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Oxygen-dependent desulphation of monomethyl sulphate by *Agrobacterium* sp. M3C

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Abstract

Agrobacterium sp. M3C, previously isolated from canal-water for its ability to grow on monomethyl sulphate, degraded this ester with stoichiometric liberation of inorganic sulphate. In contrast with the biodegradation of monomethyl sulphate in *Hyphomicrobium* sp., and of other longer-chain alkyl sulphates in *Pseudomonas* spp., the pathway in *Agrobacterium* appeared not to involve a sulphatase enzyme capable of catalysing ester-bond hydrolysis. No such sulphatase was detectable under a range of conditions of bacterial culture, or using various methods for preparing cell-extracts, or different assay conditions. There was no incorporation of ^{18}O-label from $H_2{}^{18}O$ into the liberated inorganic sulphate. No methanol was detectable during biodegradation, and the organism was incapable of growth on methanol, and did not produce methanol dehydrogenase activity when grown on monomethyl sulphate. Tracer studies using mono[^{14}C]-methyl sulphate indicated that formate serine and glycine were produced during the biodegradation. The presence of these amino acids, together with high activity of hydroxypyruvate reductase, indicated the operation of the serine pathway common in methylotrophs. Use of an oxygen electrode in conjunction with monomethyl[^{35}S]sulphate showed that release of $^{35}SO_4^{2-}$ was dependent on availability of O_2, and that there was equimolar stoichiometry among monomethyl sulphate degraded, O_2 consumed and $^{35}SO_4^{2-}$ released. A proposed pathway for the degradation involved an initial mono-oxygenation to methanediol monosulphate with subsequent elimination of SO_4^{2-} and concomitant formation of formaldehyde. The pathway was compared with degradation mechanisms for other C_1 compounds and for other sulphate esters.

Introduction

Sulphate esters of long-chain aliphatic alcohols occur both naturally (for a review see Dodgson et al. 1982) and as important components of many commercial detergent preparations (Shore & Berger 1976). The ability of microorganisms, especially bacteria, to accomplish mineralisation of these materials is manifest by the absence of significant accumulations in the environment. Biodegradation is initiated by alkylsulphatase enzymes (Dodgson & White 1983; Thomas & White 1989) that separate SO_4^{2-} ions from the long chain alcohols by a simple mechanism involving hydrolysis of the C-O-S ester linkage at either the C-O or O-S bonds.

The alkylsulphatases studied hitherto show high degrees of positional specificity (for esters of primary or secondary alcohols) and stereospecificity (for D- or L-secondary esters). However, a common feature is that although they are non-specific

insofar as they can accommodate a range of alkyl chain-lengths, none will operate on substrates below C_5. Some short-chain alkyl sulphates occur naturally, for example, propyl sulphates in avian eggs (Yagi 1964, 1966), and others are produced industrially. Monomethyl sulphate (CH_3OSO_3H) is formed in appreciable quantities during combustion of coal (Lee et al. 1980) and as a waste product following the use of dimethyl sulphate in methylation reactions in the agrochemical and dyestuffs industries (Ghisalba & Kuenzi 1983). Occurrence of such compounds in the environment, together with the known inability of long-chain alkylsulphatases to hydrolyse them, prompted a successful search for other bacteria able specifically to utilise short-chain alkyl sulphates (Crescenzi et al. 1985; White et al. 1987). Preliminary evidence indicated that propyl and butyl sulphates were degraded in a coryneform species by a sulphatase enzyme specific for C_3-C_7 esters, and other workers (Ghisalba & Kuenzi 1983; Ghisalba et al. 1986) have reported a monomethyl sulphatase in a *Hyphomicrobium* sp. MS 223 capable of growth on monomethyl sulphate. However, an *Agrobacterium* sp. isolated in our laboratory from canal-water for its ability to grow on monomethyl sulphate appeared devoid of any detectable methylsulphatase activity (White et al. 1987). This observation, together with the observation that microbial assimilation of numerous C_1 compounds is initiated by mono-oxygenation reactions, led us to investigate the possible existence of an alternative oxygen-dependent mechanism for biodegradation of monomethyl sulphate.

Materials and methods

Except where state otherwise, all chemicals were analytical grade supplied by BDH Ltd or Sigma Chemical Co. both of Poole, Dorset, U.K.

Monomethyl sulphate

Dimethyl sulphate (5 ml; 52.8 mmol; Fluka Chemicals) was dissolved in 0.5 l distilled water and maintained at 50–60° C for 1 h. Preliminary studies showed these conditions to be adequate for complete hydrolysis of the first methyl group without significant loss of the second. After neutralisation with 10 M-KOH, potassium monomethyl sulphate was recovered by freeze-drying (95% yield), and recrystallised from methanol. Elemental analysis of the product was K, 26.5% (theory 26.03%); C, 8.17 (7.99); H, 1.99 (2.01); SO_4, 64.63 (63.96). Infra red spectra (Nujol mulls) contained strong absorption bands at 1210–1260 and 770–810 cm^{-1} corresponding to vibrational modes of S-O and C-O-S linkages respectively (Lloyd et al. 1961). Absorptions at 3320 cm^{-1} characteristic of free-OH groups were absent.

