Metabolism of Arylsulphonates by Micro-organisms

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1. Species of *Pseudomonas* capable of degrading arylsulphonates and detergents of the alkylbenzenesulphonate type were isolated from sewage and river water. 2. Benzenesulphonate, benzenesulphonate and toluene-*p*-sulphonate were rapidly degraded by these organisms with the release of the sulphonate group as sulphite; detergent homologues with a chain length up to 16 carbon atoms (4-*n*-hexadecyl-benzenesulphonate) also released sulphite. Sulphite oxidation to sulphate in the medium can occur non-enzymically. 3. Growth on benzenesulphonate and toluene-*p*-sulphonate elicited a catechol 2,3-oxygenase, which effected a 'meta' cleavage of the ring. The metabolic route for benzenesulphonate was determined as: benzenesulphonate → catechol → 2-hydroxymuconic semialdehyde → formate and 4-hydroxy-2-oxovalerate → acetaldehyde and pyruvate; the enzymes catalysing these steps were all inducible. 4. Toluene-*p*-sulphonate was degraded via 2-hydroxy-5-methylmuconic semialdehyde to formate and 4-hydroxy-2-oxohexanoate and the latter was cleaved to propionaldehyde and pyruvate. Propionaldehyde and propionate were oxidized rapidly by toluene-*p*-sulphonate-grown cells but slowly by fumarate-grown organisms. 5. The specificity of the catechol 2,3-oxygenase induced by the arylsulphonates, towards catechol and the methylcatechols, varied during the purification and suggested that 3-methylcatechol was probably oxidized by a separate enzyme. Detergents of the alkylbenzenesulphonate type also induced a catechol 2,3-oxygenase in these bacteria. 6. A few isolates, after growth on benzenesulphonate, opened the ring of catechol by an 'ortho' route to form *cis-cis*-muconate. The enzymes to degrade this intermediate to β -oxoadipate were also present in induced cells.

The destruction of detergents by water-borne and soil-borne micro-organisms has become of increasing importance in recent years with the deposition into sewage and natural waters of large quantities of both domestic and industrial detergent-containing effluents. Most detergents in common usage are alkylbenzenesulphonates in which the long alkyl side chain is either highly branched (a 'hard' detergent) or unbranched (a 'soft' detergent). Microbial attack on a detergent usually commences at the methyl end of the side chain and degradation proceeds two carbon atoms at a time towards the aromatic nucleus (Swisher, 1963; Huddleston & Allred, 1963) by a mechanism that is probably analogous to fatty acid degradation. In those ('soft') detergents that undergo complete degradation, therefore, the organism must metabolize the benzene ring, the mechanism and enzymology of which, with detergent compounds, has not been examined.

To avoid possible complications from the alkyl side chains and the difficulty in obtaining substantial amounts of the pure 4-*n*-alkyl-substituted

isomers of detergent compounds, we have confined most of our initial studies to benzenesulphonate and toluene-*p*-sulphonate (Farr & Cain, 1965). These compounds showed no biochemical oxygen demand (i.e. they were not utilized in 5 days) with unadapted sewage microflora (Heukelekian & Rand, 1955), but with an adapted microflora from similar sources Ludzack & Ettinger (1960) found about half the expected biochemical oxygen demand for toluene-*p*-sulphonate and ethylbenzenesulphonate. Sawyer & Ryckman (1957) in similar experiments obtained complete recovery of the sulphonate group from these substrates as sulphate, suggesting degradation of benzene ring, but no other metabolic products were reported. Symons & del Valle-Rivera (1961), using activated-sludge cultures adapted to benzenesulphonate and toluene-*p*-sulphonate, concluded that the former was attacked at the sulphonate group but the latter through the methyl group. We have substantiated the first of these conclusions, but the present paper shows that the methyl group of toluene-*p*-sulphonate remains intact through at least five enzymic stages.

MATERIALS AND METHODS

Organisms. The bacteria used were all isolated, by elective culture methods on mineral salts medium containing 0.05% (w/v) benzenesulphonate or toluene-*p*-sulphonate, from a small sewage-purification plant at Felling (Co. Durham) receiving household sewage and domestic detergent effluents. Other isolates were made from Tyne river water heavily contaminated with industrial effluents, and from garden soils not normally exposed to detergent compounds. The isolated bacteria, all species of *Pseudomonas*, were maintained on both nutrient agar and mineral salts medium containing the aromatic carbon source (0.1%, w/v) solidified with 2% (w/v) New Zealand agar, and were subcultured monthly. Growth on benzenesulphonate and toluene-*p*-sulphonate agar was originally poor but improved with continuous subculture.

Growth of cells. Large-scale production of cells was effected in 201. fermenters or 101. flasks fitted with a sterile sampling device and containing 181. or 91. respectively of a defined medium with the following composition (per l. of deionized water): NH_4Cl , 1g.; K_2HPO_4 , 1g.; MgCl_2 , 0.1g.; organic carbon source, 1g.; trace-elements solution (Barnett & Ingram, 1955; modified by substituting chloride salts for sulphates), 10ml. Where a carbon source other than a sulphonate was employed, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1g./l.) was substituted for MgCl_2 . The medium was adjusted to pH 7.0–7.2 with KOH or H_3PO_4 and sterilized by autoclaving. An inoculum derived from the stock slopes was grown at 30° for 36 hr. in shake cultures of the appropriate medium (500 ml. in a 21. conical flask) and this culture was then used to inoculate the large flasks, which were incubated at 30°; forced aeration (approx. 31. of air/min.) was necessary with this volume of medium. Cells were harvested towards the end of the exponential phase of growth (24–30 hr.). Alkylbenzenesulphonates with a side-chain length of more than 4 carbon atoms (4-*n*-butylbenzenesulphonate) showed the foaming properties of typical detergents; growth of the bacteria on these substrates was therefore performed in 21. flasks (containing 500 ml. of medium) aerated by circular agitation in a New Brunswick Gyrotory shaker-incubator. Growth yield coefficients were determined by using tared Millipore filters as described by Cain (1966).

Preparation of extracts. Freshly harvested cells, or deep-frozen cells after thawing, were crushed by exposure for 5 min. at 0° to the output of an MSE-Mullard 60 w ultrasonic disintegrator (20 kcy./sec.) or with the Hughes (1951) bacterial press without abrasive. The crushes, suspended in water, were clarified by centrifugation at 30 000g for 20 min. and the supernatant fluid usually contained 15–25 mg. of protein/ml. Particulate preparations from the crushes were prepared by carefully removing the top pinkish layer of the sedimented pellet, washing this layer three times with buffer at 0° and recentrifuging at 100 000g for 40 min. The upper two-thirds of the resulting pellet was collected each time to avoid including coarse cell debris. The final pellet was resuspended in buffer and the particulate preparation used immediately. Some clarified extracts were recentrifuged at 144 000g for 2 hr. to give soluble fractions devoid of any particulate material; these preparations would accumulate formate from catechol. Heat treatment (55° for 5 min.) followed by centrifugation of the coagulated protein left extracts that oxidized the catechols to the 2-hydroxymuconic semialdehyde derivatives but did not degrade these products further.

To obtain preparations that accumulated 4-hydroxy-2-oxovalerate or 4-hydroxy-2-oxohexanoate, crude extracts were treated with protamine sulphate, $(\text{NH}_4)_2\text{SO}_4$ and Sephadex G-25 (100–270 mesh) as described by Dagley & Gibson (1965).

Partial purification of the catechol 2,3-oxygenase from *Pseudomonas aeruginosa* strain A was effected as follows. The clarified extract was treated with 0.2 vol. of 2% (w/v) protamine sulphate [Sigma (London) Chemical Co., London S.W. 6] added in small portions at 0° with constant stirring. After removal of the precipitate by centrifuging the supernatant was brought to 30% saturation with neutral saturated $(\text{NH}_4)_2\text{SO}_4$ solution and the small precipitate again centrifuged off. All the activity lay in the protein fraction precipitated by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 50% saturation. This precipitate was dissolved in distilled water, dialysed against several changes of water for 4 hr. and the fraction precipitated between 33% and 45% saturation with $(\text{NH}_4)_2\text{SO}_4$ collected. After being dissolved in water and dialysed for 6 hr. against frequent changes of water the enzyme was adsorbed on 2 vol. of calcium phosphate gel (25 mg. dry wt./ml.) by gentle shaking for 30 min. at 4°. The gel was centrifuged down, the pellet washed with distilled water and recentrifuged and then treated successively with 4 vol. of potassium phosphate buffers of increasing concentration and pH to elute the enzyme. Nearly all catechol 2,3-oxygenase activity was eluted with 0.05 M-phosphate buffer, pH 7.0.

The protein precipitated by $(\text{NH}_4)_2\text{SO}_4$ between 33% and 50% saturation, after being redissolved in water, was also chromatographed on a Sephadex G-200 column (25 cm. $\times$ 2 cm. diam.) developed with 0.05 M-potassium phosphate buffer, pH 7.0, and 5 ml. fractions were collected. The catechol oxygenase activity appeared in fractions 9–14, which were pooled and then treated with calcium phosphate gel. All extracts and protein fractions containing catechol 2,3-oxygenase activity were reactivated by reduction with NaBH_4 *in vacuo* immediately before their activity was tested (Taniuchi, Kojima, Kanetsuna, Ochiai & Hayaishi, 1962).

Chemicals. Synthetic 4-hydroxy-2-oxovalerate was kindly provided by Professor S. Dagley. All the pure 4-*n*-alkylbenzenesulphonate isomers were provided by Mr R. Huddleston of the Research Division of the Continental Oil Co. (Ponca City, Okla., U.S.A.). 4-Oxalocrotonic acid was a gift from Dr Y. Nishizuka. *cis-cis*-Muconic acid was synthesized from redistilled phenol by oxidation with peracetic acid (Elvidge *et al.* 1950), and β -oxoadipic acid was synthesized by the method of Bardhan (1936). (+)-Muconolactone was prepared biologically as described by Cain (1961); the racemic ($\pm$)-isomer was a gift from Professor W. C. Evans. 2,4-Dinitroanilino-maleimide was prepared from 2,4-dinitrophenylhydrazine and maleic anhydride as described by Clark-Walker & Robinson (1961). [$\text{Me-}^{14}\text{C}$]Toluene (11.1 $\mu\text{C}/\mu\text{mole}$), obtained from The Radiochemical Centre (Amersham, Bucks.), was diluted with carrier redistilled toluene (0.5 ml.) and sulphonated with conc. H_2SO_4 (Hickinbottom, 1950). The product, after recrystallization twice from ethanol, had m.p. 103° and a specific activity of 6910 counts/min./ μmole . Other chemicals were commercial products redistilled or recrystallized where necessary. All melting points were measured with a Gallenkamp Electrothermal apparatus and are uncorrected for emergent thermometer stem.

