The Bacterial Metabolism of 2,4-Xylenol

By P. J. CHAPMAN* AND D. J. HOPPER*
Department of Biochemistry, University of Hull

(Received 28 June 1968)

1. Measurements of the rates of oxidation of various compounds by a fluorescent *Pseudomonas* indicated that metabolism of 2,4-xylenol was initiated by oxidation of the methyl group *para* to the hydroxyl group. 2. 4-Hydroxy-3-methylbenzoic acid was isolated as the product of oxidation of 2,4-xylenol by cells inhibited with $\alpha\alpha'$ -bipyridyl. 3. 4-Hydroxyisophthalic acid accumulated at low oxygen concentrations when either 2,4-xylenol or 4-hydroxy-3-methylbenzoic acid was oxidized by cells grown with 2,4-xylenol. 4. When supplemented with NADH, but not with NADPH, cell extracts oxidized 4-hydroxy-3-methylbenzoic acid readily. 2-Hydroxy-5-methylbenzoic acid was not oxidized. 5. Both 4-hydroxyisophthalic acid and *p*-hydroxybenzoic acid were oxidized to β -oxoadipic acid by cell extracts supplemented with either NADH or NADPH. 4,5-Dihydroxyisophthalic acid was not oxidized. 6. From measurements of oxygen consumed and carbon dioxide evolved it was concluded that protocatechuic acid is an intermediate in the conversion of 4-hydroxyisophthalic acid into β -oxoadipic acid.

The metabolic pathways by which phenol and its three monomethyl-substituted homologues, the cresols, are degraded by micro-organisms have been examined in some detail. Dagley & Patel (1957) showed that a non-fluorescent *Pseudomonas* initiates degradation of *p*-cresol by oxidation of the methyl group with the successive formation of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid. A different route is used by certain fluorescent species of *Pseudomonas*, which form methyl-substituted catechols from cresols by hydroxylation of the benzene nucleus (Ribbons, 1966; Bayly, Dagley & Gibson, 1966). In all the above cases cleavage of the benzene ring occurs between adjacent carbon atoms, only one of which carries a hydroxyl group. This is often referred to as 'meta' fission. A general scheme of reactions for the subsequent metabolism of products of 'meta' fission was published by Dagley, Chapman, Gibson & Wood (1964).

In contrast, little is known of the bacterial metabolism of dimethyl-substituted phenols, or xylenols, although they, like the cresols, are by-products of the coal-gas industry. Leibnitz, Behrens, Striegler & Gabert (1962) demonstrated the bacterial oxidation of 2,4-xylenol to 4-hydroxy-3-methylbenzoic acid, but its complete degradation was not reported. The present paper describes the identification of a number of intermediates of

2,4-xylenol metabolism and suggests a pathway of degradation.

MATERIALS AND METHODS

Maintenance and growth of organism. The organism was isolated from Hull river mud by elective culture in liquid medium (described below) containing 2,4-xylenol as sole source of carbon for growth. Dr T. G. Mitchell kindly identified the organism as a fluorescent *Pseudomonas*. It was deposited with the National Collection of Industrial Bacteria under the collection number N.C.I.B. 9866. Stock cultures were maintained on nutrient-agar slants at 4° and subcultured monthly.

Cells for respirometric studies were grown in 2l. volumes in wide-necked 4l. Erlenmeyer flasks at 27–30° with vigorous shaking on a Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The growth medium contained (per l.): Na_2HPO_4 , 0.433 g.; KH_2PO_4 , 0.265 g.; NH_4Cl , 2.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g.; 2,4-xylenol, 0.3 g. Bacteria were harvested by centrifuging at 4400g for 20 min., washed with 0.05 M KH_2PO_4 – Na_2HPO_4 buffer, pH 7.0, and resuspended immediately before use in the same buffer to give approx. 2 mg. dry wt. of cells/ml. Larger quantities of bacteria were grown in 24l. batches in a stainless-steel vessel at 30° with vigorous aeration and stirring. Growth was followed by measurement of the turbidity at 540 m μ in a Unicam SP. 600 spectrophotometer and related to the dry wt. of bacterial cells/ml. by the use of a standard curve. When necessary, further additions of the carbon source were made by adding 100 ml. of a 4.8% (w/v) suspension of 2,4-xylenol in growth medium. Cells were harvested in a Sharples Super centrifuge and, if not used immediately, were stored at –15°.

* Present address: Department of Biochemistry, University of Minnesota, St Paul, Minn. 55101, U.S.A.

