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Initial steps in the degradation of benzene sulfonic acid, 4-toluene sulfonic acids, and orthanilic acid in *Alcaligenes* sp. strain O-1

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Abstract

Alcaligenes sp. strain O-1 grew with benzene sulfonate (BS) as sole carbon source for growth with either NH_4^+ or NH_4^+ plus orthanilate (2-aminobenzene sulfonate, OS) as the source(s) of nitrogen. The intracellular desulfonative enzyme did not degrade 3- or 4-aminobenzene sulfonates in the medium, although the enzyme in cell extracts degraded these compounds. We deduce the presence of a selective permeability barrier to sulfonates and conclude that the first step in sulfonate metabolism is transport into the cell. Cell-free desulfonation of BS in standard reaction mixtures required 2 mol of O_2 per mol. One mol of O_2 was required for a catechol 2,3-dioxygenase. When *meta* ring cleavage was inhibited with 3-chlorocatechol in desalted extracts, about 1 mol each of O_2 and of NAD(P)H per mol of BS were required for the reaction, and SO_3^{2-} and catechol were recovered in high yield. Catechol was shown to be formed by dioxygenation in an experiment involving $^{18}O_2$. 4-Toluene sulfonate was subject to NAD(P)H-dependent dioxygenation to yield SO_3^{2-} and 4-methylcatechol, which was subject to *meta* cleavage. OS also required 2 mol of O_2 per mol and NAD(P)H for degradation, and SO_3^{2-} and NH_4^+ were recovered quantitatively. Inhibition of ring cleavage with 3-chlorocatechol reduced the oxygen requirement to 1 mol per mol of OS SO_3^{2-} (1 mol) and an unidentified organic intermediate, but no NH_4^+, were observed.

Introduction

Several naphthalene sulfonates (Brilon et al. 1981b; Nörtemann et al. 1986; Wittich et al. 1988) and benzene sulfonates (Feigel & Knackmuss 1988; Locher et al. 1989b; Thurnheer et al. 1986, 1988) are totally degraded as sole or mixed carbon sources in pure and in defined mixed cultures of bacteria. Wider ranges of compounds can be desulfonated by bacteria (Zürrer et al. 1987) and possibly by algae (Luther & Soeder 1987). Two groups of degradative pathways can be distinguished: firsly, those with desulfonation as the first (or sole) catabolic reaction (Brilon et al. 1981a; Luther & Soeder 1987; Nörtemann et al. 1986; Swisher 1987; Zürrer et al. 1987; cf. Wittich et al. 1988), and secondly, those with reactions prior to desulfonation (Feigel & Knackmuss 1988; Locher et al. 1989b; cf. Wittich et al. 1988). Nevertheless, very little is known about desulfonation reactions, and only two workers have obtained desulfonation rates above the trace level of 1 to 10 μ kat per kg of protein in cell-free systems (Locher et al. 1989b; Thurnheer et al. 1986). One of the latter cases involves the

degradation of benzene sulfonate (BS), 4-toluene sulfonate (TS) and the dyestuff precursor, orthanilate (OS; 2-aminobenzene sulfonate) in *Alcaligenes* sp. strain O-1 (Thurnheer et al. 1986). Furthermore, the transport of these compounds has received little attention (cf. Leidner et al. 1980), though one may anticipate transport systems for these highly polar compounds, and a transport system for a sulfate ester has been reported (Reinert & Marzluf 1974).

We now report evidence for a selective permeability barrier to some aromatic sulfonates in *Alcaligenes* sp. strain O-1, for cell-free dioxygenation and desulfonation as the first metabolic reaction of BS, TS and OS, and for *meta* ring cleavage.

Materials and methods

Materials

The sources of the aromatic sulfonates were given elsewhere (Thurnheer et al. 1986). Benzene dihydrodiol was a gift from Prof. D.T. Gibson. 3-Chlorocatechol was kindly provided by Prof. W. Reineke. 3-Aminoveratrol was a present from Prof. H.-J. Knackmuss. 4-Nitrocatechol was from Fluka (Buchs, Switzerland); other chemicals were of the highest purity available commercially. Sephadex G-25 in PD-10 columns (Pharmacia, Upp-sala, Sweden) was used.

Apparatus and analyses

Spectrophotometric and optical density measurements (Thurnheer et al. 1986), reversed phase HPLC (Grossenbacher et al. 1986; Locher et al. 1989b), oxygen uptake measurements (Locher et al. 1989b; Zamanian & Mason 1987), capillary GC and GC-MS (Locher et al. 1989b) were done with apparatus described previously. Protein in whole cells or in extracts was measured by Lowry-type assays, and routine colourimetric tests were used for sulfate (Johnston et al. 1975) and sulfite (Grant 1947; Kondo et al. 1982).

