Studies on Sulphatases

7. A PRELIMINARY ACCOUNT OF THE GLYCOSULPHATASE OF *LITTORINA LITTOREA*

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The presence in certain tropical molluscs of an enzyme capable of liberating sulphuric acid from glucose 6-sulphate was first noted by Soda & Hattori (1931). The enzyme was a simple hydrolase (Soda & Egami, 1933) and was concentrated in the digestive glands. Subsequent work (Tanko, 1932; Buehler, Katzman & Doisy, 1951) showed a similar glycosulphatase to occur in certain bacteria but there have been no reports of a mammalian source of the enzyme. Although other carbohydrate sulphates were substrates for the enzyme, activity was greatest with glucose 6-sulphate and the name 'glucosulphatase' was adopted (Soda, 1934). However, Soda's observations must be regarded with caution since no attempt was made to compare activities at the optimum conditions for each particular substrate. For this reason the name 'glycosulphatase' is preferred by the present authors until the specificity of the enzyme is known more precisely. Between 1931 and 1948 a series of publications by Soda and his co-workers described the purification and properties of glycosulphatase, although the value of the quantitative work was limited by the insensitivity of the methods used to estimate enzyme activity. Part of this work was reviewed by Fromageot (1950) but the enzyme does not appear to have become widely known, despite its possible use in structural carbohydrate chemistry.

As part of a general study of sulphatases in this laboratory a search was made for glycosulphatase in marine molluscs common to British waters. Some of these organisms possessed weak glycosulphatase activity (see also Percival, 1949) but the large periwinkle (*Littorina littorea*) was particularly active. By using a sensitive method for the determination of inorganic sulphate (Dodgson & Spencer, 1953) it was possible to study glycosulphatase for the first time using short incubation periods (1 or 2 hr.). During these studies it became obvious that glucose 6-sulphate prepared by direct sulphonation was not pure and was unsuitable as a substrate for enzyme studies. This heterogeneity appears to have escaped the notice of other workers (Percival & Soutar, 1940; Duff, 1949; Wolfrom & Montgomery, 1950) but was noted by Egami (1940) for glucose 6-sulphate prepared by the method of Soda (1933).

METHODS AND MATERIALS

Measurement of enzyme activity. The amount of sulphate liberated by the enzyme from potassium glucose 6-sulphate was measured by the method of Dodgson & Spencer (1953). This method cannot be used with barium salts. During preliminary studies there were indications that the method gave anomalous results at high substrate concentrations. This was probably due to low blank values, since recoveries of added K_2SO_4 from concentrations of substrate greater than 0.02 M (Table 2) were high for this reason. Variation of the conditions for the precipitation of benzidine sulphate did not affect these low blank values, and it was necessary to lower the substrate concentration by diluting the incubation mixture before the addition of benzidine in order to obtain quantitative recoveries of SO_4^{2-} . Thus instead of incubating 0.6 ml. substrate with 0.6 ml. enzyme solution as described in the original method, smaller volumes (0.1 or 0.2 ml.) were used in conjunction with an equal volume of a more concentrated enzyme solution. After incubation, aqueous ethanol was added to give a final volume of 6 ml. The amount of ethanol added was such that there was a concentration of 80% (v/v) ethanol in the final mixture. This procedure fulfilled the conditions necessary for precipitating benzidine sulphate outlined by Dodgson & Spencer (1953).

Molluscs. Supplies of *Littorina littorea*, *Patella vulgata* and *Gibbula umbilicalis* were obtained from the Bristol Channel area. Other organisms were obtained from the Marine Biological Station, Plymouth. They were kept alive in aerated sea-water or, for shorter periods, in the ice chest. For enzyme assay, organisms were used as soon as possible after collection (see Dodgson, Lewis & Spencer, 1953), whilst for the preparation of enzyme concentrates they were starved for a week in order to remove food material from the visceral hump.

