Biodegradation of sulphosuccinate: direct desulphonation of a secondary sulphonate

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The bacterial biodegradation of a secondary sulphonate, sulphosuccinate, has been shown to occur by direct desulphonation. A bacterium, designated *Pseudomonas* sp. BS1, was isolated from activated sewage sludge, for its capacity to grow on sulphosuccinate as the sole source of carbon and energy. Cultures grown on sulphosuccinate were able to convert this substrate to sulphite which was subsequently oxidized rapidly to sulphate. The sequence of desulphonation and carbon-chain catabolism of sulphosuccinate was determined from measurements of the kinetics of sulphite and ¹⁴CO₂ release from specifically radiolabelled sulphydro[1,4-¹⁴C]succinate and sulphydro[2,3-¹⁴C]succinate, which were synthesized from the corresponding maleic anhydrides. When each radiolabelled compound was incubated separately with washed-cell suspensions of *Pseudomonas* BS1, sulphite was released before ¹⁴CO₂, as shown by chemical assay and radiorespirometry, respectively. Differences in the kinetics and extent of ¹⁴CO₂ release from the 1,4- and 2,3-labelled substrates were consistent with entry of the intact C₄ chain into the citric acid cycle. When carrier oxaloacetate was added to incubation mixtures containing resting-cell suspensions and radiolabelled sulphosuccinate, a radiolabelled metabolite with the same HPLC retention time as oxaloacetate accumulated. No radioactive metabolites accumulated when carrier oxaloacetate was replaced with succinate, fumarate or malate. Collectively, the data indicated co-production of sulphite and oxaloacetate from sulphosuccinate, which is interpreted in terms of an oxidative desulphonation mechanism.

Keywords: sulphonate, biodegradation, surfactant, desulphonation

INTRODUCTION

Dialkyl esters of sulphosuccinic acid [ROCO·CH₂·CH(SO₃⁻)COOR'; DASS; Fig. 1] constitute an interesting group of synthetic surfactants with commercial importance by virtue of their excellent wetting, foaming, emulsifying and dispersing properties (Linfield, 1976). Current applications include anti-fogging agents, inks, pesticide sprays, shampoos, cosmetics, dish-washing liquids and the textile industry. Production, use and waste-disposal of such widely-used compounds is likely to lead to their entry into the environment which in turn raises questions about their biodegradability.

Published studies on the biodegradation of DASS sur-

factants have been restricted to experiments with mixed microbial cultures. The primary biodegradation (i.e. the initial step which removes the surface-active properties) of DASS, measured using a range of assay methods, is virtually complete but takes from a few days up to 2–3 weeks, depending on the source of bacteria (e.g. sewage sludge or river-water) and the assay system (Swisher, 1987; Schoberl *et al.*, 1988; Hales, 1993, and references therein). In contrast, ultimate (complete) biodegradation, measured by dissolved-organic-carbon (DOC) removal, CO₂ production or release of sulphate, was incomplete, varying between 30 % and 70 % depending on the system used (Hales, 1993; Swisher, 1987). Hammerton (1956) showed that biodegradation was influenced by the nature of the alkyl chains and concluded that the ester link was not the point of bacterial attack. However, more recently Hales (1993), using hexyl/octyl sulphydro[¹⁴C]succinate with

Abbreviation: DASS, dialkylsulphosuccinate.

Fig. 1. Structures of sulphosuccinate and some sulphonated surfactants.

mixed bacterial cultures from a pilot-scale sewage-treatment plant, showed that there were probably two alternative biodegradation pathways (depending on which alkyl chain was released first); he proposed that both pathways channel substrate via sulphosuccinate (Fig. 1) for ultimate biodegradation, and that desulphonation of DASS does not occur prior to dealkylation. Because succinate itself is readily assimilated by bacteria, it would appear that the removal of the sulphonate group from sulphosuccinate is critical for ultimate biodegradation to take place. A further reason for studying the biodegradation of sulphosuccinate is that similar small aliphatic molecules containing secondary sulphonate bonds are likely to be the intermediates of metabolism of related surfactants, such as fatty acid ester sulphonates (Steber & Wierich, 1989; Masuda *et al.*, 1993) and possibly from ω/β -oxidation of secondary alkanesulphonates (see Fig. 1 for structures).

Long-chain primary aliphatic sulphonate surfactants with the $-SO_3^-$ moieties attached at terminal carbons of alkyl chains are known to be biodegraded by desulphonation via mono-oxygenation which introduces a hydroxyl group at the sulphonated carbon (Thyssen & Wanders, 1972, 1974). These authors also mentioned that dodecane 2-sulphonate was desulphonated to dodecan-2-one, but no experimental details were given. Moreover, there are abundant naturally occurring primary aliphatic sulphon-

ates such as the plant sulpholipid 6-sulpho- α -D-quinovopyranosyl-2,3-diacyl-D-glycerol (Harwood & Russell, 1984) and taurine in animals (Schram & Crokaert, 1957), and there is good evidence that these compounds undergo microbial biodegradation (Schram & Crokaert, 1957; Martelli & Benson, 1964; Martelli & Souza, 1970; Shimamoto & Berk, 1980; Uria-Nickelsen *et al.*, 1993a, b). In comparison, secondary sulphonates of biological origin have yet to be reported. Therefore, it is important to establish whether there are micro-organisms capable of degrading them and by what means this is achieved.

