Journal of General Microbiology (1987), **133**, 2137–2146. *Printed in Great Britain*

Hydroquinone as the Ring-fission Substrate in the Catabolism of 4-Ethylphenol and 4-Hydroxyacetophenone by *Pseudomonas putida* JD1

By JACQUELINE M. DARBY, DAVID G. TAYLOR AND DAVID J. HOPPER*

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth, Dyfed SY23 3DD, UK

(*Received 8 December 1986; revised 2 March 1987*)

A bacterium capable of growth on 4-ethylphenol was isolated from soil and identified as *Pseudomonas putida*. Intact cells grown on 4-ethylphenol rapidly oxidized 4-hydroxyacetophenone as well as growth substrate and the bacterium was also capable of growth on 4-hydroxyacetophenone. The initial enzymes for 4-ethylphenol catabolism were still present, although at lower activities, in succinate-grown cells which oxidized 4-ethylphenol to 4-hydroxyacetophenone. Extracts of 4-ethylphenol-grown cells oxidized 4-hydroxyacetophenone when provided with NADPH. When this activity was partially purified a stoichiometry of 1 μmol O_2 consumed per μmol of substrate was observed with the production of hydroquinone as required for a monooxygenase producing 4-hydroxyphenyl acetate followed by hydrolysis by an esterase. Cell extracts contained esterase activity and hydrolysed 4-hydroxyphenyl acetate to yield hydroquinone. Intact cells converted the analogue, acetophenone, into phenol. Hydroquinone served as the ring-fission substrate and was cleaved by an O_2-requiring reaction. The enzymes of the proposed pathway were induced by growth on 4-ethylphenol or 4-hydroxyacetophenone.

INTRODUCTION

The bacterial metabolism of alkyl-substituted phenols may proceed by initial attack on the alkyl group or, in some cases, the side-chain may be retained intact until after ring fission. This is illustrated by the aerobic pathways that have been described for *p*-cresol metabolism, which include oxidation of the methyl group to carboxyl with eventual formation of protocatechuic acid as ring-fission substrate, or initial hydroxylation of the ring to give 4-methylcatechol which is then cleaved (Dagley & Patel, 1957; Bayly *et al.*, 1966). Similar possibilities exist for metabolism of the higher homologue, 4-ethylphenol, which, like *p*-cresol, occurs naturally, often as the product of anaerobic conversions of aromatic compounds (Martin, 1982). However, if attack is on the alkyl group very different compounds will be formed and different pathways initiated depending on which of the carbons in the side-chain is oxidized.

The methylene group in the side-chain of 4-ethylphenol is the site for hydroxylation by *Pseudomonas putida* NCIB 9869, which converts the substrate into 1-(4′-hydroxyphenyl)ethanol and can oxidize this slowly to 4-hydroxyacetophenone (McIntire *et al.*, 1984). The enzyme involved is *p*-cresol methylhydroxylase which has a sufficiently broad specificity to enable it to hydroxylate 4-ethylphenol although the K_m for this substrate is much higher than that for *p*-cresol (McIntire *et al.*, 1985). A notable feature of this enzyme is that it acts by dehydrogenation of the substrate followed by hydration (Hopper, 1978) and will also catalyse the dehydrogenation of the alcohol produced, to give 4-hydroxyacetophenone from 4-ethylphenol. This compound was not metabolized further by *P. putida* NCIB 9869 but a pathway for its metabolism by an *Alcaligenes* sp. involving 4-hydroxybenzoic acid and protocatechuic acid has been described (Hopper *et al.*, 1985) (pathway 1, Fig. 1). To see if any bacteria use a *p*-cresol methylhydroxylase

type of enzyme to initiate complete 4-ethylphenol catabolism, several organisms capable of growth on this compound were isolated. The pathway used by one of these, involving attack on the alkyl group and with the uncommon ring-fission substrate, hydroquinone, as intermediate, is described in this paper.

METHODS

Organism and growth. The bacterium, strain JD1, is a short Gram-negative rod, 1–2 × 0·7 μm, with a polar flagellum. It gives a fluorescent green colour when grown on King's B medium and its properties in a range of standard cytological and biochemical tests were in agreement with its identification as a *Pseudomonas putida*. Bacteria were grown and harvested as described by Hopper *et al.* (1985). Aromatic carbon sources were at an initial concentration of 0·5 g l^{-1} and sodium succinate at 1·0 g l^{-1}.

Preparation of bacterial extracts. Crude cell extract was prepared by the procedure described previously (Hopper *et al.*, 1985). Where indicated, this was dialysed for 3 h against 30 vols 42 mM-sodium/potassium phosphate buffer pH 7·0, with hourly changes of the buffer.

DEAE-cellulose chromatography of crude extract. The 4-hydroxyacetophenone monooxygenase was partially purified by chromatography of crude extract on DEAE-cellulose (DE52). Crude extract (11 ml containing 550 mg protein) was loaded onto a DE52 column (4·5 × 2·5 cm) equilibrated with 42 mM-sodium/potassium phosphate buffer pH 7·0. It was washed with two column volumes of buffer and then eluted with a linear gradient of KCl from 0 to 0·3 M in 200 ml buffer. Fractions of 2·8 ml were collected and those containing the enzyme, fractions 21–25, were pooled.