Potassium glucose 6-sulphate. This was obtained from the barium salt prepared by direct sulphonation of glucose with chlorosulphonic acid and pyridine (Percival & Soutar, 1940) or more frequently with pyridine: SO_3 reagent thus avoiding the need to remove Cl^- (Duff, 1949). The barium salt was converted into the potassium salt by adding the requisite amount of K_2SO_4 to a concentrated aqueous solution of the barium salt. The precipitated $BaSO_4$ was separated by centrifuging and the potassium glucose 6-sulphate in the supernatant precipitated with absolute ethanol. The compound was dried at 80–90° *in vacuo*. (Found: SO_4 , 32.9; K, 13.7. Calc. for $C_6H_{11}O_6SK$: SO_4 , 32.2; K, 13.1%.)

Potassium glucose 3-sulphate. The method of Percival (1945) was modified by using pure pyridine: SO_3 as sulphonating agent. Di-isopropylidene glucose (14.9 g.) was dissolved in 100 ml. of dry pyridine and treated with 13.5 g. of pyridine: SO_3 reagent at 60° for 3 hr. with stirring. About

half the starting material was sulphated. Thereafter the method followed that of Percival (1945) except that after extraction of di-isopropylidene glucose 3-sulphate with acetone, evaporation of the acetone at room temperature gave a crystalline product without further treatment. Attempts to remove the isopropylidene groups of this compound with acid ion-exchange resins at 100° resulted in loss of ester sulphate and the original procedure was adhered to. The barium salt obtained was converted into the potassium salt as described for potassium glucose 6-sulphate. The compound was dried at 70–80° *in vacuo*.

RESULTS

The purity of potassium glucose 6-sulphate

Egami (1938, 1940, 1942) concluded that glucose 6-sulphate prepared by the direct sulphation method of Soda (1933) was impure since it was partially hydrolysed (about 15%) by hydrazine at 37·5° in the presence of acetate at pH values varying from 4·1 to 7·1. No further hydrolysis was observed after addition of more hydrazine or by prolonged incubation but after adding more glucose 6-sulphate further hydrolysis occurred to the extent of about 15% of the extra material added. By fractional crystallization of the brucine salt, Egami prepared glucose 6-sulphate which was 'hardly hydrolysed' by hydrazine. Studies on the effect of increasing substrate concentration on the activity of *Littorina* glycosulphatase during the present work suggested that glucose 6-sulphate prepared by other direct sulphation methods was also impure (see Fig. 4) and further studies confirmed this.

The action of hydrazine on potassium glucose 6-sulphate. The findings of Egami were confirmed for the substrate prepared according to Duff (1949). Potassium glucose 6-sulphate (1 ml. of 0·5M) in barium acetate:acetic acid buffer (0·5M, pH 5·3) was incubated at 37·5° with hydrazine (2 ml. of 0·5M hydrazine hydrate in the same buffer adjusted to pH 5·3) and the liberated sulphate determined gravimetrically as BaSO₄. Suitable blank determinations were made. A typical hydrolysis curve (Fig. 1) shows that hydrolysis did not cease completely after the initial rapid change. The slow hydrolysis was accompanied by a darkening of the solution and it seemed probable that some general degradation of remaining substrate was occurring. The degree of initial hydrolysis varied slightly from preparation to preparation. Addition of more hydrazine did not affect the extent of the rapid hydrolysis, but addition of more substrate after the conclusion of the initial hydrolysis resulted in a further equivalent amount of initial hydrolysis. The extent of primary hydrolysis could be substantially reduced by preparing potassium glucose 6-sulphate via the recrystallized brucine salt. In a typical experiment the product from the sulphation of 20 g. of glucose was divided into three parts. One

part was converted into the potassium salt in the usual way (preparation A), a second part was converted into the potassium salt via the once recrystallized brucine salt (B), and the third part was treated identically except that the brucine salt was recrystallized four times (C). The degree of initial hydrolysis by hydrazine was 27·4, 18·9 and 15·6%, respectively.

Fig. 1 also shows the effect of hydrazine on potassium glucose 3-sulphate under identical experimental conditions. The experiment was not continued beyond 10 days since by then the solutions had darkened considerably and the controls without hydrazine showed appreciable hydrolysis (about 8%).

