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Biodegradation of short-chain alkyl sulphates by a coryneform species

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Abstract

Coryneform B1a isolated from soil grew well on butyl-, pentyl- and hexyl-1-sulphates esters and on the corresponding parent alcohols as sole sources of carbon, with growth rates around 0.14–0.19 h^{-1}. Propyl-1-sulphate and heptyl-1-sulphate supported slower growth, and their C_1, C_2 and C_8 homologues were not utilised at all. Growth of the organism was accompanied by disappearance of butyl-1-sulphate. In the presence of resting cells, butyl-1-sulphate degradation was stoichiometric with the liberation of inorganic sulphate. Butan-1-ol was also detected but in less than stoichiometric amounts. Non-denaturing polyacrylamide gel electrophoresis of extracts of cells grown on butyl-1-sulphate, followed by incubation of gels in butyl-1-sulphate and precipitation of liberated SO_4^{2-} as $BaSO_4$, revealed a single white band of alkylsulphatase activity. Other zymograms produced in the same way but incubated with the C_5 and C_6 esters, each produced a single band of the same mobility and intensity. With the C_3 and C_7 homologues, the same band was present but considerably less intense. No alkylsulphatase band was detected for methyl, ethyl or octyl-1-sulphates. Assays of alkylsulphatase activity in crude cell-extracts indicated maximum activity towards butyl-1-sulphate at pH 7.5 and 30° C, with $K_m = 8.4 \pm 1.4$ mM and $V_{max} = 0.13 \pm 0.01$ μmol/min/mg of protein. The results indicated that degradation of short-chain alkyl sulphates in this organism was initiated by enzymic hydrolysis to the corresponding alcohol.

Introduction

Alkyl sulphates enter the environment from natural sources and as a result of industrial and agricultural activities. Long chain alkyl sulphates (ca. C_{12}) are important components of surfactant formulations (Shore & Berger 1976) and higher homologues are produced as lipid components in algal cells (Haines 1973; Mercer & Davies 1979). Much shorter homologues such as methyl sulphate also occur naturally in avian eggs, and this input is currently augmented by man-made sources which include coal fly-ash (Lee et al. 1980) and waste streams from industrial methylation reactions employing dimethyl sulphate (Ghisalba & Kuenzi 1983). Avian eggs are also sources of ethyl sulphate and propyl-2-sulphate among others (Yagi 1964). Methanol, propanol, butanol and pentanol are converted to their respective sulphate esters when incubated with rat liver (Vestermark & Bostrom 1959) or toad liver extracts (Scully et al. 1970), and this conjugation may be part of a detoxification route for elimination of toxic alcohols from mammals and amphibians.

The biodegradation pathway for the long-chain alkyl sulphate surfactants is now well established (Thomas & White 1989; White & Russell 1988) and is initiated by alkylsulphatase enzymes that catalyse hydrolysis of the C-O-S ester bond to liberate the parent alcohol and inorganic sulphate (Barrett

et al. 1980; Bartholomew et al. 1978; Bateman et al. 1986; Cloves et al. 1980; Dodgson & White 1983; Dodgson et al. 1982; Shaw et al. 1980). A common feature of these enzymes is that they are inactive with substrates of chain-lengths less than C_5. Subsequent attempts to establish the biodegradation pathways for the shorter esters have shown that oxidative as well as hydrolytic mechanisms operate. Methyl sulphate degradation is initiated by a sulphatase in *Hyphomicrobium* spp. (Ghisalba and Kuenzi 1983; Ghisalba et al. 1986) but by an oxygenation reaction in *Agrobacterium* sp. (Davies et al. 1990). Propyl-2-sulphate undergoes initial oxidation to lactate-2-sulphate (Crescenzi et al. 1985) before a specific sulphatase liberates sulphate from the latter compound (Crescenzi et al. 1984). The switch to exclusive hydrolytic cleavage should thus occur around C_4 or C_5. A coryneform bacterium able to degrade C_3 and C_4 sulphate esters has previously been isolated in this laboratory (White et al. 1987). The present paper describes experiments to establish the range of primary alkyl sulphates capable of supporting growth of this isolate, and whether or not the biodegradation of short chain alkyl sulphates is initiated by sulphate hydrolysis.

