Chemically Defined Inducers of Alkylsulphatases Present in *Pseudomonas* C12B

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When *Pseudomonas* C12B is grown on nutrient broth to the stationary phase, cell extracts contain two secondary alkylsulphatases (S1 and S2) active towards potassium decan-5-yl sulphate but not towards potassium pentan-3-yl sulphate and one primary alkylsulphatase (P1) active towards sodium dodecan-1-yl sulphate (sodium dodecyl sulphate). When 10mM-sodium hexan-1-yl sulphate is included in the nutrient broth an additional primary alkylsulphatase (P2) is produced. The S1, S2, P1 and P2 enzymes are also present in extracts of cells grown on broth containing the commercial detergent Oronite, together with an additional secondary alkylsulphatase (S3) active towards pentan-3-yl sulphate as well as decan-5-yl sulphate. The P2 primary alkylsulphatase can be induced by a number of primary and secondary alkyl sulphate esters but the induction of the S3 enzyme appears to be a more specific and complex process. Studies on the ability of different fractions separated from Oronite to act as inducers suggest that the combination of a long-chain secondary alkyl sulphate(s) and a long-chain secondary alcohol(s) is responsible for the appearance of the S3 enzyme. Potassium hexadecan-2-yl sulphate or potassium tetradecan-2-yl sulphate, in combination with either hexadecan-2-ol or tetradecan-2-ol, can serve as inducers for the enzyme. Some characteristics of these specific inducer systems have been elucidated.

Pseudomonas C12B is an organism isolated from soil obtained at the edge of a river near the outfall of a sewage disposal plant in Athens, Georgia, U.S.A. (Payne & Feisal, 1963). When grown in nutrient broth containing the detergent sodium dodecan-1-yl sulphate (sodium dodecyl sulphate), the organism produced alkylsulphatase enzymes that were capable of liberating inorganic SO_4^{2-} from dodecan-1-yl sulphate (Williams & Payne, 1964). Subsequent work (Payne *et al.*, 1965, 1967; Payne & Painter, 1971) provided the basis for the following working hypotheses: (1) *Pseudomonas* C12B, grown to the stationary phase in nutrient broth alone, produced a sulphatase enzyme active towards the primary alkyl sulphate ester, dodecan-1-yl sulphate, and towards the secondary alkyl sulphate ester, decan-5-yl sulphate; (2) under similar growth conditions but with dodecan-1-yl sulphate also present in the culture medium, a second sulphatase active towards both sulphate esters was produced; (3) when Oronite (a commercial detergent consisting of a C_{10} – C_{20} mixture of secondary alkyl sulphate esters; Chevron Chemical Co., San Francisco, Calif., U.S.A.) was substituted for dodecan-1-yl sulphate a third sulphatase appeared that was active towards decan-5-yl sulphate but not towards dodecan-1-yl sulphate. *Pseudomonas* C12B thus appeared to be capable of synthesizing a total of three alkylsulpha-

tases, two of them having dual specificity towards both primary and secondary long-chain alkyl sulphates and the other having specificity towards secondary long-chain alkyl sulphates only.

As additional substrates became available, work (Fitzgerald & Payne, 1972a) in which short-chain alkyl sulphate esters were tested as inducing agents suggested that the situation was probably more complex. Moreover, there were indications that both culture age and the nature of the inducer were factors which helped to determine the type and specificity of the alkylsulphatases produced.

The present paper deals with alkylsulphatases that are present at the stationary phase of growth and establishes that the earlier interpretations (see Payne & Painter, 1971) were oversimplified. The paper also describes a defined chemical system that is capable of inducing alkylsulphatases in a manner analogous to that of the chemically complex Oronite detergent.

Materials and Methods

Sulphate esters

Potassium decan-5-yl sulphate. This was prepared by sulphation of the parent alcohol with H_2SO_4 . Decan-5-ol (0.1mol; all secondary alcohols were purchased from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., or from K & K Laboratories Inc., Plainview, N.Y., U.S.A.) was dissolved in 600ml of dry light petroleum (b.p. 30–40°C) and

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cooled in an ice-salt mixture. Sulphuric acid (0.15 mol, sp.gr. 1.84, taken from a newly opened bottle) was added dropwise over a period of 20 min to the vigorously stirred mixture. Stirring was continued for a further 3 h in the ice-salt mixture and for 10–15 min at room temperature. Thionyl chloride (0.08 mol) was added to the mixture and the whole shaken carefully, to remove water formed during the sulphation process. Hydrogen chloride and SO_2 were then quickly removed by means of a rapid stream of dry air and the whole was concentrated *in vacuo* at 35°C. The concentrated solution was cooled and cautiously treated as rapidly as possible with 0.15 mol of KOH in 45 ml of dry methanol. This was generally sufficient to neutralize the bulk of the excess of acid and neutralization could then be completed (in practice a pH of about 8–9 was aimed for) more carefully by addition of further methanolic KOH. If the bulk of the excess of acid is not neutralized rapidly, methyl hydrogen sulphate tends to form as a result of the esterification of methanol with unchanged H_2SO_4 . The inorganic salts which precipitated out as a result of the methanolic KOH treatment were separated by filtering the hot solution at the pump and the filtrate was then concentrated *in vacuo* at room temperature, with the addition of a little ether from time to time. Once crystals started to form the whole was kept overnight at -10°C before filtering. The crystals were washed with a small amount of methanol (cooled to -10°C) and then well washed with ether before drying *in vacuo*. The material (yield 20–25% of theory) was recrystallized twice from the smallest possible volume of methanol-ether (40:60, v/v), a trace of methanolic KOH being added as necessary to be certain that the pH of the hot solution was about 8.0. This ensures that no loss of K^+ occurs during recrystallization. The final product gave analysis results for K^+ and SO_4^{2-} that agreed with a structure of anhydrous potassium decan-5-yl sulphate. The infrared spectrum was in accord with that of a secondary alkyl sulphate ester.

