Oxygenation and spontaneous deamination of 2-aminobenzenesulphonic acid in *Alcaligenes* sp. strain O-1 with subsequent *meta* ring cleavage and spontaneous desulphonation to 2-hydroxymuconic acid

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2-Aminobenzenesulphonic acid (2AS) is degraded by *Alcaligenes* sp. strain O-1 via a previously detected but unidentified intermediate. A mutant of strain O-1 was found to excrete this intermediate, which was isolated and identified by m.s., ^{1}H- and ^{13}C-n.m.r. as 3-sulphocatechol (3SC). Proteins from cell extracts of strain O-1 were separated by anion-exchange chromatography. A multicomponent oxygenase was observed to convert 1 mol each of NADH, O_2 and 2AS into 1 mol each of 3SC, NH_3 and NAD^+. The enzyme presumably catalysed formation of the ring of a 2-amino-2,3-diol moiety, and elimination in the amino group led to a rearomatization. 3SC was further degraded via *meta* ring cleavage, which could be prevented by inactivation of the 3-sulphocatechol-2,3-dioxygenase (3SC23O) with 3-chlorocatechol. In Tris buffer, the separated 3SC23O catalysed the reaction of 1 mol each of 3SC and O_2 involving a transient yellow intermediate, and release of 1 mol of sulphite and two organic products. The major product was identified by n.m.r. and by g.c./m.s. as 5-carboxypenta-2,4-dien-5-olide (CPDO), an indicator of formation of 2-hydroxymuconic acid (2HM). The second product was identified as the Z,E isomer of 2HM by comparison with authentic material. When the CPDO in the product mixture was chemically hydrolysed to (Z,E)-2HM, 1 mol of (Z,E)-2HM/mol of 3SC was observed. If oxygenation of 3SC by 3SC23O was carried out in phosphate buffer, only a single product was detected, a keto form of 2HM. This dioate was also formed from authentic (Z,E)-2HM in phosphate buffer. Formation of the natural product (Z,E)-2HM from the xenobiotic, 3SC, seems to involve oxygenation to the unstable 2-hydroxy-6-sulphonomuconic acid semialdehyde, which hydrolyses spontaneously to 2HM. There would appear to be at least one spontaneous reaction per enzyme reaction in this pathway.

INTRODUCTION

With one known exception (Laskin and Lechevalier, 1984), aromatic sulphonates are xenobiotic compounds which are produced and dispersed in the environment in multimillion tonnes annually as detergents (Berth and Jeschke, 1989), dyestuffs and additives to products as diverse as ink and engine oil (Cook and Leisinger, 1991). For a considerable period, only one mechanism for the desulphonation of aromatic compounds was generally accepted. This involved desulphonation to the corresponding catechol as an early or initial metabolic reaction preceding ring cleavage (Cain, 1981; Brilon et al., 1981; Swisher, 1987; Locher et al., 1989; Painter, 1992). One enzyme of this type has been purified and characterized, the two-component 4-sulphobenzoate 3,4-dioxygenase system (EC 1.14.12.8) from *Comamonas testosteroni* T-2 (Locher et al., 1991a). EC 1.14.12.8 is a class IA dioxygenase consisting of a reductase and an oxygenase (e.g. Mason and Cammack, 1992). A similar biochemical reaction is catalysed by *Alcaligenes* sp. strain O-1 during the desulphonation of benzenesulphonate (Thurnheer et al., 1990). These dioxygenases attack the ring at two adjacent carbon atoms, one of which carries the sulphonate substituent, to give the bisulphite-addition complex of a cyclic ketone, which decays spontaneously to form the catechol and sulphite.

An undefined mono-oxygenase is responsible for

desulphonation when aromatic sulphonates are used as sole sulphur sources by *Pseudomonas putida* S313 (Zürrer et al., 1987). Oxygenation (*ortho* cleavage) is also an essential preliminary step to desulphonation of 4-sulphocatechol, because the sulphomuconate is transformed to the relatively stable sulpholactone (4-carboxymethyl-4-sulphobut-2-en-4-olide), which is enzymically hydrolysed to maleylacetate and sulphite (Feigel and Knackmuss, 1993).

Oxygenation thus plays a critical role in destabilizing the C–SO_3^- bond of aromatic sulphonates, whether the desulphonation is concomitant with oxygenation before ring cleavage or in a hydrolytic step subsequent to oxygenolytic ring cleavage and lactonization. In a novel variant of this generality in *Alcaligenes* sp. strain O-1, we find 2-aminobenzenesulphonate (2AS) to be oxygenated to 3-sulphocatechol (3SC), which is then subject to *meta* cleavage and concomitant hydrolysis to the natural product 2-hydroxymuconate (2HM) and sulphite.

MATERIALS AND METHODS

Materials

2HM was a gift from D. W. Ribbons. It was eluted from the h.p.l.c. column (see below) at 21 min with the following u.v.-absorption spectrum (nm): max. < 190, min. 250, max. 296 ($\lambda_{max.}$ at pH 12, 350 nm). 3-Chlorocatechol (Helix Biotech,

Richmond, BC, Canada), orthanilate (> 99 %, 2AS; Tokyo Chemical Industries, Tokyo, Japan), [²H]dimethyl sulphoxide (Dr. Glaser, Basel, Switzerland), dichloromethane (> 99 %), Dowex 50W (X8 20–50 mesh) strong cation exchanger, and diethyl ether (> 99 %) and N-nitroso-N-methylurea (Fluka) for the preparation of diazomethane, and NO-bis(trimethylsilyl)-trifluoroacetamide (Pierce) were from commercial sources. DEAE-Sepharose 6B was from Pharmacia. The sources of routinely used chemicals have been described elsewhere (Thurnheer et al., 1986, 1990).