Mono[^{14}C]methyl sulphate was prepared by aqueous hydrolysis of di[^{14}C]methyl sulphate (New England Nuclear, 0.598 TBq mol^{-1}). The whole sample (9.25 MBq) was dissolved in about 10 ml of distilled water. After 3 h at 37° C, the solution was neutralised with KOH solution and rotary-evaporated to dryness. The residue was twice re-dissolved in Analar methanol and re-evaporated to remove [^{14}C]methanol. The product was purified by preparative TLC on 10 × 10 cm Silica Gel G 60 plates (E. Merck, Darmstadt) eluted with ethanol/chloroform/water (4 : 2 : 1, by vol.). The area of mono[^{14}C]methyl sulphate was located by autoradiography (see below), scraped from the plate and eluted with 6 × 1 ml portions of water. Pooled washings were freeze-dried and the residue stored at − 20° C. Authenticity of the product was established by mixing a sample with unlabelled monomethyl sulphate and repeatedly recrystallising from methanol whereupon the specific activity remained constant (1.6, 1.6, 1.5 kBq/mmol). Two dimensional TLC (see below) in ethanol/chloroform/water (4 : 2 : 1 by vol.) and ethanol/water/ammonia (8 : 5 : 1 by vol.) produced a single radioactive component.

Monomethyl [^{35}S]sulphate was prepared by reacting methanol with [^{35}S]sulphuric acid. Inorganic [^{35}S]sulphate (Amersham International, plc; 37.9 MBq; 0.9 nmol) was freeze-dried, and sulphuric acid (0.1 mmol) was added to the residue and the mixture allowed to equilibrate for 18 h at 10° C. Methanol (Analar, 0.1 mmol) was added to

the cooled (− 20° C) mixture. After 2 h at − 20° C, the mixture was neutralised with 15 μl of 10 M-KOH. Monomethyl [^{35}S]sulphate was purified from the mixture by preparative TLC as described above.

Other alkyl sulphates

Primary alkyl sulphates with chain lengths from C_2 to C_{10} were prepared by the method of Lloyd et al. (1961) and purified by recrystallization from methanol as potassium salts.

Organism and cultural conditions

Agrobacterium sp. strain M3C was isolated previously for its ability to grow on monomethyl sulphate (White et al. 1987). Culture media contained, per litre of distilled water, K_2HPO_4, 7 g; KH_2PO_4, 3 g; NH_4Cl, 0.5 g; NaCl, 0.5 g; $MgCl_2.6H_2O$, 0.15 g and 1 ml of trace elements solution. Trace elements solution contained 20 mg each of $Na_2B_4O_7.10H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $CoSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$ and $MnSO_4.H_2O$ and 1 g of $FeSO_4$ per litre distilled water. This basal salts medium (25–400 ml in 0.1–1 l Erlenmeyer flasks) was autoclaved (0.1 MPa, 121° C for 20 min) and supplemented with small volumes of filter-sterilized monomethyl sulphate solution to give a final concentration of 21 mM (0.3% w/v). Cultures were incubated at 30° C in an orbital shaker (150 rpm) and growth was monitored by culture optical density (OD_{420}). Stock cultures were maintained on plates and slopes of the same medium solidified with 1.5% Noble agar (Difco, Detroit, Mi.).

Preparation of cell suspensions and cell-extracts

Cells were harvested by centrifugation (90000 g_{av}.min, GS3 rotor in a Sorvall RC-5B centrifuge), washed and resuspended in basal salts medium. For cell extracts, cells were washed and resuspended in 100 mM-Tris/HCl pH 7.5, and then disrupted in one of three ways:

(i) by sonication at 0° C using a model B-30 Cell Disruptor (Branson Sonic Power, Inc.) pulsed in 1 s bursts for 3 min;
(ii) passage (three times) through a chilled French pressure cell (American Instrument Co., Inc.) at 126 MPa;
(iii) incubating for 1 h at 30° C with lysozyme (0.5 mg/ml) in 0.1 M phosphate buffer pH 6.5 containing 20% (w/v) sucrose and 10 mM EDTA and lysing the resulting spheroplasts by the addition of Triton X-100 to a final concentration of 0.1%.

In each case, membrane fractions were removed by centrifuging (90000 g_{av}.min). Whole cell lysates, supernatants and resuspended membrane fractions were stored at 4° C until required.

Assays

Inorganic SO_4^{2-} liberated from monomethyl sulphate was quantified routinely by the turbidimetric ($BaCl_2$/gelatin) method of Dodgson (1961) as modified by Thomas & Tudball (1967). Desulphation of monomethyl [^{35}S]sulphate during growth of *Agrobacterium* sp. M3C was followed by removing 0.1 ml samples from the culture at intervals, centrifuging to remove cells, and adding 0.1 ml of 0.1 M $BaCl_2$ to precipitate $^{35}SO_4^{2-}$ ions as $Ba^{35}SO_4$. The suspension was diluted to 0.5 ml and the precipitate separated by centrifugation, washed once in 0.5 ml distilled water and resuspended in 0.5 ml water. Radioactivities in the two supernatants, measured by mixing each with 1.5 ml of Hydroluma liquid scintillation cocktail (May & Baker, Manchester) and counting in an Intertechnique 4041 spectrometer, were used to calculate residual monomethyl sulphate. Radioactivities of the resuspended $Ba^{35}SO_4$, measured in the same way using $^{35}SO_4^{2-}$ internal standards to determine counting efficiency, were used to calculate amounts of $^{35}SO_4^{2-}$ released. Desulphation of monomethyl [^{35}S]sulphate in the oxygen electrode (see below) was monitored in a similar way.

Attempts were made to detect formaldehyde in cultures growing on monomethyl sulphate, using the pentandione assay (Sarwicki & Sarwicki 1975).

NAD(P)-Independent methanol dehydrogenase (EC 1.1.99.8) was assayed by the method used by Schar et al. (1985) to detect activity in monomethyl sulphate-degrading *Hyphomicrobium* sp.