Chromatography. TLC was done using precoated silica-gel GHLF plates (Analtech) with solvents A [toluene/dioxan/acetic acid (90:25:4, by vol.)], B [light petroleum (b.p. 60–80 °C)/ethyl formate/propionic acid (70:30:15·4, by vol.)] and C [chloroform/methanol (20:1, v/v)]. Aromatic compounds were located by viewing under UV light for materials that quenched the fluorescence of the indicator in the plates. Phenols were located by spraying with Folin–Ciocalteu's phenol reagent followed by exposure to NH_3. Ketones were located by spraying with 0·1% (w/v) 2,4-dinitrophenylhydrazine in 2 M-HCl.

GLC was performed on a Carlo-Erba HRGC 5300 (Mega Series) instrument using a WCOT fused silica CP-Wax-52 CB capillary column (25 m × 0·32 mm i.d.). Hydrogen was the carrier gas at a flow rate of 1·5 ml min^{-1}. The temperature programme started at 45 °C rising to 80 °C at a rate of 25 °C min^{-1} then to 150 °C at 10 °C min^{-1} and finally rising at the maximum rate to 200 °C where it was held for 10 min.

Spectra. UV absorption spectra were recorded with a Unicam SP8-150 spectrophotometer. IR spectra of samples mulled in Nujol were recorded with a Perkin–Elmer Infracord spectrophotometer.

O_2 consumption. Oxidations of substrates by intact cells and cell-free extracts were followed in either a conventional Warburg apparatus or an O_2 monitor (Yellow Springs Instrument Co.). For studies with intact cells, Warburg flasks contained 5·0 mg dry wt bacteria in 1·5 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, 0·3 ml 10 mM-substrate tipped in from the sidearm and 0·2 ml 20% (w/v) KOH in the centre well. O_2 uptake was measured at 30 °C. In other experiments the intact cells were replaced by cell-free extract and when an NADH-generating system was required 1 μmol NAD^+, 20 μl ethanol and 0·1 mg alcohol dehydrogenase (35·2 units) were included in the reaction mixture. For an NADPH-generating system 1 μmol $NADP^+$, 10 μmol glucose 6-phosphate and 3 units of glucose 6-phosphate dehydrogenase were used.

2,4-Dinitrophenylhydrazones. 2,4-Dinitrophenylhydrazones of reaction products were prepared as described by Cripps *et al.* (1978).

Assays. 4-Ethylphenol 2′-hydroxylase and 1-(4′-hydroxyphenyl)ethanol dehydrogenase were assayed as described for *p*-cresol methylhydroxylase by McIntire *et al.* (1985) but with 4-ethylphenol or 1-(4′-hydroxyphenyl)ethanol as substrate instead of *p*-cresol.

(4-Hydroxybenzoyl)methanol oxygenase was assayed as described by Hopper & Elmorsi (1984).

4-Hydroxybenzoate hydroxylase and protocatechuate oxygenase were assayed as described by Hopper *et al.* (1985). For catechol oxygenase, catechol replaced protocatechuate as substrate.

(4-Hydroxybenzoyl)formate decarboxylase was assayed by measuring CO_2 production in the Warburg apparatus as described by Kennedy & Fewson (1968).

Esterase activity was demonstrated with a pH-stat as described by Norris & Trudgill (1971) but with 4-hydroxyphenylacetate or methyl 4-hydroxybenzoate as substrates instead of ε-hexanolactone and with 4 mg crude extract protein. 4-Hydroxyphenyl acetate hydrolase was assayed spectrophotometrically at 30 °C. The reaction mixture, in a 1 cm pathlength cuvette, consisted of 3 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, containing 2 μmol 4-hydroxyphenyl acetate. This was made anaerobic by bubbling with N_2 and was sealed with a rubber cap. Reaction was started by injection of extract and the increase in A_{289} was followed. An increase of 0·77 was equivalent to production of 1 μmol hydroquinone.

4-Hydroxyacetophenone monooxygenase was assayed spectrophotometrically at 30 °C by following the

decrease in A_{370} due to oxidation of NADPH. The reaction mixture contained, in 1 ml 50 mM-Tris/HCl pH 8·0, 0·25 μmol NADPH, 1 μmol 4-hydroxyacetophenone and extract.

Hydroquinone oxygenase was assayed by following O_2 consumption in the O_2 monitor at 30 °C. The 3 ml reaction mixture contained 0·1 M-sodium/potassium phosphate buffer pH 7·5, 2 μmol hydroquinone and cell-free extract.

Attempts to demonstrate various NAD^+- or $NADP^+$-linked dehydrogenases spectrophotometrically were made by adding crude extract to 3 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, containing 1 μmol of the appropriate substrate and 1 μmol NAD^+ or $NADP^+$ in a 1 cm pathlength cuvette and following the increase in A_{340} or A_{370} ($\varepsilon_{370} = 2{\cdot}86 \times 10^3\ M^{-1}\ cm^{-1}$). Phenol was assayed by incubating 1 ml of sample with 5 ml 2% (w/v) Na_2CO_3 in 0·1 M-NaOH and 0·5 ml 50% (v/v) Folin–Ciocalteu's phenol reagent in water, at 30 °C for 30 min and then measuring the A_{750}.

