The Glycosulphatase of *Trichoderma viride*

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The growth of the mould *Trichoderma viride* on a defined medium containing either potassium D-glucose 6-O-sulphate or potassium D-galactose 6-O-sulphate as sole sources of both carbon and sulphur is marked by the production of an enzyme system capable of liberating inorganic SO_4^{2-} ions from either of the sulphate esters. The enzyme is not produced when the organism is grown with glucose (or galactose) and potassium sulphate or with glucose and methionine as sole sources of carbon and sulphur. Experimental conditions are described whereby inorganic SO_4^{2-} ions liberated from potassium glucose 6-O-sulphate by the growing mould appear in the culture medium after a constant lag period of 21–24 hr. The enzyme has been shown to be a simple glycosulphatase that is active towards the 6-O-sulphate esters of D-glucose and D-galactose but not towards potassium glucose 3-O-sulphate. The properties of the crude glycosulphatase show the enzyme to be appreciably different from analogous molluscan enzymes that can degrade monosaccharide sulphate esters.

The induction, repression and properties of bacterial and fungal arylsulphatase enzymes (aryl-sulphate sulphohydrolases, EC 3.1.6.1) have formed the subject of several studies (see, e.g., Harada, 1957, 1964; Harada & Spencer, 1962, 1964; Harada & Kamogawa, 1963; Rammner, Grado & Fowler, 1964). In contrast, the related microbial glycosulphatase enzymes (sugar sulphate sulphohydrolases, EC 3.1.6.3) have received relatively little attention in spite of the potential importance of such enzymes as 'tools' in the determination of the structure of naturally occurring sulphated polysaccharides.

Previous reports from these Laboratories (Lloyd, 1961, 1962a; Lloyd, Large, James & Dodgson, 1964) showed that micro-organisms were responsible for the liberation of inorganic $^{35}\text{SO}_4^{2-}$ ions that occurred when rat faecal preparations were incubated with the 6-[^{35}S]-O-sulphate esters of hexoses and N-acetylhexosamines. The micro-organism responsible for the desulphation of one of these esters, potassium D-glucose 6-[^{35}S]-O-sulphate, produced the desulphating enzyme only when grown in a medium containing the ester (Large, Lloyd & Dodgson, 1964). Unfortunately, the organism, which was tentatively assigned to the genus

Lactobacillus, had complex growth requirements and was not very suitable as a potential source of carbohydrate sulphatases. During these studies Large *et al.* (1964) also observed that the growth of the mould *Trichoderma viride* in a mineral salt solution containing potassium D-glucose 6-[^{35}S]-O-sulphate as the sole source of carbon and sulphur was accompanied by liberation of inorganic $^{35}\text{SO}_4^{2-}$ ions into the growth medium (cf. Yamashina, 1951). The present paper extends these observations and describes a glycosulphatase enzyme that is responsible for the phenomenon.

MATERIALS AND METHODS

Sulphate esters. Crude preparations of the barium salt of D-glucose 6-O-sulphate were obtained by either the chlorosulphonic acid method of Lloyd (1962b) or the pyridine- SO_3 method of Guiseley & Ruoff (1961). The crude material was then fractionated (in lg. quantities dissolved in the minimum volume of water) on a Dowex 1 (X8; Cl^- form; 200–400 mesh) anion-exchange resin column (2.5 cm. $\times$ 35.0 cm.) (cf. Meezan, Olavesen & Davidson, 1964). Unchanged D-glucose was eluted with water and sulphated materials were resolved into three fractions (detected with the anthrone reagent of Siefert, Dayton, Novio & Muntwyler, 1950) by gradient elution with 2l. of m-LiCl-0.1N-HCl in a reservoir and 670 ml. of water in a mixing chamber, with a flow rate of 0.5 ml./min. The first of these fractions contained D-glucose 6-O-sulphate and was adjusted to pH 6–7 with N-LiOH before concentrating to low bulk *in vacuo* at 35°. The concentrate was desalted by passage through a column (2.5 cm. $\times$ 40 cm.) of Dowex 50 (X8; H^+ form; 100–200 mesh) cation-exchange resin and the acid eluate was neutralized by vigorous stirring with solid Ag_2CO_3 . The