Hydroxypyruvate reductase [D-glycerate: $NAD(P)^+$ 2-oxidoreductase, EC 1.1.1.81] was assayed in a mixture containing 0.6 mM lithium hydroxypyruvate, 0.1 mM NADH or NADPH, 1 mM KCN (to inhibit NADH oxidase in crude cell-extracts) and 10 μl of strain M3C lysate (0.02 mg total protein) in a total volume of 1 ml of 0.1 M sodium phosphate buffer, pH 7.0. Rate of decrease of A_{340} was used to calculate enzyme activity (μmol NAD(P)H oxidised min^{-1} mg^{-1} protein).

Protein concentrations in cell extracts were measured by the method of Bradford (1976), using bovine serum albumin as standard.

Detection of metabolites by thin-layer chromatography and autoradiography

Cells suspended in basal salts medium were incubated with ^{14}C- or ^{35}S-labelled monomethyl sulphate at 30° C and samples removed periodically. In some experiments samples were mixed (10 : 1, v/v) with 5% sodium metabisulphite solution in attempts to trap formaldehyde. For 1-dimensional TLC, samples and authentic standards were applied as 1 μl-spots 2 cm from one edge of 10 × 10 cm plastic plates pre-coated with Silica Gel G60. Chromatograms were routinely developed in ethanol/chloroform/water (4 : 2 : 1 by vol.), then air-dried. Chromatograms were exposed to Kodak X-omat S X-ray film for up to 7 days and the films developed in Kodak Developer (diluted 1 : 9 with water) and Kodafix (diluted 1 : 4 with water).

For 2-dimensional TLC, samples were spotted in the corner (1.5 cm from adjacent edges) of a 10 × 10 cm plate and eluted in ethanol/chloroform/0.5% aqueous sodium metabisulphite (4 : 2 : 1 by vol.) in the first dimension, and methanol/water/ammonia, s.g. 0.880 (8 : 1 : 1 by vol.) in the second. [^{14}C]Formate (sodium salt) and [^{14}C]formaldehyde from Sigma Chemical Co. Ltd., and [U-^{14}C]glycine and [U-^{14}C]serine from Amersham International were used as standards.

Polyacrylamide gel zymography

Lysed cell extracts were subjected to non-denaturing polyacrylamide gel electrophoresis according to Payne et al. (1974). Extruded gels were incubated at 30° C for 4 h in solutions containing the appropriate alkyl sulphate (20 mM) and barium acetate (20 mM) in 10 mM-Tris/HCl, pH 7.5. Under these conditions, sulphatases liberating SO_4^{2-} from the supplied ester were revealed as bands of insoluble $BaSO_4$.

Gas-liquid chromatography

Cultures of *Agrobacterium* M3C growing on 0.3% monomethyl sulphate in basal salts were sampled at intervals throughout the growth period. Cells were removed by centrifugation and samples of supernatant (0.1 μl) were injected directly onto a 1.8 m × 2 mm glass column containing GP 60/80 Carbopak B coated with 5% Carbowax 20M (Supelco). The column was operated isothermally at 90° C with N_2 as carrier gas (30 ml/min) in a Perkin Elmer 8310 programmable gas chromatograph fitted with a flame ionisation detector. The detector and injection block were maintained at 230° C. The detector was calibrated with standard aqueous solutions of methanol.

^{18}O-Incorporation

Two portions (0.25 ml each) of a solution containing 25 mM monomethyl sulphate in basal salts medium were freeze-dried separately. One sample (the control) was reconstituted by adding normal distilled water, and the other by adding 0.25 ml of $H_2{}^{18}O$ (70 atom % excess, Amersham International). Cells of *Agrobacterium* M3C grown in 100 ml of 0.3% monomethyl sulphate to mid-exponential phase were harvested, washed in basal salts, and resuspended in 1 ml. Portions (0.25 ml) of the cell suspension were mixed with the $H_2{}^{18}O$-enriched and un-enriched solutions of monomethyl sulphate. After incubation for 6.7 h at 30° C to allow degradation of the ester, the reaction was stopped

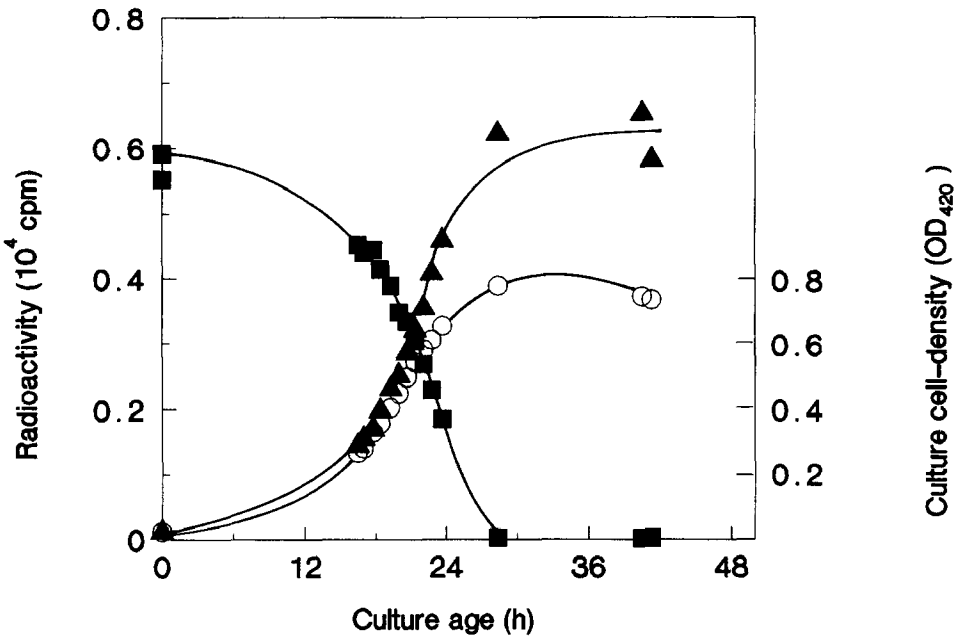

Fig. 1. Growth of *Agrobacterium* sp. M3C on 0.3% w/v monomethyl [^{35}S]sulphate. Samples (0.1 ml) removed at intervals were analysed for liberated $^{35}SO_4^{2-}$ (▲), residual monomethyl [^{35}S]sulphate (■) and culture turbidity at 420 nm (○). Radioactivity data are expressed as cpm for each component in the 0.1 ml sample.

by adding 0.1 ml of 10 M-HCl, and the cells and debris removed by centrifuging. Extent of incorporation of ^{18}O into SO_4^{2-} was assessed by infra red spectroscopy as described previously (Crescenzi et al. 1984).