Protein was assayed by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

Chemicals. (4-Hydroxybenzoyl)methanol was prepared by the procedure of Robertson & Robinson (1928). (4-Hydroxybenzoyl)formaldehyde was prepared by the SeO_2 oxidation of 4-hydroxyacetophenone by following the procedure described by Vogel (1956) for phenylglyoxal preparation from acetophenone. (4-Hydroxybenzoyl)formic acid was prepared by the cold-permanganate oxidation of 4-hydroxyacetophenone. Benzoylmethanol was prepared by treatment of phenacylbromide with acetic acid in triethylamine followed by controlled hydrolysis of the benzoylmethyl acetate produced. Phenyl acetate and methyl benzoate were prepared as described by Vogel (1956). The procedure for phenyl acetate was also used for 4-hydroxyphenyl acetate starting with hydroquinone instead of phenol but with dropwise addition of acetic anhydride to an excess of hydroquinone. Methyl 4-hydroxybenzoate, acetophenone, 4-hydroxybenzoic acid, protocatechuic acid, catechol, hydroquinone and 1-phenylethanol were from BDH. 4-Ethylphenol, 4-hydroxyacetophenone and 4-hydroxyphenylacetic acid were from Aldrich. 4-Methylcatechol was from R. Emanuel. Homogentisic acid was from Sigma and 1-(4′-hydroxyphenyl)ethanol was from NIPA Labs.

RESULTS AND DISCUSSION

Isolation of bacteria

Four bacterial strains were isolated from different soil samples by elective culture in liquid medium containing 4·1 mM-4-ethylphenol as sole carbon source. These were tested in the O_2 monitor for their abilities to oxidize 4-methylcatechol, catechol and protocatechuic acid after growth on 4-ethylphenol. Rapid oxidation of 4-methylcatechol and catechol but not protocatechuic acid was taken as an indication that the probable route for metabolism of 4-ethylphenol was by hydroxylation of the ring to give 4-ethylcatechol as the ring-fission substrate. Although this actual intermediate was not tested, catechol 2,3-oxygenases often have a broad specificity (Bayly *et al.*, 1966; Clark & Slater, 1986) and the 4-ethylcatechol oxygenase might be expected to be active with 4-methylcatechol and catechol. Three of the four strains appeared to use this pathway. The fourth strain, JD1, oxidized 4-methylcatechol only slowly and catechol not at all but there was a slow oxidation of protocatechuic acid. It apparently used a different pathway for catabolism of 4-ethylphenol and was chosen for further study. It was identified as *Pseudomonas putida*.

Oxidations by intact cells

4-Ethylphenol grown cells of *P. putida* JD1, in a Warburg apparatus, oxidized both 1-(4′-hydroxyphenyl)ethanol and 4-hydroxyacetophenone immediately at rates similar to that for the growth substrate of 3·75 μmol O_2 h^{-1} (mg dry wt)$^{-1}$ but there was no oxidation of 4-hydroxyphenylacetic acid or homogentisic acid. This suggested an initial attack on 4-ethylphenol by oxidation of the methylene group of the side-chain rather than the methyl. Protocatechuic acid, a possible ring-fission substrate in the pathway, was oxidized immediately but only slowly at first [0·8 μmol O_2 h^{-1} (mg dry wt)$^{-1}$] with the rate increasing during the incubation, a pattern typical of enzyme induction.

Lactate- or succinate-grown bacteria also oxidized 4-ethylphenol, although at only about a third of the rate for 4-ethylphenol-grown bacteria and with a lower final consumption approaching only 1 μmol O_2 (μmol substrate)$^{-1}$, but they did not oxidize 4-hydroxyacetophenone.

Table 1. *Specific activities of enzymes in cell-free extracts*

Crude extracts from 4-ethylphenol-grown (19 mg protein ml^{-1}), 4 hydroxyacetophenone-grown (14·8 mg protein ml^{-1}), 4-hydroxybenzoate-grown (14·3 mg protein ml^{-1}) and succinate-grown (17·2 mg protein ml^{-1}) bacteria were assayed as described in Methods. Specific activities are expressed as μmol substrate transformed min^{-1} (mg protein)$^{-1}$.

Enzyme	Specific activity in extracts of bacteria grown on: 4-Ethylphenol	4-Hydroxyacetophenone	4-Hydroxybenzoate	Succinate
4-Ethylphenol 2′-hydroxylase	0·15	0·22	0·064	0·064
1-(4′-Hydroxyphenyl)ethanol dehydrogenase	0·083	0·055	0·05	0·034
4-Hydroxyacetophenone monooxygenase	0·1	0·063	<0·0001	<0·0001
4-Hydroxyphenyl acetate hydrolase	0·15	0·28	0·0013	0·0024
Hydroquinone oxygenase	0·94	1·31	0·006	0·0011
4-Hydroxybenzoate hydroxylase	0·002	0·0013	0·54	<0·0001
Protocatechuate oxygenase	0·0086	0·0022	2·76	0·003
Catechol oxygenase	<0·0001	<0·0001	0·047	<0·0001

Product of 4-ethylphenol oxidation by lactate-grown bacteria

To obtain sufficient product for identification from the limited oxidation of 4-ethylphenol by lactate-grown bacteria, 90 μmol substrate was added to a suspension of bacteria (168 mg dry wt) in 40 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, in a 100 ml Erlenmeyer flask. This was incubated for 80 min on an orbital shaker at 30 °C. The bacteria were then removed by centrifuging and the supernatant solution was acidified to pH 1 with 5 M-HCl and extracted twice with an equal volume of diethyl ether. The combined extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness.

TLC of the product using solvents A and B gave a single spot, when viewed under UV light, corresponding to 4-hydroxyacetophenone (R_F 0·54 and 0·35 in A and B respectively) with no trace of starting material (R_F 0·7 and 0·93). Both the product and 4-hydroxyacetophenone gave orange spots when sprayed with 2,4-dinitrophenylhydrazine, which reacts with aldehydes and ketones. The product, crystallized from light petroleum (b.p. 100–120 °C), gave UV and IR spectra identical to those for authentic 4-hydroxyacetophenone.

