The Metabolism of Cresols by Species of *Pseudomonas*

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(Received 21 June 1966)

1. A comparison of rates of oxidation of various compounds by whole cells indicated that protocatechuate was a reaction intermediate when a non-fluorescent species of *Pseudomonas* oxidized *p*-cresol. In contrast, a fluorescent *Pseudomonas* oxidized 3-methylcatechol and 4-methylcatechol when grown with *p*-cresol, but did not oxidize protocatechuate. 2. Heat-treated extracts of the fluorescent *Pseudomonas* oxidized catechol, 3-methylcatechol and 4-methylcatechol to ring-fission products, the spectroscopic properties of which were recorded. Rates of enzymic degradation of these products were also measured. 3. Acetic acid and formic acid were obtained by the action of a Sephadex-treated extract on 3-methylcatechol and 4-methylcatechol respectively. In each case 0.8 mol. of the carboxylic acid was formed from 1.0 mol. of substrate. 4. Dialysed extracts converted 3-methylcatechol into acetaldehyde and pyruvate, with 4-hydroxy-2-oxovalerate as a reaction intermediate. 4-Methylcatechol was converted first into 4-hydroxy-2-oxohexanoate and then into propionaldehyde and pyruvate. 5. The ring-fission product of catechol was formed from phenol by a fluorescent *Pseudomonas*, that of 3-methylcatechol was formed from *o*-cresol and *m*-cresol, and the ring-fission product of 4-methylcatechol was given from *p*-cresol. Propionate was readily oxidized by these cells after growth with *p*-cresol, but this compound was not attacked when phenol, *o*-cresol or *m*-cresol served as source of carbon. 6. Cell extracts appeared to attack only one enantiomer of synthetic 4-hydroxy-2-oxohexanoate.

Dagley & Patel (1957) showed that degradation of *p*-cresol by a non-fluorescent *Pseudomonas* was initiated by oxidation of the methyl group: *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were successive metabolites in the formation of the substrate that underwent benzene ring-fission, namely protocatechuic acid. In contrast, there have been brief reports, which are confirmed in the present work, that when cresols are metabolized by certain fluorescent species of *Pseudomonas* the ring-fission substrate is a catechol that bears intact the methyl group of the cresol (Dagley, Gibson & Thomas, 1964*b*; Ribbons, 1964). In all the systems mentioned, 'meta' fissions occurred, i.e. the benzene ring was opened between adjacent carbon atoms, only one of which bore a hydroxyl group. Dagley, Chapman, Gibson & Wood (1964*a*) published a general scheme of reactions by which aromatic compounds that undergo *meta* fissions of the nucleus may be converted into pyruvate and other products. Although this generalization would embrace 3-methylcatechol and 4-methylcatechol,

no complete study to justify their inclusion in the scheme has been reported hitherto.

MATERIALS AND METHODS

Maintenance and growth of organism. The three species of *Pseudomonas* used in this work were maintained on nutrient agar slopes and were subcultured every 2 weeks. The non-fluorescent *Pseudomonas* N.C.I.B. 8893, which utilized *p*-cresol or *p*-hydroxybenzoic acid as source of carbon, was grown in the mineral salts medium of Dagley & Patel (1957). The two fluorescent organisms *Pseudomonas* (L) and *Pseudomonas* (U) (Dagley & Gibson, 1965) were grown with forced aeration at 30° in a basal medium that contained (per l.) in addition to the source of carbon: KH₂PO₄, 2g.; (NH₄)₂SO₄, 1g.; MgSO₄·7H₂O, 0.4g.; Lab-Lemco (Oxoid Division of Oxo Ltd., London), 0.3g.; adjusted to pH 7.2 with NaOH. Foaming during the growth of aerated cultures was controlled by the addition of Silicone MS Antifoam A (Hopkin and Williams Ltd., Chadwell Heath, Essex). After growth for 18 hr. cells used for preparing cell-free extracts were harvested, washed once by resuspending in phosphate buffer (KH₂PO₄, 2g./l.; pH 7.4) and then centrifuged at 3° for 10 min. at 4300 g. When the cells were needed for respirometer studies they were washed twice and suspended in phosphate buffer to give a cell density of 10 mg. dry wt./ml. The measurement

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of gas exchanges at 30° and the preparation of cell extracts of *Pseudomonas* (U) grown in the basal medium containing 0.06% (w/v) of phenol were as described by Dagley & Gibson (1965). When a cresol served as source of carbon its concentration was 0.04% (w/v). All phosphate buffers contained KH_2PO_4 adjusted to the required pH with NaOH.