Oxygen electrode experiments

A thermostated Clark-type oxygen electrode (Rank Brothers, Cambridge) contained 3 ml of basal salts medium equilibrated with air at 30° C. Cells of *Agrobacterium* M3C harvested in exponential phase and washed in basal salts, were resuspended (50-fold concentrated) and samples (50–200 μl) were introduced into the electrode. After establishing the basal rate of O_2-uptake, small volumes (5–20 μl) of concentrated substrate solutions were added. The electrode was calibrated with NADH as described by Robinson & Cooper (1970).

Results and discussion

Liberation of sulphate from monomethyl sulphate by whole cells

Growth of *Agrobacterium* sp. M3C on monomethyl [^{35}S]sulphate was accompanied by the disappearance of the ester from the growth medium and the simultaneous appearance of stoichiometric amounts of $^{35}SO_4^{2-}$ (Fig. 1). The recovery of ^{35}S (residual monomethyl [^{35}S]sulphate plus $^{35}SO_4^{2-}$ as $Ba^{35}SO_4$) averaged over all samples was 102% ± 4% of the original activity present, indicating precise stoichiometry of conversion of substrate to SO_4^{2-}. Figure 1 also shows that the amount of bacterial growth during the exponential phase was proportional to product formation, and therefore to the amount of substrate degraded.

Resting cell suspensions of strain M3C grown on monomethyl sulphate also accomplished quantitative desulphation of added monomethyl sulphate. Cells grown on other carbon sources (nutrient broth, pyruvate, methylamine) were unable to

achieve immediate desulphation, but were induced to do so after exposure to the ester.

Search for methylsulphatase activity

In view of (i) the involvement of sulphatases in the degradation of mid- to long-chain alkyl sulphates (Dodgson & White 1983; Dodgson et al. 1982), (ii) the occurrence of a monomethylsulphatase in *Hyphomicrobium* species (Ghisalba & Kuenzi 1983) and (iii) near-stoichiometric release of SO_4^{2-} from monomethyl sulphate in the present study (Fig. 1), a search was made for monomethylsulphatase activity in extracts of *Agrobacterium* sp. M3C. Lysates of cells grown on 0.2% (13.3 mM) monomethyl sulphate were tested for ability to liberate SO_4^{2-} from the ester using both the turbidimetric $BaCl_2$/gelatin assay and polyacrylamide gel zymography. No monomethylsulphatase was detected by either method under any of the following assay conditions: 4–20 mM substrate concentration, pH 7.0–7.5, incubation times from 2 min to 22 h. Under all these conditions there was also no detectable activity towards any primary alkyl sulphate homologues from C_1 to C_{10}. Cell extracts and membrane fractions prepared by sonication or French pressure cell treatment of cells grown on different concentrations of monomethyl sulphate (0.1–0.3%) or from cells harvested at different stages of batch culture (exponential or stationary phase), all failed to exhibit any methylsulphatase activity.

Further support for the absence of a sulphatase-initiated pathway was the observation that methanol (the expected co-product of sulphatase action) was not detectable by GLC either in culture media containing cells growing on 19 mM monomethyl sulphate, or in suspensions of washed resting cells in medium containing 10 mM monomethyl sulphate. Furthermore the organism did not grow on methanol as sole source of carbon under the conditions tested (1–10 mM methanol, 30° C, aerobic), and extracts of cells grown on monomethyl sulphate to mid-exponential phase lacked methanol dehydrogenase activity. In both these respects, the organism differs from *Hyphomicrobium* spp. (Ghisalba & Kuenzi 1983).

The collective evidence thus indicated that metabolism of monomethyl sulphate in *Agrobacterium* sp. M3C was not initiated by a sulphatase/dehydrogenase system, typically observed in the degradation of higher homologues.

Incorporation of ^{18}O from $H_2{}^{18}O$

Absence of a sulphatase to catalyse hydrolysis suggested that liberation of SO_4^{2-} from monomethyl sulphate might have occurred without direct participation of water molecules. To clarify this point, desulphation of the ester by whole cells was allowed to proceed to completion in aqueous solution enriched with $H_2{}^{18}O$ to 35 atom % excess, and in normal water. Liberated SO_4^{2-} was precipitated as $BaSO_4$ and analysed for ^{18}O content by infrared spectrometry (Spencer 1959). The spectrum for $BaSO_4$ produced in ^{18}O-enriched water was identical with the unenriched control, and did not show an absorbance at 961 cm^{-1} characteristic of the $[S^{18}O^{16}O_3]^{2-}$ ion. Absence of incorporation of excess ^{18}O into SO_4^{2-} showed that hydrolytic fission of the O-S bond of the C-O-S linkage did not occur. Two possibilities then remained; either the mechanism is hydrolytic at the C-O bond or separation of SO_4^{2-} occurs without the direct involvement of water molecules. Sulphatases acting on the C-O bond are a rarity confined to some long-chain alkylsulphatases (Bartholomew et al. 1977; Shaw et al. 1980; Cloves et al. 1977). The majority of sulphatases so far examined hydrolyse at the O-S bond including all mammalian and microbial arylsulphatases (Dodgson et al. 1982), choline sulphatase (Lucas et al. 1972), D-lactate 2-sulphatase (Crescenzi et al. 1984), 2,4-dichlorophenoxy ethylsulphatase (Lillis et al. 1983) and butylsulphatase (unpublished observations). The failure of *Agrobacterium* sp. M3C to break the O-S bond thus casts further doubt on the involvement of a simple sulphatase-mediated hydrolysis in this organism.