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suspension was clarified by centrifuging and again passed through a Dowex 50 column as before. The acid eluate was neutralized with 0.5N-KOH and its volume reduced to about 3 ml. *in vacuo* at 35° before being filtered through a Millipore filter (0.22 μ) to remove traces of insoluble silver salts. The clear filtrate was evaporated to a thin syrup and crystallized from aqueous ethanol at 0°. The crystalline potassium D-glucose 6-O-sulphate showed properties identical with those described by Guiseley & Ruoff (1961).

Potassium D-glucose 6-[^{35}S]-O-sulphate was prepared by the Lloyd (1962b) method, starting with 0.5 g. of D-glucose and 0.2 ml. of chloro[^{35}S]sulphonic acid (0.3 g., specific activity 5.9 mc/m-mole), and was purified as described above. The final product (0.6 g.) was stored as a dilute (60 mM) aqueous solution at -10° to minimize degradation by self-irradiation (cf. Lloyd, Balazs, Embury & Wusteman, 1965).

Potassium D-glucose 3-O-sulphate was prepared by the method of Dodgson & Spencer (1954) and potassium D-galactose 6-[^{35}S]-O-sulphate by the method of Lloyd (1962b). Choline sulphate was synthesized according to the directions of Schmidt & Wagner (1904).

Growth media. Cultures of *T. viride* were maintained by periodic subculture on nutrient-agar plates incubated at 18°, and on corn-meal-agar when prolonged periods of storage were required.

Liquid growth medium was prepared by mixing equal volumes of two sterile solutions. One of these was a basal solution, each litre of which contained KH_2PO_4 (1 g.), K_2HPO_4 (0.5 g.), NaNO_3 (3 g.), MgCl_2 (0.2 g.) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g.), plus 1 ml. of a trace-element solution, each litre of which contained FeCl_3 (0.1 g.), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.3 g.), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.072 g.), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.04 g.) and ZnCl_2 (4.2 g.). The other solution was an aqueous one containing various carbon and sulphur sources. The basal solution was adjusted to pH 6.8-7.0 (with dilute HCl or NaOH as appropriate) before being autoclaved. The second solution was sterilized by Millipore filtration.

Determination of inorganic $^{35}\text{SO}_4^{2-}$ ion liberated by growing cultures. A variety of practical difficulties made it impossible to measure the activity of the glycosulphatase enzyme during the growth phase. It was therefore necessary to take the appearance in the culture medium of inorganic $^{35}\text{SO}_4^{2-}$ ions liberated from either D-glucose 6-[^{35}S]-O-sulphate or D-galactose 6-[^{35}S]-O-sulphate as an indication of the activity of the enzyme system. Samples (5-10 μl .) were removed under aseptic conditions and submitted to electrophoresis on Whatman no. 1 paper in the presence of 0.1M-ammonium acetate-acetic acid buffer, pH 4.0, for 2 hr. at a potential gradient of 12 v/cm. (cf. Lloyd, 1960). Radioactive areas on the dried electrophoretograms were determined with a Packard 7200 Radiochromatogram Scanner and the inorganic $^{35}\text{SO}_4^{2-}$ ion content of the medium (calculated as a percentage of the total radioactivity present in the medium) was determined by measuring the areas under the appropriate peaks on the recording chart (cf. Dodgson, Lloyd & Tudball, 1961).

Assay of enzyme activity of cell-free extracts. Glycosulphatase activity of cell-free extracts of *T. viride* was assayed under a variety of conditions with the potassium salts of D-glucose 6-O-sulphate, D-glucose 6-[^{35}S]-O-sulphate, D-glucose 3-O-sulphate or D-galactose 6-O-sulphate, each dissolved in 0.04M-tris-HCl buffer at the appropriate pH.

