Microbial Degradation of Alkylbenzenesulphonates

METABOLISM OF HOMOLOGUES OF SHORT ALKYL-CHAIN LENGTH

BY AN *ALCALIGENES* SP.

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1. An organism isolated from sewage and identified as an *Alcaligenes* sp. utilized benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate as sole source of carbon and energy for growth. Higher alkylbenzenesulphonate homologues and the hydrocarbons, benzene, toluene, phenylethane and 1-phenyldodecane were not utilized. 2. 2-Phenylpropanesulphonate was metabolized to 4-isopropylcatechol. 3. 1-Phenylpropanesulphonate was metabolized to an *ortho*-diol, which was tentatively identified, in the absence of an authentic specimen, as 4-*n*-propylcatechol. 4. In the presence of 4-isopropylcatechol, which inhibited catechol 2,3-dioxygenase, 4-ethylcatechol accumulated in cultures growing on phenylethane-*p*-sulphonate. 5. Authentic samples of catechol, 3-methylcatechol, 4-methylcatechol, 4-ethylcatechol and 3-isopropylcatechol were oxidized by heat-treated extracts to the corresponding 2-hydroxyalkylmuconic semialdehydes. Ring cleavage occurred between C-2 and C-3. 6. The catechol derived from 1-phenylpropanesulphonate was oxygenated by catechol 2,3-dioxygenase to a compound with all the properties of a 2-hydroxyalkylmuconic semialdehyde, but it was not rigorously identified. 7. The catechol 2,3-dioxygenase induced by growth on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate showed a constant ratio of specific activities with catechol, 3-methylcatechol, 4-methylcatechol and 4-ethylcatechol that was independent of the growth substrate. At 60°C, activity towards these substrates declined at an identical first-order rate. 8. Enzymes of the '*ortho*' pathway of catechol metabolism were present in small amounts in cells grown on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate. 9. The catechol 1,2-dioxygenase oxidized the alkylcatechols, but the rates and the total extents of oxidation were less than for catechol itself. The oxidation products of these alkylcatechols were not further metabolized.

The microbial degradation of detergents is a problem that has received considerable attention in recent years. Among the most common surfactants incorporated into detergents in industrial and domestic usage are the linear alkylbenzenesulphonates derived from alk-2-enes. These compounds, unlike the highly branched tetrapropylene-derived alkylbenzenesulphonates which they replaced, are readily biodegradable (Swisher, 1970). Huddleston & Allred (1963) and Swisher (1963, 1964) established that oxidative attack on the linear alkylbenzenesulphonate molecule by the mixed microflora in river-water or activated-sludge samples commenced at the terminal methyl group of the side chain and was followed by stepwise degradation, two carbon atoms at a time, towards the aromatic nucleus by

the process of β -oxidation. These observations have now been substantiated in pure-culture studies with a *Pseudomonas* sp. (Bird & Cain, 1972; Bird, 1972), which degraded the C₁₁ or C₁₂ alkyl chain of 1-phenylalkanesulphonates by ω - and β -oxidation, but which was unable to degrade further the short-alkyl-chain sulphophenylalkanoates so formed.

Similarly, in a *Bacillus* sp. (Willetts & Cain, 1970, 1972) and *Cladosporium resinae* (Cain *et al.*, 1972) ω - and β -oxidation of the surfactant alkyl chain preceded cleavage of the aromatic ring. To study the degradation of the aromatic nucleus of alkylbenzenesulphonates, attention has also been directed to the microbial dissimilation of model surfactant homologues possessing short alkyl chains. Manometric studies by Heyman & Molof (1968) suggested that toluene-*p*-sulphonate and phenylethane-*p*-sulphonate, like their larger-chain-length homologues, were degraded by *Pseudomonas* C12B through an initial oxidation of the alkyl moiety, but Cain & Farr (1968)

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found that a strain of *Pseudomonas aeruginosa* degraded both benzenesulphonate and toluene-*p*-sulphonate by an initial desulphonation to form catechol and 4-methylcatechol respectively, and that these intermediates were subsequently metabolized by extra-diol ('*meta*') cleavage of the aromatic ring. Focht & Williams (1970) reported another pseudomonad that metabolized toluene-*p*-sulphonate through the intermediate formation of 3-methylcatechol, and Baggi *et al.* (1972) have shown that the aromatic ring of 3-phenylpentane can also be degraded without prior side-chain attack. The present paper reports the degradation of alkylbenzenesulphonates with an alkyl chain of less than C₄ by homologous routes involving the formation and subsequent ring cleavage of *ortho*-diols, without prior side-chain attack.

Materials and Methods

Isolation of the organism

A Gram-negative bacterium was isolated by elective culture on mineral-salts medium containing 200 µg of phenylethane-*p*-sulphonate/ml, from a small sewage-purification plant at Felling, Co. Durham, U.K. which treated purely domestic effluents on the trickle-filter principle. The organism was identified by Dr. J. M. Shewan and his colleagues at the Torry Research Station, Aberdeen, U.K. as a species of the genus *Alcaligenes* (Shewan *et al.*, 1960; Cowan & Steel, 1965) and was subsequently deposited in the National Collection of Industrial Bacteria under accession no. 10771. The isolated bacterium was maintained in the laboratory both on nutrient agar and in defined medium (see below) containing phenylethane-*p*-sulphonate (0.05%, w/v) as carbon source, but solidified where appropriate with 2% (w/v) Oxoid Agar. The isolate was subcultured at monthly intervals.

Growth of cells

Large-scale production of cells was carried out in 10-litre flasks fitted with a sterile sampling device and containing 9 litres of a defined medium of the following composition (g/litre of water): (NH₄)₂SO₄, 1; K₂HPO₄, 0.7; KH₂PO₄, 0.3; MgSO₄·7H₂O, 0.1; organic carbon source, 0.5, and trace-elements solution (1 ml) (Barnett & Ingram, 1955). The medium was adjusted to pH 7.0–7.1 with KOH or orthophosphoric acid, and sterilized by autoclaving. Inocula for 9-litre batch cultures were grown at 30°C in shake cultures in 2-litre conical flasks containing 1 litre of the appropriate medium; these inocula themselves had been started from liquid cultures (100 ml) of the same medium, or directly from stock cultures. The 9-litre batch cultures were incubated

at 30°C with forced aeration (approx. 5 litres of filtered air/min) and harvested in the exponential phase of growth (10–12 h).

Preparation of extracts

Freshly harvested cells, or frozen cells after thawing, were resuspended in 0.05 M-Tris-HCl buffer, pH 7.4, and crushed by exposure for 5 min at 0°C to the output of an MSE-Mullard 60 W ultrasonic disintegrator at 20 KHz. The crush was then centrifuged at 2500g for 15 min to remove cell debris, giving extracts with protein concentrations usually between 10 and 25 mg/ml. As a routine extracts to be assayed for catechol 2,3-dioxygenase were treated before centrifugation by adding acetone to a final concentration of 10% (v/v) to the suspending buffer to preserve this enzyme activity (Nozaki *et al.*, 1963), which was extremely labile in air both at room temperature and at 0°C. Heat treatment (55°C for 10 min) of the crude extracts followed by centrifugation to remove coagulated protein left extracts that oxidized the catechols only to the 2-hydroxymuconic semialdehyde derivatives, and did not degrade these products further (Treccani *et al.*, 1968); such heat-treated extracts were made 10% (v/v) in acetone after the centrifugation.