In a similar experiment with 1-(4′-hydroxyphenyl)ethanol as the starting material 4-hydroxyacetophenone was again identified as the product. This ketone will also serve as growth substrate for the organism, resulting in the same pattern of enzyme induction as after growth on 4-ethylphenol (Table 1). These results confirm the earlier suggestion that 1-(4′-hydroxyphenyl)-ethanol and 4-hydroxyacetophenone are the early intermediates of 4-ethylphenol catabolism.

Activities in extracts

The oxidation of NADH or NADPH by cell extracts, assayed spectrophotometrically, was not stimulated by addition of 4-ethylphenol as would have been expected for a monooxygenase type of hydroxylase utilizing one of these cofactors. Nor was there evidence, again from spectrophotometric assays, for an NAD^+- or $NADP^+$-linked dehydrogenase for 1-(4′-hydroxyphenyl)ethanol. However, when extracts were assayed for a *p*-cresol methylhydroxylase-type of enzyme, which hydroxylates by dehydrogenation followed by hydration, the results were positive with either 4-ethylphenol or 1-(4′-hydroxyphenyl)ethanol as substrate (Table 1). Such an enzyme requires an electron acceptor, which in the assay was phenazine methosulphate. *p*-Cresol methylhydroxylase from *P. putida* NCIB 9869 can oxidize 4-ethylphenol to 4-hydroxyacetophenone and it seems likely that a similar enzyme catalyses the first two steps in 4-ethylphenol metabolism in *P. putida* JD1.

Hydroxylase was also detected in succinate-grown cells, although at a lower specific activity (Table 1), in agreement with the lower rate of oxidation of 4-ethylphenol seen for intact cells grown on non-aromatic compounds. The stoichiometry of 1 μmol O_2 required for the conversion of 1 μmol 4-ethylphenol into 4-hydroxyacetophenone by the lactate-grown bacteria is also in agreement with the occurrence of two dehydrogenation reactions in the process.

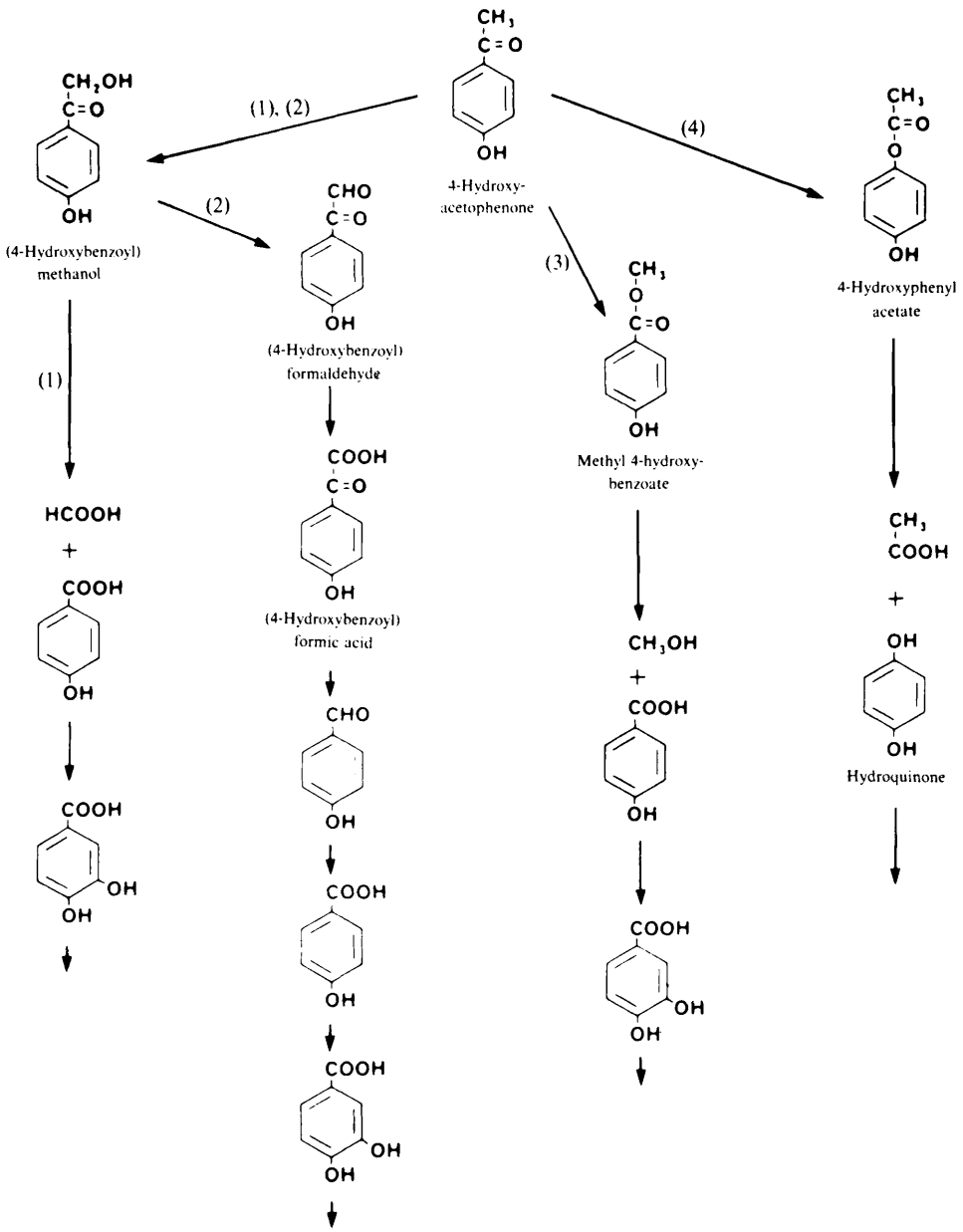

Fig. 1. Some possible pathways for the catabolism of 4-hydroxyacetophenone with a monooxygenase-catalysed first step.

