Such a mechanism requires the appropriate groups *para* to each other and all of the substrates for the enzyme have this orientation (Table 3). A similar scheme has been postulated for hydroxylation of α-conidendrin in its metabolism by *Pseudomonas multivorans*, although no enzymes have been demonstrated (Toms & Wood, 1970). *p*-Hydroxybenzyl alcohol was identified as a product, but a second dehydrogenation by the enzyme can convert this into *p*-hydroxybenzaldehyde, which was the major product under the conditions used. This second reaction is not necessarily of physiological importance, since cells also contain NAD^+-linked alcohol dehydrogenases (Keat & Hopper, 1976).

The *p*-cresol methylhydroxylase in 3,5-xylenol-grown cells (hydroxylase A) was purified, although electrophoresis on polyacrylamide gels gave three bands (Fig. 2). These were probably due to aggregation, since all three were active and a re-run of protein eluted from one band again gave three bands. Also, only one band was seen after electrophoresis on gels containing sodium dodecyl sulphate. A similar phenomenon has been reported by Pujar & Ribbons (1976) for 4,5-dihydroxyphthalate decarboxylase. The mol.wt. of 58000 for the hydroxylase from electrophoresis on sodium dodecyl sulphate-containing gels compared with 114000 by ultracentrifuge studies suggests there are two subunits of about equal molecular weight. One of these is a *c*-type cytochrome as judged by the spectrum of the alkaline pyridine haemochromogen (Bartsch, 1971) and a value of one haem per molecule of enzyme can be calculated. The cytochrome *c* subunit can be extracted with acid/acetone, although the peak at 413nm in the absorption spectrum of the yellow protein remaining after extraction indicates some residual haem. This also accounts for one of the peaks in the fluorescence excitation spectrum for this yellow protein. The remaining peaks in both spectra suggest the presence of flavin and, as is typical for a flavin, the peak at 450nm is lost on reduction. Attempts to dissociate the flavin were unsuccessful and possibly it is attached covalently to the second subunit. The involvement of the haem in the enzyme reaction was demonstrated by its reduction by substrate, and the enzyme is one of the class of proteins known as flavocytochrome *c* (Bartsch *et al.*, 1968).

A similar *p*-cresol methylhydroxylase (hydroxylase B) with the same specificity was induced by growth on *p*-cresol itself. It too converted *p*-cresol into the alcohol and aldehyde under anaerobic conditions with a requirement for an electron acceptor and again contained haem and flavin in two subunits of equal size. However, this does not appear to be the same enzyme as that in 3,5-xylenol-grown cells. The patterns of bands after electrophoresis of the purified enzymes are clearly different (Fig. 2), as also were the bands of activity after electrophoresis of crude extracts, which showed that both enzymes were induced by growth on a mixture of *p*-cresol and 3,5-xylenol. Hydroxylase B, unlike hydroxylase A, was not retained on the DEAE-cellulose column during purification and gave a lower molecular weight and slightly lower K_m values for *p*-cresol and *p*-hydroxybenzyl alcohol than hydroxylase A. The pyridine haemochromogen of hydroxylase B was reoxidized much more rapidly than that of hydroxylase A, making the recording of its spectrum more difficult.

Thus oxidation of *p*-cresol by 3,5-xylenol-grown cells is the result of gratuitous induction of a *p*-cresol methylhydroxylase. However, the physiological role of this enzyme is not known, since it is not present in *p*-cresol-grown cells, which contain a similar, but not identical, hydroxylase.

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References

Andrews, P. (1965) *Biochem. J.* **96**, 595–606

Bartsch, R. G. (1971) *Methods Enzymol.* **23**, 344–363

Bartsch, R. G., Meyer, T. E. & Robinson, A. B. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. & Sekuu, I., eds.), pp. 443–451, University of Tokyo Press, Tokyo

Byrne, G. A. (1965) *J. Chromatogr.* **20**, 528–540

Chapman, P. J. (1972) in *Degradation of Synthetic Organic Molecules in the Biosphere*, pp. 17–55, Printing and Publishing Office, National Academy of Sciences, Washington

Gholson, R. K., Baptist, J. N. & Coon, M. J. (1963) *Biochemistry* **2**, 1155–1159

Gordon, A. H. (1969) in *Electrophoresis of Proteins in Polyacrylamide and Starch Gels* (Work, T. S. & Work, E., eds.), pp. 9–13, North-Holland, Amsterdam and London

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Hopper, D. J. (1976) *Biochem. Biophys. Res. Commun.* **69**, 462–468

Hopper, D. J. & Chapman, P. J. (1971) *Biochem. J.* **122**, 19–28

Hopper, D. J. & Taylor, D. G. (1973) *J. Bacteriol.* **122**, 1–6

Hopper, D. J. & Taylor, D. G. (1977) *Biochem. J.* **167**, 155–162

Jurtshuk, P. & Cardini, G. E. (1971) *C.R.C. Crit. Rev. Microbiol.* **1**, 239–289

Keat, M. J. & Hopper, D. J. (1976) *Biochem. Soc. Trans.* **4**, 494–495

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

McKenna, E. J. & Coon, M. J. (1970) *J. Biol. Chem.* **245**, 3882–3889

Nozaka, J. & Kusunose, M. (1968) *Agric. Biol. Chem.* **32**, 1033–1039

Ornstein, L. & Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 305–650

Pastuska, G. (1961) *Z. Anal. Chem.* **179**, 355–358

Pujar, B. G. & Ribbons, D. W. (1976) *Abstr. Am. Soc. Microbiol. Annu. Meet. 76th* 145

Rosenberger, R. F. & Elsden, S. R. (1960) *J. Gen. Microbiol.* **22**, 726–739

Ruettinger, R. T., Griffith, G. R. & Coon, M. J. (1977) *Arch. Biochem. Biophys.* **183**, 528–537

Smith, I. (1960) *Chromatographic and Electrophoretic Techniques*, 2nd edn., vol. 1, pp. 291–298, W. Heinemann, London

Toms, A. & Wood, J. M. (1970) *Biochemistry* **9**, 733–740

Tonge, G. M., Harrison, D. E. F., Knowles, C. J. & Higgins, I. J. (1974) *FEBS Lett.* **58**, 293–299

Vogel, A. I. (1956) *Practical Organic Chemistry*, 3rd edn., p. 185, Longman, Green and Co., London

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317