Chemicals

Phenylethane-*p*-sulphonate was prepared by sulphonation of phenylethane with H₂SO₄ at 85–95°C. The *p*-sulphonate was crystallized from aqueous solution as its sodium salt by addition of a large excess of NaCl solution, and the filtrate further purified by recrystallization from NaCl solution (Cason & Rapoport, 1962). 1-Phenylpropane-sulphonate and 2-phenylpropanesulphonate were prepared by sulphonation with oleum of 1-phenylpropane and 2-phenylpropane respectively (Gray *et al.*, 1955). The crude sulphonate product, after neutralization with NaOH, was extracted with boiling ethanol and filtered through a pre-warmed Büchner funnel. The compound that crystallized from the supernatant at 0°C was separated, washed in ice-cold ethanol and dried. It contained small amounts of the *ortho* and *meta* isomers. Higher alkylbenzenesulphonate homologues were prepared either by the method of Gray *et al.* (1955) or were provided by Mr. R. D. Huddleston of the Research Division of the Continental Oil Co., Ponca City, Okla., U.S.A.

3-Ethylcatechol and 4-ethylcatechol were the gift of Dr. L. Barker of the Department of Fuel Science, University of Leeds, U.K.

3-Isopropylcatechol and 4-isopropylcatechol were presented by Coalite and Chemical Co., Chesterfield, Derbyshire, U.K. *cis,cis*-Muconic acid was prepared by Mr. B. E. Halsall in this Laboratory by the

oxidation of redistilled phenol with peracetic acid (Elvidge *et al.*, 1950), and (+)-muconolactone was prepared by the biological method of Cain (1961). 5-Methyl-2-picolinic acid and 4-methyl-2-picolinic acid were the gift of Dr. S. Senoh, Tohoku University, Sendai, Japan. Catechol-4-sulphonate and 2,2-dimethyl-*cis,cis*-muconic acid were the gift of Dr. M. Nozaki of the University of Kyoto, Japan. All other chemicals were obtained from commercial sources.

Determinations

Benzenesulphonate, toluene-*p*-sulphonate and phenylethane-*p*-sulphonate in growth media were determined by measuring the loss of E_{225} (caused by the aromatic ring) against a blank of the mineral-salts medium, after removal of cells by centrifugation. Catechol substances in growth and other media were determined by a method modified from Evans (1947). To samples (1 ml) of the centrifuged test materials were added in turn 1 ml of 10% (w/v) Na_2MoO_4 , 0.5 ml of 0.5 M-HCl and 0.5 ml of 0.5% (w/v) NaNO_2 . After the tubes had been shaken and left for 10 min, 1 ml of 0.5 M-NaOH was added and after 5 min the E_{510} was read against a blank of water taken through the same procedure. Growth was measured turbidimetrically at 500 nm and protein was measured by the phenol method of Lowry *et al.* (1951) with bovine serum albumin as standard. U.v.-absorption spectra of growth media and of isolated and authentic materials were measured in a Unicam SP.800 recording spectrophotometer with light-path (10 mm) and a slit width of 0.02 mm. The absorption spectra and molar extinction coefficient, ϵ , of the 'meta'-cleavage products of catechol and the substituted catechols were measured with a Unicam SP.500 spectrophotometer.

Manometric experiments

Uptake of O_2 was determined by conventional manometric methods (Umbreit *et al.*, 1964) with air as the gaseous phase.

Enzyme assays

All enzymes were assayed spectrophotometrically with a Unicam SP.800 recording spectrophotometer fitted with an SP.820 constant-wavelength attachment and a constant-temperature cuvette holder through which water at 30°C was circulated by a Braun (Melsungen, W. Germany) Circotherm unit. An SP.840 expansion unit and a Honeywell Electronic 15 slave recorder were also used to increase the extinction scale tenfold when low enzyme activities were being measured.

Catechol 2,3-dioxygenase [catechol-oxygen 2,3-oxidoreductase (decyclizing) (EC 1.13.11.2)] was

assayed spectrophotometrically in cuvettes containing, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, 145 μmol , and enzyme protein sufficient to contain about 20 munits (nmol/min). The reaction was started by addition of 0.5 μmol of substrate to the reaction cuvette, and the increase in E_{375} owing to the product followed. Specific activities with the various catechol homologues were determined by using the molar extinction coefficients of the products at 375 nm, measured experimentally. For routine assays, the rate of oxidation of the catechol substrates by catechol 1,2-dioxygenase and the rate of disappearance of the products of the catechol 2,3-dioxygenase reaction in cuvettes containing crude untreated extract were so low that they could be neglected. The further metabolism of the 2-hydroxyalkylmuconic semialdehydes was assayed in crude extracts by measuring the disappearance of the 'meta'-cleavage products of catechol or the alkylcatechols (at 33 μM) produced *in situ* by heat-treated (55°C, 10 min) extracts containing catechol 2,3-dioxygenase (Cain & Farr, 1968). Cuvettes contained, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, 145 μmol and enzyme protein sufficient to contain about 200 munits of catechol 2,3-dioxygenase. The reaction was started by adding catechol or alkylcatechol (0.1 μmol) to the reaction cuvette, and after the initial rapid formation of the appropriate 2-hydroxy-muconic semialdehyde (some 30 s) 0.05–0.1 ml of the crude extract on test was added and the decrease in E_{375} was followed. This procedure measures 2-hydroxy-muconic semialdehyde hydrolase (no EC number) and, in the absence of added NAD^+ , low activities of any 2-hydroxy-muconic semialdehyde dehydrogenase present (Sala-Trepat & Evans, 1971).

Catechol 1,2-dioxygenase [catechol-oxygen 1,2-oxidoreductase (decyclizing) (EC 1.13.11.1)] was measured by determining the accumulation of *cis,cis*-muconate at 260 nm in the presence of 1 mM-EDTA (Hegeman, 1966). The enzyme was assayed in extracts which had been diluted with 0.05 M-Tris-HCl buffer, pH 7.4, to a concentration of 10 mg of protein/ml and vigorously aerated for some 15 min at 0°C to inactivate the catechol 2,3-dioxygenase. Extracts so treated were checked for any residual catechol 2,3-dioxygenase activity before assay. Cuvettes contained, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, 145 μmol ; EDTA, 3 μmol ; enzyme protein, approx. 0.25 mg. The reaction was started by addition of catechol or alkylcatechol (0.5 μmol) to the reaction cuvette. *cis,cis*-Muconate cycloisomerase [(+)-4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing) (EC 5.5.1.1)] was determined by measuring the disappearance of *cis,cis*-muconate at 260 nm in cuvettes containing, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, 145 μmol ; MnCl_2 , 3 μmol ; enzyme protein, approx. 0.25 mg (Hegeman, 1966). The reaction was started

by adding *cis,cis*-muconate ($0.25\ \mu\text{mol}$) to the reaction cuvette. An overall measure of (+)-muconolactone isomerase and 3-oxoadipate enol-lactone hydrolase was obtained by following the disappearance of (+)-muconolactone at 230 nm (Sistrom & Stanier, 1954) in cuvettes containing, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, $145\ \mu\text{mol}$, and enzyme protein, 0.1–0.2 mg. The reaction was started by adding (+)-muconolactone ($0.5\ \mu\text{mol}$) to the reaction cuvette. A unit of activity of all these enzymes was defined as the amount causing a change of $1\ \mu\text{mol}$ of substrate or product/min at 30°C and pH 7.6.

