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Microbial Metabolism of Alkylbenzene Sulphonates

BACTERIAL METABOLISM OF UNDECYLBENZENE-*p*-SULPHONATE AND DODECYLBENZENE-*p*-SULPHONATE

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1. A study was made of the biodegradation of alkylbenzene sulphonate homologues, one of the major components of commercially marketed detergents. A *Bacillus* species was elected for growth on alkylbenzene sulphonate homologues as the sole source of carbon and sulphur. 2. The results from both whole-cell and cell-free systems indicated that the alkyl, aryl and sulphonate moieties of alkylbenzene sulphonate homologues were all further metabolized by the *Bacillus* species. 3. The alkyl side chain, after a presumed initial oxidation of the terminal methyl group, was subsequently oxidized by a β-oxidation pathway. Three enzymes of the β-oxidation pathway, i.e. acyl-CoA synthetase, acyl-CoA dehydrogenase and β-hydroxyacyl-CoA dehydrogenase, were identified in cell-free extracts of the detergent-grown *Bacillus* species. The substrate specificity of acyl-CoA synthetase indicated activity towards several alkylbenzene sulphonate homologues. 4. The sulphonate moiety was released as sulphite by a desulphonating enzyme. Some kinetic properties of this enzyme were determined. The sulphite was subsequently metabolized to either sulphate or adenosine 5′-sulphatophosphate. Two enzymes involved in sulphite metabolism, i.e. sulphite–cytochrome *c* reductase and adenosine 5′-sulphatophosphate–cytochrome *c* reductase were detected in cell-free extracts of undecylbenzene-*p*-sulphonate-grown *Bacillus* species. 5. The combined results of continuous sampling programmes monitored by both t.l.c. and sulphite appearance in the growth medium indicated that desulphonation of the aromatic moiety was the likely first step in the overall biodegradation of several alkylbenzene sulphonate homologues. 6. The presence of *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate in cells after growth on several alkylbenzene sulphonate homologues containing an odd number of carbon atoms in the side chain was confirmed by g.l.c. and t.l.c. analysis. Cells grown on several homologues containing an even number of carbon atoms in the side chain were shown to contain *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate. 7. The aromatic nucleus obtained from undecylbenzene-*p*-sulphonate was further metabolized by an oxidation sequence involving an '*ortho*-cleavage' route. 8. An overall metabolic pathway for the biodegradation of various alkylbenzene sulphonate homologues by this *Bacillus* species is proposed.

The biodegradation of synthetic detergents is of ecological importance with the increasing deposition into water systems of large quantities of both domestic and industrial detergent-containing effluents. Linear alkylbenzene sulphonates are the group of detergent compounds in most common domestic and industrial use. These compounds consist of a straight-chain alkyl side chain and an aryl sulphonate nucleus. Such *n*-alkylbenzene sulphonates constitute a homologous series, the lower members of which lack detergent properties. Several reports (Huddleston & Allred, 1963; Swisher, 1963*a*,*b*) have concluded that detergents of this type are biodegradable, the attack involving initial oxidation of the terminal methyl group of the alkyl side chain and subsequent β-oxidation of this carboxyl-substituted alkyl moiety. Previous research has been almost exclusively limited to whole-cell studies with mixed flora degrading mixtures of various linear alkylbenzene sulphonates.

Biodegradation of benzenesulphonate, the lowest homologue of the series, has been reported (Farr & Cain, 1965; Cain & Farr, 1968; Ripin *et al.*, 1971), involving release of the sulphonate group into the medium as sulphate and subsequent aromatic ring fission (Cain & Farr, 1968) by either classical '*ortho*-' (Stanier *et al.*, 1966) or '*meta*-' (Dagley & Gibson,

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1965) cleavage pathways of aromatic catabolism. Analogous catabolism of toluene-*p*-sulphonate has been recorded (Cain & Farr, 1968). In direct contrast, attack on the methyl group of toluene-*p*-sulphonate has also been reported (Kitagawa, 1956).

The present paper reports the degradation of undecylbenzene-*p*-sulphonate and dodecylbenzene-*p*-sulphonate by a pure culture of a *Bacillus* species elected for growth on undecylbenzene-*p*-sulphonate as the sole source of carbon and sulphur. Preliminary reports of the biodegradation of various alkylbenzene sulphonate homologues by this *Bacillus* species have appeared previously (Willetts & Cain, 1970; Cain *et al.*, 1971).

Materials and Methods

Organisms

Isolation. The organism used was isolated, by elective culture methods on minimal-salts medium containing 0.05% (w/v) undecylbenzene-*p*-sulphonate, from a small sewage-purification plant at Felling (Co. Durham, U.K.) receiving household sewage and domestic detergent effluents. The organism was identified as a *Bacillus* species by classical taxonomic evaluation (Breed *et al.*, 1957). The isolated bacterium was maintained on minimal-salts medium containing undecylbenzene-*p*-sulphonate (0.1%, w/v) solidified with Oxoid no. 3 agar (2%, w/v), and subcultured monthly.

Growth of cells. Large-scale production of cells was effected in 20-litre fermenters fitted with a sterile sampling device and containing 18 litres of a defined medium with the following composition, per litre of glass-distilled water: NH_4Cl, 1g; K_2HPO_4, 14.6g; KH_2PO_4, 6.8g; $MgCl_2$, 0.1g; organic carbon source, 50mg; trace element solution (Barnett & Ingram, 1955; modified by substituting chloride salts for sulphates), 10ml. Where a carbon source other than a sulphonate was used, $MgSO_4,7H_2O$ was substituted for $MgCl_2$. The medium was adjusted to pH7.0 and autoclaved for 15min at 15lb/in^2 (kN/m^2). An inoculum derived from stock slopes was grown at 30°C for 24h in shake cultures of the appropriate medium (1 litre in a 2-litre conical flask) and one such starter culture was used to inoculate each of the 20-litre flasks, which were incubated at 30°C; forced aeration (approx. 3 litres of air/min) was necessary to eliminate oxygen starvation in this volume of medium. Cells were harvested towards the end of the exponential phase of growth (20–24h). Alkylbenzene sulphonates with an alkyl side-chain length of more than four carbon atoms showed the foaming properties of typical detergents under these conditions of forced aeration and this foaming was suppressed by the addition of 10ml of silicone antifoam (BDH Chemicals Ltd., Poole, Dorset, U.K.) per 18 litres of culture medium.

Preparation of cell-free extracts. Freshly harvested cells were suspended in 0.1M-potassium phosphate buffer, pH7.0, and crushed by exposure for 5min at 0°C to the output of an MSE Mullard 60W ultrasonic disintegrator (20kHz). Cell-free extracts were prepared by centrifuging crushed suspensions at 30000*g* for 20min. The resultant supernatant was stored at −15°C until required.

Enzyme assays

All spectrophotometric assays were performed with a Unicam SP.800 recording spectrophotometer fitted with an SP.820 constant-wavelength attachment and a constant-temperature cuvette holder maintained at 30°C.