Incubation mixtures contained 0.15 ml. of the appropriate buffered substrate solution together with 0.15 ml. of a similarly buffered cell-free extract of *T. viride* mycelium prepared as described below. After incubation at 28° for 60 min. determinations were carried out for inorganic SO_4^{2-} ions, inorganic $^{35}\text{SO}_4^{2-}$ ions, D-glucose or D-galactose, depending on the circumstances and on the nature of the substrate employed. Suitable control determinations were made in which substrate and *T. viride* extract were incubated separately and mixed at the end of the incubation mixture.

(a) Determination of liberated inorganic SO_4^{2-} ions. The turbidimetric method (method B) of Dodgson (1961a) was employed for determination of liberated inorganic SO_4^{2-} ions from unlabelled substrates; inorganic $^{35}\text{SO}_4^{2-}$ ions liberated from labelled substrates were determined by the electrophoretic method described above.

(b) Determination of liberated D-glucose. The glucose oxidase-peroxidase-o-dianisidine method (Huggett & Nixon, 1957) was adapted for determination of D-glucose liberated from either potassium D-glucose 3-O-sulphate or potassium D-glucose 6-O-sulphate by the action of the glycosulphatase. Purified glucose oxidase (GOD-II) and horseradish peroxidase were commercial products (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany).

After incubation of *T. viride* extract-substrate mixtures, 3 ml. of the glucose oxidase-peroxidase-o-dianisidine reagent of Dahlqvist (1961) was added. The whole was then incubated for a further 60 min. at 37° and the intensity of the resultant colour was measured on the Unicam SP.600 spectrophotometer at 436 m μ (see Hansen, 1962). Standards containing 0-60 μg . of D-glucose were run simultaneously with each test determination. Preliminary experiments revealed (a) that glucose oxidase was without action on either D-glucose 6-O-sulphate or D-glucose 3-O-sulphate, (b) that neither ester inhibited activity of glucose oxidase towards D-glucose, and (c) that the dilution effect and change in pH achieved by the addition of the glucose oxidase reagent effectively prevented the further action of the *T. viride* extract.

(c) Determination of liberated D-galactose. This was achieved by coupling the action on D-galactose of a preparation of the D-galactose oxidase of *Polyporus circinatus* (see Avigad, Amaral, Asensio & Horecker, 1962) with peroxidase and o-dianisidine in a manner analogous to the determination of D-glucose.

Galactose oxidase (5 mg.), horseradish peroxidase (0.25 mg.) and 1 ml. of ethanol containing 10 mg. of o-dianisidine were added successively to 50 ml. of 0.5M-tris-HCl buffer, pH 7.0, and the whole was mixed thoroughly. A portion (2 ml.) of this reagent was added to 1.5 ml. of the D-galactose 6-O-sulphate-*T. viride* extract incubation mixture. After incubation for 60 min. at 37° the reaction was stopped by adding 0.1 ml. of 2N-HCl and the intensity of colour produced was measured at 436 m μ . Measurements for a calibration curve (in the range of 0-150 μg . of D-galactose) were made at the same time as the test determinations. Preliminary experiments revealed that D-galactose 6-O-sulphate did not interfere with the determination procedure either by acting as a substrate for galactose oxidase or by inhibiting the action of this enzyme.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to determine soluble protein in *T. viride* extracts.

EXPERIMENTAL AND RESULTS

Appearance of glycosulphatase activity in T. viride

In initial small-scale studies 3ml. of liquid medium containing potassium D-glucose 6[³⁵S]-O-sulphate (final concentration 30mM) as sole source of carbon and sulphur was inoculated with *T. viride* at the stage of sporulation. Cultures were grown in 15cm. x 1.5cm. Pyrex tubes at 22° without aeration. Inorganic ³⁵SO₄²⁻ ions were liberated into the culture medium during incubation, but there was an initial lag period of at least 20hr. (Fig. 1, curve A). In a number of experiments carried out under these conditions the lag period varied considerably, and on a few occasions growth occurred without inorganic ³⁵SO₄²⁻ ions appearing in the medium even after incubation for 10 days. In all experiments where inorganic ³⁵SO₄²⁻ ions appeared in the medium maximum concentrations were always attained at approx. 70hr. in spite of the variable lag period. Similar results were obtained when potassium D-galactose 6[³⁵S]-O-sulphate was used as sole carbon and sulphur source. At no time during any of the experiments could D-glucose (or D-galactose) be detected (cf. Trevelyan, Procter & Harrison, 1950) on electrophoretograms of samples of the growth media.

