Gentisic Acid and its 3- and 4-Methyl-Substituted Homologues as Intermediates in the Bacterial Degradation of *m*-Cresol, 3,5-Xylenol and 2,5-Xylenol

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1. Intact cells of a non-fluorescent *Pseudomonas* grown with *m*-cresol, 2,5-xylenol, 3,5-xylenol, 3-ethyl-5-methylphenol or 2,3,5-trimethylphenol rapidly oxidized all these phenols to completion. 3-Hydroxybenzoate and 2,5-dihydroxybenzoate (gentisate) were also readily oxidized. 2. 3-Hydroxybenzoic acid and 2,5-dihydroxybenzoic acid were isolated as products of *m*-cresol oxidation by cells inhibited by $\alpha\alpha'$ -bipyridyl. Alkyl-substituted 3-hydroxybenzoic acids and alkyl-substituted gentisic acids were formed similarly from 2,5-xylenol, 3,5-xylenol, 3-ethyl-5-methylphenol and 2,3,5-trimethylphenol. 3. When supplemented with NADH, not NADPH, extracts of cells grown with 2,5-xylenol catalysed the oxidation of all five phenols and accumulated the corresponding gentisic acids in the presence of $\alpha\alpha'$ -bipyridyl. 4. Cells of a fluorescent *Pseudomonas* grown with *m*-cresol oxidized *m*-cresol, 3,5-xylenol and 3-ethyl-5-methylphenol to completion and oxidized 2,5-xylenol and 2,3,5-trimethylphenol partially. The oxidation product of 2,5-xylenol was identified as 3-hydroxy-4-methylbenzoic acid. In the presence of $\alpha\alpha'$ -bipyridyl, 3-hydroxy-5-methylbenzoic acid and 3-methylgentisic acid were formed from 3,5-xylenol.

Previous studies have shown that *Pseudomonas* spp. convert monomethyl-substituted phenols (cresols) into catechols before they open the benzene nucleus. Some species oxidize methyl substituents to carboxyl groups before ring-cleavage, but others do not. Thus the fluorescent *Pseudomonas* of Bayly, Dagley & Gibson (1966) left these groups intact, so that *p*-cresol was oxidized to 4-methylcatechol, and *o*- and *m*-cresol both gave rise to 3-methylcatechol. By contrast, a non-fluorescent *Pseudomonas* attacked the methyl group of *p*-cresol and then used the *meta*-fission pathway of protocatechuate to degrade the resulting *p*-hydroxybenzoate (Dagley & Patel, 1957; Dagley, Geary & Wood, 1968). Similarly, another *Pseudomonas* sp. initiated attack on a dimethyl-substituted phenol, namely 2,4-xylenol, by oxidizing the methyl group situated *para* to the hydroxyl. The second methyl group was then oxidized, giving 4-hydroxyisophthalic acid, which was converted into protocatechuate and metabolized by reactions that involved *ortho*-fission of the benzene nucleus (Chapman & Hopper, 1968). In the present work,

species of *Pseudomonas* were used that initiated the degradation of cresols and xylenols by oxidizing a methyl group placed *meta* to the hydroxyl. Catechols related to these cresols were not metabolized. Instead, a second hydroxyl group was introduced *para* to the first, gentisic acid (2,5-dihydroxybenzoic acid) or an alkyl-substituted gentisate was formed, and this compound served as substrate for the ring-fission dioxygenase.

MATERIALS AND METHODS

Maintenance and growth of organisms. Two organisms were isolated from Hull river mud by elective culture in liquid media containing 2,5-xylenol and 3,5-xylenol respectively. Dr T. G. Mitchell kindly examined these organisms. The first, a non-fluorescent pseudomonad, we designated as *Pseudomonas* 2,5; the second, a fluorescent pseudomonad, was referred to as *Pseudomonas* 3,5. They were deposited with the National Collection of Industrial Bacteria, *Pseudomonas* 2,5 under the collection number N.C.I.B. 9867 and *Pseudomonas* 3,5 as N.C.I.B. 9869. Cells were grown, harvested and stored as previously described (Chapman & Hopper, 1968) except that 2,4-xylenol was replaced by the carbon sources used in the present work, each at a concentration of 0.3 g/l. The phosphate buffer used throughout was 0.05 M-KH₂PO₄-Na₂HPO₄, pH 7.0.

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Preparation of cell-free extracts. Extracts were prepared, treated with $(\text{NH}_4)_2\text{SO}_4$ and dialysed as described by Chapman & Hopper (1968).

Chemical analyses. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as a standard. Microanalyses were by Weiler and Strauss, Oxford, U.K., and by Galbraith Laboratories Inc., Knoxville, Tenn., U.S.A.

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

Chromatography. Compounds were identified and isolated by means of t.l.c. and paper chromatography as described by Chapman & Hopper (1968), whose solvents A, C, D, E and F, and detection reagents (a), (b) and (c), were also used, and are so designated in this paper.

