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Chemical Heterogeneity of Heparan Sulfate from a Human Neuroblastoma Cell Line

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The chemical heterogeneity of radiolabelled neuroblastoma heparan sulfate has been studied by ion exchange chromatography and by affinity chromatography on heparan sulfate-agarose. Although the entire population of chains shows considerable homogeneity in charge density, the deaminative cleavage products ranged in size from disaccharides to eicosasaccharides. Under appropriate conditions neuroblastoma heparan sulfate could be separated into two pools of low or high affinity for lung heparan sulfate-agarose. Analyses of periodate oxidation-alkaline elimination indicated that the high affinity chains contained larger proportions of heparin-like segments, *i.e.* iduronate-rich and *N*-sulfated ones.

Heparan sulfate occurs as side-chains of cell surface proteoglycans in many animal cells. The carbohydrate back-bone, which is composed of the repeating disaccharide $(GlyA-GlcN)_n$, shows extensive variation.* At least 9 different types of repeats are present in a large number of sequential arrangements (for references see Ref. 1). The heparan sulfate side-chains are able to self-associate which has been shown both by chromatographic and by physical methods.[2–5] Using affinity chromatography on various heparan sulfate-agarose gels as a screening method for self-affinity, a variety of heparan sulfates from lung tissue,[6] lung fibroblasts[7] and skin fibroblasts[8] have been examined. Specific interactions seems to be conveyed by special contact zones which may be placed in variable positions along the chains.[5,9,10] Whereas heparan sulfates from normal quiescent 3T3 fibroblasts are association-prone, corresponding material from SV40-transformed or polyoma- virus-transformed cells have no apparent self-affinity.[11] Structural analysis of heparan sulfates from transformed 3T3 cells have shown[12] that loss of aggregatability is accompanied by increased structural heterogeneity. Moreover, sequences that constitute putative contact zones are less frequent in such heparan sulfates.

In general, heparan sulfates from 3T3 cells and other cultured cell lines have a rather low degree of *N*- and *O*-sulfation. Recently, a heparan sulfate produced by human neuroblastoma cells was described[13] in which over 50 % of the glucosamine residues were *N*-sulfated

* Abbreviations used: GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $GlcNSO_3$, 2-sulfamido-2-deoxy-D-glucose; GlyA, hexuronic acid; IdoA, L-iduronic acid; GlcA, D-glucuronic acid; $-OSO_3$, ester sulfate; R, remnant of an oxidized and degraded GlcA residue; anMan, D-anhydromannose.

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and the polymer had an *N*/*O*-sulfate ratio of 0.95. It was suggested that a high polymer sulfation may be a characteristic feature of neural cell heparan sulfate. In the neuroblastoma cell cultures heparan sulfate was mainly associated with the cell surface. If this material has self-affinity properties, it would indicate a potentially important function for heparan sulfate in cellular recognition in the nervous system. In this connection, it is significant that the concentration of neuronal cell surface heparan sulfate increases considerably during growth of ganglionic neurones and the development of dendritic arborisations.[14] The present study was therefore undertaken to evaluate the reactivity of neuroblastoma heparan sulfate with a family of structurally related heparan sulfates as determined by affinity chromatography. Binding patterns were correlated with the chemical heterogeneity of heparan sulfate as revealed by products of periodate oxidation.

EXPERIMENTAL

Materials. Hyaluronate and chondroitin 4-sulfate were the NIH standards. Heparan sulfate subfractions HS1, HS2, HS3, HS4 and HS5 were prepared from bovine lung. Association-prone variants (HS2-A, HS3-A and HS4-A) were then obtained by gel permeation chromatography.[2,15] The procedures for coupling various heparan sulfates onto Sepharose 4B were outlined previously.[4,5] After brief oxidation (15 min) with periodate (10 % destruction of GlcA) heparan sulfates were coupled to adipic acid dihydrazide-derivatized agarose and the resulting aldimins were stabilized by reduction. Sources of enzymes, chromatography media and chemicals were as follows: Papain (16–40 BAEE units/mg), trypsin (type III), pronase and guanidinium chloride, Sigma; chondroitinase-ABC, Miles Laboratories; Sephadex and Sepharose gels, Pharmacia; DEAE-cellulose (DE-52), Whatman; Biogel P-10 (minus 400 mesh), Bio–Rad; Instagel, Packard.