This paper describes the metabolic route whereby a bacterial isolate, which is capable of growth on sulphosuccinate as the sole source of carbon and energy, accesses the carbon in this secondary sulphonate. We show that there is direct desulphonation of the growth substrate, without prior catabolism of the C_4 chain.

METHODS

Materials. Dialkyl sulphosuccinate (40:60 mixture of C_6/C_8 alkyl chains, 89% pure) used for bacterial enrichments was synthesized at Unilever Research, Port Sunlight Laboratory (Bebington, Merseyside, UK). Di(ethylhexyl) sulphosuccinate for the production of sulphosuccinate, and Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] were obtained from Sigma. [2,3- ^{14}C]Maleic anhydride (radiochemical purity > 95% and specific radioactivity 50 GBq mol $^{-1}$), and [1,4- ^{14}C]maleic anhydride (radiochemical purity > 98% and specific radioactivity 60 GBq mol $^{-1}$) for synthesis of radiolabelled sulphosuccinate were obtained from Sigma. Radiolabelled standards for HPLC analysis were obtained as follows: [1- ^{14}C]fumarate from ICN; [U- ^{14}C]malate and [1- ^{14}C]acetate from Amersham International; and [2,3- ^{14}C]succinate from Sigma.

Hionic-fluor and Opti-fluor scintillation fluids were purchased from Canberra-Packard. Nutrient agar, Noble agar and yeast extract were obtained from Difco. The API 20B test kits used for the identification of environmental bacteria were obtained from API Laboratory Products. All other chemicals (AnalaR grade) were supplied by BDH-Merck.

Isolation of a sulphosuccinate-degrading bacterium. Enrichment cultures were established in a mineral salts medium containing (g l $^{-1}$): K_2HPO_4 , 7; KH_2PO_4 , 3; NH_4Cl , 0.5; NaCl, 0.15; $MgCl_2 \cdot 6H_2O$, 0.5, and 1 ml of a trace elements solution containing 20 mg l $^{-1}$ each of $Na_2B_4O_7$, $CuSO_4 \cdot 5H_2O$, $CoSO_4 \cdot 7H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $MnSO_4 \cdot H_2O$ and $ZnSO_4$. In some early experiments the mineral medium also contained Na_2SO_4 (0.15 g l $^{-1}$). Enrichments were performed in baffled Erlenmeyer flasks (250 ml) containing 50 ml of the mineral salts medium supplemented with filter-sterilized (Millipore membrane filters, 0.2 μ m pore size) sulphosuccinate or DASS to give a final concentration of 0.1% (w/v). Flasks were inoculated with activated sewage sludge (1%, v/v) obtained from Coslech sewage works, South Glamorgan, Wales and shaken at 120 r.p.m. and 30 °C in a rotary incubator. When growth was visible, samples were subcultured (2%, v/v) into identical fresh medium. After three subculturings, samples (0.1 ml) of the liquid cultures were plated on nutrient agar and incubated at 30 °C for 48 h when individual colonies could be selected for further growth in liquid or on solid medium.

Growth of cultures and preparation of resting-cell suspensions. Cultures were grown to late-exponential phase

($OD_{420} = 1.3\text{--}1.4$) in baffled Erlenmeyer flasks (250 ml) containing 50 ml minerals salts medium supplemented with sulphosuccinate (1%, w/v) and yeast extract (0.005%, w/v) and harvested by centrifugation at 4000 g_{av} for 20 min. The bacterial cell pellet was washed twice by resuspension in mineral salts solution (50 ml) having the same composition as that of the growth medium, followed by recentrifugation as above and final resuspension in 5 ml of the salts solution to give a bacterial cell density of approximately $20\text{ mg wet wt ml}^{-1}$. For incubations with radiolabelled substrates, the cell suspensions (10 ml) were placed in sterile 100 ml Erlenmeyer flasks and shaken at 120 r.p.m. in a rotary incubator at 30°C .

Preparation of sulphosuccinate from dialkylsulphosuccinate.

Di(ethylhexyl) sulphosuccinate (150 g) was boiled under reflux in 1.5 l 0.1 M HCl for 24 h. The cooled mixture was extracted three times with light petroleum (60–80 $^\circ\text{C}$ b.p. fraction) to remove liberated alcohol. The aqueous phase was taken to dryness in a rotary evaporator and the residue redissolved in 200 ml distilled water and converted to the trisodium salt by addition of 0.2 M NaOH until the pH was 10. The solution was evaporated and the resulting solid stirred in 200 ml ethanol for 1 h. The slurry was centrifuged at 3000 g_{av} for 20 min to separate the insoluble sulphosuccinate. The supernatant was discarded, the ethanol washing repeated and the final product dried at 60°C .