Partial purification of cell extracts. To a crude cell extract at 4° was added, with stirring, 0.2 vol. of 2% (w/v) protamine sulphate; the precipitate was then removed by centrifuging at 26000 g_{av} for 20 min. at 2°. The supernatant solution was treated as follows to give extracts that are referred to as 'heat-treated', 'Sephadex-treated' and 'dialysed'. When extracts were heated at 55° for 5 min. and the resultant precipitate was removed by centrifuging, the heat-treated extract still oxidized catechol, 3-methylcatechol and 4-methylcatechol but did not metabolize the products of ring-fission. For Sephadex-treated and dialysed extracts the solution at 2° was first brought to 70% saturation by addition, with stirring, of a neutralized solution of $(\text{NH}_4)_2\text{SO}_4$ and the precipitate formed was collected on the centrifuge. For Sephadex-treated extracts the precipitate was then dissolved in phosphate buffer (KH_2PO_4 , 2 g./l.; pH 7.4) and applied to a column (20 cm. $\times$ 2 cm.) of Sephadex G-25 (fine grade; 20–80 μ). When eluted with the same buffer, the extract was free of $(\text{NH}_4)_2\text{SO}_4$ and contained 12–16 mg. of protein/ml. Sephadex-treated extracts converted 3-methylcatechol and 4-methylcatechol respectively into 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate, which were metabolized to pyruvate only slowly. To prepare a dialysed extract the precipitate formed after $(\text{NH}_4)_2\text{SO}_4$ treatment was dissolved in phosphate buffer to give a protein concentration of 20–30 mg./ml. and the solution was dialysed with stirring against three changes of the same buffer. Dialysed extracts converted 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate into pyruvate.

Chromatography. Thin-layer chromatography was carried out on Kieselgel G and Aluminiumoxid G (E. Merck A.-G., Darmstadt, Germany). Glass plates were coated to a thickness of 0.25 mm. and activated at 100° for 30 min. Whenever a compound was identified by chromatography its R_f was referred to that of authentic material run on the same chromatogram. For the detection of 2,4-dinitrophenylhydrazones of oxo acids and their lactones three solvent systems were used. These were: A, benzene-ethyl formate-propionic acid (18:1:1, by vol.) (Dagley & Gibson, 1965); B, light petroleum (b.p. 60–80°)-ethyl formate-propionic acid (70:30:15.4, by vol.) (Ronkainen, 1963); C, benzene-tetrahydrofuran-acetic acid (15:9:1, by vol.) (Byrne, 1965). The R_f values of 2,4-dinitrophenylhydrazones on Kieselgel G in solvents A, B and C were as follows: for pyruvate in A, 0.18; in B, 0.28; in C, 0.70; for 4-hydroxy-2-oxovalerate in A, 0.08; in B, 0.06, 0.19; in C, 0.67; for 4-hydroxy-2-oxohexanoate in A, 0.12; in B, 0.10, 0.27; in C, 0.74; for 4-methyl-2-oxobutyrolactone in A, 0.30; in B, 0.33, 0.49; in C, 0.81; for 4-ethyl-2-oxobutyrolactone in A, 0.40; in B, 0.47, 0.69; in C, 0.85. Where two values are given for one compound in a particular solvent they are presumably those for stereoisomers.

The 2,4-dinitrophenylhydrazines of neutral carbonyl compounds were identified on Kieselgel G and Aluminiumoxid G with the solvent light petroleum (b.p. 80–100°)-diethyl ether (7:3, v/v) (Byrne, 1965). The R_f values for the 2,4-dinitrophenylhydrazones of the following com-

pounds were: acetaldehyde, 0.22 (Kieselgel G) and 0.17 (Aluminiumoxid G); propionaldehyde, 0.34 and 0.24; butyraldehyde, 0.40 and 0.29.

4-Methyl-2-oxobutyrolactone and 4-ethyl-2-oxobutyrolactone were chromatographed on Kieselgel G with the solvent acetic acid-chloroform (1:10, v/v); the R_f values were: for 4-methyl-2-oxobutyrolactone, 0.67, and for 4-ethyl-2-oxobutyrolactone, 0.76 and 0.65, the two values probably being due to isomerization of this compound since spots of R_f value 0.76 again gave 0.76 and 0.65 when rechromatographed. Lactones were detected by spraying with a 2% (w/v) solution of 2,6-dibromo-*p*-benzoquinone-4-chloroimine in ethanol followed by heating at 100° for 5 min., when the lactones gave purple spots. Oxo acids were detected by spraying with a 0.1% 2,4-dinitrophenylhydrazine in 2N-HCl.

Acetic acid and formic acid were identified and determined by partition chromatography on water-washed silicic acid (Mallinckrodt Chemical Works, New York, N.Y., U.S.A.), as described by Bulen, Varner & Burrell (1952). Rapidly flowing columns were prepared from 100-mesh silicic acid minus fines. Fractions of volume 3 ml. were collected, 8 ml. of CO_2 -free water was added to each and the eluted acids were titrated with 11.0 mN-NaOH with phenol red as indicator. The identity of formate in reaction mixtures was confirmed by the colorimetric method of Grant (1948) as described by Dagley & Gibson (1965). The presence of acetate in reaction mixtures was confirmed by its enzymic conversion into acetyl phosphate, which forms an acetyl-ferriic hydroxamate complex (Rose, 1955). The acetic acid was steam-distilled from deproteinized reaction mixtures and after neutralizing with NaOH the distillate was evaporated to a small volume. The source of acetate kinase (ATP-acetate phosphotransferase, EC 2.7.2.1) was a cell-free extract of the organism identified by Sebald & Véron (1963) as a species of *Moraxella* (N.C.I.B. 8250; formerly designated '*Vibrio* O1'), which was grown on a mineral salts medium with acetate as the sole source of carbon.