Search for degradation intermediates

Absence of a simple hydrolytic mechanism implies

that monomethyl sulphate was modified in some way before SO_4^{2-} release occurred. Possible mechanisms fall into 2 broad groups. First, the methyl group may be transferred to an acceptor molecule (other than water) with concomitant release of SO_4^{2-}. Monoalkyl sulphates are very much less reactive as alkyl donors than dimethyl sulphate (Heiner et al. 1962) thus making non-enzymic transfer unlikely. However an enzyme-catalysed transfer remained a possibility. Second, metabolism may be oxidative via formaldehyde and formate, involving pathways characteristic of methylotrophic bacteria. Although cultures growing on monomethyl sulphate were analysed using the pentanedione assay, no formaldehyde was detected. Consequently, radio-labelled substrates and TLC analysis were employed to resolve the mechanistic possibilities. Figure 2a shows the TLC analysis of supernatants from an incubation mixture containing resuspended *Agrobacterium* sp. M3C and monomethyl [^{35}S]sulphate (4 mM, 137 kBq ml^{-1}). Disappearance of the ester was accompanied by appearance of $^{35}SO_4^{2-}$, but no other ^{35}S-labelled intermediates were detected. Similar results were obtained with supernatants from incubations with carrier-free monomethyl [^{35}S]sulphate and with ethanol extracts of whole incubation mixtures.

TLC analysis of samples from incubation mixtures containing cells and mono[^{14}C]methyl sulphate (1.70 MBq mmol^{-1}) resulted in autoradiograms showing progressive disappearance of substrate and the accompanying accumulation of several metabolites (Fig. 2b). The R_f of the major metabolite (0.33) was similar to those of formate and formaldehyde in this solvent system. Two dimensional TLCs in which the sample spots were augmented with either [^{14}C]formate or [^{14}C]formaldehyde showed that the major accumulating metabolite was in fact formate.

Radioactivity at the origin was attributed to ^{14}C incorporated into whole cells, and the areas with R_f around 0.07 had the same mobility as serine and glycine. Both of these amino acids are involved in the serine pathway in methylotrophs.

Collectively these experiments pointed towards pathways involving formate and possibly the serine pathway in dissimilation or assimilation of the carbon in monomethyl sulphate. A prerequisite of both of these pathways is the availability of formaldehyde.

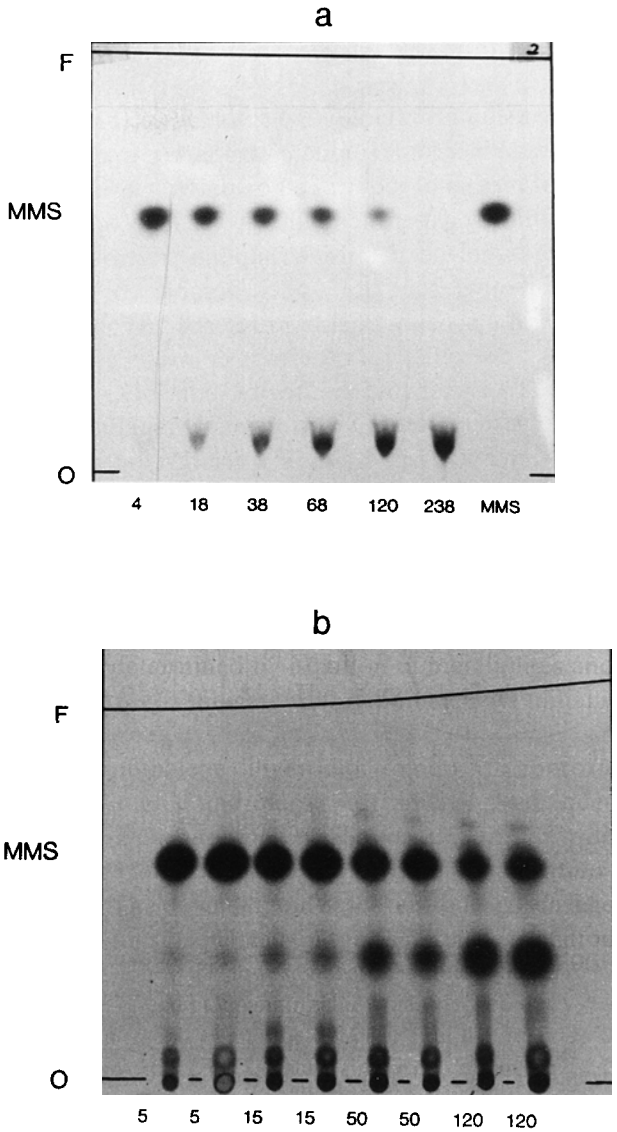

Fig. 2. TLC autoradiograms showing (a) degradation of monomethyl [^{35}S]sulphate to inorganic sulphate, (b) conversion of mono[^{14}C]methyl sulphate to ^{14}C-labelled metabolities. Numbers represent sampling times in minutes and O, F and MMS are origin, solvent front and monomethyl sulphate, respectively. Inorganic sulphate remained at the origin during chromatography.