Infra-red spectra of the product compressed in KBr discs were obtained using a Perkin-Elmer 137 Infracord infra-red spectrometer. The spectra showed absorption bands at $1190\text{--}1250\text{ cm}^{-1}$ and 1060 cm^{-1} corresponding to vibrations in the sulphonate group, and carboxylate resonances at $1550\text{--}1610\text{ cm}^{-1}$ and 1700 cm^{-1} . Minor bands at 3300 cm^{-1} were due to the presence of residual water in the dried sample.

To confirm the structure as sulphosuccinate, ^1H -nuclear magnetic resonance spectroscopy of the chemically synthesized product dissolved in D_2O was carried out using a Bruker WM 360 nuclear magnetic resonance spectrometer operating in the Fourier transform mode. A $180\text{--}\tau\text{--}90$ pulse sequence ($\tau \sim 3\text{ s}$) with a recycle time of 5 s was used to eliminate the solvent peak (HDO). Samples gave spectra that were characteristic of a compound having two geminal protons coupled to each other on a carbon atom that was attached to a chiral carbon atom carrying a single proton; no other protons were detected and the chemical-shift values were consistent with a compound having the general structure $^-\text{OOC-CH}_a\text{H}_b\text{-CH}_x(\text{SO}_3^-)\text{COO}^-$. The values of the coupling constants for the protons were $J_{ab} = 13\text{ Hz}$, $J_{ax} = 10\text{ Hz}$ and $J_{bx} = 4\text{ Hz}$, which are typical of gem- and vicinal-substituted protons. Elemental analyses of the synthesized sulphosuccinate were $\text{C} = 16.52\%$, $\text{H} = 1.71\%$, which were in close agreement with the theoretical values of $\text{C} = 17.02\%$ and $\text{H} = 1.77\%$ for the trisodium salt monohydrate.

Preparation of ^{14}C -labelled sulphosuccinate. Sulpho[1,4- ^{14}C]succinate and sulpho[2,3- ^{14}C]succinate were prepared by converting the correspondingly radiolabelled maleic anhydrides first to the dioctyl esters, then adding sulphite at the double bond, and finally removing the octyl groups by hydrolysis. Carrier maleic anhydride (0.18 g, 1.84 mmol) was dissolved in 10 ml anhydrous toluene. The appropriate radiolabelled maleic anhydride (3.7 MBq, about 0.05 mmol) was added, followed by 0.71 ml (6.5 mmol) anhydrous octanol and a few crystals of *p*-toluene sulphonic acid as catalyst. The mixture was heated under reflux for 4 h, cooled and extracted sequentially once with 15 ml NaOH (30%, w/v), twice with 15 ml water and finally

twice with 15 ml saturated NaCl. The organic phase was evaporated under reduced pressure to leave the di-octyl maleate as a yellow liquid (about 2 ml). The residue was dissolved in 2 ml ethanol and sulphonation of the double bond was achieved by the addition of 1.8 ml sodium metabisulphite (40%, w/v) and boiling under reflux for 6 h. Excess inorganic salts were precipitated from the cooled mixture by the addition of 10 ml ethanol and removed by centrifugation at 2000 g_{av} for 10 min. The supernatant was taken to dryness to leave dioctyl sulphosuccinate as a white powder. The radiolabelled surfactant was converted to sulphosuccinate by hydrolysis in 5 ml 0.2 M HCl under reflux for 24 h. The cooled solution was extracted twice with 10 ml light petroleum (60–80 $^\circ\text{C}$ b.p. fraction) to remove octanol. Sulphosuccinate was recovered by rotary evaporation of the aqueous phase and converted to the sodium salt as described for unlabelled material. Final purification was achieved by preparative TLC (20 cm $\times$ 20 cm $\times$ 2 mm layer, silica gel 60, Merck) with propan-2-ol/water (7:3, v/v) as the developing solvent.

Assay for sulphite. Sulphite was quantified by reaction with Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid); 1 mg (ml 0.1 M phosphate buffer) $^{-1}$, pH 7.0] according to the method of Johnston *et al.* (1975). Samples (1 ml) of incubations with radiolabelled substrates (see above) were removed at intervals, placed in Eppendorf tubes and centrifuged in a microcentrifuge. A portion (0.25 ml) of the supernatant was removed and diluted to 1 ml with distilled water. Ellman's reagent (0.1 ml) was added, the solution mixed and incubated at room temperature for 2 min for colour development before measuring the absorbance at 415 nm using a LKB Novaspec spectrophotometer and a reagent blank. Sulphite reacts with the disulphide form of Ellman's reagent to give 5-thio 2-nitrobenzoate: the assay is sensitive, rapid and obeys Beer's law up to a concentration of 0.1 mM. However, Ellman's reagent also gives a positive colour reaction with thiols. Therefore, control experiments were carried out routinely in which 10 ml 50 mM formaldehyde was added to the Ellman's reagent (0.1 ml as above) to remove the sulphite by formation of a formaldehyde sulfoxylate (Roy & Trudinger, 1970); in such controls the residual absorbance would be due to thiols and can be used to correct the test values.

