Sulphur Utilization during Growth of *Pseudomonas fluorescens* on Potassium D-Glucose 6-O-Sulphate

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Pseudomonas fluorescens N.C.I.B. 8248 was adapted to grow on potassium D-glucose 6-O-sulphate as the sole carbon and sulphur source. Adapted bacteria grew optimally at 37°C on 1.6% (w/v) sulphate ester and growth coincided with the disappearance of the ester from the culture medium at a rate of 2.4 mg/h per ml. Three sulphated compounds were detected in the culture fluid at the termination of growth. One of these was present in traces only and has not been identified. The second was present in somewhat greater amounts and was identified as the 6-O-sulphate ester of D-gluconate, and the major metabolite was identified as D-glycerate 3-O-sulphate. Sulphur utilization by the organism was not associated with the appearance of a glycosulphatase enzyme in the cells. However, a novel enzyme system (or systems) was present that liberated inorganic $^{35}\text{SO}_4^{2-}$ ions from dipotassium D-gluconate 6-[^{35}S]-O-sulphate and from dipotassium DL-glycerate 3-[^{35}S]-O-sulphate. Activity towards the latter substrate could not be detected when the adapted or parent *Pseudomonas* strain was cultured on D-glucose and potassium sulphate as respective carbon and sulphur sources. Some properties of the enzyme acting on the glycerate ester are recorded.

A number of studies have been made in these laboratories on glycosulphatase (sugar sulphate sulphonylhydrolase, EC 3.1.6.3), an enzyme that is of potential importance as a tool for the determination of the structures of naturally occurring sulphated polysaccharides. Particularly active preparations of the enzyme have been obtained from molluscs, but such preparations exhibited a broad substrate specificity (Lloyd, 1966) and, even when considerably purified, still contained a number of other sulphatases as well as enzymes capable of degrading carbohydrates (Dodgson, 1961; Lloyd, 1966). Consequently some attention has been paid to the possibility of inducing the formation of specific glycosulphatases in micro-organisms. For example, a bacterium tentatively assigned to the genus *Lactobacillus* was found to possess desulphating activity towards potassium D-glucose 6-O-sulphate only when grown on this ester (Large, Lloyd & Dodgson, 1964). Similarly, a glycosulphatase of relatively high specificity was induced when the mould *Trichoderma viride* was cultured with the ester serving as sole carbon and sulphur source (Lloyd, Large, Davies, Olavesen & Dodgson, 1968). In both cases a number of factors complicated attempts to define the parameters for maximum

production of this enzyme by the organisms, and consequently other bacteria capable of growing on D-glucose 6-O-sulphate have been sought as more suitable sources of glycosulphatase.

During studies on the ability of various species of *Pseudomonas* to metabolize D-glucose 6-O-sulphate it became apparent that sulphur utilization was not a consequence of the action of a glycosulphatase as had been demonstrated for *T. viride*. The present paper reports on the utilization of D-glucose 6-O-sulphate by *Pseudomonas fluorescens* N.C.I.B. 8248 and describes the partial characterization of a novel desulphating enzyme system(s) that may be involved in sulphur utilization.

MATERIALS AND METHODS

Sulphate esters. Unlabelled potassium D-glucose 6-O-sulphate was prepared on a large scale by sulphonation of D-glucose with pyridine- SO_3 and isolated from the reaction mixture as described in the Appendix (Fitzgerald, Thomas, Tudball & Winterburn, 1971).

