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Biosynthesis of Heparin

SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF *N*- AND *O*-SULPHOTRANSFERASES

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Assay methods were developed enabling separate determination of *N*- and *O*-sulphotransferase activities in an enzyme preparation from mouse mastocytoma. *N*-Desulphoheparin and chemically *N*-acetylated heparan sulphate were used as specific exogenous sulphate acceptors in the transfer of [^{35}S]sulphate residues from adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate to amino and hydroxyl groups respectively. The resulting ^{35}S-labelled polysaccharides were isolated as their cetylpyridinium complexes on filter paper. Sulphotransferases were solubilized from a mastocytoma microsomal fraction by treatment with detergent–alkali. The pH optimum for both enzymes was about 7.5. K_m with regard to adenosine 3′-phosphate 5′-sulphatophosphate was estimated to be 2×10^{-5}M for the *N*-sulphotransferase and 1×10^{-4}M for the *O*-sulphotransferase(s). The enzymes required bivalent cations for maximum activity, Mn^{2+} stimulating both the *N*- and *O*-sulphotransferase activities four- to five-fold, whereas Ca^{2+} increased the *N*- but not the *O*-sulphotransferase activity. The *O*-sulphotransferase was found to be more sensitive to heat-inactivation, 60% of the activity being lost after 1 min at 50°C, whereas only 15% of the *N*-sulphotransferase activity was lost. In contrast, the *N*-sulphotransferase was selectively inhibited (or inactivated) by NaCl; at 0.125M-NaCl concentration the *O*-sulphotransferase activity was essentially unaffected, whereas the *N*-sulphotransferase activity was depressed by 80%. These results strongly indicate that *N*- and *O*-sulphate-transfer reactions should be ascribed to different enzymes, or, alternatively, to separate and independent active sites on the same enzyme molecule.

Enzymes involved in the biosynthesis of glycosaminoglycans are located in the rough and smooth endoplasmic reticulum and in the Golgi system (Silbert, 1966; Dodgson & Rose, 1970; Rodén, 1970). The main features of the biosynthetic processes are fairly well understood. However, certain aspects of the polymerization and sulphation reactions remain to be elucidated. The sulphation process, which requires adenosine 3′-phosphate 5′-sulphatophosphate as sulphate donor, seems to proceed either after or along with polymerization; sulphated monosaccharide units are thus not incorporated into the glycosaminoglycan chain.

Transfer of [^{35}S]sulphate from adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate to endogenous or exogenous polysaccharide acceptors has been demonstrated by experiments *in vitro*, by using cell-free enzyme preparations from a number of tissues, including mouse mastocytoma (Ringertz, 1963; Silbert, 1967; Balasubramanian *et al.*, 1968), chick cartilage (Meezan & Davidson, 1967), rat brain (George *et al.*, 1970), mouse liver (Greiling *et al.*, 1972), ox lung (Foley & Baker, 1973) and hen oviduct (Suzuki & Strominger, 1960) or uterus (Johnson & Baker, 1973). Enzyme from different tissues was found to require different glycosaminoglycan acceptors for maximum sulphate-transfer activity (Rodén, 1970), suggesting the occurrence of several specific sulphotransferases. Further, the sulphation of a single polysaccharide species may well be catalysed by more than one enzyme. For example, heparin and heparan sulphate carry sulphate substituents at several positions, as sulphamino groups (C-2 of the glucosamine residues) and ester sulphate groups [C-6 of the glucosamine and C-2 of the iduronic acid residues respectively (see Lindahl & Axelsson, 1971)]. The formation of both *N*- and *O*-sulphate groups (sulphamino and ester sulphate groups respectively) was demonstrated by Balasubramanian *et al.* (1968), who studied the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to exogenous heparan sulphate and *N*-desulphoheparin, catalysed by an enzyme from mouse mastocytoma. These workers even succeeded in solubilizing the enzymes, from a 'postmicrosomal' particulate fraction, by treatment with snake venom phospholipase. However, as convenient methods for the distinction between

N- and *O*-sulphation were unavailable at the time, the properties of the corresponding enzymes were not studied further.