Analytical methods

Attenuance (a D_{500} value of 1.0 represented 160 mg of protein/l), spectrophotometric determinations and some u.v.-absorption spectra were measured with a Uvicon 820 (Kontron, Zürich, Switzerland). Reversed-phase h.p.l.c. was carried out with Pharmacia–LKB apparatus equipped with a u.v. detector and a diode array detector (Grossenbacher et al., 1986; Locher et al., 1989). The mobile phase for 2AS and 3SC was 100 mM potassium phosphate buffer pH 2.2, at a flow rate of 1 ml/min. Gradient elution was used to separate 3SC, 5-carboxypenta-2,4-dien-5-olide (CPDO) and (Z,E)-2HM; the initial conditions comprised 10 mM potassium phosphate buffer, pH 2.2, at a flow rate of 1 ml/min for 13 min, after which a linear gradient of the second eluent (B, 90 % methanol containing 10 % 10 mM potassium phosphate buffer, pH 2.2) was applied over 3 min to 80 % B. After a further 5 min the initial conditions were regenerated. F.p.l.c. was performed with Pharmacia apparatus. Polarimetric O_2-uptake measurements (Zamanian and Mason, 1987) and m.s. (Locher et al., 1991b) were carried out with apparatus described previously. The electron-impact mass spectra in g.c./m.s. were obtained with Finnigan apparatus after separation on a 13-metre DB1 (2HM) or a 10-metre PS 090 (CPDO) capillary column. ^{1}H-(300 MHz) and ^{13}C-n.m.r. (128 MHz) spectra were obtained with Bruker instrumentation. SO_4^{2-} and Cl^- were quantified by ion chromatography with a suppressor (IONPAC AS9 analytical column; Dionex). Protein in whole cells was solubilized and measured in a Lowry-type assay (Kennedy and Fewson, 1968); otherwise, protein in solution was measured by the method of Bradford (1976). A routine colorimetric test was used for sulphite (Grant, 1947; Kondo et al., 1982). NH_4^+ was determined enzymically (da Fonseca-Wollheim et al, 1974).

Data on stoichiometry represent the means of three to five determinations.

Organisms and their growth

Alcaligenes sp. strain O-1 (DSM 6325) (Thurnheer et al., 1986, 1990; Jahnke et al., 1990) was used routinely. Mutant strain Tn28, derived from strain O-1 by transposon mutagenesis (Jahnke, 1987), was also used. This mutant is unable to utilize 2AS as a source of carbon and energy, but is able to utilize 2AS as a sole source of nitrogen.

The mineral medium of Thurnheer et al. (1986) was used for growth of the wild-type strain O-1. The organism was routinely maintained at 4 °C on 6 mM 2AS/salts medium. A preculture (100 ml 6 mM 2AS/salts medium in a 500 ml Erlenmeyer flask) was inoculated with one colony from a fresh plate of homologous medium, and, when the D_{500} was 0.8, used as inoculum (1 %) for 1-litre batches of the same medium in 5-litre Erlenmeyer flasks. Cells were harvested at D_{500} of 0.7–0.9 (about 33 h) when the specific activity of 2-aminobenzenesulphonate dioxygenase system (2ASDOS) was maximal, and about 1 g wet wt. of cells/l was obtained; if cells were harvested earlier or later, much less

enzyme was obtained, analogous to the effects illustrated by Bünz and Cook (1993), Jahnke et al. (1993) and Junker et al. (1994). Cells were washed in cold 50 mM Tris/HCl buffer, pH 7.5 (set at room temperature), and stored at −20 °C.

The minimal medium used for strain O-1 was adapted for the 2AS-dependent growth of mutant Tn28. NH_4^+ was eliminated and replaced by 5 mM 2AS (a twofold excess), the limiting carbon source was 6 mM succinate, and the pH was set to 6.8 to reduce loss of the alkaline-labile unknown product. Each culture (1 litre) was inoculated with a fresh colony of the mutant from nutrient agar. Growth (D_{500}) and the u.v.-absorption spectra of cell-free culture medium were followed. When the $\lambda_{max.}$ at 298 nm (2AS) shifted to 287 nm (largely 3SC), the cells were removed by centrifugation and discarded. The supernatant was acidified to pH 2 with 5 M HCl, concentrated on a rotary evaporator (30 °C) to 25 ml and stored at 4 °C.