Desulphonating enzyme. The rate of sulphite release was measured spectrophotometrically at 342nm by using 2,4-dinitroanilinomaleimide reagent (Robinson, 1965). Reaction mixtures contained, in a final volume of 3.0ml: decylbenzene-*p*-sulphonate, 10μmol; 2,4-dinitroanilinomaleimide in acetone, 0.5μmol; tris–HCl buffer, pH7.5, 250μmol; approx. 2mg of protein as cell-free extract.

Isocitrate lyase (*EC* 4.1.3.1). This enzyme was assayed by the method of Dixon & Kornberg (1959), in which the formation of glyoxylate phenylhydrazone is followed spectrophotometrically at 324nm.

Acyl-CoA synthetase (*EC* 6.2.1.3). This enzyme was assayed by the method of Overath *et al.* (1969), involving the increase in spectrophotometric absorption at 520nm dependent on acylhydroxamate formation from the acyl-CoA product of the enzyme-catalysed reaction.

β-Hydroxyacyl-CoA dehydrogenase (*EC* 1.1.1.35). This enzyme was assayed spectrophotometrically at 340nm by following the *N*-acetyl-*S*-acetoacetylcysteamine-dependent oxidation of NADH (Overath *et al.*, 1967).

Acyl-CoA dehydrogenase (*EC* 1.3.99.3). This enzyme was assayed by the method of Green *et al.* (1954), in which the butyryl-CoA-dependent reduction of cytochrome *c* is monitored spectrophotometrically at 550nm.

Adenosine 5'-sulphatophosphate–cytochrome c reductase. This enzyme was assayed spectrophotometrically at 550nm by measuring AMP-dependent reduction of cytochrome *c* in the presence of sulphite ions (Lyric & Suzuki, 1970*b*).

Alkane 1-hydroxylase. This enzyme was measured spectrophotometrically at 340nm by following NADH oxidation in an assay based on the ω-oxidation of fatty acids (Peterson *et al.*, 1967). Reaction mixtures contained, in a final volume of 3.0ml: undecylbenzene-*p*-sulphonate or undecane, 1.5μmol; NADH, 0.5μmol; $FeSO_4$, 0.2μmol; tris–HCl buffer, pH7.5, 100μmol; approx. 2mg of protein as cell-free extract.

p-Hydroxybenzoate hydroxylase. This enzyme was assayed spectrophotometrically at 340nm by following *p*-hydroxybenzoate-dependent oxidation of NADH (Hosokawa & Stanier, 1966).

Protocatechuate 3,4-*oxygenase* (*EC* 1.13.1.3). This enzyme was assayed by the method of Cain *et al.* (1968), in which substrate disappearance is followed at 290nm.

Protocatechuate 4,5-*oxygenase* (*EC* 1.13.1.8). This enzyme was assayed by the method of Trippett *et al.* (1960), in which the production of an aldehydic ring-cleavage product is measured spectrophotometrically at 410nm in alkaline conditions.

β-Carboxymuconate-lactonizing enzyme. This enzyme was assayed by the method of Cain *et al.* (1968), in which substrate disappearance is followed at 260nm.

Catechol 1,2-*oxygenase* (*EC* 1.13.1.1). This enzyme was assayed by following at 260nm the accumulation of *cis,cis*-muconate by the method of Hegemen (1966).

Catechol 2,3-*oxygenase* (*EC* 1.13.1.2). This enzyme was determined by the increase in E_{375} when catechol is added to extracts (Cain & Farr, 1968).

(+)-4-*Carboxymethyl*-4-*hydroxyisocrotonolactone lyase* (*decyclizing*) (*EC* 5.5.1.1). This enzyme was assayed by the method of Cain *et al.* (1968), in which the disappearance of *cis,cis*-muconate is monitored at 260nm.

Sulphite–cytochrome c oxidoreductase (*EC* 1.8.3.1). This enzyme was assayed by the method of Lyric & Suzuki (1970*a*), in which the reduction of cytochrome *c* was followed spectrophotometrically at 550nm.

3,4-*Dihydroxyphenylacetate oxygenase* (*EC* 1.13.1.7). This enzyme was assayed spectrophotometrically by measuring disappearance of 3,4-dihydroxyphenylacetate at 280nm. The reaction mixture contained, in a final volume of 3.0ml: 3,4-dihydroxyphenylacetate, 1μmol; tris–HCl buffer, pH7.5, 260μmol; approx. 2mg of protein as cell-free extract.

Determinations

Sulphate in growth media and Warburg flask contents was determined by using barium chloranilate reagent (Bertolacini & Barney, 1957), after removal of cells by centrifugation.

Sulphite was similarly assayed by using 2,4-dinitroanilinomaleimide reagent (Robinson, 1965).

Catechol substances were determined by the Evans (1947) method but with the substitution of sodium molybdate for sodium tungstate.

Alkylbenzene sulphonate homologues containing seven or more carbon atoms in the alkyl side chain were determined by using a quantitative Methylene Blue test (Hammerton, 1955). Homologues (including all homologues containing six or less carbon atoms in the alkyl side chain) were alternatively determined by using Pinacryptol Yellow (Borecký, 1959).

Protein was assayed by a modified biuret method (Turner, 1966).

Respiration of washed-cell suspensions

Conventional manometric techniques (Umbreit *et al.*, 1957) were used to measure respiration of twice-washed cell suspensions. Vessels received 5–8mg dry wt. of washed cells, 50μmol of potassium phosphate buffer, pH7.0, and various amounts of substrate in a volume of 2.8ml. The centre well contained 0.2ml of 20% (w/v) KOH; oxygen consumption was measured at 30°C.

Chromatography

Paper chromatography. Compounds were applied to Whatman no. 1 paper and developed by descending chromatography in appropriate solvents. The following solvents were used: A, ethanol–aq. NH_3 (sp.gr. 0.880)–water (20:1:4, by vol.); B, butan-1-ol–acetic acid–water (4:1:5, by vol.); C, butan-1-ol–acetic acid–water (4:1:1, by vol.); D, ethyl methyl ketone–ethanol–aq. NH_3 (sp.gr. 0.880) (15:3:1, by vol.); E, butan-1-ol–ethanol–water (4:1:5, by vol.).

Phenols were detected by spraying dried chromatograms with $FeCl_3$ [0.5% (w/v) in aq. 50% (v/v) ethanol].

Alkylbenzene sulphonate homologues were detected by spraying dried chromatograms with aq. 0.05% (w/v) Pinacryptol Yellow and examining the sprayed chromatographs under u.v. light. Alkylbenzene sulphonate spots were fluorescent under u.v. light.

Gas–liquid chromatography. Samples of growth medium taken at various times during the growth cycle were prepared for g.l.c. by acidification to 1M with HCl and clarification by boiling on a hot-plate for 15–20min. After cooling, the pH was adjusted to 7.0 with NaOH. Partial purification of the sample was accomplished by passage through a 3cm×20cm charcoal column containing about 15g of charcoal. The charcoal column was subsequently eluted with 500ml of methanol–benzene (1:1, v/v) containing 1% of aq. NH_3 soln. (sp.gr. 0.880). The charcoal was then removed from the column and extracted with an additional 500ml of elution solvent by warming on a steam bath for 20–25min. After filtration, all solvents were combined and evaporated to dryness. The residue was then transferred to a small flask for desulphonation.