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 from Sigma Chemical Co., St Louis, Mo., U.S.A. Xylenols, gentisic acid and 2-hydroxy-3-methylbenzoic acid were from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; 3-hydroxy-4-methylbenzoic acid and 2-ethylphenol were from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. 2-Hydroxy-4-methylbenzoic acid was from K and K Laboratories Inc., Plainview, N.Y., U.S.A. Compounds not available commercially were synthesized as follows. 3-Methylgentisic acid was prepared by persulphate oxidation of 2-hydroxy-3-methylbenzoic acid under essentially the same conditions as those used by Updegraff & Cassidy (1949) for the analogous oxidation of o-coumaric acid to 2,5-dihydroxycinnamic acid. Recrystallization from water gave pale-yellow needles, m.p. 217–218°C (decomp.) (Found: C, 57.4; H, 4.9. Calc. for $\text{C}_8\text{H}_8\text{O}_4$: C, 57.1; H, 4.8%). Bernatek & Bø (1959) gave m.p. 216°C for 3-methylgentisic acid. 4-Methylgentisic acid, prepared by oxidizing 2-hydroxy-4-methylbenzoic acid with persulphate, gave yellow needles from water, m.p. 240–241°C (decomp.) (Found: C, 57.1; H, 4.8. Calc. for $\text{C}_9\text{H}_8\text{O}_4$: C, 57.1; H, 4.8%). Heilbron & Bunbury (1953) gave m.p. 210°C, Inouye (1954) gave m.p. 232–

234°C. 3-Ethylgentisic acid was obtained by first carboxylating 2-ethylphenol, by the method of Jones (1958), to give 3-ethyl-2-hydroxybenzoic acid which crystallized from water as white needles, m.p. 114–115°C. Letsinger & Schnizer (1951) gave m.p. 112–112.5°C. This compound was then oxidized with persulphate to give pale-yellow crystals of 3-ethylgentisic acid, m.p. 195–197°C (decomp.). Renz (1947) gave m.p. 188°C. 3,4-Dimethylgentisic acid was similarly obtained by carboxylating 2,3-xylenol to give 3,4-dimethyl-2-hydroxybenzoic acid [white crystals from aq. ethanol, m.p. 200–201°C; Divekar & Vining (1964) gave m.p. 200–202°C]. This compound was then oxidized with persulphate to give pale-yellow crystals of 3,4-dimethylgentisic acid, m.p. 213.5–215.5°C (decomp.). 3-Hydroxy-5-methylbenzoic acid was prepared by the method of Berner & Laland (1949) and gave white crystals from water, m.p. 211°C. 3,4-Dihydroxy-5-methylbenzoic acid was obtained by the limited biological oxidation of 3,5-dimethylcatechol (generously supplied by Coalite and Chemical Products Ltd., Bolsover, Chesterfield, U.K.) by resting cell suspensions of *Pseudomonas* 2,5 grown with *p*-hydroxybenzoic acid (Hopper, 1967). Chloramphenicol was a generous gift from Parke, Davis and Co. Ltd., Hounslow, Middx., U.K.

RESULTS

Oxidation of substituted phenols by intact cells of *Pseudomonas* 2,5. Washed suspensions of *Pseudomonas* 2,5 grown with *m*-cresol (I, Scheme 1) readily oxidized the following compounds, each of which contains a methyl group situated *meta* to the hydroxyl: *m*-cresol (I), 2,5-xylenol (IV), 3,5-xylenol (VII), 2,3,5-trimethylphenol and 3-ethyl-5-methylphenol. Moreover, all of these compounds were oxidized at similar rates and to a similar extent by cells grown at the expense of any member of the group. These results were not altered by chloramphenicol at 50 µg/ml. Succinate-grown cells did not attack these alkyl-substituted phenols but were able to oxidize 3-hydroxybenzoate and gentisate;

Table 1. *Rates of oxidation of phenolic compounds by intact cells of Pseudomonas* 2,5

The main compartments of Warburg flasks contained 2.5 ml of a suspension of washed cells (5 mg dry wt.) in 0.05M-phosphate buffer, pH 7.0; centre wells contained 0.2 ml of 20% (w/v) KOH, and 0.3 ml of 10 mM-substrate was tipped from the side arms. Uptake of O_2 in the absence of substrate (8 µmol of O_2 /min per g. dry wt. of cells) has been subtracted.

Substrate oxidized...	Rate of oxidation (µmol of O_2 /min per g dry wt. of cells)					
	<i>m</i> -Cresol	2,5-Xylenol	3,5-Xylenol	3-Ethyl-5-methylphenol	2,3,5-Tri-methylphenol	Succinate
Growth substrate						
<i>m</i> -Cresol	73	58	62	70	70	0
2,5-Xylenol	63	61	63	70	68	0
3,5-Xylenol	66	61	63	72	70	0
3-Ethyl-5-methylphenol	57	63	69	60	70	0
2,3,5-Trimethylphenol	66	68	67	74	73	0
3-Hydroxybenzoate	89	57	67	74	75	13
Gentisate	42	34	38	23	28	18