Preparation of cell extracts of *Alcaligenes* sp. strain O-1 and protein separation

Pellets of strain O-1 (about 10 g wet weight) were thawed and suspended in 3 vol. of 50 mM Tris/HCl buffer, pH 7.5, containing 20 % (v/v) glycerol and brought to 1 mM phenylmethane-sulphonyl fluoride. The cells were disrupted by three passages through a chilled French pressure cell (135 MPa), and whole cells and debris were removed by centrifugation (30000 g, 4 °C, 30 min). The pellet was discarded and residual membranous material in the supernatant was removed by ultracentrifugation (200000 g, 4 °C, 40 min). The red–brown supernatant was stirred on ice under a stream of N_2 and a solution of streptomycin sulphate [10 % (w/v) in 50 mM Tris/HCl buffer, pH 7.5] was added slowly (to 2 %, v/v) and stirred for 20 min before the precipitated nucleic acid was removed by centrifugation (30000 g, 4 °C, 20 min).

Components of 2ASDOS and 3-sulphocatechol-2,3-dioxygenase (3SC23O) were separated on a column of DEAE-Sepharose (15 cm × 2.6 cm) at 4 °C. The column was equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM dithiothreitol (buffer A) at 2.5 ml/min, and streptomycin-treated extract (400 mg of protein) was applied to the column; 8 ml samples were collected. Buffer A was pumped for about 30 min, followed by a linear gradient to 60 % buffer B (1 M Tris/HCl, pH 7.5, containing 0.1 mM dithiothreitol) over 140 min. Residual protein was then washed from the column, which was re-equilibrated.

Enzyme assays

2ASDOS was routinely assayed as the rate of 2AS-dependent O_2 uptake in 0.5 ml reaction mixtures containing 1 mg of protein at 30 °C. The basic assay mixture contained 37 μmol of Tris/HCl, pH 7.5, and 200 nmol of NADH, and the reaction was started by the addition of 2 μmol of 2AS. Several variants of this procedure were used. When stoichiometry of the overall reaction was being examined, desalted enzyme and different amounts of 2AS or NADH were used. When components of this multicomponent enzyme were being located in separated protein fractions, 100 μl portions of different fractions were used, irrespective of the protein concentration. The location of the components could be deduced from their colour (Mason and Cammack, 1992), the reductase being yellow and the oxygenase red. The reaction of the reductase was confirmed by its reaction with dichlorophenol-indophenol and with cytochrome c (Locher et al., 1991a).

3SC23O was routinely assayed colorimetrically as the rate of catechol-dependent formation of 2-hydroxymuconate

semialdehyde at 30 °C (Bird and Cain, 1974). The enzyme was also quantified as the rate of catechol-dependent O_2 uptake in 1 ml reaction mixtures at 30 °C. These contained 75 μM Tris/HCl, 10 nmol of $FeSO_4$ and enzyme (0.5 mg of protein), and the reaction was started by the addition of 10 μmol of catechol. When stoichiometry was being examined, different amounts of substrate were used.

Isolation of unknown products

The solution of 3SC in 10 ml of concentrated spent growth medium was adjusted to pH 1.5 with HCl, and ion pair reagent (tetrabutylammonium phosphate, 200 mM final concentration) was added. The ion pair (3SC/tetrabutylammonium) was repeatedly extracted into portions of 20 ml of dichloromethane till no yellow colour ($\lambda_{max.}$ 286 nm) remained in the aqueous phase. The dichloromethane was evaporated off, and the residue was dissolved in 5 ml of distilled water and passed through a strong cation exchanger (25 ml, in the H^+ form) to remove the tetrabutylammonium. This material was passed through the cation exchanger, in the Na^+ form, to give the sodium salt of 3SC, which was freeze-dried.

The product(s) from the *meta* cleavage of 3SC was generated at 22 °C from a 10 ml fraction of 3SC23O in Tris buffer (from the DEAE column) stirred in a flat beaker to which was added 60 nmol of $FeSO_4$ and 10 mg of 3SC in portions. At intervals, 0.5 ml portions of the mixture were placed in the O_2 electrode to confirm the activity of the preparation and then returned to the beaker. When the enzyme lost activity, the mixture was brought to pH 1 with HCl and the precipitated protein was removed by centrifugation. The solution was saturated with NaCl and extracted 10 times into equal volumes of diethyl ether. The ethereal solution was then dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. The dry residue was then examined. On occasion, smaller-scale transformations were carried out with purified 3SC23O (Junker et al., 1994) or with crude 3SC23O which had been desalted in 2.0 ml portions with a PD10 column of Sephadex G-25 (Pharmacia) equilibrated with 50 mM potassium phosphate buffer, pH 7.5. In the latter cases, a portion (0.6 ml) of the Tris-free enzyme was added to an open O_2 electrode and supplemented with 5 nmol of $FeSO_4$ and 0.6 μmol of 3SC; when the reaction stopped, the O_2 concentration rose and protein was removed by ultrafiltration or by precipitation with HCl. Samples were then examined photometrically and by h.p.l.c. Control experiments with the enzyme in Tris buffer were also performed.

RESULTS

Isolation and identification of 3SC from a mutant of *Alcaligenes* sp. strain O-1

Mutant Tn28 of strain O-1 was found to excrete a transient intermediate during growth with 2AS as sole source of nitrogen. This intermediate was identical with the unknown intermediate [coelution from h.p.l.c. column at 9.5 min; u.v.-absorption spectrum (nm): max. 200, 224 (shoulder), min. 236, max. 286] in the degradation of 2AS by strain O-1 (cf. Thurnheer et al., 1990). We thus had access to larger amounts of the unknown compound, which was purified and identified.

