

Studies on Sulphatases

28. PREPARATION OF SUBSTRATES FOR THE ASSAY OF GLYCOSULPHATASE*

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Glucose monosulphate, used as assay substrate for the study of the enzyme glycosulphatase, has been prepared by the direct action of chlorosulphonic acid or pyridine-sulphur trioxide reagent on glucose in pyridine solution (see Dodgson & Spencer, 1957). With material prepared in this way, Dodgson & Spencer (1954), during a study of the glycosulphatase of the marine mollusc *Littorina littorea*, observed anomalies in the behaviour of the enzyme toward increasing substrate concentration, which were attributed to the presence of one or more sulphated contaminants in the substrate preparation. Attempts to remove these contaminating materials were unsuccessful, and further kinetic studies were abandoned. Lloyd (1959*a*) showed that monosaccharide sulphate esters prepared by the direct action of chlorosulphonic acid invariably consist of mixtures of mono- and di-sulphate esters, together with small amounts of unchanged monosaccharides. For this reason methods were evolved for the definitive synthesis of potassium glucose 6-*O*-sulphate and potassium *N*-acetylglucosamine 6-*O*-sulphate with chlorosulphonic acid as sulphating agent. As chlorosulphonic acid yields reaction mixtures contaminated with chloride, which can be removed only with difficulty, the method is not ideally suited to the preparation of enzyme substrates for routine studies.

In the present report methods are described for the synthesis of monosaccharide monosulphate esters by a modification of the method due to Duff (1949). A preliminary account of part of this work has already been given (Lloyd, 1959*b*).

MATERIALS AND METHODS

Monosaccharides and derivatives. D-Glucose, D-galactose and D-glucosamine were commercial preparations (British Drug Houses Ltd.). D-Galactosamine was prepared from potassium chondroitin sulphate (Dodgson, Lloyd & Spencer, 1957) by the method of Roseman & Ludowieg (1954). The procedure described by the latter workers was used for the *N*-acetylation of both the glucosamine and galactosamine preparations. Several authentic carbohydrate sulphates were prepared by the chlorosulphonic

acid procedure (Lloyd, 1959*a*). Potassium glucose 6-*O*-sulphate was obtained after sulphating 1:2:3:4-tetra-*O*-acetyl-D-glucose (Reynolds & Evans, 1942), potassium galactose 6-*O*-sulphate after sulphating 1,2:3,4-di-(*O*-isopropylidene)-D-galactose and potassium *N*-acetylglucosamine 6-*O*-sulphate after sulphating 1:3:4-tri-*O*-acetyl-*N*-acetyl-D-glucosamine (Anderson & Percival, 1956).

Sulphation with pyridine-sulphur trioxide reagent. The monosaccharide (0.05 mole) was dissolved in 100 ml. of dry pyridine in the presence of 10 g. of Drierite (L. Light and Co. Ltd.) at 70°. The mixture was cooled to 37° and stirred mechanically during the addition of 0.10 mole of pyridine-sulphur trioxide reagent (Baumgarten, 1926). The reaction mixture was stirred at 37° for 12 hr., cooled to 20° and kept for 3 hr., when the crude ester sulphate separated as an oily lower layer. The upper pyridine layer was removed by decantation and the oil dissolved in the minimum amount of water at room temperature with vigorous stirring. Drierite was removed by filtration and the clear filtrate stirred at room temperature for 2 hr. The solution was cooled to 2°, and a suspension of 40 g. of Ba(OH)₂ in 100 ml. of water, previously cooled to 2°, was added with constant stirring over a period of 10 min. Precipitated BaSO₄ was removed by centrifuging at 3000 *g* and 0° for 15 min. Ba²⁺ ions were precipitated from the alkaline supernatant solution by the addition of solid CO₂ over a period of 30 min. Precipitated BaCO₃ was removed by centrifuging as before. The clear supernatant solution was distilled under reduced pressure in a rotary evaporator at 35° to remove pyridine, water being added continuously to avoid concentration. The solution was finally concentrated to 20 ml. by distillation *in vacuo* at 30°.