Potassium decan-2-, decan-3- and decan-4-yl sulphates. These were prepared as for the 5-isomer and were generally obtained in a slightly better yield and with greater initial ease of crystallization.

Potassium hexadecan-2-yl sulphate. Hexadecan-2-ol (0.04 mol) was dissolved in 180 ml of dry chloroform and the whole was cooled in a bath of crushed ice. Chlorosulphonic acid (0.06 mol) was added to the vigorously stirred solution over a period of about 20 min and stirring was then continued for a further 3–4 h. A rapid stream of dry air was passed through the mixture to drive off HCl and the whole was then concentrated to a syrup *in vacuo* at 40°C. Methanolic KOH (50 ml containing 0.06 mol of KOH) was then added rapidly but carefully to the syrup to neutralize the bulk of the acid (see above) and neutralization (to pH 8–9) was then completed more carefully by addition of further methanolic KOH. Precipitated in-

organic salts were removed by filtration of the hot methanolic mixture through a charcoal pad contained in a warm Buchner funnel. Crystals separated out on cooling overnight at -10°C and were filtered off, washed with small amounts of methanol at -10°C , followed by cold acetone and ether. The material (yield about 45%) was dried *in vacuo* and then recrystallized twice from methanol. The product gave analysis results for K^+ and SO_4^{2-} that agreed with a structure of anhydrous potassium hexadecan-2-yl sulphate. The infrared spectrum was that expected of a secondary alkyl sulphate ester.

Potassium tetradecan-2-yl sulphate. This was prepared by each of the two methods described above. The chlorosulphonic acid procedure gave somewhat better yields (41%:28%) than the H_2SO_4 method. With each method two different samples of tetradecan-2-ol (purchased from different suppliers, as mentioned in a previous section) were employed as starting materials. There were no obvious differences between the infrared spectra of any of the products although, as will be seen in a later section, some differences were apparent when different samples were used in enzyme-induction experiments.

Other sulphate esters. Sodium dodecan-1-yl sulphate and sodium hexan-1-yl sulphate were purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Potassium pentan-3-yl sulphate was kindly provided by Dr. N. Tudball, Department of Biochemistry, University College, Cardiff, U.K. All others mentioned in the text were prepared by the H_2SO_4 method described above.

Bacteria and culture conditions

Pseudomonas C12B was maintained as described by Payne & Painter (1971). In the induction experiments the organism was usually grown in 500 ml Erlenmeyer flasks containing 200 ml of 0.8% (w/v) nutrient broth. When long-chain alcohols were required as culture components the alcohol was added to the broth before sterilizing by autoclaving. When alkyl sulphate esters were tested as inducers an appropriate amount was dissolved in water, the solution was sterilized by Millipore filtration (GS 0.22 μm filter) and a 5 ml portion was then added aseptically to the sterile broth. Cultures were incubated at 30°C with shaking. Growth was followed by measuring the absorption of a sample at 650 nm in 1 cm light-path cells with the Hitachi Perkin-Elmer model 139 spectrophotometer at a slit-width of 0.1 mm.

Preparation of extracts

At the appropriate times bacteria were harvested by centrifuging cultures at 4°C. The surface of the cell pellet and the insides of the centrifuge pots were washed with cold 0.01 M-Tris-HCl buffer, pH 7.8,

without disturbing the pellet and the latter was then suspended in 1–2 ml of the cold buffer and ruptured by passage through a chilled French pressure cell operating at 126 MPa until extracts became translucent (usually three or four passes). Cell debris was removed by centrifuging in the SS-34 rotor of the Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). The rotor was operated for 1 h at 2°C and 18000g (r_{av} 3.25 in). The supernatants were then dialysed for 24 h at 4°C against several changes of buffer. Extracts could then be stored in the frozen state for several weeks without losses in alkylsulphatase activity.

Assay of sulphatase activity

Enzymically liberated SO_4^{2-} was estimated by the $BaCl_2$ -gelatin method of Dodgson (1961) as modified by Thomas & Tudball (1967) for use with relatively crude enzyme extracts. Experiments in which a check was made on the recoveries of added SO_4^{2-} from buffered solutions of various alkyl sulphate esters showed that the method could not be used with the sulphate esters of decan-1-ol, dodecan-1-ol, dodecan-2-ol, tetradecan-2-ol and hexadecan-2-ol because of interference by precipitation of the relatively insoluble barium salts of the sulphate esters. Recoveries of SO_4^{2-} from solutions of the sulphate esters of decan-2-, decan-3- and decan-4-ol were always slightly low (between 90 and 98% of theory), and from octan-1-yl sulphate always slightly high (usually about 6%). Good recoveries were obtained with the sulphate esters of decan-5-ol, nonan-2-ol, octan-2-, octan-3- and octan-4-ol, hexan-1-, hexan-2- and hexan-3-ol, pentan-3-ol and butan-1-ol.