Chemical analyses. Pyruvate was determined in the presence of 4-hydroxy-2-oxovalerate or 4-hydroxy-2-oxohexanoate by a modification of the method of Friedemann & Haugen (1943) used by Dagley & Gibson (1965). 4-Hydroxy-2-oxovalerate was determined by converting the compound into its lactone and measuring extinctions at 230 m μ in acid solution (Dagley & Gibson, 1965). The same procedure was used to determine 4-hydroxy-2-oxohexanoate since the absorption spectrum of its lactone, 4-ethyl-2-oxobutyrolactone, is very similar to that of 4-methyl-2-oxobutyrolactone. Lactonization of 90% of 4-hydroxy-2-oxohexanoate was consistently observed.

Materials. Acetaldehyde, propionaldehyde, catechol and pyruvic acid were from British Drug Houses Ltd., Poole, Dorset. 3-Methylcatechol and 4-methylcatechol were from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. All catechols were purified by sublimation and the pyruvic acid was redistilled. 4-Methyl-2-oxobutyrolactone and 4-ethyl-2-oxobutyrolactone were obtained by hydrolysis (Rossi & Schinz, 1948) of the 3-carbethoxy derivatives of the corresponding 2-oxobutyrolactones, prepared by the method of Stacey & Wagner (1952). Solutions of 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate were prepared by mild alkaline hydrolysis of their lactones (Dagley

Table 1. Rates of oxidation of various compounds by whole cells of *Pseudomonas* (U) and *Pseudomonas* N.C.I.B. 8893

The main compartments of Warburg flasks contained, in 3.0 ml., 44 μ moles of potassium phosphate buffer, pH 7.2, and washed cells (5 mg. dry wt.); centre wells contained 0.2 ml. of 20% (w/v) KOH. The gas phase was air. Each substrate (5 μ moles) was added from the side arms. Q_{O_2} values are expressed as μ l. of O_2 consumed/mg. dry wt. of cells/hr.; uptake of O_2 in the absence of substrate (Q_{O_2} 8–18) was subtracted.

Organism	Substrate Carbon source	Q_{O_2} for substrate oxidized				
		<i>p</i> -Hydroxy- benzoate	Proto- catechuate	3-Methyl- catechol	4-Methyl- catechol	<i>p</i> -Cresol
<i>Pseudomonas</i>	<i>p</i> -Cresol	133	150	0	118	141
N.C.I.B. 8893	<i>p</i> -Hydroxybenzoate	92	109	0	0	0
<i>Pseudomonas</i>	<i>p</i> -Cresol	0	3	144	108	70
(U)	<i>p</i> -Hydroxybenzoate	130	137	14	14	16

& Gibson, 1965). Hydrolysis of 4-ethyl-2-oxobutyrolactone was complete at room temperature in 3–4 hr., and, since this solution was unstable at room temperature and decomposed even when frozen, it was used immediately. Lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Absorption spectra. Infrared-absorption spectra were recorded by a Unicam SP.200 spectrophotometer and ultraviolet-absorption spectra by a Unicam SP.800A spectrophotometer.

RESULTS

Oxidation of substrates by whole cells of *Pseudomonas* (U) and *Pseudomonas* N.C.I.B. 8893. When *Pseudomonas* N.C.I.B. 8893 had been grown with *p*-hydroxybenzoate as a source of carbon, *p*-hydroxybenzoate and protocatechuate were the only substrates tested that were oxidized readily. After growth with *p*-cresol, the growth substrate and 4-methylcatechol were oxidized in addition to *p*-hydroxybenzoate and protocatechuate, whereas 3-methylcatechol was not attacked (Table 1). These observations agree with those of Dagley & Patel (1957), who proposed that protocatechuate is a reaction intermediate when *p*-cresol, *p*-hydroxybenzoate and 4-methylcatechol are oxidized by this organism. In contrast, the two methylcatechols were readily oxidized by *Pseudomonas* (U) that had been grown with *p*-cresol; however, neither *p*-hydroxybenzoate nor protocatechuate was oxidized unless *p*-hydroxybenzoate had been utilized for growth, in which case the ability to oxidize *p*-cresol and the methylcatechols was lost. Further experiments showed that *Pseudomonas* (U) oxidized catechol, 3-methylcatechol or 4-methylcatechol when grown with *o*-cresol, *m*-cresol or phenol; these compounds did not support growth of *Pseudomonas* N.C.I.B. 8893.

Oxidation of catechol and methylcatechols by heat-treated extracts of *Pseudomonas* (U). When crude extracts or Sephadex-treated extracts of *Pseudomonas* (U), grown with phenol or a cresol, were incubated at pH 7.4 with catechol, 1 μ mole of oxygen was taken up per μ mole of substrate and a yellow compound with the ultraviolet-absorption spectrum of 2-hydroxymuconic semialdehyde (Dagley & Gibson, 1965) was formed and then disappeared. With 3-methylcatechol a yellow compound also appeared and then disappeared more rapidly than that from catechol but the product from 4-methylcatechol persisted for longer periods. When extracts were heat-treated the same volumes of oxygen were consumed but the ring-fission compounds were no longer metabolized, and it was therefore possible to prepare them in solution and to measure their light-absorption. The heat-treated extract (0.5 ml.) was incubated in a Warburg flask (reaction vol. 3.0 ml.) with 130 μ moles of phosphate buffer, pH 7.4, and 5 μ moles of catechol, 3-methylcatechol or 4-methylcatechol. When uptake of oxygen ceased, 0.2 ml. of 6% (w/v) perchloric acid was added, the precipitate was removed by centrifuging and the solution extracted with ether (3 $\times$ 10 ml.). The ethereal solution was re-extracted with 0.02 M-phosphate buffer, pH 7.6 (4 $\times$ 25 ml.), and, after dilution and addition of sodium hydroxide or hydrochloric acid as required, the spectra of each ring-fission product were recorded at pH 11.0, 7.6 and 2.5. The spectra of the ring-fission products of 3-methylcatechol and 4-methylcatechol resembled those reported by Dagley, Evans & Ribbons (1960) for 2-hydroxymuconic semialdehyde, which is formed from catechol. Each compound showed a strong absorption of light between 370 and 390 $m\mu$ at pH 11.0 that was abolished on acidification to give another absorption peak at 310–320 $m\mu$. The ring-fission product of 3-methylcatechol differed from those of catechol and 4-methylcatechol in that its light-absorption at 388 $m\mu$ was more sharply