Radiorespirometry. The release of $^{14}\text{CO}_2$ during bacterial metabolism of radiolabelled substrates was quantified by radiorespirometry and liquid scintillation counting. The apparatus consisted of a series of glass scintillation vials each containing an open plastic mini-vial insert and sealed with a Teflon-coated rubber septum through which solutions could be injected aseptically. The apparatus was sterilized by autoclaving and, shortly before use, 0.35 ml 1 M KOH was added to the mini-vial to trap any $^{14}\text{CO}_2$ evolved. Washed bacterial suspension (1 ml, $20\text{ mg wet wt ml}^{-1}$; see above) was rapidly pre-mixed with substrate and added to the main compartment of the apparatus (i.e. inside the glass vial but outside the mini-vial insert) to start the incubation. The substrate was comprised of a mixture of non-radioactive and specifically radiolabelled sulphosuccinate (see above) adjusted so that the final concentration in the incubation mixture was 0.4 mM at a specific radioactivity of 2.5 kBq mmol^{-1} (equivalent to 1 kBq ml^{-1}).

Since the objective was to measure the release of both sulphite and $^{14}\text{CO}_2$, the reaction could not be stopped by the addition of trichloroacetic acid (as commonly done in radiorespirometry) because such acidification interferes with the sulphite assay. Instead, the reaction was stopped by centrifuging aliquots (0.4 ml) of the incubation mixture rapidly in an Eppendorf tube using a microcentrifuge. The radioactivity in a portion (0.25 ml)

of the supernatant was determined by liquid scintillation counting using a LKB Rackbeta liquid scintillation spectrometer with 5 ml Opti-fluor as the scintillant. The efficiency of counting was determined using the external standard channels-ratio facility on the scintillation counter, programmed using a series of control samples quenched with chloroform. Residual activity in the complete (i.e. uncentrifuged) incubation medium was determined by counting portions (100 μ l). Control experiments showed that the combined efficiency of CO_2 trapping by the KOH and scintillation counting (after correction for quenching) was only reduced to 90% by omission of the acidification step.

To quantify the $^{14}\text{CO}_2$ trapped by the KOH, the mini-vial insert was removed and the outside washed free of residual incubation mixture. Scintillation fluid (3.5 ml Hionic-fluor) was added to the contents of the mini-vial, which was stoppered and the radioactivity in $\text{K}_2^{14}\text{CO}_3$ measured. The residual radioactivity in the incubation mixture was measured by counting a portion (100 μ l).

HPLC analysis for TCA intermediates. Incubations of washed bacterial suspensions were incubated with radiolabelled sulphosuccinate as described above for radiorespirometry. Samples (100 μ l) were transferred at timed intervals into screw-cap Eppendorf tubes and boiled for 5 min to release soluble metabolites from the bacteria. The tubes were centrifuged in a microfuge and the supernatant analysed by HPLC.

Separation of metabolites of sulphosuccinate was achieved by reverse-phase HPLC using a Spherisorb C8 column (15 cm $\times$ 4.6 mm, particle size 3 μ m, Jones Chromatography, Hengoed, Wales) with a Milton Roy CM4000 solvent delivery system. The system was driven by an Axiom Chromatography 727 HPLC software package, version 3.67. Radiolabelled metabolites were detected using a Beckman 171 radioisotope detector with a flow-cell scintillation counter (flow-cell volume, 600 μ l). Samples (40 μ l) were injected via a Rheodyne injection loop. The following authentic compounds were used as ^{14}C -labelled standards for identification of metabolites: acetate, aspartate, malate, succinate and fumarate. In addition, unlabelled standards of *cis*-aconitate, citrate, oxaloacetate, pyruvate and glyoxylate, were eluted from the same chromatographic system and detected by UV absorbance at 210 nm in a Milton Roy 310 flow spectrometer.