Oxidation of 4-hydroxyacetophenone by cell extracts

Addition of NADPH to extracts of *P. putida* JD1 grown on 4-ethylphenol and 4-hydroxyacetophenone, in either the Warburg apparatus or O_2 monitor, resulted in consumption of O_2, indicative of attack by a monooxygenase. There was no oxidation if NADH replaced NADPH. Some possible pathways for the catabolism of 4-hydroxyacetophenone to ring-fission substrates, in which the first step is catalysed by a monooxygenase, are shown in Fig. 1.

Pathways (1) and (2) involve hydroxylation of the methyl group of 4-hydroxyacetophenone to give (4-hydroxybenzoyl)methanol. Pathway (1) was shown by Hopper *et al.* (1985) to occur in an

Table 2. *GLC of product from acetophenone oxidation*

The compound isolated after acetophenone oxidation by intact bacteria was identified by comparison of its retention time on GLC with retention times for putative products. GLC was performed as described in Methods.

Compound	Retention time (min)
Acetophenone	8·2
Product	10·6
Phenol	10·6
Methyl benzoate	8·05
Benzoylmethanol	12·4
1-Phenylethanol	8·8
Phenyl acetate	8·0

Alcaligenes sp. although in this organism NADH was the preferred cofactor for the hydroxylase. Pathway (2) involves complete oxidation of the methyl group to give (4-hydroxybenzoyl)formate, a known intermediate in the catabolism of 4-hydroxymandelic acid, where it undergoes decarboxylation to 4-hydroxybenzaldehyde (Gunter, 1953; Kennedy & Fewson, 1968). Both of these putative pathways lead to 4-hydroxybenzoic acid with protocatechuic acid as the ring-fission substrate, but although intact cells of *P. putida* JD1 grown on 4-ethylphenol oxidized both of these compounds the rates were low. Furthermore, when 4-hydroxybenzoate hydroxylase and protocatechuate oxygenase activities were measured in cell extracts, their specific activities in 4-ethylphenol- or 4-hydroxyacetophenone-grown bacteria were considerably lower than after growth on 4-hydroxybenzoic acid (Table 1). No (4-hydroxybenzoyl)methanol oxygenase, required for pathway (1), was detected in cell extracts nor was there any (4-hydroxybenzoyl) formate decarboxylase, or $NAD(P)^+$-linked dehydrogenases active with (4-hydroxybenzoyl) methanol or (4-hydroxybenzoyl)formaldehyde, as would be required for pathway (2).

Pathways (3) and (4) both involve the formation of an ester since the first step is catalysed by a monooxygenase that inserts an oxygen atom between two carbons, one of which bears a ketonic group. This type of reaction for the metabolism of ketonic compounds has a number of precedents (Rahim & Sih, 1966; Forney & Markovetz, 1969; Norris & Trudgill, 1971) including the conversion of acetophenone into phenyl acetate (Cripps, 1975; Cripps *et al.*, 1978). Pathway (3) can be ruled out for *P. putida* JD1 as it again leads to 4-hydroxybenzoic and protocatechuic acids and, in addition, no esterase activity towards methyl 4-hydroxybenzoate was detected in cell extracts.

Conversion of acetophenone to phenol by intact cells

The first indication that pathway (4) (Fig. 1) might be operating came from the isolation and identification of the product from the limited oxidation by intact cells of a substrate analogue, acetophenone. As oxidation was slow, sufficient chloramphenicol was added to inhibit any enzyme synthesis during the incubation. Samples were removed from the incubation mixture at intervals over a period of several hours. Bacteria were removed from each sample by centrifuging and the supernatant solution was extracted twice with an equal volume of diethyl ether. The extracts were dried over anhydrous Na_2SO_4 and examined by GLC. The initial peak for acetophenone gradually decreased over the course of the incubation, concomitant with the appearance and increase of a second peak, which had the same retention time as phenol and was clearly separated from other putative products (Table 2).

After 6 h the bacteria were removed from the remainder of the incubation mixture which was then extracted as before. The diethyl ether extract was evaporated to dryness in a test tube and the product was purified by gently heating the bottom of the tube, resulting in evaporation of material and its condensation higher up on the sides of the tube. This material gave a single peak on GLC when mixed with authentic phenol and also gave UV and IR spectra identical with those for phenol.

