Biochem. J. (1973) 135, 187–192
Printed in Great Britain

Heparan Sulphate Sulphotransferase

PROPERTIES OF AN ENZYME FROM OX LUNG

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(*Received* 9 *March* 1973)

A heparan sulphate sulphotransferase was partially purified from an ox lung homogenate by $(NH_4)_2SO_4$ precipitation. Various glycosaminoglycans were assayed as sulphate acceptors with this enzyme. The highest acceptor activity was obtained with desulphated heparin and heparan sulphate, which indicates that sulphate transfer may be to free amino groups of the substrate. Some heparan sulphate was ^{35}S-labelled by incubation with the enzyme and re-isolated. On treatment of this heparan [^{35}S]sulphate with nitrous acid and separation of the degradation products on Sephadex G-15, a major peak of radioactivity was obtained, and identified as [^{35}S]sulphate by high-voltage electrophoresis at pH 5.3. The [^{35}S]sulphate is believed to be derived from *N*-[^{35}S]sulphated groups of heparan [^{35}S]-sulphate. That the ox lung preparation contained an *N*-sulphotransferase was confirmed by the isolation of 2-deoxy-2-[^{35}S]sulphoamino-D-glucose as the major product from the flavobacterial degradation of heparan [^{35}S]sulphate.

Heparan sulphate is an acidic glycosaminoglycan that occurs in many tissues including brain (Meyer *et al.*, 1959), lung (Jorpes & Gardell, 1948), aorta (Meyer *et al.*, 1956), hen oviduct (Schiller, 1959) and tissues of patients with Hurler's syndrome (Dorfman, 1958). Its function remains unclear, although some possibly important roles have recently received attention (Kraemer, 1971).

Some features of heparan sulphate composition and structure are established (for a review, see Brimacombe & Webber, 1964). Clearly, considerable polydispersity and heterogeneity occur in the structure. For example, heparan sulphate fractions with widely different molecular sizes, degree and type of sulphation, proportion of *N*-acetyl to *N*-sulphate groups, and glucuronate to iduronate content have been isolated.

Sulphate esters are biosynthesized by transfer of sulphate groups from adenosine 3′-phosphate 5′-sulphatophosphate to a wide variety of acceptors, in reactions catalysed by specific sulphotransferases. Likewise, heparan sulphate may be sulphated by enzymes which have been reported in hen oviduct (Suzuki & Strominger, 1960) and brain (Balasubramanian & Bachhawat, 1964).

This paper reports the finding of a heparan sulphate sulphotransferase in ox lung, its partial purification and some of its properties. The main purpose of this study was to determine the location within heparan sulphate of enzymically transferred sulphate groups. Thus future studies of the enzyme's substrate specificity and purification will be facilitated.

A preliminary report of this work has been published (Foley & Baker, 1971).

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Experimental

Materials

Heparan sulphate (a fraction eluted from Dowex 1, X2, with 1.25 M-NaCl) was kindly provided by Dr. J. A. Cifonelli, Department of Pediatrics, University of Chicago, Chicago, Ill., U.S.A.

N-Desulphated heparan sulphate was prepared by hydrolysing heparan sulphate in 0.04 M-HCl at 100°C for 90 min (Jorpes *et al.*, 1950). Heparin (114 units/mg) was purchased from Wilson Laboratories, Chicago, Ill., U.S.A. *Flavobacterium heparinum* (A.T.C.C. 13125) was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., U.S.A. 2-Deoxy-2-[^{35}S]sulphoamino-D-glucose, chemically synthesized by the method of Lloyd *et al.* (1964), was a generous gift from Dr. B. Wisdom, The Queen's University, Belfast, U.K. Ox lung was collected immediately after slaughter of animals from the International Meat Co. Ltd., Dublin, and was stored at −20°C until required. Sodium [^{35}S]sulphate (up to 100 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Tryptone and soya peptone are products of Oxoid Ltd., London S.E.1, U.K. ATP (disodium salt) was purchased from Sigma Chemical Co., St. Louis,

Mo., U.S.A., and Cellex-E was obtained from Biorad Laboratories, Richmond, Calif., U.S.A.