The desulphonation technique, using phosphoric acid hydrolysis, was an extension of the method of Knight & House (1959). In this procedure, by the use of micro-equipment, the desulphonated alkylbenzenes were distilled from phosphoric acid, boiling at about 215°C, and collected on the surface of a

water trap. The oils were then removed by extraction with *n*-hexane (b.p. 67–70°C) and the extracts transferred to a test tube. The samples in *n*-hexane were then methylated by using diazomethane generated *in situ* from *N*-methyl-*N*-nitrosourea.

G.l.c. analyses of desulphonated methylated samples were made by using either an Apiezon L column (1.6m) on dimethyldichlorosilane or a diethylene glycol adipate column (1.6m) on dimethyldichlorosilane. Both columns were operated with a gas-flow rate of 75ml/min, maximum injection port temperature and a constant detector oven temperature of 250°C. Optimum results were obtained from both columns with a 10μl sample and 2×10^4 attenuation. The column temperatures were maintained at 200°C and 180°C when using Apiezon L and diethylene glycol adipate respectively. G.l.c. analyses were performed with a Pye Unicam series 104 chromatography apparatus, with an authentic sample of 4-phenylbutyrate taken through an identical preparation procedure as an internal reference peak.

Chemicals

β-Oxoadipate was synthesized by the method of Bardhan (1936). (+)-Muconolactone was prepared biologically as described by Cain (1961). 2,4-Dinitroanilinomaleimide was prepared as described by Clark-Walker & Robinson (1961).

Alkylbenzene-*p*-sulphonate homologues containing eight or more carbon atoms in the alkyl side chain were prepared by sulphonation of the corresponding alkylbenzene with fuming sulphuric acid, and fractional crystallization from water or ethanol (Adams *et al.*, 1963). Shorter alkyl-side-chain homologues were provided by the Continental Oil Co. (Ponca City, Okla., U.S.A.).

All other chemicals used were of A.R. grade or the highest purity commercially available.

Nomenclature

The compound undecylbenzene-*p*-sulphonate can be described by an alternative organic nomenclature system, in common usage in the U.S.A., as 1-phenylundecane-*p*-sulphonate.

Results

Growth of Bacillus species on various alkylbenzene-p-sulphonate homologues

The *Bacillus* species was originally elected on a medium containing undecylbenzene-*p*-sulphonate as the sole source of carbon and sulphur for growth. The isolated organism was capable of substantial growth on all homologues with an alkyl-side-chain length of 2–18 carbon atoms, and also grew well on benzenesulphonate and toluene-*p*-sulphonate. The organism was capable of growth on *p*-hydroxybenzoate and 3,4-dihydroxybenzoate, but not on *p*-hydroxyphenylacetate or 3,4-dihydroxyphenylacetate, as the sole carbon source. No growth occurred in control flasks of the mineral-salts medium alone without an added carbon source. Growth-yield coefficients for alkylbenzene sulphonates (Bauchop & Elsden, 1960) of 6.0–24.0 were obtained, which compared with values of 48.6 and 63.0 obtained from the growth of the organism on succinate and glucose respectively.

With undecylbenzene-*p*-sulphonate as the sole carbon and sulphur source, optimum growth was obtained when the culture was grown at 30°C. Maximum growth was reached within 30h; under the conditions employed, the mass-doubling time of the culture during exponential growth, as indicated by turbidity measurements, was approx. 3h.

Growth of the *Bacillus* species on undecylbenzene-*p*-sulphonate was correlated with the following parameters: (i) disappearance of the aromatic ring (measured spectrophotometrically at 235nm); (ii) disappearance of the alkyl side chain (measured by the Methylene Blue test); (iii) appearance of sulphite in the growth medium; (iv) appearance of sulphate in the growth medium.

A typical results pattern (Fig. 1) showed two distinct phases. There was an initial phase extending up to 10h after inoculation of an undecylbenzene-*p*-sulphonate-grown inoculum, during which the alkyl side chain was progressively degraded, sulphite rapidly accumulated in the medium up to a maximum concentration of approx. 0.1μmol of sulphite/ml 2h after inoculation and remained at that approximate value throughout the remainder of the growth cycle, sulphate after a lag phase of 2h accumulated up to a maximum concentration of 0.05μmol/ml 5h after inoculation and remained at that concentration throughout the growth cycle, and the aromatic nucleus showed no evidence of biodegradation. After approx. 10h of growth, by which time the degradation of the alkyl side chain had been completed according to the parameter of the Methylene Blue test, this initial phase was replaced by a secondary growth phase during which both sulphite and sulphate concentrations remained constant, and biodegradation of the aromatic nucleus commenced and progressed linearly to completion approx. 24h after the initial inoculation of *Bacillus* species. This general growth pattern was typical of the growth of *Bacillus* species on all single alkylbenzene-*p*-sulphonate homologues containing 2–18 carbon atoms in the alkyl side chain, with two qualifying provisos: (*a*) homologues containing six or less carbon atoms in the side chain were not detected by the Methylene Blue test; (*b*) the initial lag phase before biodegradation of the aromatic nucleus commenced was inversely proportional to the number of carbon atoms in the alkyl side chain.

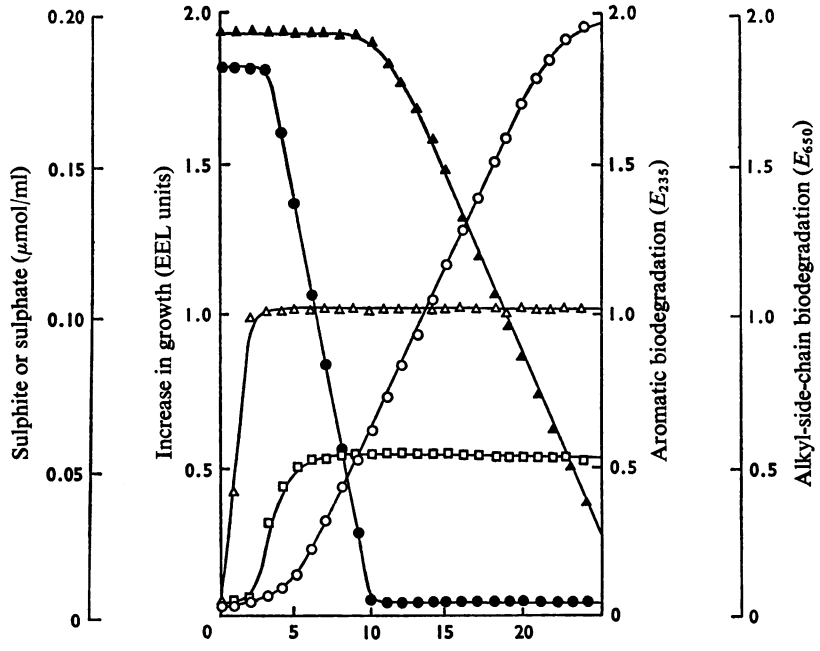


Fig. 1. *Cells from 20 litres of minimal medium, with undecylbenzene-p-sulphonate as the sole carbon and sulphur source, were harvested aseptically in the exponential phase, washed three times with sterile deionized water and resuspended in 1 litre of fresh undecylbenzene-p-sulphonate medium (pre-warmed to 30°C), and growth was allowed to continue*

Growth (○), sulphite (△) and sulphate (□) content in the medium, aromatic biodegradation (▲) and alkyl side-chain biodegradation (●) were followed, as described in the text, on samples removed aseptically at 1h intervals.