The potassium salts of pentan-3-yl sulphate and decan-5-yl sulphate were most regularly employed as assay substrates. Both were present in the incubation mixture at a final concentration of 0.018 M in the presence of 0.02 M-Tris-HCl buffer and at pH 7.8 and pH 8.2 respectively. All incubations were carried out at 30°C and incubation times varied from 10 to 100 min, depending on the substrate and the amount of enzyme present in extracts. Other substrates were occasionally employed, experimental conditions being as for pentan-3-yl sulphate.

Enzyme activity was expressed in terms of protein content of extracts and this was determined by the method of Lowry *et al.* (1951). Dry bovine albumin, fraction V powder (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), was the standard. A unit of enzyme activity is defined as 1 nmol of SO_4^{2-} liberated/min per mg of protein.

Detection of sulphatase activity on polyacrylamide-gel zymograms

The method of Payne & Painter (1971) was employed except that primary alkylsulphatase activity

was detected by incubating the extruded gel at 30°C in 0.02 M-Tris-HCl buffer, pH 7.8, containing 0.01 M-sodium dodecan-1-yl sulphate and inspecting at frequent intervals for the appearance of the white bands that denoted sulphatase activity. Secondary alkylsulphatase activity was detected by incubating a second identical gel in 0.01 M-decan-5-yl sulphate in the same buffer at the same pH. In some experiments gels were sliced vertically into halves, one of which was incubated with dodecan-1-yl sulphate and the other with decan-5-yl sulphate. It was not possible to incubate a single gel in a mixture of the two substrates because, although the enzymes active towards decan-5-yl sulphate are inactive towards dodecan-1-yl sulphate and vice versa, it appears from actual gel experiments that each type of enzyme is inhibited by the other enzyme's substrate. The nature of the white bands that denote the position of the sulphatases is still obscure and is currently under investigation. The bands do not appear when alkyl sulphate esters with chain length below C_{10} are employed.

Polyacrylamide-gel electrophoresis

The procedure of Payne & Painter (1971) was modified as follows. Buchler analytical equipment (Polyanalyst, Buchler Instruments Division, Nuclear-Chicago Corp., Fort Lee, N.J. 07024, U.S.A.) was employed, the concentration of the Cyanogum 41 solution was reduced to 6.8% and care was taken to de-gas the solution before use. Polymerization of the gels was achieved under standardized lighting conditions over a 30 min period and enzyme samples were introduced on to the gels at pH 7.8 in 10% (w/v) sucrose. Electrophoresis was at 2 mA per tube and was continued until the tracking dye was approx. 3 mm from the bottom of the gel. Cooling fluid at 4°C was circulated through the apparatus.

Fractionation of Oronite

Oronite (50 ml of a sample that had been stored at room temperature for some time) was dropped slowly into 350 ml of acetone, which was being stirred vigorously. The whole was then allowed to stand at –10°C for 2 h before removal of precipitated material by filtration at the pump and washing with acetone. The washed precipitate was dried *in vacuo* (yield 10.2 g of a white powder, fraction 1) and the filtrate and washings were pooled and concentrated *in vacuo* at 35°C. The concentrated syrup was repeatedly triturated with acetone and re-concentrated and eventually a white crystalline material separated out from the acetone solution. This was filtered at the pump, washed with acetone and dried *in vacuo* (yield 3.13 g, fraction 2). The filtrate and washings were pooled and concentrated *in vacuo* as before, when a brown oil remained (yield 5.1 g, fraction 3). A 2 ml portion of

fraction 3 was shaken with 8 ml of light petroleum (b.p. 30–60°C), when some crystalline material separated out. The material was filtered off, washed well with light petroleum and then dried *in vacuo* (yield 0.15 g of material; this fraction was not designated or examined further). The filtrate and washings were pooled and shaken in a separating funnel with three half-volumes of water. An emulsion formed on each washing and was broken by centrifuging. The light petroleum layer was separated on each occasion for re-washing. The light petroleum layer was finally concentrated *in vacuo* at 40°C to yield 1.2 ml of a yellow-brown oil with a characteristic odour (fraction 3A).

Weighed portions of the fractions were hydrolysed with 2M-HCl under reflux for 40 min before liberated SO_4^{2-} was determined gravimetrically as BaSO_4 . Care was taken to include a final washing of the BaSO_4 precipitate with hot ethanol followed by ether in order to remove free long-chain alcohol which had separated out during hydrolysis. The hydrolysed fractions had the following sulphate content: fraction 1, 58.2% (theory for $\text{Na}_2\text{SO}_4 = 67.6\%$); fraction 2, 31.8% (theory for sodium alkyl sulphates of average chain length $\text{C}_{14} = 30.4\%$); fraction 3, 14.0%; fraction 3A, nil.