The mass spectrum of the unknown compound was acquired by direct probe insertion and electron-impact ionization. The spectrum {m/z 190 [M^+ (the molecular ion)], 172 ($M^+ - H_2O$), 110 ($M^+ - SO_3$), 108 ($M^+ - H_2O - SO_2$), 107 ($M^+ - H_2O - SO_2H$), 92 ($M^+ - H_2O - SO_3$), 80 (SO_3^+), 64 (SO_2^+)} indicated a benzene ring with a sulphono and two hydroxy substituents. The ^{1}H-n.m.r.

spectrum (Table 1A) indicated a benzene ring with three adjacent ring protons and two non-identical quenchable protons at low field (hydroxy groups), which, together with the mass spectrum, suggested 3SC. This conclusion was confirmed by ^{13}C-n.m.r. (Table 1B), which shows six aromatic carbon atoms, one of which is sulphonated, two of which carry non-equivalent hydroxy substituents and three of which carry adjacent protons.

The sample of 3SC was chromatographically pure and contained neither organic impurities (^{1}H- and ^{13}C-n.m.r. spectra) nor SO_4^{2-} ions (ion chromatography), although heavy contamination with Cl^- was detected. Microanalysis of the sample gave a sulphur content of 7.2% (calc. 15.1%). The sulphonate content of the product was calculated from the sulphur content of the sample (48%), the balance being essentially NaCl. The corresponding molar absorption coefficient (ϵ_{286}) was calculated to be about 5100 $M^{-1} \cdot cm^{-1}$.

This isolation of 3SC allowed us to quantify the intermediate in experiments and direct the search for further intermediates.

2ASDOS

Crude extract of 2AS-grown cells of strain O-1 catalysed the NADH-dependent disappearance of 2AS, the release of sulphite and NH_4^+ ions and the concomitant consumption of 2 mol of O_2/mol of 2AS, as previously observed (Thurnheer et al., 1990). The activity of a catechol 2,3-dioxygenase (C23O), in samples in the O_2 electrode, was titrated out by addition of about 20 nmol of the suicide substrate 3-chlorocatechol (Klecka and Gibson, 1981; Bartels et al., 1984), such that addition of catechol caused no consumption of O_2. Disappearance of 2AS was now accompanied by the uptake of only 1.0 mol of O_2/mol of 2AS. The products, 3SC (0.9 mol/mol) and NH_4^+ ion (1.0 mol/mol), were observed, but no sulphite was detected.

The crude extract was chromatographed on the DEAE anion-exchange column [not shown; see Junker et al. (1994)] and several coloured fractions were observed, which were largely separated from the C23O (elution at about 320 mM Tris). No single fraction catalysed the 2AS-dependent uptake of O_2, but when one of a group of red fractions (oxygenase; eluted at about 165 mM Tris) was mixed with one of a group of yellow fractions (reductase; eluted at about 250 mM Tris), oxygenation of 2AS was detected. The stoichiometry, initially observed with 3-chlorocatechol-treated crude extract (see above), was confirmed with the separated proteins, and extended to 1.0 mol of O_2/mol of NADH in the presence of excess 2AS.

The reaction is thus an oxygenation of 2AS by a multicomponent putative dioxygenase with the following reagents and products:

$$NADH + H^+ + O_2 + 2AS \Rightarrow NAD^+ + 3SC + NH_3$$

3SC23O

Crude extract of 2AS-grown cells of strain O-1 catalysed the disappearance of 3SC, the release of 1.0 mol of sulphite/mol of 3SC and the concomitant consumption of 1.0 mol of O_2/mol of 3SC; the reaction mixture remained colourless. 3SC was not degraded by the extract that had been treated with 3-chlorocatechol, which implied involvement of a C23O in the degradative pathway.

The 3SC23O in separated proteins from crude extract of strain O-1 was first located as the formation of 2-hydroxymuconate semialdehyde from catechol. The (yellow) semialdehyde was not further degraded by these fractions and the reaction consumed 1 mol of O_2/mol of catechol. The protein(s) catalysing 3SC-dependent uptake of O_2 was co-eluted with the C23O, and the

Table 1   Data from ^{1}H- and ^{13}C-n.m.r. spectra to identify 3SC, (Z,E)-2HM and CPDO

The chemical under study was dissolved in dimethyl sulphoxide. Chemical shifts are reported as p.p.m. relative to tetramethylsilane. Standard data (Pretsch et al., 1989) give no information on the influence of sulphonate on ^{1}H-n.m.r. chemical shifts, so values for carboxylate were used in calculations (A). The coupling constants in parentheses (C), the attribution of the signal to a proton (C) or a carbon (D) and the literature data for ^{13}C-n.m.r. (in C^{2}H$_3$O^{2}H) (D) are from Whitman et al. (1991). A large water peak was present (E), so quenching of any ionizable group would occur. Saeki et al. (1980) give very similar data for the methyl ester of CPDO in C^{2}HCl$_3$. The coupling constants for unknown II (E) are characteristic of a *cis,cis*-dienol, as in the standard spectrum [penta-2,4-dien-5-olide (PDO) (F)], and different from those in (C).