Identification by g.l.c. of intermediates of biodegradation of alkylbenzene-p-sulphonate

Samples of the growth medium taken 18h after inoculation, during the exponential phase of growth of the *Bacillus* species on undecylbenzene-*p*-sulphonate as sole carbon and sulphur source, were analysed by g.l.c. on two different columns after prior preparation, desulphonation and methylation as described in the Materials and Methods section. Peak identifications were made by comparing relative retention times with those of authentic samples of the compounds in Tables 1 and 2, taken through an identical preparation procedure. Consistent results were obtained from several experiments and typical separations on Apiezon L and diethylene glycol adipate columns are represented in Table 1. From the almost identical patterns obtained from g.l.c. sample analyses on these two different columns, it was concluded that biodegradation of undecylbenzene-*p*-sulphonate by the *Bacillus* species involved the appearance of *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate in the growth medium.

Identical experiments with samples of growth medium taken during the growth of the *Bacillus* species on dodecylbenzene-*p*-sulphonate gave comparable results (Table 2). By analogy it was concluded that biodegradation of dodecylbenzene-*p*-sulphonate by the *Bacillus* species involved the appearance of *p*-hydroxyphenylhexanoate, *p*-hydroxyphenylbutyrate, *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate in the growth medium.

Isolation of some biodegradation products of alkylbenzene sulphonate after growth on undecylbenzene-p-sulphonate

A 20-litre culture of *Bacillus* species was inoculated and grown for 24h with undecylbenzene-*p*-sulphonate as the sole carbon source as described in the Materials and Methods section. At time-intervals of 0, 1, 3, 6, 12 and 24h after inoculation 2-litre batches

Table 1. *Gas-chromatographic analyses of samples of growth medium taken 18h after inoculation of the Bacillus species into minimal medium containing undecylbenzene-p-sulphonate as the carbon and sulphur source*

For operating conditions and sample preparations see the Materials and Methods section. Results are expressed ±S.D. with the numbers of determinations shown in parentheses.

Peak no.	Component	Analysis on Apiezon L-packed column Mean wt. (%)	Analysis on diethylene glycol adipate-packed column Mean wt. (%)
1	*p*-Hydroxybenzoate (dimethyl derivative)	11.5±0.02 (8)	10.5 ±0.19 (8)
2	*p*-Hydroxybenzoate (monomethyl derivative)	25.1±0.10 (8)	28.6 ±0.26 (8)
3	*p*-Hydroxyphenylpropionate (dimethyl derivative)	9.0±0.06 (8)	4.0 ±0.13 (8)
4	3,4-Dihydroxybenzoate (trimethyl derivative)	4.8±0.06 (8)	5.65±0.08 (8)
5	3,4-Dihydroxybenzoate (dimethyl derivative)	9.9±0.17 (8)	14.55±0.18 (8)
6	3,4-Dihydroxybenzoate (monomethyl derivative)	27.6±0.10 (8)	8.4 ±0.16 (8)
7	*p*-Hydroxyphenylpropionate (monomethyl derivative)	12.1±0.22 (8)	28.3 ±0.14 (8)

Table 2. *Gas-chromatographic analyses of samples of growth medium taken 18h after inoculation of the Bacillus species into minimal medium containing dodecylbenzene-p-sulphonate as the carbon and sulphur source*

For operating conditions and sample preparations see the Materials and Methods section. Results are expressed ±S.D. with the number of determinations shown in parentheses.

Peak no.	Component	Analysis on Apiezon L-packed column Mean wt. (%)	Analysis on diethylene glycol adipate-packed column Mean wt. (%)
1	*p*-Hydroxyphenylacetate (dimethyl derivative)	8.8±0.15 (8)	6.4±0.14 (8)
2	*p*-Hydroxyphenylacetate (monomethyl derivative)	18.8±0.12 (8)	29.0±0.06 (8)
3	3,4-Dihydroxyphenylacetate (trimethyl derivative)	4.7±0.14 (8)	4.8±0.09 (8)
4	3,4-Dihydroxyphenylacetate (dimethyl derivative)	12.1±0.17 (8)	9.2±0.11 (8)
5	3,4-Dihydroxyphenylacetate (monomethyl derivative)	32.1±0.11 (8)	32.7±0.09 (8)
6	*p*-Hydroxyphenylbutyrate (monomethyl derivative)	13.8±0.12 (8)	12.3±0.08 (8)
7	*p*-Hydroxyphenylhexanoate (monomethyl derivative)	8.7±0.09 (8)	5.6±0.13 (8)

of growth medium were sampled, and the cells in these samples immediately harvested, washed and resuspended in 10ml of glass-distilled water. Cell-free extracts, prepared by ultrasonic treatment of cell suspensions as described previously, were freeze-dried and resuspended in the minimum volume of glass-distilled water. The cell-free extracts taken at various time-intervals were then separated by one-directional paper chromatography with five different solvent systems, and alkylbenzene sulphonate and phenol metabolites were detected on the dried chromatograms by shield-spraying techniques with Pinacryptol Yellow and ferric chloride sprays respectively, as described in the Materials and Methods section.

The developed chromatograms were compared with an appropriate reference chromatogram for each solvent system by using authentic samples under identical conditions.

The results repeatedly gave these indications. (*a*) At all time-intervals examined (i.e. 0–24h) only one Pinacryptol-positive spot per chromatogram was detected. At each interval, and in all solvents, this corresponded exactly with the growth substrate undecylbenzene-*p*-sulphonate. Although this experiment was repeated several times, and on occasions time-intervals of up to 72h were included, no evidence was obtained to indicate the presence of biodegradation products of undecylbenzene-*p*-sulphonate retaining the sulphonate-substituted ring system. Although no quantitative analysis was employed, it was apparent that the intensity of the Pinacryptol-positive spot decreased progressively with increasing sampling time after the initial inoculation. (*b*) The results from ferric chloride spraying were more diverse. Repeatedly, chromatograms at the 0h and 1h time-intervals indicated no $FeCl_3$-positive spots. The chromatogram at the 3h time-interval carried two unidentified $FeCl_3$-positive spots. The chromatograms at the 6h and 12h time-intervals carried these same two unidentified $FeCl_3$-positive spots plus three additional chromogen-positive spots, which corresponded in all five solvents to authentic samples of *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate. The sample at the 24h time-interval again contained all five $FeCl_3$-positive spots, but at a visibly assessed lower concentration.