Results

Gel-electrophoresis experiments

Pseudomonas C12B was grown to the early stationary phase under three different culture conditions, namely, in nutrient broth alone, in broth containing sodium hexan-1-yl sulphate (final concentration 0.01 M) and in broth containing Oronite (final concentration 0.2%, v/v). Dialysed cell extracts were prepared as described above and subjected to polyacrylamide-gel electrophoresis. The results (Fig. 1a,b) showed that in broth alone the organism produces two secondary alkylsulphatases (S1 and S2), active towards decan-5-yl sulphate but not dodecan-1-yl sulphate, and one primary alkylsulphatase (P1) active towards dodecan-1-yl sulphate but not towards decan-5-yl sulphate. The enzymes are clearly separated from each other on the gels. Separate enzyme assays showed that cell extracts were also able to hydrolyse other secondary alkyl sulphate esters, namely, the potassium salts of decan-2-, decan-3- and decan-4-yl sulphate and octan-2-yl sulphate, but were without action on potassium pentan-3-yl sulphate. The extracts were able to hydrolyse slowly the primary alkyl sulphate ester, potassium octan-1-yl sulphate, and had even weaker activity towards hexan-1-yl sulphate (Table 1).

When hexan-1-yl sulphate was included in the culture medium gel electrophoresis showed that the extracts contained the same two secondary alkyl-

sulphatase bands (S1 and S2, active towards decan-5-yl sulphate) and the same primary alkylsulphatase band (P1, active towards dodecan-1-yl sulphate) as those from cells grown on broth alone. However, a further distinct band of enzyme activity was now present (P2) migrating ahead of the other three and active towards dodecan-1-yl sulphate but not decan-5-yl sulphate (Fig. 1d). Enzyme assay of the extracts gave results similar to those obtained with broth-grown cells except that hexan-1-yl sulphate was now rapidly hydrolysed by the extracts (Table 1).

When Oronite was included in the culture medium the four bands described above (S1, S2, P1 and P2) appeared on zymograms of cell extracts but a fifth and fast-moving band (S3) was now also present when decan-5-yl sulphate was the substrate (Fig. 1e). This enzyme is apparently a secondary alkylsulphatase since the band was absent when dodecan-1-yl sulphate was employed as substrate. Enzyme assay of cell extracts gave results similar to those obtained with extracts from cells grown in the presence of

Fig. 1. Alkylsulphatase enzyme bands present on polyacrylamide-gel zymograms of extracts of cells of *Pseudomonas* C12B

One gel of each pair was incubated at 30°C in a solution of either potassium decan-5-yl sulphate (D-5-S; gels a, c and e) or sodium dodecyl sulphate (SDS; gels b, d and f). The arrow denotes the position of migration of the Bromophenol Blue tracking dye. (a) and (b), Extracts of cells grown on nutrient broth; (c) and (d), extracts of cells grown on nutrient broth containing 10 mM-sodium hexan-1-yl sulphate; (e) and (f), extracts of cells grown on nutrient broth containing 0.2% (v/v) of Oronite. Locations of the three secondary alkylsulphatases and two primary alkylsulphatases are designated by the symbols S1, S2 and S3 and P1 and P2 respectively.

Table 1. *Alkylsulphatase activity towards primary and secondary alkyl sulphates of cell extracts of Pseudomonas C12B grown under various conditions*

Bacteria were grown to the early stationary phase and extracts were assayed under the conditions described in the text.

Additions to broth	Alkyl sulphate ...	Enzyme activity (units)			
		Decan-5-yl sulphate	Pentan-3-yl sulphate	Octan-1-yl sulphate	Hexan-1-yl sulphate
None		200.0	0	5.7	1.6
Hexan-1-yl sulphate*		160.0	0	32.0	36.0
Oronite†		203.0	5.4	37.0	39.0

* Present at a final concentration of 10 mM.

† Present at a final concentration of 0.2% (v/v).

hexan-1-yl sulphate except that pentan-3-yl sulphate was now slowly hydrolysed (Table 1).

Nature of the inducing components of Oronite

The experiments described in the previous section showed that bacteria grown in broth alone produced two secondary (S1 and S2) and one primary alkylsulphatase (P1). Addition of hexan-1-yl sulphate to the broth led to the appearance of a further primary enzyme (P2), whereas when Oronite replaced hexan-1-yl sulphate a fifth enzyme also appeared (the S3 secondary enzyme). Only the S3 enzyme could hydrolyse pentan-3-yl sulphate. Table 4 in the Discussion section summarizes these and other findings. The question now arose as to the nature(s) of the component(s) present in Oronite that were responsible for the induction of P2 and (particularly) S3. Accordingly a study was made of the ability of various fractions separated from Oronite, either alone or in combination with other materials, to induce the formation of the two enzymes in question. In calculating the amount of any fraction to be added as possible inducer, account was taken of the quantitative yields of each fraction from the Oronite fractionation procedure and these were related to the concentration of Oronite normally employed as inducer, namely 0.2% (v/v) final concentration. When pure alkyl sulphate esters (other than hexan-1-yl sulphate) were present in cultures their final concentration was 1 mM. Cells were grown to the stationary phase and cell extracts were assayed for primary and secondary alkylsulphatase activity by the usual gel-electrophoresis procedure and for protein and S3 sulphatase activity towards pentan-3-yl sulphate. The results (Table 2) showed that no pentan-3-yl sulphatase activity was present in extracts of cells when any of Oronite fractions 1, 2 or 3 were separately present in the culture medium. However, when all were present together, pentan-3-yl sulphatase activity (S3 enzyme) was observed. Combining fractions 1 and 2 did not induce the enzyme, fractions 1 and 3 pro-

duced traces of the enzyme, particularly as evidenced by gel electrophoresis, whereas fractions 2 and 3 caused reasonable amounts of the enzyme to appear.