Preparation of radiolabelled heparan sulfates. The precursors used were (^{3}H)-GlcN and $^{35}SO_4^{2-}$. Radiolabelled heparan sulfates were isolated from primary cultures of fibroblasts and from transformed or non-transformed 3T3 cells after trypsin and papain treatments as previously described.[12] In the case of human neuroblastoma cells (CHP 100) a similar approach was taken.[13] Cell-surface associated material was removed by rapid trypsinization using 0.05 % (w/v) of trypsin for 10 min at 37 °C. These extracts were chromatographed on DE-52 DEAE-cellulose (total volume, 8 ml) by elution with a linear 0–0.8 M NaCl gradient (total volume 100 ml). The (^{3}H/^{35}S)-glycosaminoglycans were pooled, digested with pronase (1 mg of enzyme per ml in 0.1 M Tris-acetate, pH 7.8, containing 5mM calcium acetate) at 37 °C over night and purified by ion exchange chromatography. Radiolabelled heparan sulfate was finally obtained after digestion with chondroitinase-ABC (Ref. 16) followed by gel filtration on a column (1 cm×30 cm) of Sephadex G-50 at 4 °C (eluant, 0.2 M pyridine–acetate, pH 5.0). The void volume fractions were pooled, dialysed against water and freeze-dried. The neuroblastoma heparan sulfate was also digested with papain and recovered by gel filtration as described for the fibroblast-derived heparan sulfates.[12]

Degradation methods. Deaminative cleavage of bonds between $GlcNSO_3$ and GlyA was performed with the pH 1.5/HNO_2 method.[17] Reaction mixtures were neutralised with 2M $NaHCO_3$. The fragments have the general carbohydrate structure GlyA-(GlcNAc–GlcA)$_n$-anMan where n=0, 1, 2... *etc.* The smallest fragment (n=0) is the disaccharide GlyA–anMan which may carry no, one or two $-OSO_3$ groups. The tetrasaccharide, GlyA–GlcNAc–GlcA–anMan, may carry up to three $-OSO_3$ groups. However, the most common positions are the end-sugars (anMan and GlyA=IdoA). Periodate oxidations were conducted with 0.02 M sodium metaperiodate/0.05 M sodium formate (pH 3.0) at 4 °C for 24 h in the dark.[18] Reactions were terminated by the addition of 0.1 vol. of 10 % (w/v) D-mannitol. After dialysis against distilled water or desalting by gel filtration, oxidized products were cleaved by alkaline elimination at pH 12.0 for 30 min at room temperature. The degradation products obtained after one cycle of oxidation have the general carbohydrate sequence GlcNAc/$GlcNSO_3$–(GlyA–GlcNAc/$GlcNSO_3$)$_n$–R where GlyA is GlcA, IdoA or IdoA–OSO_3. After reoxidation, the remaining GlcA residues are largely

degraded and the resulting oligomers are enriched with the structure $GlcNSO_3-(IdoA-GlcNSO_3)_n-R$ with occasional ester sulfate groups on IdoA.

Chromatographic methods. Radiolabelled heparan sulfates were subjected to ion exchange chromatography on columns (6 mm×140 mm) of DE-52 DEAE-cellulose that were equilibrated with 0.1 M-sodium acetate, pH 5.0. Elution was performed with a linear gradient of 0.1–2.5 M sodium acetate, pH 5.0 (total volume, 100 ml) at a rate of 3 ml/h. The shape of the gradient was checked by conductivity measurements. Gel chromatography of various degradation products was carried out either on columns (17 mm×400 mm) on Bio-Gel P-10 or on columns (8 mm×1700 mm) of Sephadex G-50, superfine grade, that were eluted with 0.5 M NH_4CHO_3 or with 0.2 M pyridine acetate, pH 5.0, respectively. Effluents were analyzed for radioactivity either in Aquasol II on a Beckman series 7500 or in Instagel on a Packard 2650 liquid-scintillation counter both with automatic quench correction.

Affinity chromatography was performed on columns (6 mm×100 mm) of various heparan sulfate-substituted agaroses. Samples were applied in 0.15 M NaCl and the columns were eluted with linear gradients of 0.15 M NaCl 1.5 M guanidine hydrochloride (total volume, 100 ml) at a rate of 3 ml/h. Effluents were analyzed for radioactivity as above.