Fig. 1. Oxidation of various compounds by washed cells of *Pseudomonas* 2,5 grown with 2,5-xylene. Each flask contained 2.5 ml of a bacterial suspension (5 mg dry wt.) in 0.05 M-phosphate buffer, pH 7.0, 0.3 ml of 10 mM substrate was in the side arm, and 0.2 ml of 20% (w/v) KOH in the centre well. Substrates were: (1) 2,3,5-trimethylphenol; (2) 3-ethyl-5-methylphenol; (3) 2,5-xylene; (4) 3,5-xylene; (5) *m*-cresol; (6) 2,3,5,6-tetramethylphenol; (7) water; (8) 3-hydroxy-4-methylbenzoate; (9) 4-methylgentisate; (10) gentisate; (11) 3-methylcatechol; (12) protocatechuate; (13) 2,3-dihydroxybenzoate; (14) 3-ethylphenol. The temperature was 30°C. The gas phase was air. (a) and (b) represent two separate experiments.

however, the last-named compounds were oxidized faster when cells were grown with phenols (Table 1). From measurements of the total amounts of oxygen consumed it was evident that, allowing for material assimilated by the cells, the substrates in Table 1 were oxidized to completion: typical results, for cells grown with 2,5-xylene, are shown in Fig. 1(a). When a methyl group was attached at C-6 of the benzene nucleus, as in 2,3,5,6-tetramethylphenol, the compound was oxidized only to a limited extent. In agreement with Scheme 1, intact cells of *Pseudomonas* 2,5, grown with 2,5-xylene, oxidized 3-hydroxy-4-methylbenzoate (V) at the same initial rate as the growth substrate (Fig. 1b). 4-Methylgentisate (VI) was oxidized at a lower, but linear, rate. However, since gentisate and 4-methylgentisate are rapidly oxidized by extracts of this organism (Hopper, Chapman & Dagley, 1971), oxidation by intact cells appears to be limited by the rates at which these substrates can gain access to intracellular enzymes. Other dihydric phenols that can serve as substrates for various ring-fission dioxygenases, namely 3-methylcatechol, protocatechuate and 2,3-dihydroxybenzoate, were oxidized very slowly, if at all, by intact cells; further, 3-ethylphenol, *o*-cresol and 2,6-xylene were not attacked, possibly because they do not possess a

methyl group placed *meta* to the hydroxyl and therefore cannot furnish the carboxyl group of a gentisic acid.

Aromatic compounds accumulated by intact cells of Pseudomonas 2,5 in the presence of $\alpha\alpha'$ -bipyridyl. Additional evidence for the reactions of Scheme 1 was obtained by isolating compounds that accumulated when *m*-cresol, 2,5-xylene and 3,5-xylene were oxidized by cell suspensions in the presence of $\alpha\alpha'$ -bipyridyl, an inhibitor of bacterial oxygenases (Chapman & Hopper, 1968). Each of three 4 litre Erlenmeyer flasks contained 1.8 g (dry wt.) of *m*-cresol-grown *Pseudomonas* 2,5 suspended in 700 ml of 0.05 M-phosphate buffer, pH 7.0, and 5 mM- $\alpha\alpha'$ -bipyridyl. The mixtures were equilibrated on a Gyrotary shaker at 30°C for 20 min and additions (100 ml of each) were then made of 0.01 M-*m*-cresol to the first, 0.01 M-2,5-xylene to the second and 0.01 M-3,5-xylene to the third suspension. Oxidation was followed in a parallel manometric experiment and, when oxygen uptake ceased, a further 100 ml of each substrate was added. A total of three additions were made in each case until oxidation ceased, and the cells were removed by centrifugation. Excess of $\alpha\alpha'$ -bipyridyl was converted into its iron chelate by addition of ferrous sulphate to each supernatant solution, which was

Scheme 1.

then acidified with M-sulphuric acid, filtered and extracted four times with 500ml of ether. For each reaction mixture, extracts were combined, dried over anhydrous sodium sulphate and evaporated to dryness to give 300–400mg of solid in each case.

Products from the oxidation of *m*-cresol. When a little of the solid, isolated from the products of oxidation of *m*-cresol, was examined by t.l.c. with solvent A and detection reagent (b) (Table 2), three spots were revealed that corresponded to *m*-cresol (I), 3-hydroxybenzoic acid (II) and gentisic acid (III). The remainder of the solid was dissolved in 50ml of 0.5M-phosphate buffer, pH 7.0, and gently shaken with 50ml of ether to remove *m*-cresol. The aqueous solution was then acidified with M-sulphuric acid, extracted vigorously four times with 50ml of ether and the combined extracts were evaporated to dryness after drying with anhydrous sodium sulphate. Preparative t.l.c. was used to isolate 3-hydroxybenzoic acid and gentisic acid from this solid, of which 30mg was applied to each plate. Chromatograms were developed in solvent A, and compounds were located as two u.v.-fluorescent bands, which were then scraped from the plate. The silica gel was eluted with ether, the solutions were filtered and dried and the ether was removed. More than 100mg of 3-hydroxybenzoic acid and a

few milligrams of gentisic acid were obtained: both products appeared homogeneous when rechromatographed. The sample of 3-hydroxybenzoic acid was recrystallized twice from water, after being decolorized with charcoal, yielding white needles, m.p. 199–200°C unchanged by admixture with authentic 3-hydroxybenzoic acid (Found: C, 60.6; H, 4.6. Calc. for $C_7H_6O_3$: C, 60.8; H, 4.4%). Weast (1968) gave m.p. 201.5°C. The i.r.- and u.v.-absorption spectra were identical with those of authentic 3-hydroxybenzoic acid. Further confirmation of identity was obtained by paper chromatography (product 1, Table 2). The small quantity of the second compound, which had the u.v.-absorption spectrum and chromatographic properties (product 2, Table 2) of gentisic acid, was crystallized from chloroform, yielding a pale-yellow solid, m.p. 199–200°C (decomp.). Heilbron & Bunbury (1953) gave m.p. 204.5°C for gentisic acid.