Table 2. *Spectroscopic properties of the products of enzymic ring-fission of catechol, 3-methylcatechol and 4-methylcatechol*

Catechol, 3-methylcatechol and 4-methylcatechol were oxidized to ring-fission products by heat-treated extracts of *Pseudomonas* (U). The amount of each product, from which molar extinction coefficients (ϵ) were calculated, was obtained from the volume of oxygen consumed in its formation.

Compound oxidized	Catechol		3-Methylcatechol		4-Methylcatechol	
pH	$\lambda_{\max.}$ (m μ)	ϵ	$\lambda_{\max.}$ (m μ)	ϵ	$\lambda_{\max.}$ (m μ)	ϵ
11.0	375	37100	388	33500	382	39600
7.6	375	33400	388	13800	382	28100
2.5	320	16200	315	19500	320	18700

reduced when the pH was decreased from 11.0 to 7.6. Other differences are summarized in Table 2; for 2-hydroxyomuconic semialdehyde, Kojima, Itada & Hayaishi (1961) have reported $\lambda_{\max.}$ 375m μ (ϵ 33000) at pH 7.5.

Metabolism of ring-fission products by Sephadex-treated extracts. The ring-fission compounds formed from the three catechols by the action of a heat-treated extract were attacked by Sephadex-treated extracts at different rates. From Fig. 1 the times taken for 75% of each compound to disappear may be measured, and for the ring-fission products of 4-methylcatechol and catechol these times were respectively 5.5 and 3.0 min. when Sephadex extract (1.0mg. of protein) was added. Since the corresponding time for the product of oxidation of 3-methylcatechol was 1.5 min. for 0.1mg. of extract protein, the relative rates of metabolism of the ring-fission compounds of 4-methylcatechol, catechol and 3-methylcatechol by Sephadex-treated extract (1.0mg. of protein) were 1:1.8:37.

Metabolism of 3-methylcatechol and 4-methylcatechol by Sephadex-treated extracts. (a) Conversion of catechols into hydroxyoxo acids. When a Sephadex-treated extract of *Pseudomonas* (U) was incubated at 30° with 10 μ moles of 3-methylcatechol, 10 μ moles of oxygen were taken up and no carbon dioxide was evolved. A compound having the light-absorption maxima of the lactone of 4-hydroxy-2-oxovaleric acid (228m μ in acid, 262m μ in alkali; Dagley & Gibson, 1965) was formed when the reaction mixture was deproteinized with perchloric acid and held at 100° for 5min. to lactonize hydroxy acids. A similar reaction occurred with 4-methylcatechol; but, although the product showed spectroscopic properties apparently identical with those of 4-methyl-2-oxobutylolactone, chromatographic differences were established as follows. After heating to lactonize, compounds were removed from reaction mixtures by continuous extraction with ether for 36hr.; each ethereal solution was then dried with anhydrous sodium sulphate and evaporated on a water bath to leave

Fig. 1. Formation and degradation by cell extracts of the products of ring-fission of: ●, catechol; ▲, 3-methylcatechol; ▼, 4-methylcatechol. Cuvettes contained, in 3ml., 0.1 m-mole of phosphate buffer, pH 7.4, and 0.1 μ mole of the respective catechol. Each reaction was started by the addition of heat-treated extract (0.6mg. of protein) and, at the point shown by arrow A, Sephadex-treated extract (1.0mg. of protein) was added to the two solutions that contained the ring-fission products of catechol and 4-methylcatechol. At B, Sephadex-treated extract (0.1mg. of protein) was added to the solution of the ring-fission product of 3-methylcatechol. Extinctions were measured at $\lambda_{\max.}$ (pH 7.6) for each compound (see Table 2).

an oily residue. Each residue was dissolved in ethanol and chromatographed on Kieselgel G with the solvent acetic acid-chloroform (1:10, v/v); a spray of 2,6-dibromo-*p*-benzoquinone-4-chloroimine revealed purple spots due to compounds that also gave a brown colour with 2,4-dinitrophenylhydrazine. The product of enzymic degradation of 3-methylcatechol had the same R_F value as synthetic 4-methyl-2-oxobutylolactone and that from 4-methylcatechol had the R_F values of 4-ethyl-2-oxobutylolactone. When 2,4-dinitrophenyl-

hydrazones were prepared directly from deproteinized solutions, lactonization at 100° being omitted, the derivatives could be extracted successively into ethyl acetate, 10% (w/v) sodium carbonate and then, after acidification, into ethyl acetate for chromatography on Kieselgel G. For the 2,4-dinitrophenylhydrazone of the oxo acid from 3-methylcatechol R_f values in solvents *A*, *B* and *C* were identical with those of the derivative of 4-hydroxy-2-oxovalerate, whereas the oxo acid from 4-methylcatechol gave a 2,4-dinitrophenylhydrazone chromatographically the same as that of 4-hydroxy-2-oxohexanoate.