These results may be interpreted by assuming that an impurity is present to the extent of about 25–30% in glucose 6-sulphate prepared by direct sulphation. The most likely main impurity would be an isomeric glucose monosulphate. The rate of hydrolysis of this impurity by hydrazine is much greater than that of glucose 6-sulphate and the hydrolysis curve for the latter is only evident when hydrolysis of the impurity is complete. It is difficult to follow this slower hydrolysis further, since it is complicated by a general non-specific hydrolysis (and possibly degradation) of substrate in the absence of hydrazine. On the other hand, glucose 3-sulphate which is chromatographically pure shows a smooth hydrolysis curve with hydrazine, as expected. Again a non-specific hydrolysis and degradation of substrate prevented the study of the curve to completion.

Fig. 1. Curves showing the rate of hydrolysis of potassium glucose 3- and 6-sulphates by hydrazine hydrate at 37·5°. The sulphate (1 ml. of 0·5M in 0·5M barium acetate:acetic acid buffer, pH 5·3) was incubated with 2 ml. of 0·5M hydrazine hydrate in the same buffer adjusted to the same pH. ○—○, potassium glucose 3-sulphate; ●—●, potassium glucose 6-sulphate.

Chromatography experiments. Mrs E. G. V. Percival (private communication) suggested that glucose sulphates might be separated by paper chromatography using the top layer of a mixture of ethyl acetate, acetic acid and water (3:1:3, v/v) as mobile phase. Chromatograms were run for 24 hr., dried at room temperature, sprayed with aniline oxalate and heated to 110° for 5–10 min. At least three spots (designated as 1, 2 and 3 in order of increasing R_f value) were obtained with potassium glucose 6-sulphate (preparation A), spot 2 being very intense and considered to be glucose 6-sulphate. None of these spots was chromatographically identical with glucose or potassium glucose 3-sulphate, although in chromatograms run for shorter periods (6–12 hr.) a faint, fast-moving spot was identified as glucose. Chromatography of equal concentrations of preparations A, B and C showed that spot 1 decreased in intensity from A to C and this was evidently the main impurity. Spot 3 was very faint and present in A only. Potassium glucose 3-sulphate appeared to be homogeneous on the paper chromatogram.

Spectroscopic observations. During preliminary studies, the ultraviolet absorption spectra of a number of preparations of glucose 6-sulphate (prepared according to Duff, 1949) revealed the presence of an absorption peak at the wavelength of maximum absorption of pyridine in water (256 m μ). Since glucose itself had no characteristic absorption

in the ultraviolet, the absorption maximum noted was presumably due to traces of contaminating pyridine or to unchanged pyridine salt which occurs as an intermediate in the preparation of glucose 6-sulphate. This impurity was not present in preparations where the treatment of the pyridine glucose 6-sulphate with barium carbonate was extended from 2 to 4 hr. (see Fig. 2). In later experiments this procedure was always followed and results quoted in this paper are for glucose 6-sulphate prepared in this way (preparation A). However, removal of the impurity with consequent removal of the associated peak absorption revealed a smaller absorption at 260 m μ . The curve was again characteristic of pyridine and was probably due to other contaminating pyridine salts since the impurity was not present in preparations B and C which had been obtained via a recrystallized brucine salt.

Soda (1933) reported that the ultraviolet absorption spectrum of potassium glucose 6-sulphate in water showed a maximum absorption at 263 m μ . The magnitude of this absorption was of the same order as that obtained at 260 m μ during the present work. Soda attributed the absorption to a straight-chain aldehyde form of glucose 6-sulphate though he noted that it was much more displaced towards shorter wavelengths than was usual for the C=O bond. Chemical tests made on several different preparations of glucose 6-sulphate in this laboratory gave no indication of the existence of a straight-chain aldehyde form. Whatever the cause of this absorption it was not due to a major impurity since it was absent from preparations B and C, although the hydrazine and chromatographic experiments showed them to be substantially impure.

Enzyme experiments. A study of the effect of increasing the concentration of substrate on the activity of the glycosulphatase present in an acetone-dried preparation of *Littorina* visceral hump gave an abnormal substrate concentration curve (Fig. 4). This curve suggested substrate heterogeneity. It must be noted that potassium glucose 6-sulphate (preparation A) was hydrolysed completely by *Littorina* glycosulphatase.

