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Molecular distinctions between heparan sulphate and heparin

Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of *N*-sulphated polysaccharides

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Heparan sulphate and heparin are chemically related $\alpha\beta$-linked glycosaminoglycans composed of alternating sequences of glucosamine and uronic acid. The amino sugars may be *N*-acetylated or *N*-sulphated, and the latter substituent is unique to these two polysaccharides. Although there is general agreement that heparan sulphate is usually less sulphated than heparin, reproducible differences in their molecular structure have been difficult to identify. We suggest that this is because most of the analytical data have been obtained with degraded materials that are not necessarily representative of complete polysaccharide chains. In the present study intact heparan sulphates, labelled biosynthetically with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$, were isolated from the surface membranes of several types of cells in culture. The polysaccharide structure was analysed by complete HNO_2 hydrolysis followed by fractionation of the products by gel filtration and high-voltage electrophoresis. Results showed that in all heparan sulphates there were approximately equal numbers of *N*-sulpho and *N*-acetyl substituents, arranged in a similar, predominantly segregated, manner along the polysaccharide chain. *O*-Sulphate groups were in close proximity to the *N*-sulphate groups but, unlike the latter, the number of *O*-sulphate groups could vary considerably in heparan sulphates of different cellular origins ranging from 20 to 75 *O*-sulphate groups per 100 disaccharide units. Inspection of the published data on heparin showed that the *N*-sulphate frequency was very high (>80% of the glucosamine residues are *N*-sulphated) and the concentration of *O*-sulphate groups exceeded that of the *N*-sulphate groups. We conclude from these and other observations that heparan sulphate and heparin are separate families of *N*-sulphated glycosaminoglycans.

Heparan sulphate and heparin are formed from similar sulphated and non-sulphated monosaccharide units, and they are the only two mammalian polysaccharides that contain *N*-sulphoglucosamine ($GlcNSO_3^-$). The initial product in the biosynthesis of both macromolecules is a non-sulphated polymer composed of alternating sequences of glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc) called 'heparan' or *N*-acetylheparosan: –GlcUAβ1→4GlcNAcα1→4–. This precursor substance is then enzymically transformed into complex sulphated derivatives. The sequence of events in the modification pathway has been mainly worked out by Lindahl and co-workers (for reviews see Lindahl & Höök, 1978; Rodén, 1980). The key step in the transformation of heparan is the conversion of GlcNAc into the unique *N*-sulphated residue. Other modifications then occur principally in the vicinity of the $GlcNSO_3^-$. GlcUA may be converted into iduronic acid (IdUA), and both the newly formed iduronate residues and $GlcNSO_3^-$ may be ester (*O*)-sulphated at C-2 and C-6 respectively. On some occasions GlcNAc residues in sequences $GlcNSO_3$-UA-GlcNAc may also acquire a sulphate residue at C-6 (Sanderson *et al.*, 1984). The end product of complete polymer-level modification is a trisulphated disaccharide of structure: IdUA(2S)α1→4$GlcNSO_3$(6S). In most mammalian heparins these structural changes are very extensive, with over 80% of the hexosamine residues appearing in the *N*-sulphamated form,

with sequences of trisulphated disaccharides making up most of the mature polysaccharide chain (Jacobsson *et al.*, 1979; Delaney & Conrad, 1983).

During the biosynthesis of heparan sulphate similar but more limited modifications seem to occur (Reisenfeld *et al.*, 1982), culminating in products that, in general, have fewer *N*- and *O*-sulphate groups than heparin and have smaller numbers of trisulphated disaccharides, and that also contain some unmodified sequences of *N*-acetylated units (Cifonelli & King, 1977; Winterbourne & Mora, 1981; Hampson *et al.*, 1983). However, there are no accepted structural criteria for distinguishing between heparan sulphate and heparin. The term 'heparan sulphate' has been traditionally used to describe heparin-like by-products of the industrial preparation of heparin from animal tissues such as bovine lung or pig mucosa (Jorpes & Gardell, 1948; Comper, 1981). The heparan sulphates in these by-products had little or no anticoagulant activity and displayed considerable heterogeneity in molecular mass and sulphate content with degree of polymer sulphation (defined here as sulphate groups per disaccharide) varying from 0.25 to 2.0, the sulphate-rich end of the spectrum merging with typical heparin degrees of sulphation, which are in the range 2.0–2.5 (Linker & Hovingh, 1973; Dietrich & Nader, 1974; Cifonelli & King, 1975; Delaney & Conrad, 1983). Although the view was expressed that heparan sulphate and heparin might be separate entities (Linker *et al.*, 1958), the chemical data led to the widely held belief that these two polysaccharides constituted a continuous series of related polysaccharides with no clear-cut distinguishing features (Jacques, 1980; Johnson, 1984).

In the early 1970s an alternative and important source of *N*-sulphated glycosaminoglycans, broadly similar in structure to heparan sulphates in commercial by-products, was identified on the surface membranes of cultured cells (Dietrich & DeOca, 1970; Kramer, 1971). Many reports have confirmed and extended these findings (for reviews see Kramer, 1979; Höök *et al.*, 1984). The intact heparan sulphate chains derived from cell cultures were larger, often considerably so, than their commercial counterparts, and there was strong evidence for high-sulphate and low-sulphate regions within a single macromolecule. It is most probable, therefore, that commercial heparan sulphates are degraded products with variable degrees of sulphation, determined by the break points in the native polymer. Analysis of such fragments would obscure any consistent chemical properties that might differentiate heparan sulphate from heparin. In order to identify characteristic structural features of heparan sulphate, the present analytical study was carried out on the intact polysaccharide chains isolated from a variety of cell cultures. The resulting data are compared with published results on heparin. The findings suggest that, although specific patterns of molecular variability are inherent in heparan sulphates from different cell types, there are relatively precise and reproducible properties of these polymers that clearly distinguish them from heparin.