Products from the oxidation of 2,5-xylenol. T.l.c. results showed that the solid after oxidation of 2,5-xylenol contained, in addition to unmetabolized substrate, three components, of which two had the chromatographic properties of 3-hydroxy-4-methylbenzoic acid and 4-methylgentisic acid respectively (V and VI, Scheme 1). From its position on these chromatograms the third com-

Table 2. *Chromatographic identification of compounds formed during the metabolism of m-cresol, 2,5-xyleneol and 3,5-xyleneol by intact cells inhibited with $\alpha\alpha'$ -bipyridyl*

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

Compound	Solvent ...	R_F				Appearance under u.v. light	Colour		
		C	D	E	F		(a)	(b)	(c)
3-Hydroxybenzoic acid		0.38	0.49	0.67	0.70	Deep blue	Blue*	Red-purple	Yellow
Product 1, from m-cresol		0.38	0.49	0.66	0.70	Deep blue	Blue*	Red-purple	Yellow
Gentisic acid		0.43	0.66	0.46	0.62	Light blue	Blue	Pale yellow	Pale mauve
Product 2, from m-cresol		0.43	0.66	0.46	0.61	Light blue	Blue	Pale yellow	Pale mauve
3-Hydroxy-4-methylbenzoic acid		0.57	0.35	0.88	0.61	Deep blue	Blue*	Purple	Orange-yellow
Product 3, from 2,5-xyleneol		0.57	0.35	0.88	0.60	Deep blue	Blue*	Purple	Orange-yellow
4-Methylgentisic acid		0.56	0.45	0.68	0.54	Light blue	Blue	Pale green	Olive-green
Product 4, from 2,5-xyleneol		0.56	0.45	0.68	0.54	Light blue	Blue	Pale green	Olive-green
3-Hydroxy-5-methylbenzoic acid		0.44	0.33	0.82	0.63	Deep blue	Blue*	Red-purple	Orange-yellow
Product 5, from 3,5-xyleneol		0.44	0.32	0.82	0.63	Deep blue	Blue*	Red-purple	Orange-yellow
3-Methylgentisic acid		0.57	0.44	0.68	0.54	Light blue	Blue	Pale green	Purple-brown
Product 6, from 3,5-xyleneol		0.57	0.45	0.68	0.54	Light blue	Blue	Pale green	Purple-brown
3-Hydroxybenzyl alcohol		—	—	0.59	0.76	U.v.-absorbing	Blue*	Mauve	Orange-yellow
Product 7, from 2,5-xyleneol		—	—	0.77	0.70	U.v.-absorbing	Blue*	Purple	Orange
3-Ethylgentisic acid		—	—	0.76	0.54	Light blue	Blue	Pale green	—
Product 8, from 3-ethyl-5-methylphenol		—	—	0.76	0.54	Light blue	Blue	Pale green	—
3,4-Dimethylgentisic acid		—	—	0.76	0.44	Light blue	Blue	Olive-green	—
Product 9, from 2,3,5-trimethylphenol		—	—	0.76	0.44	Light blue	Blue	Olive-green	—

* Denotes that it was necessary to expose to ammonia for colour development.

pound appeared to be a homologue of 3-hydroxybenzyl alcohol. An ethereal solution, which contained this alcohol and unchanged 2,5-xyleneol, was first obtained by extracting a solution of the solid in phosphate buffer, pH 7.0; 3-hydroxy-4-methylbenzoic acid and 4-methylgentisic acid were then extracted by ether after the aqueous solution had been acidified. The ether was removed from these solutions, and preparative t.l.c. was applied: acids were located by their u.v. fluorescence and the alcohol by means of reagent (b) sprayed along the edge of the chromatography plate. After elution with ether and removal of solvent we obtained 200mg of 3-hydroxy-4-methylbenzoic acid, 10mg of 4-methylgentisic acid and 1–2mg of the alcohol. Each compound isolated gave a single spot on t.l.c. plates. The first component, presumed to be a 3-hydroxy-4-methylbenzoic acid, was recrystallized twice from water, giving white needles, m.p. 207–208°C, unchanged by admixture with authentic material (Found: C, 63.0; H, 5.1. Calc. for $C_8H_8O_3$: C, 63.1; H, 5.3%). Weast (1968) gave m.p. 208.5°C. The i.r.- and u.v.-absorption spectra were identical with those of authentic 3-hydroxy-4-methylbenzoic acid, as was its behaviour on paper chromatograms (product 3, Table 2). When crystallized from chloroform, the second component, 4-methylgentisic acid, gave pale-yellow crystals, m.p. 239–240°C (decomp.), unchanged by admixture with authentic material, m.p. 240–241°C (decomp.). The i.r.- and u.v.-absorption spectra of the compound, and its behaviour on paper chromatograms (product 4, Table 2), were those of authentic 4-methylgentisic acid. The amount of the third compound originally extracted from neutral aqueous solution with ether was sufficient only for examination by paper chromatography (product 7, Table 2). When the chromatographic properties of this compound are viewed in relation to those of 3-hydroxy-4-methylbenzoic acid and 4-methylgentisic acid it is seen that there exist relationships similar to those found between 3-hydroxybenzyl alcohol and 3-hydroxybenzoic acid and gentisic acid. It is therefore suggested that product 7 was 3-hydroxy-4-methylbenzyl alcohol.