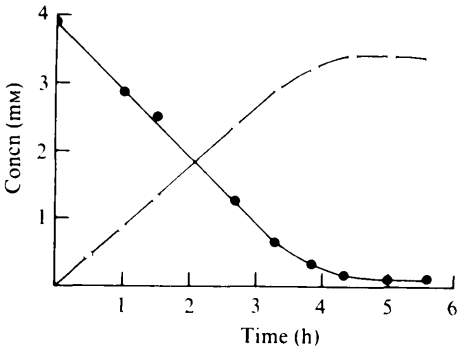

Fig. 2. Conversion of acetophenone into phenol by intact cells. A suspension of 4-ethylphenol-grown *P. putida* JD1 (180 mg dry wt) in 100 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, containing 50 μg chloramphenicol ml^{-1}, in a 250 ml Erlenmeyer flask was incubated on an orbital shaker at 30 °C. An addition of 47 mg acetophenone was made and samples were removed at intervals. Bacteria were removed from samples by centrifuging and the supernatant solutions were assayed for phenol (○) and acetophenone (●).

In a similar experiment, phenol production was followed quantitatively (Fig. 2). Phenol appearance showed good stoichiometry with acetophenone disappearance and it was clearly the major product rather than the result of a minor secondary transformation. This conversion has been described as part of the pathway for acetophenone metabolism in a species of *Arthrobacter* (Cripps, 1975) where it is catalysed by an ester-forming monooxygenase to give phenyl acetate which is then hydrolysed by an esterase to phenol and acetic acid.

Formation of hydroquinone

By analogy with this conversion of acetophenone into phenol, 4-hydroxyacetophenone would yield hydroquinone (1,4-dihydroxybenzene) with the intermediate formation of hydroxyphenyl acetate as shown in pathway 4 (Fig. 1). In support of this it was shown that growth of *P. putida* JD1 on either 4-ethylphenol or 4-hydroxyacetophenone resulted in induction of the enzymes 4-hydroxyphenyl acetate hydrolase and hydroquinone oxygenase (Table 1).

The presence of the hydroquinone oxygenase precluded the isolation of hydroquinone produced from 4-hydroxyacetophenone by intact cells or crude cell extracts and, therefore, the 4-hydroxyacetophenone monooxygenase was partially purified by chromatography of crude extract on DEAE-cellulose. This preparation was free from hydroquinone oxygenase but still contained esterase activity. A portion was used to oxidize 3 μmol 4-hydroxyacetophenone in a Warburg apparatus in a reaction mixture containing an excess of NADPH (5 μmol). Oxidation ceased after the consumption of 1 μmol O_2 (μmol substrate)$^{-1}$. The flask contents were extracted twice with an equal volume of diethyl ether and the pooled extracts dried over anhydrous Na_2SO_4 and then evaporated to dryness. When examined by TLC in solvents A and B, the product gave a single spot corresponding to hydroquinone (R_F 0·41 and 0·51 in A and B respectively). Like hydroquinone it gave a blue colour immediately with Folin–Ciocalteu's phenol reagent, characteristic of a dihydric phenol. There was no trace of starting material (R_F 0·53 and 0·62) or 4-hydroxyphenyl acetate (R_F 0·62 and 0·65), both of which required exposure to NH_3 for development of the blue colour, as expected for a monohydroxy phenol.

To demonstrate that hydroquinone was also produced from 4-hydroxyphenyl acetate this substrate was incubated with cell extract under anaerobic conditions to prevent further oxidation of the product by the hydroquinone oxygenase. A Thunberg tube containing 40 μmol 4-hydroxyphenyl acetate in 4 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, with 1 ml extract (44 mg protein) in the stopper, was alternately evacuated and filled with N_2 several times and finally left under vacuum. The cell extract was tipped in and the tube was incubated at 30 °C

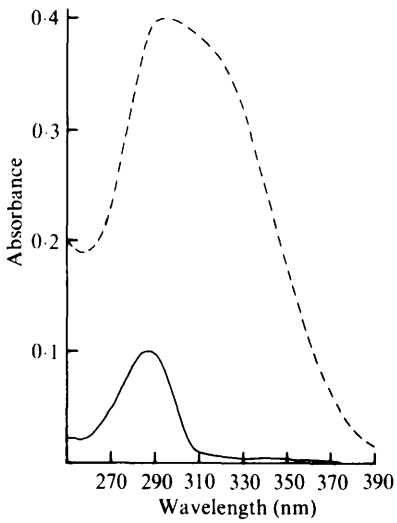

Fig. 3. Spectra of hydroquinone (——) and the product of its oxidation by dialysed crude cell-extract (– – –). A 1 cm pathlength cuvette contained 0·1 μmol hydroquinone in 3 ml 42 mM-sodium/potassium phosphate buffer pH 7·0. The spectrum was recorded against a buffer blank; 10 μl of dialysed crude extract was added to each cuvette. When there was no further change the spectrum of the product was recorded.

for 30 min. The reaction mixture was then extracted twice with an equal volume of diethyl ether. The combined extracts were dried over anhydrous Na_2SO_4, evaporated to dryness and the product examined by TLC in solvents A and C. As a control, a second tube with extract that had been heated at 100 °C for 5 min was treated in the same way. The product from the first tube gave a single spot on TLC corresponding to the hydroquinone standard when viewed under UV light. Both gave a blue colour when sprayed with Folin–Ciocalteu's phenol reagent before exposure to NH_3. There was no trace of starting material which required NH_3 before giving a blue colour with the detection reagent. The product from the control tube corresponded to the starting material with only the trace of hydroquinone present as an impurity in the original 4-hydroxyphenyl acetate.