Materials and methods

Materials

D-[1-^{3}H]Glucosamine (sp. radioactivity 2–5Ci/mmol) and $Na_2{}^{35}SO_4$ (sp. radioactivity 25–40Ci/mg) were obtained from Amersham International. Chondroitin ABC lyase and chondroitin AC lyase were from Miles Chemicals, and trypsin (3× crystallized), papain (2× crystallized) and testicular hyaluronidase (type VI-S) were from the Sigma Chemical Co. Sepharose CL-6B was from Pharmacia Fine Chemicals, Bio-Gel P-10 (–400 mesh) and Bio-Gel P-6 (200–400 mesh) were from Bio-Rad Laboratories, and DEAE-cellulose (DE-52) was from the Whatman Chemical Co. Aquasol II scintillant was from New England Nuclear. Cell-culture media (Eagle's Minimal Essential, Fischer's and Alpha) were obained from Gibco Biocult. All other reagents used were of AnalaR or AristaR grade from BDH Chemicals.

Gel chromatography

Columns used were Sepharose CL-6B (80cm × 1cm) in 0.5M-NaCl eluted at a flow rate of 4ml/h, Bio-Gel P-10 (80cm × 1.5cm) in 0.5M-NH_4HCO_3 eluted at a flow rate of 2–3ml/h, and Bio-Gel P-6 (90cm × 1.5cm) also in 0.5M-NH_4HCO_3 eluted at a flow rate of 6ml/h. All columns were pretreated with 5mg of bovine serum albumin, 1mg of chondroitin sulphate and 1mg of heparin.

Ion-exchange chromatography

Samples in phosphate-buffered saline (0.145M-NaCl/10mM-sodium phosphate buffer, pH7.2) were applied to a DEAE-cellulose column (5cm × 1cm) equilibrated in the same buffer. Several bed volumes of equilibration buffer were passed through the column, and bound radioactivity was then eluted with a linear gradient (total vol. 200ml) of 0.15M→0.8M-NaCl in 20mM-sodium phosphate buffer, pH6.8, at a flow rate of 20ml/h; 2ml fractions were collected.

High-voltage paper electrophoresis

Samples (10–20μl) of low-M_r oligosaccharides from HNO_2-treated heparan sulphate were applied as narrow strips on to a sheet of Whatman

3MM paper and dried rapidly in a stream of hot air. Each strip was also loaded with 5μl of a 2mg/ml solution of HNO_2-degraded heparin to act as a carrier. The sheet was carefully wetted with 1.6M-formic acid (pH 1.7) electrophoresis buffer, and subjected to an electric field of 4kV for 35–40min in a Locarte high-voltage electrophoresis apparatus. The sheet was then removed, rapidly dried with hot air and cut into 0.5cm strips for radioactivity determinations.

Scintillation counting

Aqueous samples (max. vol. 0.5ml) were mixed with 5ml of Aquasol II; paper strips were placed in 0.5 ml of water and shaken for 1h before addition of 5ml of Aquasol II. Radioactivity was determined in a Beckman series 7500 scintillation counter with an external-standard 'H'-number quench correction. D.p.m. values were plotted and stored by using a Hewlett–Packard 1845 computer.

Degradation of glycosaminoglycans

Chondroitin ABC lyase and testicular hyaluronidase were used as described elsewhere (Gallagher *et al.*, 1983). Heparan sulphate was degraded by using the low-pH HNO_2 procedure of Shiveley & Conrad (1976). Samples of heparan sulphate in water (50–100μl) were mixed with 0.45–0.9ml of freshly prepared HNO_2 and incubated for 10min at room temperature. The reagent was neutralized by the addition of 2M-Na_2CO_3 and stored at −20°C.

Pronase digestion

This was carried out with a 5mg/ml solution of Pronase in 0.1M-Tris/acetate buffer, pH 7.8, containing 5mM-calcium acetate, incubated overnight at 37°C. Protein was removed by precipitation with ice-cold 10% (w/v) trichloroacetic acid, and the soluble material was dialysed against water, concentrated by rotary evaporation and freeze-dried.

Cell cultures

Confluent cultures of human skin fibroblasts were kindly provided by Dr. S. Schor. The cultures were maintained at 37°C (5% CO_2 in air) in Eagle's Minimal Essential Medium supplemented with 15% (v/v) heat-inactivated foetal-calf serum, 2mM-glutamine and non-essential amino acids. Mouse bone-marrow stromal-cell cultures were kindly given by Dr. T. M. Dexter. These were cultured at 33°C in 5% CO_2 in air in Fischer's medium containing 15% (v/v) mouse serum (Dexter *et al.*, 1977). A proline-requiring Chinese-hamster ovary (CHO) cell line was obtained from Dr. P. M. Stanley and grown in our laboratory in complete Alpha medium containing 10% (v/v) heat-inactivated foetal-calf serum.