Products from the oxidation of 3,5-xyleneol. The compounds arising from 3,5-xyleneol were isolated and identified by methods similar to those used for the oxidation products of *m*-cresol and 2,5-xyleneol. Preparative t.l.c. gave 150mg of a monohydroxy acid, presumed to be 3-hydroxy-5-methylbenzoic acid (Scheme 1, VIII), and also 20mg of 3-methylgentisic acid (IX), each of which ran as a single spot on t.l.c. Two crystallizations from water gave white needles, m.p. 211°C, unchanged by admixture with authentic material, m.p. 210–211°C (Found: C, 63.5; H, 5.3. Calc. for $C_8H_8O_3$: C, 63.1; H, 5.3%). Claisen (1889) gave m.p. 210°C. The i.r.- and u.v.-absorption spectra of the biologically prepared

material, and also its behaviour on paper chromatograms (product 5, Table 2) were essentially the same as those of authentic material. The sample of 3-methylgentisic acid obtained by preparative t.l.c. was recrystallized from water, after being decolorized with charcoal, and gave pale yellow crystals, m.p. 212–213°C (decomp.) unchanged by admixture with authentic material having m.p. 217–218°C (decomp.). Heilbron & Bunbury (1953) gave 215°C and Inouye (1954), 211.5–213°C. The material formed by *Pseudomonas* 2,5 gave the same u.v.-absorption spectrum as chemically synthesized 3-methylgentisic acid and showed the same behaviour on paper chromatograms (product 6, Table 2).

Products from the oxidation of 3-ethyl-5-methylphenol and 2,3,5-trimethylphenol. The above results support the reactions of Scheme 1, in which a methyl group situated *meta* to a phenolic hydroxyl is oxidized to a carboxyl group and a second hydroxyl group is then introduced *para* to the first. Two other compounds were also investigated. In the first of these, 3-ethyl-5-methylphenol, an ethyl group has replaced one methyl group of 3,5-xyleneol, so that the catabolites predicted would be 3-ethyl-5-hydroxybenzoic acid and 3-ethylgentisic acid. The second compound, 2,3,5-trimethylphenol, is derived from 3,5-xyleneol by introducing a third methyl group at C-2, and should therefore give rise to 3,4-dimethyl-5-hydroxybenzoic acid and 3,4-dimethylgentisic acid. The procedures for isolating the products of oxidation of these two phenols were essentially the same as those employed previously when *m*-cresol, 2,5-xyleneol and 3,5-xyleneol were the substrates. Compounds were extracted with ether from incubation mixtures, in which intact cells inhibited with $\alpha\alpha'$ -bipyridyl had metabolized each phenol, and were separated and purified by preparative t.l.c. Of the two compounds isolated from 3-ethyl-5-methylphenol, the first (m.p. 176.5–177.5°C) had chromatographic properties consistent with its being 3-ethyl-5-hydroxybenzoic acid, namely R_f 0.89 and 0.62 in solvents *E* and *F* respectively, with the following colours on chromatograms: red with detection reagent (b), blue with reagent (a), but only when exposed to ammonia, and deep-blue fluorescence under u.v. light. No authentic compound was available for comparison (Found: C, 64.8; H, 6.0. Calc. for $C_9H_{10}O_3$: C, 65.0; H, 6.1%). The second compound had m.p. 195–196°C (decomp.), unchanged by admixture with authentic 3-ethylgentisic acid, m.p. 195–197°C (decomp.). Its i.r.- and u.v.-absorption spectra, and behaviour on paper chromatograms (product 8, Table 2), were those of 3-ethylgentisic acid. Two compounds were also isolated from 2,3,5-trimethylphenol. One had m.p. 208°C (Found: C, 64.7; H, 6.1. Calc. for $C_9H_{10}O_3$: C, 65.0; H, 6.1%). Divekar & Vining (1964) gave m.p. 212°C for 3,4-dimethyl-5-hydroxy-

benzoic acid. No authentic material was available, but paper chromatography showed: R_F 0.89 and 0.46 in solvents *E* and *F* respectively; deep-blue fluorescence of spots under u.v. light; a blue colour given only with ammonia, with reagent (a); mauve given with reagent (b). The other compound had m.p. 209.5–210.5°C (decomp.), unchanged by admixture with authentic 3,4-dimethylgentisic acid, m.p. 212.5–213.5°C (decomp.). The i.r.- and u.v.-absorption spectra and behaviour on paper chromatograms (product 9, Table 2) were also the same as those of this authentic material.

