Orthanilic Acid and Analogues as Carbon Sources for Bacteria: Growth Physiology and Enzymic Desulphonation

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Carbon-limited aerobic batch enrichment cultures were grown and 17 bacteria able to degrade orthanilic acid (2-aminobenzenesulphonic acid), sulphanilic acid, sulphonamide, 4-sulphobenzoic acid, and benzene-, toluene- and phenolsulphonic acids were isolated. The organisms could each use one to three of the substances. Strain O-1, a *Pseudomonas* sp., which utilized three of these compounds, was studied in detail. A complete mass balance was obtained for the growth of the organism in medium containing, for example, orthanilic acid, and a specific growth rate of 0.1 h^{-1} was observed. Cell extracts desulphonated six aromatic sulphonates. The enzyme(s) was soluble and was not synthesized in succinate-grown cells. Enzyme activity [about $40 \mu\text{kat} (\text{kg protein})^{-1}$] was dependent on the presence of catalytic amounts of NAD(P)H.

INTRODUCTION

Sulphonated aromatic compounds are major pollutants of rivers and lakes. About 10% of the organic load in the river Rhine is reported to be due to this class of compound (Malle, 1978). These substances can come from detergents, from pulp mill wastes and from the manufacture of dyestuffs, but documentation on biodegradation is as yet largely limited to information on detergents (Cain & Farr, 1968; Focht & Williams, 1970; Ripin *et al.*, 1971; Endo *et al.*, 1977; Belly & Harbison, 1980).

We observed 4-aminobenzenesulphonate as the putative undegraded product from the aerobic bacterial degradation of two azo dyestuffs (Zimmermann *et al.*, 1982, 1984). Intracellularly formed 4-aminobenzenesulphonate is excreted into the medium, where it accumulates (Kulla *et al.*, 1983). Since 4-aminobenzenesulphonate therefore appears resistant to microbial degradation, we initiated enrichments for micro-organisms that degrade this compound and other typical 1-ring sulphonated compounds in wastes from the dyestuff industry. We now report degradation of several aromatic sulphonates and their desulphonation by cell-free preparations.

METHODS

Materials. The aromatic sulphonates used (Tables 1 and 2) were from Fluka except for 4-sulphobenzoic acid, which was synthesized in the Department of Chemical Engineering and Industrial Chemistry, and 3-aminobenzenesulphonic acid, which was purchased from Eastman. The compounds were chromatographically pure and their identity was confirmed by comparing UV spectra with literature data (Weast, 1983; Sadtler, 1975) or by calculating the spectrum from basic principles (Pretsch *et al.*, 1976). All other chemicals were of reagent grade or better.

Apparatus and analyses. Spectrophotometric analyses were done with a Kontron 800 or a Bausch & Lomb Spectronic 88 spectrophotometer, each equipped with a flow-through cell of 1 cm path length. Optical density was

measured in an Eppendorf photometer. High pressure liquid chromatography (HPLC) was done with a Du Pont apparatus and a UV detector (Grossenbacher *et al.*, 1985). The aromatic sulphonates were determined by HPLC with reversed phase columns (Cook *et al.*, 1983*a*) and 100 mM-potassium phosphate buffer, pH 7.0 (or pH 2.0 for succinate and 4-sulphobenzoic acid), as the mobile phase (cf. Jandera & Churacek, 1980). The wavelength setting in the detector was 220 nm for aromatic compounds and 205 nm for succinate. Sulphite (Grant, 1947; as in the modification of Kondo *et al.*, 1982), sulphate (Johnston *et al.*, 1975) and ammonium ion (Weatherburn, 1967) were measured by routine colorimetric methods. The identity of the ammonium ion was confirmed by further reactions (Cook & Hütter, 1981). Growth was quantified as protein (Cook & Hütter, 1981); 10 µg protein ml⁻¹ was the limit of detection under these conditions. Dissolved organic carbon was assayed by standard methods (Greenberg *et al.*, 1981).

Growth medium and the isolation of organisms. The growth medium was a buffered (10 mM-potassium phosphate, pH 7.0) salts solution to which trace elements (Pfennig & Lippert, 1966; the sulphate anion was replaced by chloride, which necessitated preparing the solution in two soluble portions) and a single carbon source were added. All enrichment cultures contained 20 mM-NH₄Cl and 0.25 mM-MgSO₄ but in some experiments in pure culture, the inorganic source of nitrogen and/or sulphur was eliminated. The complete medium was autoclaved. The resulting solution was clear and no loss of sulphonate was observed.