RESULTS

Isolation and growth of organism

Attempted enrichments in mineral medium containing DASS and added sodium sulphate produced numerous isolates capable of growth on DASS, but none which could utilize sulphosuccinate as growth substrate. Synthesis of bacterial sulphatase enzymes acting on sulphate esters is often repressed by more readily assimilable sources of sulphur such as inorganic sulphate (Dodgson *et al.*, 1982). Similar repression may regulate the biosynthesis of desulphonation systems. From a second series of enrichments in medium lacking added sulphate, a mixed culture was obtained, derived from activated sewage sludge, which showed rapid and substantial biodegradation of DASS. Because DASS was now also the sole source of sulphur as well as carbon, the vigour of the growth suggested that all of the molecule, including sulphosuccinate was undergoing biodegradation. The

mixed culture was subcultured into minimal medium containing sulphosuccinate (1%, w/v, but lacking sulphate). After three transfers, the culture was plated on nutrient agar from which colonies were picked for a further round of three sub-culturings on minimal medium containing sulphosuccinate. The fastest-growing isolate was selected and was identified as a *Pseudomonas* sp., using the API 20B test kit. This isolate was designated *Pseudomonas* BS1. It grew slowly on sulphosuccinate minimal medium (doubling time, 130 h) but much more rapidly (doubling time, 22 h) when the medium was supplemented with 0.005% (w/v) yeast extract (Fig. 2). The growth curve was biphasic, and a separate experiment in which sulphosuccinate was omitted showed that the initial faster growth phase corresponded to utilization of the yeast extract. *Pseudomonas* BS1 was tested for its ability to grow on DASS, fatty acid ester sulphonate, secondary alkane sulphonate, C_1 – C_6 linear primary alkane-sulphonates, sulphoacetic acid, benzene sulphonate and *p*-toluene sulphonate. Even when incubation periods were extended over several weeks, no growth was observed on any of these compounds, thus indicating the inability of these compounds to serve as sole source of carbon and energy for growth.

Metabolic studies

Resting-cell suspensions of *Pseudomonas* BS1 (10-times concentrated relative to the volume of the harvested culture) rapidly released sulphite from sulphosuccinate (Fig. 3). Conversion was complete within 25 min, after

Fig. 2. Growth of *Pseudomonas* BS1 on sulphosuccinate (0.1%, w/v) supplemented with 0.005% (w/v) yeast extract (●) and on 0.005% yeast extract alone (■). Growth was monitored by measurement of OD₄₂₀.

Fig. 3. Release of sulphite from sulphosuccinate by *Pseudomonas* BS1. Resting bacterial cell suspensions (20 mg wet wt ml⁻¹) of cultures grown on sulphosuccinate were incubated with 0.49 mM sulphosuccinate, and the release of sulphite was quantified using the chemical assay of Johnston *et al.* (1975).

Fig. 4. Production of sulphite (●) and ¹⁴CO₂ (■) from sulphonyl[2,3-¹⁴C]succinate by *Pseudomonas* BS1. Sulphite release was quantified chemically and ¹⁴CO₂ by radiorespirometry.

which the sulphite concentration rapidly decreased. Separate experiments indicated that disappearance of sulphite was coincident with formation of inorganic sulphate (data not shown).

When sulphite release and ¹⁴CO₂ production from sulphonyl[2,3-¹⁴C]succinate were measured simultaneously, sulphite concentration again reached a maximum after 25 min and decreased thereafter (Fig. 4). In contrast, ¹⁴CO₂ release did not begin until sulphite release was well advanced, and reached its maximum value (about one-half of the total radiolabel present) in 3 h.

Fig. 5. Production of sulphite (●) and ¹⁴CO₂ (■) from sulphonyl[1,4-¹⁴C]succinate by *Pseudomonas* BS1. Sulphite release was quantified chemically and ¹⁴CO₂ by radiorespirometry.

In parallel experiments using sulphonyl[1,4-¹⁴C]succinate, sulphite release was again the initial event (Fig. 5) but ¹⁴CO₂ production differed in two respects from that observed with the 2,3-labelled substrate. Firstly, there was a much shorter lag period between sulphite liberation and ¹⁴CO₂ production and secondly, 90% of the total label added was oxidized to ¹⁴CO₂ (as compared to 40–50% for the 2,3-¹⁴C-labelled compound). Because sulphite and ¹⁴CO₂ were produced rapidly and with an apparently small lag, a further experiment was performed to establish with certainty the presence of the lag. Increasing the sulphosuccinate concentration to 1 mM (2.5 kBq ml⁻¹) slowed the overall process and confirmed the presence of a lag period (data not shown).

HPLC analysis of biodegradation products

When the boiled aqueous extracts of incubation mixtures of sulphonyl[1,4-¹⁴C]succinate with resting-cell suspensions of *Pseudomonas* BS1 were analysed by radio-HPLC, there was a progressive disappearance of the starting radio-labelled compound (Fig. 6a). Similar results were obtained with sulphonyl[2,3-¹⁴C]succinate (data not shown). By 2.5 h all of the starting material had been metabolized, and with the exception of a small amount of material with a retention time of 6.1 min, there were no major accumulations of metabolic breakdown products. Separate additions of the citric acid cycle C₄ intermediates, succinate, malate or fumarate, as unlabelled carriers, slowed the rate of biodegradation of radiolabelled sulphosuccinate, but did not cause the accumulation of any radiolabelled metabolites. In contrast, the addition of 3 mM carrier oxaloacetate resulted in significant accumulation of the compound with a retention time of 6.1 min (Fig. 6b). This retention time was the same as that of standard oxaloacetate; it was also close to malate (5.8 min) and acetate (6.0 min), but not aspartate (3.0 min), succinate (10.5 min) and fumarate (11.6 min).

