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Localization by Electron Microscopy of Alkylsulphatases in Bacterial Cells

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Alkylsulphatases have been localized in cells of two bacterial isolates using transmission electron microscopy. Cells were incubated with the appropriate alkyl sulphate ester in the presence of Ba^{2+} ions. Inorganic sulphate liberated by alkylsulphatases was precipitated at the site of liberation as BaSO_4 . Electron microscopy of thin sections was used to locate electron-dense grains which were identified by energy dispersive analysis of X-rays (EDAX) as BaSO_4 . The long-chain primary alkylsulphatases of the detergent-degrading bacterium *Pseudomonas* C12B, active on C_6 – C_{14} primary alkyl sulphates, were located on the outer cell-wall. There was no activity inside the cells. In contrast, the short-chain (C_3 – C_7) alkylsulphatase in a coryneform isolated for its ability to grow on but-1-yl sulphate was located entirely in the cytoplasm. The butylsulphatase was apparently associated with granules of poly- β -hydroxybutyric acid. The different locations for the long- and short-chain alkylsulphatases may be related to the relative potential toxicities of their ester substrates.

INTRODUCTION

Sulphate esters of long-chain primary alcohols and alcohol ethoxylates (e.g. sodium dodecyl sulphate, sodium dodecyl triethoxy sulphate) feature as important components of a variety of household and industrial detergent formulations (Shore & Berger, 1976). Despite their widespread use, these surfactants are not troublesome pollutants because they are readily degraded by bacteria present in sewage treatment plants, soils and receiving waters (Swisher, 1987; Cain, 1976, 1981). A major catabolic pathway for the degradation in competent bacteria is initiated by alkylsulphatase enzymes that liberate SO_4^{2-} ions from the $-\text{CH}_2-\text{OSO}_3^-$ ester linkage (Payne, 1963; Williams & Payne, 1964; Hales *et al.*, 1986). The enzymes responsible, and those involved in the degradation of the isomeric secondary alkyl sulphates ($\text{CH}-\text{OSO}_3^-$ ester linkage) and related compounds, have received considerable attention in the Cardiff laboratories, especially those in the detergent-degrading bacterium *Pseudomonas* C12B (see Dodgson & White, 1983, for a review). A common feature of several of the alkylsulphatases that have been purified and characterized so far is that they will accommodate a range of alkyl chain-lengths but none below C_5 , despite the occurrence of such alkyl sulphates in the environment (White *et al.*, 1987). Recent studies (White *et al.*, 1987; Crescenzi *et al.*, 1984, 1985) have shown that there are indeed bacteria present in the environment capable of utilizing short-chain (C_1 – C_4) alkyl sulphates as sole sources of carbon and energy. However, of these esters, butyl and propyl sulphates were alone in undergoing sulphatase-mediated hydrolysis as the initial step. The organism responsible was a coryneform rod, isolated from garden soil and designated B1a, which produced an alkylsulphatase active only on relatively short-chain (C_3 – C_7) primary alkyl sulphates (White *et al.*, 1987).

Knowledge of the cellular location of microbial sulphatases is generally inadequate and has been based mainly on osmotic shock and lysozyme/EDTA treatments or on indirect evidence obtained, for example, from enzyme purification procedures (see Dodgson *et al.*, 1982 for a

Abbreviation: EDAX, energy dispersive analysis of X-rays.

review). Such experiments are not always conclusive but the collective evidence indicates that most bacterial arylsulphatases, choline sulphatase and long-chain alkylsulphatases are at the cell periphery or in the periplasmic space. Electron microscopy has previously been used to locate arylsulphatases in mammalian (Hopsu-Havu & Helminen, 1974; Zemelman *et al.*, 1985) and fungal cells (Garrison *et al.*, 1975). The present report describes an electron microscopic examination of the location of long- and short-chain alkylsulphatases in bacterial cells which exploits the capacity of liberated SO_4^{2-} to precipitate Ba^{2+} or Pb^{2+} ions at the site of liberation.