Samples of the 2,4-dinitrophenylhydrazone of each lactone were obtained by incubating 100 μ -moles of 3-methylcatechol or 4-methylcatechol with Sephadex-treated extract (300 mg. of protein) in 20 ml. of 0.1M-phosphate buffer, pH 7.4, at 30°. The catechols were added in 20 μ mole portions to reaction mixtures agitated on a rotary shaker (150 rev./min.) over a period of 1 hr.; 10 ml. of 5N-hydrochloric acid was added, the mixtures were held at 100° for 5 min. and then cooled in ice and the protein was removed by centrifuging. Addition of 25 ml. of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid gave yellow precipitates when the solutions were kept overnight at 30°. The 2,4-dinitrophenylhydrazones were collected and dissolved in ethyl acetate, their solutions were shaken with 10% (w/v) sodium carbonate and, after the ethyl acetate layers had been dried over anhydrous sodium sulphate, each was applied separately as a band to the base of a layer (0.5 mm. thick) of Kieselgel G. The chromatograms were developed with solvent *A* and bands with the respective R_f values of the 2,4-dinitrophenylhydrazones of 4-methyl-2-oxobutyrolactone and 4-ethyl-2-oxobutyrolactone were eluted with ethyl acetate. The solvent was evaporated and the 2,4-dinitrophenylhydrazones were crystallized from benzene-light petroleum (b.p. 60–80°). The compound from the enzymic decomposition of 3-methylcatechol gave 8 mg. of yellow crystals, m.p. 170–172°, with an infrared-absorption spectrum that showed the same absorption peaks as that of the 2,4-dinitrophenylhydrazone of synthetic 4-methyl-2-oxobutyrolactone (m.p. 172–174°). The compound from 4-methylcatechol gave 5 mg. of yellow crystals (m.p. 161–164°) with the infrared-absorption spectrum of the 2,4-dinitrophenylhydrazone of synthetic 4-ethyl-2-oxobutyrolactone (m.p. 168–171°). The identity of these compounds was confirmed by chromatography on Kieselgel G in solvents *A*, *B* and *C*.

Chemical evidence for the formation of 4-hydroxy-2-oxohexanoate was obtained by incubating 100 μ -moles of 4-methylcatechol with Sephadex-treated extract, deproteinizing and lactonizing with hydro-

chloric acid as before. The solution was then brought to pH 9 with sodium hydroxide and held at 60° in a vessel fitted with inlet and outlet tubes for air which was first passed through 0.1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. The stream of air passed through the solution of the lactone and thence through a second tube containing the same solution of 2,4-dinitrophenylhydrazine. After 2 hr. an orange precipitate was collected and crystallized from ethanol: the crystals had m.p. 152–153°, unchanged when mixed with authentic 2,4-dinitrophenylhydrazone of propionaldehyde. The infrared-absorption spectra of the two samples were identical, as were R_f values when chromatographed on Kieselgel G and Aluminiumoxid G with the solvent light petroleum (b.p. 80–100°)–diethyl ether (7:3, v/v).

After propionaldehyde had been removed by the air stream the solution was acidified with hydrochloric acid and 15 ml. of 0.1% 2,4-dinitrophenylhydrazine was added: a yellow precipitate was collected on standing overnight at 30°. The compound was dissolved in ethyl acetate, extracted into 10% (w/v) sodium carbonate and then into ethyl acetate after acidification. Chromatography in solvents *A*, *B* and *C* gave spots with R_f values identical with those of the 2,4-dinitrophenylhydrazone of pyruvic acid.

(b) Conversion of 3-methylcatechol into acetic acid and 4-methylcatechol into formic acid. Two reaction mixtures that contained 20 ml. of 0.1M-phosphate buffer, pH 7.4, Sephadex-treated extract (150 mg. of protein) and 100 μ -moles of 3-methylcatechol or 4-methylcatechol respectively were incubated at 30° on a rotary shaker (120 rev./min.) for 45 min. Each solution was deproteinized with 10 ml. of 5N-sulphuric acid, extracted continuously with ether for 36 hr. and the ethereal solutions were shaken with 15 ml. of 11mN-sodium hydroxide. For the reaction product from 3-methylcatechol, later identified as acetic acid, it was sufficient to evaporate the sodium hydroxide solution to 0.5 ml., acidify with 5N-sulphuric acid and apply to a column of silicic acid (14 cm. $\times$ 1.4 cm.) prepared as described by Bulen *et al.* (1952). However, we found that, although the acids were eluted separately when they were applied singly, pyruvic acid was not well separated from formic acid by our columns when present in mixtures, so that pyruvate interfered with the determination of formate. Accordingly, when formate was determined as a metabolite of 4-methylcatechol, the pyruvate formed as a minor product was converted enzymically into lactate, which was readily separated from formate on our columns. The alkaline solution obtained after extracting the products of degradation of 4-methylcatechol was brought to pH 7.4