Fig. 6. HPLC analysis of the biodegradation of sulpho[1,4- ^{14}C]succinate by resting cell suspensions of *Pseudomonas* BS1 in (a) the absence or (b) the presence of excess unlabelled oxaloacetate (3 mM). Each trace is labelled with the time (min) at which the sample was taken.

DISCUSSION

There are numerous reports of the desulphonation of aromatic and primary aliphatic sulphonates (Thyssen & Wanders, 1974; Shimamoto & Berk, 1980; Cain, 1987; Swisher, 1987; Cook & Leisinger, 1991; Uria-Nickelsen *et al.*, 1993a, b). In comparison, understanding of the biodegradative pathways of secondary sulphonates is meagre and this paper presents for the first time a direct demonstration of the desulphonation of a secondary sulphonate. Earlier work (Steber & Wierich, 1989) had shown that mixed bacterial cultures degraded α -sulpho-fatty acid (C_{16}) methyl ester via ω/β -oxidation to produce short-chain sulphonated dicarboxylic acids which have structures similar to that of sulphosuccinate. Although these intermediates were found to undergo ultimate biodegradation, the nature and timing of the desulphonation step was not established in those experiments. Thyssen & Wanders (1974), in their study of primary aliphatic sulphonate biodegradation, mention

that dodecane 2-sulphonate underwent desulphonation to dodecan-2-one, but experimental details were not provided.

The present demonstration of the sequential liberation of sulphite then $^{14}\text{CO}_2$ from either 2,3- or 1,4-labelled sulphosuccinates (Figs 4 and 5) provides clear evidence that desulphonation precedes disruption of the C_4 chain. Thus, the carbon chain of the sulphosuccinate remains intact during desulphonation which in turn means that sulphite is removed from a secondary carbon atom.

The sulphite released was converted rapidly to sulphate, and the speed of this oxidation is believed to be the cause of considerable inter-experimental variability in the maximum percentage of sulphite released from both the specifically radiolabelled sulphosuccinates. Sulphite oxidation was presumed to be due to the action of sulphite oxidase which has been detected in a number of other micro-organisms (Cohen & Fridovich, 1971; Siegel, 1975); spontaneous oxidation of sulphite in air can also occur (McCord & Fridovich, 1969). Despite the rapid appearance of sulphate, there was no doubt that the primary product of desulphonation was sulphite. This is consistent with initial steps in the desulphonation of arylsulphonates and primary aliphatic sulphonates (White & Russell, 1993).

Biodegradation of sulpho[^{14}C]succinate was accompanied by transient formation of small amounts of a radiolabelled metabolite (Fig. 6a, retention time 6.1 min), the concentration of which was markedly increased by the presence of unlabelled oxaloacetate during biodegradation (Fig. 6b). The possible identities of this metabolite deduced from HPLC analysis were oxaloacetate (6.1 min), acetate (6.0 min) or malate (5.8 min). Acetate can be eliminated on theoretical grounds, since a mechanism for desulphonation which entrains simultaneous C–C bond cleavage to acetate is difficult to envisage. An oxidative mechanism for the desulphonation would give oxaloacetate, whereas hydrolysis would be required to give malate. The latter seems less likely, in view of the stability of the C–S bond to hydrolysis (Wagner & Reid, 1931) and the fact that oxidation is required to metabolize primary alkane sulphonates (White & Russell, 1993). Moreover, under anaerobic conditions, dialkylsulphosuccinate undergoes hydrolytic removal of alkyl chains but no desulphonation (Hales, 1993), indicating that the latter is not a hydrolytic process. Thus, oxaloacetate seems to be the most likely identity of the ^{14}C -labelled metabolite, and this proposition is supported by the experimental evidence. Firstly, the retention time of oxaloacetate, amongst those likely products, was the one which matched most closely that of the radioactive metabolite. Secondly, there was enhanced accumulation of the metabolite only in the presence of carrier oxaloacetate, but not with carrier malate, succinate or fumarate.

The formation of oxaloacetate from sulphosuccinate provides an explanation for the kinetics and extent of production of $^{14}\text{CO}_2$ (Figs 4 and 5). When the substrate was 1,4-labelled, $^{14}\text{CO}_2$ production was fast and extensive (approaching 90%, see Fig. 5). This is consistent with the

Fig. 7. Proposed reaction mechanism for the desulphonation of sulphosuccinate.

well-known transformations of the citric acid cycle in which the citrate synthesized is 'biologically' asymmetrical: the C₄ of oxaloacetate becomes the C₁ carboxyl of isocitrate, and the C₁ of oxaloacetate becomes the carboxyl attached at C₃ of isocitrate. These two carboxyls are lost sequentially in the conversion of isocitrate → 2-oxoglutarate → succinate. Thus, the succinate produced from [1,4-¹⁴C]oxaloacetate in the first round of the citric acid cycle is unlabelled and all radioactivity has been converted to ¹⁴CO₂.