The present work was undertaken to establish experimental conditions for the selective incorporation of sulphate into *N*- and *O*-sulphate groups respectively, thus enabling characterization of the separate enzymes. The methods developed were applied to a study on sulphotransferases involved in the biosynthesis of heparin.

Materials and Methods

Materials

A mast-cell tumour (Furth *et al.*, 1957) was maintained in (A/Sn×Leaden)F_1 mice, as described by Ögren & Lindahl (1971).

Heparin (prepared from pig intestinal mucosa; stage 14) was purchased from Wilson Laboratories, Park Forest South, Ill., U.S.A., and purified as described by Lindahl *et al.* (1965). The purified material contained 2.4 sulphate residues/disaccharide unit. Samples of dermatan sulphate and chondroitin 4-sulphate, prepared from bovine aorta, [samples DSIII and CS I (see Iverius, 1971)] and heparan sulphate (0.46 sulphate residue/disaccharide unit), isolated from human aorta [sample II (see Iverius, 1971)] were kindly given by Dr. P.-H. Iverius of this Institute. Acetylation of free amino groups in heparan sulphate and heparin was accomplished by treatment of the polysaccharides with acetic anhydride (Danishefsky & Steiner, 1965); the resulting preparations will be referred to as *N*-acetylated heparan sulphate and *N*-acetylated heparin respectively. All polysaccharides subjected to chemical *N*-acetylation were completely devoid of unsubstituted amino groups, as indicated by gel chromatography before and after treatment with HNO_2 (reaction B; see below). *N*-Desulphoheparin was prepared by heating the purified heparin in 0.04M-HCl (1mg/ml) at 100°C for 90min, followed by dilution with water and freeze-drying (Wolfrom & Shen Hahn, 1961). Keratan sulphate, prepared from bovine cornea (Laurent & Anseth, 1961), was kindly provided by Professor T. C. Laurent of this Institute.

Mono- and di-sulphated uronosylanhydromannose reference disaccharides were isolated from heparin, after deaminative degradation of the polysaccharide (Höök *et al.*, 1974).

Carrier-free inorganic $^{35}SO_4^{2-}$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate (specific radioactivity 12μCi/μmol) was prepared by the method of Balasubramanian *et al.* (1967).

Pronase was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and used as a 1% (w/v) solution in water. Dextran (Sephadex G-50) and agarose (Sepharose 6B) gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Cetylpyridinium chloride was a product of AB Recip, Stockholm, Sweden.

Analytical methods

Uronic acid was determined by the carbazole method of Bitter & Muir (1962). Protein was estimated as described by Lowry *et al.* (1951) with serum albumin as standard. Radioactivity was determined with a Packard model 2001 liquid-scintillation counter. Insta-Gel (Packard Instruments Corp., La Grange, Ill., U.S.A. was used as scintillation medium.

High-voltage electrophoresis was conducted on Whatman 3MM paper in 0.08M-pyridine–0.046M-acetic acid, pH5.3, at 80V/cm. Papers were stained with a silver-dip reagent (Smith, 1960); radioactive components were detected with a Packard model 7201 radiochromatogram scanner.

Gel chromatography was performed on columns (1cm×90cm or 1cm×100cm) of Sephadex G-50 equilibrated with 0.12M-pyridine–0.10M-acetic acid or with 1M-NaCl respectively, or on a column (1cm×85cm) of Sepharose 6B equilibrated with 2% (v/v) Tween-20 in 0.05M-Hepes* buffer, pH7.4. All the columns were eluted at a rate of 4ml/h; fractions of volume 2.0–2.5ml were collected.