with sulphuric acid and 2 ml. of *m*-phosphate buffer, pH 7.4, were added together with amounts of lactate dehydrogenase and NADH sufficient to reduce all the pyruvate, the concentration of which had been previously determined by the method of Friedemann & Haugen (1943). A solution containing known amounts of acetic acid and pyruvic acid was taken through the same extraction procedure as the products of metabolism of 3-methylcatechol and the acids were chromatographed and titrated; likewise, a solution of formic acid and pyruvic acid was treated as described for the metabolic products arising from 4-methylcatechol. By this procedure, losses during extraction or chromatography could be corrected for directly. Acids were eluted from the silicic acid columns with solutions equilibrated with 0.5*N*-sulphuric acid and applied in the following sequence: (1) 85 ml. of 5% (v/v) butanol in chloroform, (2) 50 ml. of 15% (v/v) butanol in chloroform, (3) 100 ml. of 25% (v/v) butanol in chloroform. These solvents did not elute the lactones of 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate. 3-Methylcatechol was converted enzymically into acetate (Fig. 2); an oxo acid that could be reduced completely to lactate on addition of lactate dehydrogenase and NADH was also formed. 4-Methylcatechol gave formate but the additional acidic material was not completely accounted for as pyruvate since some oxo acid remained after treatment with lactate dehydrogenase. The identity of this oxo acid, which did not appear as a product of reaction from 3-methylcatechol under the conditions used, has not yet been established. From the data of Fig. 2 the metabolism of 100 μ moles of 3-methylcatechol yielded 82 μ moles of acetate, and, since there was a complete recovery of acetate from a mixture of 100 μ moles of acetic acid and 25 μ moles of pyruvate taken through the same procedure, Sephadex-treated extracts metabolized 1.0 μ mole of 3-methylcatechol to 0.8 μ mole of acetate. Also from Fig. 2 100 μ moles of 4-methylcatechol gave 74 μ moles of formate; and from a mixture of 100 μ moles of formic acid and 50 μ moles of pyruvic acid (converted into lactate for chromatography after extraction) 90 μ moles of formate were recovered; hence 1.0 μ mole of 4-methylcatechol was metabolized to 0.8 μ mole of formate.

In the following experiments, acetic acid and formic acid were confirmed as the respective products of action of Sephadex-treated extracts on 3-methylcatechol and 4-methylcatechol. (1) A 'particulate fraction' was prepared from disrupted cells of *Pseudomonas* (U) and then incubated with solutions of the metabolic products contained in Warburg flasks. Oxygen was consumed and carbon dioxide evolved from the solutions remaining after 4-methylcatechol had been oxidized, but there was

Fig. 2. Chromatography on silicic acid. Carboxylic acids were extracted from reaction mixtures after enzymic degradation of 4-methylcatechol (a) and 3-methylcatechol (b); the acids were then eluted and titrated with NaOH. The pyruvate formed from 4-methylcatechol was converted into lactate before this mixture was applied to the column. Maximum quantities of the following authentic acids were eluted in the fractions (3 ml.) at points indicated by arrows: (1) acetate; (2) formate; (3) pyruvate; (4) lactate; (5) an unidentified oxo acid that did not react with lactate dehydrogenase.

no gas exchange for those from 3-methylcatechol. We confirmed the observation of Dagley & Gibson (1965) that these 'particulate fractions' could oxidize formate but not acetate, pyruvate or 4-hydroxy-2-oxovalerate; 4-hydroxy-2-oxohexanoate was not attacked. (2) The volatile acids were steam-distilled from the acidified enzymic reaction mixtures, distillates were neutralized with sodium hydroxide and the solutions evaporated to about 1 ml. On treatment with magnesium and then with chromotropic acid (Dagley & Gibson, 1965) the product from 4-methylcatechol gave a pink compound with a spectrum in the region 350–650 $m\mu$ identical with that obtained when authentic formic acid was taken through the same procedure. This pink compound was not given by metabolites from 3-methylcatechol. (3) The volatile acid from 3-methylcatechol reacted with ATP in the presence of acetate kinase to give a compound that formed a purple ferric hydroxamate complex as described in the Materials and Methods section. The product from 4-methylcatechol did not show these reactions.

Metabolism of 3-methylcatechol and 4-methylcatechol by dialysed extracts. Dialysed extracts of *Pseudomonas* (U) oxidized the catechols to pyruvate and neutral carbonyl compounds that were identified as follows. To a stirred reaction mixture of 20 ml. of 0.1 *M*-phosphate buffer, pH 7.4, contain-

ing dialysed extract (500mg. of protein) were added 100 μ moles of 3-methylcatechol in 20 μ mole amounts at 5min. intervals and the reaction was allowed to proceed for 1hr. A stream of air that had first passed through 0.1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid was blown through the solution and passed into a second tube containing the same solution of 2,4-dinitrophenylhydrazine where a yellow precipitate was collected (yield, 20mg.) and then crystallized from ethanol. The crystals had m.p. 147°, unchanged when mixed with an authentic sample of the 2,4-dinitrophenylhydrazone of acetaldehyde; the infrared-absorption spectra of the samples were identical, as were the R_f values when chromatographed on Kieselgel G or Aluminiumoxid G with the solvent light petroleum (b.p. 80–100°)—diethyl ether (7:3, v/v). A similar experiment was performed with 100 μ moles of 4-methylcatechol except that the temperature of the reaction mixture was raised from 30° to 60° before the air stream was passed to remove volatile products. The orange 2,4-dinitrophenylhydrazone that was collected and crystallized from ethanol (yield, 10mg.) had m.p. 153–154°, unchanged by admixture with a sample of the authentic 2,4-dinitrophenylhydrazone of propionaldehyde; the infrared-absorption spectra of the samples were identical, as were the R_f values when chromatographed.