Oxidation of phenols by cell extracts. Although *m*-cresol, 2,5-xyleneol, 3,5-xyleneol, 2,3,5-trimethylphenol and 3-ethyl-5-methylphenol were readily oxidized by intact cells grown with 2,5-xyleneol (Fig. 1a), ammonium sulphate-treated extracts (Chapman & Hopper, 1968) of the same cells failed to oxidize any of these compounds until supplemented with NADH. When this coenzyme was generated in the reaction mixture from added yeast alcohol dehydrogenase, ethanol and NAD⁺, extracts catalysed the immediate oxidation of all five substituted phenols with the consumption of 2.5–3.0 μ mol of oxygen/ μ mol of substrate. There was no oxidation when NADH was replaced by NADPH, which was generated from glucose 6-phosphate dehydrogenase, glucose 6-phosphate and NADP⁺. Cell extracts also catalysed the oxidation of gentisic acid, with the consumption of 1.0 μ mol of oxygen/ μ mol of substrate. This uptake of oxygen was abolished by 0.5 mM- $\alpha\alpha'$ -bipyridyl, so that oxygen consumption was decreased (Fig. 2) and gentisic acids accumulated in solution when various phenols were oxidized by cell extracts in the presence of this inhibitor. To investigate the products formed when cell-free oxidations were inhibited with $\alpha\alpha'$ -bipyridyl, reactions were set up as in Fig. 2 with *m*-cresol, 2,5-xyleneol, 3,5-xyleneol and 3-hydroxy-4-methylbenzoic acid as substrates. When oxygen uptake ceased, the contents of each flask were washed into a tube and protein was precipitated with 3 ml of 30% (w/v) metaphosphoric acid. The precipitate was removed by centrifugation, each solution was extracted three times with 10 ml of ether and, after drying with anhydrous sodium sulphate, the ether was removed by evaporation from the pooled extracts to give a small amount of solid. Each of these samples gave one major spot, corresponding to a gentisic acid, when examined by t.l.c. in solvent *A*. When re-chromatographed on paper (Table 2) it was confirmed that *m*-cresol (I, Scheme 1) gave gentisic acid (III); 2,5-xyleneol (IV) gave 4-methylgentisic acid (VI); 3-hydroxy-4-methylbenzoic acid (V) also gave 4-methylgentisic acid; 3,5-xyleneol (VII) gave 3-methylgentisic acid (IX). The preliminary thin-layer chromatograms of the products of enzymic oxidation of 2,5-

Fig. 2. Oxidation of 2,5-xyleneol and 3-hydroxy-4-methylbenzoic acid by cell-free extracts of *Pseudomonas* 2,5 grown with *m*-cresol. Reaction mixtures were provided with NADH, generated by enzymic reduction of NAD⁺; the endogenous oxidation (200 μ l of O₂/h) has been subtracted. Each flask contained: 1.0 ml of 0.05 M-phosphate buffer, pH 7.0, 1.0 ml of cell extract (20 mg of protein), 0.1 ml of 10 mM-NAD⁺, 0.1 ml of 0.2 M-ethanol, 0.1 ml of alcohol dehydrogenase (1 mg/ml); 0.5 ml of 10 mM-substrate was in the side arm, and 0.2 ml of 20% (w/v) KOH in the centre well. The temperature was 30°C. The gas phase was air. Substrates were: ○ and ●, 2,5-xyleneol; △ and ▲, 3-hydroxy-4-methylbenzoic acid. Reactions occurred in the presence (●, ▲) and absence (○, △) of 0.5 mM- $\alpha\alpha'$ -bipyridyl.

xyleneol and 3,5-xyleneol also showed small spots due to 3-hydroxy-4-methylbenzoic acid and 3-hydroxy-5-methylbenzoic acid respectively.

Oxidation of substituted phenols by intact cells of *Pseudomonas* 3,5. Washed suspensions of *Pseudomonas* 3,5 grown with 3,5-xyleneol oxidized 3,5-xyleneol, 3-ethyl-5-methylphenol, *m*-cresol, 2,3,5-trimethylphenol and 2,5-xyleneol (Fig. 3a). In this respect they resembled *Pseudomonas* 2,5 grown with either 2,5-xyleneol or 3,5-xyleneol (Table 1). They differed insofar as *Pseudomonas* 2,5 oxidized both 2,3,5-trimethylphenol and 2,5-xyleneol virtually to completion (Fig. 1a), whereas *Pseudomonas* 3,5 oxidized these compounds only to a limited extent (Fig. 3a). This is in accord with the observation that *Pseudomonas* 3,5 grew only at the expense of *m*-cresol, 3,5-xyleneol and 3-ethyl-5-methylphenol, and not with 2,5-xyleneol or 2,3,5-trimethylphenol. Moreover, 2,3,5,6-tetramethylphenol, which was oxidized to a limited extent by *Pseudomonas* 2,5, was not attacked at all by *Pseudomonas* 3,5. The latter oxidized *m*-cresol to completion when grown with 3,5-xyleneol, although the rate was lower than those for 3,5-xyleneol and 3-ethyl-5-methylphenol.