Preparation of cell-free extracts. Extracts were prepared by suspending each 1g. of wet cells in 3ml. of ice-cold 0.05M-KH₂PO₄-Na₂HPO₄ buffer, pH 7.0, cooling the suspension in an ice-water bath and disrupting the bacteria in a 60 W MSE ultrasonic disintegrator for $\frac{1}{2}$ min. periods for a total of 3 min. The extracts were centrifuged at 0° at 39000g for 10 min. and the supernatant solution, referred to as crude extract, was retained. Such extracts contained 35–40 mg. of protein/ml. To 1 vol. of crude extract, 2.33 vol. of ice-cold saturated (NH₄)₂SO₄ soln. was slowly added with stirring to adjust the extract to 70% saturation with (NH₄)₂SO₄. After 1 hr. at 0° the protein precipitate was removed by centrifuging at 25000g for 10 min. at 0°. The pellet was redissolved in a volume of cold 0.05M-KH₂PO₄-Na₂HPO₄ buffer, pH 7.0, equal to that of the original crude extract, and dialysed with stirring for 3 hr. against two changes of 100 vol. of the same buffer. This extract is referred to as an (NH₄)₂SO₄-treated extract.

Chemical analyses. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as a standard. β -Oxoadipic acid was detected by the Rothera (1908) reaction and was determined manometrically by decarboxylation with 4-aminoantipyrine (Sistrom & Stanier, 1953). *o*-Dihydroxy phenols were determined by the method of Evans (1947). Microanalyses were by Weiler and Strauss, Oxford.

Infrared-absorption spectra. Samples (5 mg.) were milled in Nujol, and their spectra were recorded with a Unicam SP.200 spectrophotometer.

Ultraviolet-absorption spectra. Samples were dissolved in 66 mM-KH₂PO₄-Na₂HPO₄ buffer, pH 7.0, and their spectra were recorded with a Unicam SP.800 spectrophotometer.

Chromatography. Thin-layer chromatography was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany). For analytical work glass plates were coated to a thickness of 0.25 mm. and activated for 30 min. at 110°. For preparative work layers 1 mm. thick were dried overnight at room temperature and activated for 1 hr. at 110°. Phenolic compounds were separated by using solvent A: benzene-dioxan-acetic acid (90:25:4, by vol.) (Pastuska, 1961). For 2,4-dinitrophenylhydrazones of oxo acids solvent B was used: ethyl formate-light petroleum (b.p. 60–80°)-propionic acid (30:70:15.4, by vol.) (Ronkainen, 1963). Ascending paper chromatography on Whatman no. 1 paper was carried out with the following solvents. Solvents C, D, E and F were used to separate phenolic acids, and solvent C was also used for 2,4-dinitrophenylhydrazones: solvent C, butan-1-ol-ethanol-1.5N-(NH₄)₂CO₃ buffer, (40:11:19, by vol.) (Dagley, Fewster & Happold, 1952); solvent D, propan-2-ol-aq. NH₃ sp.gr. (0.88)-water (20:1:2, by vol.) (Smith, 1960); solvent E, benzene-acetic acid-water (125:72:3, by vol.) (Smith, 1960); solvent F, 5% (w/v) sodium formate-formic acid (200:1, v/v) (Smith, 1960).

Detection reagents. Chromatograms of phenolic compounds were first viewed under u.v. light to locate fluorescent and u.v.-absorbing material. Phenols were then located by spraying the chromatograms with one of the following reagents: (a) Folin-Ciocalteu reagent (British Drug Houses Ltd., Poole, Dorset) followed by exposure to NH₃ vapour (Mitchell & Davis, 1954); (b) diazotized *p*-nitroaniline (Smith, 1960); (c) diazotized sulphanilic acid (Smith, 1960); (d) 2% (w/v) FeCl₃ in 0.02N-HCl (Smith, 1960). Chromato-

grams of 2,4-dinitrophenylhydrazones were sprayed with ethanolic 10% (w/v) KOH.

Examination of reaction mixtures. Reaction mixtures containing cell extracts were deproteinized by the addition of 0.5 vol. of 30% (w/v) metaphosphoric acid. The protein precipitate was removed by centrifuging at 25000g for 10 min. Oxo acids in such mixtures were identified by chromatography of their 2,4-dinitrophenylhydrazones. These were prepared by incubation of the deproteinized mixture with 0.1% 2,4-dinitrophenylhydrazine in 2N-HCl for 30 min. at 30°. This was extracted into ethyl acetate, which was re-extracted into 10% (w/v) Na₂CO₃. This solution was washed with ethyl acetate, acidified and extracted into ethyl acetate, which was then dried over anhydrous Na₂SO₄ and evaporated to dryness.

