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J. Ruff · T. Hitzler · U. Rein · A. Ritter · A. M. Cook

Bioavailability of water-polluting sulfonoaromatic compounds

Abstract Highly substituted arenesulfonates are chemically stable compounds with a range of industrial applications, and they are widely regarded as being poorly degradable. We did enrichment cultures for bacteria able to utilise the sulfonate moiety of 14 compounds, and we obtained mixed cultures that were able to desulfonate each compound. The products formed were usually identified as the corresponding phenol, but because we could not obtain pure cultures, we followed up these findings with quantitative work in pure cultures of, e.g., *Pseudomonas putida* S-313, which generated the same phenols from the compounds studied. Many of these phenols are known to be biodegradable, or to be subject to binding to soil components. We thus presume that the capacity to degrade aromatic sulfonates extensively is widespread in the environment, even though the degradative capacity is spread over several organisms and conditions.

Introduction

Non-reacted naphthalenesulfonate monomers are significant components (15%) of the commercial formaldehyde condensate formulations used to modify the flow characteristics of liquid cement (Dodson 1990). Other naphthalenesulfonates, with benzenesulfonates, are precursors for dyestuffs, optical brighteners, pesticides, resins, plasticisers etc. (e.g. Suter et al. 1997), and all these products are disposed of in waste dumps. The sulfonates are then found in the leachates from these dumps (Suter et al. 1997) and many sulfonates are found in surface waters (Lindner et al. 1994), where they are described as being difficult to remove during the preparation of potable water (Lange et al. 1995). These compounds have raised environmental concern for some time, as was highlighted in a review propagating extreme physicochemical treatments (200 °C, 200 bar, titanium-lined vessels) (Bretscher 1981).

The degradation of organosulfonates as carbon sources for growth is characterised by organisms with narrow substrate ranges (one to three sulfonated compounds) (Cook et al. 1998), though exceptions are known (see Haug et al. 1991) and some naphthalenedisulfonates are biodegradable (e.g. Wittich et al. 1988). In contrast, bacteria capable of desulfonating a wide range of compounds are readily found under sulfate-limiting conditions (Zürrer et al. 1987; see also Key et al. 1998), where a monooxygenation is responsible in aerobic bacteria (Dudley and Frost 1994; Zürrer et al. 1987) and an as yet unknown mechanism has been observed in anaerobic bacteria (Denger and Cook 1999).

We now report that many contaminative arenesulfonates are desulfonated to compounds known to be biodegradable, so that coupled systems should lead to their complete degradation or immobilisation.

J. Ruff · T. Hitzler · U. Rein · A. M. Cook (✉)
Department of Biology, The University,
D-78457 Konstanz, Germany
e-mail: Alasdair.Cook@uni-konstanz.de
Tel.: +49-7531-884247
Fax: +49-7531-882966

A. Ritter
Swiss Federal Institute for Materials Testing and Research,
CH-9001 St. Gallen, Switzerland

Materials and methods

Materials

Chemicals used as sulfur substrates were 4-chlorobenzenesulfonate (CLBS), 2-nitrobenzenesulfonate (*o*NBS), 3-nitrobenzenesulfonate (*m*NBS), 4-nitrobenzenesulfonate (*p*NBS), 4-nitrotoluene-2-sulfonate (NTS), 5-amino-2-chlorotoluene-4-sulfonate (ACT), 1,5-naphthalenedisulfonate (15NS), 1,6-naphthalenedisulfonate (16NS), 2,6-naphthalenedisulfonate (26NS), 2,7-naphthalenedisulfonate (27NS), 8-amino-1,5-naphthalenedisulfonate (8A15NS), 3-amino-1,5-naphthalenedisulfonate (3A15NS), 6-amino-1,3-naphthalenedisulfonate (6A13NS), 3-amino-2,7-naphthalenedisulfonate (3A27NS), 4,4′-dinitrostilbene-2,2′-disulfonic acid-disodium salt (DNS) and 4,4′-diaminostilbene-2,2′-disulfonic acid (DAS). All were purchased from TCI (Tokyo) at the highest purity available.