Materials and methods

Except where otherwise stated, all chemicals were analytical grade supplied by Merck or Sigma Chemical Co., both of Poole Dorset, UK.

Alkyl sulphates

Potassium methyl sulphate was prepared from dimethyl sulphate (Fluka, Glossop, UK) as described elsewhere (Davies et al. 1990). Other primary alkyl sulphates (C_2–C_9) were synthesised as their potassium salts by the method of Lloyd et al. (1961). Butyl-1-sulphate prepared in this way yielded the following analysis (% by weight): K content by flame photometry, found 19.7 (theoretical 20.3); sulphate content by gravimetric analysis as $BaSO_4$ after acid hydrolysis of the ester, 61.8 (62.7); carbon by combustion analysis, 24.8 (25.0); hydrogen by combustion analysis, 4.61 (4.68). Infra-red spectroscopy in Nujol mulls using a Perkin Elmer 257 grating spectrometer revealed strong bands at 1210–1260 cm^{-1} corresponding to S-O stretching vibrations, and at 770–810 cm^{-1}corresponding to the O-S bond on the C-O-S ester linkage (Lloyd et al. 1961).

Organism and cultural conditions

The isolation of coryneform B1a from soil has been described elsewhere (White et al. 1987). The organism was maintained by monthly transfers on Noble agar/basal salts medium (see below) containing 10 mM butyl-1-sulphate as sole source of carbon. The organism was grown routinely in batch cultures at 30 °C with shaking (120 rpm). When carbon sources other than alkyl sulphates were to be used, the basal salts medium contained (g/l): K_2HPO_4, 3.5; KH_2PO_4, 1.5; NH_4Cl, 0.5; NaCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.15; Na_2SO_4, 0.5. When alkyl sulphates were used as carbon sources, the basal salts medium was modified by doubling the concentrations of both phosphate salts (to buffer the acid produced during ester hydrolysis) and omitting the sulphate (provided by the ester). Alkyl sulphates were sterilised by filtration (0.2 μm filter) and added aseptically to pre-autoclaved basal salts medium. Growth was monitored by measuring culture optical densities (A) at 420 nm. For individual growth curves, specific growth rates were determined from the exponential phase of each growth curve by linear regression of ln(A) values to time t according to the equation $\ln(A) = B + \mu \cdot t$ where μ is the specific growth rate and B is a constant. The data for growth on different concentrations [S] of butyl sulphate was analysed collectively by assuming Monod growth kinetics, i.e. $\mu = \mu_{max}[S]/(K_s+[S])$ where μ_{max} is the maximum specific growth rate and K_s is the half-saturation constant for growth. Combining this with the exponential equation for growth, and assuming [S] is approximately constant over the exponential growth period, leads to the equation $A = A^{\circ} \cdot \exp(\mu_{max}[S]t/(K_s+[S]))$. Computer regression was achieved us-

ing the Marquadt-Levenberg algorithm in the SigmaPlot® software package, Version 4.0 (Jandel Scientific, California, USA).

Cell suspensions and cell extracts

Cells were harvested by centrifugation (11,600 × g for 15 minutes), washed three times by resuspension in 0.1 M Tris-HCl, pH 7.5, and resuspended in the same buffer to give a cell-suspension concentrated (usually 5-fold) relative to the original culture. When cell extracts were required for gel zymography, washed cells were resuspended at 40-fold concentration in ice-cold 3.3 mM Tris-40 mM glycine, pH 8.3, then sonicated (Cell Disruptor Type 7533a, Branson Sonic Power Company, Connecticut, USA) for 3 minutes at 0 °C. Portions (1 ml) were centrifuged at high speed in an Eppendorf microfuge for 10 minutes to remove cell debris, and the supernatants each mixed with 0.2 ml bromophenol blue tracker dye (0.4% w/v in 50% v/v glycerol) prior to loading onto electrophoresis gels.