Materials. All melting points are uncorrected for the emergent thermometer stem. Alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1), glucose 6-phosphate dehydrogenase (glucose 6-phosphate-NADP+ oxidoreductase, EC 1.1.1.49), NAD+ and NADP+ were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Compounds not commercially available were synthesized as follows. 4,5-Dihydroxyisophthalic acid was prepared from 4-hydroxyisophthalic acid (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.) by the method of Hunt, Jones & Lindsey (1956). 4-Hydroxy-3-methylbenzoic acid and 5-methylprotocatechuic acid (3,4-dihydroxy-5-methylbenzoic acid) were prepared by the limited biological oxidation of 2,4-xylenol (Hopkin and Williams Ltd., Chadwell Heath, Essex) and 3,5-dimethylcatechol (a generous gift from Coalite and Chemical Products Ltd., Bolsover, Chesterfield, Derbys.) respectively by resting cell suspensions of a non-fluorescent species of *Pseudomonas*, N.C.I.B. 9867, grown with *p*-hydroxybenzoic acid (Hopper, 1967). 4-Hydroxy-3-methylbenzoic acid had m.p. 173–174° and u.v.-absorption properties identical with those described by Dagley & Patel (1957) (Found: C, 59.2; H, 5.8; loss at 100°, 5.7. Calc. for C₉H₈O₃. $\frac{1}{2}$ H₂O: C, 59.6; H, 5.6; H₂O, 5.6%). Heilbron (1953) gave m.p. 174–175° for 4-hydroxy-3-methylbenzoic acid. 5-Methylprotocatechuic acid had m.p. 217–218° (Found: C, 54.5; H, 5.1; loss at 100°, 5.3. Calc. for C₈H₈O₄. $\frac{1}{2}$ H₂O: C, 54.2; H, 5.1; H₂O, 5.1%).

RESULTS

Experiments with intact cells. Since cells grown with 2,4-xylenol readily oxidized 4-hydroxy-3-methylbenzoate, *p*-cresol, *p*-hydroxybenzoate and protocatechuate (Fig. 1) it appeared that the methyl group *para* to the hydroxyl group was oxidized to a carboxyl group, as Smith, Jones & Evans (1952) and Dagley & Patel (1957) found for the bacterial metabolism of *p*-cresol. There was no evidence for oxidation of the *o*-methyl group as neither 2-hydroxy-5-methylbenzoic acid nor 4-hydroxyisophthalic acid was oxidized.

Isolation of intermediates. The total oxygen consumption of cell suspensions of the organism, grown with 2,4-xylenol and oxidizing this substrate, was considerably decreased in the presence of 10 mM- α -bipyridyl. To accumulate the products of this limited oxidation in amounts suitable for

Fig. 1. Oxidation of various compounds by washed cells grown with 2,4-xyleneol. Each flask contained 2.5 ml. of a bacterial suspension (approx. 5 mg. dry wt.) in 0.05 M-phosphate buffer, pH 7.0, 0.3 ml. of 10 mM substrate, tipped from the side arm, and 0.2 ml. of 20% (w/v) KOH in the centre well. Substrates were: (1) 2,4-xyleneol; (2) 4-hydroxy-3-methylbenzoic acid; (3) 5-methylprotocatechuic acid; (4) 2-hydroxy-5-methylbenzoic acid; (5) 4-hydroxyisophthalic acid; (6) water; (7) *p*-cresol; (8) *p*-hydroxybenzoic acid; (9) protocatechuic acid; (10) catechol; (11) gentisic acid; (12) 2,3-dihydroxybenzoic acid. The temperature was 30°. The gas phase was air.

complete characterization the reaction was scaled up.

A 10 g. sample of wet cells grown with 2,4-xyleneol was resuspended in 700 ml. of 0.5 M-phosphate buffer, pH 7.0, containing 10 mM- $\alpha\alpha'$ -bipyridyl in a 4 l. Erlenmeyer flask. The mixture was equilibrated on a Gyrotary shaker at 30° for 15 min., and then 100 ml. of 10 mM-2,4-xyleneol was added. Oxidation was followed in a parallel manometric experiment and when oxygen uptake ceased a further 100 ml. of 10 mM substrate was added. A total of four additions were made. After oxidation was complete, as shown by the parallel manometric experiment (approx. 150 min.), the cells were removed by centrifuging and the excess of $\alpha\alpha'$ -bipyridyl was converted into its iron chelate by the addition of ferrous sulphate to the supernatant solution. This was then acidified with 2 N-sulphuric acid and extracted three times with 500 ml. of ether. The combined extracts were dried over anhydrous sodium sulphate and then evaporated to dryness, yielding about 500 mg. of material. Thin-layer chromatography with solvent A showed two major spots. The first (R_F 0.71) gave an orange colour with diazotized *p*-nitroaniline, as did authentic 2,4-xyleneol (R_F 0.72). The second spot (R_F 0.49) corresponded to 4-hydroxy-3-methylbenzoic acid (R_F 0.49), both giving a yellow colour with diazotized *p*-nitroaniline. The acid was isolated by preparative

thin-layer chromatography with solvent A. The material was located on the plates as a u.v.-absorbing band. The silica gel was scraped off and eluted with ether, and the extract was filtered, dried over anhydrous sodium sulphate and then evaporated to dryness. The solid was recrystallized twice from water, after being decolorized with charcoal, yielding white needles, m.p. 172–173°, unchanged by admixture with authentic 4-hydroxy-3-methylbenzoic acid (Found: C, 59.4; H, 5.7; loss at 100°, 5.9. Calc. for $C_9H_8O_3 \cdot \frac{1}{2}H_2O$: C, 59.6; H, 5.6; H_2O , 5.6%). Heilbron (1953) gave m.p. 174–175°. The i.r.- and u.v.-absorption spectra were identical with those of authentic 4-hydroxy-3-methylbenzoic acid. Further confirmation of the identity of the compound was obtained by paper chromatography (product 1, Table 1).