Radioactivity measurements. Radioactivity measurements were carried out with a Tri-Carb liquid-scintillation spectrometer (model 3003, with model 526 automatic control; Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). The scintillator used in the earlier experiments consisted of 2,5-diphenyloxazole (5g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.3g.) in toluene (11.). Samples were counted in triplicate at 0° in standard 20 ml. glass screw-capped vials containing 0.1 ml. of sample, 3 ml. of scintillator solution and 2 ml. of ethanol for 10–100 min., depending on the radioactivity of the samples. A minimum of 5000 counts was measured. Background counts were obtained from triplicate 100 min. counts and averaged 16 counts/min. The degree of quenching by the samples and overall counting efficiency were determined by using an internal toluene standard.

In later experiments the new scintillator 2-(4-*tert.*-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole [Ciba (A.R.L.), Duxford, Cambs.], which is particularly useful with quenched samples (Scales, 1967), was used. 2-(4-*tert.*-Butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole (8g.) in toluene (500 ml.) was mixed with methanol (500 ml.); 6 ml. volumes of this scintillator formed a completely miscible solution with up to 0.6 ml. of an aqueous sample. The counting efficiency of toluene-*p*-sulphonate was 58.9%, that of the intensely coloured 2-hydroxy-5-methylmuconic semialdehyde was 15.4% and that of propionaldehyde dinitrophenylhydrazone was 53.5%. The recorded specific activities for these compounds are corrected for these efficiencies.

The incorporation of label from [*Me*-¹⁴C]toluene-*p*-sulphonate into propionaldehyde by growing bacteria was followed by harvesting an exponential-phase culture of *P. aeruginosa*, washing and resuspending the cells in 50 ml. of fresh growth medium, prewarmed to 30° and containing 2 mM-toluene-*p*-sulphonate. When exponential growth had been re-established (90–160 min.), labelled substrate (270 μ moles, 11.1×10^6 counts/min.) was injected and 5 ml. samples of the growing culture were pipetted into 5 ml. of hot dinitrophenylhydrazine solution (0.2%, w/v, in *N*-HCl) at intervals from 10 sec. onwards. After standing at room temperature overnight, the solutions were extracted with ethyl acetate and the ethyl acetate layers were re-extracted with three 0.3 vol. portions of Na₂CO₃ (10%, w/v) to remove acidic dinitrophenylhydrazones, unchanged toluene-*p*-sulphonic acid and other acidic materials. The ethyl acetate layers, containing neutral dinitrophenylhydrazones, were concentrated and chromatographed separately on thin-layer plates in solvent Y (see below) with appropriate markers. Bands corresponding to propionaldehyde dinitrophenylhydrazone were scraped from the plates, and each derivative was eluted with ethyl acetate and concentrated. Samples (0.3 ml.) were counted in triplicate (100 min.) and the propionaldehyde dinitrophenylhydrazine content of the solution was estimated by drying several millilitres to constant weight.

Determinations. Sulphate in growth media and Warburg flask contents was determined on 1 ml. samples by the barium chloroanilate method of Bertolacini & Barney (1957), after removal of cells by centrifugation; the chloroanilic acid was measured at 332 μ m in a Unicam SP. 500 spectrophotometer. In some earlier experiments sulphite was determined as sulphate by oxidizing 3 ml. samples of the test material with 0.5 ml. of '100 vol.' (approx. 30%, w/v) H₂O₂ for 1 hr. at

room temperature. Excess of peroxide was destroyed by boiling until no iodine was released when a drop of the treated solution was added to acidified KI. Sulphate was then determined in a 2 ml. sample and corrected for the original dilution; the difference between samples before and after peroxide treatment was used as a measure of sulphite.

In most experiments, sulphite was detected and determined by the highly specific test of Robinson (1965) in which inorganic sulphite is treated with an acetone solution of 2,4-dinitroanilinomaleimide. The bright-yellow addition compound moves as a discrete spot chromatographically and was used to detect sulphite qualitatively. No colour was given with sulphate, sulphide, recrystallized thiosulphate or dithionate, dithionite, cysteine, GSH, sulphur suspensions or colloidal sulphur, benzenesulphinic acid, benzene-sulphonate, phosphate or chloride. Quantitative determinations were made by adding 0.1 ml. of 2,4-dinitroanilinomaleimide (0.1 M in acetone) to a 1 ml. test sample and diluting to 10 ml. with deionized water. After the mixture had stood for 10 min. the colour was read at 335 μ m against a blank of deionized water taken through the same procedure. A linear calibration curve was obtained over the range 0–1 μ mole of sulphite. Phosphate, sulphate and chloride did not interfere at the concentrations in which they occurred in growth media and incubation mixtures.

Catechol substances were determined by the Evans (1947) method but with the substitution of sodium molybdate for sodium tungstate, and pyruvate by the method of Friedmann & Haugen (1943) with xylene as the final extractant. Formate was detected qualitatively, after reduction to formaldehyde with Mg ribbon and HCl, by the chromotropic acid method of Grant (1948), and was determined by the more sensitive acetylacetone method of Nash (1953) after steam-distillation of the formate from the acidified contents of Warburg flasks. Distillate (25 ml.) was collected (97 $\pm$ 2% recovery), neutralized with 0.5 N-NaOH and concentrated *in vacuo* to 1 ml., and 0.5 ml. of this distillate was treated with acetylacetone, after reduction with Mg ribbon (about 100 mg.) and 0.01 N-HCl (0.5 ml.).

Acetaldehyde was originally determined by the method of Hullin & Noble (1953) but samples required two or more copper-lime treatments to remove the pyruvate, which interfered. Most quantitative determinations were performed by the Stotz (1943) method in which the acetaldehyde was first steam-distilled from the reaction mixture. 2-Hydroxymuconic semialdehyde and *cis*-*cis*-muconate were determined by measuring *E*₃₇₅ and *E*₂₆₀ respectively at pH 7.5; ϵ for the former is 32 800 and for the latter 17 200.

4-Hydroxy-2-oxovalerate was determined after conversion into 4-methyl-2-oxobutylolactone by 0.83 N-HCl at 100° for 3 min. (Dagley & Gibson, 1965). The free acid has only end-absorption in the ultraviolet but the lactone derivative shows *E*_{max.} at 228 μ m in acid and at 262 μ m in alkaline solution. Synthetic 4-hydroxy-2-oxovalerate (0–1 μ mole) was used as standard. In experiments to confirm that the free acid and not its lactone was the natural intermediate, the acid present in incubation flasks was determined as the 2,4-dinitrophenylhydrazone by the Friedmann & Haugen (1943) method but with the time of incubation with the acid 2,4-dinitrophenylhydrazine reagent restricted to 3 min. at 30° before extraction into ethyl acetate and then Na₂CO₃ solution. Long incubation in acid solution (50–150 min. at 30° or 3 min. at 100°)

caused lactonization to yield the 2,4-dinitrophenylhydrazone of 4-methyl-2-oxobutylolactone, which, because it has no free carboxyl group, could not be extracted into carbonate (Dagley & Gibson, 1965). The appearance on chromatograms of only the 2,4-dinitrophenylhydrazone of 4-hydroxy-2-oxovalerate when prolonged incubation with reagent and heating were omitted showed that the lactone was an artifact of the procedure and not an enzymic reaction product. 4-Hydroxy-2-oxohexanoate was determined similarly (Bayly, Dagley & Gibson, 1966).

Pyruvate (0.5 ml. samples) was determined enzymically by measuring spectrophotometrically at 340 m μ the amount of NADH oxidation in the presence of muscle lactate dehydrogenase (Sigma type 340-10, 0.05 ml.). Acetate was measured by the acetokinase enzymic method of Rose (1955). The enzyme was prepared from a 10l. culture of *Moraxella calcoacetic* (*Vibrio* O1, N.C.I.B. 8250) grown on the defined medium with 0.1% (w/v) sodium acetate as the sole carbon source. The packed cells were washed in 0.1 M-phosphate buffer containing 0.5 mM-cysteine and extracts prepared in the Hughes press. The crude extract converted approx. 1.5 μ moles of acetate/hr./mg. of protein to its hydroxamate at 30° under the assay conditions of Rose (1955).

Protein was usually determined by the biuret procedure of Gornall, Bardawill & David (1949), but throughout the purification procedure for the catechol 2,3-oxygenase the E_{260} and E_{280} values of suitably diluted samples of each fraction were used (Warburg & Christian, 1941).

Enzyme assays. Catechol 1,2-oxygenase (EC 1.13.1.1) was determined by following at 260 m μ the accumulation of *cis-cis*-muconate in the presence of mM-EDTA (Hegeman, 1966). Catechol 2,3-oxygenase (EC 1.13.1.2) was determined by the increase in E_{375} when catechol or its methyl homologues were added to extracts. Lactonizing enzyme [(+)-4-carboxymethyl-4-hydroxyisocrotonolactone lyase (deacylizing), EC 5.5.1.1], (+)-muconolactone isomerase and β -oxoadipate enol lactone hydrolase (EC numbers not assigned) were assayed by the methods of Hegeman (1966). All spectrophotometric assays were performed with a Unicam SP.800 spectrophotometer fitted with the SP.820 constant-wavelength attachment and a constant-temperature cuvette holder maintained at 30°. Cuvettes contained: substrate, 0.5 μ mole; tris-HCl buffer, pH 7.5, 300 μ moles; extract, 0.05 ml. (approx. 0.1–1.0 mg. of protein); water to a total volume of 3 ml. 2-Hydroxymuconic semialdehyde hydrolase in crude extracts was determined by following the rate of disappearance of 0.3 μ mole of substrate at 375 m μ in tris-HCl buffer, pH 7.5. The aldolase cleaving 4-hydroxy-2-oxovalerate to pyruvate and acetaldehyde was determined by coupling it with excess of lactate dehydrogenase and following the rate of oxidation of NADH. Cuvettes contained: tris-HCl buffer, pH 7.5, 250 μ moles; MgSO₄, 1 μ mole; NADH, 1 μ mole; synthetic ($\pm$)-4-hydroxy-2-oxovalerate, 2 μ moles; lactate dehydrogenase (Sigma type 340-10, 8000 units/ml.) 0.05 ml.; dialysed extract, 0.2 ml. (2–5 mg. of protein). The rates of NADH oxidation in the absence of 4-hydroxy-2-oxovalerate and in the absence of extract were determined and subtracted.

Alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) (C. F. Boehringer und Soehne G.m.b.H., Mannheim, West Germany) and lactate dehydrogenase (lactate-NAD oxidoreductase, EC 1.1.1.27) (Sigma) were commercial products.