Radiolabelling and extraction of cell-surface glycosaminoglycans

Confluent cultures were incubated for 72h with [^{3}H]glucosamine (10μCi/ml) and $Na_2{}^{35}SO_4$ (20μCi/ml). The medium was removed and the cell layers were carefully washed twice with warm (37°C) phosphate-buffered saline. Cell-surface heparan sulphates were then prepared by incubating the cultures with trypsin (50μg/ml in phosphate-buffered saline), which detached most of the cells from the culture surface. Then 0.25vol. of soya-bean trypsin inhibitor (0.25mg/ml in phosphate-buffered saline) was added, the cell suspension was centrifuged (500*g* for 5min) and the supernatant was removed. The cell pellet was resuspended in phosphate-buffered saline and centrifuged again, the supernatant was mixed with the previous one and the whole trypsin extract was stored at −20°C.

Results and discussion

Preparation of intact heparan sulphate chains

Details are presented below of the heparan sulphates from human skin fibroblasts, mouse bone-marrow stromal cells and CHO cells. Analyses of human neuroblastoma cells and bovine endothelial cells have already been published by us (Hampson *et al.*, 1983; Winterbourne *et al.*, 1983), but are referred to on occasions in the text, together with data of others on hepatocyte (Akasaki *et al.*, 1975; Oldberg *et al.*, 1977) and mouse fibroblast (Winterbourne & Mora, 1981) heparan sulphates.

The heparan sulphates examined in this study were radiolabelled biosynthetically by incubation of cultured cells for 72h with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$. After incubation, the radioactive sulphated proteoglycans were extracted from the surfaces of the various cell cultures by brief treatment with trypsin; residual polypeptide was removed by exhaustive proteolysis with Pronase, and the free chains were partially resolved from hyaluronic acid and from dermatan sulphate and chondroitin sulphate by chromatography on DEAE-cellulose (Gallagher *et al.*, 1983). Fig. 1 shows a characteristic separation for the heparan sulphate glycosaminoglycans from bone-marrow stromal cells. Heparan sulphate (peak II in Fig. 1) from all but one of the other cell types used was also eluted before chondroitin sulphate (peak III in Fig. 1). The exception was the neuroblastoma heparan sulphate, which was co-eluted with chondroitin sulphate (Hampson *et al.*, 1983). Fractions corresponding to heparan sulphate were pooled, de-

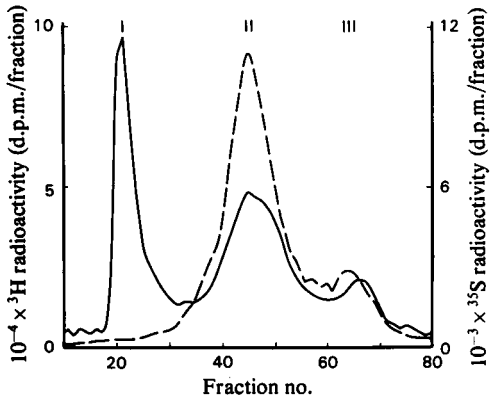

Fig. 1. *Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose*

Heparan sulphate (peak II) can be partially resolved from hyaluronic acid (peak I) and from chondroitin sulphate and dermatan sulphate (peak III) by NaCl-concentration-gradient elution from DEAE-cellulose. In this example, radioactive materials were extracted, by using trypsin, from the cell surface of cultured bone-marrow stromal cells previously incubated for 72h with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$. Pronase-digested samples were applied to the DEAE-cellulose column (1.0cm × 5cm) in phosphate-buffered saline. After a washing with 20ml of this starting buffer (up to fraction no. 10), a linear gradient of NaCl was developed from 0.15M to 0.8M in a total volume of 200ml; 2ml fractions were collected. ——, ^{3}H radioactivity; — —, ^{35}S radioactivity. The peak maximum value for heparan sulphate was reached at about 0.4M-NaCl.

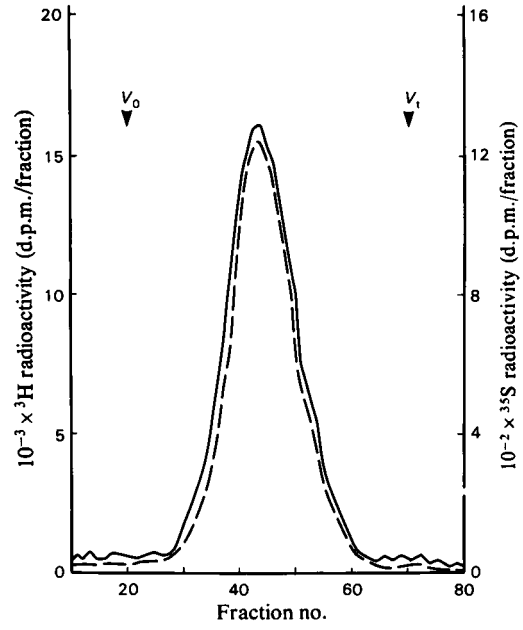

Fig. 2. *Gel filtration of heparan sulphate on Sepharose CL-6B*

Heparan sulphate (1ml), prepared as described in Fig. 1, was treated with chondroitinase ABC and dialysed. The treated sample was applied to a Sepharose CL-6B column (80cm × 1cm) eluted with 0.5M-NaCl. The flow rate was 4ml/h, and 1ml fractions were collected. V_0 void volume; V_t, total volume. ——, ^{3}H radioactivity; — —, ^{35}S radioactivity.

salted, concentrated by rotary evaporation and freeze-dried.