Comparison of chromatograms from identical experiments with nonylbenzene-*p*-sulphonate, heptylbenzene-*p*-sulphonate, pentylbenzene-*p*-sulphonate and propylbenzene-*p*-sulphonate used as alternative sole carbon sources for growth indicated that: (*a*) in all cases only one Pinacryptol-positive spot was detected, which always corresponded to the growth substrate; (*b*) in all cases *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate were identified as $FeCl_3$-positive reaction products on chromatograms; (*c*) one of the two unidentified $FeCl_3$-positive spots identified on chromatograms after growth on undecylbenzene-*p*-sulphonate [see (*b*) above] was specific to that growth substrate, and the second spot was recorded only when nonylbenzene-*p*-sulphonate, but not heptylbenzene-*p*-sulphonate, pentylbenzene-*p*-sulphonate or propylbenzene-*p*-sulphonate, was used as an alternative growth substrate to undecylbenzene-*p*-sulphonate.

Isolation of some biodegradation products of alkylbenzene sulphonate after growth on dodecylbenzene-p-sulphonate

An identical series of experiments with alkylbenzene sulphonate detergent homologues containing an even number of carbon atoms in the alkyl side chain produced a comparable set of results and conclusions, i.e.: (*a*) in all cases the only Pinacryptol-positive compound present corresponded to the original growth substrate; (*b*) $FeCl_3$-positive spots occurred in all samples, and the only rigorously identified spots were always *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate; (*c*) after growth on dodecylbenzene-*p*-sulphonate as carbon source two $FeCl_3$-positive compounds accumulated in the cells, which were not present after growth on alkylbenzene sulphonate homologues containing eight, six, four or two carbon atoms in the alkyl side chain. One of these unidentified $FeCl_3$-positive compounds also accumulated in cells after growth on decylbenzene-*p*-sulphonate as carbon source.

Oxidation of alkylbenzene-p-sulphonate homologues and other aromatic compounds by washed suspension and cell-free extracts of Bacillus species

Washed suspensions of *Bacillus* species after growth in mineral-salts medium with undecylbenzene-*p*-sulphonate as the carbon and sulphur source were capable of rapid oxidation of all alkylbenzene-*p*-sulphonate isomers tested containing one (toluene-*p*-sulphonate) to 18 (octadecylbenzene-*p*-sulphonate) carbon atoms in the alkyl side chain. The rate of oxygen uptake by such suspensions ranged from 26nmol of O_2/min per mg dry wt. of cells to 60nmol of O_2/min per mg dry wt. of cells, the lower values being associated with alkylbenzene sulphonate homologues containing 12 or more carbon atoms in the alkyl side chain. Washed suspensions of undecylbenzene-*p*-sulphonate-grown *Bacillus* species were more selective in the oxidation of a number of related aromatic compounds. Thus whereas rapid oxidation of *p*-hydroxyphenylvalerate, *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate was recorded, a low oxidation rate was recorded with *p*-hydroxyphenylbutyrate, and no oxidation was recorded with *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate.

Similar rates of oxygen uptake were recorded with equivalent substrates by washed suspensions of *Bacillus* species after growth in minimal-salts medium with dodecylbenzene-*p*-sulphonate as the carbon and sulphur source, but with a number of important exceptions. Such cells exhibited slow oxidation of *p*-hydroxyphenylvalerate and no oxidation of *p*-hydroxyphenylpropionate, *p*-hydroxybenzoate, 3,4-dihydroxybenzoate and toluene-*p*-sulphonate. However, in contrast to undecylbenzene-*p*-sulphonate-grown cells, washed suspensions of dodecylbenzene-*p*-sulphonate-grown cells rapidly oxidized *p*-hydroxyphenylbutyrate, *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate.

Enzymes induced by growth on undecylbenzene-p-sulphonate and dodecylbenzene-p-sulphonate

The combined results from the experiments so far reported suggested that the pattern of biodegradation of alkylbenzene sulphonate by the *Bacillus* species involved the following outline steps: (1) replacement of the *para*-substituted sulphonate grouping of the alkylbenzene sulphonate molecule by a *para*-substituted hydroxyl grouping; (2) progressive removal of the alkyl side chain; (3) biodegradation of the aromatic nucleus.

These three molecular transformations were examined at the enzyme level in alkylbenzene sulphonate-grown *Bacillus* species.

Desulphonation. An inducible desulphonating enzyme present in alkylbenzene sulphonate-grown cell-free extracts was partially characterized kinetically. The enzyme had a sharp pH optimum at pH 7.5 in tris–HCl buffer. After growth on undecylbenzene-*p*-sulphonate the specific activity recorded under the conditions of the assay was typically 30nmol of sulphite released/min per mg of protein, and the apparent Michaelis constant for undecylbenzene-*p*-sulphonate was 12mM.

The enzyme concentration was increased 100-fold by growth on undecylbenzene-*p*-sulphonate compared with growth on a number of other carbon sources, including succinate, acetate, glycerol, malate, glucose and, most significantly, undecylbenzene. The enzyme was completely repressed by the addition of sulphite, but not of sulphate or thiosulphate, at concentrations as low as 0.5mM, to the normally sulphite-free growth medium. However, when assayed in cell-free extracts prepared from medium containing no exogenous sulphite, the enzyme was not inhibited by sulphite concentrations as high as 30mM in the reaction mixture.

The enzyme induced by growth on undecylbenzene-*p*-sulphonate was active with a number of homologues containing both more and less than 11 carbon atoms in the alkyl side chain: assuming the activity with undecylbenzene-*p*-sulphonate to be 100%, the activity with the longer-chain compound, heptadecylbenzene-*p*-sulphonate, was 90% and the activity with the shorter-chain compound, nonylbenzene-*p*-sulphonate, was 170%.

Two key enzymes associated with the subsequent metabolism of sulphite were also detected in cell-free extracts of alkylbenzene-*p*-sulphonate-grown *Bacillus* species. The concentration of sulphite–cytochrome *c* oxidoreductase (Lyric & Suzuki, 1970*a*) was increased sixfold by growth on various alkylbenzene sulphonate homologues compared with growth on media containing undecylbenzene as the sole carbon source and either cysteine or methionine as the sole sulphur source. The enzyme had a sharp pH optimum at pH 8.8 in tris–HCl buffer. The specific activity after growth on undecylbenzene-*p*-sulphonate was typically 17nmol of cytochrome *c* reduced/min per mg of protein. Adenosine 5′-sulphatophosphate cytochrome *c* reductase (Lyric & Suzuki, 1970*b*), the second key enzyme present in alkylbenzene sulphonate-grown extracts associated with subsequent sulphite metabolism, was constitutive in the *Bacillus* species. The pH optimum of this enzyme was also 8.8 in tris–HCl buffer, and the specific activity measured under the standard assay conditions in alkylbenzene sulphonate-grown cell-free extract was 33nmol of cytochrome *c* reduced/min per mg of protein.

Alkyl side-chain oxidation. Biodegradation of alkyl chains by micro-organisms has been widely recorded in Nature (McKenna & Kallio, 1965). Although many molecular variations have been recognized, the basic pattern apparent in most cases involves an initial oxidation of the terminal methyl group of the alkyl side chain to a carboxylic acid grouping. Subsequent biodegradation of the oxidized alkyl side chain has been recorded, involving either β-oxidation (Lynen, 1955) or α-oxidation (Martin & Stumpf, 1959).