(A) ^{1}H-n.m.r. of unknown I (3SC)

Proton	δ (p.p.m.)		J_1 (Hz) (obs.)	J_2 (Hz) (obs.)
	Observed	Calculated		
1 (OH)	8.8			
2 (OH)	10.5			
4	6.9	7.3	7.8	1.7
5	6.6	7.1	7.8	
6	6.7	6.9	7.8	1.6

(B) ^{13}C-n.m.r. of unknown I (3SC)

Nucleus	δ (p.p.m.)	
	Observed	Calculated
1	145.4	143.9
2	142.1	140.4
3	131.2	132.1
4, 5, 6	(116.3, 117.1, 117.7)	

(C) ^{1}H-n.m.r. of (Z,E)-2HM

Proton(s)	δ (p.p.m.)		J_1 (Hz) (obs.)	J_2 (Hz) (obs.)
	Observed	Calculated		
1, 6 (COOH)	12.8			
2 (OH)	9.8			
3	6.2	6.3	12 (12)	
4	7.5	7.5	15 (15)	12 (12)
5	6.0	6.1	16 (15)	

(D) ^{13}C-n.m.r. of (Z,E)-2HM

Nucleus	δ (p.p.m.)	
	Observed	Literature
1	165	167
2	147	148
3	108	109
4	137	140
5	122	122
6	168	171

(E) ^{1}H-n.m.r. of unknown II (CPDO)

Proton(s)	δ (p.p.m.)		J_1 (Hz) (obs.)	J_2 (Hz) (obs.)
	Observed	Calculated		
2	6.6	6.4	8.4	
3	7.6	7.6	9.4	6.6
4	7.1	7.0	6.6	

(F) ^{1}H-n.m.r. of PDO from the literature

| Proton(s)	δ (p.p.m.)	J_1 (Hz)	J_2 (Hz)
2	6.4	6.3	
3	7.6	9.4	6.3
4	6.4	9.4	5.0
5	7.8	5.0	

3SC

(Z,E)-2HM

CPDO

PDO
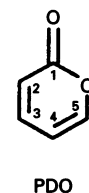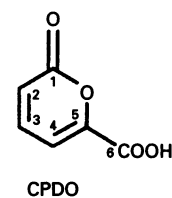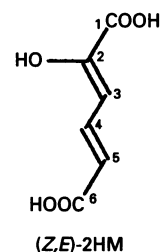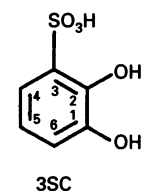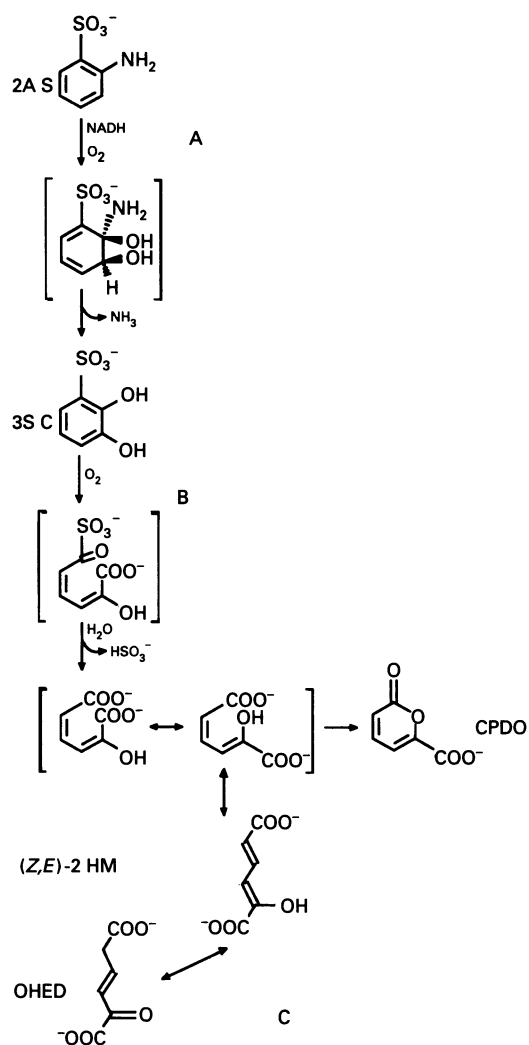
Figure 1 Degradation of 2AS, via 3SC and the natural product 2HM, to OHED

Steps A, B and C are enzyme-catalysed. Reaction C can be catalysed by e.g. phosphate buffer, at a lower rate than the enzymic reation (Whitman et al., 1991). We believe the other reactions to be spontaneous. The spontaneous reaction to CPDO occurs in Tris buffer.

reactions were later shown to be catalysed by the same protein (Junker et al., 1994); 1 mol of O_2 was consumed and 1 mol of sulphite ion released/mol of 3SC and the reaction remained colourless. When milligram amounts of 3SC were used as a substrate for the separated C23O, a transient yellow colour was observed over the course of a few seconds. The product(s) was colourless. We thus postulated 2,3-dioxygenation of 3SC to an unstable (yellow) sulphonoaldehyde which hydrolysed spontaneously to sulphite and 2HM (Figure 1).