Preparation of microsomal enzymes

A microsomal fraction (105000*g* precipitate) was prepared from homogenized mouse mastocytoma tissue (20000*g* supernatant) as reported previously (Lindahl *et al.*, 1973). Microsomal enzymes were solubilized essentially as described by Helting (1971). The particulate fraction was suspended in 0.05M-Tris–acetate buffer, pH7.4, containing 1mM-EDTA and 0.07M-KCl (20mg of protein/ml) and centrifuged at 105000*g* for 60min; the resulting pellet was resuspended in the same buffer (20mg of protein/ml) by sonication, and Tween-20 was added to a concentration of 2%, by volume. The mixture was adjusted to pH10.4 by the addition of 12M-NH_3 and was then quickly readjusted to pH7.4 with concentrated acetic acid. Particulate material was separated from the solubilized components by centrifugation at 105000*g* for 60min. The supernatant was dialysed against 0.05M-Hepes buffer, pH7.4, containing 2% (v/v) Tween-20, and stored at −20°C until used. All operations were performed at +4°C.

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*'-yl)ethanesulphonic acid; Mes, 2-(*N*-morpholino)-ethanesulphonic acid.

Incubation of solubilized enzymes with adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate and exogenous polysaccharide acceptors

Standard incubation mixtures (250μl) consisted of 0.5mg of solubilized microsomal protein, 200μg of exogenous polysaccharide, and 0.05μmol of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate in 0.05M-Hepes buffer, pH7.4, containing 10mM-$MgCl_2$, 10mM-$MnCl_2$ and 5mM-$CaCl_2$. Incubations were carried out at 37°C for 30min and were terminated by heating at 100°C for 2min. The incubation mixtures were digested with Pronase (15μl) at 55°C for 15h. After heat inactivation (100°C, 2min) precipitated protein was removed by centrifugation. Deviations from the standard incubation conditions are described in the legends to Tables and Figures.

Quantification of ^{35}S-labelled polysaccharide

The incorporation of [^{35}S]sulphate into polysaccharide was estimated by either of the following procedures. Portions (100μl) of the Pronase-treated incubation mixtures (see above) were chromatographed on Sephadex G-50 in 1M-NaCl; ^{35}S-labelled polysaccharide was eluted at the void volume of the column, well separated from labelled low-molecular-weight components. Alternatively, 100μl samples were spotted on strips of Whatman 3MM paper; the strips were washed with five changes of 1% (w/v) cetylpyridinium chloride in 0.05M-NaCl, cut into small pieces and subjected to liquid-scintillation counting (Wasteson *et al.*, 1973).

Degradation of polysaccharides with HNO_2

Degradation of polysaccharides with HNO_2 was performed by reactions A and B respectively, as described by Lindahl *et al.* (1973). The two procedures, differing with respect to the concentrations of NO_2^- and acetic acid used, permit selective deamination of the polysaccharide. Reaction A (treatment with 0.24M-$NaNO_2$ in 1.8M-acetic acid for 80min at room temperature) cleaves the glycosidic bonds of *N*-unsubstituted or *N*-sulphated glucosamine residues, with concomitant release of *N*-sulphate groups as inorganic SO_4^{2-}. Reaction B (treatment with 3.9M-$NaNO_2$ in 0.28M-acetic acid for 10min) attacks the glycosidic bonds of *N*-unsubstituted glucosamine units only.

Results

Incorporation of [^{35}S]sulphate into exogenous glycosaminoglycan acceptors

During incubation of *N*-desulphoheparin or of *N*-acetylated heparan sulphate with adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, in the presence of solubilized protein from the mastocytoma microsomal fraction, [^{35}S]sulphate was incorporated into the polysaccharides. The labelled products were recovered either by gel chromatography or by precipitation with cetylpyridinium chloride on filter paper. A comparison between the two methods of quantification showed consistently lower recoveries with the precipitation method (Table 1); this discrepancy applied particularly to the ^{35}S-labelled *N*-desulphoheparin. However, as the losses were fairly reproducible, the latter method, enabling

Table 1. *Estimation of ^{35}S incorporation into N-desulphoheparin (a) and N-acetylated heparan sulphate (b)*

Incubations were performed with solubilized enzyme, under standard conditions, for various periods of time. ^{35}S-labelled polysaccharides were quantified after gel chromatography on Sephadex G-50 or after precipitation on filter paper with cetylpyridinium chloride. For further experimental details, see the text.