Liquid medium containing either potassium D-glucose 6[³⁵S]-O-sulphate or the corresponding galactose analogue was inoculated with a washed inoculum of *T. viride* previously grown on either compound and shown to possess glycosulphatase activity. Under these conditions there was no lag in the appearance of inorganic ³⁵SO₄²⁻ ions in the culture medium and a maximum concentration was again reached after about 70hr. (Fig. 1, curves B and C).

These preliminary experiments indicated that an enzyme able to cause the desulphation of mono-sulphate esters of D-glucose and D-galactose was produced when *T. viride* was grown in the presence of potassium D-glucose 6[³⁵S]-O-sulphate and suggested that the enzyme was not a constitutive one. Confirmation of this last point was obtained as follows. *T. viride* was grown on a large scale in three different media, one of which contained potassium glucose 6-O-sulphate (30mM) as sole carbon and sulphur source, another contained D-glucose (30mM) and potassium sulphate (30mM), and the third contained D-glucose (30mM) and DL-methionine (30mM). In all instances growth was at 22° with aeration (reciprocal shaker) in 1l. flat-bottomed flasks each containing 250ml. of medium. Mycelia were harvested after 10–14 days by centrifuging at 2° and washed twice by suspension in aq. 0.9% potassium chloride followed by centrifuging. The washed mycelium was frozen at –15° and ground in

Fig. 1. Liberation of inorganic ³⁵SO₄²⁻ ions from mono-saccharide 6[³⁵S]-O-sulphate esters during growth of *T. viride* in liquid medium. Experimental details are described in the text. Curve A (○), mycelium grown on either potassium D-glucose 6[³⁵S]-O-sulphate or potassium D-galactose 6[³⁵S]-O-sulphate; curve B (●), mycelium grown on potassium D-glucose 6[³⁵S]-O-sulphate and reinoculated into fresh medium containing the same [³⁵S]sulphate ester; curve C (□), mycelium grown on potassium D-galactose 6[³⁵S]-O-sulphate and reinoculated into fresh medium containing the same [³⁵S]sulphate ester; curve D (△), mycelium grown on potassium D-glucose 6[³⁵S]-O-sulphate under the standard conditions described (see the text) for the establishment of a constant lag period.

a mortar at –5° with one-fifth of its wet weight of water-washed powdered alumina. Acid- and water-washed Ballotini beads (solid glass, no. 12, diam. 170–180mμ) could be substituted for powdered alumina, but a large number of other solubilization techniques that were tried were unsuccessful. The resulting powder was extracted with 3 vol. of ice-cold 0.04M-tris-hydrochloric acid buffer, pH 7.9, and the extract was cleared by centrifuging. Extracts were assayed for glycosulphatase activity with potassium D-glucose 6-O-sulphate as substrate under optimum conditions (see below). Extracts of mycelia grown on potassium glucose 6-O-sulphate possessed glycosulphatase activity. In contrast, extracts of mycelia grown on D-glucose and potassium sulphate or on D-glucose and DL-methionine were completely devoid of the enzyme. In similar experiments it was shown that the enzyme was present in extracts of mycelia grown in the presence of potassium D-galactose 6-O-sulphate (30mM) as sole carbon and sulphur source, but was not present if a mixture of D-galactose (30mM) and potassium sulphate (30mM) was substituted. The presence of the 6-O-sulphate esters of D-glucose or D-galactose in the medium thus appears to be a prerequisite for the production of glycosulphatase.