Cleavage of hydroquinone

Crude extract of *P. putida* JD1 catalysed rapid oxidation of hydroquinone when assayed in the O_2 monitor (Table 1). Oxidation of hydroquinone was also followed spectrophotometrically by repeated scanning of the spectrum when crude extract was added to a cuvette containing hydroquinone. There was a rapid increase in absorbance with a slight shift in λ_{max} to give a spectrum (Fig. 3) similar to that reported by Larway (1965) for the ring-fission product from hydroquinone. This he identified as γ-hydroxymuconic semialdehyde for which he reported a λ_{max} of 293 nm at pH 7·0.

The requirement for oxygen in the process was demonstrated with a similar reaction mixture made anaerobic by gassing with O_2-free argon and sealing the cuvette with a rubber cap. Injection of extract through the cap resulted in very little change in the spectrum but on introduction of O_2 there was a rapid increase in absorbance as before. Addition of a small amount of NAD^+ to the cuvette resulted in complete abolition of the spectrum, and this is in accord with the report by Larway (1965) that the γ-hydroxymuconic semialdehyde is converted into β-oxoadipic acid by cell extract in the presence of NAD^+. This was confirmed by identifying the product from the oxidation of hydroquinone in the presence of NAD^+. A reaction mixture containing 5 ml crude extract, 5 ml 42 mM-sodium/potassium phosphate buffer pH 7·0, 5 μmol NAD^+ and 25 μmol hydroquinone was incubated with shaking at 30 °C for 30 min. A small portion of the reaction mixture was then tested for ketones by the Rothera test

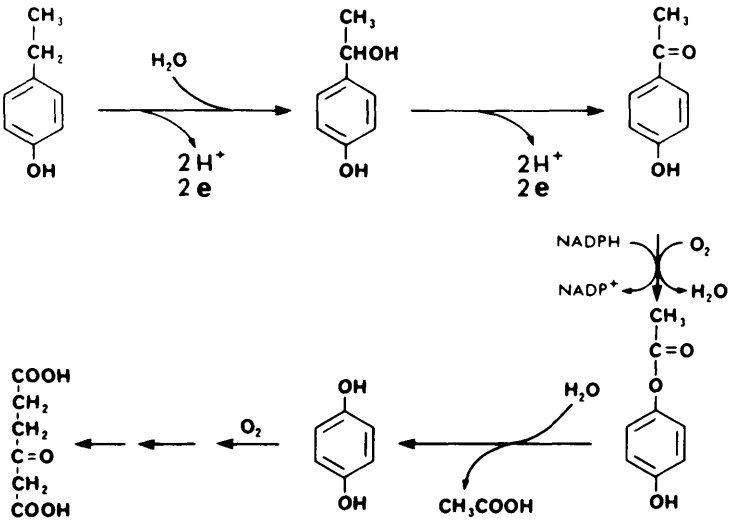

Fig. 4. Proposed pathway for 4-ethylphenol catabolism by *P. putida* JD1.

(Rothera, 1908) and gave a strong positive reaction. The rest of the reaction mixture was acidified with 5 M-HCl, the protein precipitate removed by centrifuging and the 2,4-dinitrophenylhydrazine derivative of the product in the supernatant solution prepared. The 2,4-dinitrophenylhydrazone of the product corresponded with that for authentic β-oxoadipic acid after TLC in solvents A and B (R_F 0·35 and 0·64 in A and B respectively) and they had identical absorption spectra in neutral and alkaline solution. No 2,4-dinitrophenylhydrazones were extracted from a control reaction mixture without hydroquinone.

Conclusions

The pathway proposed for 4-ethylphenol catabolism by *P. putida* JD1 is shown in Fig. 4. The presence of the relevant enzymes in cell extracts has been demonstrated and their specific activities were increased by growth on 4-ethylphenol over those in bacteria grown on aliphatic compounds (Table 1).

The initial steps appear to be catalysed by a *p*-cresol methylhydroxylase-type of enzyme, but one probably better adapted for substrates with larger side-chains than those previously studied. The specific activities of the enzymes for the first two steps were still at appreciable levels in cells grown on non-aromatic compounds in contrast to those for the rest of the enzymes in the pathway which were barely measurable. This allowed sufficient accumulation of 4-hydroxyacetophenone to itentify it as the product of oxidation of 4-ethylphenol.

The metabolism of 4-hydroxyacetophenone by this organism was via a different pathway from that used by an *Alcaligenes* sp. (Hopper *et al.*, 1985) and paralleled the route described by Cripps (1975) for acetophenone catabolism, resulting in formation of hydroquinone rather than phenol. Indeed, the outline of the pathway was established because the enzymes for 4-hydroxyacetophenone breakdown were of sufficiently broad specificity to convert acetophenone into phenol.

One of the unusual features of this pathway is the occurrence of hydroquinone as the ring-fission substrate. Reports abound of other dihydric phenols serving as ring-fission substrates in the metabolism of aromatic compounds (Dagley, 1975). Catechol and protocatechuic acid are found as the final aromatic intermediates in pathways for a range of different benzenoid compounds and similarly the *p*-dihydric phenol, gentisic acid, and its homologues are well-established ring-fission substrates (Chapman, 1972). Oxidative cleavage of these intermediates and subsequent breakdown to central metabolites have been thoroughly studied and are well understood. In contrast there have been relatively few reports of hydroquinone in this role.

Those that have appeared include its cleavage by a bacterium growing on hydroquinone itself (Larway, 1965; Larway & Evans, 1965) and identification as an intermediate in the catabolism of *p*-nitrophenol by a *Moraxella* sp. (Spain *et al.*, 1979). To these can now be added its role as the ring-fission substrate in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone. The pathway for metabolism of hydroquinone to central metabolites has never been described in detail although Larway (1965) identified γ-hydroxymuconic semialdehyde as the product of ring-fission and showed its conversion into β-oxoadipic acid by cell extracts. The results described for hydroquinone oxidation by *P. putida* JD1 are in agreement with this but the details have still to be elucidated.