Incubation time (min)	^{35}S-labelled polysaccharide ($10^{-3}\times$c.p.m.) estimated by: Gel chromatography (a)	Gel chromatography (b)	Precipitation procedure (a)	Precipitation procedure (b)
15	1.43	4.18	0.89	3.22
30	3.38	7.72	1.48	5.95
60	6.12	9.74	3.07	7.97
120	7.59	11.15	3.33	9.07

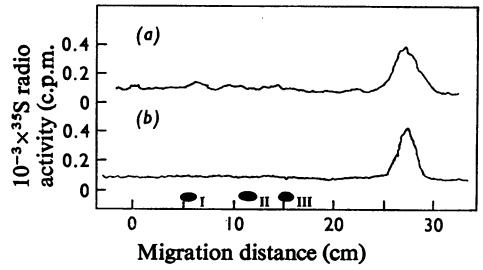

Fig. 1. *High-voltage electrophoresis of ^{35}S-labelled N-desulphoheparin, after deaminative cleavage with HNO_2*

N-Desulphoheparin was incubated under standard conditions (see the Materials and Methods section). After digestion with Pronase the incubation mixture was chromatographed on Sephadex G-50 in 0.12M-pyridine–0.10M-acetic acid. ^{35}S-labelled polysaccharide was recovered from the concentrated void-volume fractions by precipitation with 70% (v/v) ethanol. The degradation products obtained after treatment of the polysaccharide with HNO_2 (reaction A; see the Materials and Methods section) were analysed by high-voltage paper electrophoresis at pH5.3. (*a*) Sample; (*b*) inorganic [^{35}S]-sulphate. Additional standards: (I) glucuronic acid; (II) monosulphated uronosylanhydromannose; (III) disulphated uronosylanhydromannose.

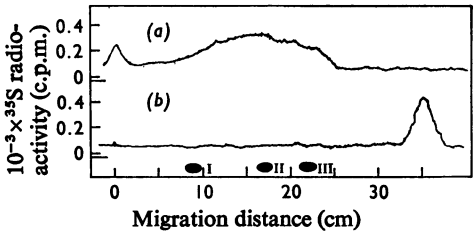

Fig. 2. *High-voltage electrophoresis of ^{35}S-labelled N-acetylated heparan sulphate, after deaminative cleavage with HNO_2*

N-Acetylated heparan sulphate was incubated under standard conditions (see the Materials and Methods section). (*a*) Sample; (*b*) inorganic [^{35}S]sulphate. For additional experimental details, as well as a description of standards, see the legend to Fig. 1.

Table 2. *Incorporation of [^{35}S]sulphate into various exogenous polysaccharide acceptors*

Incubations were carried out under standard conditions (see the Materials and Methods section), with solubilized enzyme.

Acceptor	^{35}S incorporated (c.p.m.)
N-Desulphated heparin	1570
N-Acetylated heparan sulphate	12060
Dermatan sulphate	1900
N-Acetylated heparin	140
Chondroitin 4-sulphate	430
Keratan sulphate	130
No acceptor present	80

simple and rapid determinations of ^{35}S-labelled polysaccharide, was used throughout this investigation. Duplicate samples, analysed by this method invariably differed by less than 10%.

The positions of the [^{35}S]sulphate groups incorporated into *N*-desulphoheparin and *N*-acetylated heparan sulphate were determined by deaminative degradation (reaction A; see the Materials and Methods section), followed by paper electrophoresis at pH 5.3. *N*-Desulphoheparin was thus found to act as acceptor for the transfer of sulphate to amino groups only, since all polysaccharide-bound radioactivity was released as inorganic [^{35}S]sulphate (Fig. 1). In contrast, none of the [^{35}S]sulphate transferred to *N*-acetylated heparan sulphate appeared as inorganic sulphate after deamination; all ^{35}S-labelled degradation products migrated like oligosaccharides in the electrophoresis system used (Fig. 2). It is thus concluded that the sulphate residues transferred to *N*-desulphoheparin and to *N*-acetylated heparan sulphate were specifically incorporated into