Chromatography. Keto acids were chromatographed on Whatman no. 4 paper as their 2,4-dinitrophenylhydrazone derivatives in the following solvents: *A*, 2-methylbutan-2-ol–propanol-1-ol–aq. NH₃ (sp.gr. 0.88) (65:5:30, by vol., upper layer); *B*, propan-2-ol–aq. NH₃ (sp.gr. 0.88)–water (20:1:2, by vol.); *C*, butan-1-ol–ethanol–0.5 N-NH₃ (7:2:1, by vol.); *D*, 2-methylbutan-2-ol–ethanol–water (5:1:4, by vol., upper layer); *E*, benzene–ethyl formate–propionic acid (18:1:1, by vol.). The 2,4-dinitrophenylhydrazones of neutral carbonyl compounds (and free dinitrophenylhydrazine) ran with the front in these solvents, so they were chromatographed on thin-layer plates (20 cm. $\times$ 10 cm.) of Kieselgel G (0.3 mm. thick) activated by heating at 100° for 30 min. The solvent used was: *Y*, diethyl ether–*n*-hexane (1:3, v/v); formaldehyde 2,4-dinitrophenylhydrazone, acetaldehyde 2,4-dinitrophenylhydrazone and propionaldehyde 2,4-dinitrophenylhydrazone gave R_F values of 0.17, 0.22 and 0.36 respectively in this system. The solvents of Petrowitz (1962) and Reio (1958) were used to separate catechol and 4-methyl-2-oxobutylolactone. The former [solvent *F*; chloroform–methanol–acetic acid (45:8:4, by vol.)] gave R_F values of 0.55 and 0.67 for catechol and the lactonic compound respectively. The latter [solvent *G*; chloroform–methanol–water–formic acid (100:20:19:0.8, by vol.)] gave R_F values of 0.62 and 0.85 respectively. Dihydric phenols, phenolic acids and other aliphatic acids were run in the following solvents: *H*, ethanol–aq. NH₃ (sp.gr. 0.88)–water (20:1:4, by vol.); *J*, butan-1-ol–acetic acid–water (4:1:5, by vol.).

Phenols and acids were detected by spraying with either FeCl₃ [0.5% (w/v) in aq. 50% (v/v) ethanol] or 2,6-dichloroquinone-4-chloroimide [2% (w/v) in ethanol]. Catechol and its homologues gave brown spots and 4-methyl-2-oxobutylolactone green spots with the latter spray. Aliphatic acids were detected by spraying with bromophenol blue [2% (w/v) in aq. 70% (v/v) ethanol]. The yellow addition product of sulphite with 2,4-dinitroanilinomaleimide was chromatographed in solvent *K* [propan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (6:3:1, by vol.)] and was visible without treatment. α -Picolinic (pyridine-2-carboxylic) acid and its methyl homologues were chromatographed in the following solvents: *M*, butan-1-ol–formic acid–water (8:3:2, by vol.); *N*, butan-1-ol–aq. 65% (v/v) methanol (1:1, v/v) (Hashizuma, 1954). They were detected by their quenching of u.v. light. All paper chromatography was performed by the descending method and all thin-layer chromatography by the ascending technique. Marker compounds were always run with experimental material.

RESULTS

Growth of P. aeruginosa on the arylsulphonates

Twelve strains of *Pseudomonas* were found capable of utilizing benzenesulphonate and toluene-*p*-sulphonate as sole source of carbon, sulphur and energy, but fell into two groups according to their enzymic constitution. Nine of the strains, identified as *P. aeruginosa* by the criteria of Stanier, Palleroni & Doudoroff (1966), were found to degrade the benzene ring by a 'meta' cleavage route, the remaining three by an 'ortho' cleavage route (see

Fig. 1. Conversion of the sulphonate group into sulphite and sulphate in growing cultures of *P. aeruginosa* strain A. Cells from 1 l. of defined medium, with sodium benzenesulphonate as sole carbon and sulphur source, were harvested aseptically in the exponential phase, washed three times with sterile deionized water and resuspended in 1 l. of fresh benzenesulphonate medium (pre-warmed to 30°), and growth was allowed to continue. Growth, benzenesulphonate, sulphite and sulphate content in the medium were followed on samples removed aseptically at 1 or 2 hr. intervals. ○, Benzenesulphonate; ▲, sulphite; ●, sulphate; ----, growth.

below). One strain (A and B respectively) from each group of isolates was used for detailed metabolic studies. None of these 12 isolates was capable of growth on 4-ethylbenzenesulphonate or derivatives with an alkyl chain length of up to 18 carbon atoms (4-*n*-octadecylbenzenesulphonate). Five other isolates that were able to grow well on 4-*n*-octadecylbenzenesulphonate were also obtained from the appropriate elective cultures. These bacteria (I-V), also *Pseudomonas* spp., were capable of continued growth on all the alkylbenzenesulphonates with an alkyl chain length of 2 to 18 carbon atoms and grew equally well on benzenesulphonate and toluene-*p*-sulphonate.

Growth of strain A on benzenesulphonate was correlated with disappearance of the aromatic ring (measured spectrophotometrically at 225 mμ) and the appearance of sulphite in the growth medium. Sulphite began to appear as early as the lag phase of growth (Fig. 1); it rapidly disappeared, to be replaced by sulphate, when exponential growth commenced. Control experiments in uninoculated aerated media showed that sulphite oxidation to sulphate could readily occur non-enzymically under the neutral or slightly alkaline growth conditions used. Sulphite was also detected in cultures with toluene-*p*-sulphonate, 4-*n*-butylbenzenesulphonate, 4-*n*-hexylbenzenesulphonate and 4-*n*-octadecylbenzenesulphonate as substrates. Sulphite never appeared in growth media or incubations of washed cells in buffer when sulphate was supplied as the sole sulphur source.

Growth yield coefficients for benzenesulphonate

Table 1. *Sulphite formation from benzenesulphonate by washed suspensions of P. aeruginosa strain A*

Warburg flasks contained 10 μmoles of benzenesulphonate and 0.5 ml. of washed cells (equivalent to 28.5 mg. dry wt.). Experimental flasks and control flasks (without substrate) were removed at intervals, the contents rapidly centrifuged at 0° and the supernatants assayed for residual benzenesulphonate and sulphite (see the Materials and Methods section). Results are corrected for the appropriate control value.

Time (min.)	Benzenesulphonate remaining (μmoles)	Sulphite formed (μmoles)	Sulphate formed (μmoles)
0	10	0	0
15	8.5	1.5	0
30	6.7	3.0	0
90	2.7	6.5	0
135	0	8.2	0
180	0	8.0	0.3

(Bauchop & Elsdon, 1960) of 8.0–22.0 were obtained with different organisms. These values are low compared with that (59.2) obtained with the same bacterium grown with benzoate, even though there was apparently complete assimilation of the available substrate.

Oxidation of arylsulphonates by washed suspensions and cell-free extracts of P. aeruginosa

Washed suspensions of strain A or B, after growth on either compound, rapidly oxidized

benzenesulphonate or toluene-*p*-sulphonate with an oxygen uptake about 60% of the theoretical value. Benzenesulphinatase was also rapidly oxidized without a lag by benzenesulphonate- or toluene-*p*-sulphonate-grown cells and took up an additional 0.5 mole of oxygen/mole of substrate, consistent with the oxidation of the sulphinatase group to sulphonate and then sulphite. Benzenesulphonate-grown cells also oxidized *p*-fluorobenzenesulphonate. Under these conditions, sulphite was the only initial product (Table 1). Sulphate added to washed suspensions oxidizing fumarate or catechol under the same conditions was not reduced to sulphite. The formation of sulphite was confirmed by co-chromatography of the 2,4-dinitroanilinomaleimide derivative with an authentic sample in solvent *K*.

Some crude extract preparations, supplemented with GSH (10mM) and NADPH or an NADPH-generating system [isocitrate, 20 μ moles; NADP⁺, 2 μ moles; isocitrate dehydrogenase (Boehringer), 100 μ g. of protein] oxidized benzenesulphonate (10 μ moles) with an oxygen uptake and sulphite production satisfying the following equation:

The enzyme was very labile, however, and consistent results have not been obtained.

Metabolism of benzenesulphonate by P. aeruginosa strain A

Isolation of catechol. Washed suspensions of strain A, grown with benzenesulphonate, oxidized only catechol among several phenols and phenolic acids examined, but catechol was never detected in growing cultures by the sensitive Evans (1947) test. Its rate of oxidation by washed suspensions was some 40% faster than that of benzenesulphonate, so that it was unlikely ever to accumulate in well-aerated cultures. Hegeman (1966) showed, however, that there was frequently a significant lag in the appearance of enzymes behind the first sequential inductive step (benzenesulphonate conversion into catechol, in this case), so that catechol should accumulate transiently if fumarate-grown cells were exposed to benzenesulphonate under suitable conditions.

Cells from a 5l. culture grown with fumarate for 24 hr. were harvested, washed and resuspended to high density in 50 ml. of 0.05M-phosphate buffer, pH 7.0, containing benzenesulphonate (0.02M). This suspension was shaken at 210 rev./min. in air at 30° on a New Brunswick Gyrotory shaker and 1 ml. samples were examined at intervals for catechol substances (Sleeper, 1951). A strong

positive test was obtained at 27 hr. The flask contents were centrifuged and the supernatant (pH 7.0) was extracted with three 0.5 vol. portions of ether. The ether extracts were dried over anhydrous sodium sulphate and evaporated to dryness and the residue was recrystallized from ethyl acetate-benzene. The recrystallized product (12 mg.), m.p. 101°, did not depress the m.p. of authentic catechol (104°) on admixture. It chromatographed as catechol in solvents *F* (R_F 0.56) and *G* (R_F 0.62) and gave the same colour (red-brown) as catechol when sprayed with 2,6-dichloroquinone-4-chloroimide. The visible spectrum of the derivative of the isolated material in the Evans (1947) test was identical with that given by the derivative of authentic catechol ($E_{\max}$. at 480 μ) and the natural product was oxidized to 2-hydroxymuconic semialdehyde ($E_{\max}$. at 375 μ , pH 10.0) by a purified catechol 2,3-oxygenase preparation. The u.v. spectra of the natural product and catechol were also identical.

Conversion of catechol into 2-hydroxymuconic semialdehyde. Crude extracts from cells of strain A grown with benzenesulphonate rapidly oxidized catechol with the consumption of 1 mole of oxygen/mole of substrate and no carbon dioxide production. Addition of extracts to catechol was followed by the immediate appearance of a strong yellow colour, which on prolonged incubation (3–4 hr.) eventually disappeared without further gaseous exchange. The yellow material had the u.v.-spectra characteristics of 2-hydroxymuconic semialdehyde (Dagley & Stopher, 1959; Kojima, Itada & Hayaishi, 1961).