Organisms, growth medium and enzyme assays

Most experiments were done with *Alcaligenes* sp. strain O-1 (Thurnheer et al. 1986; Thurnheer et al. 1988); the taxanomy of the organism is discussed elsewhere (Jahnke et al. 1990). *Pseudomonas putida* strains mt2 (ATCC 23793), F1 and G7 were used; the latter were kindly provided by Profs. D.T. Gibson and H.-J. Knackmuss, respectively. Defined salts-medium (Thurnheer et al. 1986) was used for growth of all organisms. Cultures were harvested in the late exponential phase, washed twice and stored frozen (Thurnheer et al. 1986). Experiments with cells in suspension were done at 5 mg of cell-protein per ml. Suspensions of cells were disrupted in a French pressure cell and the supernatant fluid after centrifugation was used for analyses (Thurnheer et al. 1986).

The optimized assay for desulfonation of OS in orthanilate-grown cells was routinely done at 30° C in 5 ml reaction mixtures (initial volume), from which samples were taken at intervals for the determination of organic and inorganic compounds. The mixture contained 250 μmol Tris-HCl buffer, pH 7.2, 7.5 μmol OS, 0.75 μmol NADH and 30 mg of protein, with which the reaction was started. Most experiments in the oxygen electrode were done with 5 ml reaction mixtures and were started by the addition of 0.25 μmol of aromatic substrate. Some work was done in 1 ml reaction mixtures (cf. Zamanian & Mason 1987) which contained 50 μmol Tris-HCl buffer, pH 7.5, 2 mg of protein, 1 to 3 nmol 3-chlorocatechol, 250 nmol sulfonoaromatic substrate and was started by additions of 20 nmol of NADH; the additions of chlorocatechol were just enough to titrate out the activity of catechol 2,3-dioxygenase, which allowed the NAD(P)H requirement of the desulfonation reaction to be measured when desalted extract was used.

Experiments with $^{18}O_2$ were done at 30° C in 10-ml reaction mixtures contained in 125 ml flasks which were gassed with nitrogen, evacuated and filled with mixtures of nitrogen and isotopes of oxygen (cf. Locher et al. 1989b). The reaction mixtures contained 450 μmol potassium phosphate

buffer, pH 7.2, whole cells (60 mg of protein), 20 μmol of 3-chlorocatechol, and 30 μmol of BS, with which the reaction was started. The reaction was monitored by HPLC, and when BS was exhausted, cells were removed by centrifugation (30,000 g, 20 min, 4° C) and catechol was extracted from the supernatant fluid in two 5-ml portions of diethyl ether, which was evaporated off. The residual material was dissolved in 0.5 ml of methanol and examined directly by GC and GC-MS (Locher et al. 1989b).

Catechol 1,2-dioxygenase (Hegemann 1966), catechol 2,3-dioxygenase (and 4-methylcatechol 2,3-dioxygenase) (Cain & Farr 1968), *cis, cis* muconate lactonizing enzyme (Hegemann 1966), 2-hydroxymuconate semialdehyde hydrolase and 4-oxalcrotonate dehydrogenase (Sala-Trepat & Evans 1971) were assayed by standard methods. The behaviour of protocatechuate and other compounds in crude extract was followed by HPLC, analogous to the assay for desulfonation of OS.

Synthesis of 3- and 4-aminocatechol

Catechol was acetylated to 1-O-acetylcatechol, which was nitrated (Schulz & Hecker 1973). Products (10 g) were applied to a column (60 by 8 cm) of silica gel (60–80 mesh) and 3-nitro-1-O-acetylcatechol (500 mg) was eluted with a mixture of hexane and ether (2 : 1 v/v) as the first of many products. This intermediate was converted to 3-nitrocatechol (81% yield), which was reduced to 3-aminocatechol hydrochloride (39% yield) (Schulz & Hecker 1973), whose identity was confirmed by ^{13}C-NMR (147.68, 141.40, 120.65, 119.78, 116.73 and 114.88). 4-Nitrocatechol was reduced to 4-aminocatechol, whose identity was confirmed by ^{13}C-NMR (149.65, 149.15, 125.06, 118.71, 116.77 and 113.01).