To accumulate later intermediates a second technique, that of incubating cells with 2,4-xyleneol under conditions of limited aeration, was used. A 10 g. sample of wet cells grown with 2,4-xyleneol was resuspended in 970 ml. of 0.05 M-phosphate buffer, pH 7.0, and shaken with 30 ml. of 0.1 M-2,4-xyleneol at 30° in a 1 l. narrow-necked Erlenmeyer flask. The contents were tested periodically for *o*-dihydroxy phenols, but even after 36 hr. the result was negative. The cells were then removed by centrifuging and the supernatant was acidified to pH 2.0 with 2 N-sulphuric acid, filtered and extracted

Table 1. *Chromatographic identification of 4-hydroxy-3-methylbenzoic acid, 4-hydroxyisophthalic acid and protocatechuic acid, isolated as intermediates of 2,4-xynol metabolism*

See the text for details of the solvent systems *C*, *D*, *E* and *F* and the detection reagents (a), (b), (c) and (d).— Not determined.

Compound	Solvent ...	R_F				Appearance under u.v. light	Reagent ...	Colour			
		<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>			(a)	(b)	(c)	(d)
4-Hydroxy-3-methylbenzoic acid	0.44	0.27	0.87	—	—	u.v.-absorbing	Blue	Purple	Orange-yellow	—	—
Product 1	0.44	0.27	0.87	—	—	u.v.-absorbing	Blue	Purple	Orange-yellow	—	—
4-Hydroxyisophthalic acid	0.12	0.12	0.83	0.56	—	Light blue	Pale blue	Faint yellow	No colour	Pink	—
Product 2	0.11	0.12	0.82	0.55	—	Light blue	Pale blue	Faint yellow	No colour	Pink	—
Protocatechuic acid	0.14	0.05	0.30	0.56	—	Deep blue	Blue	Olive-green	Pale tan	Blue-green	—
Product 3	0.14	0.05	0.30	0.54	—	Deep blue	Blue	Olive-green	Pale tan	Blue-green	—
4,5-Dihydroxyisophthalic acid	0.08	0.02	0.21	—	—	—	Blue	Pale purple-grey	No colour	Purple	—

three times with 500 ml. of ether. The combined extracts were dried over anhydrous sodium sulphate and evaporated to yield about 200 mg. of solid. Thin-layer chromatography with solvent *A* showed the presence of 2,4-xynol with a tailing spot (R_F 0.28) that fluoresced bright blue under u.v. light and gave a pale-yellow colour with diazotized *p*-nitroaniline. These characteristics corresponded with those of 4-hydroxyisophthalic acid (R_F 0.29). 5-Methylprotocatechuic acid (R_F 0.35) gave a mauve-brown colour with the detection reagent. The residual 2,4-xynol was removed by dissolving the products in 100 ml. of 0.05 M-phosphate buffer, pH 7.0, extracting with ether and discarding the ether layer. The buffer was then acidified to pH 2.0 and re-extracted three times with 0.5 vol. of ether. The combined extracts were dried over anhydrous sodium sulphate and evaporated to dryness. The solid, which gave a single spot on a thin-layer chromatogram, was recrystallized from water after being boiled with charcoal. This gave white crystals, m.p. 303–305° (decomp.), not depressed when mixed with authentic 4-hydroxyisophthalic acid (Found: C, 52.8; H, 3.0. Calc. for $C_8H_6O_5$: C, 52.8; H, 3.3%). Heilbron (1953) gave m.p. 310°. The isolated material gave i.r.- and u.v.-absorption spectra identical with those of authentic 4-hydroxyisophthalic acid. Further confirmation of its identity was obtained from paper chromatography (see product 2 in Table 1).

In a similar experiment conducted on a smaller scale in a 50 ml. flask 4-hydroxy-3-methylbenzoic acid was used as substrate. The products were examined after 12 hr. by thin-layer chromatography, which showed that 4-hydroxyisophthalic acid was also formed from this substrate.

Isolation of protocatechuic acid. Although 2,4-xynol-grown cells did not oxidize exogenous 4-hydroxyisophthalic acid, the organism grew with this substrate, after a lag, when transferred from 2,4-xynol medium. These cells oxidized 4-hydroxyisophthalic acid, *p*-hydroxybenzoic acid and protocatechuic acid at similar rates. To accumulate later intermediates, cells grown with 4-hydroxyisophthalic acid were used to oxidize this compound under the same conditions of limited aeration as those described above for the isolation of 4-hydroxyisophthalic acid. After incubation for 9 hr. the Evans (1947) test showed the presence of an *o*-dihydroxy compound, the concentration of which increased up to 24 hr., when the flask was removed from the shaker and its contents were treated as in the previous experiment. An examination of the products by thin-layer chromatography in solvent *A* showed two compounds, one of which corresponded to the starting material. The other (R_F 0.32) gave a brown colour with diazotized *p*-nitroaniline, as did protocatechuic acid (R_F 0.30). 2,3-Dihydroxybenzoic acid also gave a brown colour, with R_F 0.46. There was no evidence for *p*-hydroxybenzoic acid (R_F 0.46). The two acids were separated by preparative thin-layer chromatography. The protocatechuic acid band was scraped off, eluted with ether and evaporated to dryness to yield a few milligrams of solid, which gave a positive Evans test. It was identified as protocatechuic acid by paper chromatography (Table 1, product 3).