Extracts, purified by ammonium sulphate fractionation and dialysed, or after heat treatment (see the Materials and Methods section), accumulated the yellow oxidation product and did not degrade it further; the use of an automatic titrator showed that this oxidation resulted in the formation of 1 g. ion of H⁺/mole of substrate. The reaction product was prepared on a large scale and isolated essentially as described by Kojima *et al.* (1961). It had m.p. 210° (decomp.); Dagley & Morgan (1962) gave 210–214° (decomp.) for the synthetic material (Found: C, 50.4; H, 4.4. Calc. for C₆H₆O₄: C, 50.7; H, 4.3%). On adding excess of ammonium chloride and then acidifying and warming, the isolated material exhibited a u.v. spectrum similar to that of α -picolinic acid. It also formed a 2,4-dinitrophenylhydrazone derivative that darkened at 190° and gave no distinct m.p.; this derivative had a visible spectrum in 0.1N-sodium hydroxide or in methanol identical with that of authentic material. The u.v. spectra of the isolated material in acid ($E_{\max}$. at 320 μ) and alkali ($E_{\max}$. at 375 μ) were identical with those reported for 2-hydroxymuconic semialdehyde. These results are

consistent with the ring-fission product being 2-hydroxymuconic semialdehyde (5-formyl-2-oxopent-4-enoic acid). We also detected this product spectrophotometrically in the supernatant from washed suspensions of cells incubated with benzenesulphonate.

Conversion of catechol into 4-hydroxy-2-oxovalerate and formate. 4-Hydroxy-2-oxovalerate was identified as an intermediate in catechol metabolism by pseudomonads (Dagley, Chapman, Gibson & Wood, 1964; Dagley & Gibson, 1965). Its formation from catechol by benzenesulphonate-grown cells of strain A was observed with that fraction of crude extracts precipitated between 30% and 70% saturation with ammonium sulphate followed by Sephadex treatment (see the Materials and Methods section). Two Warburg flasks containing 10 μ moles of catechol, 0.5 ml. of Sephadex-treated soluble extract and 250 μ moles of phosphate buffer, pH 7.2, were incubated until all the yellow colour of 2-hydroxymuconic semialdehyde had disappeared (3 hr.). The flask contents were acidified with 0.5 ml. of 5*N*-hydrochloric acid, heated at 100° for 5 min., cooled in ice and the precipitated protein was centrifuged off. The supernatants were then examined spectrophotometrically, with the contents of a control flask (no substrate) as the blank. The spectrum of the compound produced enzymically from catechol was similar to that given by synthetic 4-methyl-2-oxobutyrolactone itself or synthetic 4-hydroxy-2-oxovalerate taken through the acid and heat treatment described above, which converts it into the lactone.

The remaining supernatant solution was extracted with ether, and the ether fractions were pooled, dried over anhydrous sodium sulphate and evaporated to dryness, leaving a small residue with the characteristic odour of 4-methyl-2-oxobutyrolactone. This residue was dissolved in a little ethanol and applied to paper chromatograms together with the authentic material and catechol. The enzymic product had R_F values of 0.67 in solvent *F* and 0.85 in solvent *G*, and gave a green colour on spraying with 2% 2,6-dichloroquinone-4-chloroimide. Authentic 4-methyl-2-oxobutyrolactone had the same R_F values and colour reaction. From larger-scale incubations (100 μ moles of catechol), the 2,4-dinitrophenylhydrazone derivative of 4-methyl-2-oxobutyrolactone was isolated as yellow crystals. They had m.p. 169–171°; Bayly *et al.* (1966) gave m.p. 172–174° (Found: C, 44.2; H, 3.5; N, 18.5. $C_{11}H_{10}N_4O_6$ requires: C, 44.5; H, 3.1; N, 19.0%).

The natural intermediate was identified as 4-hydroxy-2-oxovalerate, and not its lactone, by chromatography of the 2,4-dinitrophenylhydrazone derivatives produced by short-term (3–4 min.) incubation of reaction products with the acidic

Table 2. *Formation of 4-hydroxy-2-oxovalerate and formate from catechol by Sephadex-treated extracts of P. aeruginosa strain A*

Warburg flasks contained in a total volume of 3 ml.: phosphate buffer, pH 7.0, 200 μ moles; catechol, 5 μ moles; Sephadex-treated extract from *P. aeruginosa* strain A, 13 mg. of protein. Flasks were incubated at 30° and, at intervals, the reaction was stopped in individual flasks by the addition of 0.5 ml. of 6% (w/v) HClO₄. Determinations of 2-hydroxymuconic semialdehyde, formate and 4-hydroxy-2-oxovalerate were made on the deproteinized flask contents.

Time (min.)	2-Hydroxymuconic semialdehyde formed (μ moles)	4-Hydroxy-2- oxovalerate formed (μ moles)	Formate formed (μ moles)
0	0	0	0
2	4.6	0.5	0.3
5	2.9	1.6	1.5
10	0.6	1.5	1.8
15	0.4	2.6	2.4
25	0	2.7	3.2
30	0	3.4	3.8
40	0	3.6	4.3

dinitrophenylhydrazine reagent (see the Materials and Methods section).

Formate was the other product of catechol oxidation by Sephadex-treated extracts (Table 2). It was first identified by ether-extracting the products formed enzymically from 10 μ moles of catechol in Warburg flasks, and then extracting them back into alkali. This solution was reduced with dilute hydrochloric acid and magnesium and then treated with 4,5-dihydroxynaphthalene-2,7-disulphonic (chromotropic) acid (Grant, 1948). The pink compound produced gave a visible spectrum identical with that of formate taken through the same procedure. No pink derivative was formed without the reduction step, showing that the enzymic product was not formaldehyde.

The product of catechol oxidation was, like formate, steam-volatile and the condensate (after reduction) reacted with acetylacetone to give a pink product, the spectrum of which was identical with that given by authentic formate taken through this procedure (Nash, 1953); again there was no colour development without the reduction stage. The enzymic product was converted into carbon dioxide by a partially purified formate hydrogen-lyase preparation derived from anaerobically grown *Escherichia coli* (Wood & Gest, 1957), which did not attack formaldehyde.

The crude-extract preparations failed to accumulate formate stoicheiometrically; when centrifugation was increased to 144 000*g* for 2 hr., however, the clear supernatant did not metabolize formate further. A thrice-washed particle fraction, derived

Fig. 2. Oxidation by washed particulate preparations of formate produced from catechol metabolism. Double-side-arm Warburg flasks contained in a total volume of 3 ml.: phosphate buffer pH 7.0, 150 μ moles; catechol, 5 or 10 μ moles; KOH, 0.2 ml. in centre well; one side arm contained Sephadex-treated soluble extract (15.0 mg. of protein), the other contained a washed-particle preparation of *P. aeruginosa* strain A derived from the same cell crush (0.2 ml., 4.4 mg. dry wt.). The reaction was started by tipping the soluble extract and oxygen uptake (○) followed until no further change occurred. At 30 min. (arrow) the particle fraction was tipped, when a second oxygen uptake occurred owing to formate oxidation (▲). A control flask containing only formate (5 μ moles) (△) showed no oxygen uptake until the particle fraction was tipped. Other control flasks containing acetate, 4-hydroxy-2-oxovalerate, pyruvate or no other substrate showed no oxygen uptake with either soluble or particulate enzymes. Results are corrected for controls without substrate.

from the upper pink layer of the pellet and re-suspended in phosphate buffer, pH 7.0, oxidized formate to carbon dioxide (0.5 mole of oxygen/mole of substrate), but did not oxidize catechol, acetate, pyruvate or 4-hydroxy-2-oxovalerate. The enzymology of the system was confirmed by an experiment similar to that performed by Dagley & Gibson (1965). Catechol (5 μ moles) in double-side-arm Warburg flasks was oxidized by a Sephadex-treated soluble fraction until oxygen uptake had ceased (4.9 μ moles) and the yellow colour due to 2-hydroxymuconic semialdehyde had disappeared (0.5 hr.). The particulate fraction was then tipped from the second side arm, when a second uptake of oxygen was observed (equivalent to 2.2 μ moles), owing to formate oxidation (Fig. 2).

Conversion of catechol and 2-hydroxymuconic semialdehyde into pyruvate and acetaldehyde. Crude extracts of benzenesulphonate-grown strain A, on prolonged incubation with catechol or 2-hydroxymuconic semialdehyde, yielded pyruvate and acetaldehyde. Pyruvate was identified by chromatography of the 2,4-dinitrophenylhydrazone derivatives extracted from Warburg-flask contents at the end of 3 hr. incubation of crude extracts with 5 μ moles of catechol. Pyruvic 2,4-dinitrophenyl-

hydrazone and the derivative of the enzymically formed product co-chromatographed at R_F 0.39 and 0.67 and at R_F 0.58 and 0.70 (two values are for the two stereoisomers) in solvents B and D, and both gave a reddish-brown colour on spraying with 0.1 N-sodium hydroxide. The visible spectra of both authentic and natural derivatives produced in the Friedmann & Haugen (1943) test and measured in 0.1 N-sodium hydroxide were identical [$E_{\max}$. at 442 m μ and 510 m μ (shoulder)]. A large-scale preparation of the 2,4-dinitrophenylhydrazone of the keto acid derived from 150 μ moles of catechol incubated for 4 hr. with crude extract gave a product, m.p. 218°, which did not depress the m.p. (219°) of authentic pyruvic 2,4-dinitrophenylhydrazone. When NADH (20 μ moles) and purified muscle lactate dehydrogenase (0.5 ml.) (8000 units/ml.) were added to incubation mixtures of catechol (5 μ moles) and extract in Warburg flasks, no pyruvate was detected by the Friedmann & Haugen (1943) method. Chromatography of the ether extracts of such incubations always showed material with the same R_F as lactate. Similarly, when excess of NADH was added to a sample of the products of catechol (5 μ moles) metabolism contained in a cuvette with 200 μ moles of tris-hydrochloric acid buffer at pH 7.5 and the changes in E_{340} were followed in a recording spectrophotometer, there was an immediate fall in E_{340} on the addition of purified lactate dehydrogenase owing to oxidation of the NADH by pyruvate present in the reaction mixtures. Control incubations, which had contained either no catechol or boiled extract, showed negligible NADH oxidation. There is little doubt therefore that pyruvate is one product of catechol metabolism.