A survey of cell-free extracts of undecylbenzene-*p*-sulphonate-grown *Bacillus* species, covering a wide range of pH conditions and metal-ion activators, failed to reveal any evidence of an NADH- or NADPH-dependent alkane 1-hydroxylase possibly involved in the initial oxidation of the terminal carbon atoms of the alkyl side chain of several alkylbenzene sulphonate homologues, or straight-chain alkanes (Peterson *et al.*, 1967). The failure of g.l.c. analysis of the growth medium during the growth of the *Bacillus* species on undecylbenzene-*p*-sulphonate to detect any compounds containing an even number of carbon atoms in the alkyl side chain was indirect evidence against the existence of an α-oxidation mechanism in this micro-organism.

However, three key enzymes of the classical β-oxidation pathway (Lynen, 1955), i.e. acyl-CoA synthetase, acyl-CoA dehydrogenase and β-hydroxyacyl-CoA dehydrogenase, were present in cell-free extracts of *Bacillus* species after growth on several different alkylbenzene sulphonate homologues (Willetts & Cain, 1972). Some kinetic properties of these three enzymes from undecylbenzene-*p*-sulphonate-grown cells are presented in Table 3. The concentrations of all three enzymes were increased to similar activities during growth on the straight-chain hydrocarbon undecane and the fatty acid undecanoic acid, but were present at much lower concentrations during growth on either succinate or acetate. The substrate specificity of acyl-CoA synthetase after growth on undecylbenzene-*p*-sulphonate, undecanoic acid or undecane was remarkably similar (Table 4) and indicated that the enzyme induced by undecylbenzene-*p*-sulphonate was probably involved in the biodegradation of a wide range of alkyl chains rather than the alkyl side chain of alkylbenzene sulphonate

Table 3. *Kinetic properties of some enzymes of β-oxidation in cell-free extracts of the Bacillus species*

Cell-free extracts were prepared from undecylbenzene-*p*-sulphonate-grown *Bacillus* species except where otherwise indicated. Assay conditions were as described in the Materials and Methods section. The specific activities of acyl-CoA synthetase are expressed as nmol of hydroxamate produced/min per mg of protein. The specific activities of acyl-CoA dehydrogenase are expressed as nmol of cytochrome *c* reduced/min per mg of protein. The specific activities of β-hydroxyacyl-CoA dehydrogenase are expressed as nmol of NAD^+ reduced/min per mg of protein.

Enzyme	Optimum pH	K_m (mM)	Carbon source ...	Max. recorded specific activity			
				Undecylbenzene-*p*-sulphonate	Undecylbenzene	Undecanoic acid	Succinate
Acyl-CoA synthetase	8.5	Oleate = 0.15		9.2 (oleate)	9.4 (oleate)	9.6 (oleate)	0.9 (oleate)
		Valerate = 0.16		22.8 (butyryl-CoA)	16.6 (butyryl-CoA)	17.6 (butyryl-CoA)	0.2 (butyryl-CoA)
Acyl-CoA dehydrogenase	7.0	Butyryl-CoA = 0.4					
		Nonanoyl-CoA = 0.34					
β-Hydroxyacyl-CoA dehydrogenase	6.8	*N*-Acetyl-*S*-acetoacetyl-cysteamine = 0.21		26.1	19.2	15.9	0.6

homologues in particular. Indirect evidence for the operation of a β-oxidation pathway of alkyl-side-chain biodegradation in alkylbenzene sulphonate-grown *Bacillus* species was the 90-fold increase in the activity of isocitrate lyase (maximum specific activity 115nmol of glyoxylate produced/min per mg of protein) during growth on undecylbenzene-*p*-sulphonate compared with growth on succinate. The induction of high activities of isocitrate lyase was characteristic of the presence in alkylbenzene sulphonate-grown extracts of a metabolic sequence producing significant quantities of acetyl-CoA.

Aromatic ring fission. The oxidative cleavage of a wide range of aromatic compounds, to open-chain compounds with an equivalent number of carbon atoms, and the subsequent catabolism of these open-chained products of ring fission to CO_2, has been recognized among many genera of organisms in Nature (Gibson, 1969). The initial attack has, in nearly all cases, involved the introduction of two hydroxyl groupings on to adjacent carbon atoms in the aromatic ring. Subsequent molecular-oxygen-dependent cleavage of the dihydroxy aromatic derivative to an open-chain molecule has been by one of two cleavage pathways (Dagley *et al.*, 1960): (*a*) '*meta* cleavage', involving oxidative cleavage of the aromatic ring adjacent to the dihydroxy substitution; (*b*) '*ortho* cleavage', involving oxidative cleavage of the aromatic ring between the two adjacent hydroxyl groupings.

A report of aromatic fission preceded by an initial dehydrogenation of the ring compound has appeared (Dutton & Evans, 1969), but the widespread occurrence of such an alternative ring-fission mechanism remains to be proved.

From the results so far presented, the biodegradation intermediate of alkylbenzene sulphonate homologues containing an odd number of carbon atoms in the alkyl side chain, which was initially susceptible to aromatic fission, would be predicted to be *p*-hydroxybenzoate. The equivalent susceptible molecule produced by biodegradation of alkylbenzene sulphonate homologues containing an even number of carbon atoms in the alkyl side chain would be predicted to be *p*-hydroxyphenylacetate, as no evidence for the alternative accumulation of phenol was obtained by g.l.c.

Examination of cell-free extracts of the *Bacillus* species grown on undecylbenzene-*p*-sulphonate revealed the presence of several enzymes of the '*ortho*-cleavage' pathway necessary to oxidize *p*-hydroxybenzoate to equimolar concentrations of succinate and acetyl-CoA. Some kinetic properties of these enzymes are summarized in Table 5. These enzymes were induced to approximately equivalent specific activities only by growth on alkylbenzene sulphonate homologues containing an odd number of carbon

Table 4. *Substrate specificity of acyl-CoA synthetase in cell-free extracts of Bacillus species after growth on undecylbenzene-p-sulphonate, undecane or undecanoic acid as the sole carbon source*

Assay conditions are as described in the Materials and Methods section. The specific activity of acyl-CoA synthetase is expressed as nmol of hydroxamate produced/min per mg of protein.

Substrate in assay	Carbon source ...	Activity of cell-free extract: Undecylbenzene-*p*-sulphonate	Undecane	Undecanoic acid
Undecylbenzene-*p*-sulphonate		8.6	4.4	4.9
Heptylbenzene-*p*-sulphonate		8.4	3.6	3.7
Pentylbenzene-*p*-sulphonate		5.6	2.2	2.6
Propylbenzene-*p*-sulphonate		5.0	1.8	2.2
p-Hydroxyphenylpropionate		7.2	6.4	6.6
p-Hydroxyphenylacetate		3.0	3.8	3.0
5-Phenylvalerate		6.6	6.0	6.9
4-Phenylbutyrate		6.0	5.0	5.9
3-Phenylpropionate		5.2	4.4	5.0
Undecanoate		10.2	8.6	9.6
Valerate		8.6	5.2	6.8
Butyrate		6.2	4.0	5.4

Table 5. *Kinetic properties of some enzymes of aromatic ring fission induced by growth of the Bacillus species on undecylbenzene-p-sulphonate*

Assay conditions are as described in the Materials and Methods section. The specific activities are expressed as nmol of specific parameter/min per mg of protein (for the specific parameter measured for each individual enzyme, see the appropriate reference in the Materials and Methods section). —, Not tested.