Results

Selective permeability of the cell membrane for sulfonates

Strain O-1 can utilize only three benzene sulfonates for growth (BS, TS and OS), whereas cell extracts can desulfonate at least seven substrates (Thurnheer et al. 1986), so we suspected that there was selective permeability of the cell membrane towards benzene sulfonates. This idea was supported when non-growing suspensions of cells from BS-salts medium failed to desulfonate the four non-growth substrates for the enzyme (4-hydroxybenzene sulfonate, 4-sulfobenzoate, metanilate [3-aminobenzene sulfonate] and sulfanilate [4-aminobenzene sulfonate], each of 6 mM). Cultures of strain O-1 were then grown in N-limited BS-salts medium containing different potential N-sources. The enzyme(s) catalyzing desulfonation were thus present, and growth, shown here as substrate disappearance, was rapid if 2 mM ammonium ion was the source of nitrogen (Fig. 1a). Growth was equally rapid if 50% of the ammonium ion was replaced by OS, which also disappeared (Fig. 1b), and which was utilized for growth. If orthanilate was replaced by sulfanilate or metanilate (Fig. 1c, d), the latter compounds were not desulfonated, though the intracellular enzyme was active on BS for at least eight days. We presume that the cell membrane of strain O-1 is impermeable to aromatic sulfonates and contains one or more transport systems, each specific for three or less aromatic sulfonates.

Desulfonation of benzene sulfonate and p*-toluene sulfonate, and ring cleavage of the reaction products*

Conditions for the desulfonation of BS in extracts of OS-grown cells were optimized for substrate, cofactor and buffer concentrations, for temperature, pH and for the nature of the buffer (not shown). A specific rate of about 160 μkat per kg of protein was obtained (at 5 mg of protein per ml of reaction mixture), whether measured as substrate

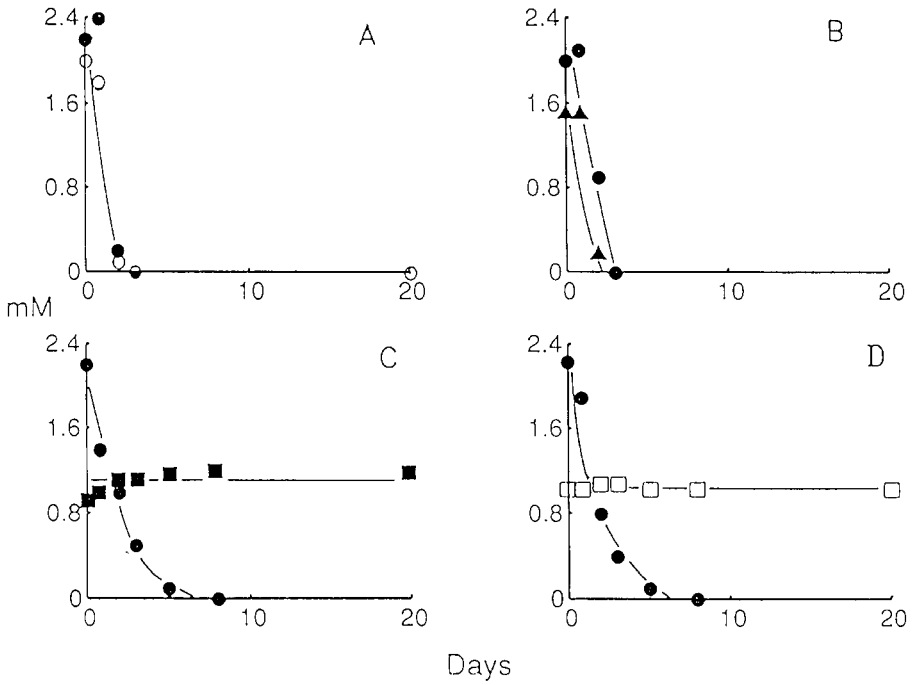

Fig. 1. Concentrations of benzene sulfonate(s) and the nitrogen source(s) in cultures of *Alcaligenes* sp. strain O-1 growing in nitrogen-limited BS-salts medium containing different potential sources of additional nitrogen. The organism was pregrown in BS-salts medium and growing cells were washed in nitrogen-free salts-medium and used as inoculum (1% v/v) for these experiments. BS (●), OS (▲), metanilate (■) and sulfanilate (□) were present where shown; ammonium (○) was present in each reaction, but was degraded within two days in each case and is usually not shown because so many data points overlap.

disappearance or sulfite release, some fourfold higher than non-optimized rates (cf. Thurnheer et al. 1986). Desulfonation showed an absolute requirement for molecular oxygen (Thurnheer et al. 1986). Allowing 1 mol of O_2 per mol for ring cleavage (see below), 1 mol of O_2 was consumed per mol of BS desulfonated (Table 1, 'Uptake of O_2); left hand column). Desulfonation also showed an absolute requirement for NAD(P)H: NADH was used routinely in reaction mixtures because the rate was some twofold higher than with NADPH. NADH was required in catalytic amounts only, so an efficient recycling of NADH must be available in the system.