In comparison, when the substrate was sulpho[2,3-¹⁴C]succinate a different pattern of ¹⁴CO₂ production occurred (Fig. 4), which can also be explained by the known metabolic conversions of oxaloacetate via the citric acid cycle. Conversion of sulpho[2,3-¹⁴C]succinate to [2,3-¹⁴C]oxaloacetate would lead to ¹⁴C incorporation at the C₂ and C₃ positions of 2-oxoglutarate and thence into C₁ and C₂ of succinate. Thus, no ¹⁴CO₂ would be released in the first round of the citric acid cycle, in keeping with the distinct 15–20 min lag observed for this specifically radiolabelled sulphosuccinate (Fig. 4). Moreover, the succinate molecule is symmetrical and leads effectively to the formation of [U-¹⁴C]oxaloacetate after the first round of the citric acid cycle. Therefore, in the second and subsequent rounds of oxidation by the citric acid cycle, only that part of the label at the C₁ and C₄ positions is converted to ¹⁴CO₂. In addition, repeated passage of radiolabel through metabolic intermediates such as 2-oxoglutarate and oxaloacetate during successive rounds of the cycle allows radiolabel to be diverted away

from ¹⁴CO₂ production and into biomass. This accounts for the lower conversion of radiolabel to ¹⁴CO₂ from 2,3-labelled sulphosuccinate compared with the 1,4-labelled substrate.

The formation of sulphite and oxaloacetate as initial products of sulphosuccinate metabolism is consistent with an oxidative mechanism (Fig. 7). In this mechanism, substrate undergoes oxidation at C₂ to yield 2-sulphomalate. The HO-C-SO₃⁻ structure is equivalent to the sulphite adduct of a carbonyl group, which can undergo spontaneous reformation of the carbonyl with elimination of sulphite. This mechanism is parallel to that proposed for desulphonation of primary aliphatic sulphonates (Thyssen & Wanders, 1972, 1974; Kelly & Smith, 1990; Baker *et al.*, 1991) except that the carbonyl forms an α-keto acid, rather than an aldehyde. Masuda *et al.* (1993) have proposed that the biodegradation of methyl α-sulphosuccinate derived from α-sulphotetradecanoic acid methyl ester in mixed cultures from activated sewage sludge, yields oxaloacetate and sulphate. However, this conclusion was based solely on the late release of sulphate during surfactant biodegradation and the tentative identification of methyl α-sulphosuccinate as an intermediate. The authors did not present any evidence that identified the C₄ desulphonation product as oxaloacetate, nor did they consider the possibility of formation of sulphite as an intermediate, nor indicate any mechanism for the oxidative cleavage proposed.

The oxidative mechanism in Fig. 7 is distinct from that proposed for bacterial metabolism of sulphoacetaldehyde, which is mediated by a thiamine pyrophosphate-dependent sulphoacetaldehyde sulphydrylase (Kondo & Ishimoto, 1974, 1975) to produce sulphite and acetate. The latter mechanism involves covalent bonding between TPP and the aldehydic group of the substrate, a mechanism which is untenable for the catabolism of sulphosuccinate which lacks an aldehydic functional group. The mechanism proposed here for sulphosuccinate biodegradation is also distinct from that suggested for bacterial desulphonation of sulphoacetate (Martelli & Souza, 1970) which involves a 'double' oxidation of both carbon and sulphur moieties to yield glycollate and sulphate.

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REFERENCES

- Baker, S. C., Kelly, D. P. & Murrell, J. C. (1991). Microbial degradation of methanesulphonic acid: a missing link in the biogeochemical sulphur cycle. *Nature* **350**, 627.
- Cain, R. B. (1987). Biodegradation of anionic surfactants. *Biochem Soc Trans* **15**, 7S–22S.
- Cohen, H. J. & Fridovich, T. (1971). Hepatic sulfite oxidase. Purification and properties. *J Biol Chem* **246**, 359–366.