Oxygen-dependent metabolism of monomethyl sulphate

Conversion of monomethyl sulphate to formaldehyde and inorganic sulphate without the intermediacy of methanol requires an oxidative mechanism. Preliminary experiments showed that resting cells which achieved extensive sulphate release from monomethyl sulphate under aerobic conditions (50% in 6 h) were unable to release any sulphate under anaerobic, but otherwise equivalent, conditions. To investigate the involvement of O_2 in the process, degradation of monomethyl sulphate by *Agrobacterium* sp. M3C in a Clark-type oxygen electrode, was examined. Addition of pure monomethyl sulphate caused an immediate acceleration of O_2-uptake above the basal rate. The accelerated phase ended almost equally abruptly after about 10 min. The effect was repeatable with subsequent additions of substrate and the excess amount of O_2 used in the accelerated phase was equimolar with the amount of ester added (see Experiment 1 in Table 1). Addition of methanol in place of its sulphate ester caused no acceleration in O_2-uptake, confirming the absence of methanol from the degradation pathway.

In order to relate SO_4^{2-} release to O_2-uptake, oxygen electrode experiments were performed using monomethyl [^{35}S]sulphate. After the addition of monomethyl [^{35}]sulphate, small samples (50 μl) were withdrawn at intervals from the electrode chamber without perturbing the O_2 concentration, and analysed for $^{35}SO_4^{2-}$. Figure 3 shows the data corrected for small amounts of SO_4^{2-} present in the original ester preparation. Clearly O_2-uptake and $^{35}SO_4^{2-}$ release occurred simultaneously and completion of $^{35}SO_4^{2-}$ release and return of the O_2-uptake rate to the basal rate were also simultaneous after 5–6 min. These events were repeatable upon the addition of further aliquots of substrate. A total of three sequential additions of substrate were made, each raising the monomethyl sulphate concentration in the electrode by 0.056 mM. After metabolism of the third aliquot the system was reduced to an anaerobic state (15 min). Further addition of monomethyl sulphate (16.4 min) did not lead to an immediate release of $^{35}SO_4^{2-}$ as had occurred with previous additions; only when O_2 was readmitted (18 min) was release of $^{35}SO_4^{2-}$ resumed. Subsequent addition of monomethyl sulphate to the re-aerated system (29 min) caused the usual simultaneous and stoichiometric $^{35}SO_4^{2-}$ release and O_2-uptake. Table 1 (Experiment 2) shows the overall changes in O_2 and SO_4^{2-} concentrations in the medium accompanying degradation of each aliquot of monomethyl sulphate added at the intervals shows in Fig. 3. Within the limits of experimental variation, the mean values of changes in O_2 and SO_4^{2-} were equal to each other and to the amount of monomethyl sulphate added. The total sulphate liberated was also equal to the total monomethyl sulphate added.

Two specific activities associated with the biodegradation of monomethyl sulphate were monitored at various stages of batch culture: first, the activity for SO_4^{2-}-liberation; second, that part of oxygen up-take dependent on the addition of, and stoichiometric with, monomethyl sulphate. Harvested cells, washed and resuspended, were assayed separately for these activities, and the results

Table 1. Stoichiometric relationship among the amounts of substrate added, oxygen consumed and inorganic sulphate released during degradation of monomethyl sulphate by *Agrobacterium* sp. M3C.

Monomethyl sulphate added (mM)	O_2 consumed (mM)	Sulphate released (mM)
Experiment 1		
0.067	0.061	nd
0.067	0.061	nd
0.067	0.061	nd
Means 0.067	0.061	nd
Totals 0.201	0.183	nd
Experiment 2		
0.056	0.044	0.041
0.056	0.049	0.058
0.056	0.054	0.058
0.056	–[a]	0.048
0.056	0.064	0.083
Means 0.056	0.053 ± 0.008	0.058 ± 0.016
Totals 0.280	–[a]	0.288

[a] O_2-uptake by bacteria was not measurable during admission of air to the electrode cell; see Fig. 3.
nd not determined

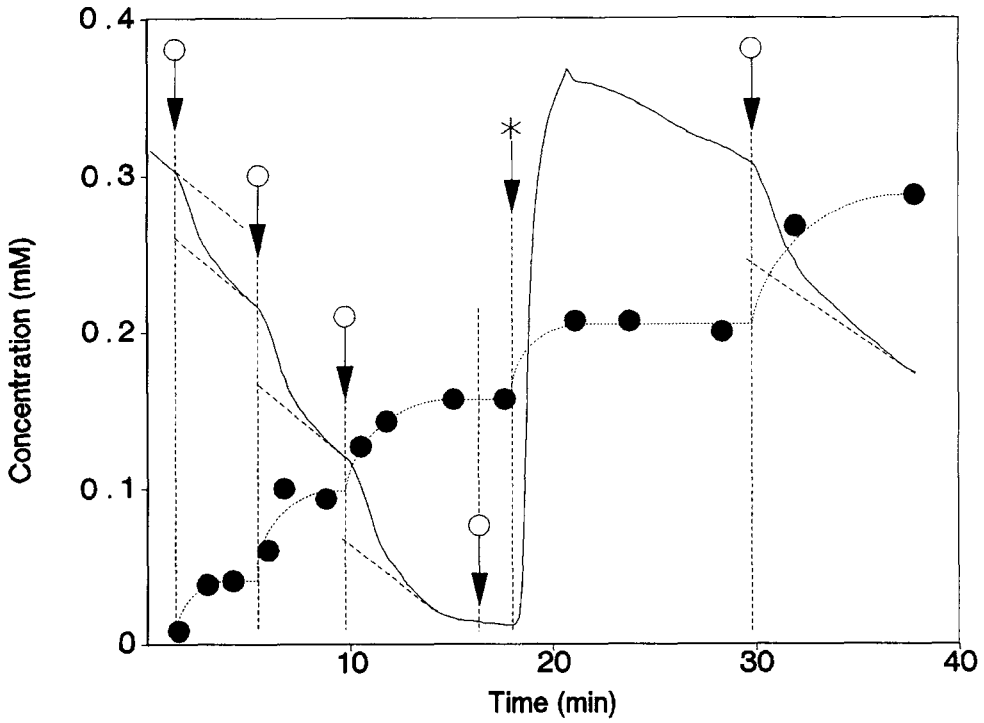

Fig. 3. Oxygen up-take (solid line) and $^{35}SO_4^{2-}$ release (●) during a series of additions of monomethyl [^{35}S]sulphate (0.056 mM increments at arrows marked O) to a suspension of *Agrobacterium* sp. M3C. Air was re-admitted to the electrode chamber at the arrow marked *.