Paper chromatography

Both ascending and descending methods were used with benzene-acetic acid-water (3:3:1, by vol.) as solvent. After drying and examining under u.v. light, spots were detected by spraying with one of the following reagents: (1) diazotized sulphanilic acid (Pauly reagent), freshly prepared as described by Smith (1960), or (2) 0.4% (w/v) FeCl_3 in 0.02 M-HCl (Smith, 1960).

Gas-liquid chromatography

All g.l.c. was conducted on a Pye model 64 (series 104) gas chromatograph with separately heated injection port and heated flame-ionization detectors. Gas effluents were monitored with a Honeywell Elektronik 15 chart recorder. O_2 -free N_2 was used as a carrier gas. Samples ($10\ \mu\text{l}$) from a solution of the test material in dry ether (2 ml) were injected into one of the following columns: (i) a $1.52\text{ m} \times 6.5\text{ mm}$ internal diam. ($5\text{ ft} \times \frac{1}{4}\text{ in.}$) circular glass column containing Apiezon L as the stationary phase (Pye-Unicam Ltd., Cambridge, U.K.; catalogue no. 12440/2/10 APL) operated at 200°C with a carrier-gas flow of 75 ml/min; (ii) a $2.1\text{ m} \times 6.5\text{ mm}$ internal diam. ($7\text{ ft} \times \frac{1}{4}\text{ in.}$) circular glass column of diethylene glycol adipate as stationary phase (Pye-Unicam Ltd., catalogue no. 12442/2PT/10 DEGA) operated at 180°C with a carrier-gas flow of 75 ml/min. The solvent, diethyl ether, was eluted within 1 min of injection. Aromatic acids, hydroxy aromatic acids, phenols and catechols were separated by g.l.c. as their methyl esters. The test material was methylated by the method of Schlenk & Gellerman (1960) as described by Burchfield & Storrs (1962), in which diazomethane [generated by the reaction of *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide with 40% (w/v) NaOH] was the methylating agent. After methylation catechol, 3-methylcatechol, 3-ethylcatechol, 3-isopropylcatechol and 4-isopropylcatechol consistently produced two distinct peaks on both columns (i) and (ii), corresponding to the *O*-monomethyl and *OO*-dimethyl ether derivatives.

4-Methylcatechol and 4-ethylcatechol, after methylation, consistently produced two peaks on the diethylene glycol adipate column, but only a single peak was resolved on Apiezon L. In all g.l.c. analyses, 4-phenylbutyric acid was used as an internal standard and taken through the methylation procedures. For purposes of characterization, the retention times of compounds were expressed relative to this standard, thus:

Retention ratio =

$$\frac{\text{retention time of methylated experimental compound}}{\text{retention time of methyl 4-phenylbutyrate}}$$

Results

Growth of Alcaligenes strain 2d on alkylbenzenesulphonates

Cultures of strain 2d maintained on yeast extract or succinate grew in basal medium containing either benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate as sole source of carbon and energy, after a lag of some 20–40 h. Growth was correlated with the disappearance of the aromatic ring, measured spectrophotometrically at 225 nm. Higher alkylbenzenesulphonate homologues were not utilized. Among a variety of other aromatic compounds tested, benzene, toluene, phenylethane, benzoate, *p*-hydroxybenzoate, phenol and 4-methylphenol did not support growth of this micro-organism.

Oxidation of alkylbenzenesulphonates and related compounds by washed cell suspensions

Washed suspensions of cells grown with benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate oxidized each of these compounds at high rates and without a lag (Fig. 1). 1-Phenylpropane-sulphonate, phenol-*p*-sulphonate and benzenesulphinic acid were more slowly oxidized. Among a number of aromatic acids and hydroxy aromatic compounds tested, only catechol, 3-methylcatechol, 4-methylcatechol and 4-ethylcatechol were rapidly oxidized without a lag by cells grown on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate, at rates between 70 and $125\ \mu\text{l}$ of O_2 /h per mg dry wt. of cells. With these substrates the initial rapid O_2 uptake was accompanied by the immediate appearance of an intense yellow coloration, consistent with the cleavage of the catechols by a catechol 2,3-dioxygenase. Succinate-grown cells oxidized catechol and the alkylcatechols at much lower initial rates ($6\text{--}10\ \mu\text{l}$ of O_2 /h per mg dry wt. of cells) which gradually increased during incubation, and did not oxidize the aromatic sulphonates at all.

Fig. 1. Oxidation of alkylbenzenesulphonates by washed suspensions of cells grown on phenylethane-*p*-sulphonate

Warburg flasks contained, in a total volume of 2.5 ml: phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.0, 150 μmol ; cell suspension equivalent to 12.0 mg dry wt.; substrate 3 μmol ; 20% (w/v) KOH, 0.2 ml in centre well. Incubation was at 30°C. ●, Phenylethane-*p*-sulphonate; ○, toluene-*p*-sulphonate; Δ, benzenesulphonate; ▲, 1-phenylpropane-sulphonate; ■, 2-phenylpropanesulphonate; □, 1-phenylbutanesulphonate and 1-phenyldodecane-*p*-sulphonate. The endogenous rate of 8.1 μl of O_2/h per mg dry wt. of cells is subtracted.