METHODS

Sulphate esters. Unless stated otherwise, all chemicals were AnalaR grade from BDH or Sigma. Primary alkyl sulphates were synthesized as potassium salts by the method of Lloyd *et al.* (1961) and re-crystallized from methanol. Esters were characterized and authenticated by elemental analyses (C, H, K, and S as sulphate) and infra-red spectroscopy.

Bacteria and cultural conditions. Isolation and maintenance of *Pseudomonas* C12B (NCIB 11753) and coryneform rod B1a have been described elsewhere (Payne & Feisal, 1963; White *et al.*, 1987). *Pseudomonas* C12B was grown in 0.3% (w/v) nutrient broth (Difco) alone or in 0.3% (w/v) nutrient broth supplemented with 4 mM-hex-1-yl sulphate. Coryneform B1a was grown in a basal salts medium containing (l^{-1}): K_2HPO_4 , 7 g; KH_2PO_4 , 3 g; NH_4Cl , 0.5 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15 g; NaCl , 0.5 g, and supplemented with 10 mM-but-1-yl sulphate as sole source of carbon and energy. In both cases bacteria were grown in batch culture with shaking at 120 r.p.m. and 30 °C to late-exponential phase (15 h for both organisms).

Preparation of cells for electron microscopy. Cells from 400 ml cultures were harvested by centrifuging (174 000 g min at r_{av} 9.53 cm and 4 °C) and fixed by resuspending the sedimented cells in 20 ml 100 mM-sodium cacodylate, pH 7.4, containing 1% (v/v) glutaraldehyde. After incubation for 30 min, cells were washed by centrifuging and resuspending in cacodylate buffer at 4 °C for 10 min. The washing step was repeated four times and cells were left in the final washing medium for 1 h at 4 °C. Washed cells were recovered by centrifuging and resuspended in a preincubation medium containing 24 mM- BaCl_2 in 100 mM-Tris/HCl, pH 7.5, for 45 min at 4 °C. Cells were centrifuged and resuspended in incubation medium containing 12 mM-alkyl sulphate (but-1-yl sulphate for coryneform B1a; oct-1-yl sulphate for *Pseudomonas* C12B) plus 24 mM- BaCl_2 7% (w/v) sucrose in 100 mM-Tris/HCl, pH 7.5. After incubation at 30 °C for 30 min, cells were washed twice in cacodylate buffer pH 7.4 before post-fixation (60 min at 4 °C) in 1% (v/v) osmium tetroxide in the same buffer. Post-fixed cells were washed once in cacodylate buffer, centrifuged and dehydrated by sequential resuspension for 10 min each in increasing concentrations of ethanol in water (50%, 70%, 80% and 95% v/v, and four times in pure ethanol). Samples of both isolates were also processed through two separate control regimes, which were the same as the procedure described above with the following modifications: control I, sulphate ester was omitted from the final incubation medium; control II, sulphate ester and BaCl_2 were omitted from the preincubation and incubation media.

Preparation and electron microscopy of thin sections. Sedimented cells from the last dehydration step were embedded in resin by infiltration of the pellet with a solution containing 50% (v/v) LR White Hard Grade acrylic resin (London Resin Co.) in ethanol for 12 h, followed by curing in undiluted LR White Hard resin at 60 °C for 48 h. Thin sections (120 nm) were cut from the resin block using glass knives on a Reichart ultramicrotome. In some cases cells were double stained with 2% (w/v) uranyl acetate (Watson, 1958) and 2% (w/v) lead citrate (Reynolds, 1963), each saturated with BaCl_2 , to reveal fine detail of cell ultrastructure.

The cut sections were examined using a Philips 400T electron microscope operated at 80 kV, in the transmission mode, with the beam current at 50 μA . Elemental composition of selected areas was established using an energy dispersive analysis of X-rays (EDAX) microanalysis system, with a tilt angle of 24°. Semi-quantitative analysis was achieved using the EDAX Thin Section Software, version 2.2S.

Light microscopy. Coryneform B1a was examined by phase contrast light microscopy for inclusions of poly- β -hydroxybutyric acid or lipid. Cells grown on but-1-yl sulphate were stained with 0.3% (w/v) Sudan Black in 70% ethanol for 15 min, followed by counter-staining with 9.5% (w/v) aqueous Safranin (Norris & Swain, 1971). Stained cells were examined in a Reichert Biovar phase contrast microscope.