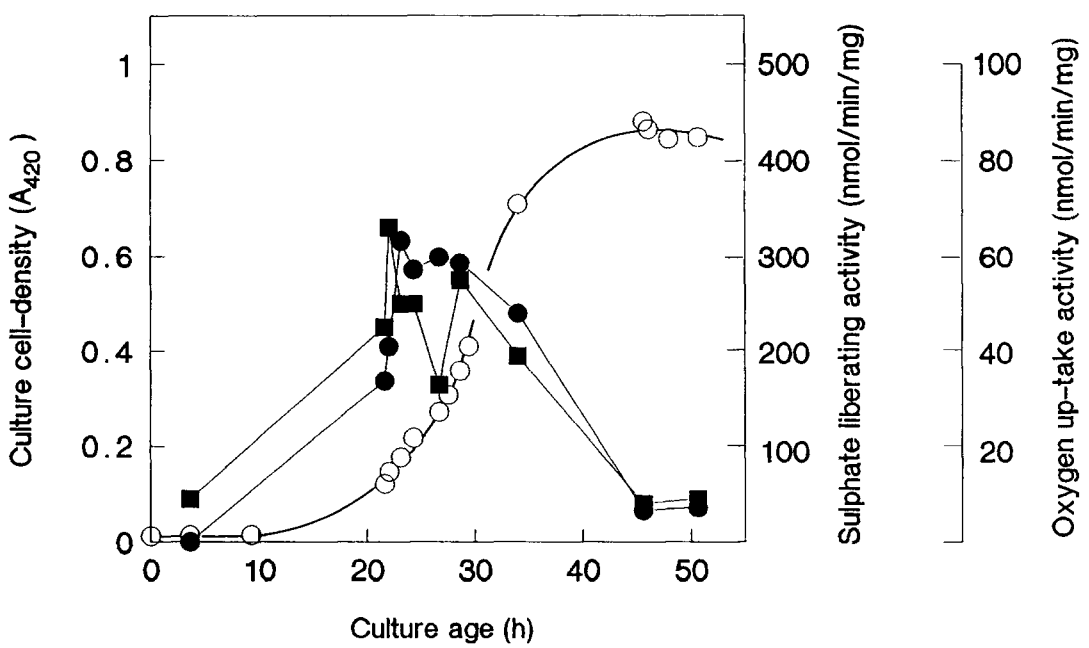

Fig. 4. Monomethyl sulphate-dependent oxygen up-take (●) and sulphate-liberating (■) activities at various stages of growth (○) in batch culture of *Agrobacterium* sp. M3C. Monomethyl sulphate oxygenation and sulphate-liberating activities were measured at 0.32 mM and 5.0 mM concentrations of substrate, respectively.

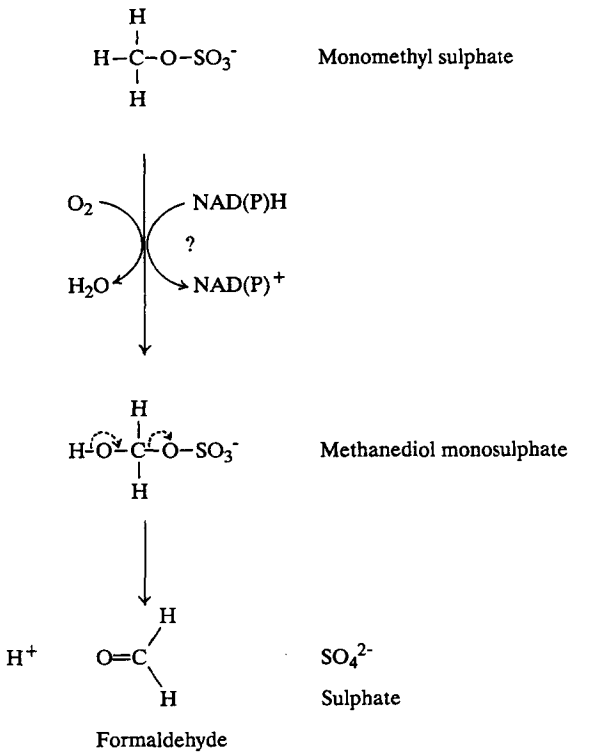

Fig. 5. Proposed pathway for desulphation of monomethyl sulphate by *Agrobacterium* sp. M3C.

were expressed as specific activities (nmol/min per mg of protein in the cells). The results (Fig. 4) show that the profiles of specific activity for each of these functions were similar throughout the batch culture cycle. Coefficient of correlation between SO_4^{2-} release and monomethyl sulphate-dependent O_2 uptake activities was $r = 0.81$ ($P < 0.01$).