Removal of the greater part of the unchanged monosaccharide was achieved as follows. The concentrated solution containing the crude monosaccharide sulphate was passed through a Dowex 50 column (H⁺ form; 100–200 mesh; cross-linkage × 4; 30 cm. × 2 cm.) at 2° at a flow rate of 1 ml./min. The acid eluate and washings were combined (40 ml.) and treated with a saturated ethanolic solution of brucine (20 ml.) at room temperature. The mixture was then concentrated to dryness under reduced pressure at 35°, a precipitate of brucine which appeared during the concentration procedure being removed by filtration. The residue was dissolved in the minimum amount of water at 50° with vigorous stirring, cooled to 25° and absolute ethanol (about 250 ml.) added. The mixture was cooled to 5° and kept for 2 hr. The resulting precipitate was filtered and recrystallized twice from aqueous ethanol as described above.

For conversion into the potassium salt the brucine derivative was dissolved in the minimum amount of water and passed through a Dowex 50 column (H⁺ form as before).

* Part 27: Dodgson & Powell (1959).

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The acid eluate and washings were adjusted to pH 7.4 with 0.05M-KOH and then concentrated to small volume by distillation under reduced pressure at 35°. The potassium salt was precipitated by the addition of 5 vol. of ethanol, the precipitate was collected by filtration and, after being washed with absolute ethanol and ether, finally dried *in vacuo* at 30° over CaCl₂.

Paper chromatography. The homogeneity of the reaction products was checked by descending chromatography on Whatman no. 3MM paper for 48 hr. at 20° with butan-1-ol-acetic acid-water, 50:12:25 (by vol.). Spots were detected by spraying with silver nitrate (Trevelyan, Procter & Harrison, 1950) or aniline hydrogen phthalate (Partridge, 1949). Hexosamines and derivatives were demonstrated by spraying with Elson-Morgan reagents (Partridge, 1948). For the detection of sulphuric acid esters the chromatograms were sprayed with 10% (v/v) of perchloric acid in ethanol, followed by heating for 5 min. at 80–85° in a moist (water vapour) atmosphere. The inorganic sulphate so released was then detected by spraying with BaCl₂ followed by sodium rhodizonate (Burma, 1953).

Filter-paper electrophoresis. Zone electrophoresis on filter-paper strips (32 cm. in length) was carried out in horizontal tanks with a multipoint paper support. Several volatile buffer systems, chosen to avoid time-consuming desalting procedures, were examined during initial stages of the study. Best separations of monosaccharide monosulphate from the contaminating disulphate and parent compounds were obtained after electrophoresis in ammonium acetate-acetic acid buffer (0.1M; pH 4.0). For analytical purposes, Whatman no. 3MM filter paper was employed, the applied potentials being varied in the range 110–400 v over periods of 2–12 hr.

Filter-paper electrophoresis was also employed for small-scale preparative procedures on Whatman no. 17 or Whatman Seed Test papers. Before use, the sheets were washed by downward irrigation with 0.01N-HCl for 48 hr., with water for 48 hr. and finally with the buffer to be used for the separation for a further 24 hr. The sheets were finally dried in a warm air stream.

The material to be separated was applied as a narrow zone across the width of the thick filter-paper strip (32 cm. × 11 cm.) from a self-filling micropipette. On Whatman no. 17 paper it was possible to apply a maximum of 100 mg./strip, and on Seed Test paper this could be increased to 175 mg./strip. However, with the latter there was considerable variation in the efficiency of the separation since marked zone-spreading occurred with some samples of the paper. Contact between the ends of the thick filter-paper strip and the electrolyte chambers of the electrophoresis apparatus was made with Whatman no. 1 wicks.

After passage of the current the damp filter paper was removed from the tank and placed on a clean glass plate. A piece (32 cm. × 11 cm.) of Whatman no. 1 paper was placed on top of the strip and the two were pressed evenly together by applying gentle pressure with a soft rubber roller. As the upper strip absorbed moisture from the lower one, traces of material from the separated zones were transferred to it. After the upper strip had been sprayed, the thick filter-paper strip could be returned to the tank if separation was found to be incomplete.