RESULTS AND DISCUSSION

Long-chain primary alkylsulphatases of Pseudomonas C12B

When grown on nutrient broth alone, *Pseudomonas* C12B produces a single alkylsulphatase enzyme (designated P1) active towards primary alkyl sulphate surfactants of chain-length C_6 – C_{14} and towards certain aryl sulphates (Bateman *et al.*, 1986). The addition of a primary alkyl sulphate (e.g. hex-1-yl sulphate) to the culture medium induces synthesis of an additional but

distinct primary alkylsulphatase, P2 (Cloves *et al.*, 1980*a, b*). The P2 enzyme, while similar to P1 in its activity towards C₆–C₁₄ primary substrates, is quite different in its mechanism of action (C–O cleavage of the C–O–S ester linkage, cf. O–S cleavage for the P1 enzyme) and its inability to hydrolyse aryl sulphates and butoxyethyl sulphate (Bateman *et al.*, 1986). In the present work, both sets of cultural conditions were used to allow comparison of the locations of the P1 and P2 enzymes.

Fig. 1 shows typical electron micrographs of *Pseudomonas* C12B grown on nutrient broth alone and so containing only the P1 enzyme. Cells pre-incubated with BaCl₂ then incubated in a solution containing BaCl₂ and oct-1-yl sulphate showed abundant production of crystalline electron-dense material (Fig. 1*a*). Some deposits were present as aggregates of crystals in the surrounding medium whereas others were apparently associated with the cell surface, but there were no deposits in the cytoplasm. Cells that were treated in a similar way but with oct-1-yl sulphate omitted from the incubation medium produced no such deposition (Fig. 1*b*), showing that the precipitation did not arise from interaction of Ba²⁺ with other compounds present in the cells or the medium. Because barium salts of short-chain alkyl sulphates (<C₉) are soluble in aqueous media, we conclude that the electron-dense grains correspond to highly insoluble barium sulphate deposited following enzymic liberation of SO₄²⁻ from oct-1-yl sulphate.

Grains attached to the cells were found only at the cell periphery, indicating that the enzyme responsible (P1) was located there. Previous studies have shown that the P1 enzyme is not liberated into the extracellular medium by the cells (Bateman, 1985). Thus the grains observed in the surrounding medium have arisen from a sloughing of grains originally formed at the cell surface. The sloughing and aggregation of the crystals probably arose during the cell-washing and centrifugation steps.

Attempts were made to define more closely the location of BaSO₄ deposits in the cell envelope, in relation to the inner and outer cell-membranes. Higher magnification (Fig. 1*c*) confirmed the absence of the BaSO₄ deposits from the cytoplasm but, because these cells had not been double-stained with uranyl acetate/lead citrate, the ultrastructural details were still not apparent. Uranyl acetate/lead citrate double staining of cells previously stained for sulphatase with Ba²⁺/oct-1-yl sulphate revealed much more structural detail (Fig. 1*d, e*). However the staining procedure also tended to dislodge the bulk of the crystalline deposits from the cell periphery. This phenomenon has been observed previously during experiments to locate arylsulphatases in mammalian cells (Hopsu-Havu & Helminen, 1974; Kalimo *et al.*, 1967) when it was attributed to partial solution of BaSO₄ in the staining medium. Despite this problem, it was still apparent in the present experiments that those crystals remaining associated with the cells were attached to the outside of the outer cell-wall. This observation is consistent with the results of cell-washing experiments (Bateman, 1985) which showed that P1 alkylsulphatase activity was released from *Pseudomonas* C12B by treatment with lysozyme/sucrose/EDTA mixtures, the combination of which is known to be necessary for effective removal of outer cell-walls from Gram-negative bacteria (Nikaido & Vaara, 1985; De Maagd & Lugtenberg, 1987). The collective evidence thus indicates an outer cell-wall location for the P1 long-chain alkylsulphatase.