Potassium D-glucose 6-[^{35}S]-O-sulphate (specific radioactivity approx. 11.6 $\mu\text{Ci/mg}$) was initially supplied by Mr D. I. Bain and had been prepared by a method involving the use of heavy metals (cf. Lloyd *et al.* 1968). To eliminate any remaining traces of such metals the preparation was treated in the following manner. The ester (0.13 g) was dissolved in water and passed through a column (4.0 cm $\times$

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35.0 cm) of Dowex 50 (X8; H⁺ form; 100–200 mesh). The acid eluate was re-treated with regenerated resin in a similar manner. The eluate from this refractionation was adjusted to pH 9.0 with 0.5M-KOH and immediately re-adjusted to pH 7.4 with acetic acid. The potassium salt was precipitated by the addition of 5 vol. of ethanol (96%, w/v) and collected by centrifugation. The material was washed with ethanol followed by acetone and dried *in vacuo* at room temperature over CaCl₂. The ester was stored at -20°C as an aqueous 5 mM solution and was stable under these conditions for up to 12 months (cf. Lloyd, Balazs, Embery & Wusteman, 1966).

Dipotassium D-glucuronate 6-[³⁵S]-O-sulphate was prepared by the pH-controlled bromine oxidation of potassium D-glucose 6-[³⁵S]-O-sulphate, a method analogous to that described by Horecker (1957) for the preparation of 6-phosphogluconate. Potassium D-glucose 6-O-sulphate (0.5 g) was dissolved in 20 ml of an aqueous 5 mM solution of the corresponding ³⁵S-labelled ester, and bromine (0.5 ml) was added dropwise with constant stirring over a period of 30 min. During this period and for a further 3.5 h the pH was maintained between 4.9 and 5.8 by adding 0.5M-KOH. Subsequently the excess of bromine was removed by gassing the reaction mixture with N₂ for 60 min. The pH was then adjusted to pH 9.0 with 0.5M-KOH and then immediately readjusted to pH 7.4 with dilute acetic acid. The solution was concentrated to low bulk *in vacuo* at 30°C and the ester was precipitated by the dropwise addition of 3 vol. of ethanol (96%, v/v). Attempts to speed up the precipitation procedure led to the production of an oil. The precipitate was separated, washed with ethanol and dried *in vacuo* at room temperature over CaCl₂. The yield was 0.31 g and the specific radioactivity was approx. 0.14 μCi/mg. The preparation migrated as a single component on electrophoresis and on paper chromatography in six different solvent systems. The i.r. spectrum of the product was identical with that of unlabelled D-glucuronate 6-O-sulphate prepared by direct sulphonation of D-glucosylactone (Lloyd, Large, Tudball, Wusteman & Dodgson, 1965c). The preparation was stored as an aqueous 80 mM solution at -20°C and was stable for about 45 weeks, after which time traces of other ³⁵S-containing components could be detected.

Dipotassium D-glycerate 3-O-sulphate and dipotassium DL-glycerate 3-[³⁵S]-O-sulphate were prepared as described in the Appendix (Fitzgerald *et al.* 1971).

Culture conditions. *Pseudomonas fluorescens* (National Collection of Industrial Bacteria strain 8248) was stored as a stock culture on tryptic soy agar slants (Difco dehydrated) at 4°C. The adapted strain was maintained by periodic subculture at 37°C on solid synthetic media made by mixing at 45°C Difco agar (1.5 g/50 ml) and D-glucose 6-O-sulphate (1.6 g/50 ml), each dissolved in the standard medium.

The standard liquid medium contained carbon and sulphur in the form and concentration described for each experiment. The other components of this medium were as follows: KH₂PO₄ (3 g), K₂HPO₄ (7 g), NH₄Cl (1 g), MgCl₂·6H₂O (0.3 g), NaCl (1 g) and water (1 litre). When D-glucose 6-O-sulphate was the carbon and sulphur source the medium was sterilized by Millipore filtration (0.22 μm). On other occasions sterilization was by autoclaving. The pH of the medium was 7.0. Cells were grown in shake culture on a Gallenkamp Orbital Incubator operating at

250 rev./min at a temperature and an approximate flask-to-culture volume ratio specified for each experiment. In each case the size of the inoculum was adjusted so that after inoculation the culture medium had an initial extinction (650 nm) of approx. 0.02 (Unicam SP. 600).