Glycosulphatase activity in molluscs

Using arbitrary experimental conditions (0.01M potassium glucose 6-sulphate in 0.5M acetate at pH 5.2), suspensions of the visceral humps of a number of common marine molluscs were tested for glycosulphatase activity. The results are recorded in Table 1. The activities probably represent only a fraction of the actual activity since the experimental conditions used were not optimum.

Preparation of a glycosulphatase concentrate. In spite of the relatively high concentration of glycosulphatase in *Littorina* compared with that in the

Fig. 2. Ultraviolet absorption of aqueous solutions (1%, w/v) of various samples of potassium glucose 6-sulphate prepared by direct sulphation of glucose. Curve 1 is for the compound prepared according to Duff (1949); curve 2 is for a similar preparation after prolonged treatment with BaCO₃ (preparation A, see text); curve 3 is for preparation A purified via a recrystallized ($\times 4$) brucine salt; curve 4 is a reference curve for glucose (Hopkin & Williams Ltd., A.R.)

Table 1. *Glycosulphatase activity of suspensions of marine molluscs*

Substrate, 0.01 M potassium glucose 6-sulphate in 0.5 M acetate, pH 5.2, incubated for 2 hr. at 37.5°

Organism	Organ	SO ₄ ²⁻ liberated/ g. of tissue (μg.)
<i>Osilinus linneatus</i>	Visceral hump	282
<i>Calliostoma zizyphinum</i>	Visceral hump	215
<i>Gibbula umbilicalis</i>	Visceral hump	0
<i>Patina pellucida</i>	Whole organism	0
<i>Patella vulgata</i>	Visceral hump	96
<i>Buccinum undatum</i>	Posterior digestive gland	0
<i>B. undatum</i>	Anterior digestive gland	0
<i>B. undatum</i>	Mucous gland	0
<i>Littorina littorea</i>	Visceral hump	997

other molluscs examined, it was convenient to make a concentrated enzyme preparation before studying the enzyme. The visceral humps of periwinkles were dissected out, washed quickly in ice-cold distilled water and macerated in ice-cold acetone. After being filtered at the pump, the solid material was again macerated with ice-cold acetone and the debris removed, washed with ice-cold acetone and dried *in vacuo* (yield 9.0 g. from 46 g. visceral hump). The powder (8.5 g.) was macerated in 120 ml. of ice-cold distilled water and cold acetone was added slowly (stirring) until the concentration of acetone was 20% (v/v). Precipitated material was removed by centrifuging at 0° and cold acetone was added to the supernatant to give a final concentration of 60% (v/v). After standing at 0° for a few minutes to allow flocculation, the precipitate was separated, washed well with cold acetone and dried *in vacuo*. The light-coloured powder obtained (2.0 g.) was stable when kept in an air-tight bottle at 0°. One g. of this powder liberated 80 000 μg. of SO₄²⁻ from 0.07 M potassium glucose 6-sulphate (preparation A) in 0.5 M acetate, pH 5.85 in 1 hr. at 37.5°. The nitrogen content was 11% (w/w).

The effect of pH on enzyme activity. The optimum pH for the enzyme was 5.85 in 0.5 M acetate when the concentration of substrate (preparation A) was 0.01 M (Fig. 3). The impure nature of the substrate was not reflected in this curve. Soda (1936) records the optimum pH of a preparation of glycosulphatase obtained from *Triton nodiferus* as 5.1–5.2 when the substrate was 0.044 N barium glucose 6-sulphate.

The effect of substrate concentration on enzyme activity. At pH 5.85 in 0.5 M acetate an abnormal substrate concentration curve was obtained (Fig. 4). This curve is probably due to the impure nature of the substrate (preparation A), although interpretation is difficult in the absence of more precise information about the substrate. Moreover, the possibility of more than one glycosulphatase being present has not been excluded.

Fig. 3. pH activity curve for the glycosulphatase of a concentrate prepared from the visceral hump of *Littorina*. The concentration of potassium glucose 6-sulphate was 0.01 M in 0.5 M acetate and incubations were for 1 hr. at 37.5°.

Fig. 4. Substrate concentration activity curve for the glycosulphatase of a concentrate prepared from the visceral hump of *Littorina* acting on potassium glucose 6-sulphate in 0.5 M acetate, pH 5.85, during 1 hr. at 37.5°.