- Cook, A. M. & Leisinger, T. (1991).** Desulfonation of aromatic compounds. In *Proceedings of the International Symposium on Environmental Biotechnology*, part 1, pp. 115–122. Edited by H. Verachtert & W. Verstraete. Antwerp: Royal Flemish Society of Engineers.
- Dodgson, K. S., White, G. F. & Fitzgerald, J. W. (1982).** *Sulfatases of Microbial Origin*. Boca Raton: CRC Press.
- Hales, S. G. (1993).** Biodegradation of the anionic surfactant dialkyl sulphosuccinate. *Environ Chem Toxicol* **12**, 1821–1828.
- Hammerton, C. (1956).** Synthetic detergents and water supplies. II. Chemical constitution of anionic surface active compounds and their susceptibility to biochemical oxidation. *Proc Soc Water Treat Exam* **5**, 160–174.
- Harwood, J. L. & Russell, N. J. (1984).** *Lipids in Plants and Microbes*. London: George Allen and Unwin.
- Johnston, J. B., Murray, R. & Cain, R. B. (1975).** Microbial metabolism of aryl-sulfonates. A reassessment of colorimetric methods for the determination of sulfite and their use in measuring desulfonation of aryl- and alkylbenzenesulfonates. *Antonie Leeuwenhoek* **41**, 493–511.
- Kelly, D. P. & Smith, N. A. (1990).** Organic sulphur compounds in the environment. Biochemistry, microbiology and ecological aspects. *Adv Microb Ecol* **11**, 345–385.
- Kondo, H. & Ishimoto, M. (1974).** Requirement for thiamine pyrophosphate and magnesium for sulfoacetaldehyde sulfo-lyase activity. *J Biochem* **76**, 229–231.
- Kondo, H. & Ishimoto, M. (1975).** Purification and properties of sulfoacetaldehyde sulfo-lyase, a thiamine pyrophosphate-dependent enzyme forming sulfite and acetate. *J Biochem* **78**, 317–325.
- Linfield, W. M. (1976).** Sulfopolycarboxylic acid derivatives. In *Anionic Surfactants*. Surfactant Science Series vol. 7, part II, pp. 405–443. Edited by W. M. Linfield. New York: Marcel Dekker.
- McCord, J. M. & Fridovich, T. (1969).** The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethylsulfoxide and oxygen. *J Biol Chem* **244**, 6056–6063.
- Martelli, H. L. & Benson, A. A. (1964).** Sulfocarbohydrate metabolism. I. Bacterial production and utilization of sulfoacetate. *Biochim Biophys Acta* **93**, 169–171.
- Martelli, H. L. & Souza, S. M. (1970).** Biochemistry of sulfonic compounds. III. Formation of a two-carbon compound during the oxidation of sulfoacetate by a pseudomonas strain. *Biochim Biophys Acta* **208**, 110–115.
- Masuda, M., Odake, H., Miura, K., Ito, K., Yamada, K. & Oba, K. (1993).** Biodegradation of 2-sulfonatofatty acid methyl ester. (α -SFMe). II. Biodegradation pathways of α -SFMe. *J Jpn Oil Chem Soc* **42**, 905–909.
- Roy, A. B. & Trudinger, P. A. (1970).** *The Biochemistry of Inorganic Sulphur Compounds*. London: Cambridge University Press.
- Schoberl, P., Bock, K. J. & Huber, L. (1988).** Ökologisch relevante Daten von Tensiden in Wasch- und Reinigungsmitteln. *Tenside Surf Deterg* **25**, 86–98.
- Schram, E. & Crokaert, R. (1957).** Etude du métabolisme de la taurine chez le rat. Formation de sulfate. *Biochim Biophys Acta* **26**, 300–308.
- Shimamoto, G. & Berk, R. S. (1980).** Taurine catabolism. II. Biochemical and genetic evidence for sulfoacetaldehyde sulfolase involvement. *Biochim Biophys Acta* **632**, 121–130.
- Siegel, L. (1975).** Biochemistry of the sulfur cycle. In *Metabolism of Sulfur Compounds*, pp. 217–286. Edited by D. M. Greenberg. New York: Academic Press.
- Steber, J. & Wierich, P. (1989).** The environmental fate of fatty acid α -sulfolmethyl esters. *Tenside Surf Deterg* **26**, 406–411.
- Swisher, R. D. (1987).** *Surfactant Biodegradation*. Surfactant Science Series, vol. 18. New York: Marcel Dekker.
- Thyssen, G. J. E. & Wanders, T. H. (1972).** Degradation of n -alkane-1-sulfonates by *Pseudomonas*. *Antonie Leeuwenhoek* **38**, 53–63.
- Thyssen, G. J. E. & Wanders, T. H. (1974).** Initial steps in the degradation of n -alkane-1-sulfonates by *Pseudomonas*. *Antonie Leeuwenhoek* **40**, 25–37.
- Uria-Nickelsen, M. R., Leadbetter, E. R. & Godchaux, W. (1993a).** Sulphonate utilization by enteric bacteria. *J Gen Microbiol* **139**, 203–208.
- Uria-Nickelsen, M. R., Leadbetter, E. R. & Godchaux, W. (1993b).** Sulphonate-sulfur assimilation by yeasts resembles that of bacteria. *FEMS Microbiol Lett* **114**, 73–78.
- Wagner, F. C. & Reid, E. E. (1931).** The stability of the carbon-sulphur bond in some aliphatic sulphonic acids. *J Am Chem Soc* **53**, 3407–3413.
- White, G. F. & Russell, N. J. (1993).** Biodegradation of anionic surfactants and related compounds. In *Biochemistry of Microbial Degradation*, pp. 143–177. Edited by C. Ratledge. Dordrecht: Kluwer.

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