Phenols (4-chloro-, 2-, 3- or 4-nitro) were from Merck or Fluka. Glassware was cleaned thoroughly and care taken to exclude extraneous sulfur (e.g. Laue et al. 1996).

Inocula for enrichment cultures were from activated sludge from sewage treatment plants in Konstanz, Germany (largely communal) and Ludwigshafen, Germany (largely industrial).

Analytical methods

Substrates and products were determined by isocratic reversed-phase high-pressure liquid chromatography (HPLC) (Laue et al. 1996) or by ion-pair chromatography (Lange et al. 1995) as indicated in the text; the apparatus included a diode array detector. Chromatograms were initially evaluated with wavelength settings of 215 nm for benzenesulfonates, 230 nm for the naphthalenedisulfonates, and 245 nm for the amino-naphthalenedisulfonates. The degradation product of NTS or 27NS was purified on a semi-preparative HPLC column and examined by gas chromatography–mass spectrometry (GC-MS) (Kölbener et al. 1996; Rein and Cook 1999). Protein was assayed by a Lowry-type method (Cook and Hütter 1981).

Organisms and growth conditions

Enrichment cultures, with attempts to isolate pure cultures of bacteria, were done as described by Zürrer et al. (1987). The organisms we routinely used were *Pseudomonas putida* strains S-313 (DSM 6884) and S-832 (DSM 6883) (Zürrer et al. 1987). Initial experiments were done with the phosphate-buffered medium of Zürrer et al. (1987). The sulfur-free acetate-Tris-buffered salts medium of Laue et al. (1996) gave the same products with negligible background growth, and became our standard medium. Sulfur was provided at 50 μM, except for disulfonates, where the initial sulfonate concentration was 30 μM. Cultures were grown in screw-capped tubes on a roller at 30 °C. Samples were taken at 3-day intervals for 9 days: bacteria were removed by centrifugation and the protein content measured, and 100-μl portions of the supernatant fluid were examined by HPLC.

Results

Enrichment cultures able to utilise CLBS, oNBS, mNBS, pNBS, NTS, ACT, 15NS, 16NS, 26NS, 27NS, 8A15NS, 3A15NS, 6A13NS, or 3A27NS as sole source of sulfur for growth were readily obtained, as evidenced by growth, and by both substrate utilisation and product formation. Frequently, these products were transient, and after growth ceased, the products tended to disappear within hours or days. The sulfonate substrates in sterile control experiments were stable; experiments with DNS and DAS were abandoned, because the compounds were unstable.

However readily we obtained enrichment cultures, we experienced great difficulty in obtaining pure cultures. One unidentified bacterium utilising CLBS was isolated (and later lost) and the sulfonate was converted quantitatively to the biodegradable 4-chlorophenol (see Discussion). We made no attempt to re-isolate the lost strain because *P. putida* S-313 (Table 1) and strain S-832 (not shown) catalysed the same reaction.

Given the difficulty in obtaining pure cultures, we chose to extend our use of the established desulfonative organisms, *P. putida* S-313 and S-832, to confirm the idea that complex sulfonates can be converted to biodegradable materials. The organisms desulfonated each mononitrobenzenesulfonate to the corresponding nitrophenol (Table 1). The two organisms catalysed the same reactions found in the mixed cultures, sometimes to different extents (all enrichments involved complete turnover of substrate), as with strain S-832, which fully desulfonated pNBS (not shown), whereas strain S-313 did not (Table 1). In contrast to the chemical stability of the parent compounds (see above), the products of desulfonation tended to disappear, especially visible with 4-nitrophenol (Table 1). The fact that the same product disappeared equally fast (or not at all) with all cultures, whether mixed or pure, suggests that this represented the chemical properties of the product and not degradative abilities of the organisms.

The more complex NTS was converted to 2-nitrocresol, which was stable under these conditions, and which we identified by GC-MS. The most complex benzenesulfonate studied, ACT, was subject to ready and complete desulfonation, but the identity of the desulfonation product(s) is unknown. The medium was coloured brown, so we presume polymerisation of a portion of the putative chloroaminocresol formed. The only product we observed chromatographically (Table 1) was exceptional in having a higher apparent polarity than its precursor; we presume that the zwitterionic ACT formed an effectively neutral, ion-paired second ring with correspondingly improved interaction with the stationary phase, whereas the putative product was positively charged and smaller.