The appropriate regions of the electrophoresis strip were then cut out, clamped between glass plates and eluted with

water. The eluates were concentrated to dryness by freeze-drying over KOH. The residue was taken up in the minimum amount of water, passed through a Dowex 50 column (H⁺ form; 100–200 mesh), and the acid eluate and washings were pooled and adjusted to pH 7.4 with 0.05M-KOH and concentrated *in vacuo*. Ethanol (5 vol.) was added to the solution and the precipitated material allowed to flocculate. The precipitate was collected by centrifuging at 2000 g and 0° for 10 min., washed with absolute ethanol and ether and finally dried *in vacuo* at 30° over CaCl₂.

Cellulose-column electrophoresis. Zone electrophoresis was carried out in externally cooled columns at 4° (Porath, 1956). On an analytical scale the column dimensions were 100 cm. × 1.5 cm., and for preparative purposes the dimensions were 80 cm. × 3 cm. Packing of the ethanolysed cellulose powder was performed as described by Flodin & Kupke (1956), after removal of gases from the buffer slurry at 10° under reduced pressure. The cellulose bed was then washed with 5 hold-up vol. of buffer before use.

When electrophoresis was complete the column was eluted with buffer at 40 ml./hr. (analytical scale) or at 100 ml./hr. (preparative scale). The position of displaced zones was determined quantitatively by the anthrone procedure (Fairbairn, 1953). Fractions corresponding to homogeneous elution peaks were pooled, concentrated to small volume by distillation *in vacuo* at 35° and, after freeze-drying over KOH, treated as described for the isolation of material after zone electrophoresis on thick paper.

Analysis. All samples were dried at 40° *in vacuo* over CaCl₂ before analysis. For the determination of ester sulphate, samples were hydrolysed under reflux with 4N-HCl for 8 hr. The hydrolysates were concentrated to dryness *in vacuo* over KOH at 50°, the residue was dissolved in water and inorganic sulphate estimated by the method of Lloyd (1959c). Nitrogen was determined with the micro-Kjeldahl apparatus (Markham, 1942) and potassium by the flame photometer.

EXPERIMENTAL AND RESULTS

Preparation of monosaccharide sulphates by the pyridine-sulphur trioxide procedure. Table 1 records the average yields of crude monosaccharide sulphates obtained as the potassium salts after the sulphation of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine as described earlier. Paper-chromatographic analysis of the reaction products indicated the presence of three components in each case, two containing ester sulphate and a third identical in chromatographic mobility with the parent compound (Table 1).

The sulphation procedure described differs from that of Duff (1949) and Dodgson & Spencer (1954, 1957) in several respects. As preliminary modifications the concentration of pyridine-sulphur trioxide reagent was increased and the reaction was performed in the presence of Drierite, resulting in an average increase in yield of 27% (by wt.) for crude potassium glucose sulphate. To compare the relative concentrations of the components, samples of crude glucose sulphate, prepared by the original

Table 1. Average yields of crude monosaccharide sulphate esters obtained by the pyridine-sulphur trioxide procedure

Chromatographic mobilities (R_G) are related to that of glucose, which is taken as unity.

| Ester | Yield of crude potassium salt (g./10 g. of starting material) | R_G of components | | |
|--|---|---------------------|-----------------|-------|
| | | Parent compound | Ester sulphates | |
| | | | Di- | Mono- |
| Glucose sulphate | 8.2 | 1.0 | 0.66 | 0.42 |
| Galactose sulphate | 8.9 | 0.95 | 0.59 | 0.42 |
| <i>N</i> -Acetylglucosamine sulphate | 6.8 | 1.25 | 0.76 | 0.54 |
| <i>N</i> -Acetylgalactosamine sulphate | 6.5 | 1.15 | 0.71 | 0.51 |

and modified procedures, were chromatographed, and the separate components were eluted and estimated by the anthrone procedure. The concentration of the monosulphate component prepared by the modified method corresponded to a 16% increase in yield, as compared with the concentration of this component in mixtures obtained by the earlier methods.