RESULTS

The ion exchange chromatography profiles of the ^3H-labelled heparan sulfates derived from cultures of skin and lung fibroblasts, from transformed and non-transformed 3T3 cells as well as from a human neuroblastoma cell line are shown in Fig. 1. All preparations had been

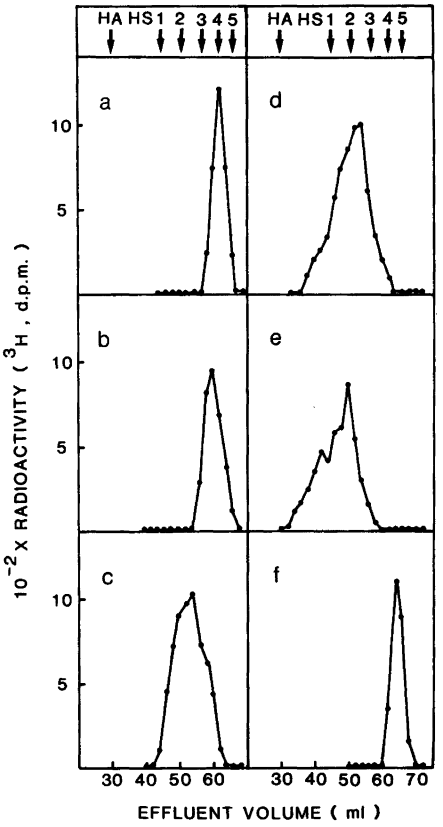

Fig. 1. Ion exchange chromatography profiles of (^3H) heparan sulfates from human embryonic skin (a) and lung fibroblasts (b), mouse 3T3 fibroblasts (c), SV40-transformed (d) and polyomatransformed 3T3 cells (e) and a human neuroblastoma cell line (f). The precursor was (^3H) GlcN. Heparan sulfates were isolated as described in the Experimental section. The elution positions of hyaluronate (HA) and five heparan sulfate subfractions (HS1–5) from bovine lung are indicated above the top panels.

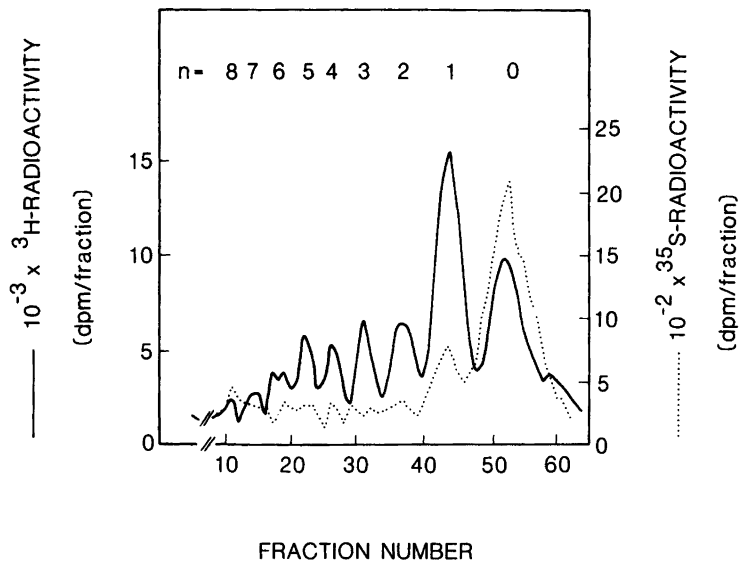

Fig. 2. Gel chromatography Bio-gel P10 of deaminative cleavage products of neuroblastoma heparan sulfate. ($^3H/^{35}S$)-Heparan sulfate was isolated as described in the Experimental section. The positions of the homologous saccharides are indicated by n=0, 1, 2... *etc*, where n is the number of intact repeating disaccharides.

treated with papain to ensure that single chains were obtained. The heparan sulfate from neuroblastoma cells was of the highest charge density eluting almost at the position of the HS5 heparan sulfate standard (Fig. 1f). Moreover, the neuroblastoma material was relatively homogeneous compared with *e.g.* heparan sulfates from virus-transformed 3T3 cells (Figs. 1d and e).

To examine the structural features of the neuroblastoma heparan sulfate preparation used in these experiments, degradation with HNO_2 was performed. The deaminative cleavage products of the trypsin-pronase treated material were subjected to gel permeation chromatography on Bio-Gel P-10 (Fig. 2). A series of saccharides ranging from di-saccharide to almost eicosa-saccharide were resolved. The di- and tetra-saccharide components (n=0 and 1 in Fig. 2) constituted 60 % of the degradation products reflecting the high degree of polymer *N*-sulfation.