To confirm the identity of the electron-dense deposits as BaSO₄, elemental compositions were established by EDAX analysis of the deposits and the surrounding areas labelled A, B and C in Fig. 1*a*). The deposits at site A contained an abundance of both barium and sulphur (Fig. 2*a*). Semi-quantitative analysis of the peak intensities indicated a Ba/S atomic ratio of 1.1–1.3, in good agreement with the value of 1.0 expected for BaSO₄. Site B, outside the cell and away from the BaSO₄ deposits, contained traces of phosphorus and sulphur (Fig. 2*b*). Site C, inside the cell, contained appreciable amounts of phosphorus and some calcium but very little sulphur or barium (Fig. 2*c*). A virtually identical EDAX spectrum (but totally lacking in barium) was obtained for the cytoplasm of cells incubated in the absence of both barium ions and sulphate ester. EDAX analysis of crystals attached at the outer cell envelope of double-stained sections (Fig. 1*d, e*) indicated the presence of Ba but the sulphur K_α line (2.307 keV) was masked by a very strong signal from the lead (M_α, 2.345 keV) in the double-staining medium.

When cells were grown on nutrient broth supplemented with hex-1-yl sulphate to induce

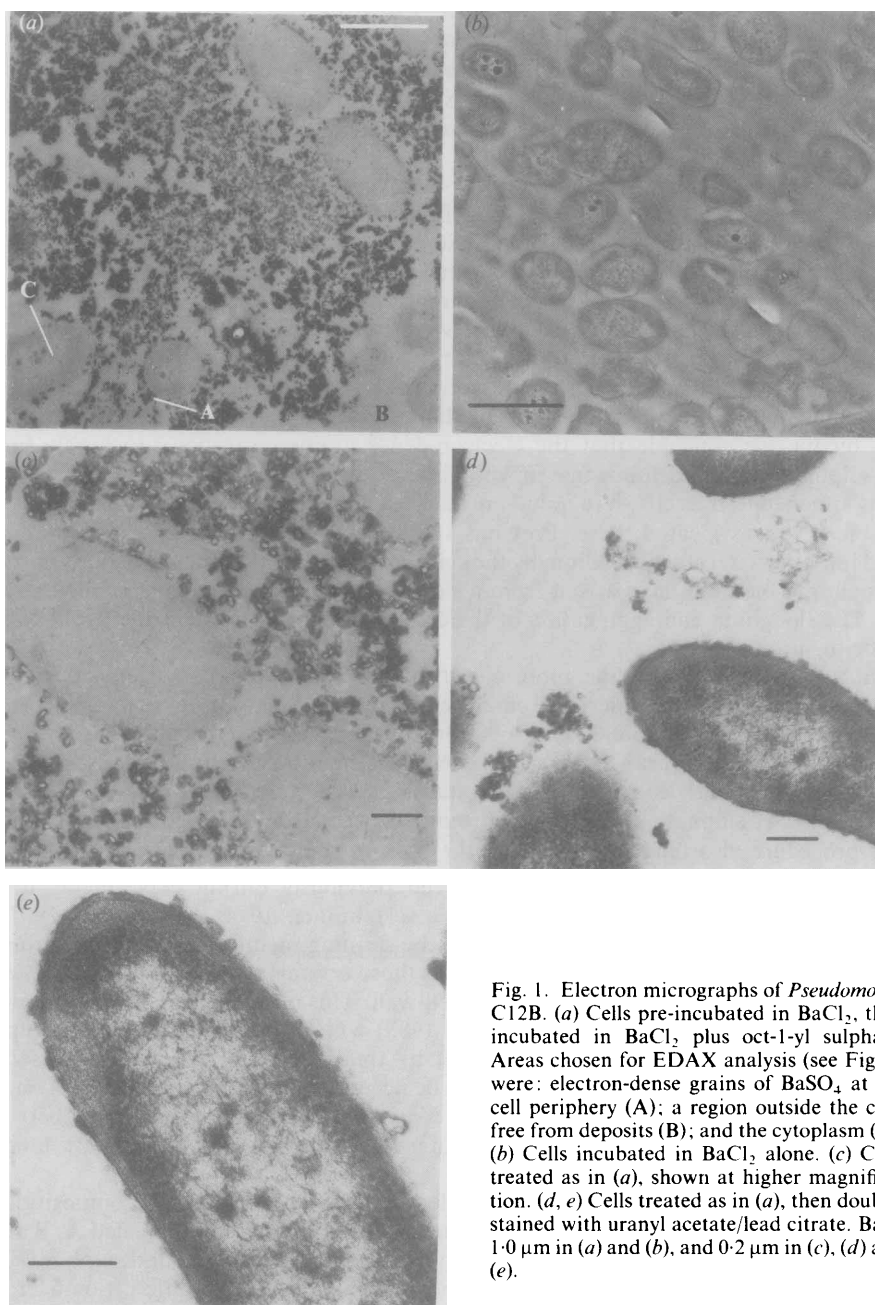


Fig. 1. Electron micrographs of *Pseudomonas* C12B. (a) Cells pre-incubated in BaCl_2 , then incubated in BaCl_2 plus oct-1-yl sulphate. Areas chosen for EDAX analysis (see Fig. 2) were: electron-dense grains of BaSO_4 at the cell periphery (A); a region outside the cells free from deposits (B); and the cytoplasm (C). (b) Cells incubated in BaCl_2 alone. (c) Cells treated as in (a), shown at higher magnification. (d, e) Cells treated as in (a), then double-stained with uranyl acetate/lead citrate. Bars, $1.0 \mu\text{m}$ in (a) and (b), and $0.2 \mu\text{m}$ in (c), (d) and (e).