Table 2. Recoveries of K_2SO_4 added to solutions of potassium glucose 6- and 3-sulphates in 0.5M acetate at pH 5.85

Substrate	Molarity of substrate	Time of standing of precipitated benzidine sulphate at 0° (hr.)	K_2SO_4 (μ g.)	
			Added	Recovered
Potassium glucose 6-sulphate	0.01	18	26.25	26.3
	0.02	18	26.25	26.2
	0.03	18	26.25	28.0
	0.05	18	26.25	33.4
	0.07	18	26.25	41.2
Potassium glucose 3-sulphate	0.01	18	20.3	7.8
	0.01	3	20.3	9.5
	0.02	18	20.3	7.4
	0.02	3	20.3	10.4

Activities of other enzymes in the concentrate. Other sulphatase enzymes were present in the concentrate. Arylsulphatase activity measured at the optimum conditions (Dodgson *et al.* 1953) was 1047000 arylsulphatase units/g. when potassium *p*-acetylphenyl sulphate was the substrate. Chondrosulphatase was also present, 1 g. of the powder liberated 5430 μ g. SO_4^{2-} from sodium chondroitin sulphate in 1 hr. at 37.5° under arbitrary experimental conditions (1.65%, w/v, substrate in 0.5M acetate at pH 5.75). Drs S. R. Stitch and I. D. K. Halkerston (private communication) have demonstrated the presence in our preparation of an enzyme capable of liberating SO_4^{2-} from sodium dehydro-isoandrosterone sulphate (see also Stitch & Halkerston, 1953). The β -glucuronidase content was 55000 *p*-chlorophenol units/g. (see Dodgson *et al.* 1953).

Potassium glucose 3-sulphate as a glycosulphatase substrate. Since the method of preparation gave chromatographically pure potassium glucose 3-sulphate the possibility of using this compound as a substrate was considered. It was readily attacked by the glycosulphatase preparation, 90 mg. being hydrolysed in 96 hr. at 37.5° by 0.2 g. enzyme powder in the presence of barium acetate:acetic acid buffer, pH 5.85. The liberated sulphate was determined indirectly (Soda, 1936) as barium sulphate. However, potassium glucose 3-sulphate was less stable than the corresponding 6-sulphate and was slowly hydrolysed under the conditions necessary for the determination of sulphate by the benzidine method (Dodgson & Spencer, 1953). Table 2 shows the recovery of inorganic sulphate added to solutions of potassium glucose 3-sulphate in the presence of 0.5M acetate at pH 5.85. In all cases recoveries were low, but the amount of sulphate present in both 'test' and 'blank' determinations was abnormally high and it was obvious that, even at 0° the substrate was unstable in the presence of trichloroacetic acid. For this reason

potassium glucose 3-sulphate was an unsuitable substrate for glycosulphatase studies using the benzidine method.

DISCUSSION

The existence of glycosulphatase in molluscs common to British waters has now been established with certainty. The large periwinkle, available in quantity in most fish markets, forms a convenient and active source of the enzyme and stable acetone-dried concentrates are readily prepared. The presence of aryl-, alkyl- and chondro-sulphatases and β -glucuronidase in this concentrate suggests its possible value as a tool in the elucidation of the structure of important naturally occurring sulphate esters and in the analysis of urinary steroids. Aryl-, chondro- and glyco-sulphatases appear to be different enzymes since Soda, Katsura & Yoda (1940) were able to separate them to some degree. On the evidence available, it is not possible to distinguish between alkylsulphatase (Stitch & Halkerston, 1953) and glycosulphatase in mollusc preparations, although Soda (1936) noted that glycosulphatase was not a general alkylsulphatase since it did not hydrolyse potassium ethyl sulphate. The specificity of the sulphatases is vague and their nomenclature must be regarded as provisional.

The role of these enzymes in the living mollusc is still obscure, though it is tempting to suggest connexions between glycosulphatase and the polysaccharide sulphates present in the seaweeds on which many molluscs feed. However, preliminary experiments indicate that the enzyme preparations show little activity towards certain of these substrates. The separation and purification of these sulphatases is a necessary preliminary to studies on their physiological significance since little is known about the specificity of the enzymes, and in the living organism they may well have no connexion with substrates which have been used for their study *in vitro*.