Heparan sulphate fractions, freed of contaminating glycosaminoglycans by treatment with chondroitinase ABC, were eluted as single peaks on Sepharose CL-6B (Fig. 2). The example shown is the bone-marrow heparan sulphate, which was reasonably homogeneous and had a $K_{av.}$ of about 0.45, corresponding to an M_r of about 30000 (Wasteson, 1971). Skin fibroblast and neuroblastoma heparan sulphates (Hampson *et al.*, 1983) were eluted a little earlier on Sepharose CL-6B, again as single peaks with $K_{av.}$ values of 0.28–0.35.

Frequency of occurrence of N-sulphate groups

A sensitive and specific means for examining the structure of heparan sulphate is provided by the use of low-pH (<2.0) HNO_2, which causes quantitative release of *N*-sulphate groups and hydrolysis of the glycosidic linkage between the deaminated sugar and hexuronic acid. Ring contraction leads to the formation of anhydromannose at the reducing ends of the oligosaccharide fragments (Shiveley & Conrad, 1976).

The amount of free sulphate released by HNO_2 will be equivalent to the number of *N*-sulphate groups, and the fragmentation pattern will be determined by both the number and proximity of these residues in the intact polysaccharide. Ester (*O*-)sulphates are unaffected by HNO_2, and so the proportion of [^{35}S]sulphate found as free sulphate compared with oligosaccharide-bound sulphate (these may be separated by high-voltage electrophoresis; see below) gives the *N*-/*O*-sulphate ratio for heparan sulphate.

In order to minimize losses of material, heparan sulphates prepared by ion-exchange chromatography (Fig. 1) were incubated with HNO_2 directly without prior removal of the glycosaminoglycans that were not completely separated by the DEAE-cellulose column. These contaminants were easily identified as HNO_2-resistant components on gel filtration.

Heparan sulphates, prepared as shown in Fig. 1, were treated with HNO_2 and fractionated according to molecular size by chromatography on Bio-Gel P-10 (Fig. 3). Radioactive material that appeared in the void volume was completely

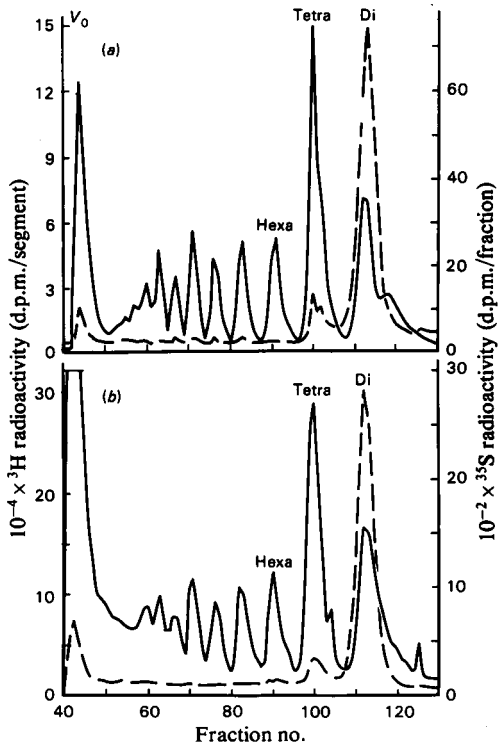

Fig. 3. *Gel filtration on Bio-Gel P-10 of oligosaccharides produced by complete HNO_2 hydrolysis of heparan sulphate*

Heparan sulphate from skin fibroblasts (*a*) and bone-marrow stromal cells (*b*) prepared by DEAE-cellulose chromatography were treated with low-pH (<2.0) HNO_2 and applied to a Bio-Gel P-10 (~400 mesh) column (80cm × 1.5cm). The column was eluted with 0.5M-NH_4HCO_3 at 3ml/h; 1ml fractions were collected. ——, ^3H radioactivity; — —, ^{35}S radioactivity. A graded series of oligosaccharides was observed from disaccharides (labelled Di) to a nine-disaccharide fragment emerging around fraction no. 60. Material eluted in and immediately after the void volume (V_0) (fractions 40–55) was degraded to disaccharides by chondroitinase ABC. If HNO_2 treatment was excluded, radioactivity emerged almost entirely in the void volume, although some 'tailing' was noted in the bone-marrow samples (36). Elution positions of the di-, tetra- and hexa-saccharide peaks was determined by calibration with low-M_r products of chondroitinase ABC and testicular-hyaluronidase digestions of chondroitin sulphate as previously described (Hampson *et al.*, 1983). Fractions corresponding to disaccharides and tetrasaccharides were pooled, freeze-dried and analysed by high-voltage electrophoresis.

degraded by treatment with chondroitinase ABC, showing that it consisted of a combination of hyaluronic acid and galactosaminoglycans. In the fractionating range of the Bio-Gel P-10 column (fractions 50–120) there is a striking similarity in the radioactivity profiles between skin fibroblast (Fig. 3*a*) and bone-marrow (Fig. 3*b*) heparan sulphates. The size of the oligosaccharide products of HNO_2 treatment is shown in square brackets in the following sequence:

$$GlcNSO_3^-\text{-}[(UA\text{-}GlcNAc)_n\text{-}UA\text{-}GlcNSO_3^-]\text{-}UA\text{-}R$$