Dodgson & Spencer (1954) reported that samples of potassium glucose sulphate prepared according to Duff (1949) exhibited an absorption peak in the ultraviolet at the wavelength of maximum absorption of pyridine in water. This was presumed to be due to traces of a pyridine salt occurring as an intermediate in the preparation of glucose sulphate. The concentration of this component was substantially decreased after prolonged treatment with aqueous BaCO_3 , but it was completely removed only after repeated recrystallization of the brucine salt of the ester. In the present method removal of this pyridine complex, as indicated by the disappearance of the ultraviolet absorption, was achieved by stirring the acid aqueous solution of the ester at room temperature and neutralizing with aqueous $\text{Ba}(\text{OH})_2$. No change in yield of the glucose monosulphate component of the mixture, determined chromatographically as before, was observed after incorporation of this step.

Separation of sulphated products by paper electrophoresis. Complete separation of monosaccharide monosulphate from the corresponding disulphate and parent monosaccharide could be achieved on Whatman no. 3MM paper in 2 hr. at 400 v in 0.1M-ammonium acetate buffer, pH 4.0. The separation was facilitated by applying a hydrodynamic counterflow by raising the level of the buffer in the anode chamber by 3 cm. over that in the cathode chamber. Similar conditions were used to separate 300 mg. of crude potassium glucose sulphate preparation on Whatman no. 17 paper in 4.5 hr. The slowest-moving component isolated from the strip was identified as glucose. The fastest-moving component had R_G 0.42 and was glucose di-*O*-sulphate (Found: ester SO_4^{2-} , 46.3; K, 18.3. Calc. for $\text{C}_6\text{H}_{10}\text{O}_{12}\text{S}_2\text{K}_2$: ester SO_4^{2-} , 46.1; K, 18.7%).

The remaining component had R_G 0.66 and was glucose mono-*O*-sulphate (Found: ester SO_4^{2-} , 33.4; K, 13.2. Calc. for $\text{C}_6\text{H}_{11}\text{O}_9\text{SK}$: ester SO_4^{2-} , 32.2; K, 13.1%). Crude preparations of the ester sulphates of galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine could be separated under the same electrophoresis conditions.

Separation of sulphated products by electrophoresis on cellulose columns. The crude salt (0.5 g.), dissolved in 5 ml. of water, was converted into the free acid by passage through a Dowex 50 column (H^+ form; 100–200 mesh; 7 cm. \times 1 cm.). The acid eluate and washings were combined (9 ml.) and 2 ml. of cresol red was added as indicator. After draining the buffer (0.1M-ammonium acetate, pH 4.0) in the electrophoresis tube to the level of the cellulose bed, a 5 ml. portion of water was added to the column and allowed to drain in under gravity flow. The sample was then added to the cellulose bed and allowed to drain in as before. Flow through the column was stopped and the space above the cellulose bed washed thoroughly by the successive addition and removal of five 20 ml. portions of water. Finally, a fresh 5 ml. portion of water was added to the column and allowed to drain in slowly. The red indicator band was then displaced slowly down the column to the 30 cm. position by the addition of buffer.

The buffer in the electrode vessels was levelled and current was applied from a stabilized power source to yield a potential difference of 400 v, the electrode at the lower end of the electrophoresis tube being made the positive pole in the system. During the first 2 hr. the current rose from 15.0 to 19.5 mA, this period being marked by a distinct sharpening of the indicator zone, which decreased to a width of 0.75 cm. (cf. Porath, 1956). The period of zone-sharpening was also marked by a change in the colour of the indicator band, this change occurring bandwise from the lower edge of the band, until the latter was completely yellow in colour.

When zone-sharpening was complete, the level of buffer in the cathode chamber was lowered by 4 cm. to induce a hydrodynamic counterflow. This

difference in levels was maintained for 28 hr. The electrophoresis was then stopped and the separated zones were displaced with buffer. Eluates corresponding to elution peaks were examined by paper chromatography. The results are represented in Fig. 1.

On a preparative scale 4 g. of the potassium salt of the crude monosaccharide sulphate, dissolved in 15 ml. of water, was converted into the free acid as described above. The sample was applied to the column between two 10 ml. zones of water and displaced to the 30 cm. position with buffer. A potential difference of 500v was applied for 4 hr. The level of buffer in the cathode chamber was then lowered by 4 cm., this difference in levels being maintained throughout the remaining 26 hr. of the separation. Separated zones were then displaced and the components isolated for analysis. Results of typical separations are given below. The

components (disulphate, monosulphate and parent compound) are designated according to the order of elution from the column.