Methods

High-voltage paper electrophoresis. A Gilson model D instrument was employed. The buffer used was pyridinium acetate, pH5.3 [pyridine (100ml) and acetic acid (32ml) in 10 litres of water]. All separations were on Whatman 3MM paper.

Paper chromatography. Paper chromatograms were developed in either solvent A (isobutyric acid–aq. 0.5M-NH_3, 5:3, v/v) or in solvent B (butan-1-ol–acetic acid–water, 10:3:6, by vol.). Reducing compounds were located with $AgNO_3$–NaOH reagent (Smith, 1960).

Determination of protein. This was done by the method of Lowry *et al.* (1951). Crystalline bovine plasma albumin, purchased from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., was used as a standard.

Measurement of radioactivity on paper. Paper (2.5cm×3.8cm) bearing radioactive material was immersed in scintillation fluid (10ml) containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene and counted for radioactivity in a Packard Tri-Carb scintillation spectrometer, model 3375.

Preparation of adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate. The biosynthetic method as described by McEvoy & Carroll (1971) was used.

Preparation of a 'heparitinase' fraction from F. heparinum. Cells of *F. heparinum* were grown, with agitation, in a medium containing tryptone (17g), soya peptone (3g), NaCl (5g), K_2HPO_4 (2.5g) and heparin (150mg) in a volume of 1 litre at pH6.5. After a 24h growth period at room temperature, cells were harvested by centrifugation, washed with 0.1M-sodium acetate, pH7.0, and freeze-dried. The freeze-dried cells were suspended in 0.1M-sodium acetate, pH7.0, at a concentration of 20mg/ml and sonicated, with a Soniprobe (1130A 20kHz) sonicator, three times for 2min with the temperature kept below 20°C. The sonicate was centrifuged at 10000*g* for 30min to remove most cellular debris, and then the supernatant was centrifuged at 100000*g* for 1.5h.

Preparation of heparan sulphate sulphotransferase from ox lung. Ox lung (55g) was cut into cubes, minced, suspended in 4vol. of 20mM-sodium phosphate buffer, pH7.0, and then homogenized for 2min at maximum speed in a Waring Blendor at 4°C. The homogenate was passed through three layers of cheesecloth and the filtrate was freeze-dried (weight 6.7g). This preparation could be stored at −18°C *in vacuo* over silica gel without appreciable loss of heparan sulphate sulphotransferase activity.

To re-solubilize the enzyme, the freeze-dried material (2g) was suspended at 20mg/ml in 20mM-sodium phosphate buffer, pH7.0, and stirred for 18h at 4°C. The suspension was centrifuged at 25000*g* for 45min and the supernatant, which contained most of the enzyme activity, was separated. Solid $(NH_4)_2SO_4$ was added to this supernatant to give a final concentration of 40% saturation and the mixture was centrifuged at 10000*g* for 15min. The 40% saturation supernatant was adjusted to 60% saturation by addition of solid $(NH_4)_2SO_4$ and centrifuged again. This 40–60%-satd. $(NH_4)_2SO_4$ pellet, which contained most of the heparan sulphate sulphotransferase activity, was dissolved in 3ml of 20mM-sodium phosphate buffer, pH7.0, and dialysed against the same buffer for 12h at 4°C. This preparation could be stored at −18°C with little loss of activity and was used as 'heparan sulphate sulphotransferase' in the experiments detailed below.