The number of disaccharides in each oligosaccharide fragment is $n+1$. Since tetrasaccharides ($n=1$) and disaccharides ($n=0$) were the major hydrolytic fragments (Fig. 3), most of the *N*-sulphate groups in heparan sulphate are present in alternating sequences with GlcNAc-containing disaccharides, or as uninterrupted sequences. When two or more *N*-acetylated disaccharides occur in sequence (i.e. $n \geqslant 2$), HNO_2 treatment produces relatively large oligosaccharides, which in practice range in size from hexasaccharides (labelled Hexa in Fig. 3) to the largest identifiable fragment, occurring in relatively small quantities, which is a nine-disaccharide structure containing a sequence of eight *N*-acetylated units (fractions 56–60 in Fig. 3). These results demonstrate the strong tendency of *N*-sulphated and *N*-acetylated disaccharides to be segregated in heparan sulphate to produce *N*-sulphate-rich and *N*-acetyl-rich domains along the polysaccharide chain.

The gel-filtration profile of HNO_2 scission can be used to calculate the proportions of glucosamine residues with *N*-sulphate or *N*-acetyl substituents (Hampson *et al.*, 1983). Values of 47% and 50% *N*-sulpho derivatives are obtained for skin fibroblasts and bone-marrow stroma heparan sulphates, the remaining glucosamines being in the *N*-acetylated form. These values, and corresponding data for other heparan sulphates, are given in Table 1. Although the substitution of the amino groups varies to some degree, the values fall within a fairly narrow range. Without exception oligosaccharides produced by complete HNO_2 hydrolysis of heparan sulphates cited in Table 1 gave gel-filtration profiles that resembled those shown in Fig. 3. One may therefore conclude that all intact heparan sulphates so far examined express broadly equivalent frequencies and distributions of *N*-sulphated and *N*-acetylated hexosamine units irrespective of the cell and species of origin.

Topographical relationships of N-sulphate and ester (O-)sulphate groups

Gel filtration of HNO_2-treated heparan sulphate showed that the greater part of the [^{35}S]sulphate was found in the disaccharide peak, with smaller quantities in the tetrasaccharides (Figs. 3*a* and 3*b*). [^{35}S]Sulphate in the disaccharide fraction consists of both free sulphate from *N*-sulphamino

Table 1. *Content and distribution of sulphate groups in heparan sulphate*

Heparan sulphates are listed in increasing order of their concentrations of *O*-sulphate groups. References to data for the different types are as follows: endothelial cells (Winterbourne *et al.*, 1983), normal and transformed mouse fibroblasts (Winterbourne & Mora, 1981), granulosa cells (Yanagishita & Hascall, 1983) and neuroblastoma cells (Hampson *et al.*, 1983). No single publication contained all the necessary data for hepatocytes (cell type no. 11 in Table). The values given were taken from results of Akasaki *et al.* (1975), which showed that 50% of the glucosamine units were *N*-sulphated, and the *O*-sulphation was calculated to be 75% by using the data from Oldberg *et al.* (1977), who found that the *N*-/*O*-sulphate ratio for hepatocyte heparan sulphate was 0.66. Both these papers were in agreement that the hepatocyte polysaccharide was highly sulphated. The hepatocyte results reported by Akasaki *et al.* (1975) and Oldberg *et al.* (1977) were obtained with freshly isolated cells or tissues. However, Bienkowski & Conrad (1984) have characterized the heparan sulphate from an established liver-derived cell line in which degree of polymer sulphation is low, due mainly to the small concentration of *O*-sulphate groups (cell type no. 2 in Table). Data for the remaining heparan sulphates are given in the present study. Values for *N*- and *O*-sulphate groups shown here are also plotted in Fig. 7 for comparison with corresponding results with heparin.

Cell type	*N*-Sulphate (groups/100 disaccharide units)	*O*-Sulphate (groups/100 disaccharide units)	*N*-/*O*-Sulphate ratio	Polymer sulphation (groups/disaccharide)
(1) Endothelial (bovine)	38	19	2.0	0.57
(2) Liver-derived cell line (rat)	40	20	2.0	0.60
(3) Skin fibroblasts (human)	47	27	1.72	0.74
(4) Mouse fibroblasts (clone 215-CSC, Simian-virus-40-transformed)	47	32	1.47	0.79
(5) Mouse fibroblasts (clone 219, tumour cell line)	50	34	1.47	0.84
(6) Bone-marrow stroma (mouse)	50	39	1.28	0.89
(7) Mouse fibroblast (clone 210C, normal cells)	47	41	1.15	0.88
(8) Granulosa (mouse)	45	45	1.0	0.90
(9) Neuroblastoma (human)	51	47	1.08	0.98
(10) Chinese-hamster ovary (CHO)	42	72	0.58	1.14
(11) Hepatocyte (rat)	50	75	0.66	1.25

groups and ester (*O*-)sulphates bound to the sugar residues of the disaccharides; in the tetrasaccharides [^{35}S]sulphate is exclusively in the form of *O*-sulphate (see below).

Since di- and tetra-saccharide products of HNO_2 hydrolysis are obtained from regions with the highest content of *N*-sulphate groups, these findings confirm the conclusions from other studies that the *N*- and *O*-sulphate residues are near neighbours in the heparan sulphate chain. As discussed elsewhere (Winterbourne *et al.*, 1983), these results show that in heparan sulphates with tetrasaccharides as the largest *O*-sulphated fragment the maximum theoretical spacing between *N*- and *O*-sulphated sugars is a single monosaccharide unit.