For assays of alkylsulphatase activity, chilled cell suspensions were disrupted by passage (3 times) through a French Pressure Cell (American Instrument Co., Maryland, USA). Cell debris was removed by centrifugation (36,700 × g, 25 minutes) and the supernatant stored on ice before use.

Alkylsulphatase activity

The dependence of butyl-1-sulphatase activity on temperature, pH and substrate concentration were examined. Temperature dependence was measured by incubating extracts (French Pressure Cell) with 50 mM butyl-1-sulphate in 0.1 M Tris-HCl, pH 7.5, at the desired temperature in the range 20–37 °C. Dependence on substrate concentration was measured in the range 0.5–20 mM butyl-1-sulphate in the same buffer at pH 7.5 and 30 °C. For the pH-profile, aliquots of late-exponential-phase cells were resuspended in 0.1 M Tris-HCl buffer at each test pH (6.5, 7.5, 8.0, 8.5) and sonicated to disrupt cells. After centrifugation, supernatants were mixed and incubated with substrate in the corresponding buffer. In all experiments, the final mixture (0.2 ml) was incubated for 5 minutes, then the reaction stopped by addition of 0.05 ml of 15% w/v trichloroacetic acid, and the liberated inorganic sulphate determined by the $BaCl_2$/gelatin method (see below).

Analysis of inorganic sulphate

Inorganic sulphate released from butyl-1-sulphate by whole cells or cell extracts was measured using the turbidimetric $BaCl_2$/gelatin method "B" of Dodgson (1961) as modified by Thomas and Tudball (1967).

Analysis of butyl-1-sulphate

Residual butyl-1-sulphate was measured using the methylene blue procedure as described by Hayashi (1975). This method is usually employed for analysis of long-chain alkyl sulphates and other anionic surfactants but we found that it could also be used for shorter esters including butyl-1-sulphate. The working range for the assay was about 0.02–0.20 μmol of butyl-1-sulphate contained in any volume up to 1 ml.

Analysis of butan-1-ol

Samples from resting cell suspensions incubated with 10 mM butyl-1-sulphate were centrifuged to remove cells, and 1 μl aliquots of supernatant injected directly onto a 2 m × 2 mm gas chromatography column (5% Carbowax 20 M on 60/80 Carbopack B) installed in a Perkin Elmer 8310 Gas Chromatograph with a flame ionisation detector. The carrier gas was N_2 and the oven temperature was 100 °C. Pure butan-1-ol was used as a calibration standard.