synthesis of the P2 enzyme in addition to P1, the micrographs were very similar to those in Fig. 1. This finding of a common location for P1 and P2 at the outer cell envelope confirms the tentative conclusion reached by Fitzgerald & Laslie (1975) on the basis of cell-washing and osmotic-shock treatments.

Butylsulphatase in coryneform B1a

Coryneform B1a was isolated for its ability to utilize but-1-yl sulphate as sole source of carbon and energy. Cells grown on but-1-yl sulphate, then incubated with the ester and BaCl_2 together,

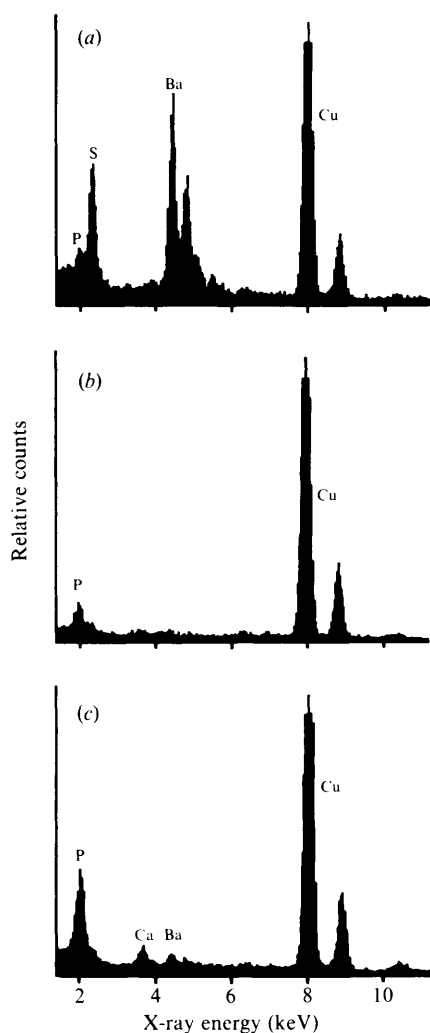


Fig. 2. EDAX spectra for sections of *Pseudomonas* C12B shown in Fig. 1*a*. Analysis of (a) electron-dense deposits outside the cells at A; (b) in the region outside the cells free from deposits at B and (c) inside the cells at C.

yielded the electron micrograph in Fig. 3*a*). These sections (unstained with uranyl acetate/lead citrate) contained some very electron-dense granules but in contrast with *Pseudomonas* C12B, the grains were located entirely in the cytoplasm and no grains were observed outside the cells. Cells of strain B1a incubated with Ba^{2+} but not but-1-yl sulphate lacked the very dense deposits (Fig. 3*b*), showing that deposition was dependent on liberation of SO_4^{2-} from the ester. The deposits were identified as BaSO_4 by EDAX analysis (Fig. 4*a*), which showed strong signals from barium and sulphur in proportions very similar to those obtained for the BaSO_4 deposits in *Pseudomonas* C12B. In addition there was evidence for the presence of phosphorus and small amounts of calcium, expected for the cell interior. In effect, the spectrum corresponded to superimposition of the spectrum for BaSO_4 grains and the bacterial cell interior observed for *Pseudomonas* C12B.