The CHO-cell heparan sulphate differed from that in skin fibroblasts and bone-marrow stroma because in this case all the HNO_2-released oligosaccharides contained *O*-sulphate groups (Fig. 4) (N.B.: these materials were fractionated on Bio-Gel P-6 rather than Bio-Gel P-10, and so the largest oligosaccharides were not resolved).

This could indicate some slackening of control of polymer *O*-sulphation in CHO cells. Alternatively it might represent the synthesis of a separate class of heparan sulphate. In studies on heparan sulphate from bovine lung *O*-sulphate residues have been found in tetrasaccharides (Sanderson *et al.*, 1984) and larger oligosaccharides (Sanderson, 1984) isolated after complete HNO_2 scission. Interestingly, n.m.r. analysis showed that in these fragments the sites of *O*-sulphation were confined to C-6 of the reducing anhydromannose or C-6 of the GlcNAc adjacent to the non-reducing uronic acid. These *O*-sulphate groups are therefore still within one monosaccharide of a $GlcNSO_3$ unit in the intact polysaccharides. The structure of the CHO-cell heparan sulphate could be rather like that of heparan sulphate from bovine lung.

Polymer sulphations: proportions of ^{35}S radioactivity as N-sulphate and O-sulphate groups

The amounts of ^{35}S label in heparan sulphate in the form of *N*- and *O*-sulphate groups may be determined by high-voltage electrophoresis of HNO_2-degraded material. At pH 1.6 (1.7M-formic acid) the ionization of carboxy groups is almost

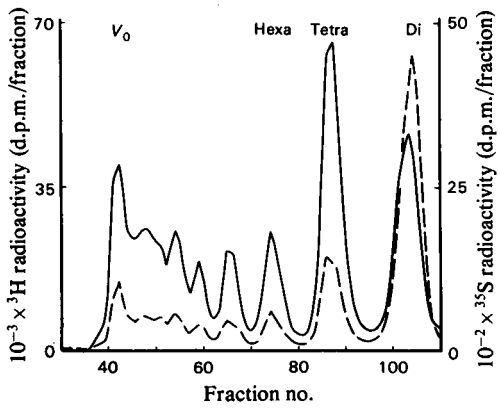

Fig. 4. *Gel filtration on Bio-Gel P-6 of CHO-cell heparan sulphate after treatment with HNO_2*

Heparan sulphate was prepared from trypsin extracts of CHO-cell surfaces as described in Fig. 1. In this case, contaminating glycosaminoglycans were removed before HNO_2 treatment. The Bio-Gel P-6 (200–400 mesh) column (90cm × 1.5cm) was eluted with 0.5M-NH_4HCO_3 at a flow rate of 6ml/h; 1.25ml fractions were collected. ——, ^3H radioactivity; — —, ^{35}S radioactivity. The column was calibrated as described for the Bio-Gel P-10 column in Fig. 3 legend. Di- and tetra-saccharide peaks were pooled, freeze-dried and analysed by high-voltage electrophoresis.

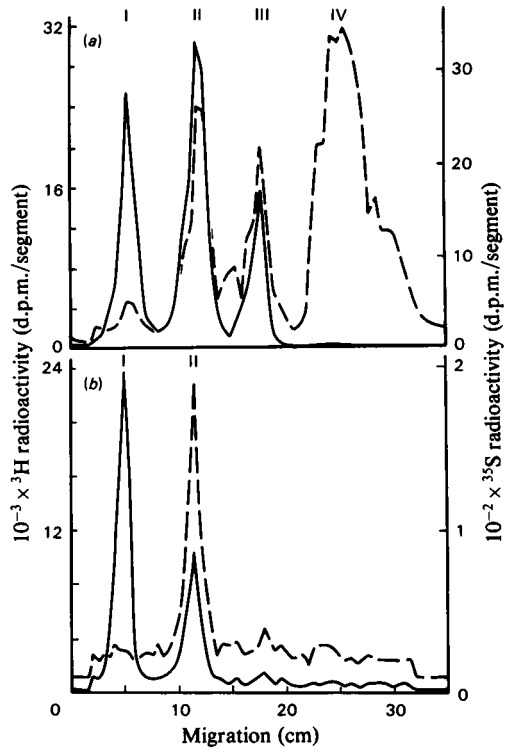

Fig. 5. *High-voltage electrophoresis of low-M_r products from HNO_2 hydrolysis*

Disaccharides (*a*) and tetrasaccharides (*b*) from HNO_2-treated bone-marrow heparan sulphate (Fig. 3) were subjected to paper electrophoresis on Whatman 3MM paper at 4kV for 40min in 1.6M-formic acid (pH 1.7). ——, ^3H radioactivity; — —, ^{35}S radioactivity. In (*a*), peak I is non-sulphated disaccharide, peaks II and III are monosulphated and disulphated disaccharides respectively, and peak IV is free [^{35}S]sulphate. In (*b*) the tetrasaccharides in peak I are non-sulphated and in peak II they are monosulphated. Peak II in (*a*) corresponded to chondroitin sulphate disaccharides. The system was further calibrated by using well-characterized disulphated disaccharides from heparin and monosulphated and disulphated tetrasaccharides from heparan sulphate kindly provided by Dr. P. Sanderson and Dr. I. Nieduszynski (Sanderson *et al.*, 1984).