Gel zymography

Non-denaturing polyacrylamide gel electrophore-

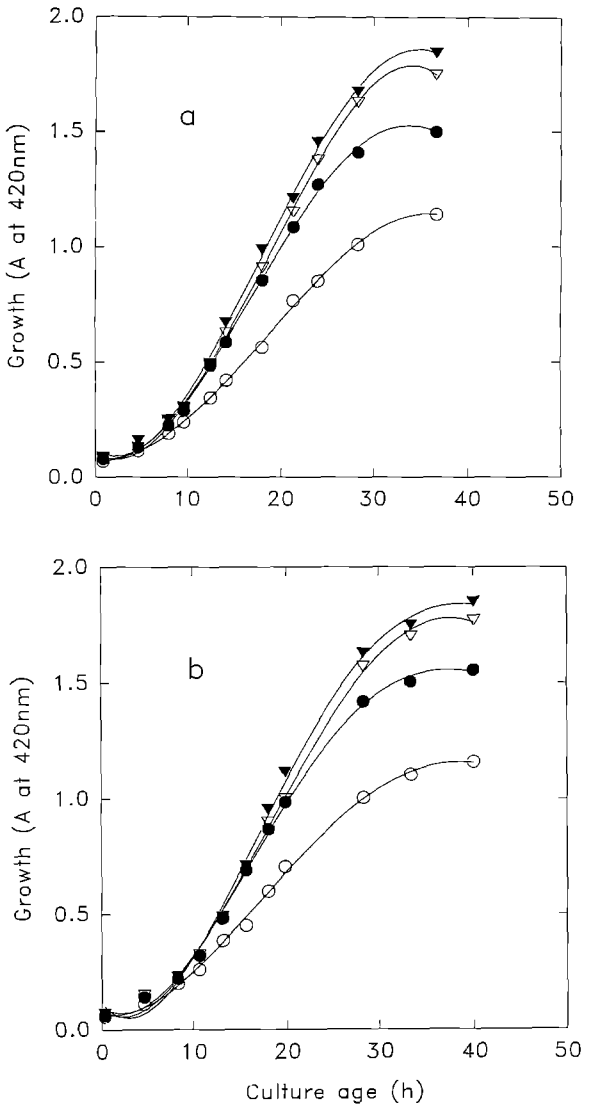

Fig. 1. Growth of coryneform B1a on different concentrations of (a) butyl-1-sulphate and (b) butan-1-ol. ○5 mM; ●10 mM; ▽15 mM; ▼20 mM.

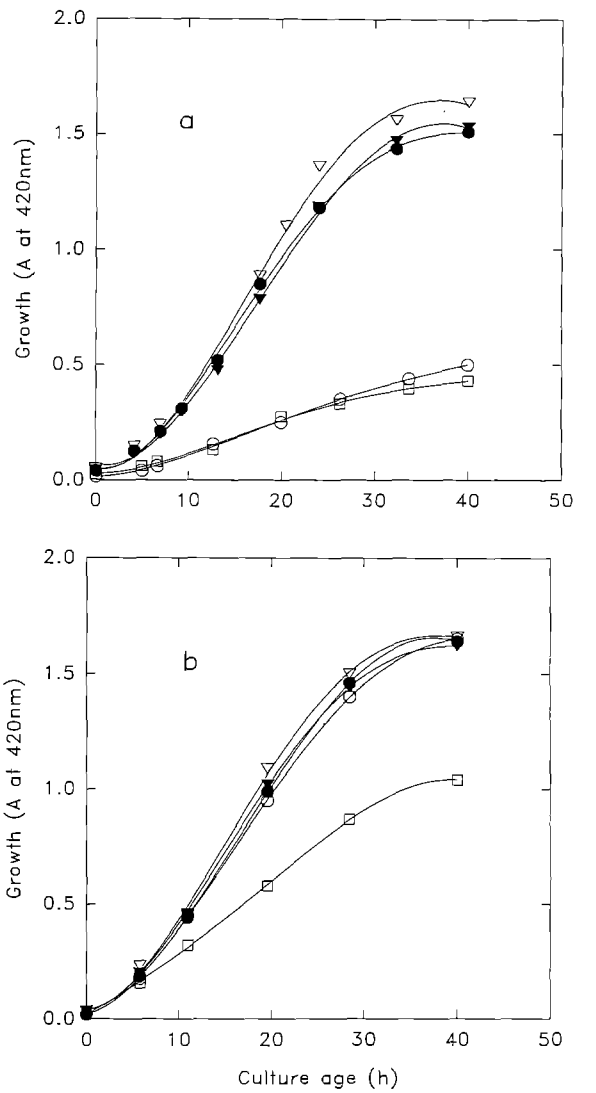

Fig. 2. Growth of coryneform B1a on (a) homologous alkyl-1-sulphate esters and (b) their parent alcohols. All substrates were initially present at 10 mM. ○C_3; ●C_4; ▽C_5; ▼C_6; □C_7.

sis was performed by the method of Payne et al. (1974). Gel zymograms were developed by incubating the extruded rod-gels in 0.1 M Tris-HCl, pH 7.5, containing 20 mM primary alkyl sulphate (C_1–C_9) and 20 mM barium acetate. Covered tubes were incubated at 30 °C until enzyme bands were revealed in the gels as white bands of barium sulphate formed by precipitation of inorganic sulphate liberated from the ester.