REFERENCES

BAYLY, R. C., DAGLEY, S. & GIBSON, D. T. (1966). The metabolism of cresols by species of *Pseudomonas*. *Biochemical Journal* **101**, 293–301.

CHAPMAN, P. J. An outline of reaction sequences used for the bacterial degradation of phenolic compounds. In *Degradation of Synthetic Organic Molecules in the Biosphere*, pp. 17–55. Edited by P. J. Chapman & S. Dagley. Washington DC: Printing and Publishing Office, National Academy of Sciences.

CLARKE, P. H. & SLATER, J. H. (1986). Evolution of enzyme structure and function. In *The Bacteria*, vol. X, p. 99. Edited by J. R. Sokatch & L. N. Ornston. London: Academic Press.

CRIPPS, R. E. (1975). The microbial metabolism of acetophenone and some chloroacetophenones by an *Arthrobacter* species. *Biochemical Journal* **152**, 233–241.

CRIPPS, R. E., TRUDGILL, P. W. & WHATELEY, J. G. (1978). The metabolism of 1-phenylethanol and acetophenone by *Nocardia* T5 and an *Arthrobacter* species. *European Journal of Biochemistry* **86**, 175–186.

DAGLEY, S. (1975). A biochemical approach to some problems of environmental pollution. In *Essays in Biochemistry*, vol. II, pp. 81–138. Edited by P. N. Campbell & W. N. Aldridge. London: Academic Press.

DAGLEY, S. & PATEL, M. D. (1957). Oxidation of *p*-cresol and related compounds by a *Pseudomonas*. *Biochemical Journal* **66**, 227–233.

FORNEY, F. W. & MARKOVETZ, A. J. (1969). An enzyme system for aliphatic methyl ketone oxidation. *Biochemical and Biophysical Research Communications* **37**, 31–38.

GUNTER, S. E. (1953). The enzymatic oxidation of *p*-hydroxymandelic acid to *p*-hydroxybenzoic acid. *Journal of Bacteriology* **66**, 341–346.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry* **177**, 751–766.

HOPPER, D. J. (1978). Incorporation of [^{18}O]water in the formation of *p*-hydroxybenzyl alcohol by the *p*-cresol methylhydroxylase from *Pseudomonas putida*. *Biochemical Journal* **175**, 345–347.

HOPPER, D. J. & ELMORSI, E. A. (1984). Cleavage of formate from ω,4-dihydroxyacetophenone. An unusual oxygen-requiring reaction in the bacterial catabolism of 4-hydroxyacetophenone. *Biochemical Journal* **218**, 269–272.

HOPPER, D. J., JONES, H. G., ELMORSI, E. A. & RHODES-ROBERTS, M. E. (1985). The catabolism of 4-hydroxyacetophenone by an *Alcaligenes* sp. *Journal of General Microbiology* **131**, 1807–1814.

KENNEDY, S. I. T. & FEWSON, C. A. (1968). Enzymes of the mandelate pathway in bacterium NCIB 8250. *Biochemical Journal* **107**, 497–506.

LARWAY, P. F. (1965). *The oxidative metabolism of certain phenols by microorganisms*, PhD. thesis, University College of North Wales, Bangor.

LARWAY, P. F. & EVANS, W. C. (1965). Metabolism of quinol and resorcinol by soil pseudomonads. *Biochemical Journal* **95**, 52P.

MARTIN, A. K. (1982). The origin of urinary aromatic compounds excreted by ruminants. 3. The metabolism of phenolic compounds to simple phenols. *British Journal of Nutrition* **48**, 497–507.

MCINTIRE, W., HOPPER, D. J., CRAIG, J. C., EVERHART, E. T., WEBSTER, R. V., CAUSER, M. J. & SINGER, T. P. (1984). Stereochemistry of 1-(4′-hydroxyphenyl)ethanol produced by hydroxylation of 4-ethylphenol by *p*-cresol methylhydroxylase. *Biochemical Journal* **224**, 617–621.

MCINTIRE, W., HOPPER, D. J. & SINGER, T. P. (1985). *p*-Cresol methylhydroxylase: assay and general properties. *Biochemical Journal* **228**, 325–335.

NORRIS, D. B. & TRUDGILL, P. W. (1971). The metabolism of cyclohexanol by *Nocardia globarula* CL1. *Biochemical Journal* **121**, 363–370.

RAHIM, M. A. & SIH, C. J. (1966). Mechanisms of steroid oxidation by microorganisms. XI Enzymatic cleavage of the pregnane side chain. *Journal of Biological Chemistry* **241**, 3615–3623.

ROBERTSON, A. & ROBINSON, R. (1928). Experiments on the synthesis of anthrocyanins. *Journal of the Chemical Society*, 1460–1472.

ROTHERA, A. C. H. (1908). Note on the sodium nitroprusside reaction for acetone. *Journal of Physiology* **37**, 491–494.

SPAIN, J. C., WYSS, O. & GIBSON, D. T. (1979). Enzymatic oxidation of *p*-nitrophenol. *Biochemical and Biophysical Research Communications* **88**, 634–641.

VOGEL, A. I. (1956). *Practical Organic Chemistry*, 3rd edn. London: Longman, Green.