Glucose sulphate. Component 1, yield 0.8 g., R_G 0.42 (Found: ester SO_4^{2-} , 46.32; K, 18.8. Calc. for $C_6H_{10}O_{12}S_2K_2$: ester SO_4^{2-} , 46.1; K, 18.7%). Component 2, yield 2.3 g., R_G 0.66 (Found: ester SO_4^{2-} , 33.2; K, 12.9. Calc. for $C_6H_{11}O_9SK$: ester SO_4^{2-} , 32.2; K, 13.1%). Component 3, yield 0.22 g., R_G 1.0, was identified as glucose.

Galactose sulphate. Component 1, yield 0.52 g., R_G 0.42 (Found: ester SO_4^{2-} , 46.0; K, 18.9. Calc. for $C_6H_{10}O_{12}S_2K_2$: ester SO_4^{2-} , 46.1; K, 18.7%). Component 2, yield 2.25 g., R_G 0.59 (Found: ester SO_4^{2-} , 32.9; K, 13.3. Calc. for $C_6H_{11}O_9SK$: ester SO_4^{2-} , 32.2; K, 13.1%). Component 3, yield 0.09 g., R_G 0.95, was identified as galactose.

N-Acetylglucosamine sulphate. Component 1, yield 0.72 g., R_G 0.54 (Found: N, 3.9; ester SO_4^{2-} , 41.4; K, 16.8. Calc. for $C_8H_{13}O_{12}NS_2K_2$: N, 3.1; ester SO_4^{2-} , 42.2; K, 17.2%). Component 2, yield 2.1 g., R_G 0.76 (Found: N, 4.4; ester SO_4^{2-} , 28.1; K, 12.1. Calc. for $C_8H_{14}O_9NSK$: N, 4.1; ester SO_4^{2-} , 28.3; K, 11.5%). Component 3, yield 0.12 g., R_G 1.24, was identified as *N*-acetylglucosamine.

N-Acetylgalactosamine sulphate. Component 1, yield 0.12 g., R_G 0.51 (Found: N, 3.7; ester SO_4^{2-} , 42.8; K, 16.8. Calc. for $C_8H_{13}O_{12}NS_2K_2$: N, 3.1; ester SO_4^{2-} , 42.2; K, 17.2%). Component 2, yield 2.2 g., R_G 0.71 (Found: N, 3.8; ester SO_4^{2-} , 28.1; K, 11.2. Calc. for $C_8H_{14}O_9NSK$: N, 4.1; ester SO_4^{2-} , 28.3; K, 11.5%). Component 3, yield 0.19 g., R_G 1.13, was identified as *N*-acetylgalactosamine.

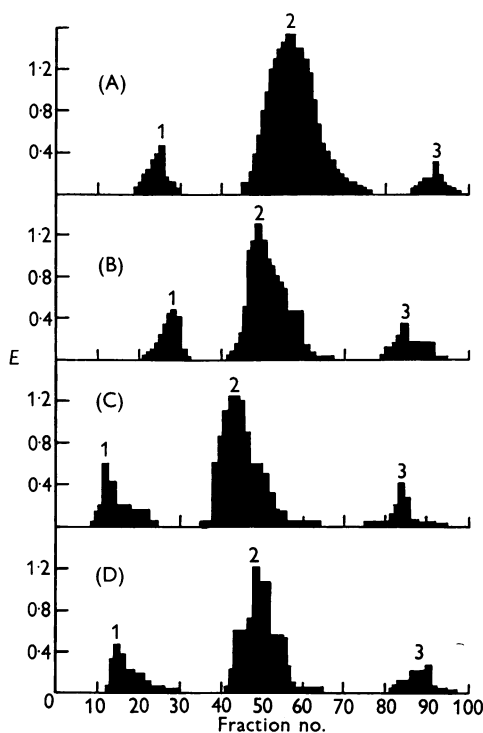


Fig. 1. Zone electrophoresis of crude sulphate esters on cellulose columns. The histograms represent extinctions of fractions in the anthrone procedure (Fairbairn, 1953). (A) Glucose sulphate: component 1, R_G 0.42; component 2, R_G 0.66; component 3, R_G 1.0. (B) Galactose sulphate: component 1, R_G 0.42; component 2, R_G 0.59; component 3, R_G 0.95. (C) *N*-Acetylglucosamine sulphate: component 1, R_G 0.54; component 2, R_G 0.76; component 3, R_G 1.25. (D) *N*-Acetylgalactosamine sulphate: component 1, R_G 0.51; component 2, R_G 0.71; component 3, R_G 1.15.