Results

Growth curves

Figure 1a shows growth curves for coryneform B1a utilising different concentrations of butyl-1-sulphate as sole source of carbon. Growth yield and growth rate appeared to increase with increasing concentration. Simultaneous regression of the data

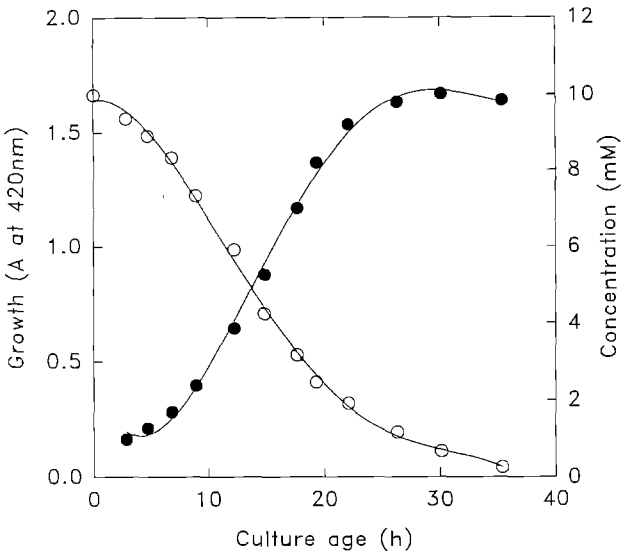

Fig. 3. Disappearance of butyl-1-sulphate (○) during growth of coryneform B1a (●).

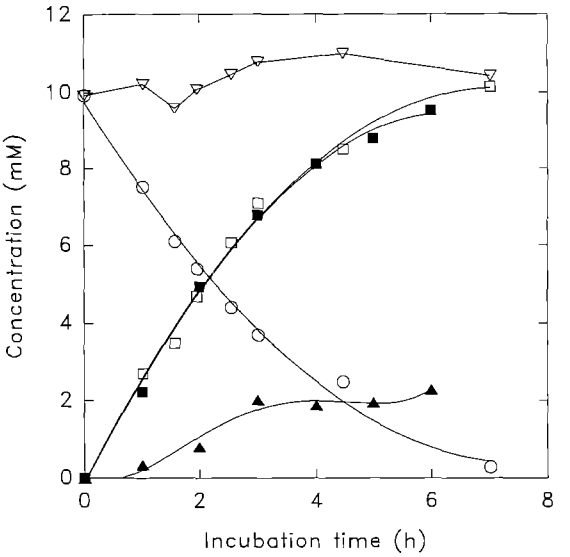

Fig. 4. Product formation during the biodegradation of butyl-1-sulphate by resting-cell suspensions of coryneform B1a. Experiment 1 (open symbols): ○ butyl-1-sulphate; □ inorganic sulphate; ▽ butyl-1-sulphate plus inorganic sulphate. Experiment 2 (filled symbols): ■ inorganic sulphate; ▲ butan-1-ol.

in the early exponential phase of all four curves to a single Monod rate equation yielded $\mu_{max} = 0.176 \pm 0.005$ (SD) h^{-1} and $K_s = 1.46 \pm 0.20$ mM. Very similar curves (Fig. 1b) and growth rates were obtained for growth on the same molar concentrations of butan-1-ol, for which $\mu_{max} = 0.165 \pm 0.004$ (SD) h^{-1} and $K_s = 0.93 \pm 0.14$ mM.