No organic products from BS were observed in the standard reaction mixtures, but we presumed catechol to be involved because of the very high levels of catechol 2,3-cleavage induced under these conditions (22-fold higher than the desulfonation; Table 1). The possibility that benzene dihydrodiol might be involved as an intermediate in the degradation of BS was rendered unlikely, because this dihydrodiol was not transformed by strain O-1 whereas a high oxidation rate was observed with *P. putida* F1 grown in benzene-salts medium. We were unable selectively to inhibit ring cleavage with α,α′-dipyridyl, H_2O_2, *o*-nitrophenol, *o*- or *m*-phenathroline or pyridine, but attained non-reversible inhibition with 3-chlorocatechol (cf. Bartels et al. 1984; Klecka & Gibson 1981). We thus had preliminary evidence for suicide inactivation of a catechol 2,3-dioxygenase, and we used this effect to study the desulfonation in the absence of the subsequent metabolic reactions.

BS, in reaction mixtures containing 3-chlorocatechol, was converted to a compound which co-chromatographed (HPLC) with authentic catechol, and whose identity as catechol was confirmed by its spectral properties (Table 2), by co-chromatography (GC) with authentic material and by GC-

MS (see below). The yield of product was 90–95%, and under these conditions only 1 mol of O_2 per mol of BS was consumed (Table 1). Concomitantly, 1 mol of sulfite per mol of BS was released. The stoichiometry of the requirement for NADH had to be examined in desalted extract, because small molecules in crude extract sufficed to allow transformation of about 1 mM OS to sulfite. Desalted extract containing inhibited catechol 2,3-dioxygenase and an excess of BS utilized about 0.8 mol of O_2 per mol of NADH. Our data indicate that, during desulfonation, 1 mol each of BS, O_2, and NAD(P)H are converted to 1 mol each of sulfite, catechol and $NAD(P)^+$.

The catechol produced from BS in the presence of $^{16}O_2$ or $^{16}O_2 + {}^{18}O_2$ was examined by GC-MS. In the presence of $^{16}O_2$, a molecular ion of m/z = 110 was observed, and the fragmentation pattern ($M^+ - H_2O$, $M^+ - CHO$, $M^+ - H_2O - CO$, $M^+ - CHO - CO$) confirmed the identity of the catechol. When the mixture of isotopes was used, two molecular ions were observed, at 110 and 114. There was no significant peak at 112. These data confirm that desulfonation is catalyzed by dioxygenation.

Degradation of catechol had an absolute requirement for stoichiometric amounts of molecular oxygen (1 mol per mol of catechol; Table 1). The product was yellow (near pH 7) and co-chromatographed (HPLC) with, and was spectrally identical to authentic 2-hydroxymuconate semialdehyde (Table 2). No *ortho* cleavage or *cis,cis*-muconate lactonizing enzyme was observed (Table 1), though we could assay both activities in *P. putida* G7. 2-Hydroxymuconate semialdehyde was subject to a low rate of transformation in desalted extracts of strain O-1 (Table 1), but was degraded much more rapidly on the addition of NAD^+ (Table 1). We interpret these data as low activity of a 2-hydroxymuconate semialdehyde hydrolase and higher activity of 4-oxalcrotonate dehydrogenase and that the latter is the source of NADH for continued rapid desulfonation in the presence of sub-stoichiometric amounts of NADH (see above). Strain O-1 thus catalyzes *meta* ring cleavage of catechol, which is degraded largely by the oxalcrotonate branch of the *meta* pathway.

Table 1. Specific activities of selected enzymes in, and oxygen uptake by, extracts of *Alcaligenes* sp. strain O-1 grown in orthanilate-salts medium.

Substrate	Enzyme reaction	Specific activity in cell extracts (μkat kg^{-1} of protein)	Uptake of O_2[a] (mol mol^{-1} substrate)	
			3-Chlorocatechol	
			absent	present
Benzene sulfonate	Desulfonation	160	2.1	1.0
p-Toluene sulfonate	Desulfonation	90	2.2	1.0
Orthanilate	Desulfonation	160	1.9	1.0
Benzene dihydrodiol[b]	Dehydrogenase	< 10		
Catechol	2,3-Dioxygenase	3600	1.0	0.0
2-Hydroxymuconate semialdehyde	Hydrolase	80	na[c]	na
	Dehydrogenase	220	na	na
Catechol	1,2-Dioxygenase[d]	< 10	na	na
cis,cis-muconate	Lactonization	< 10	na	na
4-Methyl-catechol	2,3-Dioxygenase	4800	1.0	0.0

[a] These data are from extracts of OS-grown cells, but the same values were observed with extracts from BS-grown cells. Extracts of TS-grown cells displayed a very high rate of endogenous respiration and were not used. The data represent the mean of 3 values; SD 6%.