Standardization of the lag period. A complex interplay of factors may influence the appearance of inorganic SO₄²⁻ ions in the culture medium; for example, the transport of materials into and out of the mould, the rate of aeration, the sulphur and

carbon reserves in the organism, the utilization of liberated inorganic SO_4^{2-} ions and so on. Inorganic $^{35}\text{SO}_4^{2-}$ ions are certainly used for synthetic purposes by the growing organism. Thus when *T. viride* was grown in medium containing D-glucose (0.05M) and inorganic $^{35}\text{SO}_4^{2-}$ ions of high specific activity ($\text{Na}_2^{35}\text{SO}_4$, 1.0mc/ml.) chromatography of mycelial extracts showed the presence of several radioactive components. The most prominent of these had the same chromatographic and electrophoretic properties as choline sulphate. This ester is known to act as an important reserve store of sulphur in a large number of fungi (Harada & Spencer, 1960).

Investigations of the effects of reserve sulphur stores and various other factors on the appearance and concentrations of inorganic $^{35}\text{SO}_4^{2-}$ ions in the growth medium are currently being investigated in these Laboratories, but it is now possible to define conditions under which the growth of *T. viride* is accompanied by a constant lag period with respect to the appearance of inorganic $^{35}\text{SO}_4^{2-}$ ions into the medium. The organism was first grown in liquid medium containing 5.5mM-D-glucose and 1.0mM-potassium sulphate without aeration. The mycelium was established in this medium for 7 days and was subcultured into fresh medium three times during this period. Mycelium was then inoculated into 3ml. of liquid medium containing potassium D-glucose 6- ^{35}S -O-sulphate (30mM). Growth was carried out in tapered centrifuge tubes (1.7cm. $\times$ 1.1cm.) modified with a side-arm adapter for inoculation and removal of samples and an inlet and an outlet for aeration with sterile air. The tubes were aerated in a water bath at 28°. Under these circumstances the lag period for appearance of inorganic $^{35}\text{SO}_4^{2-}$ ions in the medium was constant at 21–24hr. from experiment to experiment (total of 20 experiments). When the maximum concentrations in the medium were attained approx. 40% of the total radioactivity present in the medium was in

the form of inorganic $^{35}\text{SO}_4^{2-}$ ions (Fig. 1, curve D). This value was lower than that usually obtained under the conditions employed in the preliminary induction experiments. Glycosulphatase continued to be produced when either 30mM-D-glucose or 30mM-potassium sulphate (or both) was incorporated into the growth medium in addition to potassium D-glucose 6- ^{35}S -O-sulphate.

When spores of *T. viride* were inoculated into liquid medium containing 30mM-potassium D-glucose 6- ^{35}S -O-sulphate and grown under the conditions outlined above, the lag period for the appearance of inorganic $^{35}\text{SO}_4^{2-}$ ions in the medium was no longer constant. However, the concentrations obtained were consistently higher than those recorded when mycelium was used as inoculum.

Properties of the glycosulphatase

For these studies *T. viride* was grown in medium containing potassium D-glucose 6-O-sulphate according to the large-scale procedure described above. The mycelial extract obtained could be stored in the freeze-dried state for up to 3 months. It was shown that these extracts liberated hexose and inorganic SO_4^{2-} ions from potassium glucose 6-O-sulphate in approximately equimolar amounts (Table 1). Similar findings were made with potassium D-galactose 6-O-sulphate, though the quantitative agreement was less satisfactory in this case (Table 1). The enzyme is thus established as a simple sulphatase (sulphohydrolase) liberating hexose and inorganic SO_4^{2-} ions in equimolar amounts.

The optimum temperature for the enzymic reaction was 28° and activity decreased sharply at either side of this temperature (Fig. 2). The effects of substrate concentration and pH on enzyme activity were determined at 28° by following the liberation of D-glucose and inorganic SO_4^{2-} ions from potassium D-glucose 6-O-sulphate and of inorganic SO_4^{2-} ions from potassium D-galactose

Table 1. *Liberation of inorganic SO_4^{2-} ions, D-glucose and D-galactose during the hydrolysis of D-glucose 6-O-sulphate and D-galactose 6-O-sulphate by T. viride extracts*

The appropriate hexose 6-O-sulphate (20mM) was incubated with extracts of *T. viride* mycelium (grown on potassium D-glucose 6-O-sulphate) in 0.04M-tris-HCl buffer, pH 7.9, at 28°. Parallel determinations for D-glucose, D-galactose and inorganic SO_4^{2-} ions were made as described in the text.