Homologous sulphate esters (C_1–C_8) and their corresponding alcohols were compared as growth substrates, all at 10 mM initial concentration. Compounds at the extremes of this chain length range (C_1, C_2 and C_8) were not able to support growth, either as sulphate esters or as the parent alcohols. For the remaining sulphate esters, maximum cell yields (Fig. 2a) were obtained with the C_4–C_6 homologues, with C_3 and C_7 supporting slower growth. Growth on equimolar concentrations of the corresponding primary alcohols produced the curves in Fig. 2b. With the exception of heptanol, the growth curves for the growth-supporting alcohols C_3–C_6 were very similar to each other and to the curves for the best growth-supporting esters (Fig. 2a). From Fig. 2b cells appeared to grow more slowly on C_7 than on the C_3–C_6 alcohols, but still faster than on heptyl-1-sulphate.

Substrate utilisation

When coryneform B1a was grown on butyl-1-sulphate, there was a close correlation between the amount of substrate degraded and the culture cell-density (Fig. 3). Furthermore, when a resting-cell suspension of butyl-1-sulphate-grown cells was incubated with butyl-1-sulphate, the amount of ester degraded was stoichiometric with the amount of sulphate liberated (Fig. 4). In a parallel experiment, supernatant medium was analysed simultaneously for inorganic sulphate and butan-1-ol (Fig. 4). Inorganic sulphate was produced stoichiometrically as before but butan-1-ol was detected in less than stoichiometric amounts.

Gel zymography

Non-denaturing polyacrylamide gel electrophoresis of crude extracts of coryneform B1a, followed by staining for alkylsulphatase activity produced the zymograms shown in Fig. 5. The opaque white

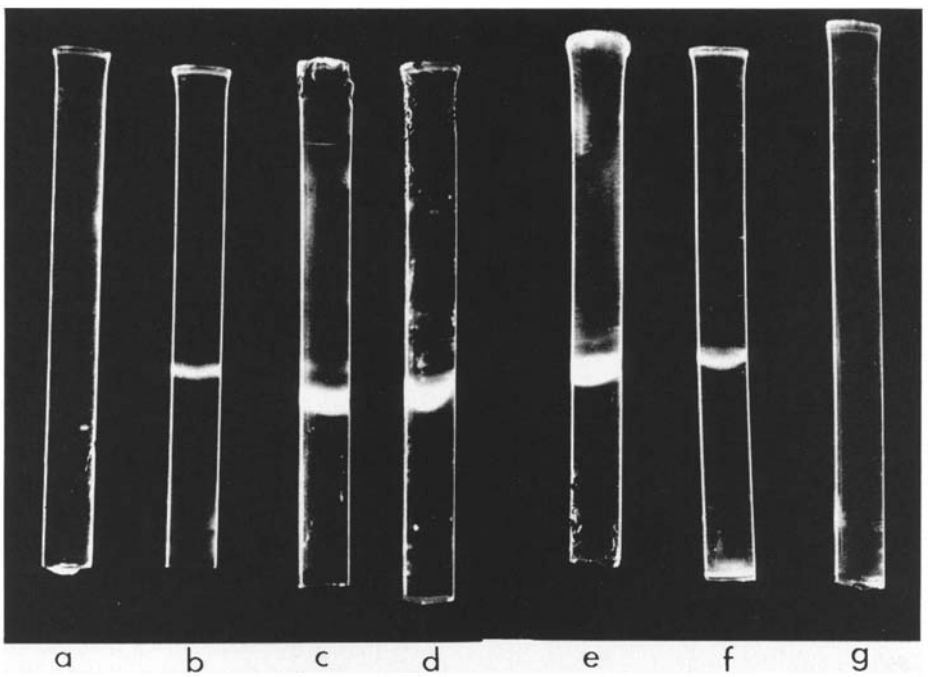

Fig. 5. Gel zymograms of short-chain alkylsulphatase activity in extracts of coryneform B1a grown on butyl-1-sulphate. After non-denaturing electrophoresis, extruded gels were incubated in medium containing Ba^{2+} ions and (a) ethyl sulphate, (b) propyl-1-sulphate, (c) butyl-1-sulphate, (d) pentyl-1-sulphate, (e) hexyl-1-sulphate, (f) heptyl-1-sulphate, (g) octyl-1-sulphate.