[b] This activity was measured in whole cells only.

[c] na: no assay

[d] This activity was tested when the *meta* enzyme was inhibited. In the absence of inhibitor (3-chlorocatechol) an artefact due to the overlap of the UV-spectra of the semialdehyde product of *meta* cleavage, and of the muconate product of *ortho* cleavage, led to the apparent presence of the catechol-1,2-dioxygenase.

Strain O-1 degraded benzoate via 2-hydroxymuconate semialdehyde, which was identified as described in the previous paragraph. In contrast to work with desulfonation, however, it was very difficult to obtain the catechol 2,3-dioxygenase from benzoate-grown cells in cell-free extract, where this enzyme activity was unstable. We presume there to be isofunctional catechol 2,3-dioxygenases in this organism. The presence of benzoate did not cause any detectable oxygen uptake in extracts of orthanilate-grown cells, whereas sulfonates were readily oxygenated. Benzoate was thus not a substrate for the desulfonation system. In contrast, naphthalene carboxylate is a substrate for cells of *Pseudomonas* sp. strain A3 able to desulfonate naphthalene sulfonates (Brilon et al. 1981b).

The degradation of TS was analogous to that of BS. 1 mol each of TS, NAD(P)H and O_2 were converted to about 1 mol each of 4-methylcatechol (Table 2), sulfite and, presumably, $NAD(P)^+$. Methylcatechol was subject to *meta* ring cleavage (Table 2) and the semialdehyde was presumably degraded largely via a methyl-oxalcrotonate. No protocatechuate dioxygenase was observed, so we presume all methylcatechol to be degraded directly via *meta* ring cleavage.

All experiments in this section were done with cell extracts, unless otherwise indicated (Table 1). These experiments were repeated with whole cells (data not shown), which differed from cell extracts only in releasing the sulfonate moiety as the sulfate and not the sulfite ion.

Table 2. Data from the UV-spectra of substrates and intermediates observed during degradation of aromatic sulfonates by *Alcaligenes* sp. strain O-1. Compounds in reaction mixtures were separated by reversed-phase HPLC (i.e. the most polar first) with 100 mM potassium phosphate, pH 2.2, as the mobile phase (unless otherwise stated) and the spectra were recorded with a diode array detector.

Substance	T_R[a] (min)	Data from UV-spectra (nm)[b]						
		max	min	max	min	max	min	max
Benzene sulfonate	16.9	*193*	202	211				
cis-Benzene dihydrodiol	>30	199	225			*259*		
Catechol (A)[c]	>30	*189*		217s	245	276		
Catechol (B)[d]	>30	*189*		217s	245	276		
2-Hydroxymuconate semialdehyde (A)[e]	5.6	*200*	212	220			292	374
2-Hydroxymuconate semialdehyde (B)[e]	5.6	*200*	212	220			292	374
p-Toluene sulfonate	>30	*196*	206	220				
4-Methylcatechol (A)	>30	*200*			240	280		
4-Methylcatechol (B)	>30	*200*			240	280		
2-Hydroxy-methyl-muconate semialdehyde (A)[e]	9.0	*205*					300	383
2-Hydroxy-methyl-muconate semialdehyde (B)[e]	9.0	*205*					300	383
Orthanilate	9.4	*193*	199	203	226	237	263	298
Desulfonated orthanilate (B)	8.9	*200*		224s	236	286		
3-Aminocatechol (A)	4.6	*202*		216s	243	272		
4-Aminocatechol (A)	4.1	*197*		214s	245	276		
1,2,3-Trihydroxy-benzene (A)	13.2	*199*		223s				
1,2,4-Trihydroxy-benzene (A)	9.9	*195*		224s	236	288		
4-Aminoresorcinol (A)	4.8	*197*		216s	241	273		
2-Aminophenol (A)	7.3	*192*	202	213	235	269		
3-Aminophenol (A)	6.2	*192*	205	215	238	270		

[a] Values over 30 min are less accurate and were used here only to facilitate comparisons of UV-spectra; routine determinations involved isocratic conditions with methanol in the mobile phase or gradient elutions.

[b] The value in italics represents the highest molar absorbance. The use of the addendum 's' indicates a shoulder.

[c] A, Authentic material.

[d] B, Biological product in an enzyme preparation: catechol was derived from BS, methylcatechol from TS and the semialdehydes from the appropriate catechol.