Acetaldehyde formed from catechol in incubation mixtures was detected by thin-layer chromatography of the neutral 2,4-dinitrophenylhydrazones left in the ethyl acetate layer of the Friedmann & Haugen (1943) test after extraction of acidic components (e.g. pyruvate) with carbonate. The natural product co-chromatographed with acetaldehyde in solvent Y on thin-layer plates. Acetaldehyde was confirmed by its isolation from the distillate of an incubation mixture of catechol (200 μ moles), crude extract (0.75 g. of protein) and 30 ml. of 0.1 M-phosphate buffer in a closed flask provided with forced aeration. The air stream was passed first through an acidic 1% (w/v) 2,4-dinitrophenylhydrazine solution, then through the incubation mixture maintained at 30° and then into a second solution of acidic 2,4-dinitrophenylhydrazine (Dagley & Gibson, 1965), where a yellow precipitate collected. The precipitate was washed and recrystallized (6 mg.) from aqueous ethanol, giving m.p. 147°, unchanged by admixture with authentic acetaldehyde 2,4-dinitrophenylhydrazone.

Fig. 3. Products of catechol metabolism by crude extracts of benzenesulphonate-grown cells employing the 'meta' pathway. Flasks contained: phosphate buffer, 200 μ moles; catechol, 5 μ moles; extract (equivalent to 20 mg. of protein) from *P. aeruginosa* strain A. They were incubated with shaking at 30°. Individual flasks were removed at intervals, and the reaction mixtures deproteinized with 10% (w/v) trichloroacetic acid (0.5 ml.) and assayed for the products. ●, 2-Hydroxymuconic semialdehyde; ○, pyruvate; ▲, acetaldehyde; △, formate.

Table 3. Enzymic cleavage of synthetic 4-hydroxy-2-oxovalerate by dialysed extracts of *P. aeruginosa* strain A

A series of Warburg flasks contained in a total volume of 3 ml.: tris-HCl buffer, pH 8.5, 250 μ moles; synthetic 4-hydroxy-2-oxovalerate, 5.0 μ moles; $MgSO_4$, 5.0 μ moles; dialysed extract of *P. aeruginosa* strain A, 15 mg. of protein. Incubation, with shaking, at 30° was stopped by the addition of 0.5 ml. of 6% (w/v) $HClO_4$, and the mixtures were cooled in ice and centrifuged at 0°. Acetaldehyde was determined on 0.3 ml. portions; the remainder was heated at 100° for 5 min. to convert any remaining substrate into 4-methyl-2-oxobutylolactone and cooled, and the lactone and pyruvate were determined in samples of the cooled mixture. Control flasks containing boiled enzyme or no substrate showed no acetaldehyde or pyruvate. N.D., Not determined.

Time (min.)	4-Hydroxy-2-oxovalerate remaining (μ moles)	Acetaldehyde formed (μ moles)	Pyruvate formed (μ moles)
0	5.0	0	0
1	N.D.	0.3	0.2
3	3.8	0.7	0.6
5	3.2	0.9	0.8
10	N.D.	1.2	1.1
15	2.0	1.5	1.3
20	2.4	1.7	1.4
30	2.4	1.7	1.4

Fig. 3 shows the conversion of catechol into these end products by crude extracts. The stoichiometry of the conversion of catechol into pyruvate and

Table 4. Activities of enzymes of the 'meta' pathway in extracts of *P. aeruginosa* strain A

Enzyme	Sp. activity (m μ moles of substrate converted/min./mg. of protein) in extracts of cells grown with	
	Benzene-sulphonate	Fumarate
Catechol 2,3-oxygenase	66.7	0
2-Hydroxymuconic semialdehyde hydrolase	3.5	0.015
4-Hydroxy-2-oxovalerate aldolase	27.5	2.0
4-Oxalocrotonate system*	17.0	0

* Measures the overall activity of the enzyme system converting oxalocrotonate into pyruvate, acetate and CO_2 determined by measuring the rate of pyruvate formation with lactate dehydrogenase.

acetaldehyde has been thoroughly examined by Dagley *et al.* (1964) and Dagley & Gibson (1965), so no attempt was made in this instance to achieve theoretical yields.

Enzymic cleavage of synthetic 4-hydroxy-2-oxovalerate to acetaldehyde and pyruvate. Synthetic 4-hydroxy-2-oxovalerate was converted into pyruvate and acetaldehyde by crude or dialysed extracts of benzenesulphonate-grown strain A. Dagley & Gibson (1965) have shown that only one enantiomorph of the synthetic material (which is asymmetric around C-4) was utilized by their organism. Similarly *P. aeruginosa* strain A utilized only one enantiomorph, but stoichiometric accumulation of the products did not occur with dialysed extracts (Table 3).

Effect of growth substrates on enzyme activities. In many pseudomonads catechol 2,3-oxygenase is probably a constitutive enzyme (Evans, Fernley & Griffiths, 1965; Griffiths, Rodrigues, Davies & Evans, 1964), but fumarate-grown cells of *P. aeruginosa* had no detectable catechol 2,3-oxygenase. The other enzymes of the 'meta' route were also strictly inducible in this organism (Table 4) except that 4-hydroxy-2-oxovalerate aldolase had basal activities in fumarate-grown cells of some 5–7% of that found in fully induced cells. This level of activity was more than sufficient to account for the slow utilization of 4-hydroxy-2-oxovalerate observed in induction experiments performed with washed suspensions of fumarate-grown cells (Farr & Cain, 1968).

4-Oxalocrotonate as an intermediate in catechol metabolism. The metabolism of a specimen of synthetic 4-oxalocrotonate was examined with the benzenesulphonate-utilizing bacterium because this compound has been implicated in catechol degrada-

Table 5. *Products of catechol and 4-oxalocrotonate metabolism by extracts of P. aeruginosa strain A*

Double-side-arm Warburg flasks contained in a total volume of 3 ml.: tris-HCl buffer, pH 7.5, 200 μ moles; substrate, 2–5 μ moles; NAD⁺, 1–2.5 μ moles (adjusted to give half of the catechol concentration); KOH in centre well, or H₂SO₄ (3 N) in the second side arm, 0.2 ml.; extract of *P. aeruginosa* strain A, 27.0 mg. of protein for Expt. 1, 4.03 mg. for Expt. 2, 6.5 mg. for Expt. 3, 18.6 mg. for Expt. 4 and 8.7 mg. for Expt. 5. Incubation times were 2 hr. for Expts. 1 and 2, 90 min. for Expt. 4 and 3 hr. for Expts. 3 and 5. At the end of incubation, the reaction was stopped by boiling the flask contents for 5 min. in stoppered tubes, the denatured protein was centrifuged off and pyruvate, formate and acetate were determined in samples of the supernatant. 4-Hydroxy-2-oxovalerate was measured separately in a sample that was acidified before heating (see the Materials and Methods section). N.D., Not determined.

Expt. no.	Type of extract	Substrate available (μ moles)	NAD ⁺ added	O ₂ consumed (μ moles)	CO ₂ evolved (μ moles)	4-Hydroxy-2-oxovalerate formed (μ moles)	Formate formed (μ moles)	Pyruvate formed (μ moles)	Acetate formed (μ mole)
1	Dialysed	Catechol (5)	No	5.05	Nil	Nil	N.D.	4.3	Nil
		Catechol (5)	Yes	4.70	2.6	Nil	N.D.	N.D.	N.D.
2	Dialysed	Catechol (2.5)	Yes	2.60	1.5	Nil	N.D.	0.92	0.4
		Oxalocrotonate (2)	No	Nil	1.2	Nil	Nil	0.9	Nil
3	Dialysed	Oxalocrotonate (2)	Yes	Nil	1.6	0.2	N.D.	1.7	Nil
		Catechol (2)	No	1.8	Nil	1.6	1.7	0.2	Nil
4	Sephadex-treated	Catechol (4)*	No	3.7	Nil	3.2	3.1	1.3	Nil
		Catechol (4)†	Yes	5.3	1.25	3.6	0.8	1.05	Nil
		Oxalocrotonate (2)	No	0.1	1.10	0.9	Nil	0.1	Nil
5	Sephadex-treated	Oxalocrotonate (2)	Yes	0.2	1.65	1.75	Nil	Nil	Nil

* 0.4 μ mole of 2-hydroxymuconic semialdehyde also remained.

† 0.07 μ mole of 2-hydroxymuconic semialdehyde also remained at 90 min. At 15 min. 3.9 μ moles of O₂ were consumed and no CO₂ was evolved; the additional O₂ and CO₂ were slowly consumed and evolved over the next 75 min.

tion by a *Pseudomonas* sp., extracts of which, when supplemented with NAD, required oxygen in addition to that necessary for ring fission and, in contrast with *Pseudomonas* U (Dagley & Gibson, 1965), produced carbon dioxide and acetate instead of formate and acetaldehyde from catechol (Nishizuka, Ichiyama, Nakamura & Hayaishi, 1962).

Crude extracts of cells grown on benzenesulphonate were able to convert 4-oxalocrotonate into pyruvate (Table 4), but this activity was completely absent from extracts derived from fumarate-grown cells. Pyruvate was confirmed as one product by chromatography of the acidic 2,4-dinitrophenylhydrazones from the reaction cuvettes in solvents *B*, *C* and *D*. Sephadex-treated extracts, which accumulate 4-hydroxy-2-oxovalerate from catechol, decarboxylated 4-oxalocrotonate to this keto acid (Table 5), the identity of which was confirmed spectrophotometrically by its conversion into 4-methyl-2-oxobutylolactone and by chromatography of the 2,4-dinitrophenylhydrazone in solvents *B*, *C* and *D* (R_F values of 0.49, 0.63 and 0.65 respectively). Dialysed extracts, which metabolized catechol as far as pyruvate, also released carbon dioxide from oxalocrotonate, but they showed no oxygen uptake in these incubations even after supplementation with NAD⁺, nor was acetate detected. The formation of acetate from oxalo-

crotonate requires the consumption of 1 g. atom of oxygen/mole of substrate for the re-oxidation of NADH formed in oxidizing 4-hydroxy-2-oxovalerate to acetopyruvate (Nishizuka *et al.* 1962).

Catechol itself consumed 1 mole of oxygen/mole of substrate oxidized and released no carbon dioxide when it was metabolized by dialysed extracts to 4-hydroxy-2-oxovalerate or to formate, pyruvate and acetaldehyde (cf. Dagley & Gibson, 1965). The addition of NAD⁺ in substrate quantities to incubation mixtures containing even very large amounts of dialysed extract (27.0 mg. of protein) failed to elicit any significant oxygen uptake above that required for ring fission (complete for 5 μ moles of catechol in 10 min. at this protein concentration) over a 3 hr. period, but a slow evolution of carbon dioxide was usually observed. The addition of NAD⁺ accelerated the disappearance of accumulated 2-hydroxymuconic semialdehyde in the incubation mixtures compared with flasks containing no NAD⁺, and samples removed at 10, 30 and 60 min. revealed the presence of oxalocrotonate when chromatography of the 2,4-dinitrophenylhydrazones was carried out. The identity of this material with oxalocrotonate dinitrophenylhydrazone was confirmed by comparing their spectra in 0.1 N-sodium hydroxide; both natural and synthetic derivatives showed $E_{\max}$.