Preparation of cell extracts. Cells were grown at 37°C in 2-litre Erlenmeyer flasks each containing 500 ml of standard medium. Bacteria were collected by centrifuging, washed twice in 50 mM-tris-HCl buffer, pH 7.5, and disrupted with an MSE Ultrasonic Disintegrator (100 W) at full power for 10 min. The temperature of the suspension during treatment was maintained below 10°C. Cell debris was removed by centrifuging and the clear supernatants were dialysed for 9 h against 5 mM-tris-HCl buffer, pH 7.5 (six changes), and stored at -20°C.

Determination of enzyme activity. An electrophoretic assay procedure based on that described by Dodgson & Tudball (1961) was used to determine desulphating activity of cell extracts towards radioactively labelled substrates. Assay mixtures contained sulphate ester (50 μl), dialysed extract (50 μl) and buffer (25 μl) in the final concentrations and pH described for each study. The procedure differed from the published one only with respect to the electrophoresis, which was carried out for 1.5 h at 22 V/cm in 50 mM-barium acetate-acetic acid buffer, pH 4.5. Under these circumstances liberated inorganic ³⁵SO₄²⁻ ions remained at the origin. One enzyme unit is defined as the amount of enzyme necessary to liberate 1 μmol of inorganic ³⁵SO₄²⁻ ion/h.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1961) was employed to determine soluble protein in dialysed cell extracts. Dry bovine serum albumin was the standard.

Measurement of D-glucose 6-O-sulphate concentration in the culture medium. The disappearance of D-glucose 6-O-sulphate (as hexose) during growth was followed by assaying culture fluid samples by the phenol-H₂SO₄ method for carbohydrate (cf. Dubois, Gilles, Hamilton, Rebers & Smith, 1956) as modified by Montgomery (1961). Samples were first clarified by centrifuging and filtering through a Millipore filter (0.22 μm). The control consisted of a sample of the standard medium without the sulphate ester. The calibration curve (prepared from the ester) was linear in the range 0–160 μg, the colour yield being about 56% of that obtained with D-glucose on an equimolar basis. The phenol-H₂SO₄ reagent did not react with D-glucuronate 6-O-sulphate or with D-glycerate 3-O-sulphate, in agreement with the results of Montgomery (1961) on the general specificity of this reagent. The method does not distinguish between D-glucose and its 6-O-sulphate ester but no sulphatase capable of producing the former from the latter appears in *P. fluorescens* (see later) and it is therefore assumed that only intact sulphate ester is measured under these circumstances. As a further check on the validity of the method the disappearance of ester was also followed by including (in small-scale experiments) D-glucose 6-[³⁵S]-O-sulphate in the standard medium and scanning electrophoretograms of culture samples as described below. The results obtained by the two methods were in good agreement.

Electrophoresis and chromatography. Homogeneity of sulphate esters and identity of intermediates was checked by paper electrophoresis (Whatman no. 1 paper, 0.1M-sodium acetate-acetic acid buffer, pH 4.5, at 22 V/cm for

1.5h) and by descending chromatography at 18°C on Whatman no. 1 paper in sealed pre-equilibrated tanks lined with the same paper. The following solvents were employed: solvent 1, butan-1-ol-acetic acid-water (4:1:2, by vol.); solvent 2, ethyl acetate-acetic acid-water (3:3:1, by vol.); solvent 3, ethanol-methanol-water (9:9:2, by vol.); solvent 4, 2-methoxyethanol-pyridine-acetic acid-water (8:4:1:1, by vol.); solvent 5, 2-methoxyethanol-ethylmethylketone-3M-NH₃ (7:2:3, by vol.); solvent 6, ethyl acetate-formamide-pyridine (6:4:1, by vol.).

Localization and quantitative determination of radioactive areas on papers was as described by Dodgson, Lloyd & Tudball (1961).