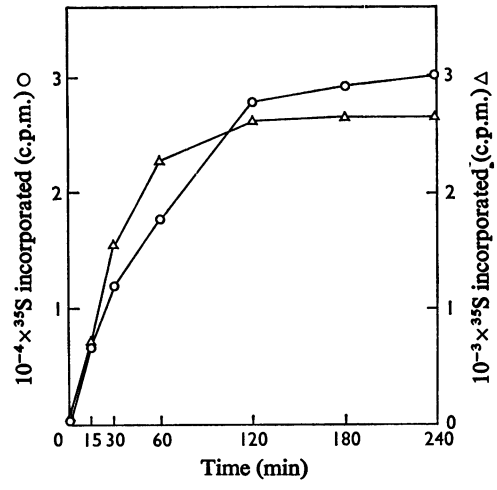

Fig. 3. *Time-course of incorporation of [^{35}S]sulphate into N-desulphoheparin* (△) *and N-acetylated heparan sulphate* (○)

Except for the variations in incubation time, incubations were carried out by the standard procedure (see the Materials and Methods section), with solubilized enzyme.

Table 3. *Recovery of N- and O-sulphotransferase in subcellular fractions from homogenized mouse mastocytoma tissue*

The total enzyme activities and protein contents of the various fractions are expressed as percentages related to the starting material (20000*g* supernatant).

Subcellular fraction	Total activity of: *N*-Sulpho-transferase (%)	*O*-Sulpho-transferase (%)	Total protein (%)
105000*g* pellet	86	79	6
105000*g* supernatant	14	21	94
Solubilized fraction of 105000*g* pellet	65	195	4
Non-solubilized residue of 105000*g* pellet	16	24	3

N-sulphate and *O*-sulphate groups respectively.

Under standard incubation conditions with solubilized enzyme (see below), *N*-acetylated heparan sulphate was found to be a far more effective sulphate acceptor than was *N*-desulphoheparin (Table 2). Of other glycosaminoglycans tested (Table 2), appreciable amounts of [^{35}S]sulphate were also incorporated into dermatan sulphate and, although to a lesser extent, into chondroitin sulphate. *N*-Acetylated heparin and keratan sulphate were essentially inactive as exogenous sulphate acceptors.

Assays of N- and O-sulphotransferase activities

The time-course of [^{35}S]sulphate incorporation into *N*-desulphoheparin and *N*-acetylated heparan sulphate respectively, under standard incubation conditions, is shown in Fig. 3. With both *N*- and *O*-sulphotransferase, the incorporation of radioactivity was linear with time for approx. 30min and ceased after about 2h. During the initial 30min period approx. 0.7nmol of *N*-sulphate and 6nmol of *O*-sulphate respectively were incorporated per mol of substrate disaccharide. The decrease in incorporation rate with incubation time is probably due to hydrolytic destruction of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate (Balasubramanian *et al.*, 1968). The amount of sulphate incorporated, during 30min of standard incubation, was proportional to protein concentration up to at least 3 mg/ml. Under optimum conditions, including appropriate concentrations of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate and bivalent metal ions (see below), the procedure developed could thus be used for separate and quantitative determinations of *N*- and *O*-sulphotransferase activities.

Analysis of particulate and solubilized mastocytoma sulphotransferases

A 20000*g* supernatant from homogenized mouse mastocytoma tissue was fractionated as described in Table 3. The *N*- and *O*-sulphotransferase activities in the various fractions were estimated by the assay procedure described above. A major portion of both sulphotransferase activities present in the 20000*g* supernatant was particle-bound, as approx. 80% of the recovered activities sedimented on centrifugation at 105000*g*. Treatment of the microsomal fraction (105000*g* pellet) with detergent and alkali (see the Materials and Methods section) afforded appreciable solubilization of the sulphotransferases. The total recovery of *N*-sulphotransferase in the soluble fraction (105000*g* supernatant) was approx. 76% of the microsomal enzyme activity, corresponding to a 16-fold increase in specific activity (based on protein content; Table 3). Solubilization of the *O*-sulphotransferase more than doubled the enzyme activity, with a 50-fold increase in specific activity (Table 3). The increase in *O*-sulphotransferase activity on solubilization cannot be readily explained at present, but may possibly reflect the relative inaccessibility of the particulate enzyme to the macromolecular substrate used.