When acetaldehyde and propionaldehyde had been removed, 15ml. of 5N-hydrochloric acid was added to each incubation mixture, the solutions were held at 100° for 5min. and precipitates were removed by centrifuging. After 25ml. of 0.1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid had been added to each solution, yellow precipitates were formed on standing overnight at 30° and these were collected separately, dissolved in ethyl acetate, extracted with 10% (w/v) sodium carbonate and re-extracted into ethyl acetate on acidifying. The solutions were dried with anhydrous sodium sulphate, the ethyl acetate was removed by evaporation and the 2,4-dinitrophenylhydrazones were crystallized from acetic acid. The yellow crystals obtained from the metabolism of 3-methyl-

catechol had m.p. 219–221°, those from 4-methylcatechol had m.p. 216–218°, and both compounds had R_f values identical with those of a sample of authentic 2,4-dinitrophenylhydrazone of pyruvic acid when chromatographed on Kieselgel G in solvents A, B and C. The infrared-absorption spectra of the authentic material and the two samples were identical.

The metabolism of 4-hydroxy-2-oxohexanoate to propionaldehyde and pyruvate closely resembled the enzymic degradation of 4-hydroxy-2-oxovalerate to acetaldehyde and pyruvate (Dagley & Gibson, 1965) insofar as the rate of disappearance of the substrate was increased by about one-third on adding mm-magnesium sulphate. The rate of the reaction also increased continuously as the pH was increased to 8.5, but non-enzymic degradation of 4-hydroxy-2-oxohexanoate in alkaline solutions precluded an accurate determination of the pH optimum of the enzymic reaction. As was the case for 4-hydroxy-2-oxovalerate, only one isomer of synthetic 4-hydroxy-2-oxohexanoate appeared to be attacked by dialysed extracts (Table 3).

Formation of ring-fission compounds from cresols by whole cells. If it is assumed that the first reaction in the metabolism of a cresol by *Pseudomonas* (U) or *Pseudomonas* (L) is hydroxylation to give a methyl-substituted catechol, then *m*-cresol could give rise to either 3-methylcatechol or 4-methylcatechol. Four washed suspensions (2mg. dry wt./ml.) of *Pseudomonas* (U) grown with phenol, *o*-cresol, *m*-cresol and *p*-cresol were shaken at 30° in phosphate buffer (2g./l., pH 7.2) with their respective growth substrates, each at a concentration of 7 μ moles/ml. After 5min., when a yellow compound had appeared in each reaction mixture, 0.1 vol. of 2N-hydrochloric acid was added and the cells were removed by centrifuging. Each solution was shaken with an equal volume of ether and each ether layer was extracted with the same volume of saturated sodium hydrogen carbonate; light-absorption was then measured at pH 2.5 and 7.6. Single peaks that appeared at the wavelengths listed in Table 2 indicated that a ring-fission product had been formed from catechol by cells

Table 3. *Enzymic degradation of synthetic 4-hydroxy-2-oxohexanoate*

Reaction mixtures at 30° contained, in 3ml., 120 μ moles of tris buffer, pH 8.1, 4.5 μ moles of 4-hydroxy-2-oxohexanoate and dialysed extract (20mg. of protein). Reactions were stopped at various times by additions of 0.5ml. of 10% (w/v) HClO₄, heated at 100° for 5min. and amounts of lactonized 4-hydroxy-2-oxohexanoate remaining were determined spectroscopically. The non-enzymic loss of substrate, determined by incubation with boiled extract, was 0.3 μ mole in 60 min.; 2.0 μ moles of pyruvate were formed enzymically in 60 min.

Time (min.)	0	5	10	20	30	40	50	60
4-Hydroxy-2-oxohexanoate (μ moles)	4.5	3.5	3.2	2.6	2.4	2.3	2.3	2.3

grown with phenol, from 3-methylcatechol by cells grown with *o*-cresol and *m*-cresol, and from 4-methylcatechol by *p*-cresol-grown cells. These observations were supported by measurements of rates of oxidation of propionate, an expected metabolite of 4-methylcatechol but not of catechol or 3-methylcatechol. Propionate (5 μ moles/3 ml. of reaction mixture) was oxidized without a lag period by *Pseudomonas* (L) grown with *p*-cresol, whereas the rate of oxidation of propionate when phenol, *o*-cresol or *m*-cresol had served as the carbon source for growth was essentially the same as the uptake of oxygen with no addition of substrate. The results for suspensions of *Pseudomonas* (U) were similar, except that oxidation of propionate by cells grown with phenol, *o*-cresol and *m*-cresol commenced after they had been exposed to propionate for 20 min. whereas similar suspensions of *Pseudomonas* (L) still failed to oxidize this substrate after exposure for 1 hr.