Fractions 1 and 2 or any combination containing at least one of these fractions gave a P2 enzyme band but fractions 3 and 3A gave no such band. Indeed only the S1 band could be detected in these particular cases.

Hexadecan-2-yl sulphate was unable alone to induce the S3 enzyme but caused the P2 enzyme to appear. However, when either fraction 3 or 3A was also present the S3 enzyme appeared (Table 2).

When tetradecan-2-yl sulphate was tested as a possible inducer no significant amounts of the S3 enzyme appeared, as judged by assay against pentan-3-yl sulphate, but incubating acrylamide gels for a period of 18 h with decan-5-yl sulphate revealed a faint S3 enzyme band. Considerable S3 activity was obtained, however, when Oronite fraction 3 or 3A was also present in the growth medium (Table 2). It will be seen from a section below that there was considerable variability in the results obtained with tetradecan-2-yl sulphate.

The sulphate esters of dodecan-2-ol, decan-2-ol, octan-2-ol and pentan-3-ol, either alone or in combination with Oronite fraction 3A, failed to induce the S3 enzyme (Table 2).

The collective results suggested that the induction of the S3 enzyme by Oronite was a complex process involving at least two factors. One of these factors was a sulphate ester(s) present in Oronite fractions 1 and (particularly) 2 and the other was not a sulphate ester since it was present in fraction 3A, a fraction devoid of such esters. Gas-liquid chromatography (kindly performed by Dr. W. R. Finnerty) showed fraction 3A to be extremely complex in character, but the pattern obtained was indicative of the presence of several long-chain secondary alcohols, not an unexpected finding since hydrolysis of solutions of secondary alkyl sulphate esters does occur on storage. The possibility therefore existed that induction of enzyme S3 could be achieved by a combination of a

Table 2. *Effects of various compounds on the production of primary and secondary alkylsulphatases by Pseudomonas C12B*

Bacteria were grown to the early stationary phase. The symbols S1, S2 and S3 refer to the three secondary alkylsulphatases described in the text and P1 and P2 to the two primary alkylsulphatases. Where no concentration is quoted for inducers the appropriate amounts employed may be deduced from the text. Where the concentration of an Oronite fraction appears in the table, the molarity is an approximation based on the assumption of an average alcohol chain length of C₁₂. A zero value for enzyme activity towards pentan-3-yl sulphate signified a value less than 0.3 unit, which is about the limits of experimental error of the assay method employed. Presence or absence of enzyme bands on electrophoretograms is denoted by + or – and no attempt has been made to assess intensity.

Additions to broth	Concn. (mM)	Enzyme activity towards pentan-3-yl sulphate (units)	Presence of alkylsulphatase bands on gel electrophoretograms				
			S1	S2	S3	P1	P2
None		0	+	+	–	+	–
Oronite		5	+	+	+	+	+
Fraction 1		0	+	+	–	+	+
Fraction 2		0	+	+	–	+	+
Fraction 3		0	+	–	–	–	–
Fraction 3A		0	+	–	–	–	–
Fractions 1+2+3		2	+	+	+	+	+
Fractions 1+2		0	+	+	–	+	+
Fractions 1+3		1	+	+	+	+	+
Fractions 2+3		2	+	+	+	+	+
Pentan-3-yl sulphate	1.0	0	+	+	–	+	+
+ Oronite fraction 3A	1.0	0	+	+	–	+	–
Octan-2-yl sulphate	1.0	0	+	+	–	+	+
+ Oronite fraction 3A	1.0	0	+	+	–	+	–
Decan-2-yl sulphate	1.0	0	+	+	–	+	+
+ Oronite fraction 3A	1.0	0	+	+	–	+	–
Dodecan-2-yl sulphate	1.0	0	+	+	–	+	+
+ Oronite fraction 3A	1.0	0	+	+	–	–	–
Dodecan-2-yl sulphate	0.5	0	+	+	–	+	+
	2.0	0	+	+	–	+	+
	3.0	0	+	+	–	+	+
	5.0	0	+	+	–	+	+
Tetradecan-2-yl sulphate	1.0	0	+	+	+	+	+
+ Oronite fraction 3	1.0	5	+	+	+	+	+
+ Oronite fraction 3A	1.0	5	+	+	+	+	+
Hexadecan-2-yl sulphate	1.0	0	+	+	–	+	+
+ Oronite fraction 3	1.0	5	+	+	+	+	+
+ Oronite fraction 3A	1.0	6	+	+	+	+	+
Hexadecan-2-yl sulphate	0.5	0	+	+	–	+	+
	0.7	0	+	+	–	+	+
	2.0	0	+	+	–	+	+
	3.0	0	+	+	–	+	+

long-chain secondary alkyl sulphate ester and a long-chain secondary alcohol.