totally suppressed and electrophoretic mobility depends on the charged sulphate residues. Before electrophoresis the degraded products were fractionated by gel filtration (Fig. 3) to separate di- and tetra-saccharides, since these were usually the only components that contained [^{35}S]sulphate. Fig. 5 shows data for the bone-marrow heparan sulphate: in the disaccharide fraction four radioactive species were identified. The most mobile component (peak IV), labelled with ^{35}S only, was free sulphate, and the peak of ^3H radioactivity near the origin (peak I) was non-sulphated disaccharide. The ^3H-/^{35}S-labelled fractions (peaks II and III) were monosulphated and disulphated disaccharides. The electrophoretic profile for the tetrasaccharides shows only two components, representing non-sulphated and monosulphated species (peaks I and II respectively). From these plots an *N*-/*O*-sulphate ratio of 1.28 can be calculated for the bone-marrow heparan sulphate. Since 50% of the disaccharides are *N*-sulphated, the degree of sulphation of this heparan sulphate is 0.89.

The mean polymer sulphations of heparan sulphates from different cell types has been examined by similar methods (Table 1), and the values obtained show considerable variation. For example, the polymer sulphation of neuroblastoma heparan sulphate, although varying slightly between membrane and secretory forms, is relatively high at 1.0, and the sulphate groups are about equally divided between *N*-sulphate and *O*-sulphate (Hampson *et al.*, 1983, 1984). The most highly sulphated heparan sulphate described in the literature to date is that from hepatocyte membranes, in which there is a molar excess of *O*-sulphate over *N*-sulphate (Oldberg *et al.*, 1977). By contrast the degree of sulphation of endothelial-cell and skin-fibroblast heparan sulphates is quite low (0.57 and 0.74 respectively). This range of sulphate

concentrations is found despite the relative constancy in the frequency of occurrence of *N*-sulphate substituents. Clearly the variability in sulphate content of heparan sulphate is due principally to differences in the number of *O*-sulphate groups. Differential capacities for polymer *O*-sulphation are genetically determined phenotypic properties, because the cultured cells reproduced their characteristic heparan sulphates through many cell doublings. Cell-lineage-related structural polymorphism of cell-surface heparan sulphate may be indicative of specific roles for this polysaccharide in normal cell growth and development (Gallagher & Hampson, 1984).

Disposition of O-sulphate groups in HNO_2-released oligosaccharides

In view of the importance of *O*-sulphate in determining the observed heterogeneity of different heparan sulphate preparations, it is useful to consider their disposition in the native polysaccharide. On average about 80% of the *O*-sulphate groups were in the disaccharides after HNO_2 hydrolysis, with the remaining 20% in the tetrasaccharides. Analyses of the sulphation of the disaccharides by high-voltage electrophoresis (as described in Fig. 5) of different heparan sulphates studied in this laboratory are given in histogram form in Fig. 6. The sulphate-rich heparan sulphate from neuroblastoma cells yielded mainly sulphated disaccharides (both mono- and di-sulphated forms), whereas in the less-sulphated skin fibroblast material sulphated disaccharides were less abundant and there was a rather high content of non-sulphated components. The hepatocyte heparan sulphate has an exceptionally high proportion of disulphated disaccharides (Kjellen *et al.*, 1983), and these structures will be largely responsible for the strong polyanionic properties of the polysaccharide. The CHO-cell heparan sulphate also has a high content of sulphate (Table 1), largely due to the *O*-sulphate groups in the *N*-acetyl-rich domains of the polysaccharide (Fig. 4) rather than high *O*-sulphation of the *N*-sulphated sequences (Fig. 6).

Our data are incomplete on the *O*-sulphation in the tetrasaccharides. Nevertheless comparison of the bone-marrow components (Fig. 6) with data published on equivalent fractions from neuroblastoma (Hampson *et al.*, 1983) and liver cells (Kjellen *et al.*, 1983) shows that significant variations occur in the most abundant of the oligosaccharide fragments in HNO_2 hydrolysates. Bone-marrow heparan sulphate tetrasaccharides are predominantly non-sulphated, whereas the neuroblastoma fraction was mainly mono-sulphated, together with a small proportion of disulphated species. Sulphated tetrasaccharides were also found in relatively high amounts in the liver cell heparan sulphate.

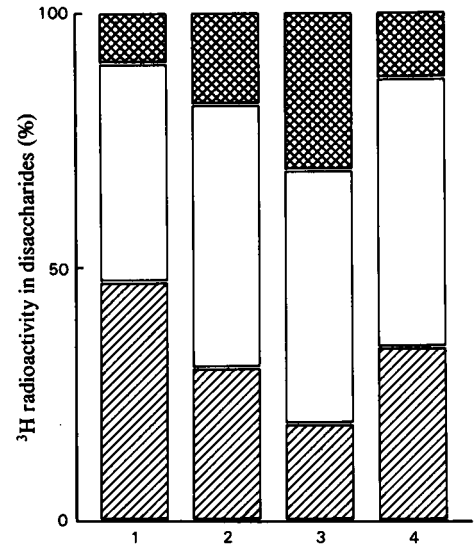

Fig. 6. *Comparison of disaccharide products released by HNO_2 from different cell-surface heparan sulphates*
Disaccharides were prepared from HNO_2-treated heparan sulphates by chromatography on Bio-Gel P-10 or Bio-Gel P-6 and fractionated into non-sulphated (▨), mono-sulphated (□) and disulphated (▩) species by using high-voltage electrophoresis (Fig. 5). Heparan sulphates were prepared from cell cultures of skin fibroblasts (1), bone marrow (2), neuroblastoma (3) and CHO-cell line (4).