bands consist of insoluble barium sulphate produced as a result of enzymic liberation of sulphate from alkyl sulphates and its subsequent precipitation with Ba^{2+}. Barium salts of alkyl sulphates are soluble under these conditions, so the white bands serve to locate alkylsulphatase activity. Of the 9 gels stained in this way (one for each of the C_1–C_9 homologous series of primary alkyl sulphates), 4 gels corresponding to C_1, C_2, C_8 and C_9, contained no alkylsulphatase bands. In contrast, the gels incubated with C_3 to C_7 alkyl sulphates each produced a single band of the same mobility. Bands for C_4–C_6 were stained more strongly than those for C_3 and C_7. Extracts of cells grown on nutrient broth instead of butyl-1-sulphate produced no bands with any of the ester substrates.

Alkylsulphatase activity

Table 1 shows the effects of substrate concentration, pH and temperature on the butyl-1-sulphatase activity of crude extracts of cells grown on butyl-1-sulphate. Activity increased with increasing substrate concentration according to Michaelis-

Table 1. Butyl-1-sulphatase activity in crude cell-extracts of coryneform B1a under different conditions of substrate concentration, pH and temperature.

Substrate concentration (mM)	pH	Temperature (°C)	Activity (μmol/min/mg)
0.5	7.5	30	0.005
1.0	7.5	30	0.014
2.0	7.5	30	0.023
5.0	7.5	30	0.047
10.0	7.5	30	0.077
15.0	7.5	30	0.084
20.0	7.5	30	0.089
50.0	7.5	20	0.048
50.0	7.5	25	0.065
50.0	7.5	30	0.075
50.0	7.5	37	0.064
10.0	6.5	30	0.065
10.0	7.5	30	0.080
10.0	8.0	30	0.078
10.0	8.5	30	0.071

Menten kinetics. Computer regression analysis of the kinetic data to the usual hyperbolic equation yielded $K_m = 8.4 \pm 1.4$ mM and $V_{max} = 0.13 \pm 0.01$ μmol of substrate converted/min/mg of protein. Maximum activity over a 5-minute incubation period was obtained at 30 °C and pH 7.5. Under these conditions, release of inorganic sulphate was linear with time for approximately 30 minutes, corresponding to about 25% hydrolysis of the 10 mM butyl-1-sulphate initially present.

Discussion

The close correlation between the growth of coryneform B1a and disappearance of butyl-1-sulphate, and the liberation of stoichiometric amounts of inorganic sulphate, suggested that there was no accumulation of other intermediates still retaining the sulphate group, and that separation of sulphate from the ester was the first step in the biodegradation pathway. Hydrolytic separation of the sulphate group from the ester should lead to formation of butan-1-ol, and this was indeed detected in incubations initially containing high (10 mM) concentrations of butyl-1-sulphate (Fig. 4). In contrast to inorganic sulphate, butan-1-ol was detected in less than stoichiometric amounts, and this presumably reflects further metabolism of the alcohol by the cells.

Initiation of biodegradation of butyl-1-sulphate by hydrolysis to butan-1-ol is also consistent with the growth kinetic experiments which showed that μ_{max} and K_s were very similar for butan-1-ol and its sulphate ester. This is consistent with a mechanism in which the two compounds are on the same pathway, and are not separated by a step which is rate-limiting for growth. The half saturation concentration (K_s) for growth on butyl-1-sulphate is about an order of magnitude lower than the K_m of 8.4 mM for butyl-1-sulphate hydrolysis, thus showing that the latter is not rate-limiting for growth under the conditions tested.