[e] In this case the mobile phase was 10 mM potassium phosphate buffer, pH 6.7.

Desulfonation of orthanilate and ring cleavage of the reaction product

The rate of desulfonation of OS in extracts of OS-grown cells was about 160 μkat per kg of protein, under our standard conditions, whether measured as substrate disappearance, sulfite ion formation or release of ammonium ion, and was thus some 23% of the rate observed in growing cells (0.7 mkat/kg of protein; Thurnheer et al. 1986). In contrast to the behaviour of e.g., catechol 2,3-dioxygenase (Fig. 2, inset), the specific activity of the desulfonative enzyme(s) was not independent of the protein concentration when the standard assay was used (Fig. 2). This observation was confirmed with data from an oxygen electrode, which was a more sensitive assay and showed a higher specific activity such that, at about 4 mg of protein ml^{-1}, the same specific activity was obtained in cell extracts as was required in growing cells. The low or zero activity at low protein concentration suggests a multi-component system typical of a ring-activating dioxygenase (Gibson et al. 1982; Mason 1988). Desulfonation in the extract required 2 mol of O_2 per mol of OS (Table 1) and NAD(P)H in the standard reaction mixture, and no organic intermediates were observed.

3-Chlorocatechol prevented further metabolism of the product from the initial attack on OS, which required 1 mol of O_2 (Table 1) and about 1.2 mol of NAD(P)H per mol of OS. This attack was a desulfonation and the sulfonate moiety was recovered as sulfate from whole cells, whereas no ammonium ion was released (Fig. 3). An organic product was released from OS (Table 2), and it chromatographed close to the educt, OS, which suggested that an ionized group (i.e., the amino group) was still present after dioxygenation and cleavage of the C-S bond. We thus presume the metabolism of OS to involve initial dioxygenation followed by *meta* ring cleavage.

The unidentified product was unstable. It turned brown within minutes at pH 7.5 and within 6 h at pH 6.9; it was stable at 4° C in 100 mM potassium phosphate buffer, pH 2. The compound was degraded (at pH 6.9 and in the absence of 3-chlorocatechol) within 5 min by cell-free extracts of strain

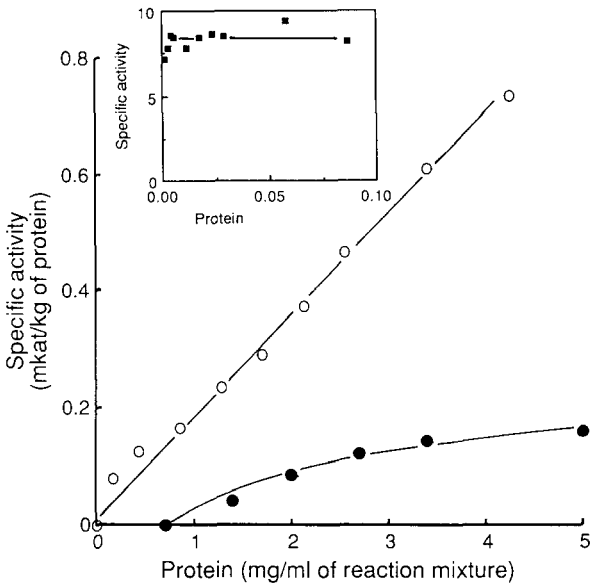

Fig. 2. Desulfonation of orthanilate and *meta* dioxygenation of catechol in extracts of orthanilate-grown cells of *Alcaligenes* sp. strain O-1 as a function of protein concentration. Crude extracts of OS-grown strain O-1 were prepared and reaction mixtures were set up with different protein concentrations. The reaction rate at 30° C was calculated from the rate of release of sulfite ion in 5 ml reaction mixtures (●), as oxygen uptake in 1 ml reaction mixtures (○), or, for catechol 2,3-dioxygenase, from product formation (■). Net oxygen uptake, corrected for endogenous reactions, was considered to represent desulfonation and ring cleavage, and rates of desulfonation were taken as 50% of the net rate of oxygen uptake. The axes for the inset have the same units as the major graph.

O-1 grown in OS-salts medium; as with OS as substrate, no yellow colour (a hydroxymuconate semialdehyde) was observed by eye, in contrast to the reaction with BS and TS. The unknown was not degraded by extracts from succinate-grown cells. The unknown was thus an intermediate in the degradative pathway of OS, or readily converted to an intermediate, and the product of putative ring cleavage was not 2-hydroxymuconate semialdehyde but possibly an aminated derivative thereof. We have been unable to isolate or identify the unknown compound, but is neither 3-or 4-aminocatechol, or several other putative products (Table 2).