Table 6. *Enzymes of the 'ortho' cleavage pathway induced in P. aeruginosa strain B by growth on benzenesulphonate*

Assays were conducted at 30° in cuvettes containing: tris-HCl buffer, pH 7.5, 300 μ moles; substrate, 0.5 μ mole; enzyme, 0.25 mg. of protein from benzenesulphonate-grown organisms and 3.0 mg. of protein from fumarate-grown organisms. β -Oxadipate enol lactone was generated from β -carboxymuconate in solution with the appropriate purified enzymes of *P. putida* A3.12 (Ornston, 1966).

Enzyme	Sp. activity (μ moles of substrate converted/min./mg. of enzyme protein) in cells grown on	
	Benzene-sulphonate	Fumarate
Catechol 1,2-oxygenase	1.33	0
Muconate-lactonizing enzyme	0.61	0
Muconolactone isomerase	1.44	0
β -Oxadipate enol lactone hydrolase	1.08	0.04

at 400 $m\mu$ and a distinct shoulder at 510 $m\mu$. 4-Hydroxy-2-oxovalerate dinitrophenylhydrazine and pyruvate dinitrophenylhydrazone were also evident. Small amounts of acetate were detected at the end of incubations with catechol and dialysed extracts supplemented with NAD⁺ (Table 5).

Metabolism of benzenesulphonate by P. aeruginosa strain B

Extracts of three of the original benzenesulphonate isolates, although showing an oxygen uptake with catechol, did not develop the typical yellow colour of 2-hydroxymuconic semialdehyde; one of them (strain B) was examined further. Catechol was oxidized by dialysed extracts of this organism with the uptake of 1 mole of oxygen/mole of substrate and the formation of *cis-cis*-muconate, characterized by its absorption maximum at 257 $m\mu$ and the hyperchromic shift of the peak to 265 $m\mu$ on boiling for 30 min.; the product co-chromatographed with *cis-cis*-muconate in solvents *H* and *J*. It was isolated from a large-scale preparation as described by Cain (1966) and gave m.p. 195°. Strain B thus contained a catechol 1,2-oxygenase.

In the presence of tris-hydrochloric acid buffer (Ornston, 1966) crude extracts converted catechol, *cis-cis*-muconate, (+)-muconolactone and β -oxoadipate enol lactone into β -oxoadipate (Table 6). The enzymes catalysing these reactions were strictly inducible except that low levels of β -oxoadipate enol lactone hydrolase activity were measurable in extracts derived from fumarate-grown cells (approx.

2% of that in fully induced cells). Extracts of this strain very slowly attacked 4-hydroxy-2-oxovalerate after growth of the organism on fumarate, benzoate or benzenesulphonate.

Metabolism of toluene-p-sulphonate by P. aeruginosa strain A

Growth on toluene-*p*-sulphonate induced in washed cells of strain A the ability to oxidize only catechol, 3-methylcatechol and 4-methylcatechol; other likely intermediates such as *p*-cresol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate and protocatechuate were oxidized only after lag periods in excess of 60 min. The catechols were not oxidized by fumarate-grown cells. Structural considerations suggested that 4-methylcatechol should be the natural intermediate in toluene-*p*-sulphonate metabolism, so evidence for the formation of this compound or its metabolites was sought.

4-Methylcatechol formation from toluene-p-sulphonate. 4-Methylcatechol was oxidized by competent organisms faster than toluene-*p*-sulphonate, and so would not be expected to accumulate in growing cultures. No positive Evans (1947) test was recorded in any toluene-*p*-sulphonate-grown culture, even when modified by decreasing the aeration or rendering the growth medium iron-free by the methods of Waring & Werkman (1943). Stationary incubations of toluene-*p*-sulphonate (1–10 mM) with washed cells derived from fumarate-grown (as described above) or toluene-*p*-sulphonate-grown cultures did not accumulate significant catechol material. A faint spot with the same *R_F* and colour reaction with 2,6-dichloroquinone-4-chloroimide as 4-methylcatechol was obtained on chromatograms of the neutral ether-soluble material from such incubations on only one occasion.

Growing cultures in the early exponential phase (12–18 hr.) and incubation mixtures of toluene-*p*-sulphonate and washed suspensions of cells from 36 hr. cultures frequently developed a yellow colour in the medium; this material was extractable into ether after acidification and showed a sharp absorption maximum at 382 $m\mu$ in alkaline solution (pH 10.0) disappearing on acidification with the simultaneous appearance of a new peak at 320 $m\mu$. These absorption maxima are characteristic of the methyl-2-hydroxymuconic semialdehyde produced by catechol 2,3-oxygenase cleavage of 4-methylcatechol (Bayly *et al.* 1966; see below). This material co-chromatographed with the product of 4-methylcatechol oxidation isolated from incubations with the same washed-cell suspensions (Fig. 4).

An isotope-trapping experiment with 2-hydroxy-5-methylmuconic semialdehyde was performed to confirm the conversion of [*Me*-¹⁴C]toluene-*p*-sulphonate into this aliphatic product. The

Fig. 4. Formation of methyl-2-hydroxymuconic semialdehyde from toluene-*p*-sulphonate and 4-methylcatechol by washed suspensions. The incubation mixtures (in 250 ml. conical flasks) contained: phosphate buffer, 2 m-moles; substrate, 40 μ moles; cell suspension of *P. aeruginosa* strain A (equivalent to 660 mg. dry wt.). The flasks were shaken at 145 oscillations/min., 8.0 cm. throw, in a Mickie Metabolic Shaker Bath at 30°. Samples were removed at intervals, the cells centrifuged off and samples assayed for their methyl-2-hydroxymuconic semialdehyde content at pH 11.0. $\blacktriangle$, Toluene-*p*-sulphonate; $\circ$, 4-methylcatechol; Δ , *p*-toluic acid; $\bullet$, no substrate.

incubation mixture contained in a total volume of 60 ml.: phosphate buffer, pH 7.2, 5.85 m-moles; [*Me*-¹⁴C]toluene-*p*-sulphonate, 270 μ moles (6910 counts/min./ μ mole); washed cells equivalent to 0.82 g. dry wt. Samples were removed at intervals, the cells removed by centrifugation and the supernatants made alkaline (pH 11.0) and examined for the ring-fission product. When this reached its maximum (0.8 μ mole), unlabelled 2-hydroxy-5-methylmuconic semialdehyde (5.6 μ moles) was added and the reaction stopped after a further 10 min. with 5 ml. of 4*N*-sulphuric acid. The semialdehyde was re-isolated and chromatographed as bands on thin-layer plates. Ether, used as the solvent, left unchanged toluene-*p*-sulphonic acid at the origin but the semialdehyde moved with *R_F* 0.2. The appropriate bands were removed, and the semialdehyde was eluted with ethyl acetate and rechromatographed in ether on plates. The bands were then eluted with potassium

phosphate, pH 11.0, and samples containing a known quantity of semialdehyde (calculated from its extinction at pH 11.0, 382 m μ , ϵ = 39 500) counted in triplicate in the scintillation counter. The re-isolated 2-hydroxy-5-methylmuconic semialdehyde had a corrected specific activity of 758 counts/min./ μ mole, equivalent, after allowing for dilution with unlabelled material, to a specific activity of 6065 counts/min./ μ mole in the original metabolic product. These results showed that some 88% of the toluene-*p*-sulphonate was metabolized through the semialdehyde intermediate.

Initial ring-fission product of 4-methylcatechol oxidation: 2-hydroxy-5-methylmuconic semialdehyde. The formation of the yellow compounds in methylcatechol oxidations by crude extracts occurred very rapidly with the consumption of 1 mole of oxygen/mole of substrate; their subsequent disappearance was much slower. The product of 3-methylcatechol oxidation had $E_{\max}$. at 390 m μ in alkali and has been identified with 2-hydroxy-6-oxohepta-2,4-dienoic acid by Ribbons (1966).

The product of 4-methylcatechol oxidation had $E_{\max}$. at 382 m μ in alkali (ϵ = 39 500) and at 320 m μ at pH 3.0 (ϵ = 20 500), and should be either 2-hydroxy-4-methyl- or 2-hydroxy-5-methylmuconic semialdehyde, depending on the position of ring cleavage.

The ring-fission product was isolated as follows. 4-Methylcatechol (0.5 m-mole, added in portions of 5 μ moles), heat-treated cell extract (2.2 g. of protein) and 100 ml. of 0.1 M-phosphate buffer, pH 7.5, were incubated with forced aeration and magnetic stirring at 30° until 0.2 ml. samples of the reaction mixture (diluted to 1 ml. with water) no longer gave a positive Evans (1947) test. At this time the yellow colour of the incubation mixture was intense. The solution was acidified to pH 3.0 with orthophosphoric acid, and the precipitated protein spun off, the supernatant nearly saturated with sodium chloride and extracted with several 0.5 vol. portions of ether. After being dried over anhydrous sodium sulphate, the combined ether fractions were evaporated to dryness, the temperature not being allowed to rise above 35°, and the residue (53 mg.) was recrystallized from ethyl acetate–light petroleum (b.p. 60–80°). The product had m.p. 162° (Found: C, 54.2; H, 5.4. Calc. for C₇H₈O₄: C, 53.8; H, 5.2%). It formed a 2,4-dinitrophenylhydrazone derivative, m.p. 168° (decomp.) (Found: N, 21.9. Calc. for C₁₉H₁₆N₈O₁₀: N, 21.7%). These results are consistent with a methyl-2-hydroxymuconic semialdehyde but do not distinguish between the 4- and 5-methyl isomers.

In the presence of excess of NH₄⁺ in acid conditions, 2-hydroxymuconic semialdehydes are converted into picolinates (Trippett, Dagley & Stopher, 1960; Dagley, Evans & Ribbons, 1960). When the

isolated ring-fission product of 4-methylcatechol was allowed to stand with 0.1N-ammonium chloride in dilute hydrochloric acid overnight, a compound with a spectrum identical with that of

5-methyl-2-picolinic acid at neutral and acid pH values was produced; this spectrum was readily distinguished from that given by 4-methyl-2-picolinic acid (Fig. 5). The unknown material co-chromatographed with 5-methylpicolinic acid in solvents *J*, *M* and *N* with R_F values of 0.59, 0.65 and 0.61 respectively. 4-Methylpicolinic acid had R_F values of 0.51, 0.55 and 0.53 respectively in these solvents. In a larger-scale preparation, the solution of the picolinic compound derived from warming approx. 200 μ moles of the 4-methylcatechol ring-fission product with excess of acidic ammonium chloride was evaporated to dryness *in vacuo*, the picolinate derivative extracted continuously from the residue with hot benzene for 10 hr. and the bulk of the benzene distilled off. The unknown product crystallized from the concentrate on cooling. It had m.p. 162°; Oliveto (1963) gives m.p. 163–164° for 5-methyl-2-picolinic acid and m.p. 140° for the 4-methyl isomer. The isolated material was treated with just above the calculated quantity of dilute hydrochloric acid to convert it into the hydrochloride and evaporated to dryness *in vacuo* over potassium hydroxide pellets. The hydrochloride had m.p. 201° and did not depress the m.p. of authentic 5-methyl-2-picolinic acid hydrochloride (m.p. 203–204°) provided by Dr S. Senoh. The m.p. of authentic 4-methyl-2-picolinic acid hydrochloride (180–182°) was depressed some 30° on admixture with an equivalent amount

Fig. 5. Ultraviolet spectra of the methylpicolinic acids at pH 7.0 (a) and pH 2.0 (b). —, Ring-fission product of 4-methylcatechol after treatment with NH_4Cl in N-HCl overnight; ----, 5-methyl-2-picolinic acid; ·····, 4-methyl-2-picolinic acid.