Isolation of alkylcatechols from suspensions oxidizing 1-phenylpropanesulphonate and 2-phenylpropanesulphonate

The accumulation of catechol from benzoate (Sleeper, 1951) and benzenesulphonate (Cain & Farr, 1968) has been accomplished by incubating thick suspensions of non-induced cells in buffer containing these substrates. The basis of this technique was explained by Hegeman (1966), who found a lag in the appearance of enzymes at each step in an inductive sequence; consequently catechol accumulated transiently after the enzymes catalysing its formation had been induced. To detect accumulation of catechol substances in cultures of *Alcaligenes* 2d, washed cells grown on phenylethane-*p*-sulphonate were suspended to high density (50–80 mg dry wt./ml) in 100 ml of 0.05M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.0, in conical flasks (250 ml) containing 1-phenylpropanesulphonate, 2-phenylpropanesulphonate or 1-phenylbutane-*p*-sulphonate at 500 $\mu\text{g}/\text{ml}$

Fig. 2. Accumulation of catechols from 1-phenylpropane-sulphonate and 2-phenylpropanesulphonate

Conical flasks (250 ml vol.) containing 100 ml of 0.05M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.0, and 500 μg of 1-phenylpropanesulphonate, 2-phenylpropanesulphonate or 1-phenylbutanesulphonate/ml or no substrate were inoculated with a washed suspension of cells (1.08 g dry wt.) derived from a phenylethane-*p*-sulphonate culture. Samples (3 ml) were removed at intervals, centrifuged to remove cells, and 1 ml quantities assayed for *o*-diphenols by the method of Evans (1947). Accumulation of *o*-diphenol was followed: ○, in the 2-phenylpropanesulphonate culture; ●, in the 1-phenylpropanesulphonate culture; Δ, in the 1-phenylbutane-*p*-sulphonate culture; and ▲, in the no-substrate control culture. The scale on the abscissa was calibrated with authentic 4-isopropylcatechol.

final concentration; these substrates did not support growth of strain 2d. A control flask contained all the components except substrate. All flasks were incubated at 30°C for alternating 4 h stationary and shaking periods. Samples were removed at intervals and tested for aromatic *o*-diphenols by the Evans (1947) test. A positive response to this test was obtained immediately from the culture containing 2-phenylpropanesulphonate and, after some 7 h incubation, from that containing 1-phenylpropanesulphonate (Fig. 2). After incubation for 9.5 h a portion (50 ml) of the flask contents of each incubation was centrifuged to remove cells, concentrated to small volume in a rotary-film evaporator, and after careful neutralization to pH 7.0, was extracted three times with an equal volume of diethyl ether. The remaining aqueous layer was acidified to pH 2.0 with 2M-HCl and re-extracted three times again with equal volumes of diethyl ether. After drying over anhydrous Na_2SO_4 , the ether extracts were concentrated by evaporation. Paper chromatography of the neutral extracts from the 2-phenylpropanesulphonate and 1-phenylpropanesulphonate incubations yielded, in each case, spots with intense u.v. absorption and specific colour reactions at an R_f value of 0.76. This

R_F value was given only by authentic 4-isopropylcatechol of the several available markers run on the chromatogram. Because authentic material was not available, the R_F of 4-*n*-propylcatechol was predicted from a plot of $\log R_F$ (obtained on the same chromatogram with catechol, 4-methylcatechol and 4-ethylcatechol) versus the number of carbon atoms in the alkyl chain of these compounds. The predicted R_F (0.77) was close to those of the natural products. The colour reactions of the unknown compounds, produced by spraying the chromatograms with diazotized sulphanilic acid (reagent 1) or acidic 2% (w/v) FeCl_3 (reagent 2), were also characteristic of those produced by 4-alkylcatechols. With reagent (1) the 4-alkylcatechols and the experimental spots gave a pale-brown colour that was quite distinct from the grey-brown colour produced by catechol itself and by 3-methylcatechol. Similarly, with reagent (2), 4-alkylcatechols and the experimental spots gave a pale-green colour, whereas catechol and 3-methylcatechol produced grey and grey-brown spots respectively. The control flask, similarly extracted and chromatographed, gave no spots. No aromatic or hydroxyaromatic acids were found in the acidic ether extracts of these incubations.

Further samples of the neutral ether extracts were chromatographed on Whatman no. 3 paper, the spots were located by their u.v. quenching, cut out and then extracted with ether. After drying over anhydrous Na_2SO_4 , methylation with diazomethane and then g.l.c., the ethereal extract of the 2-phenylpropanesulphonate incubation produced two peaks on Apiezon L with retention ratios (relative to the methyl ester of 4-phenylbutyrate) of 0.91 and 1.01. These values were very close to those (0.91 and 1.00) given by the *O*-methyl and *OO*-dimethyl derivatives of authentic 4-isopropylcatechol. On diethyleneglycol adipate the unknown material showed retention ratios of 1.21 and 1.28, identical with those given by the two methyl ethers of 4-isopropylcatechol. The 2-phenylpropanesulphonate metabolite had in aqueous solution a light-absorption maximum at 279 nm, in which it was identical with 4-isopropylcatechol, but distinct from 3-isopropylcatechol (273 nm). No increase in extinction in the region of 350–400 nm was observed when samples of the 2-phenylpropanesulphonate metabolite were incubated in 0.05 M-Tris-HCl buffer, pH 7.6, with extracts containing high catechol 2,3-dioxygenase activity. This metabolite was, however, found to inhibit catechol 2,3-dioxygenase activity in such extracts in a manner identical with that of authentic 4-isopropylcatechol (described later in this paper). 3-Isopropylcatechol, in contrast, caused no such inhibition. This metabolite therefore was identified as 4-isopropylcatechol.

The 1-phenylpropanesulphonate metabolite had, in aqueous solution, a light-absorption maximum at 280 nm, indistinguishable from that of 4-methyl-

catechol and 4-ethylcatechol, but distinct from that of catechol (275 nm) and 3-methylcatechol (273 nm). Samples of the 1-phenylpropanesulphonate metabolite were oxidized by a crude catechol 2,3-dioxygenase preparation to a yellow product with light-absorption maxima at 381–382 nm (pH 7.6) and 319 nm (pH 2.0). These values are identical with the observed maxima (both at 382 and 319 nm) for the extra-diol-cleavage products of 4-methylcatechol and 4-ethylcatechol, but quite distinct from those (375 and 319 nm; 388 and 314 nm) produced when catechol and 3-methylcatechol respectively were incubated with the same extract. After prolonged incubation with extract, the extinction at 381–382 nm produced by the 1-phenylpropanesulphonate metabolite slowly disappeared, suggesting that this product was further metabolized. Subsequent efforts to accumulate catechol substances from *Alcaligenes* cultures utilizing 1-phenylpropanesulphonate proved unsuccessful, although 4-isopropylcatechol readily accumulated in 2-phenylpropanesulphonate cultures. Since the 2-phenylpropanesulphonate metabolite corresponded by a number of tests to 4-isopropylcatechol, the 1-phenylpropanesulphonate metabolite would, by analogy, be 4-*n*-propylcatechol; all the available evidence supported this conclusion. No catechol substances were detected in or isolated from the 1-phenylbutane-*p*-sulphonate incubation mixture.

*Accumulation of 4-ethylcatechol from phenylethane-*p*-sulphonate*

The ability of 4-isopropylcatechol to inhibit catechol 2,3-dioxygenase was utilized to effect the accumulation from 1-phenylpropanesulphonate, phenylethane-*p*-sulphonate and toluene-*p*-sulphonate of catechol substances in sufficient quantity to permit their identification. Conical flasks (250 ml) containing 100 ml of 0.05 M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.0, 10 μg of 4-isopropylcatechol/ml and 500 μg of benzenesulphonate, toluene-*p*-sulphonate, phenylethane-*p*-sulphonate or 1-phenylpropanesulphonate/ml were incubated for 12 h at 30°C with a washed suspension of cells (1.2 g dry wt.) grown on phenylethane-*p*-sulphonate. The flask contents were centrifuged to remove cells and, after concentration, the supernatant (pH 7.0) was extracted three times with an equal volume of diethyl ether. The ethereal extracts were dried over anhydrous Na_2SO_4 , concentrated and then chromatographed.