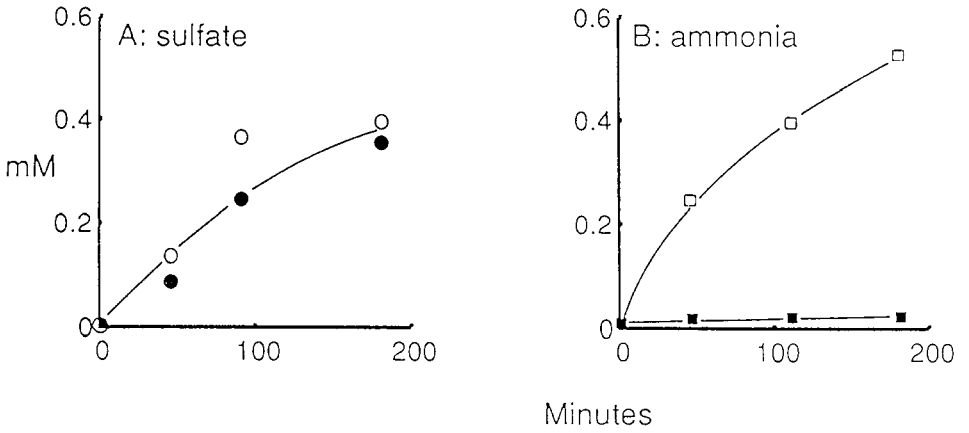

Fig. 3. Effect of 3-chlorocatechol on the release of the sulfonate and amino groups from orthanilate by orthanilate-grown cells of *Alcaligenes* sp. strain O-1. The organism was harvested from 6 mM OS-salts medium during growth. Cells were washed, suspended to 100 mg wet wt. of cells per ml in 50 mM potassium phosphate buffer, pH 7.2, equilibrated to 30° C for 15 min with or without 2 mM 3-chlorocatechol, and reactioons were started by the addition of orthanilate to 0.5 mM (A) or 1 mM (B). The data are corrected for basal levels of release of sulfate ion (0.02 mM in 180 min) or ammonium ion (0.4 mM in 180 min) from cells in the absence of orthanilate: the presence of 3-chlorocatechol had no effect on the release of ammonium in control experiments with strain O-1. Open symbols, no inhibitor present; filled symbols, 2 mM 3-chlorocatechol present to inhibit ring cleavage.

Discussion

The data illustrate that transport is the first specific step in the degradation of BS, TS or OS by *Alcaligenes* sp. strain O-1. The first metabolic reaction is desulfonation, in contrast to a later desulfonation in the degradation of TS in *Comamonastestosteroni* T-2 (discussed in Locher et al. 1989b).

Our data on the desulfonation of BS demonstrate the involvement of dioxygenation in desulfonation; these quantitative data are analogous to, and augment our work with 4-sulfobenzoate dioxygenase (Locher et al. 1989b), and they confirm quantitatively a mechanism indicated by Cain & Farr (1968). Our evidence argues against a desulfonated dihydrodiol intermediate (Table 1), with corresponding regeneration of NADH during oxidation to catechol. Rather the data support a mechanism whereby a sulfodihydrodiol (Fig. 4) formed on dioxygenation is degraded to yield the catechol without an NAD-linked oxidation (Brilon et al. 1981b). It is as yet unclear whether the putative intermediate exists only at the catalytic centre of the dioxygenase, is desulfonated by a separate protein or decays spontaneously. The nature of the degradation of OS is still unclear. In contrast to the initial deamination of 4-aminobenzene sulfonate to 4-sulfocatechol in a two-member consortium and in a pure culture (Feigel & Knackmuss 1988; Locher et al. 1989a; Locher et al. 1989b), we observed initial oxygenation and desulfonation of OS in strain O-1 (Fig. 3, Table 1) yielding sulfite and an unidentified organic intermediate.

The data reconfirm that the sulfonate moiety of a sulfonoaromatic is released as sulfite on cleavage of the aromatic C-S bond (cf. Johnston et al. 1975). The uncertainty of data derived from whole cells is emphasized, because growing cells of strain O-1 excrete large amounts of sulfite from sulfonates (Thurnheer et al. 1986), whereas the cell suspensions in this study release only sulfate.

We hypothesize that the desulfonation is a multicomponent enzyme, because of the kinetics we observe (Fig. 2). This corresponds to preliminary data from a partial purification of the desulfonative enzyme complex for BS in another bacterium (Kondo et al. 1982). The idea is analogous to the suggestion of Brilon et al. (1981a), that the desulfonation of naphthalene sulfonate is carried out by a mutant naphthalene dioxygenase with a wide substrate specificity; naphthalene dioxygenase is a multi-component system (Haigler & Gibson 1990).