Fig. 3. Oxidation of various compounds by washed cells of *Pseudomonas* 3,5 grown with either 3,5-xyleneol, (a) and (c), or with *m*-cresol, (b). Each flask contained 2.5 ml of a bacterial suspension (5 mg dry wt.) in 0.05 M-phosphate buffer, pH 7.0; 0.3 ml of 10 mM substrate was in the side arm, and 0.2 ml of 20% (w/v) KOH in the centre well. Substrates were: (1) 3,5-xyleneol; (2) 3-ethyl-5-methylphenol; (3) *m*-cresol; (4) 2,3,5-trimethylphenol; (5) 2,5-xyleneol; (6) 2,3,5,6-tetramethylphenol; (7) water; (8) 3-hydroxy-5-methylbenzoic acid; (9) 3-methylgentisic acid; (10) gentisic acid; (11) 3,4-dihydroxy-5-methylbenzoic acid; (12) 3-methylcatechol; (13) 5-hydroxyisophthalic acid. The temperature was 30°C. The gas phase was air. (a), (b) and (c) represent three separate experiments.

However, when *Pseudomonas* 3,5 was grown with *m*-cresol, only the growth substrate, out of six phenols tested, was rapidly oxidized to completion (Fig. 3b); the others were rapidly oxidized to limited extents. The two species appeared to oxidize these phenols by the same metabolic pathways (Scheme 1); and although their capabilities differed this could be accounted for by differences either in the specificities of the enzymes involved, or in requirements for enzymic de-repression. Thus 3-hydroxy-5-methylbenzoate was again oxidized readily (Fig. 3c). 3-Methylgentisate and gentisate were oxidized at lower but significant rates, as was observed when *Pseudomonas* 2,5 oxidized 4-methylgentisate and gentisate (Fig. 1b). 5-Hydroxyisophthalate, which would arise from the oxidation of both methyl groups of 3,5-xyleneol, was not attacked. 3-Methylcatechol and 3,4-dihydroxy-5-methylbenzoate, possible ring-fission substrates for metabolic pathways involving catechols, were oxidized very slowly. In support of the reactions of Scheme 1, two products were obtained from the oxidation of 3,5-xyleneol by suspensions of *Pseudomonas* 3,5 inhibited by $\alpha\alpha'$ -bipyridyl. The methods of isolation, involving ether extraction and preparative t.l.c., and also the conditions for oxidation, were the same as

those used for *Pseudomonas* 2,5 except that the concentration of $\alpha\alpha'$ -bipyridyl was decreased from 5 to 1 mM. One compound, obtained as white crystals, m.p. 211°C, had the same chromatographic properties (Table 2) as authentic 3-hydroxy-5-methylbenzoic acid, m.p. 211°C. The second compound, also in the form of white crystals, had the same m.p., 220–221°C (decomp.), and chromatographic properties as 3-methylgentisic acid. The i.r.-absorption spectrum of each compound was identical with that of the corresponding authentic material. The product formed by limited oxidation of 2,5-xyleneol (Fig. 3a) was also isolated as follows. A suspension of 7 g (wet wt.) of 3,5-xyleneol-grown *Pseudomonas* 3,5 in 700 ml of 0.05 M-phosphate buffer, pH 7.0, was shaken at 30°C for 15 min in a 4 litre Erlenmeyer flask, and 100 ml of 0.01 M-2,5-xyleneol was then added. No inhibitor was used in this experiment. When uptake of oxygen ceased, as indicated by a parallel manometric experiment, further additions were made, to a total of 300 ml. The cells were then removed by centrifuging, the solution was acidified with *m*-sulphuric acid, filtered and extracted three times with 250 ml of ether. These extracts were combined and dried over anhydrous sodium sulphate, the ether was removed by

evaporation and the solid residue (400mg) when crystallized from water gave white needles, m.p. 208–209°C, unchanged by admixture with authentic 3-hydroxy-4-methylbenzoic acid (Found: C, 63.0; H, 5.1. Calc. for $C_8H_8O_3$: C, 63.1; H, 5.3%).

DISCUSSION

Before any aromatic compound can serve as ring-fission substrate for a microbial dioxygenase, one minimal structural requirement must be satisfied, namely, the benzene nucleus to be cleaved must bear at least two hydroxyl groups. The pathways of catabolism for aromatic compounds most thoroughly investigated are those involving catechols, in which these two hydroxyl groups are borne by adjacent carbon atoms. In contrast, those pathways involving gentisate (2,5-dihydroxybenzoate) have received relatively little attention. Lack (1959, 1961) made a careful study of the enzymes that degrade gentisate, but information relating to its formation has been obtained by less direct methods, by showing that rates of oxidation by intact cells were compatible with the metabolic pathways proposed, or that cell extracts contained the enzymes for degrading gentisate. Thus gentisate appears to be an intermediate in the degradation of *m*-hydroxybenzoate (Tanaka, Sugiyama, Yano & Arima, 1957; Walker & Evans, 1952) and of 2-naphthol (Walker, 1964) by *Pseudomonas* spp., and also of anthranilate and 5-hydroxyanthranilate by *Achromobacter* (Ladd, 1962, 1964). Further, Walker & Lippert (1965) isolated gentisate from cultures of a pseudomonad metabolizing 2-naphthol, and Wheelis, Palleroni & Stanier (1967) showed that *Pseudomonas acidovorans* converted *m*-hydroxybenzoate into gentisate by a specific NADPH-dependent hydroxylase.