Comparisons of the sulphation of heparan sulphate and heparin

The content of *N*- and *O*-sulphate groups of a variety of heparan sulphates has been compared (Fig. 7) with published chemical analyses on several heparin fractions carried out by Taylor *et al.* (1973). The data in that paper were considered to be representative of the sulphate compositions of the many different heparin preparations that have been studied in the past 20 years or so. The distinction in polymer sulphation between heparan sulphate and heparin is clearly displayed in the plots of *N*-sulphation against *O*-sulphation (Fig. 7). Heparan sulphates all fall within a relatively narrow median concentration range for *N*-sulphate groups, but they express a wide spectrum of *O*-sulphate concentrations. In contrast, most of the heparin samples have 80% or more of their glucosamine residues in the *N*-sulphated form and most preparations contain one or more *O*-sulphate groups per disaccharide. Whale heparin is somewhat exceptional in that the *N*-sulphate content (74 *N*-sulphate groups per 100

Table 2. *Differences in structure between heparan sulphate and heparin*

Sulphate values are given as the number of sulphate groups per 100 disaccharide units. *N*-Acetylated disaccharide sequences have been found in heparin only in the protein linkage region (Lindahl, 1966; Cifonelli & King, 1972). Trisulphated disaccharides may be solitary in heparan sulphate (Cifonelli & King, 1977). In view of the differences in *N*-sulphate content, heparan sulphate and heparin will also differ in iduronic acid content (see the introduction).

Property	Sulphate (groups/100 disaccharide units)	
	Heparan sulphate	Heparin
Polymer sulphate	60–125	200–250
N-Sulphate	40–50	80–100
O-Sulphate	20–75	>100
N-Acetylated disaccharides	Sequences > Solitary	Solitary ≫ Sequences
Trisulphated disaccharides	Infrequent	Frequent

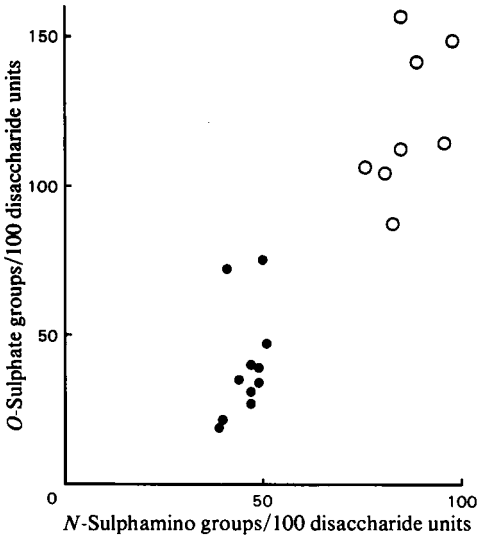

Fig. 7. *Sulphation parameters of heparan sulphate and heparin*

Different samples of heparan sulphate (●) and heparin (○) are plotted as *N*-sulphation against *O*-sulphation. The data points for heparan sulphates are listed in increasing order of their *O*-sulphate contents in Table 1. The values for heparin refer to a series of different fractions prepared and analysed by Taylor *et al.* (1973). These included three different preparations of bovine lung heparin (BLH) and two samples of pig mucosal heparin (PMH). In order of increasing *O*-sulphation (the actual values are given in parentheses) the heparin fractions are: clam heparin (0.86), HMH-II (1.04), whale heparin (1.06), BLH-III (1.12), BLH-II (1.14), PMH-I (1.41), BLH-I (1.48) and bovine mucosal heparin (1.56).

disaccharides) is below the normal heparin range but still well above that found in heparan sulphate. We would stress that all the heparin analyses have been carried out on degraded materials. Nevertheless it is evident that in intact heparin chains the vast majority of the glucosamine units are *N*-sulphated (Robinson *et al.*, 1978), and it may be that the degree of heterogeneity in mature heparin is less than that suggested from Fig. 7. The foregoing and other differences between heparan sulphate and heparin are summarized in Table 2. Such findings permit the conclusion that heparan sulphate and heparin are distinct and separate families of *N*-sulphated glycosaminoglycans: they do not constitute a graded series of polysaccharides with continuously variable sulphate compositions.

The restricted range of *N*-sulphate contents found in heparan sulphate is relevant to the proposals that similar components could be precursors of the formation of heparin (see the introduction). Studies on heparin biosynthesis in a microsomal preparation from mastocytoma cells (Jacobsson & Lindahl, 1980; Riesenfield *et al.*, 1980) have led to the identification of a polysaccharide intermediate that is virtually devoid of *O*-sulphate groups even though 70–80% of the GlcN residues are *N*-sulphated. This immature heparin therefore contains a much higher content of *N*-sulphate groups than mature heparan sulphates with their full complements of *O*-sulphate groups. It thus seems improbable that heparan sulphate-like constituents are intermediates in the pathway of heparin biosynthesis. It is reasonable to assume that the production of these two complex polymers is controlled by different mechanisms.

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