DISCUSSION

The separation of monosaccharide monosulphates from contaminating disulphate and parent compounds has been attempted in several ways. Unchanged glucose has been removed from glucose sulphate preparations by treating the crude sulphate product with yeast, which metabolizes the glucose but has no action on glucose sulphate, or by repeated recrystallization of the monosaccharide sulphate as the brucine salt (Soda, 1933). The former method is time-consuming and the latter (Dodgson & Spencer, 1954) does not result in the complete removal of the contaminating monosaccharide. Neither procedure can be used for the removal of the disulphate compound. Unsuccessful attempts have been made to remove the sulphated impurity by preferential hydrolysis with hydrazine and by selective precipitation of contaminating disulphate with 5-aminoacridine (A. G. Lloyd, unpublished results).

Methods have been reported for the fractionation of crude monosaccharide sulphates by cellulose-

column chromatography (Lloyd, 1959*a*; Turvey & Clancy, 1959) and anion-exchange resins (Turvey & Clancy, 1959). Cellulose-column chromatography is generally unsuitable for the handling of large quantities of material and requires reparation of the cellulose bed for each separation. Anion-exchange-resin chromatography, although of use in the fractionation of glycoside sulphates, has only limited uses for the purification of reducing monosaccharide sulphates, since degradative changes are catalysed (Dr K. S. Dodgson, personal communication). Zone electrophoresis on cellulose columns has been used successfully for the purification of the adenosine phosphosulphates obtained by the carbodi-imide procedure (Reichard & Ringertz, 1957). The principal advantage of zone electrophoresis for this purpose is that the cellulose bed may be used repeatedly without reparation. Further, the conditions required for separation are easily reproducible and the application of only moderately high potential differences allow the rapid separation of the components of the mixture. Purification of crude monosaccharide sulphates on a 10 g. scale may be readily achieved with 6 cm. diameter columns.

Evidence about the exact location of the sulphate group in *N*-acetylgalactosamine sulphate is still incomplete. Preliminary examination of the behaviour of the compound in the Elson-Morgan reaction (Lloyd, 1959*a*) suggested that the sulphate group occupies position 6 of the hexosamine molecule. Unsuccessful attempts to prepare triphenylmethyl (trityl) compounds from authentic glucose 6-*O*-sulphate, galactose 6-*O*-sulphate and *N*-acetylglucosamine 6-*O*-sulphate on the one hand, and *N*-acetylgalactosamine monosulphate, on the other hand, may be taken as additional evidence in support of this postulate (A. G. Lloyd, unpublished results). The inability to form trityl compounds from acetylglucopyranose isolated from *Bacillus megaterium* (Duff, Webley & Farmer, 1957; Duff & Webley, 1958) or from cerebron sulphate (Nakayama, 1951) has been taken as a criterion for the establishment of 6-*O*-substitution in these compounds. That this is true has been shown by chemical synthesis of the former (Duff & Farmer, 1958) and methylation studies with the latter (Thannhauser, Fellig & Schmidt, 1955). Further evidence for the location of the sulphate group on position 6 in *N*-acetylgalactosamine monosulphate has been obtained from the infrared spectra of this and related compounds (Lloyd & Dodgson, 1959).

SUMMARY

1. Methods have been developed for the preparation of the crude potassium salts of the sulphates of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine.

2. Pure potassium salts of glucose 6-*O*-sulphate, galactose 6-*O*-sulphate, *N*-acetylglucosamine 6-*O*-sulphate and *N*-acetylgalactosamine monosulphate have been obtained by zone electrophoresis on cellulose columns.

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