Scheme 1. Origin of the methyl-2-hydroxymuconic semialdehydes and methylpicolinates according to the position of ring fission in 4-methylcatechol.

of the isolated material. Formation of 5-methyl-2-picolinate from the ring-fission product indicated that ring cleavage of 4-methylcatechol occurred at the 2,3-bond [position (2) in Scheme 1], and not at the 1,6-bond [position (1) in Scheme 1], as had been found in 'meta' cleavage of protocatechuate (Trippett *et al.* 1960).

Conversion of 4-methylcatechol and 2-hydroxy-5-methylmuconic semialdehyde into 4-hydroxy-2-oxohexanoate and formate. Extracts after Sephadex treatment converted 4-methylcatechol or its ring-fission product into a compound with u.v. spectra in acid and alkali almost identical with those of 4-hydroxy-2-oxovalerate. The product derived from 2-hydroxy-5-methylmuconic semialdehyde, however, was distinguished from 4-hydroxy-2-oxovalerate by the different R_F values of the 2,4-dinitrophenylhydrazones in solvents *C* and *E*; the R_F values of the 2,4-dinitrophenylhydrazones of its lactone, formed by heating in dilute acid for 5 min., were also different from those of 4-methyl-2-oxobutyrolactone dinitrophenylhydrazone, but the R_F values observed agreed closely with those recorded for the 2,4-dinitrophenylhydrazones of 4-hydroxy-2-oxohexanoate and its lactone, 4-ethyl-2-oxobutyrolactone, by Bayly *et al.* (1966).

A sample of 4-ethyl-2-oxobutyrolactone dinitrophenylhydrazone was prepared by incubating 150 μ moles of 2-hydroxy-5-methylmuconic semialdehyde (or 4-methylcatechol), in 25 μ mole portions, with Sephadex-treated extract (350 mg. of protein) at 30° at pH 7.5 until the yellow colour had disappeared. The product was isolated as described by Bayly *et al.* (1966) and gave m.p. 169°; Bayly *et al.* (1966) recorded 172–174°. No authentic material was available for determinations of mixed m.p. (Found: N, 17.5. Calc. for $C_{12}H_{14}N_4O_7$: N, 17.2%).

Addition of dialysed extracts to the material accumulated by Sephadex-treated extracts from 2-hydroxy-5-methylmuconic semialdehyde or 4-methylcatechol led to its disappearance with the concomitant formation of pyruvate and propionaldehyde.

These products were also formed on mild alkaline hydrolysis (pH 9.5) of the lactone of the natural product with sodium hydroxide (Bayly *et al.* 1966), which strongly suggests the identity of the keto-acid with 4-hydroxy-2-oxohexanoate.

Formate, the other product of metabolism of 2-hydroxy-5-methylmuconic semialdehyde by Sephadex-treated extracts (Table 7), was identified in these incubation mixtures by the chromotropic acid and acetylacetone colorimetric procedures (see the Materials and Methods section) and confirmed by paper chromatography with an authentic marker in alkaline solvent *H*. It was not oxidized by fumarate-grown cells, but was rapidly attacked

Table 7. *Metabolism of 4-methylcatechol and 2-hydroxy-5-methylmuconic semialdehyde by Sephadex-treated extracts of P. aeruginosa strain A*

Warburg flasks contained in a total volume of 3 ml.: phosphate buffer, pH 7.0, 200 μ moles; 4-methylcatechol, 5 μ moles; Sephadex-treated extract of *P. aeruginosa* strain A, 150 mg. of protein. Flasks were incubated with shaking at 30° and at intervals the reaction was stopped in individual flasks by the addition of 0.5 ml. of 6% (w/v) $HClO_4$. Residual 2-hydroxy-5-methylmuconic semialdehyde, formate and 4-hydroxy-2-oxohexanoate were determined on the deproteinized flasks contents.

Time (min.)	2-Hydroxy-5-methylmuconic semialdehyde remaining (μ moles)	4-Hydroxy-2-oxohexanoate formed (μ moles)	Formate formed (μ moles)
10	2.75	1.40	1.60
15	2.50	1.45	2.00
20	2.45	1.60	2.75
30	1.08	1.72	4.25
40	0.67	2.00	4.25
60	0.33	1.40	3.60

Table 8. *Oxidation of propionaldehyde, propionate and formate by washed suspensions of P. aeruginosa strain A*

Warburg flasks contained in a total volume of 3 ml.: phosphate buffer, pH 7.0, 200 μ moles; substrate, 5 μ moles; washed suspension of cells, 0.5 ml. (equivalent to 19.7 mg. dry wt. from a toluene-*p*-sulphonate-grown culture and 18.9 mg. dry wt. from a fumarate-grown culture); KOH, 0.2 ml. in centre well. Incubation was at 30°.

Substrate	Activity (μ l. of O_2 /hr./mg. dry wt.) of cells grown with	
	Toluene- <i>p</i> -sulphonate	Fumarate
Propionaldehyde	27.8	8.6
Propionate	21.9	7.3
Formate	27.5	0

by cells from toluene-*p*-sulphonate-grown cultures (Table 8).

Metabolism of the methylcatechols by crude extracts. Reference to the general scheme for the degradation of catechols proposed by Dagley *et al.* (1964) suggested that 4-methylcatechol should be degraded to pyruvate, propionaldehyde and formate; extracts of toluene-*p*-sulphonate-grown cells produced these end products from 4-methylcatechol and 2-hydroxy-5-methylmuconic semialdehyde. Crude extracts of toluene-*p*-sulphonate-grown cells converted both methylcatechol isomers into pyruvate in a reaction in which a strong yellow colour rapidly appeared and slowly faded in the incubation mixture. The products from the oxidation of 4-methylcatechol (10 μ moles) by crude extracts (10 mg. of protein) after 3 hr. incubation at 30° in Warburg flasks

Scheme 2. Pathways of benzenesulphonate and toluene-p-sulphonate metabolism by *P. aeruginosa*.

containing phosphate buffer, pH 7.2 (250 μ moles), were treated with acidic 0.1% 2,4-dinitrophenylhydrazine, and the acidic and neutral dinitrophenylhydrazones, after extraction into ether, were separated by extracting the former into sodium carbonate solution. Paper chromatography of this acidic fraction (after acidifying the carbonate layer and re-extraction of the 2,4-dinitrophenylhydrazones into chloroform) with appropriate markers revealed pyruvic dinitrophenylhydrazone. This was isolated from a large-scale incubation of 4-methylcatechol (100 μ moles) and crude extracts (1.2 g. of protein) by the methods described for catechol degradation. The recrystallized product had m.p. 216° and did not depress the m.p. of authentic material.

The 2,4-dinitrophenylhydrazone fraction not extracted by carbonate was chromatographed on thin-layer plates in solvent Y, and material with the same R_F (0.35) as propionaldehyde 2,4-dinitrophenylhydrazone was consistently found. A larger-scale experiment, similar to that used for acetaldehyde 2,4-dinitrophenylhydrazone preparation (see above), was set up but with 4-methylcatechol (200 μ moles) and 1.5 g. of extract protein from toluene-*p*-sulphonate-grown cells in 0.1 M-phosphate buffer, pH 7.5, in the reaction vessel. In the second dinitrophenylhydrazine solution a small crystalline precipitate developed, and was collected, washed and dried. This material (4 mg.), after recrystallization from aqueous ethanol, had m.p. 154–155° and did not depress the m.p. of authentic propionaldehyde 2,4-dinitrophenylhydrazone admixture.

The methyl group of toluene-*p*-sulphonate should appear as the methyl group of propionaldehyde if the former substrate is metabolized through 4-methylcatechol (Scheme 2). The incorporation by growing bacteria of label from [*Me*-¹⁴C]toluene-*p*-sulphonate (6910 counts/min./ μ mole) into propionaldehyde (as its dinitrophenylhydrazone) was followed as described in the Materials and Methods section; maximum incorporation recurred some 40 sec. after adding labelled substrate, was maintained at this level for 5 min. and then fell away slowly. The corrected specific activity of this material was calculated as 52 counts/min./ μ mole. The heavy dilution of label in this experiment is partly accounted for by the exponential growth of the organism on unlabelled substrate before addition of labelled sulphonate. The identity of the labelled dinitrophenylhydrazone with the propionaldehyde derivative was confirmed by pooling the samples, followed by chromatography and radioautography; and also by adding authentic material and recrystallizing to constant specific activity. Pyruvate dinitrophenylhydrazone, separated from the neutral derivatives by carbonate extraction and, after acidification, by paper chromatography, had no

radioactivity derived from [*Me*-¹⁴C]toluene-*p*-sulphonate, in agreement with the metabolic route outlined in Scheme 2.

Oxidation of propionaldehyde and propionate by washed cells. Propionaldehyde arises only by 'meta' cleavage of 4-methylcatechol, not from catechol itself or the 3-methyl isomer. It would be expected therefore that extracts producing this intermediate should also have the ability to oxidize it further. An NADP-mediated aldehyde dehydrogenase active against propionaldehyde was detected in extracts of toluene-*p*-sulphonate-grown cells. Washed cells from toluene-*p*-sulphonate-grown cultures also showed some threefold higher respiratory activity towards propionaldehyde and propionate than that of similar suspensions of fumarate- or benzenesulphonate-grown cells (Table 8).

Specificity of the catechol 2,3-oxygenase induced by arylsulphonates. Bayly *et al.* (1966) have found that *Pseudomonas* U, grown on phenol, yielded crude extracts that readily oxidized catechol and both methylcatechol isomers. Evans *et al.* (1965) and Ribbons (1966) have also described catechol 2,3-oxygenases of apparently low specificity.