Neuroblastoma heparan sulfate was subjected to affinity chromatography on a variety heparan sulfate-agaroses. Affinity was only seen with HS4-A-agarose (Fig. 3); no binding was observed with HS2-A, HS3-A or HS5-substituted gels (results not shown). As reported elsewhere [11] heparan sulfates from other cultured cells will bind to some of these affinity matrices, *e.g.* lung fibroblast heparan sulfate to HS4-A and 3T3-heparan sulfates to HS3-A or HS2-A. The neuroblastoma heparan sulfate that was obtained after trypsin and pronase treatments gave the affinity profile shown in Fig. 3a. It is seen that this material displays higher affinity than the heparan sulfate preparation that has also been digested with papain (Fig. 3b). As this suggested that the pronase-treated material was of higher molecular weight than the papain-treated one, the molecular size was estimated by gel chromatography on Sepharose CL-4B under dissociative conditions (4 M guanidine hydrochloride). Although the trypsin and pronase-treated heparan sulfate eluted in the position of single chains from

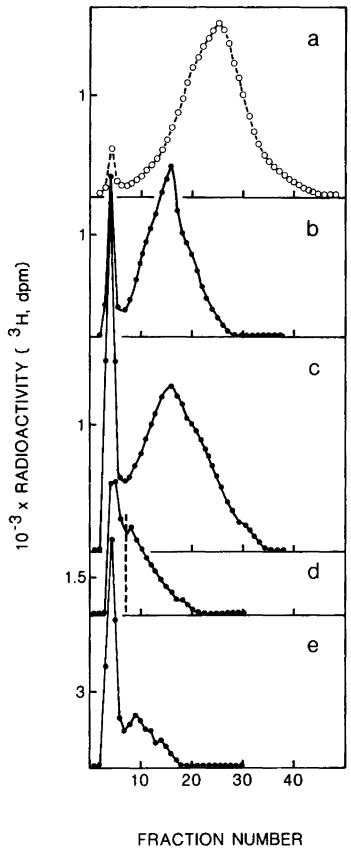

Fig. 3. Affinity chromatography on HS4-A heparan sulfate-agarose of neuroblastoma (^{3}H) heparan sulfate after trypsin and pronase digestions (a), after subsequent papain digestion (b) and in the presence of increasing amounts of carrier heparan sulfate (c-e). (^{3}H)-Heparan sulfate was isolated from the cells after trypsin and pronase digestions and subjected to affinity chromatography directly (○ in a) or after further papain degradation (● in b-e). The latter samples were chromatographed without carrier (b) and after addition of 200 μg (c), 300 μg (d) or 400 μg (e) of HS4-A heparan sulfate. Elution and analysis were performed as described in Experimental. Fractions were pooled as indicated by the vertical dashed line in d.

lung tissue or skin fibroblasts [8] suggesting an M_r of approximately 20 000, the papain-treated heparan sulfate was more retarded with an M_r of approximately 10 000 (results not shown). To further investigate the strength of binding between neuroblastoma heparan sulfate and the bovine lung HS4-A heparan sulfate, increasing amounts of carrier HS4-A were mixed with the sample before application to the affinity column (Figs. 3b–e). It is seen that increasing proportions of the labelled material were displaced from the affinity matrix with increasing amounts of carrier. In the presence of 400 μg of carrier heparan sulfate (corresponding to approximately 5 mg/ml during application) the neuroblastoma heparan sulfate interacted poorly with the immobilized chains (Fig. 3e).

In the presence of 300 μg of carrier heparan sulfate (Fig. 3d) the neuroblastoma material was partially bound to the affinity matrix. To investigate possible structural differences between bound and unbound heparan sulfate the two fractions were separately degraded by periodate oxidation-alkaline elimination. In this procedure GlcA residues of $(GlcA-GlcNAc)_n$-segments are cleaved and the resulting fragments may be resolved gel filtration. The unbound chains (Fig. 4a) afforded mainly saccharides of the structure $GlcNAc/GlcNSO_3-(GlyA-GlcNSO_3)_n-R$ with $n=0-2$ (GlyA is GlcA, IdoA or $IdoA-OSO_3$). Saccharides with $n>3$ (see bar) were scarce. In contrast, the bound chains (Fig. 4c) yielded a large proportion of extended saccharides with the peak position at $n=4$ and 5. Reoxidation of this material (see bar in Fig. 4c) followed by scission in alkali yields