The routine fate of assimilated sulfur in bacterial cells under sulfate starvation is biomass, largely as the amino acids methionine and cysteine (e.g. Roberts et al. 1955), which can be quantified as protein (e.g. Kertesz et al. 1994b). The molar growth yield with our control substrates, 4-toluenesulfonate and sulfate, was about 3.5 kg protein $(\text{mol S})^{-1}$ (a normal value; Kertesz et al. 1994b), and the same values, allowing for experimental error, were observed for the other compounds, regardless of the strain used (Table 1). So the fate of the sulfonate moiety from the sulfonates in these experiments was indeed biomass.

Naphthalenesulfonates, especially the disulfonates with additional substituents, have generally proven difficult to degrade (Haug et al. 1991; Nörtemann et al. 1986, 1994; Wittich et al. 1988). Of the eight disulfonates we examined in enrichment cultures, all of which are subject to quantitative desulfonation (see above), one was not attacked by the established strains of *P. putida* and one was subject to only partial turnover, but all others were quantitatively desulfonated (Table 2) as judged by substrate disappearance and molar growth yields. We anticipated that the products would be naphthols (Zürrer et al. 1987), and we were able to confirm this in the case of the product from 27NS, where a mass spectrum of the product identified it as a naphthalene diol [M^+, 160 (base peak); M^+-H_2O, 142; M^+-CHO, 131], presumably naphthalene-2,7-diol. The products of the other disulfonates had similar chromatographic

Table 1 Desulfonation of benzenesulfonates by *P. putida* S-313. [*ND* not determined, *cc* cochromatography (HPLC), *UV*, UV spectrum, *GC-MS* gas chromatography-mass spectrometry]

Sulfur source for growth	Substrate utilisation (%)	Molar growth yield [kg protein (mol S)$^{-1}$]	Product	Identification	Yield of product (%)
4-Chlorobenzenesulfonate	100	ND	4-Chlorophenol	cc, UV	90
2-Nitrobenzenesulfonate	90	4.4	2-Nitrophenol	cc, UV	80
3-Nitrobenzenesulfonate	82	3.3	3-Nitrophenol	cc, UV	20
4-Nitrobenzenesulfonate	90	3.3	4-Nitrophenol	cc, UV	2–94
4-Nitrotoluene-2-sulfonate	75	3.4	4-Nitrocresol	GC-MS	ND
5-Amino-2-chlorotoluene-4-sulfonate	100	3.2	Unknown	None	ND

properties, but were less stable and were not identified. Transient intermediates of polarity between substrate and product presumably represented the appropriate sulfonaphthols. The naphthalene diols were relatively unstable in solution, in contrast to the stable parent compounds, and this was most extreme with the putative aminonaphthalenediols.

Discussion

Most xenobiotic organosulfonates which we and others have examined as sulfur sources for growth of aerobic bacteria, are subject to desulfonation (Table 1, Table 2; Dudley and Frost 1994; Kertesz et al. 1994b; Key et al. 1998; King and Quinn 1997; Laue et al. 1996; Rein and Cook 1999; Seitz and Leadbetter 1995; Zürrer et al. 1987), so we believe that there is an enormous potential for desulfonation in the natural environment, apart from those organisms which degrade natural and xenobiotic products as carbon sources. This is perhaps not surprising, given the large percentage of sulfonates in soil and sediment sulfur, and the facts that humic materials are sulfonates and subject to rapid flux in soil (reviewed in Cook et al. 1998). What is less obvious in these reports is the frequency with which pure cultures are not obtained (e.g. the LAS-utilising culture in Kertesz et al. 1994b). Our experience over the last 15 years (A.M. Cook, unpublished) is over-represented by the 14 rapidly growing enrichments (see above) from which we picked one single colony able to desulfonate the compound in the enrichment, regardless of the additions we made to the medium. The loss of degradative ability in cultures on storage (50% glycerol at −80 °C), represented here in a CLBS culture, is also not uncommon (A.M. Cook, unpublished). We thus suspect that organisms like *P. putida* S-313, which grow reproducibly in the laboratory under sulfur-limited conditions, are rarities.