EXPERIMENTAL AND RESULTS

Adaptation of P. fluorescens for growth on D-glucose 6-O-sulphate. The standard medium containing D-glucose 6-O-sulphate (0.8%, w/v) as sole carbon and sulphur source was inoculated with a washed 16h tryptic soy broth suspension. Bacteria were adapted at 30°C in 6ml of medium contained in 25ml Erlenmeyer flasks. Growth (turbidimetric measurement at 650nm) began after an initial lag period of 78h and the culture extinction was 1.8 at the termination of growth (90h after incubation). Subsequent subcultures, with the same standard medium and unwashed inocula taken initially from the primary culture, resulted in a stable lag period of between 21 and 24h. There was no further significant alteration in either the maximum growth titre or the duration of the phase of linear growth. Loss of the capacity for pigment formation (visible and fluorescent) accompanied growth under these

conditions and this capacity was not retained by several subcultures in the standard medium containing D-glucose (1.0%, w/v) and potassium sulphate (1.0%, w/v) as the sole carbon and sulphur sources. The temperature required for maximum growth differed between the adapted strain and cells taken directly from the tryptic soy agar slant. Whereas the adapted strain grew well at 37°C in the standard medium containing D-glucose and potassium sulphate or D-glucose 6-O-sulphate, the parent strain grew poorly at this temperature. Preliminary examination of colonial morphology on tryptic soy agar plates revealed no visible difference between these strains and no further attempt was made to characterize the adapted strain.

Influence of D-glucose 6-O-sulphate concentration and temperature. The maximum culture extinction attained at the termination of growth of the adapted strain at 30°C increased with increasing concentration of D-glucose 6-O-sulphate (Fig. 1b). Between the substrate concentrations 0.4 and 1.6% (w/v) this increase was linear (Fig. 1a).

A concentration of 1.6% substrate was chosen for subsequent study. Temperature had relatively little influence on maximum culture extinction at this concentration, although the duration and slope of the phase of linear growth was affected (Fig. 2a). Linear growth is here defined as that phase of growth in which there is an approximately constant increase in culture extinction with time. When the slope was employed to assess growth a linear response to increasing temperature (up to 38°C) was observed (Fig. 2b).

Growth on D-glucose 6-O-sulphate as the sole

Fig. 1. Influence of D-glucose 6-O-sulphate concentration on the growth of adapted *P. fluorescens* at 30°C when the sulphate ester is serving as sole carbon and sulphur source (a). In (b) the individual growth curves for each concentration of ester are recorded. These concentrations, reading from the top downwards, were as follows (w/v): 2.8, 2.0, 1.6, 1.2, 0.8 and 0.4%. For the sake of clarity some of the experimental points have been omitted from (b).

Fig. 2. Growth of adapted *P. fluorescens* on 1.6% (w/v) D-glucose 6-O-sulphate at various temperatures. The values quoted in (b) are those derived from the slopes of those portions of the curves in (a) that are shown as unbroken lines.

carbon and sulphur source was accompanied by disappearance of the ester from the standard medium, indicating that growth was dependent on ester utilization. Instances where this may not necessarily be the case have been documented and have been related to substrate impurity (see, e.g., Payza & Korn, 1956). Fig. 3(a) correlates the inverse relationship between growth and the utilization of D-glucose 6-O-sulphate when the adapted strain was cultured on 1.6% (w/v) ester at 37°C. During the linear phase of growth the ester disappeared at a rate of 2.4 mg/h per ml of culture fluid until approx. 12% remained at the termination of growth. It has already been stated that temperature influenced growth rate rather than maximum growth titre (see Fig. 2a). An analogous temperature effect on substrate utilization was observed when adapted bacteria were cultured at 30°C on 1.6% (w/v) D-glucose 6-O-sulphate. At this temperature the rate of disappearance of the ester was considerably decreased (1.0 mg/h per ml of culture fluid) although approximately the same amount of ester (14.5%) remained at the termination of growth. Fig. 3(a) further shows that little or no growth occurred when the adapted strain was cultured in the standard medium in the absence of D-glucose 6-O-sulphate.