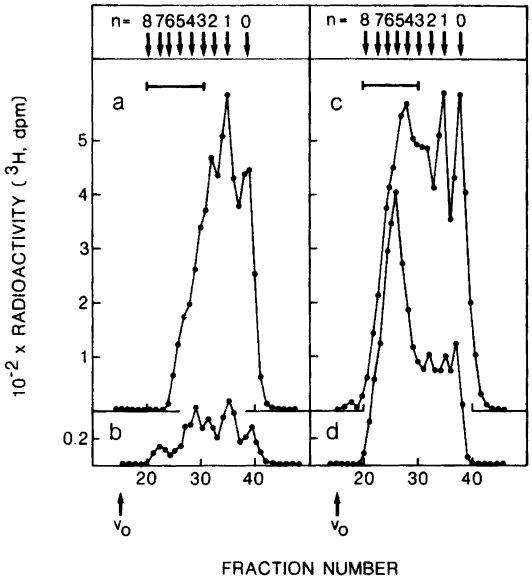

Fig. 4. Gel chromatography on Sephadex G-50 of oxidation–degradation products of (^{3}H) heparan sulfate from neuroblastoma cells. (^{3}H)-Heparan sulfate was isolated after extensive proteolysis (trypsin, pronase and papain) and subjected to affinity chromatography on HS4-A-agarose with the addition of 300 μg of carrier heparan sulfate (Fig. 3d). Two fractions were pooled (unbound and bound material) and they were subsequently oxidised with periodate and cleaved with alkali in two cycles. Degradation products of unbound material are shown in a and b and those of bound material in c and d. The elution positions of the various saccharides are indicated by arrows above the top panels. From both series (a and c) oligomers with $n>3$ were recovered (see bar), reoxidised with periodate and cleaved in alkali (second cycle; b and d).

saccharides which have the structure $GlcNSO_3-[IdoA((+-OSO_3)-GlcNSO_3]_n-R$ as GlcA is degraded upon reoxidation.[18] As shown in Fig. 4d most of the oligosaccharides derived from the affinity-bound heparan sulfate contained IdoA or $IdoA-OSO_3$ and had $n=5-6$. There were also smaller saccharides with $n=1-3$ derived from shorter segments that had been surrounded by GlcA-containing repeats. Mainly the latter type of fragments were obtained after re-oxidation of saccharides from the unbound chains (Fig. 4b).

DISCUSSION

The carbohydrate back-bones of heparan sulfate and heparin are the same, *i.e.* $(GlyA-GlcN)_n$ with β-D or α-L-linked GlyA and α-D-linked GlcN. In heparan sulfate the variability is quite formidable (see the second paragraph of this work). However, only certain sequential arrangements of repeating units are permitted. In the general formula for heparan sulfate, written as

$$[(IdoA/GlcA-GlcNAc)_m-(GlcA/IdoA-GlcNSO_3)_n]_p,$$

the first repeating unit (m) has IdoA when the latter is linked at its C-4 to a $GlcNSO_3$ of the preceding unit. Furthermore, in the succeeding repeating unit (n) the GlyA must be GlcA if

n=1. However, if n>2, internally positioned GlyA may be either GlcA or IdoA. If the GlcNAc-containing repeats have m>2 internally positioned GlyA is always GlcA. Ester-sulfates which are not indicated in the formula are often located to C-6 of $GlcNSO_3$ and occasionally to C-2 of IdoA. The neuroblastoma heparan sulfate chains have an M_r approximately 10 000 which means that the total number of repeats is approximately 20. As half of the repeating units contain $GlcNSO_3$ there are approximately 10 GlyA−$GlcNSO_3$ and 10 GlyA−GlcNAc disaccharides in the molecule.[13] The prevailing arrangement of GlyA−$GlcNSO_3$ was as single or double re- peats, *i.e.* n=1 or 2. However, n could reach a value of 5 or 6 as indicated by the results of periodate oxidation. The deaminative cleavage products ranged in size from di- to eicosasaccharide. Therefore, m in the above formula may range from 1−8 or 9 but with a prevalence for the lower numbers. It should be noted that segments of GlyA−GlcNAc with, *e.g.* m=5 are usually nonsulfated. As all the heparan sulfate chains show strikingly uniform charge density (Fig. 1) low- or non-sulfated segments must be combined with high-sulfated ones, *e.g.* IdoA(−OSO_3)−$GlcNSO_3$(−OSO_3) in relatively constant proportions. The most frequent sequential arrangement of the two major types of repeating units should be an alternating or mixed one with m=1−2 and n=1−2, *i.e.* p=5−10.