Aerobic batch enrichment cultures (50 ml, non-sterile in shaken 500 ml Erlenmeyer flasks) containing about 30 mM-carbon were used to obtain isolates able to utilize a given aromatic sulphonate as a sole source of carbon and energy for growth. Inocula for enrichments were prepared from three groups of sewage: (a) ten small sewage works receiving largely rural and domestic wastes, (b) eight large sewage works receiving largely domestic but also industrial wastes, and (c) two industrial sewage works used for wastes containing sulphonates. Cultures were evaluated (optical density and substrate disappearance) after about a week and transferred to fresh homologous medium as appropriate. After three subcultures, positive enrichments were streaked on Plate Count agar plates (Difco) and a representative of each colony type was inoculated into appropriate sterile selective medium. When an isolate which degraded the aromatic sulphonate gave rise to a single colony type three successive times on Plate Count agar, it was considered pure and was transferred and maintained in a screw-cap tube on a slant of selective medium solidified with 1.5% (w/v) agar. Limited taxonomy was done to ascertain that the strains were unlikely to be pathogens (Krieg & Holt, 1984) and Oxiferm Tubes (Roche) were used.

Quantification of growth and substrate utilization. Bacterial growth yields with limiting carbon sources were measured at 30 °C with 50 ml cultures in 500 ml Erlenmeyer flasks mounted on an orbital shaker (2–3 revolutions s⁻¹). Media containing different carbon concentrations were inoculated (0.3%, v/v) and samples (each 5 ml) for protein and substrate concentrations were taken immediately and at the end of growth (i.e. exhaustion of substrate). Samples for substrate determination were centrifuged (23 000 g for 20 min at 4 °C) and the supernatant fluid was stored frozen in a screw-cap vial.

Growth kinetics with 3 mM-substrate were followed at 30 °C in 300 ml cultures in 2 litre Fernbach flasks. Inocula were exponentially growing cultures in homologous medium. Despite the fact that 3 mM-sulphate was released during the experiment, exogenous sulphate (0.25 mM) had to be provided to allow growth. Samples were taken at intervals to measure protein, and substrate and inorganic ions. Sulphite had to be measured immediately, because it oxidized spontaneously to sulphate during storage. Material for other tests was stored frozen.

Preparation of cell extracts and enzyme assay. Cells were grown in 1 litre portions of medium in 2 litre Fernbach flasks and harvested (10 000 g for 20 min at 4 °C) during exponential growth because the specific activity of desulphonation decreased in the stationary phase. They were washed twice in 0.9% NaCl solution and the resulting pellet could be stored frozen although there was a loss of activity (about 10%) on each thawing. The pellet was suspended at 200 mg (wet wt) per ml of chilled 50 mM-potassium phosphate buffer, pH 7.0, and cells were disrupted by three passages through a French press (Aminco) at about 120 MPa. The suspension of disrupted cells was centrifuged at 30 000 g for 1 h at 4 °C, and the supernatant fluid was used for assays of enzyme activity either directly or after desalting on a column of Sephadex G-25 (PD 10; Pharmacia).

Enzyme assays were routinely done in 5 ml reaction mixtures, which were shaken at 30 °C in a water bath. They contained 250 µmol potassium phosphate buffer, pH 7.0, 2.5 µmol substrate, 0.5 µmol NADH (unless otherwise stated), and the reaction was started by the addition of about 8 mg protein. Samples (0.5 ml) were taken at intervals, acidified (trichloroacetic or perchloric acid to a final concentration of 0.5 M), and protein was removed by centrifugation (10 000 g for 2 min at ≤ 4 °C). Sulphite was assayed in 0.4 ml portions of the trichloroacetic acid supernatant fluid. Substrate disappearance was determined by HPLC after removal of perchloric acid as the potassium salt. Under these conditions substrate disappearance and sulphite liberation proceeded linearly up to an incubation time of 20 min. No substrate disappeared or gave rise to sulphite in the absence of enzyme and no sulphite or interference in HPLC determinations was observed in cell extracts. Experiments under anaerobic conditions were done as described by Cook *et al.* (1984) with the additional control that reaction mixtures were confirmed to be active after re-addition of oxygen.