Oxidation of compounds by cell extracts. Crude cell extracts catalysed the rapid oxidation of protocatechuic acid (24 μ l. of oxygen/hr./mg. of protein) and carbon dioxide evolution was also observed. 5-Methylprotocatechuic acid was oxidized at about one-quarter of this rate (5.4 μ l.

Fig. 2. Oxidation of various compounds by an $(\text{NH}_4)_2\text{SO}_4$ -treated extract of cells grown with 2,4-xylene (endogenous oxidation subtracted, $20 \mu\text{l./hr.}$). Each flask contained 1.5 ml. of cell extract (approx. 20 mg. of protein/ml.), 0.8 ml. of 0.05 M-phosphate buffer, pH 7.0, 0.5 ml. of 10 mM substrate, tipped from the side arm, and 0.2 ml. of 20% (w/v) KOH in the centre well. The temperature was 30° . The gas phase was air. Substrates were: (1) protocatechuic acid; (2) 5-methylprotocatechuic acid; (3) 4,5-dihydroxyisophthalic acid; (4) 2,3-dihydroxybenzoic acid.

of oxygen/hr./mg. of protein). Similar results were obtained with ammonium sulphate-treated extracts, as is shown in Fig. 2. Oxidation of protocatechuic acid by ammonium sulphate-treated extracts did not require the presence of Fe^{2+} ions and was not further stimulated by the addition of Fe^{2+} ions at a concentration of 6.6 mM, nor was it inhibited by 10 mM- $\alpha\alpha'$ -bipyridyl. These extracts did not catalyse the oxidation or decarboxylation of 4-hydroxyisophthalic acid, 4,5-dihydroxyisophthalic acid, salicylic acid or 2,3-dihydroxybenzoic acid.

However, when NADH or NADPH was included in the system 4-hydroxyisophthalic acid was readily oxidized. *p*-Hydroxybenzoic acid was also readily oxidized under these conditions, though at a slightly lower rate (Fig. 3), and in both cases carbon dioxide was evolved. No oxidation of 4,5-dihydroxyisophthalic acid, 5-hydroxyisophthalic acid or salicylic acid was observed. The NADH was generated in the reaction mixture from yeast alcohol dehydrogenase, ethanol and NAD^+ . Similarly NADPH was generated from glucose 6-phosphate dehydrogenase, glucose 6-phosphate and NADP^+ .

In the presence of the NADH-generating system, 4-hydroxy-3-methylbenzoic acid was also oxidized by an ammonium sulphate-treated extract

Fig. 3. Oxidation of 4-hydroxyisophthalic acid (●), *p*-hydroxybenzoic acid (○) and 4,5-dihydroxyisophthalic acid (▲) by an extract of cells, grown with 2,4-xylene, provided with NADPH (endogenous oxidation subtracted, $80 \mu\text{l./hr.}$). Each flask contained 1.5 ml. of an $(\text{NH}_4)_2\text{SO}_4$ -treated extract centrifuged for 1 hr. at 150 000g (approx. 25 mg. of protein/ml.), 0.7 ml. of 0.1 M-phosphate buffer, pH 7.0, 0.1 ml. of 10 mM- NADP^+ , 0.1 ml. of 0.2 M-glucose 6-phosphate, 0.1 ml. of glucose 6-phosphate dehydrogenase (30 units/ml.), 0.3 ml. of 10 mM substrate, tipped from the side arm, and 0.2 ml. of 20% (w/v) KOH in the centre well. The temperature was 30° . The gas phase was air.

with the consumption of $2.5 \mu\text{moles}$ of oxygen/ μmole of substrate (Fig. 4). There was no oxidation in the presence of NADPH. By contrast, 2-hydroxy-5-methylbenzoic acid was not oxidized; 2,4-xylene was oxidized slowly in the presence of either NADH or NADPH.

Products of oxidations by cell extracts. After deproteinization the reaction mixtures obtained from the oxidation of protocatechuic acid, 4-hydroxyisophthalic acid and *p*-hydroxybenzoic acid all gave persistent purple colours in the Rothera reaction, an indication of β -oxoadipic acid. When examined by paper chromatography with solvent *C* the 2,4-dinitrophenylhydrazone derivatives prepared from these reaction mixtures all had the same R_F value (0.67) as laevulinic 2,4-dinitrophenylhydrazone (prepared similarly from β -oxoadipic acid). Thin-layer chromatography with solvent *B* also revealed the close similarity of these derivatives (R_F 0.46) to that of laevulinic acid (R_F 0.47). The identification of the reaction product as β -oxoadipic acid was further confirmed by its catalytic decarboxylation with 4-aminoantipyrine, a method

also used for its determination in an experiment to determine the stoichiometry of oxygen consumption, carbon dioxide evolution and oxo acid formation. For this experiment an ammonium

sulphate-treated extract was centrifuged at 0° for 30 min. at 150 000g in an MSE Superspeed 50 preparative ultracentrifuge, to decrease NADPH oxidase activity. The results are given in Table 2.