Assay of heparan sulphate sulphotransferase. The conditions employed were similar to those described by Suzuki & Strominger (1960). The incubation mixture contained (except where otherwise stated): enzyme (200μg of protein), *N*-desulphated heparan sulphate (100μg), adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate (approx. 2×10^5 d.p.m.; sp. radioactivity 15mCi/mmol), $MgCl_2$ (0.2μmol), KF (2μmol) and sodium phosphate buffer, pH7.0 (2μmol), in a final volume of 100μl. Incubations were at 37°C for 1h and the reaction was stopped by heating at 100°C for 3min. After centrifugation, 20μl of the supernatant was applied to a chromatogram and developed for 40h in solvent A. The area around the origin was cut out and assayed for [^{35}S]sulphate by scintillation counting. By using acceptor-less incubated and non-incubated controls it was found that little, if any, [^{35}S]sulphate transfer to endogenous acceptors took place.

Preparation of heparan [^{35}S]sulphate. An incubation mixture was prepared on a scale one hundred times that given for 'Assay of heparan sulphate sulphotransferase'. Incubation was for 3h. The mixture was then heat-treated, centrifuged, and the supernatant chromatographed in solvent A. Heparan [^{35}S]sulphate, which remains origin-bound, was eluted from the paper with water and freeze-dried. The product (sp. radioactivity approx. 3×10^6 d.p.m.; 10mg dry wt.) was dissolved in water (1.0ml) and stored at −20°C.

Acetylation of heparan [^{35}S]sulphate. The method was essentially as described by Fraenkel-Conrat (1957).

A portion (0.5 ml) of the heparan [^{35}S]sulphate preparation was added to saturated sodium acetate soln. (0.5ml) at 4°C. Five 10μl portions of acetic anhydride were added with mixing during 1h, while the pH was maintained at 8–9. To remove Na^+, the solution was passed through a column (5cm×0.5cm) of Dowex 50 (X8; H^+ form) and subsequently washed with water (5ml). Effluent and washings were

combined, freeze-dried, dissolved in water (0.5ml) and stored at −20°C.

Results

Heparan sulphate sulphotransferase in rat tissues

A young male rat (120g) was killed and kidney, lung, small intestine, liver and brain were removed. Homogenates of each tissue were made immediately in 4vol. of 20mM-sodium phosphate buffer, pH7.0. Samples (20μl) of each homogenate, containing 200μg of protein, were assayed for heparan sulphate sulphotransferase as described in the Experimental section. Only brain and lung preparations contained marked activity (Table 1). An ox lung homogenate was similarly assayed and found to be active. For convenience subsequent studies of heparan sulphate sulphotransferase were made with the ox lung enzyme only.

Some properties of the ox lung heparan sulphate sulphotransferase were determined by using a 40–60%-satd. $(NH_4)_2SO_4$ fraction. The enzyme was incubated in a final concentration of 20mM-sodium citrate–phosphate buffer over the pH range shown in Fig. 1. The optimum pH for sulphate transfer to *N*-desulphated heparan sulphate was in the range 6.2–6.4. Therefore assay of the enzyme was subsequently carried out at pH6.4.

Sulphate incorporation into *N*-desulphated heparan sulphate is directly proportional to the time of incubation during the first 60min at 37°C. There is a linear relationship between enzyme activity and amount of enzyme up to a protein concentration of 600μg. Routine enzyme assays were carried out at 37°C for 1h with 200μg of protein per incubation tube. Sulphate transfer to *N*-desulphated heparan sulphate increased up to an adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate concentration of 180μM (Fig. 2). Adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate of constant specific radioactivity was used in this experiment. Routine assay of the enzyme activity was carried out at an adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate concentration of 100μM.

Optimum substrate concentration was established for both *N*-desulphated heparan sulphate and *N*-desulphated heparin (Fig. 3). Routine enzyme assays were carried out with *N*-desulphated heparan sulphate at a final concentration of 1mg/ml.

The incorporation of sulphate into some potential acceptors was investigated (Table 2). Incubation conditions were the same as given under 'Assay of heparan sulphate sulphotransferase', except that the

Table 1. *Distribution of heparan sulphate sulphotransferase in rat tissues*

Details of incubation conditions are given in the text.