Occurrence and identification of intermediates in cultures. The adapted organism was cultured at 37°C in 20 ml of standard medium containing

Fig. 3. (a) Relationship between culture extinction and utilization of D-glucose 6-O-sulphate during growth of *P. fluorescens* at 37°C on 1.6% (w/v) D-glucose 6-O-sulphate. ○, Culture extinction; ●, utilization of D-glucose 6-O-sulphate; □, culture extinction of the inoculated standard medium in the absence of D-glucose 6-O-sulphate. (b) Relationship between culture age and desulphating activity in *P. fluorescens* grown as for (a). ■, Desulphation of DL-glycerate 3[³⁵S]-O-sulphate; △, D-gluconate 6[³⁵S]-O-sulphate; ▲, D-glucose 6[³⁵S]-O-sulphate. Extracts were incubated with 20 mM substrate at 30°C for 4 h in 50 mM-glycylglycine-NaOH buffer, pH 7.5. ----, Culture pH.

Fig. 4. Liberation of some [^{35}S]sulphated intermediates into the culture fluid during growth of *P. fluorescens* on D-glucose 6[^{35}S]-O-sulphate as the sole carbon and sulphur source. ●, Culture extinction; ○, D-glycerate 3[^{35}S]-O-sulphate (metabolite C); □, D-gluconate 6[^{35}S]-O-sulphate (metabolite B); △, unidentified [^{35}S]sulphated intermediate (metabolite A).

60 mM-D-glucose 6[^{35}S]-O-sulphate (mixture of 55 mM unlabelled and 5 mM labelled) as sole carbon and sulphur source. Paper electrophoresis of cell-free culture fluid samples taken at time-intervals during growth revealed the progressive release of three ^{35}S -labelled intermediates (Fig. 4). At the termination of growth about 13% of the radioactivity had disappeared from the medium, 50% was present as starting material and the three metabolites made up the remainder. Metabolite A (electrophoretic mobility relative to inorganic SO_4^{2-} ions 0.80) occurred to the extent of 1% of the total radioactivity of the medium at the termination of growth and has not yet been identified. Metabolite B (4% yield at the termination of growth) had an electrophoretic mobility (relative to inorganic SO_4^{2-} ions 0.74) and chromatographic mobilities in the six different solvents listed in the Materials and Methods section that were identical with those of authentic D-gluconate 6[^{35}S]-O-sulphate. This compound has previously been recognized in the culture fluid when *T. viride* was grown on D-glucose 6[^{35}S]-O-sulphate (Lloyd *et al.* 1965a,b,c).

Metabolite C was present in considerable amounts in the culture fluid (32% of total radioactivity of the medium) at the termination of growth. The fluid was clarified by centrifugation and subsequent filtration through a Millipore filter (0.22 μm). The filtrate was freeze-dried and the material dissolved in 0.2 ml of water and subjected to thick-layer chromatography on Silica Gel G with solvent 2. The major metabolite (R_F 0.35) separated from metabolites A (R_F 0.08) and B (R_F 0.04), but was not entirely resolved from D-glucose 6[^{35}S]-O-sulphate

(R_F 0.2). The appropriate area of the thick-layer was scraped off and suspended in aq. 50% (v/v) acetone (approx. 0.5 g of dry gel/ml). The suspension was filtered through Whatman no. 1 filter paper, and material retained by the paper was air-dried and re-extracted twice with aqueous acetone as before. The pooled filtrates were concentrated to low bulk and resubjected to thick-layer chromatography with solvent 6. Metabolite C (R_F 0.28) was completely separated from the contaminating traces of D-glucose 6[^{35}S]-O-sulphate (R_F 0.89). The purified metabolite was eluted from the gel as before and the solution was concentrated to approx. 0.4 ml by freeze-drying. Radioactive material in this preparation was homogeneous on electrophoresis and chromatography. Electrophoretic mobility (relative to inorganic $^{35}\text{SO}_4^{2-}$ ions 0.86) and chromatographic mobilities in solvents 1–6 were identical with those exhibited by an authentic sample of DL-glycerate 3[^{35}S]-O-sulphate. Further confirmation of the identity of metabolite C came from isotope-dilution analysis, in which the labelled intermediate was mixed with an excess of unlabelled D-glycerate 3-O-sulphate and recrystallized. Material of constant specific radioactivity was obtained through four successive recrystallizations.