To confirm the operation of a hydrolytic mechanism, cell-free extracts were shown to be capable of liberating sulphate from butyl-1-sulphate without the addition of any oxidative, reductive or other cofactors or substrates. This was true for direct assays for sulphate release from the ester (Table 1) catalysed by cell-free extracts and also in gel zymograms (Fig. 5) in which the enzyme activity was further separated from other proteins and cellular constituents. Studies with the purified enzyme to be published elsewhere have confirmed the absence of any requirement for added cofactors, thus eliminating the possibility of reductive or oxidative desulphation.

Enzymic hydrolysis of the sulphate ester linkage is known to initiate the bacterial biodegradation of other homologous sulphate esters of longer chain length (Thomas and White 1989; White and Russell 1988). Such compounds are frequently used in domestic and industrial surfactants, and bacteria competent in their biodegradation are widely distributed in the environment (Anderson et al. 1988; White et al. 1989). Detailed biochemical studies have established that initial hydrolysis to the long-chain alcohol is followed by oxidation through the aldehyde to the fatty acid and subsequent β-oxidation and/or incorporation into lipids (Thomas and White 1989). It is reasonable to suppose that butan-1-ol produced by coryneform B1a follows a similar pathway.

Gel zymograms for extracts of B1a showed a single band of the same mobility for each of the C_3–C_7 alkyl sulphates, indicating that a single enzyme protein is responsible for all the activities. The staining was confined to the C_3–C_7 esters and was most intense with the C_4–C_6 substrates, which is in contrast to the long-chain alkylsulphatases previously purified from *Pseudomonas* spp. The latter enzymes show increasing activity from C_6 to C_{12} primary alkyl sulphates with little or no activity below C_6 (Bateman et al. 1986; Cloves et al. 1980; Lillis et al. 1983). Thus although there is some overlap in substrate specificity at C_6–C_7 for the *Pseudomonas* and coryneform B1a enzymes, they clearly cover different chain length ranges and have different chain-length optima.

Growth on the C_3–C_6 alcohols produced identical curves whereas growth on C_7 was somewhat slower (Fig. 2). Growth experiments using the corresponding sulphate esters gave identical curves for the C_4–C_6 chain length but the C_3 and C_7 esters

were significantly less effective as growth substrates compared to their parent alcohols. The substrate specificity of the alkylsulphatase apparent from the gel zymograms may account for this discrepancy. Thus the lower alkylsulphatase activity towards C_3 and C_7 esters (Fig. 5) may make their hydrolysis rate-limiting for growth. If this is the case, then by-passing this step, supplying the cells directly with C_3 or C_7 alcohols should produce faster growth, as was indeed the case (Fig. 2).

Some other short chain-length homologues show greater variability in their mechanisms of biodegradation by bacteria. Propyl-2-sulphate first undergoes oxidation to lactate-2-sulphate before this compound serves as a substrate for hydrolytic cleavage of sulphate group (Crescenzi et al. 1984; Crescenzi et al. 1985). Methyl sulphate biodegradation is initiated by hydrolysis in *Hyphomicrobium* spp. (Ghisalba and Kuenzi 1983; Ghisalba et al. 1986), but in *Agrobacterium* sp. mono-oxygenation to methandiol monosulphate occurs first with subsequent elimination of sulphate to yield formaldehyde (Davies et al. 1990). Thus the finding that methyl sulphate, the C_6–C_{14} long-chain esters, and now butyl-1-sulphate all conform to the hydrolytic mechanism, throws the oxidative exceptions for C_1 and secondary C_3 esters into even stronger relief.

Studies are currently in progress to purify and characterise the short-chain alkylsulphatase in coryneform B1a, and it will be interesting in due course to compare its properties with those of the other alkylsulphatase enzymes studied hitherto.

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