Tissue	[^{35}S]Sulphate transferred to *N*-desulphated heparan sulphate (d.p.m.)
Brain	9180
Lung	10830
Kidney	60
Liver	345
Small intestine	60

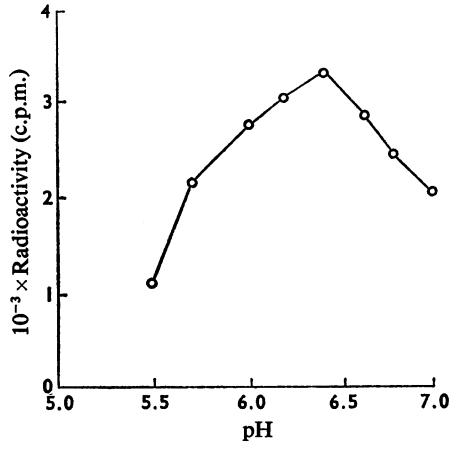

Fig. 1. *Effect of pH on enzyme activity*

Details are given in the text.

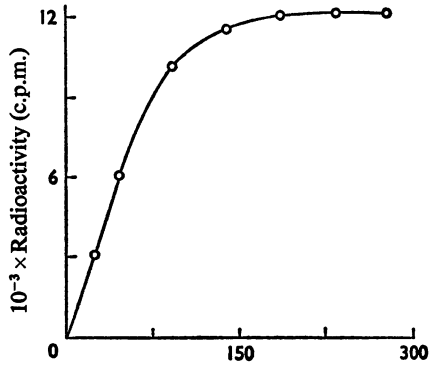

Fig. 2. *Heparan [^{35}S]sulphate formation as a function of adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate concentration*

Details are given in the text.

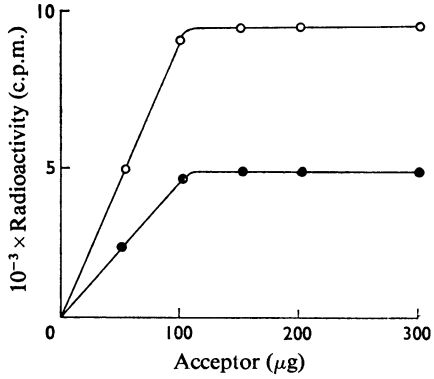

Fig. 3. *Influence of sulphate acceptor concentration on the enzymic sulphation of N-desulphated heparan sulphate* (●) *and N-desulphated heparin* (○)

Details are given in the text.

Table 2. *Effect of N-desulphation on the acceptor properties of heparan sulphate and heparin*

Details are given in the text.

Substrate	[^{35}S]Sulphate incorporation (d.p.m.)
None	685
Heparin	605
N-Desulphated heparin	11805
Heparan sulphate	4795
N-Desulphated heparan sulphate	9075

acceptor was varied. That the *N*-desulphated forms of both heparin and heparan sulphate were good acceptors strongly suggests that sulphate transfer is to free amino groups of glucosamine residues.

Treatment of heparan [^{35}S]sulphate with nitrous acid

Heparan [^{35}S]sulphate (5μl; approx. 1×10^4 d.p.m.) was dissolved in water (0.25ml) and incubated at room temperature with $NaNO_2$ (5%, w/v; 0.25ml) and acetic acid (33%, v/v; 0.25ml) for 3h. Nitrous acid was then removed by repeated evaporations with methanol (Cifonelli, 1968). After concentration to 0.1ml, the preparation was applied to a Sephadex G-15 column (41cm × 1cm) and developed with 0.1M-pyridine acetate, pH5.0. Column fractions (1.0ml) were assayed for radioactivity (Fig. 4). Most radioactivity was eluted betweeen 17ml and 20ml, which coincides with the elution volume of inorganic sulphate. High-voltage electrophoresis at pH5.3 confirmed that the radioactivity in the peak was