Table 1. Growth and utilization of sulphonated aromatics by pure cultures

Carbon source	Substrate spectrum of isolate:								
	B-1	O-1	O-2	S-1	S-3	P-2	P-3	T-2	PSB-4
Benzenesulphonate	+	+	—	—	—	—	+	—	—
2-Aminobenzenesulphonate	—	+	+	—	—	—	—	—	—
3-Aminobenzenesulphonate	—	—	—	—	—	—	—	—	—
4-Aminobenzenesulphonate	—	—	—	+	+	—	—	—	—
4-Hydroxybenzenesulphonate	—	—	—	+	—	+	+	—	—
4-Methylbenzenesulphonate	+	+	+	—	—	—	—	+	—
4-Sulphobenzoate	—	—	—	—	—	—	—	+	+
4-Aminobenzenesulphonamide	—	—	—	—	+	—	—	—	—

RESULTS AND DISCUSSION

Enrichment and isolation of organisms

Enrichment cultures were prepared, each of which contained one of three different inocula and one of eight sulphonates. All the sulphonates were observed to be chemically stable. From these enrichments several pure cultures were isolated (Table 1). The inoculum derived from eight small rural sewage works yielded no enrichments whereas that from ten large municipal centres yielded eight enrichments and the inoculum from two sewage plants for sulphonated chemical wastes yielded nine cultures. Six of the eight compounds listed in Table 1 yielded enrichments; 3-aminobenzenesulphonate and 4-aminobenzenesulphonamide did not, although one pure culture from another enrichment was able to utilize the latter compound (Table 1). Pure cultures (17, all bacteria) were isolated, each of which quantitatively utilized the carbon and energy source originally present in the enrichment. Nine different substrate spectra were observed (Table 1); several taxonomically independent strains had the same substrate spectrum.

Although biodegradation of substituted benzenesulphonates can be easily obtained by direct batch enrichment from native samples (Table 1), organisms degrading these compounds are not uniformly distributed in the environment; they appear to occur where evolutionary pressure can be expected, i.e. in wastes where such compounds are continuously available. Some of these degradative activities are already known (Cain & Farr, 1968; Focht & Williams, 1970; Ripin *et al.*, 1971) but this is the first report of growth with 2-aminobenzenesulphonate, 4-aminobenzenesulphonate or 4-sulphobenzoate as sole source of carbon in pure culture.

The isolates tended to show a narrow substrate spectrum for the compounds tested, but seven of the eight substrates tested could be degraded by at least some of the isolates (only 3-aminobenzenesulphonate was not utilized). Six strains utilized only one compound (that on which they were enriched), ten strains could utilize two substrates and one isolate (O-1) could utilize three substrates (Table 1). We then concentrated our work largely on strain O-1, in part because of its substrate spectrum and in part because of the ready extractability of its ring desulphonation activity.

Strain O-1 was a Gram-negative, oxidase positive, motile rod which grew well near pH 7 and utilized acetate as a carbon source. These and other characteristics (reactions in Oxiferm Tubes and polar monotrichous flagellum) allowed its identification as a strain of *Pseudomonas* (Krieg & Holt, 1984).

Growth physiology and enzymic desulphonation

Growth of strain O-1 was proportional to the concentration of substrate supplied (up to 36 mM-carbon) and yields of 5.9, 6.7, 5.6 and 6.0 g protein (mol carbon)⁻¹ were observed for succinate, and benzene-, 2-aminobenzene- and 4-methylbenzenesulphonate, respectively. These yields are in the normal range for heterotrophic utilization of a carbon source (Cook *et al.*, 1983b), whether the carbon source is a natural product or an aromatic sulphonate, and the negligible dissolved organic carbon after growth confirmed that the substrate was completely converted to cell material and CO₂.

Fig. 1. Growth of *Pseudomonas* sp. strain O-1 with 2-aminobenzenesulphonate as sole carbon and energy source. ●, Protein; ○, 2-aminobenzenesulphonate; □, sulphate; ■, sulphite; △, ammonium ion.