Induction of enzyme formation by potassium hexadecan-2-yl sulphate and hexadecan-2-ol or tetradecan-2-ol

The S1, S2, P1 and P2 enzyme bands were present on gel-electrophoretograms of extracts of cells grown on broth containing hexadecan-2-yl sulphate at concentrations from 0.5 to 3.0mM, but there was no evidence of the S3 band or of enzyme activity towards pentan-3-yl sulphate (Table 2). However, when 0.1mM-hexadecan-2-ol was also included in the

growth medium the S3 band and the pentan-3-yl sulphatase activity appeared. When hexadecan-2-ol alone was present in the broth only the S1, S2 and P1 enzyme bands appeared on gel electrophoresis and pentan-3-yl sulphate was not hydrolysed. Fig. 2(a) shows that when the concentration of hexadecan-2-yl sulphate was kept at 0.7mM maximum enzyme activity towards pentan-3-yl sulphate was obtained at a hexadecan-2-ol concentration of 1mM.

When the concentration of hexadecan-2-ol was kept constant at 1mM there was an increase in enzyme activity towards pentan-3-yl sulphate as the concentration of hexadecan-2-yl sulphate was increased (Fig. 2b). Fig. 3 shows the activities of the S3 enzyme at

Fig. 2. Alkylsulphatase activities towards pentan-3-yl sulphate of extracts of *Pseudomonas* C12B grown on nutrient broth containing hexadecan-2-yl sulphate and hexadecan-2-ol or tetradecan-2-ol

Experimental details are given in the text. (a) Cells grown on broth containing 0.7 mM-hexadecan-2-yl sulphate and increasing amounts of either Δ , tetradecan-2-ol, or $\blacktriangle$, hexadecan-2-ol. (b) Cells grown on broth containing increasing amounts of hexadecan-2-yl sulphate and either $\circ$, 1 mM-tetradecan-2-ol, or $\bullet$, 1 mM-hexadecan-2-ol.

various stages during growth when hexadecan-2-ol and the corresponding sulphate ester were present in the culture medium at concentrations of 1.0 mM and 0.7 mM respectively. Maximum activity was generally obtained at about 22–24 h after inoculation (Fig. 3). Growth was greater in the presence of the alcohol and there was always some indication of a diauxic effect.

Similar results were obtained when tetradecan-2-ol was substituted for hexadecan-2-ol (Figs. 2a, 2b and 3).

Effects of other alcohols and related compounds

Extracts of cells grown in nutrient broth containing 0.7 mM-potassium hexadecan-2-yl sulphate failed to contain the S3 enzyme when the following compounds were also present in the broth: dodecan-2-ol and decan-2-ol (both at final concentrations of 0.1, 1.0 and 2.0 mM); dodecan-1-ol, tetradecan-1-ol and hexadecan-1-ol; dodec-1-ene, tetradec-1-ene and hexadec-1-ene; dodecanoate and hexadecanoate (all at 1.0 mM). However, S3 enzyme activity was obtained when tetradecanoate was present.

Fig. 3. Time-course of induction of S3 alkylsulphatase active towards pentan-3-yl sulphate

Experimental details are given in the text. $\square$, Growth and $\blacksquare$, S3 alkylsulphatase activity of extracts of cells grown in nutrient broth containing 0.7 mM-hexadecan-2-yl sulphate. Δ , Growth and $\blacktriangle$, alkylsulphatase activity of extracts of cells grown on broth containing 0.7 mM-hexadecan-2-yl sulphate and 1.0 mM-hexadecan-2-ol. $\circ$, Growth, and $\bullet$, alkylsulphatase activity of extracts of cells grown on broth containing 0.7 mM-hexadecan-2-yl sulphate and 1.0 mM-tetradecan-2-ol.

Induction of enzyme formation by potassium tetradecan-2-yl sulphate

Experiments in which tetradecan-2-yl sulphate (1.0 mM) was employed as a possible inducer of the S3 enzyme gave conflicting results, depending on the preparation of the ester that was selected for study. With a total of seven different preparations (see Table 3 for details) employed as inducer in the absence of tetradecan-2-ol or hexadecan-2-ol, enzyme activity varied from zero to 7.8 units/min per mg of protein. All preparations gave roughly the same enzyme activity when tetradecan-2-ol (1.0 mM) was also present in the growth medium (Table 3). Extremely small amounts of the alcohol (0.05 mM) were apparently required to potentiate the induction (Table 3) and the possibility existed that those tetradecan-2-yl sulphate preparations that gave good activity were contaminated with small amounts of free tetradecan-2-ol. Some support for this possibility came from an experiment in which the effects of increasing the concentration of tetradecan-2-yl sulphate (preparation 3,

Table 3. Induction of S3 alkylsulphatase active towards pentan-3-yl sulphate by different preparations of tetradecan-2-yl sulphate in the presence and absence of tetradecan-2-ol

Starting material for the chemical synthesis of the tetradecan-2-yl sulphate was purchased from the two sources described in the text. These sources are referred to as A (Aldrich Chemical Co.) and B (K & K Laboratories) in the table. Except where otherwise stated the concentrations of the inducers were 1 mM.