Desulphation of certain sulphate esters by P. fluorescens. Extracts were prepared from samples of adapted cells taken at time-intervals during growth at 37°C on D-glucose 6-O-sulphate (1.6%, w/v) and examined for their ability to desulphate D-gluconate 6[^{35}S]-O-sulphate, DL-glycerate 3[^{35}S]-O-sulphate and D-glucose 6[^{35}S]-O-sulphate. Fig. 3(b) shows that maximum desulphating activity towards the sulphate esters of gluconate and glycerate occurred just before the onset of linear growth and linear D-glucose 6-O-sulphate utilization by the organism. All extracts failed to liberate inorganic $^{35}\text{SO}_4^{2-}$ ions from D-glucose 6[^{35}S]-O-sulphate under a variety of experimental conditions, including those that were optimum for the assay of the glycosulphatase from *T. viride* (Lloyd *et al.* 1968).

Extracts made from adapted cells taken 25 h after inoculation showed the highest specific activity towards both D-gluconate 6[^{35}S]-O-sulphate (0.12 enzyme unit/mg of protein) and DL-glycerate 3[^{35}S]-O-sulphate (0.61 enzyme unit/mg of protein), and such extracts were employed for subsequent studies designed primarily to confirm the enzymic nature of the desulphation process. Activity towards DL-glycerate 3[^{35}S]-O-sulphate was characterized by sharp temperature, pH and substrate concentration optima (Figs. 5a, 5b and 5c respectively). The substrate curve was of particular note in view of its atypical shape. To eliminate the possibility of artifact the influence of time was examined at the optimum concentration of the

Fig. 5. (a) Effect of temperature on desulphating activity of extracts of adapted *P. fluorescens* cells towards 1 mM-DL-glycerate 3[35 S]-O-sulphate in 50 mM-glycylglycine-NaOH buffer, pH 6.5. (b) Effect of pH on desulphating activity of extracts when incubated at 34°C with 1 mM-DL-glycerate 3[35 S]-O-sulphate in the following 50 mM buffers: sodium acetate-acetic acid, pH 4-6; glycylglycine-NaOH, pH 6.5-8.5. (c) Substrate concentration-activity curve for extracts acting on DL-glycerate 3[35 S]-O-sulphate at 34°C in 50 mM-glycylglycine-NaOH buffer, pH 6.5. In all cases substrate-enzyme mixtures were incubated for 2 h and liberated inorganic $^{35}\text{SO}_4^{2-}$ was measured. All extracts were prepared from adapted cells grown for 25 h at 37°C in 1.6% (w/v) D-glucose 6-O-sulphate.

Fig. 6. Time-activity curves for the desulphation of various concentrations of DL-glycerate 3[35 S]-O-sulphate by an extract of adapted cells of *P. fluorescens*. $\circ$, 0.4 mM-Sulphate ester; Δ , 0.9 mM-sulphate ester; $\square$, 2.0 mM-sulphate ester. Incubation was at 34°C in 50 mM-glycylglycine-NaOH buffer, pH 6.5.

ester and at concentrations on either side of the optimum that yielded similar activities (Fig. 6). In all cases activity was approximately linear with respect to time up to 4 h.

Desulphation of both DL-glycerate 3[35 S]-O-sulphate and D-gluconate 6[35 S]-O-sulphate was abolished in boiled extracts, and exhaustive dialysis of extracts against 50 mM-glycylglycine-NaOH

buffer, pH 7.5, had no effect on activity towards either substrate. The influence of various potential inhibitors and activators on enzyme activity towards the glycerate ester is recorded in Table 1.