2HM

The vial containing standard 2HM was labelled solely with the structural formula of (E,Z)-2HM (see Figure 2 for nomenclature). This material was pertrimethylsilylated and analysed by g.c./m.s. One major peak and three trace peaks, all with identical mass spectra which supported the structure, were observed. We presume that all four isomers $(E,Z$-, E,E-, Z,Z- and Z/E-) were detected. The ^{13}C-n.m.r. spectrum of the standard was essentially
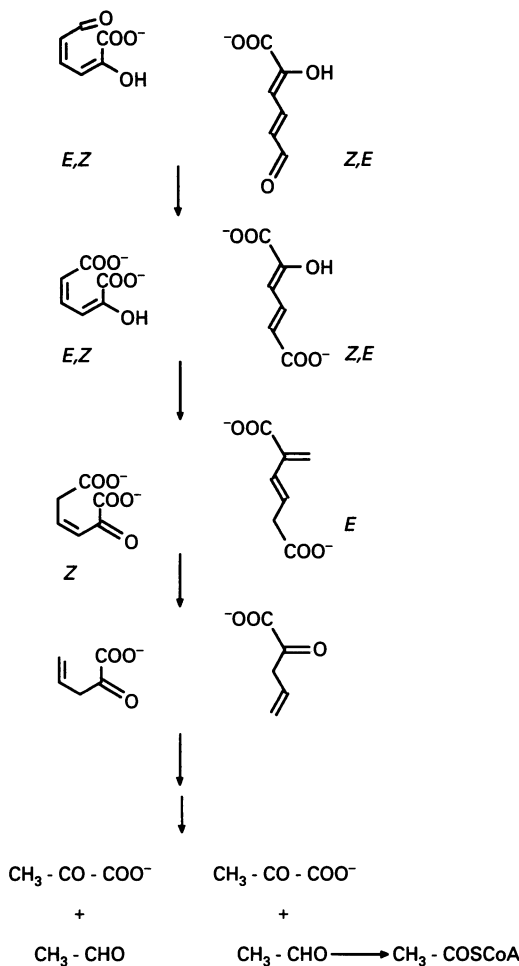
Figure 2 Normal depiction of the oxalocrotonate arm of the *meta* pathway for the degradation of catechol and a version showing C=C bonds in largely established conformations

The textbook version of reactions in the *meta* pathway, on the left-hand side of the Figure (e.g. Bayly and Barbour, 1984), is based on research carried out before n.m.r. became a routine tool. R. C. Bayly (personal communication) has confirmed that those structures simply reflect the ring structure from which they are derived. The use of *cis* and *trans* nomenclature for C=C bonds, which is valid in the *ortho* pathway because each of the two carbons is bound to a hydrogen and an additional carbon atom, cannot be used unambiguously in the *meta* pathway, so the *E,Z* system is needed to define the configuration of the C=C bonds (e.g. Pine, 1987). Whereas there are solid data for (Z,E)-2HM and (E)-OHED (Whitman et al., 1991, 1992; Lian and Whitman, 1993), the (Z,E)-muconate semialdehyde is still hypothetical. However, the oxo–enol tautomerism which permits 2HM to attain the Z,E configuration is equally operative in the semialdehyde. The depiction of acetyl-CoA as one end point of this *meta* pathway stems from Powlowski et al. (1993).

identical with that published for (Z,E)-2HM (Table 1D), and negligible organic impurities were detected. We confirmed the Z,E conformation of the standard with both the chemical shifts and the coupling factors in ^{1}H-n.m.r. (Table 1C), again with negligible organic impurities. The chemical synthesis used for the standard involves an alkaline hydrolysis, so isolation of the Z,E isomer is to be expected. The isomers detected by g.c./m.s. were presumably in equilibrium with the major organic component. The (Z,E)-2HM standard was assayed to be 24 % pure, given the ϵ_{350} at pH 12 of 21000 (Saeki et al., 1980) and we presumed the impurities to be inorganic matter.

The reaction of 3SC23O with 3SC was initially examined in Tris buffer. One product, II, was clearly detected by h.p.l.c.

[elution at 10 min; u.v.-absorption spectrum (nm): max. 196, min. 215, max. 224, min. 248, max. 299 (λ_{max} at pH 12, 350 nm)] whereas the presence of an additional product, III, was confirmed after methylation with diazomethane and g.c./m.s. (electron-impact ionization. Product III was 2HM $\{m/z\ 200\ [M^+$ (the molecular ion)], 185 $(M^+-CH_3)\}$. The major product, II, was tentatively identified as CPDO from the ^{1}H-n.m.r. spectrum (Tables 1E and 1F). This identification was confirmed by g.c./m.s. of methylated product II $\{m/z\ 154\ [M^+$ (the molecular ion)], 95 $(M^+-COOCH_3)$, which supports the structure and corresponds to published data (Saeki et al., 1980). The variation of the u.v.-absorption spectrum of product II with pH was also analogous to that published and illustrated for CPDO (Saeki et al., 1980): carboxylate anion at pH 7.5, free acid at pH 1.0, hydrolysis to the enolate trianion of (Z,E)-2HM at pH 12, protonation to (Z,E)-2HM dianion at pH 7.5, and then protonation to (Z,E)-2HM diacid at pH 1.0, or rearrangement to 2-oxohex-3-ene-1,6-dioate (OHED) at pH 7.5 [see Whitman et al. (1991)].