The present work has established that gentisic acid, or substituted gentisic acids, are formed when *m*-cresol, or xylenols, are oxidized by two species of *Pseudomonas*. In accordance with Scheme 1, gentisic acid, 4-methylgentisic acid and 3-methylgentisic acid were isolated from $\alpha\alpha'$ -bipyridyl-inhibited cell suspensions of *Pseudomonas* 2,5 when *m*-cresol, 2,5-xyleneol and 3,5-xyleneol respectively were oxidized. The proposed precursors of these acids, namely 3-hydroxybenzoic acid, 3-hydroxy-methylbenzoic acid and 3-hydroxy-5-methylbenzoic acid, were also isolated. 3-Ethyl-5-methylphenol and 2,3,5-trimethylphenol were metabolized by similar reaction sequences, since the first of these phenols gave rise to 3-ethyl-5-hydroxybenzoic acid and 3-ethylgentisic acid, and the second gave 3,4-dimethyl-5-hydroxybenzoic acid and 3,4-dimethylgentisic acid. Although the complete range of compounds was not investigated with *Pseudomonas* 3,5, this organism oxidized 3,5-xyleneol

by similar reactions, since 3-hydroxy-5-methylbenzoic acid and 3-methylgentisic acid were formed by the action of $\alpha\alpha'$ -bipyridyl-inhibited cell suspensions.

These results show that degradation by both species of *Pseudomonas* was initiated by oxidizing a methyl group situated *meta* to the hydroxyl group of the phenol, thereby forming a 3-hydroxybenzoic acid. When this position was occupied by an ethyl group, as in 3-ethylphenol (Fig. 1b), the oxidation did not occur; moreover, methyl groups at other positions in the benzene nucleus were not oxidized, at least initially, since it was possible to isolate 3-methylgentisic acid, 4-methylgentisic acid and 3,4-dimethylgentisic acid from cultures (Table 2). The five phenols that were readily oxidized by intact cells of *Pseudomonas* 2,5 (Fig. 1a) were not attacked by cell extracts unless NADH was furnished. It therefore appears that oxidation of a methyl group, to form an alcohol, was initiated by an NADH-dependent hydroxylase, and this conclusion was supported by the isolation of product 7 from 2,5-xyleneol (Table 2), which had the properties expected for 3-hydroxy-4-methylbenzyl alcohol. The subsequent oxidation of this alcohol, first to the aldehyde and then to the acid, could re-furnish the NADH required for the initial hydroxylation step.

When grown with succinate, intact cells of *Pseudomonas* 2,5 oxidized 3-hydroxybenzoate and gentisate at slow but appreciable rates, whereas they failed completely to oxidize any of the phenolic compounds tested (Table 1). It appears that the ability to metabolize these substituted phenols is entirely dependent on the prior induction of enzymes that attack the relevant methyl group. Experiments with both intact cells and cell extracts demonstrated that when this enzyme system was induced by exposure to any one of these phenols, the ability to metabolize the rest was simultaneously acquired. However, when a methyl group was introduced at C-6, as in 2,3,5,6-tetramethylphenol (Fig. 1a), the extent of oxidation was restricted.

The enzymes of *Pseudomonas* 3,5 showed more restricted substrate specificities than those of *Pseudomonas* 2,5. Growth with 3,5-xyleneol conferred the ability to oxidize all the phenols tested except 2,3,5,6-tetramethylphenol; but 2,3,5-trimethylphenol and 2,5-xyleneol, which were oxidized to completion by *Pseudomonas* 2,5 (Fig. 1a), were oxidized only to a limited extent by *Pseudomonas* 3,5 (Fig. 3a) and consequently did not support the growth of the latter organism. This restricted degradation may be ascribed to two factors. First, provided that the group is in the *meta* position relative to the hydroxyl, the enzyme system that oxidizes the methyl group to the carboxyl has the same broad substrate specificity in *Pseudomonas*

3,5 as it does in *Pseudomonas* 2,5, and this reaction accounts for the uptake of oxygen observed. Second, the hydroxylases that convert 3-hydroxybenzoates into the corresponding gentisates in *Pseudomonas* 3,5 show a much more restricted range of substrate specificities than the corresponding enzyme(s) in *Pseudomonas* 2,5. Accordingly, the hydroxylase that functions in the degradative pathway of 3,5-xynol does not attack the corresponding monohydroxy acid formed by *Pseudomonas* 3,5 from 2,5-xynol. This was demonstrated by showing that 3-hydroxy-4-methylbenzoic acid accumulated, and was not further metabolized, when 2,5-xynol was oxidized by *Pseudomonas* 3,5 grown with 3,5-xynol. By contrast, these same cells could only be made to accumulate 3-hydroxy-5-methylbenzoic acid from 3,5-xynol by adding $\alpha\alpha'$ -bipyridyl. In *Pseudomonas* 3,5 the enzymes that degrade gentisic acids are also more specific for their substrates than those in *Pseudomonas* 2,5 (Hopper *et al.* 1971).

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