Hydroxypyruvate reductase has been used previously to indicate the operation of the serine pathway in the assimilation of formaldehyde in methylotrophic bacteria (Large 1983). Specific activity of this enzyme (μmol min^{-1} mg^{-1} of protein) in crude extracts of *Agrobacterium* sp. M3C was 1.60 and 3.75 using NADH and NADPH as cofactors, respectively. Presence of hydroxypyruvate reductase is not itself proof of the operation of the serine pathway because Bamforth & Quayle (1977) have reported a constitutive enzyme in *Paracoccus denitrificans* growing on methanol by way of the ribulose bisphosphate pathway. Nevertheless the specific activity in strain M3C is 1–2 orders of magnitude higher than in *P. denitrificans* (0.031–0.111 and 0.052 μmol min^{-1} mg^{-1} for NADH and NADPH, respectively) and very similar to that of *Pseudomonas* AM1 (1.57 μmol min^{-1} mg^{-1}), which is known to assimilate formaldehyde via the serine pathway (Large & Quayle 1963).

Conclusions

The mechanism depicted in Fig. 5 is proposed for the metabolism of monomethyl sulphate in *Agrobacterium* sp. M3C. This scheme accounts for the experimental observations described, namely

- the absence of an appropriate sulphatase;
- equimolar stoichiometry among substrate added, sulphate released and O_2 consumed;
- lack of incorporation of ^{18}O from $H_2{}^{18}O$ into SO_4^{2-};
- the dependence of SO_4^{2-} release on the immediate availability of O_2; and
- the coincident production of sulphate-liberating and monomethyl sulphate-dependent O_2-uptake systems during batch culture.

The scheme is also compatible with the conversion of monomethyl sulphate to formate, and possibly, intermediates of the serine pathway for which HCHO is a precursor; and with the presence of high activities of the serine pathway enzyme, hydroxypyruvate reductase. The mechanism involves an initial mono-oxygenation at a single carbon atom. Mono-oxygenases are known to be involved in metabolism of C_1 compounds including methane and a number of its derivatives (Colby et al. 1977; Stirling & Dalton 1979; Anthony 1982; Large 1983; De Bont et al. 1981; Suylen et al. 1986). Evidence is also accumulating that the mono-oxygenases of the ubiquitous methanotrophs may be involved in metabolism of a much wider range of complex organic compounds (Higgins et al. 1980). The mono-oxygenases examined hitherto depend on NAD(P)H to supply reducing equivalents. Attempts to establish NAD(P)H-dependent activity in cell extracts in the present study were unsuccessful, presumably reflecting the instability often observed for these enzyme systems (see, for examples, De Bont et al.

1981; Colby & Dalton 1978). The product of monooxygenation of monomethyl sulphate is methanediol monosulphate (hydroxymethyl sulphate). Substituted methanols of this kind are considered to be very unstable (Colby et al. 1977) and to break down by a non-enzymic elimination reaction to yield formaldehyde. For example, dichloromethane is degraded in *Hyphomicrobium* sp. strain DM22 via *S*-hydroxymethyl glutathione which decomposes in this way to produce formaldehyde and regenerate reduced glutathione (Kohler-Staub & Leisinger 1985; Stucki et al. 1981). The analogous methanediol monosulphate is likely to be equally labile because SO_4^{2-}, like glutathione, is an excellent leaving group. A similar mechanism involving initiation by a monooxygenase has been proposed for degradation of dimethyl sulphide and dimethyl sulphoxide in *Hyphomicrobium* sp. (De Bont et al. 1981). Formaldehyde formed from monomethyl sulphate may then be assimilated by the serine or ribulose phosphate pathways or dissimilated via formate to CO_2 (Antony 1982; Large 1983; Colby et al. 1979). Absence of detectable formaldehyde in cultures was not unexpected because other workers have similarly failed to detect formaldehyde postulated as an intermediate in the catabolism of, for example, methane (Colby et al. 1977) and methanethiol (De Bont et al. 1981). Attwood and Quayle (1984) have also discussed the importance of effective formaldehyde-removal pathways in the control of its pool size in methylotrophic organisms because it is a ubiquitous and central intermediate, yet highly toxic at all but the lowest concentrations.

Methane monooxygenases are known to occur in both soluble and membrane-bound forms. Although we have not yet been able to detect monomethyl sulphate monooxygenase activity in either fraction, it is interesting that monomethyl sulphate is degraded in *Hyphomicrobium* species by a membrane-bound enzyme, albeit via a sulphatase rather than an oxygenase mechanism (Ghisalba et al. 1986).

The pathway in Fig. 5 contrasts with that known to account for microbial degradation of long-chain ($> C_4$) alkyl sulphates for which the initiating step is sulphatase-catalysed hydrolysis of ester to alcohol before sequential oxidation via aldehyde/ketone to fatty acid (Thomas & White 1989; Dodgson & White 1983). A sulphatase-initiated pathway has also been proposed for metabolism of monomethyl sulphate for *Hyphomicrobium* sp. (Ghisalba et al. 1986). On the other hand, there are precedents in which the degradation of sulphate esters is initiated by reactions other than sulphatase-mediated hydrolysis. Thus propan-2-yl sulphate metabolism in *Ps. syringae* strain GG is initiated by an oxidation reaction, not at the sulphated carbon, but at an adjacent terminal methyl group to produce D-lactate-2-sulphate before enzymic desulphation (Crescenzi et al. 1984, 1985). Glucose-6-sulphate metabolism in *Ps. fluorescens* sp. also involves oxidation (in this case via the Entner Doudoroff pathway) before desulphation occurs (Fitzgerald & Dodgson 1971a, b). In addition, while alkyl ethoxy sulphate sufactants may undergo direct desulphation, there is also good evidence that both in pure bacterial cultures and in mixed environmental systems, ether cleavage of glycol ether bonds, and/or ω-/α-oxidation of the alkyl chain usually precedes the removal of sulphate (Hales et al. 1986; Griffiths et al. 1986). Collectively these studies and the present work show that while sulphatase-initiated pathways are common, the degradation of sulphate esters may begin with alternative, often oxidative steps, before desulphation occurs.

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