DISCUSSION

After growth with *p*-cresol as source of carbon, cell suspensions of *Pseudomonas* N.C.I.B. 8893 oxidized protocatechuate readily, whereas *Pseudomonas* (U) oxidized catechol and the two methylcatechols but failed to attack protocatechuate (Table 1). Similarly, cell extracts of *Pseudomonas* N.C.I.B. 8893 that oxidized protocatechuate did not attack catechol, 3-methylcatechol or 4-methylcatechol (S. Dagley & P. J. Geary, unpublished work), compounds that were oxidized readily by cell extracts of *Pseudomonas* (U) grown with phenol. It therefore appears that, whereas *Pseudomonas* N.C.I.B. 8893 initiates the degradation of *p*-cresol by oxidizing the methyl group to carboxyl as Dagley & Patel (1957) proposed, *Pseudomonas* (U) leaves the methyl group intact and first hydroxylates the benzene nucleus to give a catechol. Although specificity is exhibited in the hydroxylation of a cresol insofar as *m*-cresol was metabolized through the ring-fission product of 3-methylcatechol and not of 4-methylcatechol, the ring-cleavage of the catechols appears to be catalysed

by the same enzyme, namely catechol 2,3-oxygenase (catechol-oxygen 2,3-oxidoreductase, EC 1.13.1.2), since 3-methylcatechol and 4-methylcatechol, as well as catechol, were oxidized by extracts of *Pseudomonas* (U) grown with phenol. The crystalline catechol 2,3-oxygenase described by Nozaki, Kagamiyama & Hayaishi (1963) attacked both catechol and 4-methylcatechol, and Evans, Fernley & Griffiths (1965) have suggested that this same enzyme is responsible for ring-fission of polynuclear substrates. This lack of specificity appears to extend to those enzymes that degrade ring-fission products since catechol, 3-methylcatechol and 4-methylcatechol were all converted into pyruvate by dialysed extracts: indeed, the ring-fission product of 3-methylcatechol was attacked much faster than 2-hydroxymuconic semialdehyde (Fig. 1), as Ribbons (1966) has also observed.

The subsequent metabolism of the ring-fission products accords with the general scheme of Dagley *et al.* (1964b), which predicts that 3-methylcatechol would yield acetate, acetaldehyde and pyruvate and that formate, propionaldehyde and pyruvate would arise from 4-methylcatechol. The fission of the benzene nucleus between C-3 and C-4 of 3,4-dihydroxytoluene (4-methylcatechol), which we have established by identifying products of enzymic degradation, stands in contrast with the fission that occurs between C-4 and C-5 of 3,4-dihydroxybenzoate (protocatechuate) as shown by Trippett, Dagley & Stopher (1960). This difference might be expected from the mechanism of ring-cleavage proposed by Hayaishi (1964), in which enzymic oxidation of a catechol is initiated by withdrawal of an electron from one of the two hydroxyl groups by a perferryl ion: an electron would be more readily available from a group attached at C-4 than at C-3 for 3,4-dihydroxytoluene, and more available at C-3 than at C-4 for 3,4-dihydroxybenzoate. The attacks on the nucleus would therefore be initiated at different points, and in consequence cleavage would eventually take place between adjacent carbon atoms situated in different positions relative to the methyl or the carboxyl substituent.

Scheme 1.

Details of the reaction sequence for the enzymic degradation of 2-hydroxymuconic semialdehyde and related compounds have not yet been elucidated. Conversion of the enol to a keto form (I) as in Scheme 1 was suggested by Dagley *et al.* (1960) to account for the changes in light-absorption that resulted from changes of pH. However, Shaw, Borkenhagen & Talalay (1965) have isolated L-2-amino-*cis*-4-hexenoic acid, which arises from a fission of the benzene nucleus when certain steroids are metabolized by bacteria. From their work it appears to be more probable that a proton would be taken up at C-5 to give compound (II), which would then be expected to undergo ready hydrolytic cleavage of formate. Similarly, succinate and acetate would arise respectively by hydrolytic cleavages of the ring-fission products of 2,3-dihydroxy- β -phenylpropionate (Dagley, Chapman & Gibson, 1965) and of 3-methylcatechol in the present studies. A reaction of the same type occurs when 4(5),9(10)-diseco-3-hydroxyandrosta-1(10),2-diene-5,9,17-trione-4-oic acid is cleaved by microbial enzymes to give 3 α -H-4 α -(3'-propionic acid)-7 α -methylhexahydro-1,5-indanediol (Gibson, Wang, Sih & Whitlock, 1966). Two pathways (*a* or *b*; Scheme 1) might be proposed for the formation of 2-hydroxy-4-oxovalerate; (*b*) shows a compound (III) that, like L-2-amino-*cis*-4-hexenoic acid, possesses an ethylenic linkage between C-4 and C-5. A decision between these alternatives is not possible from the present evidence but we have shown (Fig. 2) that an unidentified oxo acid was formed when 4-methylcatechol was degraded by Sephadex-treated extracts. No such compound was detected when 3-methylcatechol was metabolized, but the ring-fission product of 3-methylcatechol is degraded so rapidly (Fig. 1) that accumulation of reaction intermediates may be precluded.

We thank the Agricultural Research Council for their financial support.

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