Fig. 4. Oxidation of compounds by an $(\text{NH}_4)_2\text{SO}_4$ -treated extract of cells, grown with 2,4-xyleneol, provided with NADH or NADPH (endogenous oxidation subtracted, 294 $\mu\text{l./hr.}$ with NADH and 173 $\mu\text{l./hr.}$ with NADPH). Each flask contained 1.9 ml. of cell extract (approx. 20 mg. of protein/ml.), 0.1 ml. of 0.05 M-ferrous ammonium sulphate, 0.5 ml. of 10 mM substrate, tipped from the side arm, and 0.2 ml. of 20% (w/v) KOH in the centre well. Reactions provided with NADH contained 0.1 ml. of 15 mM-NAD⁺, 0.1 ml. of 0.2 M-ethanol and 0.1 ml. of alcohol dehydrogenase (1.0 mg./ml.). Those provided with NADPH contained 0.1 ml. of 15 mM-NADP⁺, 0.1 ml. of 0.2 M-glucose 6-phosphate and 0.1 ml. of glucose 6-phosphate dehydrogenase (30 units/ml.). The temperature was 30°. The gas phase was air. Substrates were: 4-hydroxy-3-methylbenzoic acid with NADH (1) or with NADPH (2); 2-hydroxy-5-methylbenzoic acid with NADH (3) or with NADPH (4); 2,4-xyleneol with NADH (5) or with NADPH (6).

DISCUSSION

The ready oxidation of 4-hydroxy-3-methylbenzoic acid by bacteria grown with 2,4-xyleneol and their failure to oxidize 2-hydroxy-5-methylbenzoic acid suggested that degradation of 2,4-xyleneol may be initiated by oxidation of the methyl substituent *para* to the carboxyl group. However, in view of the fact that *p*-cresol was also rapidly oxidized by such cells it was not possible to decide whether an alternative pathway existed that involved oxidation of the *o*-methyl substituent and loss of the resulting carboxyl group or whether *p*-cresol oxidation was catalysed by enzymes of low specificity. Nor was it possible from these preliminary experiments to evaluate the significance of *p*-hydroxybenzoic acid and protocatechuic acid oxidation. Accordingly experiments were undertaken to isolate and identify intermediates.

The accumulation of 4-hydroxy-3-methylbenzoic acid by cells oxidizing 2,4-xyleneol in the presence of $\alpha\alpha'$ -bipyridyl confirmed that oxidation of the *p*-methyl group occurred. This acid has been described as a product of 2,4-xyleneol oxidation by a *Pseudomonas* sp. with the transient formation of 4-hydroxy-3-methylbenzyl alcohol and 4-hydroxy-3-methylbenzaldehyde (Leibnitz *et al.* 1962). These authors, however, did not describe its further metabolism. It is noteworthy that Dagley & Patel (1957) reported that cells of a *Pseudomonas* sp. capable of degrading *p*-cresol via *p*-hydroxybenzoic acid and protocatechuic acid would also oxidize the *p*-methyl substituents of other phenols such as 2,4-xyleneol and 3,4-xyleneol with the accumulation of the corresponding carboxylic acids. It is therefore probable that the enzymes catalysing oxidation

Table 2. *Stoichiometry of the oxidation of protocatechuic acid, 4-hydroxyisophthalic acid and p-hydroxybenzoic acid by cell extracts*

The protocatechuic acid flask contained 1.0 ml. of 0.1 M-phosphate buffer, pH 7.0, 1.5 ml. of extract (26.5 mg. of protein/ml.) and 0.3 ml. of 10 mM-protocatechuic acid. The other flasks contained 0.7 ml. of 0.1 M-phosphate buffer, pH 7.0, 1.5 ml. of cell extract (26.5 mg. of protein/ml.), 0.1 ml. of 10 mM-NADP⁺, 0.1 ml. of glucose 6-phosphate dehydrogenase (30 units/ml.), 0.1 ml. of 0.4 M-glucose 6-phosphate and 0.3 ml. of 10 mM substrate. The centre well contained 0.2 ml. of 20% (w/v) KOH for measurements of O₂ uptake or 0.2 ml. of 5 N-H₂SO₄, tipped after completion of the reaction, for CO₂ evolution. The temperature was 30°. Corrections were made for gas changes occurring in control flasks lacking the aromatic substrates.