Enzyme	Optimum pH	K_m (mM)	Sp. activity: Growth on undecylbenzene-*p*-sulphonate	Growth on succinate
p-Hydroxybenzoate hydroxylase	7.9	0.6	28	0.2
β-Carboxymuconate-lactonizing enzyme	7.7	0.16	190	5
Catechol 1,2-oxygenase	7.2	2.4	0	0
Catechol 2,3-oxygenase	7.5	1.6	0.2	0
cis,cis-Muconate-lactonizing enzyme	7.2	3.4	0	0
Protocatechuate 3,4-oxygenase	7.4	—	41.2	0.8
Protocatechuate 4,5-oxygenase	7.6	—	0	0
3,4-Dihydroxyphenylacetate oxygenase	7.6	—	0	0

atoms in the alkyl side chain. Thus whereas all homologues containing 3,5, 7, 9, 11 or 13 carbon atoms in the alkyl side chain induced these enzymes to an approximately equivalent specific activity, growth on homologues containing 2, 4, 6, 8, 10, 12 or 14 carbon atoms in the alkyl side chain, as well as a wide range

of alternative carbon sources such as acetate, succinate, glucose, malate and oleate, induced only insignificant activities of these same enzymes.

Equivalent enzymological examination of cell-free extracts of the *Bacillus* species grown on dodecylbenzene-*p*-sulphonate revealed the presence of an oxygenase enzyme metabolizing dihydroxyphenylacetate. This enzyme was induced (maximum specific activity under assay conditions = 30.6nmol of 3,4-dihydroxyphenylacetate utilized/min per mg of protein) to an approximately equivalent specific activity by all alkylbenzene sulphonate homologues containing an even number of carbon atoms in the alkyl side chain. These results contrasted with the failure of alkylbenzene sulphonate homologues containing an odd number of carbon atoms in the alkyl side chain, or acetate, succinate, malate, glucose or oleate when used as alternative carbon sources, to induce this oxygenase to any significant extent.

Discussion

The overall pattern of the results obtained from several diverse experimental approaches indicates that the generalized pathway for the biodegradation of alkylbenzene sulphonate homologues containing an odd number of carbon atoms in the alkyl side chain by the *Bacillus* species tested is as shown in Scheme 1. The generalized pathway for the biodegradation of alkylbenzene sulphonate homologues containing an even number of carbon atoms in the alkyl side chain by the *Bacillus* species is directly comparable, resulting in the production of 3,4-dihydroxyphenylacetate before aromatic cleavage, the end products of which, however, remain to be characterized.

Similar biodegradation pathways for alkylbenzene sulphonate homologues have been postulated previously (Swisher, 1963*b*), but in all cases these predictions were based almost exclusively on results obtained by using washed-cell suspensions of mixed flora degrading mixtures of various alkylbenzene sulphonate homologues.

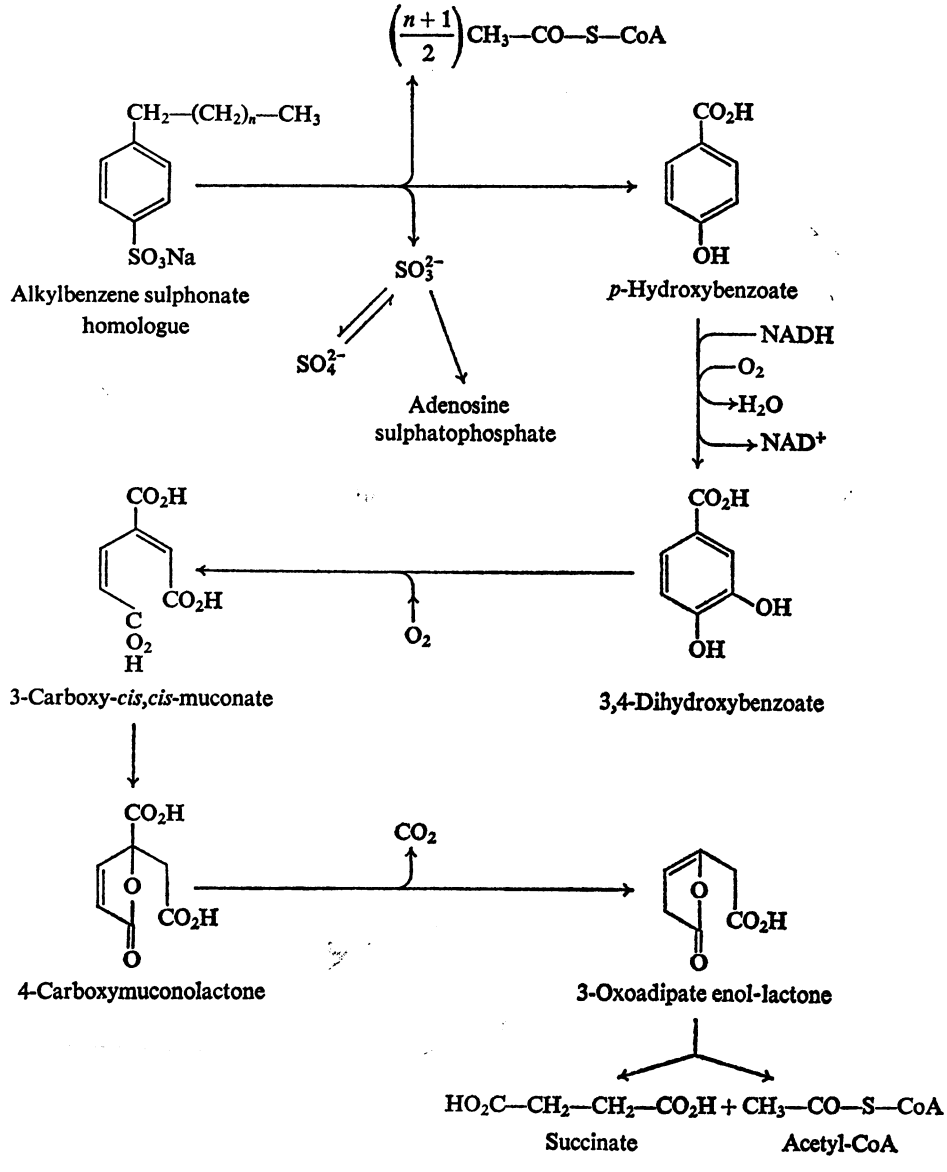
The results presented in the present paper, which include confirmation of the presence of implicated enzymes by direct assay and identification of biodegradation intermediates by both g.l.c. and t.l.c., provide direct evidence for the operation of such catabolic pathways in cells of *Bacillus* species growing on alkylbenzene sulphonate homologues as the sole source of carbon and sulphur. The nature of the initial oxidation of the terminal methyl group of the alkyl side chain remains to be identified at the enzyme level in cell-free extracts of alkylbenzene sulphonate-grown *Bacillus* species. Several available electron donors failed to mediate this reaction in cell-free extracts of alkylbenzene sulphonate-grown *Bacillus* species. The possibility remains that this oxidation is mediated by an unidentified species-specific electron donor acting in a similar manner to the recorded role of rubredoxin in the terminal oxidation of methyl groups by *Pseudomonas oleovorans* (Peterson *et al.*, 1967).