4-Ethylcatechol (R_F 0.68) was clearly identified by paper chromatography as a product of phenylethane-*p*-sulphonate metabolism in the 4-isopropylcatechol-inhibited cultures, and a weak spot (R_F 0.58) corresponding to 4-methylcatechol was detected in the extract from the similarly treated toluene-*p*-sulphonate incubation mixture. These spots had the typical colour reactions described above for 4-substituted alkylcatechols. No spots distinguishable from

4-isopropylcatechol were observed on chromatograms of the ether extract from the cultures containing 1-phenylpropanesulphonate or from the cultures containing benzenesulphonate, inhibited with 4-isopropylcatechol, though the solvent system used could not discriminate between 4-*n*-propyl- and 4-isopropylcatechol.

The identification of 4-ethylcatechol was confirmed, after methylation, by g.l.c. On Apiezon L, both the natural product and the authentic material produced a single peak with a retention ratio of 0.81. On diethyleneglycol adipate, two peaks were resolved, with retention ratios of 0.75 and 1.20 for the natural intermediate and 0.76 and 1.19 for the authentic material.

G.l.c. of the ether extract from the 1-phenylpropanesulphonate incubation inhibited with 4-isopropylcatechol revealed, in addition to peaks identified as 4-isopropylcatechol itself (retention ratios 0.91 and 1.00 on Apiezon L; 1.21 and 1.28 on diethyleneglycol adipate), distinct peaks on both Apiezon L and diethyleneglycol adipate at retention ratios near to those predicted for 4-*n*-propylcatechol from plots of log (retention ratio of authentic 4-alkylcatechols) versus the number of carbon atoms in the alkyl chain of these compounds. These values were: on Apiezon L, predicted 1.11, actual 1.14; on diethyleneglycol adipate, predicted 0.94 and 1.50, actual 0.91 and 1.46.

Ether extracts of phenylethane-*p*-sulphonate and 1-phenylpropanesulphonate incubations conducted in the absence of 4-isopropylcatechol, and ether extracts of a no-substrate control incubation, after identical treatment, showed no evidence of a 4-alkylcatechol by paper chromatography or g.l.c. The implication that 4-ethylcatechol and 4-methylcatechol were intermediates in the metabolism of phenylethane-*p*-sulphonate and toluene-*p*-sulphonate respectively was further substantiated by the observation of a transient yellow coloration in early exponential-phase cultures of *Alcaligenes* 2d growing with these substrates (due to compounds with an extinction maximum at pH 12.0 of 381–382 nm), identical with that produced when authentic 4-ethylcatechol or 4-methylcatechol was incubated with a crude catechol 2,3-dioxygenase preparation.

Metabolism of catechol and the alkylcatechols by cell-free extracts

Crude extracts of *Alcaligenes* 2d grown with phenylethane-*p*-sulphonate were treated at 55°C for 10 min to destroy catechol 1,2-oxygenase and 2-hydroxymuconic semialdehyde hydrolase activities. These extracts rapidly oxidized catechol, 3-methylcatechol, 4-methylcatechol and 4-ethylcatechol with an oxygen uptake corresponding to 1 mol of O₂/mol of substrate, forming compounds of intense yellow

colour with light-absorption maxima at pH 7.6 as follows: catechol product (2-hydroxymuconic semialdehyde), 375 nm (ϵ 33 500); 3-methylcatechol product (2-hydroxy-6-oxohepta-2,4-dienoic acid), 388 nm (ϵ 14 700); 4-methylcatechol product (2-hydroxy-5-methylmuconic semialdehyde), 382 nm (ϵ 28 200) and 4-ethylcatechol product (2-hydroxy-5-ethylmuconic semialdehyde), 382 nm (ϵ 25 200). 3-Isopropylcatechol, but not 4-isopropylcatechol, was slowly oxidized by these same extracts, producing a yellow compound with a light-absorption maximum at pH 7.6 of 393 nm. After prolonged incubation with crude extract all of the oxidation products slowly disappeared, suggesting 2-hydroxyalkylmuconic semialdehyde hydrolase activity. This decrease in metabolite concentration was also noted with washed cell suspensions.

Induction and properties of catechol 2,3-dioxygenase

The inducible catechol 2,3-dioxygenase was present in cells of *Alcaligenes* 2d grown with benzenesulphonate, toluene-*p*-sulphonate and phenylethane-*p*-sulphonate, in amounts some 50–60-fold greater than in succinate-grown cells (Table 1). The activity was very labile in air, both at room temperature and at 0°C. In common with the observations of Nozaki *et al.* (1963) and Farr (1968), however, preparation of the extracts in buffer containing 10% (v/v) acetone stabilized enzymic activity to the extent that only some 10% of the activity was lost during a period in which 99% of the activity was lost in untreated extracts. Inactivated extracts could be partially reactivated by anaerobic reduction with NaBH₄ (Taniuchi *et al.*, 1962), but for most purposes extracts to be assayed for catechol 2,3-dioxygenase were treated as a routine with 10% (v/v) acetone immediately after sonication and before centrifugation.

The position of ring cleavage in the catechol substrate was established by treating the product of 4-methylcatechol oxidation by heat-treated extracts of *Alcaligenes* 2d with excess of NH₄Cl under acid conditions at room temperature for 2 weeks (Trippett *et al.*, 1960; Cain & Farr, 1968). Under these conditions, 2-hydroxy-5-methylmuconic semialdehyde, the 2,3-cleavage product, slowly forms 5-methylpicolinic acid, whereas 2-hydroxy-4-methylmuconic semialdehyde, the 1,6-cleavage product, is converted into 4-methylpicolinic acid. The experiment was carried out spectrophotometrically and the product was identified as 5-methylpicolinic acid exactly as described by Cain & Farr (1968). The 4-methylcatechol must therefore have been cleaved between the proximal C-2 and C-3 positions to produce 2-hydroxy-5-methylmuconic semialdehyde.

Catechol 2,3-dioxygenase in crude extracts of *Alcaligenes* 2d grown on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate con-

Table 1. *Activities of enzymes of the 'meta'-cleavage pathway of catechol metabolism in Alcaligenes 2d*

Cells were harvested in exponential phase, and extracts were prepared, treated and assayed immediately as described in the Materials and Methods section. The values presented for catechol 2,3-dioxygenase in cells grown on benzenesulphonate, toluene-*p*-sulphonate and phenylethane-*p*-sulphonate are the mean values obtained from at least four separate batches of extract and for all others the mean of at least two separate batches.