Source of tetradecan-2-ol	Preparation no.	Method of preparation	Enzyme activity towards pentan-3-yl sulphate (units)	
			Tetradecan-2-yl sulphate	Tetradecan-2-yl sulphate + tetradecan-2-ol
A	1	H ₂ SO ₄	0	5.8
B	2	H ₂ SO ₄	1.6	5.8
A	3	H ₂ SO ₄	1.0	6.2
A	4	H ₂ SO ₄	2.5	7.5
				7.2*
				7.2†
A	5	H ₂ SO ₄	7.8	
B	6	ClSO ₃ H	6.8	5.8
A	7	ClSO ₃ H	6.9	4.8

* 0.05 mM-Tetradecan-2-ol.

† 0.01 mM-Tetradecan-2-ol.

Fig. 4. Alkylsulphatase activity towards pentan-3-yl sulphate of extracts of *Pseudomonas C12B* grown on nutrient broth containing increasing amounts of tetradecan-2-yl sulphate

Experimental details are given in the text. Preparation 3 of tetradecan-2-yl sulphate (see Table 3) was employed.

low inducing capacity at 1.0 mM concentration, see Table 3) on induction of the S3 enzyme was examined. At concentrations of 1.0 mM and below, very little activity towards pentan-3-yl sulphate was induced but substantial activity was obtained at higher concentrations (Fig. 4). However, further recrystallization of those ester preparations that induced considerable amounts of enzyme did not significantly decrease the extent of the induction.

Fig. 5. Time-course of induction of S3 alkylsulphatase active towards pentan-3-yl sulphate

Experimental details are given in the text. Preparation 3 of tetradecan-2-yl sulphate (see Table 3) was employed as inducer. □, Growth and ■, alkylsulphatase activity of extracts of cells grown on nutrient broth containing 1.0 mM-tetradecan-2-yl sulphate. ○, Growth and ●, alkylsulphatase activity of extracts of cells grown on broth containing 1.0 mM-tetradecan-2-yl sulphate and 1.0 mM-tetradecan-2-ol.

The time-course of induction of the S3 enzyme by a mixture of 1.0mM-tetradecan-2-ol and 1.0mM-tetradecan-2-yl sulphate (preparation 3, see Table 3) was similar to that given by the same alcohol and hexadecan-2-yl sulphate (Figs. 5 and 3).

Discussion

It is now clear from the present work and that of Fitzgerald & Payne (1972*a,b*) that the earlier working hypotheses of Payne & Painter (1971) concerning alkylsulphatase induction in *Pseudomonas* C12B were oversimplified. It has now been shown that the organism grown in nutrient broth alone produces one enzyme (P1) hydrolysing long-chain primary alkyl sulphate esters and two enzymes (S1 and S2) hydrolysing long-chain secondary alkyl sulphate esters (see Table 4). It is still not clear whether these three enzymes are induced by components present in the nutrient broth or whether they are enzymes that are repressed when the organism is grown on synthetic media containing simple oxidizable growth-supporting carbon sources (cf. Fitzgerald & Payne, 1972*b*). Some preliminary attempts have been made to deplete the nutrient broth of possible inducers by either prolonged dialysis (the dialysed broth subsequently being supplemented by an appropriate salts mixture) or by prolonged extraction with methanol or chloroform or both. In the former case the organism did not grow and in the latter case the same enzymes were produced as with unextracted nutrient broth.

Fitzgerald & Payne (1972*a*) and Fitzgerald *et al.* (1974) have shown that the stationary-phase cells grown on broth containing 10mM-sodium hexan-1-yl sulphate possess an additional enzyme (P2) active towards primary alkyl sulphates. There were also some indications from the enzyme-assay studies of these workers that other related primary sulphate esters might induce this particular enzyme. The present work has confirmed some of these findings and has established by the zymogram procedure that a variety of secondary alkyl sulphates can also induce the P2 enzyme (see Table 4). It will be interesting to pursue this matter further to establish the precise limits of the range and type of sulphate ester that can serve as an inducer for this particular enzyme.

The findings for the induction of the enzyme exhibiting activity towards potassium pentan-3-yl sulphate (the S3 enzyme, Table 4) suggests a close degree of inducer specificity and an oddly complex mechanism. The studies with Oronite fractions and with hexadecan-2-yl sulphate provide clear evidence that two factors are involved in these particular cases. One of these is a long-chain secondary alcohol and the other is a long-chain secondary alkyl sulphate ester. At present there is no clue as to why such a combination should be necessary, although one could put forward possible reasons. For example, the presence of the secondary alcohol may induce an enzyme(s) that is then able to modify the alkyl chain of the alkyl sulphate ester; the modified sulphate ester thus formed might then induce the S3 enzyme. Other possibilities involving transport could be envisaged.