Properties of solubilized sulphotransferases

Both *N*- and *O*-sulphotransferases showed pH optima around 7.5.

The effects of bivalent cations (Mn^{2+}, Mg^{2+}, Ca^{2+}) on enzyme activity are illustrated in Fig. 4. In the presence of 10mM-Mn^{2+}, both enzymes were 3–4 times more active than in the absence of this ion. (The expression 'both enzymes' is not appropriate if more than one *O*-sulphotransferase is involved, but has been used occasionally in the text for conciseness.) Mg^{2+} ions had little effect on either enzyme, whereas Ca^{2+} selectively promoted *N*-sulphation. Standard incubations were designed to involve optimum concentrations of the actual metal ions.

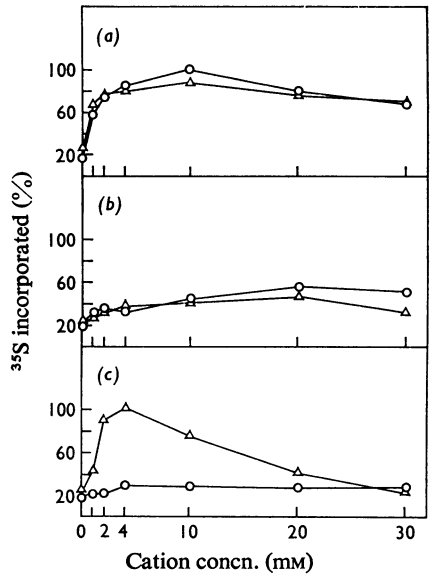

Fig. 4. *Incorporation of* [^{35}S]*sulphate into N-desulphoheparin* (△) *and N-acetylated heparan sulphate* (○) *as a function of metal ion concentration*

Incubations were carried out under standard conditions (see the Materials and Methods section), with solubilized enzyme, except that the mixture of metal ions used was replaced by Mn^{2+} (*a*), Mg^{2+} (*b*) or Ca^{2+} (*c*). Enzyme activities are given in % of ^{35}S incorporation obtained under standard conditions.

Assays performed at different concentrations of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate afforded linear Lineweaver–Burk plots for both enzyme activities. The calculated K_m values for *N*- and *O*-sulphotransferases were 2×10^{-5} M and 1×10^{-4} M respectively.

Gel chromatography showed partial inclusion ($K_{av.}$ about 0.4) of the solubilized enzymes into Sepharose 6B. Although some purification was achieved, the enzymes were not separated from each other.

Effects of salt and temperature

Addition of KCl to the incubation medium caused a marked inhibition (or inactivation) of both *N*- and

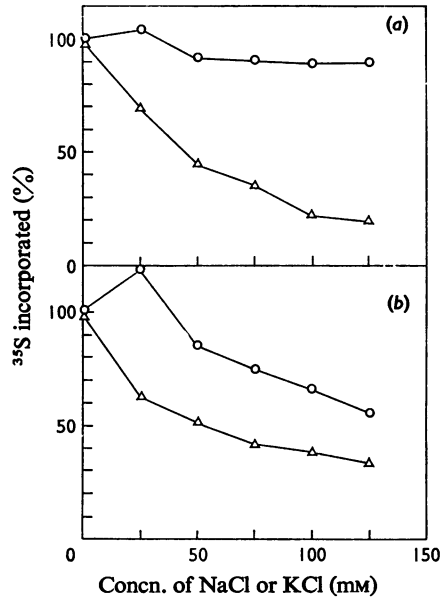

Fig. 5. *Incorporation of* [^{35}*S*]*sulphate into N-desulphoheparin* (△) *and N-acetylated heparan sulphate* (○) *as a function of NaCl* (*a*) *or KCl* (*b*) *concentration*

Incubations were performed under standard conditions (see the Materials and Methods section), except that various amounts of NaCl or KCl were included in the incubation mixtures. Enzyme activities are given in % of the ^{35}S incorporation obtained under standard conditions.