CPDO was not degraded by crude extract that degraded 3SC, whereas (Z,E)-2HM disappeared. CPDO may be seen as an indicator of the presence of 2HM (Saeki et al., 1980; Müller and Lingens, 1980; cf. Eaton and Ribbons, 1987), specifically (Z,Z)-2HM, before lactonization. The mixture of products II and III was brought to pH 12, to hydrolyse CPDO and convert all C=C double bonds into the *trans* configuration, and then acidified. One product was obtained, and its ^{1}H- and ^{13}C-n.m.r. spectra were identical with those of authentic (Z,E)-2HM (Tables 1C and 1D); 1.1 mol of (Z,E)-2HM/mol of 3SC was formed.

We were concerned about the low recovery of the putative product of the reaction, 2HM, coupled with the high recovery of CPDO, because it was unclear whether CPDO was an enzymic or a spontaneous product. Whitman et al. (1991) demonstrated that phosphate buffer facilitates rapid isomerization of (Z,E)-2HM, so we examined the degradation of 3SC in phosphate buffer. As controls, the behaviour of (Z,E)-2HM in Tris and in phosphate buffer was examined. The control in phosphate buffer yielded a single product (IV) with the same u.v.-absorption spectrum (max. at 235 nm, shoulder at 290 nm) as OHED (Figure 1), the isomerization product from (Z,E)-2HM (Saeki et al., 1980; Whitman et al., 1991). The control in Tris buffer yielded a mixture of CPDO and (Z,E)-2HM, but no product IV. The reaction of 3SC23O with 3SC in phosphate buffer yielded solely product IV. We conclude that formation of non-metabolized CPDO does indeed indicate the presence of (Z,Z)-2HM during the degradation of 3SC but that isomerization to (Z,E)-2HM follows in the cell (Figure 1). (Z,E)-2HM would then be degraded enzymically via 4-oxalocrotonate tautomerase (Whitman et al., 1991).

The overall reaction of 3SC23O, before isomerization by '4-oxalocrotonate tautomerase' or phosphate buffer [see Chen et al. (1992)] is thus:

$$3SC + O_2 + H_2O \Rightarrow (Z,E)\text{-2HM} + HSO_3^-$$
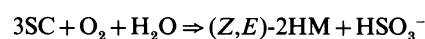
2AS is thus degraded in two stoichiometric oxygenation reactions to a natural product which we isolated as (Z,E)-2HM, and which is an intermediate in the *meta* degradative pathway of catechol.

DISCUSSION

Desulphonation in this degradative sequence resembles those in the few defined pathways, inasmuch as introduction of an O_2 substituent to the carbon atom carrying the sulphono group destabilizes the $C-SO_3^-$ bond. The destabilization is direct, in that no stable sulphonated intermediate is formed, though the

transient yellow colour observed when large amounts of 3SC were desulphonated would suggest that the putative sulphonosemialdehyde (Figure 1) is released from the enzyme surface before decaying. The fact that pure preparations of C23O carry out the reaction shows that no other enzyme plays a role in the formation of 2HM. This is thus the third class of oxygenase (EC 1.13.11.-) directly destabilizing a $C-SO_3^-$ bond, the first being a defined multicomponent dioxygenase (EC 1.14.12.8) (Locher et al., 1991a) and the second an undefined mono-oxygenase (Zürrer et al., 1987). We presume the loss of sulphite after enzymic introduction of O_2 to be spontaneous in each case.

The exploitation of oxygenation to eliminate an otherwise stable substituent at an aromtic ring would appear to be common (Engesser et al., 1989). Bernhardt et al. (1973, 1988) probably demonstrated the first example (EC 1.14.99.15), where a phenolate is the leaving group; the enzyme will also release 4-aminobenzoate and 4-mercaptobenzoate from N-methyl-4-aminobenzoate and S-methyl-4-mercaptobenzoate respectively. Enzymes of EC 1.14.14.-, cytochromes P-450, catalyse similar apparently spontaneous release of anions as a result of oxygenation (Sariaslani, 1991). The spontaneous release of Cl$^-$ from an aryl halide by enzymes EC 1.14.12.9 and EC 1.14.12.13 has been well defined (Markus et al., 1986; Fetzner et al., 1992), as has desulphonation (Locher et al., 1991a) and loss of a phenolate to cleave the ether linkage in dibenzofuran (Bünz and Cook, 1993; cf. Wilkes et al., 1992). Less-well defined dixoygenases are involved in removing aromatic nitro groups (Spanggord et al., 1991; Suen and Spain, 1993a) and cleaving the heterocycle from dibenzothiophene (van Afferden et al., 1993). The dioxygenases of EC 1.14.11- and 1.13.11.- catalyse apparently spontaneous releases of phenolates (Fukumori and Hausinger, 1993; Pfeiffer et al., 1993). Several flavin mono-oxygenases cause apparently spontaneous release of anions. Pentachlorophenol dehalogenase (EC 1.14.13.50) has an especially wide substrate range which also includes nitrile, nitro and amino groups (Xun et al., 1992; Topp et al., 1992); Suen and Spain (1993b) report another enzyme of this class that removes a nitro group and Spanggord et al. (1991) cite others. What is still unclear is to what extent this apparent spontaneity is real and what role, if any, is played by the several oxygenases after oxygenation.