Some important conclusions can be drawn by combining results from the different experimental approaches employed.

(*a*) The sequence of molecular events resulting in the ring-desulphonation step. The evidence from the continuous-sampling experiment indicates that desulphonation is an early step in the overall biodegradation pattern, but the broad substrate specificity of the desulphonating enzyme induced by growth on undecylbenzene-*p*-sulphonate is indirect evidence that this molecular transformation could occur either before or after partial removal of the alkyl side chain by β-oxidation. However, the results obtained by continuous t.l.c. monitoring of the *Bacillus* species growing on a number of separate alkylbenzene sulphonate homologues indicate that the sole sulphonated molecule detected corresponds, in all cases, to the initial growth substrate. Thus desulphonation of the aromatic ring appears to occur before progressive β-oxidation of the alkyl side chain during growth of the *Bacillus* species on alkylbenzene sulphonate homologues.

(*b*) The sequence of molecular events resulting in the ring-fission step. The evidence from the continuous-sampling experiments indicates that ring fission occurs relatively late in the overall biodegradation pattern. Confirmation at the enzyme level that dihydroxy ring substitution is an essential preliminary to ring fission, combined with the identification of 3,4-dihydroxybenzoate and 3,4-dihydroxyphenylacetate as the only dihydroxy-substituted intermediates present after growth on alkylbenzene sulphonate homologues containing an odd-numbered alkyl side chain and an even-numbered alkyl side chain respectively, is evidence that desulphonation plus total alkyl-side-chain removal both appear to be essential preliminary molecular transformations to aromatic fission in cell-free extracts of alkylbenzene sulphonate-grown *Bacillus* species.

(*c*) A comparison of the t.l.c. sampling patterns after growth on undecylbenzene-*p*-sulphonate, nonylbenzene-*p*-sulphonate, heptylbenzene-*p*-sulphonate, pentylbenzene-*p*-sulphonate and propylbenzene-*p*-sulphonate indicates that the two unidentified $FeCl_3$-positive spots detected after growth on undecylbenzene-*p*-sulphonate are possibly *p*-hydroxy-1-phenylundecane (or the equivalent carboxylic acid, *p*-hydroxy-1-phenylundecanoate) and *p*-hydroxy-1-phenylnonanoate [see (*a*) above]. A similar approach indicates that the two unidentified $FeCl_3$-positive spots recorded after growth on dodecylbenzene-*p*-sulphonate are possibly *p*-hydroxy-1-phenyldodecane (or the equivalent carboxylic acid,



Scheme 1. *Proposed pathway of alkylbenzene sulphonate degradation by the Bacillus species*

p-hydroxy-1-phenyldodecanoate) and *p*-hydroxy-1-phenyldecanoate [see (*a*) above]. No evidence has been recorded for an accumulation of the $FeCl_3$-positive intermediates *p*-hydroxy-1-phenylheptanoate or *p*-hydroxy-1-phenylvalerate after growth on undecylbenzene-*p*-sulphonate. The possibility remains that this phenomenon is associated with a small pool size of these proposed intermediates during the growth of *Bacillus* species on undecylbenzene-*p*-sulphonate.

(*d*) The substrate specificity of the enzyme acyl-CoA synthetase, involved in the biodegradation of the alkyl side chain of alkylbenzene sulphonate homologues, indicates that this enzyme is not specifically induced by growth on alkylbenzene sulphonate homologues, but is associated with the β-oxidation of long-chain alkyl molecules in general, including hydrocarbons and fatty acids.

(*e*) The substrate specificity of the aromatic fission enzymes, as recorded both directly by their selective

induction profiles and indirectly by the oxidation specificity recorded in the manometry experiments, indicates separate enzymes for the biodegradation of *p*-hydroxybenzoate and *p*-hydroxyphenylacetate by alkylbenzene sulphonate-grown *Bacillus* species. The possibility of separating some of these activities remains to be examined directly.

One problem which remains to be further investigated is the aromatic oxidation sequence involved in the biodegradation of 3,4-dihydroxyphenylacetate, the terminal aromatic product of biodegradation of all alkylbenzene sulphonate homologues containing an even number of carbon atoms in the alkyl side chain as evidenced by both g.l.c. and t.l.c. analyses. Evidence from both manometric studies and the enzyme-induction profile indicates the presence of an enzyme system ensuring some further metabolism of this compound.

In a species of *Pseudomonas* in which the metabolism of 3,4-dihydroxyphenylacetate was thoroughly investigated (Adachi *et al.*, 1964) the characterized pathway involved an initial oxygen-dependent '*meta*-type' ring cleavage, and the δ-carboxymethyl-α-hydroxymuconic semialdehyde produced was excreted into the medium without subsequent transformation. The failure of the *Bacillus* species to grow on *p*-hydroxyphenylacetate or 3,4-dihydroxyphenylacetate as the sole carbon source, in contrast to the significant growth recorded with *p*-hydroxybenzoate and 3,4-dihydroxybenzoate as the sole carbon source, is indirect evidence that 3,4-dihydroxyphenylacetate is not completely oxidized by the *Bacillus*. It is also indirect evidence that 3,4-dihydroxyphenylacetate is not subjected to an α-oxidation and subsequently totally oxidized via the '*ortho*-cleavage' pathway metabolizing 3,4-dihydroxybenzoate. The failure of dodecylbenzene-*p*-sulphonate or any other alkylbenzene sulphonate homologue containing an even number of carbon atoms in the side chain to induce significant activities of any of the enzymes of the '*ortho*-cleavage' pathway of 3,4-dihydroxybenzoate catabolism is direct evidence against such a transformation.

Ring fission of benzenesulphonate by a comparable '*ortho*-cleavage' pathway in several elected *Pseudomonas* species has been previously recorded, although other isolated organisms were also reported that accomplished fission of this compound by the alternative '*meta* cleavage' (Cain & Farr, 1968). Sulphite was detected in all cases as an intermediate in sulphonate-group metabolism (Cain & Farr, 1968). However, a recent report identified sulphate, but failed to detect sulphite, as a metabolite of benzenesulphonate by washed cell suspensions of *Pseudomonas testosteroni* H-8 (Ripin *et al.*, 1971). The comparability of the results obtained with *P. testosteroni* H-8 with those obtained with the *Bacillus* species must remain in some doubt, as the *Pseudomonas* species was unable to oxidize any alkylbenzene sulphonate homologues containing more than two carbon atoms in the alkyl side chain.

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