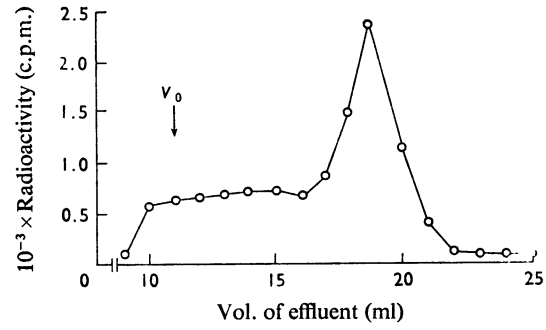

Fig. 4. *Gel-filtration pattern from Sephadex G-15 of products obtained after reaction of heparan [^{35}S]-sulphate with nitrous acid*

Details are given in the text.

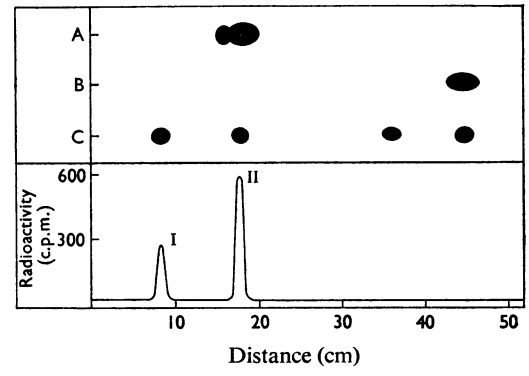

Fig. 5. *Tracing of chromatographic separation of 'heparitinase'-degradation products of acetylated heparan [^{35}S]sulphate*

A, Authentic 2-deoxy-2-sulphoamino-D-glucose; B, *N*-acetylhexosamine; C, products of a 12h incubation mixture. The chromatogram was stained with $AgNO_3$–NaOH reagent. Experimental details are given in the text.

largely associated with inorganic sulphate. A relatively small amount of radioactive material, presumably ^{35}S-labelled oligosaccharides, was eluted earlier than the [^{35}S]sulphate.

Degradation of heparan [^{35}S]sulphate by 'heparitinase' from F. heparinum

Acetylated heparan [^{35}S]sulphate (10μl; approx. 2×10^4 d.p.m.) and heparan sulphate (7.5mg) were incubated in 3.0ml of 0.1M-sodium acetate buffer, pH7.0, with 'heparitinase' (30mg of protein) at 25°C for 12h. The mixture was then heated at 100°C for

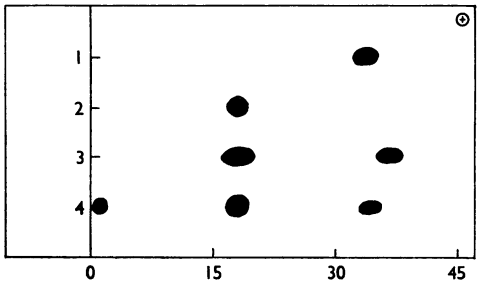

Fig. 6. *Tracing of high-voltage electrophoretogram of 'heparitinase'-degradation products of heparan [^{35}S]-sulphate*

Electrophoresis was carried out at 53 V/cm for 40 min. 1, Fraction I from 12 h incubation mixture; 2, fraction II from 12 h incubation mixture; 3, authentic 2-deoxy-2-sulphoamino-D-glucose; 4, products of a 12 h incubation mixture. The electrophoretogram was stained with $AgNO_3$–NaOH reagent.

3 min to denature and precipitate protein, centrifuged, and the supernatant separated and concentrated to 0.5 ml.

A portion (50 μl) was chromatographed in solvent A. Radioactive zones of the chromatogram were detected and measured by cutting it into 1 cm segments and scintillation counting. A duplicate chromatogram, after development, was stained with the $AgNO_3$–NaOH reagent. The results are illustrated in Fig. 5, and show fractionation of the radioactive products into two zones (labelled I and II), both of which are symmetrical. The 12 h incubation mixture contained four distinct Ag-staining products, one of which has an R_F identical with that of the major radioactive peak II and of authentic 2-deoxy-2-sulphoamino-D-glucose. A zero-time incubation (not shown in Fig. 5) contains origin-bound radioactivity only and no migrating Ag-staining material.