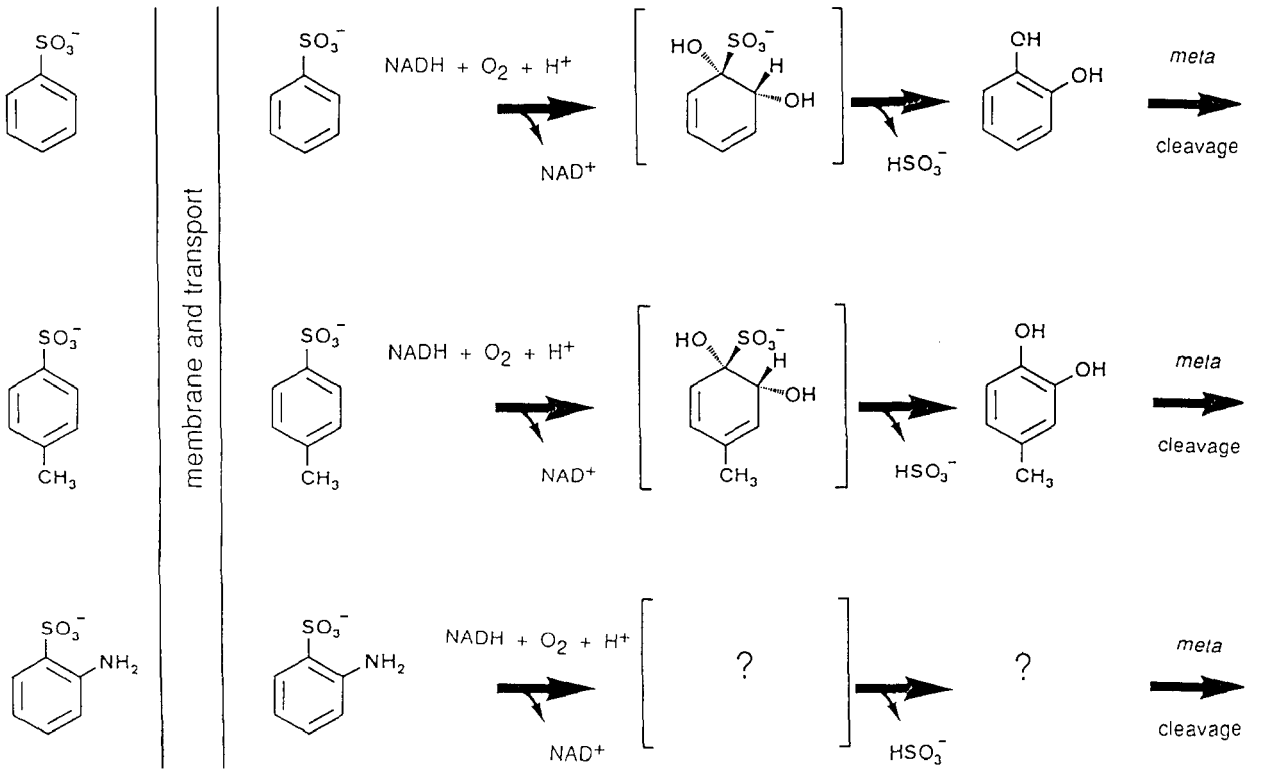

Fig. 4. Initial steps in the degradation of BS, TS and OS in *Alcaligenes* sp. strain O-1. The structures in square brackets are hypothetical intermediates.

A claim for a single-component desufonative enzyme seems to have been made (Stavskaya et al. 1987). The enzyme reaction was not defined, and an unverified, indirect assay was used which involved boiling the extract prior to a sulfite assay. We find boiling to destroy sulfite and wonder whether a non-specific sulfite assay detected a protein degradation product.

The term 'specific activity' has been used in this paper, but it is clear from Fig. 2 that it is being used incorrectly, because one basic requirement for this term is not met, namely independence of protein concentration. This observation complicates comparisons amongst different papers on multi-component dioxygenases, or even amongst different preparations of one enzyme system.

Strain O-1 apparently degrades its sulfonated substrates by only one pathway. Thus catechol is degraded via the *meta* pathway and not by the *ortho* pathway (Table 1), and the methylcatechol is degraded by *meta* cleavage and not through conversion to protocatechuate. However, the organism does seem to contain isofunctional enzymes for some reactions, e.g., the catechol 2,3-dioxygenases observed during growth on BS and benzoate. We wonder how many transport systems, dioxygenases (sulfite-forming) and ring cleavage enzymes are synthesized.

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