Further kinetic studies on glycosulphatase depend on the preparation of pure potassium glucose 6-sulphate since the presence of impurities in the compound prepared by the usual means is a complicating factor. It is possible that other conclusions regarding the properties of the 6-sulphate might need modification in view of these more recent findings. Thus Soda (1933) observed that the velocity of the mutarotation of salts of the 6-sulphate was unusual and not that of a first order change, the initial rapid mutarotation not being maintained. Various conclusions as to the structure of glucose 6-sulphate were made from these experiments although the unusual curves were probably due to the presence in his preparations of substantial amounts of a second glucose monosulphate.

Attempts have been made to purify glucose 6-sulphate by preferential hydrolysis of the impurity with hydrazine followed by recovery of the unhydrolysed 6-sulphate. A number of different methods of recovering the unhydrolysed glucose 6-sulphate were tried, but the final product was always heavily contaminated with hydrazine and the contamination persisted even after repeated purification by precipitation from aqueous solution by ethanol. The unsuitability of potassium glucose 3-sulphate as a glycosulphatase substrate when using the benzidine method is disappointing since the manner of preparation ensures a pure product of known structure. The compound may be of use as a substrate with other methods of estimating enzyme activity since it is readily hydrolysed by the enzyme. However, glucose 3-sulphate is appreciably less stable than the 6-sulphate even when stored at 0°. The preparation of pure glucose 6-sulphate by other methods is being studied in this laboratory while in the meantime the purification of glycosulphatase is being followed using the impure 6-sulphate as enzyme substrate.

SUMMARY

1. Glycosulphatase has been shown to be present in appreciable amounts in the visceral hump of the large periwinkle, *Littorina littorea*.

2. A stable acetone-dried concentrate of the enzyme has been made. Alkyl-, aryl- and chondrosulphatases and β -glucuronidase are also present in the concentrate.

3. The findings of Egami (1942) as to the impure nature of potassium glucose 6-sulphate prepared by direct sulphonation of glucose and used as a substrate for the study of glycosulphatase have been confirmed and extended.

4. Potassium glucose 3-sulphate is readily hydrolysed by glycosulphatase, but is unstable under the conditions necessary for determining sulphate by the benzidine method.

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REFERENCES

- Buehler, H. J., Katzmman, P. A. & Doisy, E. A. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 3.
 Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953). *Biochem. J.* **55**, 253.
 Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
 Duff, R. B. (1949). *J. chem. Soc.* p. 1597.
 Egami, F. (1938). *J. chem. Soc. Japan*, **59**, 1034.
 Egami, F. (1940). *J. chem. Soc. Japan*, **61**, 592.
 Egami, F. (1942). *J. chem. Soc. Japan*, **63**, 763.
 Fromageot, C. (1950). *The Enzymes*, vol. 1, pt. 1, p. 517. New York: Academic Press Inc.
 Percival, E. G. V. (1945). *J. chem. Soc.* p. 119.
 Percival, E. G. V. (1949). *Quart. Rev. chem. Soc.* **3**, 369.
 Percival, E. G. V. & Soutar, T. H. (1940). *J. chem. Soc.* p. 1475.
 Soda, T. (1933). *Bull. chem. Soc. Japan*, **8**, 37.
 Soda, T. (1934). *Bull. chem. Soc. Japan*, **9**, 83.
 Soda, T. (1936). *J. Fac. Sci. Tokyo Univ.* **3**, 150.
 Soda, T. & Egami, F. (1933). *Bull. chem. Soc. Japan*, **8**, 148.
 Soda, T. & Hattori, C. (1931). *Bull. chem. Soc. Japan*, **6**, 258.
 Soda, T., Katsura, T. & Yoda, O. (1940). *J. chem. Soc. Japan*, **61**, 1227.
 Stitch, S. R. & Halkerston, I. D. K. (1953). *Nature, Lond.*, **172**, 398.
 Tanko, B. (1932). *Biochem. Z.* **247**, 486.
 Wolfrom, M. L. & Montgomery, R. (1950). *J. Amer. chem. Soc.* **72**, 2859.