Substrate	Uptake of O ₂ ($\mu\text{moles}/\mu\text{mole}$ of substrate)	Evolution of CO ₂ ($\mu\text{moles}/\mu\text{mole}$ of substrate)	β -Oxadipic acid formed ($\mu\text{mole}/\mu\text{mole}$ of substrate)
Protocatechuic acid	0.98	1.02	0.98
4-Hydroxyisophthalic acid	1.85	1.87	0.93
<i>p</i> -Hydroxybenzoic acid	1.8	1.06	0.90

Scheme 1. Proposed pathway for 2,4-xyleneol metabolism.

of 2,4-xyleneol to 4-hydroxy-3-methylbenzoic acid that we have observed also catalyse a similar oxidation of *p*-cresol.

Although 5-methylprotocatechuic acid was only slowly oxidized by intact cells we had considered this compound a likely intermediate, formed by ring hydroxylation of 4-hydroxy-3-methylbenzoic acid in a manner analogous to the formation of protocatechuic acid from *p*-hydroxybenzoic acid (Hosokawa & Stanier, 1966). The identification of further intermediates rendered this hypothesis untenable. Accumulation of 4-hydroxyisophthalic acid when poorly aerated cell suspensions oxidized 2,4-xyleneol showed that oxidation of the *o*-methyl substituent also occurred. However, since 2-hydroxy-5-methylbenzoic acid was not oxidized by 2,4-xyleneol-grown cells it seems probable that oxidation of the *o*-methyl substituent occurred only after the *p*-methyl group had been oxidized (Scheme 1). Our results support this conclusion since 4-hydroxyisophthalic acid was also formed from 4-hydroxy-3-methylbenzoic acid when cell incubations were conducted so as to limit aeration. Although intact cells did not oxidize 4-hydroxyisophthalic acid, enzymes for its further metabolism were demonstrated in cell extracts. Accordingly failure of cells to oxidize this compound must be ascribed to its inability to enter the cell. That cells are able to take up 4-hydroxyisophthalic acid, presumably after the induction of a permease, was shown by their ability to grow at the expense of this compound. When cells were grown with this substrate, protocatechuic acid and *p*-hydroxy-

benzoic acid were also rapidly oxidized. Moreover, when aeration was limited, cells incubated with 4-hydroxyisophthalic acid accumulated small quantities of protocatechuic acid. No *p*-hydroxybenzoic acid was detected. These observations are in accord with earlier results and provide further evidence that 2,4-xyleneol is degraded via 4-hydroxyisophthalic acid and protocatechuic acid (Scheme 1).

Experiments with cell-free extracts provided additional support for this pathway. Thus extracts of 2,4-xyleneol-grown cells contained considerable protocatechuate 3,4-oxygenase activity, as shown by the stoichiometry of oxygen consumption, carbon dioxide evolution and β -oxoadipic acid formation, all of which are consistent with the pathway of protocatechuic acid metabolism through β -carboxy-*cis-cis*-muconic acid $\rightarrow$ γ -carboxy-muconolactone $\rightarrow$ β -oxoadipic acid enol lactone $\rightarrow$ β -oxoadipic acid, as described by Ornston & Stanier (1966) in *Pseudomonas putida*. The lack of inhibition of this enzyme by $\alpha\alpha'$ -bipyridyl and the failure of ammonium sulphate precipitation to effect a separation of iron and apoenzyme are also characteristic of protocatechuate 3,4-oxygenase (Hayaishi, 1966).

There is no evidence that *p*-hydroxybenzoic acid is formed from 4-hydroxyisophthalic acid, although both compounds appear to be metabolized via protocatechuic acid, as shown by their oxidation by cell extracts supplemented with either NADH or NADPH with the formation of stoichiometric amounts of β -oxoadipic acid and carbon dioxide. Thus cell extracts alone failed to catalyse the

decarboxylation of 4-hydroxyisophthalic acid. Only in the presence of reduced nicotinamide nucleotides was decarboxylation observed, and then it was always accompanied by oxygen consumption. The conversion of 4-hydroxyisophthalic acid into protocatechuic acid must therefore involve either oxidation before decarboxylation or simultaneous oxidative decarboxylation. The only possible intermediate derived from 4-hydroxyisophthalic acid that would yield protocatechuic acid on decarboxylation is 4,5-dihydroxyisophthalic acid. Decarboxylation of a similar compound, 4,5-dihydroxyphthalic acid, to give protocatechuic acid was described by Ribbons & Evans (1960) as a step in the bacterial metabolism of phthalic acid. 4,5-Dihydroxyisophthalic acid was not decarboxylated by our cell extracts and, under conditions where both 4-hydroxyisophthalic acid and protocatechuic acid were oxidized to β -oxoadipic acid, no oxidation of 4,5-dihydroxyisophthalic acid was observed. It therefore appears that 4-hydroxyisophthalic acid is converted into protocatechuic acid by an oxidative decarboxylation reaction (Scheme 1) which is dependent on reduced nicotinamide nucleotide, and which may be analogous to that catalysed by salicylate hydroxylase (Katagiri, Yamamoto & Hayaishi, 1962).