	Soluble protein present in incubation mixture ($\mu\text{g.}$)	Incubation time (hr.)	Product formed in incubation mixture (μmole)		
			Inorganic SO_4^{2-} ions	D-Glucose	D-Galactose
Potassium D-glucose 6-O-sulphate	14.3	1	0.143	0.153	—
	28.5	1	0.261	0.234	—
Potassium D-galactose 6-O-sulphate	30.2	3	0.144	—	0.112
	60.3	3	0.219	—	0.178

Fig. 2. Effect of temperature on the activity of *T. viride* glycosulphatase toward potassium D-glucose 6-*O*-sulphate (20mM) in 0.04M-tris-HCl buffer, pH 7.9. Enzyme activity was determined by measuring D-glucose liberation.

6-*O*-sulphate (see Fig. 3). Under the optimum conditions there was a linear relationship between enzyme activity and enzyme concentration up to a concentration of 0.4mg. of soluble protein present in the incubation mixture. The time-enzyme activity curve was also linear. The enzyme preparation failed to liberate either D-glucose or inorganic SO_4^{2-} ions from potassium D-glucose 3-*O*-sulphate under a variety of different experimental conditions.

Table 2 shows the effect of various common enzyme activators and inhibitors on the activity of *T. viride* glycosulphatase towards potassium glucose 6-*O*-sulphate. With some of the compounds tested it was possible to measure both liberated D-glucose and inorganic SO_4^{2-} ions, but in other cases the added compounds interfered with the determination of either liberated inorganic SO_4^{2-} ions or D-glucose.

DISCUSSION

The studies reported here confirm the preliminary observations of Yamashina (1951) that moulds of

Fig. 3. (a) Substrate concentration-activity curves for *T. viride* glycosulphatase acting on potassium D-glucose 6-*O*-sulphate and potassium D-galactose 6-*O*-sulphate in 0.04M-tris-HCl buffer, pH 7.9. ●, Potassium D-glucose 6-*O*-sulphate degradation measured by inorganic SO_4^{2-} ion liberation; ■, potassium D-glucose 6-*O*-sulphate degradation measured by D-glucose liberation; ▲, potassium D-galactose 6-*O*-sulphate degradation measured by inorganic SO_4^{2-} ion liberation. (b) Effect of pH on the activity of *T. viride* glycosulphatase towards potassium D-glucose 6-*O*-sulphate (20mM) and potassium D-galactose 6-*O*-sulphate (20mM) in 0.04M-tris-HCl buffer. ●, Potassium D-glucose 6-*O*-sulphate degradation measured by inorganic SO_4^{2-} ion liberation; ■, potassium D-glucose 6-*O*-sulphate degradation measured by D-glucose liberation; ▲, potassium D-galactose 6-*O*-sulphate degradation measured by inorganic SO_4^{2-} ion liberation. In all cases incubation was for 60 min. at 28°.

Table 2. *Effect of some common enzyme inhibitors and activators on T. viride glycosulphatase*

Results are expressed as percentages of the activity of the enzyme in the standard assay in the absence of added activator or inhibitor. The added compound was adjusted to pH 7.9 before use. Assays were made with potassium D-glucose 6-O-sulphate (20 mM) as substrate and the liberation of D-glucose or inorganic SO_4^{2-} ions (or both) was determined.