Comparison of Fig. 1*a*) and 3*a*) revealed that the grain sizes for BaSO_4 deposited in *Pseudomonas* C12B were larger than in strain B1a. Crystal size is known to depend on the degree

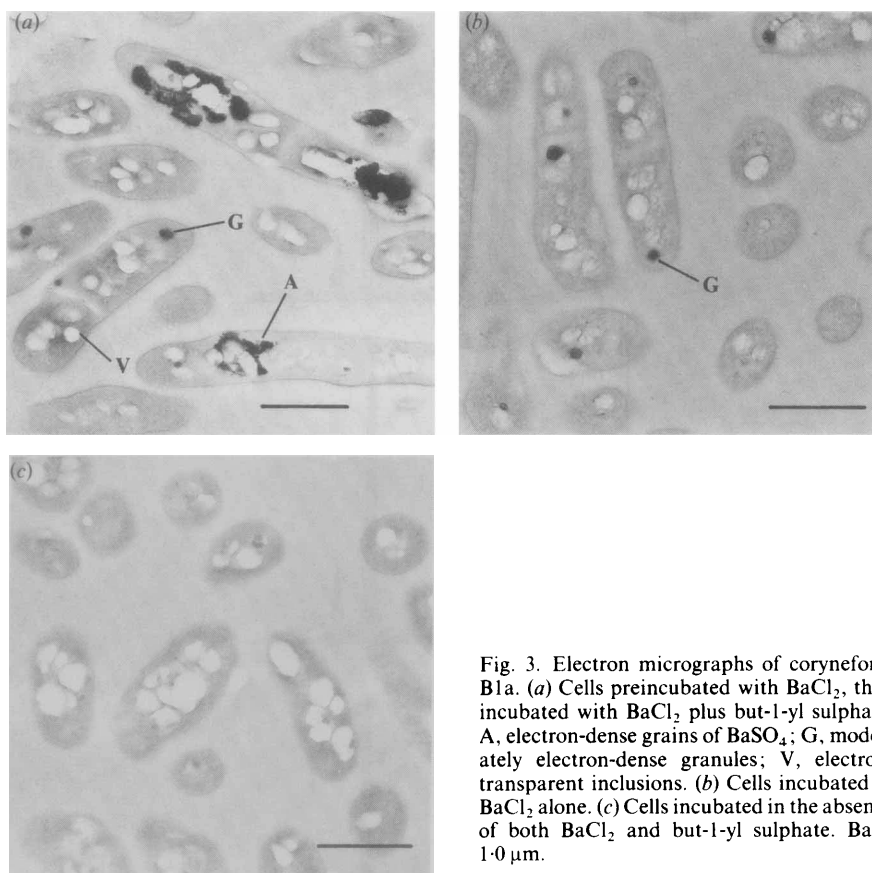


Fig. 3. Electron micrographs of coryneform B1a. (a) Cells preincubated with BaCl_2 , then incubated with BaCl_2 plus but-1-yl sulphate. A, electron-dense grains of BaSO_4 ; G, moderately electron-dense granules; V, electron-transparent inclusions. (b) Cells incubated in BaCl_2 alone. (c) Cells incubated in the absence of both BaCl_2 and but-1-yl sulphate. Bars, $1.0\ \mu\text{m}$.