Fractions I and II were eluted with water from a duplicate chromatogram and electrophoresed on paper at pH 5.3 (Fig. 6). Fraction II was homogeneous and had a mobility identical with that of 2-deoxy-2-sulphoamino-D-glucose. Fraction I also appeared homogeneous, and in this system migrated further from the origin than fraction II. In solvent B, fraction II again migrated as 2-deoxy-2-sulphoamino-D-glucose.

Discussion

The assay of various rat tissue homogenates for heparan sulphate sulphotransferase activity clearly demonstrated maximal activities in brain and lung preparations. The presence of this enzyme in lung has not previously been reported, but is not unexpected, as lung is a source of heparan sulphate, which is presumably synthesized *in situ*. Subsequently, the enzyme has been found in and partially purified from ox lung. It is possible that other enzymes of heparan sulphate biosynthesis may conveniently be purified and studied from this same source.

Some factors influencing the rate of the enzyme reaction (e.g. pH, adenosine 3′-phosphate 5′-sulphatophosphate concentration) have been studied. Thus it is possible to estimate enzyme activity and the relative efficiency of substrates under apparently optimum conditions. *N*-Desulphated heparin appears, at all concentrations used, to be a better substrate than *N*-desulphated heparan sulphate, so the enzyme may be responsible primarily for the *N*-sulphation of heparin rather than heparan sulphate.

Treatment of heparan sulphate or heparin with nitrous acid under the conditions described causes hydrolysis of sulphoamino linkages with release of inorganic sulphate. Some 60% of the [^{35}S]sulphate label of enzymically prepared heparan [^{35}S]sulphate is liberated as inorganic [^{35}S]sulphate by applying this procedure. As *O*-sulphate esters are stable to this treatment, it is evident that 60% of the [^{35}S]sulphate label enzymically transferred was to amino groups of heparan sulphate.

To verify that 2-deoxy-2-[^{35}S]sulphoamino-D-glucosyl residues were present in the heparan [^{35}S]-sulphate, the latter was digested with a *F. heparinum* preparation. Such a preparation contains enzymes that degrade heparin (or heparan sulphate) to low-molecular-weight products including 2-deoxy-2-sulphoamino-D-glucose (Dietrich, 1968). The major [^{35}S]sulphate-labelled product from heparan [^{35}S]-sulphate had the same R_F value as authentic 2-deoxy-2-sulphoamino-D-glucose in three separation systems (paper electrophoresis at pH 5.3 and paper chromatography in solvents A and B). This isolated fraction appeared homogeneous and identical with 2-deoxy-2-sulphoamino-D-glucose.

Silbert (1967) showed that a microsomal preparation from mouse mastocytoma in the presence of adenosine 3′-phosphate 5′-sulphatophosphate catalyses the concomitant and approximately equimolar *N*-deacetylation and *N*-sulphation of a heparin precursor. The sulphotransferase enzyme from lung, as reported here, and the mouse mastocytoma system as studied by Balasubramanian *et al.* (1968), catalyse the sulphation of free amino groups. Furthermore, recent work by U. Lindahl (personal communication) shows that newly synthesized heparin bears free amino groups and that *N*-sulphate groups may be formed by sulphation of such preformed free amino groups. Therefore it seems that *N*-deacetylation and *N*-sulphation are not obligatorily linked.

The products of nitrous acid treatment of heparan

[^{35}S]sulphate included some [^{35}S]sulphate-labelled oligosaccharide fragments (Fig. 4), in which the [^{35}S]sulphate must be *O*-ester linked. Thus it follows that the ox lung preparation, although mainly transferring sulphate to free amino groups of heparan sulphate, possesses some *O*-sulphotransferase activity. The location in heparan [^{35}S]sulphate of these sulphate ester groups has been the subject of a previous report (Foley & Baker, 1973).

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