Compound	Concn. (mM)	Enzyme activity (%)	
		Sulphate determination	Glucose determination
NaF	20	0	9
$\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$	20	82	85
$\text{Na}_4\text{P}_2\text{O}_7$	10	—	97
$\text{Na}_2\text{B}_4\text{O}_7$	10	—	13
Hydroxylamine	20	53	—
EDTA (disodium salt)	20	—	91
MgCl_2	20	92	94
MnCl_2	20	98	118

the genus *Trichoderma* can utilize D-glucose sulphate (presumed to be mainly the 6-O-sulphate) as a source of both carbon and sulphur for growth. In *T. viride* this process is accompanied by the production of an enzyme of the 'glycosulphatase' type that can degrade potassium D-glucose 6-O-sulphate to yield stoichiometric amounts of D-glucose and inorganic SO_4^{2-} ions (cf. Dodgson & Spencer, 1957).

Previous work on glycosulphatases has been mainly restricted to enzymes of molluscan origin (cf. Dodgson & Spencer, 1957), those from *Charonia lampas* and *Littorina littorea* having been studied most extensively. Though the properties of glycosulphatase from both of these sources are closely comparable, the glycosulphatase produced by *T. viride* has distinctively different characteristics. Thus the mould enzyme exhibits maximum activity at pH 7.8–7.9, whereas the enzymes from *Charonia* and *Littorina* both have pH optima in the region 5.2–5.9 (cf. Takahashi, 1960*a,b*; Dodgson, 1961*b*; Dodgson & Lloyd, 1961). Further, the hydrolysis of D-glucose 6-O-sulphate and of D-galactose 6-O-sulphate both proceed at the same optimum pH value when catalysed by *T. viride* glycosulphatase. In contrast, *Littorina* glycosulphatase exhibits two distinct optimum pH values (pH 5.2 and 5.9 respectively) when assayed with these substrates.

With regard to substrate specificity both of the molluscan enzymes hydrolyse potassium D-glucose 3-O-sulphate, a property that could not be demonstrated for *T. viride* glycosulphatase. This may reflect a basic specificity difference between the molluscan and mould enzymes, but a second possibility should not be ignored, namely that

molluscan preparations contain more than one glycosulphatase. Some point is given to this possibility by the observation (Yamagata, Kawamura & Suzuki, 1966) that preparations of chondrosulphatase from *Proteus vulgaris* (see Dodgson & Lloyd, 1957) actually contain two sulphatases, one specific for chondroitin 4-sulphate and the other for the isomeric 6-sulphate.

The effect of temperature on the enzymic hydrolysis of the 6-O-sulphate esters and the action of the selected enzyme inhibitors and activators also indicate that the glycosulphatase activity of *T. viride* is associated with a protein molecule having a pattern of behaviour completely different from that of either *Littorina* or *Charonia* glycosulphatases.

The production of glycosulphatase by *T. viride* depends on the presence of either D-glucose 6-O-sulphate or D-galactose 6-O-sulphate in the growth medium. Even in the presence of concentrations of D-glucose and inorganic SO_4^{2-} ions that are adequate for growth, the introduction of D-glucose 6-O-sulphate into the medium still leads to the production of the glycosulphatase. The elucidation of the processes leading to the production of the enzyme will be of intrinsic interest, particularly in relation to the results of the studies by Harada & Spencer (1962, 1964), Harada & Kamogawa (1963), Harada (1957) and Rammler *et al.* (1964) on the induction and repression in micro-organisms of the analogous arylsulphatase enzymes. However, many factors appear to complicate the study of glycosulphatase production in *T. viride*. Some of these have been mentioned above, but perhaps the most difficult to resolve is concerned with the practical problem of measuring enzyme production during growth, particularly during the early stages. Measurement of the rate of liberation of inorganic $^{35}\text{SO}_4^{2-}$ ions into the medium is unsatisfactory since it takes no account of the phenomenon of sulphate transport and of the utilization of liberated inorganic SO_4^{2-} ions for synthetic purposes. Unfortunately, the resolution of the difficulty depends on measurement of the actual concentrations of enzyme within the mycelium and a satisfactory method for achieving this in small-scale experiments has not yet been developed. At present, large-scale experiments in the numbers required to define the parameters necessary for enzyme production are prohibited by the difficulty of synthesizing the large quantities of potassium glucose 6-O-sulphate that would be required.

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