Occurrence of desulphation activity in *P. fluorescens* grown under other conditions. Low amounts of desulphating activity towards D-gluconate 6[35 S]-O-sulphate (specific activity 0.017 enzyme unit/mg of protein) were present in adapted cells, which were subsequently grown at 37°C in the standard medium containing D-glucose (1.0%, w/v) as carbon source and potassium sulphate (1.0%, w/v) as sulphur source. Some activity was also present when the parent *Pseudomonas* strain was cultured under the same conditions (specific activity 0.012 enzyme unit/mg of protein; specific activities recorded were the highest found in culture age-enzyme activity experiments and assay conditions were as stated in the legend to Fig. 3b). Desulphation of DL-glycerate 3[35 S]-O-sulphate and D-glucose 6[35 S]-O-sulphate was never detected under these circumstances, with optimum assay conditions for the desulphation of each ester (cf. Fig. 5 and Lloyd *et al.* 1968).

DISCUSSION

The results of the present study (see also Fitzgerald & Dodgson, 1970) at first sight suggest that the initial utilization of D-glucose 6-O-sulphate by *P. fluorescens* may occur via D-gluconate 6-O-sulphate and hence by means of an enzyme identical

Table 1. *Effect of certain compounds on the desulphation of DL-glycerate 3[³⁵S]-O-sulphate by P. fluorescens*

An extract of adapted cells was employed as enzyme source and incubation was for 2 h at 34°C in the presence of 0.9 mM-sulphate ester and 50 mM-glycylglycine-NaOH buffer, pH 6.5.

Compound(s)	Concn. (mM)	Enzyme activity (%)
No addition	—	100.0
NaF	20	108.0
EDTA (disodium salt)	20	45.5
MgCl ₂	20	77.7
MnCl ₂	20	94.5
DL-Glyceric acid (calcium salt)	20	56.9
Na ₂ SO ₄	20	103.3
Sodium borohydride	5	0.0
Na ₂ HPO ₄ -NaH ₂ PO ₄ buffer (pH 6.5)	50	11.1

with or analogous to D-glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49). Lloyd *et al.* (1965b) have previously shown that D-glucose 6-O-sulphate can be converted into the corresponding gluconate sulphate by purified yeast D-glucose 6-phosphate dehydrogenase, although at a rate of only about 2% of that exhibited by the enzyme towards its natural substrate. However, although the existence of this type of enzyme in *P. fluorescens* has previously been noted (Wood & Schwerdt, 1953), preliminary experiments with strain N.C.I.B. 8284 indicate that there is no requirement for NADP⁺ for the conversion of D-glucose 6-O-sulphate into the corresponding gluconate sulphate. There are further indications that the system responsible for this transformation in strain N.C.I.B. 8248 may be analogous to the glucose dehydrogenase [glucose-(acceptor) oxidoreductase, EC 1.1.99.a; see Barman (1969)] reported to be present in some members of the genus *Pseudomonas* (Hauge, 1966).

Subsequent utilization of D-gluconate 6-O-sulphate would presumably follow routes analogous to the hexose monophosphate or Entner-Doudoroff pathways. Much work remains to be done before the pathways of utilization are precisely defined, but note might be taken of the relatively unique occurrence of the Entner-Doudoroff pathway in pseudomonads (Enter & Doudoroff, 1952; De Ley, 1960, 1962). Moreover, preliminary experiments have already indicated the existence in extracts of adapted and unadapted cells of *P. fluorescens* of a potent gluconate dehydrogenase [D-gluconate-(acceptor) oxidoreductase, EC 1.1.99.3] and an enzyme system is present in adapted cells only that is capable of oxidizing D-gluconate 6-O-sulphate in an analogous fashion. Indeed the latter system has complicated efforts to define precisely the conditions required for maximum desulphating activity of extracts towards D-gluconate 6-O-sulphate and this particular difficulty has still not been resolved.