P. aeruginosa strain A, after growth on toluene-*p*-sulphonate, attacked all the catechols, but catechol itself was oxidized by crude extracts some four to five times as rapidly as the two methylcatechols, the rates of oxidation of which were virtually identical. The isolation of the oxidation product characteristic of 4-methylcatechol as the natural intermediate, however, suggested that separate enzymes might be responsible for oxidation of the various catechols. Partial purifications of the 2,3-oxygenase from toluene-*p*-sulphonate-grown cells by two methods effected a 17-fold purification over five steps (Table 9). The ratio of activity on catechol to that on 4-methylcatechol remained constant at about 4.5:1, but there were marked variations in the ratios of the activities on catechol or 4-methylcatechol to that on 3-methylcatechol during purification, which suggests that the 3-methyl isomer may be oxidized by a separate enzyme. These observations were reinforced by growing the same organism on benzenesulphonate, which induced a 2,3-oxygenase with a slightly different spectrum in crude extracts. In this instance, catechol and 4-methylcatechol were again oxidized at rates in the approximate ratio 5:1, but negligible activity for the 3-methyl isomer was elicited. Further, an examination of the kinetics of thermal inactivation of catechol 2,3-oxygenase in crude extracts showed that the losses of activity for both catechol and 4-methylcatechol paralleled each other (Fig. 6). Activity for 3-methylcatechol in these extracts, however, also fell off at a similar rate.

None of the catechol 2,3-oxygenases induced by the arylsulphonates attacked 3,4-dihydroxy-

Table 9. *Purification of catechol 2,3-oxygenase from toluene-p-sulphonate-grown cells of P. aeruginosa strain A*

All samples were reactivated by the borohydride reduction method (Taniuchi *et al.* 1962) immediately before assay. Specific activity is expressed as units/mg. of protein (Kojima *et al.* 1961).

Fraction	Sp. activity (units/mg. of protein) with catechol	Proportions of sp. activities (catechol:4-methylcatechol:3-methylcatechol)
A. 1. Ultrasonic extract	2.0	1.0:23:0.23
2. Supernatant from protamine sulphate (0.2 vol.)	2.2	1.0:22:0.22
3. (NH ₄) ₂ SO ₄ (30–50% satd.)	3.5	1.0:21:0.12
4. (NH ₄) ₂ SO ₄ (33–45% satd.)	5.0	1.0:20:0.09
5. Adsorption on calcium phosphate gel and elution with 0.05 M-phosphate buffer, pH 7.0	34.4	1.0:21:0.06
B. 1. Ultrasonic extract	2.5	1.0:25:0.21
2. Protamine sulphate and (NH ₄) ₂ SO ₄ (33–45% satd.)	8.5	1.0:21:0.11
3. Fractions (5 ml.) 9–12 from Sephadex G-200 column pooled	41.0	1.0:23:0.12
4. Eluate from calcium phosphate gel (as in A)	54.5	1.0:23:0.06

Fig. 6. Kinetics of heat inactivation of the catechol 2,3-oxygenase induced by growth on toluene-*p*-sulphonate in crude cell-free extracts of *P. aeruginosa* strain A. Samples (3 ml.) of crude extract (2.5 mg. of protein) in phosphate buffer, pH 7.0, were heated at 70° for various times, the extracts were then cooled in ice and the percentage activity remaining in the extracts was tested against: ●, catechol; ▲, 3-methylcatechol; ○, 4-methylcatechol. Cuvettes contained in a total volume of 3 ml.: substrate, 1 μ mole; extract 0.5 ml.; phosphate buffer, pH 7.2, 300 μ moles. The increase in E_{375} was followed for 10 min.

Table 10. *Catechol 2,3-oxygenase induced by growth of a Pseudomonas (isolate I) on alkylbenzene-sulphonates*

Cells were harvested in the exponential phase and the extracts assayed immediately with catechol. All extracts were treated with borohydride (see the Materials and Methods section) to elicit maximum oxygenase activity.

Carbon source	Sp. activity (μ moles of product formed/min./mg. of protein)
Toluene- <i>p</i> -sulphonate	210
4-Ethylbenzenesulphonate	81
4- <i>n</i> -Butylbenzenesulphonate	10.5
4- <i>n</i> -Decylbenzenesulphonate	9.5
4- <i>n</i> -Dodecylbenzenesulphonate	7.7
4- <i>n</i> -Hexadecylbenzenesulphonate	4.0
4-(Oct-2-yl)benzenesulphonate	4.5
Tetradecyl sulphate	0
Fumarate	0

benzoate (protocatechuate), nor did this substrate act as a competitive inhibitor of catechol or 3- or 4-methylcatechol oxidation. Catechol, on the other hand, was a strong competitive inhibitor of the analogous protocatechuate 4,5-oxygenase (Cain, 1962; Ribbons & Evans, 1962).

Griffiths *et al.* (1964) have indicated that at least some of their bacteria have constitutive catechol 2,3-oxygenases. This is not the case with the *P. aeruginosa* strain used here; fumarate-grown cells,

derived from inocula carried on nutrient agar since isolation, had no detectable oxygenase of either 'meta' or 'ortho' type (Table 4). Several detergents of various alkyl chain lengths will, however, induce this enzyme (Table 10).

DISCUSSION

The degradation by micro-organisms of arylsulphonates has been largely overlooked except for a brief report (J. R. Simpson, unpublished work quoted by Evans, 1958) where catechol was implicated by sequential induction methods as an intermediate in benzenesulphonate metabolism. Although they have no detergent properties themselves, benzenesulphonate and toluene-*p*-sulphonate are useful in establishing the likely fate of the benzene ring of alkylbenzenesulphonates. We have demonstrated for instance that benzenesulphonate, toluene-*p*-sulphonate and alkylbenzenesulphonates with an alkyl chain length of up to 16 carbon atoms all released their sulphonate group originally as sulphite on degradation. Further, growth of some *Pseudomonas* spp. (I-V) on these substrates induced a catechol 2,3-oxygenase in each case, whereas a similar aliphatic straight-chain substrate, tetradecyl sulphate, elicited no such enzyme (Table 10). The fall in specific activity of the oxygenase with growth on compounds of increasing chain length is consistent with the aromatic ring's providing correspondingly less of the available carbon for cell material and energy requirements.

The large majority of our isolates degraded the benzene ring by a 'meta' cleavage of those catechols implicated as intermediates in arylsulphonate breakdown. The arylsulphonates thus resemble the cresols in tending to select out this type of cleavage. The metabolic route for catechol cleavage by arylsulphonate-grown cells paralleled that established by Dagley & Gibson (1965) for phenol-grown pseudomonads, and so we have not considered it necessary to demonstrate stoichiometry for every stage in the metabolic sequence.

The situation for 4-methylcatechol metabolism, however, was less clear. The ring-fission product of this substrate by a 'meta' cleavage mechanism can result in the formation of either 2-hydroxy-4-methyl- or 2-hydroxy-5-methyl-muconic semialdehyde (Treccani, Fiechi, Baggi & Galli, 1965), depending on the position of ring cleavage (Scheme 1). Subsequent metabolism distinguishes the site of ring fission, if the general scheme for catechol breakdown (Dagley *et al.* 1964) holds true for this bacterium, because 4-hydroxy-2-oxohexanoate can arise only from 2-hydroxy-5-methylmuconic semialdehyde; the 4-methyl isomer would yield 4-hydroxy-4,4-dimethyl-2-oxobutyrates. The identification of 4-hydroxy-2-oxohexanoate as an

intermediate in 4-methylcatechol metabolism by *P. aeruginosa* strain A shows that the initial ring-fission product that we have isolated must be 2-hydroxy-5-methylmuconic semialdehyde, and thus establishes the site of ring cleavage. The conversion of the ring-fission product into 5-methyl-2-picolinic acid, rather than the 3- or 4-methyl isomers, confirms this site of cleavage and also establishes that the general scheme for catechol breakdown by a 2,3-bond cleavage holds true for this organism. 2-Hydroxy-5-methylmuconic semialdehyde has also been identified as the product of 4-methylcatechol oxidation by *P. desmolyticum* (Canonica, Fiechi, Kienle, Scala & Treccani, 1966).

Although 4-methylcatechol formation has not been consistently demonstrated, the conversion of toluene-*p*-sulphonate and 4-methylcatechol into 2-hydroxy-5-methylmuconic semialdehyde and the observation that extracts only oxidize 4-methylcatechol after growth with the arylsulphonates strongly suggests that 4-methylcatechol is the natural intermediate in toluene-*p*-sulphonate degradation. Further evidence is provided by the increased activity shown towards propionaldehyde and propionate by washed cells after growth on toluene-*p*-sulphonate, since these compounds are characteristic of 4-methylcatechol metabolism by the 'meta' route.

The specificity of the catechol 2,3-oxygenase effecting the 'meta' cleavage in different bacterial species also appears quite variable. Evans *et al.* (1965) found that the ring-cleavage enzyme of a pseudomonad grown on any one of naphthalene, anthracene or phenanthrene opened the rings of 1,2-dihydroxyanthracene, 3,4-dihydroxyphenanthrene or catechol itself by fission adjacent to, rather than between, the hydroxyl groups, the action of a typical 2,3-oxygenase. They concluded that a common enzyme of low specificity catalysed all these reactions. Bayly *et al.* (1966) have found that their *Pseudomonas* U after growth on phenol yielded extracts that readily oxidized catechol, 3-methylcatechol and 4-methylcatechol; 4-methylcatechol was the most rapidly oxidized of the three dihydric phenols, but its ring-fission product was degraded the slowest. Ribbons (1966) found a similar spectrum of activity with *o*-cresol-grown cells of *P. aeruginosa* T1 except that the ring-fission product of 3-methylcatechol was degraded so rapidly that it never accumulated.

Our own *P. aeruginosa*, strain A, shows a clear distinction in partially purified extracts of toluene-*p*-sulphonate-grown cells between the ability to oxidize catechol and 4-methylcatechol, the rates of attack on which remain constant at 4.5:1 over a 17-fold purification, and the ability to oxidize 3-methylcatechol, which falls off markedly during purification. Further, we found that no enzymic

activity for the 3-methyl isomer was induced if the cells were grown with benzenesulphonate. These results parallel those of Nozaki, Kagamiyama & Hayaishi (1963), whose crystalline metapyrocatechase (catechol 2,3-oxygenase) from benzoate-grown *P. arvilla* attacked catechol and 4-methylcatechol at equal rates but did not metabolize 3-methylcatechol at all.

The position of 4-oxalocrotonate as an intermediate in catechol metabolism remains obscure, but it is clear that it cannot be regarded as a direct metabolite even though the benzenesulphonate-grown bacterium has the enzymic capacity to degrade it. Although it appears in NAD⁺-supplemented incubations, the necessary decarboxylation step to 4-hydroxy-2-oxovalerate (a reaction not observed in the conversion of catechol into this keto acid) precludes its direct participation in the overall reaction sequence (Scheme 2).

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