Cultures of strain O-1 grew exponentially with benzenesulphonate ($\mu = 0.2 \text{ h}^{-1}$), 2-aminobenzenesulphonate ($\mu = 0.1 \text{ h}^{-1}$) or 4-methylbenzenesulphonate ($\mu = 0.07 \text{ h}^{-1}$) as sole source of carbon and energy. Representative results with 2-aminobenzenesulphonate are shown in Fig. 1. Substrate utilization was complete and concomitant with growth. Correspondingly, the sulphonate group was released quantitatively and recovered as sulphate. During growth, high transient accumulation of sulphite was observed. Ammonium ion was released from 2-aminobenzenesulphonate during growth and the balance of the nitrogen was calculated to be in cell material (cf. Cook *et al.*, 1983b).

The newly-available determinations by HPLC allow direct determinations of substrate utilization to aid in the mass balance and confirm complete utilization of the appropriate compound. These results indicate that it is feasible to treat biologically the aromatic sulphonates in wastes from chemical syntheses.

Crude extracts from strain O-1 were prepared by disruption of whole cells in a French press (disruption by sonication was also possible). The enzyme activity was observed only in the supernatant fluid (30000 g or 100000 g) and not in either particulate fraction. The specific activity in this unoptimized enzyme assay (Table 2) was lower than the 0.5–1.5 mkat (kg protein) $^{-1}$ calculated for growing cells. Extracts from cells which had been grown with aromatic sulphonates desulphonated many substrates, whereas extracts from succinate-grown cells had no activity (Table 2). Benzoate-grown cells had low desulphonation activities for three substrates only. The capacity for desulphonation is clearly therefore inducible and the different ratios of activities observed after growth on different substrates imply the presence of more than one desulphonating enzyme. We do not know yet whether lack of transport systems or lack of degradative pathways or some other factor causes the number of growth substrates to be lower than the number of substrates for the desulphonating enzyme(s).

The range of compounds subject to desulphonation by active extracts included the three growth substrates as well as three substrates on which the strain was unable to grow. Thus 3-aminobenzenesulphonate, which was not utilized as a carbon source by any strain (Table 1), was desulphonated by the cell extract. But whereas 4-aminobenzenesulphonate was a good substrate for desulphonation, neither the corresponding amide nor the naphthalene analogue was degraded (Table 2). Aromatic sulphonates were converted stoichiometrically to sulphite, which was oxidized very slowly to sulphate in cell extracts; the organic product(s) has not been identified.

Desulphonation by cell extracts was observed to have a cofactor requirement. The removal of small molecules from the crude extract by chromatography on Sephadex G-25 eliminated the activity [27 $\mu\text{kat (kg protein)}^{-1}$], which could be restored by the addition of 0.1 mM-NAD(P)H,

Table 2. Enzymic desulphonation observed in cell-free extracts of *Pseudomonas* sp. strain O-1

Substrate in enzyme assay	Desulphonation [$\mu\text{kat (kg protein)}^{-1}$] observed in extracts from cells grown on:				
	Succinate	Benzoate	Benzene-sulphonate	2-Amino-benzene-sulphonate	4-Methyl-benzene-sulphonate
Benzenesulphonate	<1	8	100	40	70
2-Aminobenzenesulphonate	<1	1	30	30	60
3-Aminobenzenesulphonate	<1	<1	30	5	30
4-Aminobenzenesulphonate	<1	<1	40	8	20
4-Hydroxybenzenesulphonate	<1	<1	70	20	40
4-Methylbenzenesulphonate	<1	2	80	20	90
4-Aminobenzenesulphonamide	<1	<1	<1	<1	<1
1-Amino-4-sulphono-naphthalene	<1	<1	<1	<1	<1

though NADH gave a threefold higher specific activity [$40 \mu\text{kat (kg protein)}^{-1}$]. No other cofactor tested [NAD(P), FAD] replaced NAD(P)H. NAD(P)H functioned catalytically, because 0.1 mM-cofactor sufficed for the desulphonation of 3 mM-substrate. No desulphonation occurred under anoxic conditions, so we presume the involvement of (di)oxygenases.

Enzymic ring desulphonation is still a rarity. Brilon *et al.* (1981) elucidated a mechanism for the desulphonation of naphthalene sulphonates but obtained no cell-free desulphonation. Two groups (Willettts & Cain, 1972; Heyman & Molof, 1968) observed desulphonation in crude extract and Kondo *et al.* (1982) reported cell-free desulphonation of benzenesulphonate in separated fractions. Our cell-free extracts have similarities to the NAD(P)H-coupled dioxygenase described by Gibson *et al.* (1982).

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