Operation of an Entner-Doudoroff-type pathway in the utilization of D-glucose 6-O-sulphate would be expected to yield one C₃ fragment (pyruvate) that would be available directly for energy purposes. There would also be reasonable grounds for theorizing that the remaining C₃ fragment could not be metabolized further than glycerate 3-O-sulphate and, of course, this particular ester appears in the culture medium in large quantities. Further transformation of this compound by a mechanism analogous to that operating with phosphoglycerate phosphomutase in the conversion of glycerate 3-phosphate into the corresponding 2-isomer would appear to present insuperable problems (cf. Pizer, 1962). Utilization of the fragment could occur after the intervention of a desulphating enzyme system, but, judged from the quantitative results obtained in the present work, such an intervention occurs on a relatively small scale, sufficient to supply only the sulphur requirements for growth.

The present work has also established that the ability of a micro-organism to utilize D-glucose 6-O-sulphate does not necessarily imply the presence of a glycosulphatase (cf. the growth of *T. viride* on the ester; Lloyd *et al.* 1968). In the present instance it is not certain whether the situation simply reflects the unique major pathway for glucose utilization found in pseudomonads (Entner-Doudoroff pathway) or whether other factors may be involved. Ability to utilize D-glucose 6-O-sulphate as a carbon and sulphur source is certainly not a characteristic feature of the genus *Pseudomonas* and the indications from preliminary studies are that this capacity is restricted to certain strains of *P. fluorescens*.

Because a glycosulphatase could not be detected under either adapted or unadapted conditions of culture it was necessary to consider other esters as possible sources of sulphur for growth. It has previously been reported (Fitzgerald & Dodgson, 1970) that the sulphate esters of gluconate and glycerate can supply sulphur for growth of the unadapted parent strain and this suggested that

they were not necessarily end products of sulphur metabolism in this bacterium. The present finding that the esters can serve as substrates for the desulphating activity of extracts of adapted *P. fluorescens* cells further substantiates this view and suggests that either one or both may serve as sources of sulphur *in vivo* during growth on D-glucose 6-O-sulphate. However, other sulphated intermediates on the pathway to D-glycerate 3-O-sulphate could also represent the substrate *in vivo* for a desulphating enzyme of broad specificity.

The coincidence of the enzyme specific-activity profiles towards the sulphate esters of gluconate and glycerate respectively, in relationship to culture age (see Fig. 3b), suggests that a single enzyme with dual specificity is responsible. However, other known carbohydrate sulphatases of microbial origin [e.g. the glycosulphatase of *T. viride* (Lloyd *et al.* 1968) and the two chondrosulphatases of *Proteus vulgaris* (Yamagata, Kawamura & Suzuki, 1966)] have shown relatively high substrate specificity. Further, the fact that extracts of adapted cells grown on glucose and potassium sulphate show desulphating activity only towards D-gluconate 6-O-sulphate suggests two distinct enzyme systems. No attempt has yet been made to test this possibility further.

Apart from the novel substrate requirement for desulphating activity (cf. Dodgson & Spencer, 1957) in extracts of adapted *P. fluorescens*, the way in which the desulphating enzyme(s) appeared during growth of the organism (see Fig. 3b) was unusual and indicative of an enzyme(s) of the rate type I variety according to the classification of microbial enzymes suggested by Gale (1943, 1952). The observation that desulphating activity toward DL-glycerate 3-O-sulphate occurred only under conditions in which the ester was present as an intermediate in the degradation of D-glucose 6-O-sulphate (Fitzgerald & Dodgson, 1970) suggests that the responsible enzyme may be induced.

It cannot yet be assumed that the enzyme system(s) responsible for the desulphation of the glycerate or gluconate esters is a simple hydrolase (sulphatase). It has previously been emphasized (Dodgson *et al.* 1961) that sulphate liberation does not necessarily imply the action of a sulphatase (see, e.g., the enzymic release of sulphate from L-serine O-sulphate; Thomas & Tudball, 1967; Tudball, Thomas & Fowler, 1969). In the present instance preliminary efforts to establish the exact mechanism of the desulphation process have been hindered by the crude nature of the enzyme extracts (cf. Dodgson & Tudball, 1961) and by the ready utilization of glycerate and gluconate by *P. fluorescens*. Further clarification of the situation must await purification of the enzyme(s).

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