Enzyme assayed	Specific activities (nmol/min per mg of protein) in extracts derived from cells grown with			
	Benzenesulphonate	Toluene- <i>p</i> -sulphonate	Phenylethane- <i>p</i> -sulphonate	Succinate
Catechol 2,3-dioxygenase	1860	1716	1560	32
2-Hydroxymuconic semialdehyde hydrolase	9.1	7.6	7.5	1.7

tently oxidized catechol and its alkyl-substituted derivatives (0.17 mM) at the following rates relative to catechol (1.97 μ mol/min per mg of protein, equivalent to 1): 3-methylcatechol, 1.1; 4-methylcatechol, 0.4; 4-ethylcatechol, 0.5; 3-isopropylcatechol, 0.02 and 4-isopropylcatechol, 0. No value for ϵ was available for 4-*n*-propylcatechol, but the rate (ΔE_{382} /min per mg of protein, 30°C, pH 7.6) at which the catechol metabolite (approx. 0.15–0.2 mM) of 1-phenylpropanesulphonate was oxidized by extracts of phenylethane-*p*-sulphonate-grown cells was some 40% of that (ΔE_{375} /min per mg of protein) for catechol (0.17 mM) under the same conditions.

When the kinetics of thermal inactivation of catechol 2,3-dioxygenase were examined in crude extracts of cells grown with benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate, loss of activity against the alkylcatechols declined at parallel rates in each case (Fig. 3).

Catechol 2,3-dioxygenase in heat-treated extracts did not oxidize 4-isopropylcatechol, catechol-4-sulphonate, 3,4-dihydroxybenzoate, orcinol, quinol or pyrogallol, but of these compounds only 4-isopropylcatechol inhibited catechol 2,3-dioxygenase activity. Simultaneous addition of equal concentrations (0.17 mM) of catechol and 4-isopropylcatechol to an assay mixture was accompanied by a decrease in the initial rate of enzyme activity; this rate fell progressively with time and all activity appeared to cease after about 4% of the available catechol had been oxidized (Figs. 4a and 4b). Addition of further catechol to final concentrations of 0.17 or 1.7 mM caused no resumption of activity, but oxidation was restored by the further addition of fresh enzyme. Simultaneous addition of catechol and 4-isopropylcatechol in the ratio 10/1 (1.7 mM:0.17 mM) to an enzyme preparation produced a similar reaction curve, but loss of activity was now considerably delayed (Fig. 4c). Enzyme preincubated with 0.17 mM-4-isopropylcatechol for 1 min showed no activity towards catechol (Fig. 4d). These observations suggested that catechol

Fig. 3. *Kinetics of heat inactivation of catechol 2,3-dioxygenase in crude extracts of cells grown with phenylethane-*p*-sulphonate*

Samples of extract (1 ml, containing 13.9 mg of protein) in phosphate buffer (Na_2HPO_4 – KH_2PO_4), pH 7.0, were heated at 60°C for various periods of time and the extracts then cooled in ice and assayed immediately for activity with the following substrates: ○, catechol; ●, 3-methylcatechol; △, 4-methylcatechol; ▲, 4-ethylcatechol.

2,3-dioxygenase was irreversibly or pseudo-irreversibly (Webb, 1963) inhibited by 4-isopropylcatechol.

2-Hydroxymuconic semialdehyde hydrolase activity

This enzyme activity was present in all crude extracts examined, but in amounts some 4–5-fold

greater in cells grown on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate than in cells grown on succinate (Table 1). The relative activities of the enzyme towards the extra-diol-cleavage products were approximately the same in extracts of cells grown on benzenesulphonate,

Fig. 4. Effect of 4-isopropylcatechol on the activity of catechol 2,3-dioxygenase

Extracts were derived from cells grown on benzenesulphonate and were pretreated with acetone to protect activity against O_2 inactivation. Both cuvettes contained, in a total volume of 3 ml: Tris-HCl buffer, pH 7.6, 145 μ mol, and extract, 0.025 mg of protein. The reaction was started by adding to the reaction cuvette: catechol, 0.5 μ mol at A; catechol, 0.5 μ mol, and 4-isopropylcatechol, 0.5 μ mol (premixed), at B; catechol, 5 μ mol at C; extract, 0.025 mg of protein, at D; catechol, 5 μ mol, and 4-isopropylcatechol, 0.5 μ mol (premixed), at E; 4-isopropylcatechol, 0.5 μ mol at F. The increase in E_{375} was determined in a Unicam SP. 800 spectrophotometer. Similar results were observed with untreated extracts.

toluene-*p*-sulphonate or phenylethane-*p*-sulphonate, and were at the following rates relative to the catechol product (9.3 nmol/min per mg of protein, equivalent to 1): 3-methylcatechol product, 2.1; 4-methylcatechol product, 0.8; 4-ethylcatechol product, 0.5. The rate of oxidation of 3-isopropylcatechol was very slow, but in the presence of high protein concentrations (approx. 1 mg) in the cuvette and 3-isopropylcatechol at 0.033 mM, it was possible to observe slow disappearance of the extra-diol-cleavage product of this compound at a rate estimated at some 2% of the rate for the catechol product.

*Oxidation of butyraldehyde and butyrate by washed suspensions of Alcaligenes 2d grown with phenylethane-*p*-sulphonate*

Reference to the general scheme of Dagley *et al.* (1964) for the degradation of catechols by extra-diol-cleavage suggests that the only product characteristic of 4-ethylcatechol metabolism would be butyraldehyde. Cells grown with phenylethane-*p*-sulphonate in fact showed greater respiratory activity towards butyraldehyde and its oxidation product butyrate than did cells grown with benzenesulphonate or yeast extract (Table 2).

Catechol 1,2-dioxygenase and enzymes of the 'ortho'-pathway of catechol degradation in Alcaligenes

Extracts obtained from *Alcaligenes* 2d grown on phenylethane-*p*-sulphonate, and vigorously aerated for some 15 min at 0°C to inactivate catechol 2,3-dioxygenase, still oxidized catechol with an oxygen uptake corresponding to 1 mol of O_2 /mol of substrate, accumulating a product which possessed light-absorption maxima at 258 nm (pH 7.6) and 261 nm

Table 2. Oxidation by washed suspensions of some putative products of 4-ethylcatechol metabolism by the 'meta'-cleavage pathway

Warburg flasks contained in a total volume of 2.5 ml: phosphate buffer ($Na_2HPO_4-KH_2PO_4$), pH 7.0, 150 μ mol; substrate, 3 μ mol; suspension of washed cells, 0.5 ml; KOH, 0.2 ml in the centre well. The dry wts. of cells/flask were as follows: Expt. 1, 7.4 mg; Expt. 2, 6.7 mg; Expt. 3, 7.9 mg. The corresponding endogenous rates of O_2 uptake were: Expt. 1, 8.5; Expt. 2, 7.9 and Expt. 3, 6.2 μ l of O_2 /h per mg dry wt. of cells and are subtracted. n.t., Not tested. (Propionate and acetaldehyde are included for comparison as typical products of 3-ethylcatechol metabolism by a 'meta'-cleavage pathway.)