The products of desulfonation indicated in Table 1 were independent of the culture used, so the common desulfonation reaction, a monooxygenation (Dudley and Frost 1994; Zürrer et al. 1987), is widespread. The chlorophenol (Table 1) is biodegradable under aerobic and anaerobic conditions (e.g. Commandeur and Parsons 1994), so the capacity to degrade CLBS totally occurs in nature, if in different organisms grown under different conditions. The mononitrophenols are also known to be biodegradable (Spain 1995), so desulfonation can pave the way to complete degradation, which is otherwise found rarely for mNBS as a sole source of carbon (Kölbener et al. 1994).

The fact that some compounds are not always fully desulfonated (e.g. pNBS by strain S-313, Table 1) is presumably a function of the K_m values of this particular set of transport and desulfonation enzymes, so given the wide range of desulfonative organisms found in nature (Dudley and Frost 1994; Kertesz et al. 1994b; King and Quinn 1997; Seitz and Leadbetter 1995) together with enrichment cultures involving complete substrate disappearance, other organisms have more suitable desulfonative enzymes.

Table 2 Desulfonation of naphthalenesulfonates by *P. putida* S-313

Sulfur source for growth	Substrate utilisation (%)	Molar growth yield [kg protein (mol S)$^{-1}$]	Putative product
1,5-Naphthalenedisulfonate	100	3.0	1,5-Naphthalene diol[a]
1,6-Naphthalenedisulfonate	25	ND	
2,6-Naphthalenedisulfonate	100	4.2	2,6-Naphthalene diol[a]
2,7-Naphthalenedisulfonate	100	3.5	2,7-Naphthalene diol[b]
8-Amino-1,5-naphthalenedisulfonate	100	3.4	8-Amino-1,5-naphthalene diol
3-Amino-1,5-naphthalenedisulfonate	100	3.0	3-Amino-1,5-naphthalene diol
6-Amino-1,3-naphthalenedisulfonate	0	0	
3-Amino-2,7-naphthalenedisulfonate	100	3.3	3-Amino-2,7-naphthalene diol

[a] This compound had similar retention time to the 2,7-diol
[b] The mass spectrum supported the presence of a diol, and the 2,7-naphthalene diol is proposed, because only direct substitution of the sulfono group with the hydroxy group has been observed in these reactions (Dudley and Frost 1994; Kertesz et al. 1994b; Zürrer et al. 1987)

We presume that the formation of these phenols (Table 1) in soil could lead not only to degradation, but also to binding to clay and to humic fractions (Daun et al. 1998; Thorn et al. 1996) and to polymerisation reactions (Bollag et al. 1992). The latter is presumably indicated both in the rapid disappearance of 4-nitrophenol (Table 1) and the product(s) from ACT. We similarly hypothesise that the stable 2-nitrocresol (Table 1) will be immobilised on clay surfaces, humic materials, possibly via polymerisation after reduction of the nitro group (Bollag et al. 1992; Daun et al. 1998). All the naphthalene derivatives formed by desulfonation (Table 2) are unstable, so we anticipate that they, too, tend to polymerise or bind or sorb to the soil matrix.

The detail of the regulation of these desulfonative enzymes is still emerging (Gallardo et al. 1997; Hummerjohann et al. 1998; Kertesz et al. 1994a), but preliminary evidence for this regulation in nature is clear-cut (Mazel and Marlière 1989). The combination of desulfonation and the generalisation that compounds without the sulfonate group are much more likely to be generally bioavailable (Wellens 1990) suggests that, in the long term, waste streams and leachates containing sulfonates may become more amenable to biotreatment.

The detection of arenesulfonates in the leachate of dumps of building waste (Suter et al. 1997) presumably means that sulfonates leach steadily from cement. Given the relatively low surface area of untreated cement on portions of buildings subject to weathering, the losses from the buildings should not be large. And given markedly lowered levels of deposition of atmospheric sulfur from fossil-fuelled power stations (Schnug and Beringer 1998), it seems likely that micro-organisms in soils will indeed express desulfonation enzymes, analogous to the sulfur scavenging represented in Mazel and Marlière's work (Mazel and Marlière 1989).

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