The only evidence for the involvement of *p*-hydroxybenzoic acid as an intermediate is the demonstration of its oxidation by cells and cell extracts; that is, cells grown with 2,4-xyleneol or 4-hydroxyisophthalic acid contain *p*-hydroxybenzoate hydroxylase activity. A similar finding has been reported by Wheelis, Palleroni & Stanier (1967), who found that gentisate oxygenase, maleylpyruvate isomerase and fumarylpyruvate hydrolase were gratuitously induced in *Pseudomonas testosteroni* by *m*-hydroxybenzoic acid, although this compound was metabolized exclusively through protocatechuic acid. Ornston (1966) showed that in *Pseudomonas putida* the synthesis of two enzymes required for protocatechuic acid metabolism, β -carboxy-*cis-cis*-muconate-lactonizing enzyme and γ -carboxymuconolactone decarboxylase, is induced by β -oxoadipic acid or its CoA thiol ester. Consequently when inducer is formed by other metabolic pathways both enzymes are gratuitously induced. Further experiments are necessary before it can be established whether 4-hydroxyisophthalic acid is a gratuitous inducer of *p*-hydroxybenzoate hydroxylase.

The sequence of reactions whereby 4-hydroxyisophthalate is formed from 2,4-xyleneol is apparently initiated by oxidation of the *p*-methyl substituent to a carboxyl group before the *o*-methyl substituent undergoes a similar oxidation (Scheme 1). This is supported by our observation that 4-hydroxy-3-methylbenzoate was rapidly oxidized

by cell extracts supplied with NADH whereas 2-hydroxy-5-methylbenzoate was not. NADPH could not replace NADH in this oxidation. Oxidation of 2,4-xyleneol, although catalysed only slowly by cell extracts, is less specific in its requirements for reduced nicotinamide nucleotides. Both this oxidation and that of 4-hydroxy-3-methylbenzoate presumably involve initial hydroxylation of aromatic methyl substituents. The *o*-methyl hydroxylase, however, appears more sensitive to $\alpha\alpha'$ -bipyridyl inhibition. Dagley & Patel (1957) obtained bacterial extracts that catalysed a similar reaction with *p*-cresol as substrate, but no requirement for reduced nicotinamide nucleotides was reported.

We thank Professor E. A. Dawes for his interest throughout the course of the work, and Reckitt and Sons Ltd. for their interest and for financial support for D. J. H.

REFERENCES

- Bayly, R. C., Dagley, S. & Gibson, D. T. (1966). *Biochem. J.* **101**, 293.
- Dagley, S., Chapman, P. J., Gibson, D. T. & Wood, J. M. (1964). *Nature, Lond.*, **202**, 775.
- Dagley, S., Fewster, M. E. & Happold, F. C. (1952). *J. Bact.* **63**, 327.
- Dagley, S. & Patel, N. D. (1957). *Biochem. J.* **66**, 227.
- Evans, W. C. (1947). *Biochem. J.* **41**, 373.
- Hayaishi, O. (1966). *Bact. Rev.* **30**, 720.
- Heilbron, I. (1953). *Dictionary of Organic Compounds*, 3rd ed., vol. 2, pp. 777, 832. New York: Oxford University Press.
- Hopper, D. J. (1967). Ph.D. Thesis: University of Hull.
- Hosokawa, K. & Stanier, R. Y. (1966). *J. biol. Chem.* **241**, 2453.
- Hunt, S. E., Jones, J. I. & Lindsey, A. S. (1956). *J. chem. Soc.* p. 3099.
- Katagiri, M., Yamamoto, S. & Hayaishi, O. (1962). *J. biol. Chem.* **237**, pc2413.
- Leibnitz, E., Behrens, U., Striegler, G. & Gabert, A. (1962). *Z. allg. Mikrobiol.* **2**, 81.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mitchell, F. L. & Davies, R. E. (1954). *Biochem. J.* **56**, 690.
- Ornston, L. N. (1966). *J. biol. Chem.* **241**, 3800.
- Ornston, L. N. & Stanier, R. Y. (1966). *J. biol. Chem.* **241**, 3776.
- Pastuska, G. (1961). *Z. anal. Chem.* **179**, 355.
- Ribbons, D. W. (1966). *J. gen. Microbiol.* **44**, 221.
- Ribbons, D. W. & Evans, W. C. (1960). *Biochem. J.* **76**, 310.
- Ronkainen, P. (1963). *J. Chromat.* **11**, 228.
- Rothera, A. C. H. (1908). *J. Physiol.* **37**, 491.
- Sistrom, W. R. & Stanier, R. Y. (1953). *J. Bact.* **66**, 404.
- Smith, B. S. W., Jones, J. D. & Evans, W. C. (1952). *Biochem. J.* **50**, xxviii.
- Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, pp. 291–298. London: W. Heinemann (Medical Books) Ltd.
- Wheelis, M. L., Palleroni, N. J. & Stanier, R. Y. (1967). *Arch. Mikrobiol.* **58**, 302.