Table 4. *Substrate specificities and inducer requirements of primary and secondary alkylsulphatases present in extracts of cells of Pseudomonas C12B grown to the stationary phase*

Designation of alkylsulphatase	Type of alkylsulphatase	Ability to hydrolyse sulphate esters					Inducer required to be added to nutrient broth
		Dodecan-1-ol	Octan-1-ol	Hexan-1-ol	Decan-5-ol	Pentan-3-ol	
P1	Primary	+	+	+	—	—	None
P2	Primary	+	+	+	—	—	One of: Oronite; pentan-1-yl sulphate*; hexan-1-yl sulphate; octan-1-yl sulphate*; pentan-3-yl sulphate; octan-2-yl sulphate; decan-2-yl sulphate; dodecan-2-yl sulphate; tetradecan-2-yl sulphate; hexadecan-2-yl sulphate
S1	Secondary	—	—	—	+	—	None
S2	Secondary	—	—	—	+	—	None
S3	Secondary	—	—	—	+	+	One of: Oronite; tetradecan-2-yl sulphate† + tetradecan-2-ol; hexadecan-2-yl sulphate + tetradecan-2-ol; hexadecan-2-yl sulphate + hexadecan-2-ol; hexadecan-2-yl sulphate + tetradecanoate

* Results from Fitzgerald *et al.* (1974).

† Some preparations of this sulphate ester did not require the additional presence of tetradecan-2-ol (see the text).

The degree of inducer specificity is also of interest because there appears to be a lower limit (C_{14}) below which the 2-yl sulphates fail to cause induction in the presence of Oronite fraction 3A (Table 2). Unfortunately, it has not yet been possible to obtain 2-yl sulphate esters of chain length greater than C_{16} . Similarly, although either hexadecan-2-ol or tetradecan-2-ol could serve as a co-inducer in the presence of hexadecan-2-yl sulphate, the corresponding secondary alcohols of lower chain length (C_{12} and C_{10}) could not. Again tetradecanoate could replace tetradecan-2-ol or hexadecan-2-ol as co-inducer but hexadecanoate or dodecanoate could not. This relatively high inducer specificity is of considerable interest and perhaps not entirely unexpected as a number of different investigators have pointed to the C_{10} – C_{12} chain length as a critical length in some cases of the microbial degradation and/or utilization of alkanes and alkenes, below which degradation-utilization is frequently impaired (e.g. Finnerty *et al.*, 1962; Makula & Finnerty, 1968; Kester & Foster, 1963; Klug & Markovetz, 1971).

The lack of consistency in results obtained when attempts were made to employ tetradecan-2-yl sulphate as sole inducer requires comment. Those preparations of tetradecan-2-yl sulphate which at 1 mM concentration induced only weak activities of the S3 enzyme nevertheless induced substantial activities when the concentration was increased above 2 mM (Fig. 4). No such effect was observed when hexadecan-2-yl sulphate was tested as sole inducer. Comment has already been made that the presence of traces of free parent alcohol as a contaminant of tetradecan-2-yl sulphate preparations could be responsible for the variable results obtained, particularly since, in contrast with the case of hexadecan-2-yl sulphate, large induction responses are obtained with extremely small amounts of tetradecan-2-ol (Table 3). However, at least one further possibility exists, namely that tetradecan-2-yl sulphate is itself a substrate for one or both of the two secondary alkylsulphatase enzymes (S1 and S2) present in extracts of cells grown on broth alone. Thus the organism might itself be capable of producing tetradecan-2-ol from the corresponding sulphate ester in sufficient amounts to cause co-induction of the S3 enzyme. In such a case either or both of the S1 and S2 enzymes should appear in cell extracts before the S3 enzyme during growth. This possibility has not yet been tested because of the absence of information about the precise specificities and properties of the three enzymes that would enable them to be assayed independently. At the present time it is still not known whether the S1 or S2 enzymes can hydrolyse tetradecan-2-yl sulphate. Methods currently available for assaying secondary alkylsulphatase activity cannot operate with tetradecan-2-yl sulphate as substrate and some preliminary experiments in which the ester has been employed as a pos-

sible substrate for gel-electrophoresis assay have yielded negative (and therefore equivocal) results. Resolution of this point must await the preparation of ^{35}S -labelled tetradecan-2-yl sulphate and for the present the anomalies associated with the ability of the ester to induce the S3 enzyme still remain. Meanwhile, there is now available a well-defined system, in the shape of hexadecan-2-yl sulphate plus hexadecan-2-ol, for the induction of the S3 enzyme and the reasons for the inducing ability of Oronite is at least partly resolved in a satisfactory manner.

Before much further progress can be made with our understanding of the induction of such a variety of related enzymes in a single micro-organism it will be necessary to know much more about the relative specificity of the various enzymes. It is already known that the S3 enzyme is the only secondary alkylsulphatase present that can hydrolyse both pentan-3-yl sulphate and decan-5-yl sulphate. Attempts to separate the various enzymes for detailed study are currently in progress.

Finally, it should be stressed once again that the stationary-phase enzymes only have been considered in this work and there are some indications from the work of Fitzgerald & Payne (1972a) that other sulphatase enzymes might also be produced at different stages of growth and by other sulphate esters.

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