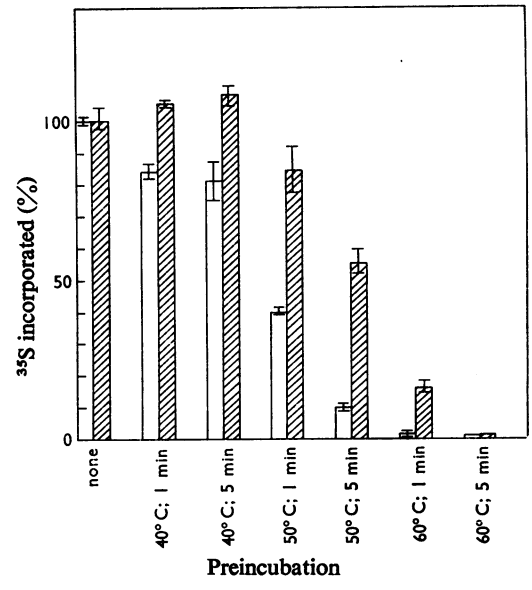

Fig. 6. *Incorporation of* [^{35}*S*]*sulphate into N-desulphoheparin* (*hatched bars*) *and N-acetylated heparan sulphate* (*open bars*) *after preincubation of enzyme at elevated temperatures*

Standard incubation mixtures with solubilized enzymes but no adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate added, were preincubated at the temperatures and for the times indicated. Preincubation was terminated by immersion in ice–water. After the addition of adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate, incubation at 37°C was performed by the standard procedure. Enzyme activities are given in % of the ^{35}S incorporation obtained with untreated enzyme under standard conditions.

O-sulphotransferases (Fig. 5). In contrast, NaCl exerted an inhibitory (or inactivating) effect on the *N*-sulphotransferase only, leaving the *O*-sulphotransferase essentially unaffected (Fig. 5). The *O*-sulphotransferase was more sensitive to increased temperature, 60% of the activity being lost after 1 min of preincubation at 50°C, whereas only 15% of the *N*-sulphotransferase was lost (Fig. 6).

Discussion

Previous studies have demonstrated the transfer of [^{35}S]sulphate from adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate to exogenous, intact or *N*-desulphated heparin or heparan sulphate, catalysed by cell-free preparations (Balasubramanian *et al.*, 1968; George *et al.*, 1970; Foley & Baker, 1973; Johnson & Baker, 1973). Evidence was obtained for the formation of *N*- as well as *O*-[^{35}S]sulphate groups. However, the methods used to detect such substituents were not adapted to selective quantitative analysis on a routine basis. Therefore characterization of the corresponding *N*- and *O*-sulphotransferases has been hampered by lack of appropriate assay procedures.

In the present investigation, transfer of [^{35}S]-sulphate exclusively into *N*-sulphate groups of *N*-desulphoheparin and into *O*-sulphate groups of *N*-acetylated heparan sulphate respectively was achieved by using a solubilized microsomal enzyme preparation from mouse mastocytoma tissue. Quantification of incorporated [^{35}S]sulphate was achieved by a simple precipitation procedure (Wasteson *et al.*, 1973). Assay methods for separate determination of *N*- and *O*-sulphotransferase activities could thus be designed.

The solubilized sulphotransferases had different kinetic properties. The *N*-sulphotransferase thus had a K_m value, with regard to adenosine 3′-phosphate 5′-sulphatophosphate about five times lower than that of the *O*-sulphotransferase(s). Whereas both *N*- and *O*-sulphotransferases were markedly stimulated by Mn^{2+} [but, in contrast with the results

of Balasubramanian *et al.* (1968), not by Mg^{2+}] only the former enzyme responded to Ca^{2+} (Fig. 4). The *N*- and *O*-sulphotransferases also differed with regard to the effects of NaCl (Fig. 5) and increased temperature (Fig. 6). These results indicate that the *N*- and *O*-sulphate-transfer reactions should be ascribed to different enzymes, or alternatively, to separate and independent active sites on the same molecule. The occurrence of more than one *O*-sulphotransferase appears likely [as *O*-sulphate groups occur in more than one position in the heparin molecule (see Lindahl & Axelsson, 1971)], but cannot be inferred from the results obtained in the present study.

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