In the context of the spontaneous release of anions, we presume the first reaction in the degradative pathway of 2AS (Figure 1) to involve dioxygenation to yield an unstable diol intermediate which rearomatizes spontaneously with the release of NH_3. Strain O-1 grown under the conditions used in this work synthesizes only one multicomponent oxygenase that we can detect (Junker et al., 1994). This enzyme catalyses dioxygenation of benzenesulphonate (Thurnheer et al., 1990), and we currently presume the enzyme system to be a dioxygenase with all substrates. We thus term the enzyme the 2-aminobenzene-sulphonate dioxygenase system (2ASDOS). This hypothesis will have to be confirmed with the purified enzyme system. However, not all reactions involving oxygenases and release of an 'anion' need involve spontaneous reactions. Anthranilate mono-oxygenase (EC 1.14.13.35) is reported to deaminate enzymically (Powlowski et al., 1987).

The spontaneous reactions in the degradation of 3SC are not limited to the release of anions. Our results, together with published data, seem to call into question the standard representation of the *meta* pathway shown in textbooks (Bayly and Barbour, 1984; Assinder and Williams, 1990), where carbon–carbon double bonds are depicted in *cis* configuration. We should then be able to detect (E,Z)-2HM as the product from 3SC (Figure 1). What we detect in Tris buffer is CPDO, where the Z,Z configuration of the double bonds is stabilized by

lactonization, together with traces of (Z,E)-2HM. When we follow the lead of Whitman et al. (1991) and use phosphate buffer for the reaction, no C=C bond in the configuration pertaining to the ring is detected. Depictions of the *meta* pathway reflecting the structure and the aromatic educt thus seem to be inaccurate, because any E,Z structure will rapidly and spontaneously isomerize to be predominantly Z,E (Figure 2).

CPDO (or a derivative) has been detected by several groups as an indicator of 2HM (or a derivative) (Müller and Lingens, 1980; Saeki et al., 1980; Eaton and Ribbons, 1987), and may be linked to the use of Tris buffer in which (multicomponent) oxygenases are often more active than in phosphate buffer. Harayama et al. (1989) re-emphasized the instability of the *meta* intermediates, but still used the convention of the conformation of the aromatic educt. Modern n.m.r. methodology (Whitman et al., 1991, 1992; Lian and Whitman, 1993) now allows us to present the correct isomers with full justification (Figure 2). Indeed, the name 4-oxalocrotonate was used frequently for an oxo tautomer of 2HM, and shown in the *cis* configuration, whereas the C=C bond in crotonate is the *trans* form (Budavari, 1989). It should also be noted that our use of the term '2-hydroxymuconate' is also a convention. This is the form in which the compound is isolated after acidification to allow extraction into organic solvent. In solution, other tautomers are also present in equilibrium (Whitman et al., 1991, 1992; Lian and Whitman, 1993).

Whereas CPDO is not involved in the *meta* pathway from catechol (e.g. Kersten et al., 1982), a substituted CPDO can be an intermediate in the degradation of protocatechuate (Kersten et al., 1982). Pfeiffer et al. (1993) report CPDO as a product in the degradation of diphenyl ether, where lactonization causes spontaneous loss of the phenolate; we wonder whether this reaction would proceed in phosphate buffer totally free of Tris buffer, and whether the degradative pathway *in vivo* may not include an esterase.

The biochemist tends to expect biochemical pathways to be purely enzyme-catalysed (Kornberg, 1990), though at least one exception, dehydroquinate synthase, is known to catalyse reactions that are followed by non-enzymic steps (Knowles, 1989). A glance at Figure 1, in the light of the above discussion, shows that in the first three biochemical reactions known or expected to occur, three and possibly four spontaneous reactions occur. This ratio is presumably an exception to the rule.

The data in this paper partially contradict an earlier publication, which indicates desulphonation before deamination (Thurnheer et al., 1990). The latter data were from experiments with whole cells and were subject to two major problems: (a) high background releases of each ion, especially NH_4^+ from the cells, and (b) difficulties of adequately inhibiting 3SC23O without inactivating 2ASDOS. Three of four experiments gave the results we published, whereas one experiment resembled the data given here. The present data were obtained with essentially zero background and complete mass balances, and are internally consistent with the organic compounds we identified. We now suspect that Thurnheer et al. (1990) achieved incomplete inhibition of the 3SC23O and that the background release of NH_4^+ from whole cells was more variable than was realized.

The degradative pathway of 2AS forms a bridge between the longer-known desulphonations, those occurring before ring cleavage, and that occurring subsequent to ring cleavage (see the Introduction). The desulphonation here occurs as a result of ring cleavage. The pathway has many similarities to that of 4-aminobenzenesulphonate (Feigel and Knackmuss, 1993). The first step is deamination at the aromatic ring. The second step is a ring-cleavage reaction with a novel substrate (3- or 4-sulphocatechol). In our case, the desulphonation is apparently

spontaneous, whereas the *ortho* ring-cleavage product from 4-sulphocatechol requires a further enzymic step, lactonization, to prepare the $C-SO_3^-$ bond for enzymic hydrolysis.

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