Oxidation (μ l of O_2 consumed/h per mg dry wt. of cells) of substrates by washed cell suspensions originally grown with

Respiratory substrate	Phenylethane- <i>p</i> -sulphonate (Expt. 1)	Benzenesulphonate (Expt. 2)	Yeast extract (Expt. 3)
Formate	12.0	n.t.	12.7
Pyruvate	33.8	n.t.	31.6
Propionate	18.9	n.t.	25.7
Acetaldehyde	30.0	33.4	29.4
Butyraldehyde	53.1	35.8	29.3
Butyrate	55.0	n.t.	23.6

Table 3. Activities of enzymes of the 'ortho'-cleavage pathway of catechol metabolism in *Alcaligenes 2d*

Cells were harvested in exponential phase, and extracts were prepared, treated and assayed immediately as described in the Materials and Methods section. Values presented in all cases are the mean of at least two separate batches.

Enzyme assayed	Specific activities (nmol/min per mg of protein) in extracts of cells grown with			
	Benzenesulphonate	Toluene- <i>p</i> -sulphonate	Phenylethane- <i>p</i> -sulphonate	Succinate
Catechol 1,2-dioxygenase	22	29	20	1
<i>cis,cis</i> -Muconate cycloisomerase	18	12	14	0
(+)-Muconolactone isomerase + 3-oxoadipate enol-lactone hydrolase	3	3	2	0

Fig. 5. Oxidation of alkylcatechols by a crude catechol 1,2-dioxygenase preparation from *Alcaligenes 2d*

An extract of cells grown on phenylethane-*p*-sulphonate was treated by vigorous aeration at 0°C for 15 min to inactivate catechol 2,3-oxygenase (checked by spectrophotometric assay with catechol at 375 nm). Warburg flasks contained, in a total reaction volume of 2.5 ml: Tris-HCl buffer (pH 7.6), 135 μmol; substrate, 3 μmol; MnCl₂, 2.5 μmol; extract, 1.3 ml (12.0 mg of protein); 20% (w/v) KOH, 0.2 ml in centre well. Incubation was at 30°C in air. ○, Catechol; △, 4-methylcatechol; ▲, 4-ethylcatechol; ●, 4-isopropylcatechol; □, 3-methylcatechol.

(pH 2.0), identical with those of authentic *cis,cis*-muconate, the product of an intra-diol-ring fission of catechol. Extracts of cells grown with benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate, when similarly treated, all slowly accumulated products with light-absorption maxima in the region of 260 nm from a range of alkylcatechols. Similar results were obtained when the substrates were purified by paper chromatography to ensure that low activities with the alkylcatechols did not

result from possible traces of catechol. The relative activities towards the alkylcatechols showed no significant variations between extracts grown on benzenesulphonate, toluene-*p*-sulphonate or 1-phenylethane-*p*-sulphonate and were consistently as follows: catechol (ΔE_{260} 0.431/min per mg of protein equivalent to 1); 4-methylcatechol, 0.1; 4-ethylcatechol, 0.06; 4-isopropylcatechol, 0.06; 3-methylcatechol, 0.04; 3-isopropylcatechol, 0.18. Catechol-4-sulphonate and 4-nitrocatechol were not oxidized.

Growth on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate led to the induction of catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase and (+)-muconolactone isomerase with 3-oxoadipate enol-lactone hydrolase (measured together in a coupled assay) in amounts over 20-fold that found in succinate-grown cells (Table 3). Crude extracts of phenylethane-*p*-sulphonate-grown cells supplemented with 1 mM-MnCl₂, in which the 2,3-dioxygenase had been inactivated by aeration, slowly oxidized 4-methylcatechol, 4-ethylcatechol, 4-isopropylcatechol and 3-isopropylcatechol without the appearance of a yellow colour characteristic of extra-diol-cleavage, but with an O₂ uptake that ceased at less than 1 mol of O₂/mol of substrate (Fig. 5). After 90 min incubation, a violet Rothera-positive reaction (Rothera, 1908) characteristic of 3-oxoadipate was obtained from the extract incubated with catechol itself, but no reaction was obtained from the extracts incubated with the alkylcatechols. A substituted muconate, 2,2-dimethyl-*cis,cis*-muconate, was not metabolized by these extracts.

Washed suspensions of cells grown on phenylethane-*p*-sulphonate and resuspended in 0.05 M-phosphate buffer (Na₂HPO₄-KH₂PO₄), pH 7.0, containing 4-isopropylcatechol (70 μM) to inhibit the catechol 2,3-dioxygenase, also accumulated from alkylcatechols products which had the spectrophotometric characteristics of the corresponding alkylmuconates (light-absorption maximum near 260 nm). Whereas *cis,cis*-muconate itself was further metabolized by these suspensions (rapid loss of E_{260}

in the spectrophotometer and an O₂ uptake on *cis,cis*-muconate in the Warburg respirometer of 39 μ l of O₂/h per mg dry wt. of cells) or extracts obtained from them, the alkylmuconate products formed from 3- and 4-methylcatechol, 4-ethylcatechol, 3- and 4-isopropylcatechol were not subsequently metabolized over a period of 48 h. There was therefore no evidence to suggest that any of the alkylcatechols could be further degraded to keto acids.

Discussion

The identification of 4-ethylcatechol in phenylethane-*p*-sulphonate cultures inhibited with 4-isopropylcatechol, the metabolism of authentic 4-ethylcatechol by whole cells and cell-free extracts to the typical extra-diol ring-fission product, the subsequent hydrolysis of this compound and the induction of the appropriate enzyme activities during growth in phenylethane-*p*-sulphonate are all consistent with the biodegradation of phenylethane-*p*-sulphonate proceeding by way of an extra-diol cleavage of 4-ethylcatechol (Scheme 1). By similar reasoning, toluene-*p*-sulphonate and benzenesulphonate would appear to be degraded by the homologous routes involving intermediate formation of 4-methylcatechol and catechol respectively (cf. Cain & Farr, 1968), although catechol itself was never detected, even in benzenesulphonate cultures containing 4-isopropylcatechol to inhibit the catechol 2,3-dioxygenase. Failure of cells to accumulate catechol under these conditions probably arose from the metabolism of this compound by the catechol 1,2-dioxygenase, which was not inhibited by 4-isopropylcatechol.

Although the phenylpropanesulphonates did not support growth of *Alcaligenes* 2d, the evidence presented indicates that these compounds may be metabolized to the corresponding 4-propylcatechols. The *p*-sulphonated isomers are implicated by analogy with phenylethane-*p*-sulphonate and toluene-*p*-sulphonate metabolism as the source of these catechols, but the observations do not preclude the possibility that the contaminating *m*-sulphonates, if metabolized, may yield the same product, or may be the source of the catechols isolated in these experiments. Observations with cell extracts suggest that 1-phenylpropanesulphonate may be further metabolized, at least as far as hydrolysis of the extra-diol-cleavage product, because the catechol product of 1-phenylpropanesulphonate metabolism was oxidized by extracts to a compound closely resembling 2-hydroxymuconic semialdehyde, which slowly disappeared on prolonged incubation with extracts.