of supersaturation at the time of precipitation (Von Weimarn, 1925), i.e. the particle size of a precipitate decreases with increasing concentration of the reacting solutions (see Vogel, 1961). In the present case, particle size will be inversely related to the rate of SO_4^{2-} liberation from the esters. Various factors may affect the rate of ester hydrolysis including the rate of diffusion/transport of substrate to the enzyme, and local concentration of substrate in relation to its K_m , but the dominant factor is likely to be the amount of enzyme in each cell. The total octylsulphatase activity of the P1 enzyme in crude extracts of cells of *Pseudomonas* C12B is about $0.03\ \mu\text{mol SO}_4^{2-}\ \text{released min}^{-1}\ (\text{mg protein})^{-1}$ (Bateman, 1985) whereas the butylsulphatase activity in strain B1a is about $0.09\ \mu\text{mol min}^{-1}\ (\text{mg protein})^{-1}$ (unpublished observations). Thus the slower rate of SO_4^{2-} release from octyl sulphate in *Pseudomonas* C12B is likely to produce larger crystals.

Cells of strain B1a incubated with Ba^{2+} (either with or without sulphate ester) contained isolated granules of moderately electron-dense material (Fig. 3a, b), which were absent from cells lacking Ba^{2+} (Fig. 3c). EDAX analysis (Fig. 4b) indicated the presence of very little sulphur in these deposits but large amounts of phosphorus together with barium and calcium. Clearly these areas are not related to butylsulphatase activity, and the abundance of phosphorus suggests that they may be polyphosphate storage granules (Dawes & Senior, 1973).

A feature common to all the electron micrographs of strain B1a is the presence of electron-transparent inclusions. Grains of BaSO_4 , indicating the location of butylsulphatase, were clustered around these inclusions. Phase contrast light microscopy of cells stained with Sudan

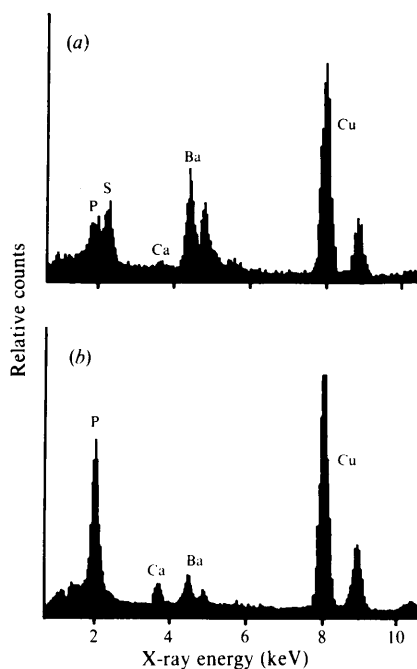


Fig. 4. EDAX spectra for sections of coryneform B1a shown in Fig. 3. (a) Electron-dense grains A. (b) Moderately electron-dense granules G.

Black revealed numerous black areas within the pale reddish cells, indicating that the inclusions probably correspond to poly- β -hydroxybutyric acid (or possibly lipid granules). These observations collectively raised the possibility that the C_4 chain of the butanol liberated by the sulphatase may be incorporated intact (after dehydrogenation and possibly β -hydroxylation but without fragmentation) into the granular material. Growth on butanol has been shown previously to lead to poly- β -hydroxybutyric acid production (Stevenson & Socolofsky, 1966) but whether or not the C_4 chain remains intact is unknown; this is currently occupying our attention.

Long- and short-chain alkylsulphatases: comparison of locations

This study has shown quite different cellular locations for long- and short-chain alkylsulphatases, which, while differing in chain-length specificity, are nevertheless both key enzymes in initiating the assimilation of carbon from alkyl sulphate esters. Evidently their different cellular locations are not dictated simply by the fact that they both initiate carbon assimilation from alkyl sulphates. Perhaps more important is the relative toxicities of long-chain and short-chain alkyl sulphates, the former but not the latter being very efficient surfactants and potentially damaging to cell membranes, proteins and sub-cellular structures. A location for long-chain alkylsulphatases in the outer cell-wall is rational because it eliminates the threat to the cell while still allowing assimilation of the carbon as the long-chain (non-surfactant) alcohol. No such protection is necessary against the short-chain esters, and the elaboration of an exocyttoplasmic enzyme would be wasteful. Moreover, advantage may accrue to the cells in producing butanol at the site of its possible incorporation into storage forms of carbon.

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