A high proportion of alternating arrangements of the disaccharide repeats GlcA/IdoA−GlcNAc, GlcA−$GlcNSO_3$ and IdoA−$GlcNSO_3$ is commonly found in the putative contact zones for self-association.[3–5,9] The neuroblastoma heparan sulfate which contains such repeats had affinity for a heparan sulfate-agarose gel (Fig. 3). The affinity was specific for the HS4-A type. Neuroblastoma heparan sulfate was easily displaced from the affinity matrix by HS4-A type heparan sulfate. This effect has not been observed with radiolabelled heparan sulfates from other cells.[4,7,8] Therefore, the structure of neuroblastoma heparan sulfate is probably only partially related to that of the HS4-A subtype. In the latter type of heparan sulfate the mixed/alternating arrangements of GlcA−GlcN and IdoA−GlcN occurs in the N-sulfated segments.[6] In neuroblastoma heparan sulfate blocks of GlcA−GlcNAc alternates with blocks of IdoA(−OSO_3)−$GlcNSO_3$. Moreover, the neuroblastoma heparan sulfates must be quite heterogeneous despite a relatively uniform charge density. That fraction of heparan sulfate which did not bind to the affinity matrix when 300 μg of carrier HS4-A was added showed the greatest similarity with this type of heparan sulfate, *i.e.* both GlcA−GlcN and IdoA−GlcN repeats were present in the *N*-sulfated regions. The material that was bound under the same conditions contained a large proportion of extended regions with IdoA(+−OSO_3)−$GlcNSO_3$−disaccharides. This subpopulation of heparan sulfate chains was displaced in the presence of higher amounts (400 μg) of carrier heparan sulfate. Although neuroblastoma heparan sulfate had generally a weak affinity for the HS4-A type of heparan sulfate it cannot be excluded that other forms of heparan sulfate, *e.g.* chains with a high proportion of IdoA(−OSO_3)-containing units, could interact strongly with this material. Anyhow, under the present conditions the neuroblastoma heparan sulfate could be separated into two pools, one which was similar to the HS4-A type of fibroblast heparan sulfate and one which was heparin-like in as much as it contained disulfated or trisulfated disaccharide units (see also 13).

The heparan sulfate studied here was presumably part of a native proteoglycan located to the pericellular coat of the neuroblastoma cells. Large, neurite-associated glycoconjugates have been isolated from rat neuroblastoma cells.[19] The glycosaminoglycan side-chains of this proteoglycan were not fully characterized, but could be a lowsulfated form of heparan sulfate. Proteoheparan sulfates are also present in cholinergic synaptic vesicles,[20,21] but the

Table 1. Structure and properties of heparan sulfates (HS) from various sources.

Source	$-(GlcA-GlcNAc)_n-$ %	$-(IdoA-GlcNAc-GlcA-GlcNSO_3)_n-$ %	$-(IdoA-GlcNSO_3)_n-$ | SO_4	Binding to HS-agarose
Bovine lung	55–80	10–25	5–10	strong and specific
Rat brain	50–80	>5	20–40	unknown
Human neuroblastoma	50	25	25	weak

side-chains have not yet been analyzed in detail. The heparan sulfate of rat brain has been degraded by nitrous acid and the products have been examined.[22] The structural features of heparan sulfates from lung, brain and neuroblastoma are summarized in Table 1 (see also Ref. 7). Most heparan sulfate chains contain extended sections with GlcA–GlcNAc repeats, usually non-sulfated but, in the case of rat brain,[22] heavily ester-sulfated. The neuroblastoma heparan sulfate did not contain sulfate in these regions. The major difference between the neuroblastoma heparan sulfate and the fibroblast-derived material[7] is probably a high proportion of IdoA($-OSO_3$)–$GlcNSO_3$ repeats in the former material. The latter feature could also be responsible for the weaker tendency to aggregate on the part of the neuroblastoma chains.

It was noted that the neuroblastoma heparan sulfate preparation obtained after papain digestion was of lower molecular weight than the corresponding material prepared after trypsin and pronase digestions. It is possible that the heparan sulfate side-chains are clustered together on the protein core, preventing trypsin and pronase from access to the polypeptide core. Papain may be able to cleave the peptide chain between every polysaccharide attachmentpoint. Alternatively, the effect of papain could be due to the reducing conditions under which digestion is performed. The protein core may contain disulfide-bonded segments.

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