The catechol 2,3-dioxygenase effecting the extra-diol cleavage in *Alcaligenes* 2d had low specificity, as has been reported in other organisms. An enzyme from *Pseudomonas* U (Bayley *et al.*, 1966) and

Pseudomonas aeruginosa (Cain & Farr, 1968) attacked both catechol and 4-methylcatechol, and the crystalline enzyme from *Pseudomonas arvilla* (Nozaki *et al.*, 1963) also attacked catechol and 4-methylcatechol at about equal rates. Evans *et al.* (1965) found that 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 'meta'-fission and concluded that a catechol 2,3-dioxygenase of low specificity catalysed all these reactions. Extracts of *Pseudomonas putida* grown with thymol (2-isopropyl-5-methylphenol) rapidly oxidized 3-isopropylcatechol and 3-isopropyl-6-methylcatechol by an extra-diol cleavage (Chamberlain & Dagley, 1968), but because 3-methylcatechol, 4-methylcatechol, 4-isopropylcatechol and catechol itself were only slowly oxidized by the same extracts Chamberlain & Dagley (1968) postulated that the cleavage of 3-isopropylcatechol and 3-isopropyl-6-methylcatechol was catalysed by a 1,6- rather than a 2,3- dioxygenase. Horvath (1970) subsequently identified a ring cleavage of 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol between the C-1 and C-6 position by extracts of *Achromobacter* sp. Because of the relatively high basal amounts of catechol 2,3-dioxygenase in succinate-grown cells (32 nmol/min per mg of protein) it was thought possible that a constitutive as well as one or more inducible catechol 2,3-dioxygenases might have been involved in the cleavage of catechol and the alkylcatechols in *Alcaligenes* 2d. All activity, however, appeared to be due to a single enzyme, since the ratio of activity on catechol and its homologues remained constant, irrespective of the growth substrate. Further, at 60°C activity towards catechol, 3-methylcatechol, 4-methylcatechol and 4-ethylcatechol in crude extracts decreased at parallel rates. This result may be somewhat equivocal, however, in the light of a similar experiment by Cain & Farr (1968), who observed the same phenomenon with catechol, 3-methylcatechol and 4-methylcatechol oxidation by extracts of *Ps. aeruginosa* grown on toluene-*p*-sulphonate, yet found that activity against 3-methylcatechol could be separated during enzyme purification.

The inhibition of catechol 2,3-dioxygenase by 4-isopropylcatechol has not previously been reported, but was utilized in the present study to accumulate alkylcatechols from their precursor alkylbenzenesulphonates. The obvious structural homology between catechol and 4-isopropylcatechol, together with the observation (Fig. 4c) that, by increasing tenfold the concentration of the catechol added simultaneously with 4-isopropylcatechol, the progressive loss of catechol 2,3-dioxygenase activity could be delayed, suggested that inactivation of the enzyme followed binding of 4-isopropylcatechol at the active site. Surprisingly, a double-reciprocal

plot of the data suggested a classical competitive inhibition, yet once the enzyme was inhibited by 4-isopropylcatechol no reversal of the inhibition could be effected even by large amounts of catechol (Fig. 4b). Further experimentation is needed to clarify these phenomena.

Although the enzymes of the 'ortho'-cleavage pathway were induced together with the 'meta'-cleavage pathway enzymes during growth on benzenesulphonate, toluene-*p*-sulphonate or phenylethane-*p*-sulphonate, the 1,2-dioxygenase was present in amounts only some 2% of that of the 2,3-dioxygenase in fully induced cells. Further, the full 'ortho'-cleavage pathway did not function in the subsequent metabolism of the alkylcatechols to respiratory metabolites. These observations are consistent with the view discussed by Hegeman & Rosenberg (1970), Dagley (1971), Ornston (1971) and Murray *et al.* (1972) that the 'ortho'-cleavage pathway functions primarily in the utilization of catechol itself or substrates metabolized to catechol, whereas the 'meta'-cleavage pathway accounts for the complete catabolism of alkylcatechols. The 1,2-dioxygenase from *Alcaligenes* 2d was able, however, to tolerate the introduction of an alkyl group into the aromatic ring of catechol. It was not unique in this respect, for Nakazawa *et al.* (1963) and Kojima *et al.* (1967) have described purified catechol 1,2-dioxygenases from *Brevibacterium fuscum* and a *Pseudomonas* sp. respectively that oxygenated catechol, 3-methylcatechol and 4-methylcatechol. The metabolism of chlorocatechols to *cis,cis*-chloromuconates by crude cell extracts from a pseudomonad (Evans & Moss, 1957) and an *Arthrobacter* sp. (Tiedje *et al.*, 1969) has also been reported. The inability of the 'ortho'-cleavage pathway to accept alkylcatechols is due rather to the extreme specificity of the next enzyme in the sequence, muconate cycloisomerase, which cannot utilize substituted muconates (Ornston, 1971) as was found also in the present study.

The degradation of the homologous linear alkylbenzenesulphonates of the surfactant type, with a side-chain of five or more carbon atoms, proceeds usually by initial attack on the side chain (Bird, 1972). On the other hand, with a side-chain length of less than four carbon atoms, biodegradation commences at the ring, as shown in the present paper. Growth on any one of the three homologous 1-phenylalkanesulphonates simultaneously adapted organisms to the rapid oxidation of the other two, and of the corresponding 4-alkylcatechols. The catechol 2,3-dioxygenase in extracts of *Alcaligenes* 2d competently oxygenated 4-*n*-alkylcatechol homologues with up to only three alkyl carbon atoms, but it is now evident that the specificity of the other catechol 2,3-dioxygenases or 1,6-dioxygenases from different organisms may well extend to homologues of much longer chain length, as has been suggested by the work of Baggi

et al. (1972), who isolated and identified from cultures of *Pseudomonas* strains utilizing 3-phenylpentane, 7-ethyl-2-hydroxy-6-oxonona-2,4-dienoic acid, the 'meta'-cleavage product of a putative *o*-diphenol substituted with a C₅ alkyl chain. Evidence for the formation of the appropriate 'meta'-cleavage compound in cultures of *Pseudomonas* species utilizing 4-phenylheptane (Baggi *et al.*, 1972) and 1-phenyloctane-*p*-sulphonate (R. B. Cain & J. B. Johnston, unpublished work) suggests that the possibility of ring attack without an alkyl oxidation on linear alkylbenzenesulphonates of the surfactant type can no longer be precluded, though the phenomenon is very uncommon.

Note Added in Proof (Received 1 April 1974)

Since this paper was accepted, 4-*n*-propylcatechol has been synthesized by the method of Späth & Quientiski (1928), and shown to be identical in all chemical and physical properties with the product of 1-phenylpropanesulphonate metabolism by the *Alcaligenes* sp.

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