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Structure of heparan sulphate from human brain, with special regard to Alzheimer's disease

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Heparan sulphate (HS) was isolated after proteolytic digestion of cerebral cortex, obtained at autopsy, of patients with Alzheimer's disease (AD) and of control subjects. Deaminative cleavage in combination with selective radiolabelling procedures showed that the N-acetylated regions in the intact polysaccharides ranged from isolated residues to ~ 10 consecutive N-acetylated disaccharide units, without any apparent difference between AD and control HS. The yield of disaccharide deamination products was slightly higher with AD than with control HS, suggesting a differential distribution of N-sulphate groups. Separation of the disaccharides by anion-exchange h.p.l.c. yielded four mono-O-sulphated and one di-O-sulphated disaccharide; these components occurred in strikingly similar proportions in all cerebral HS preparations (except polysaccharide from neonatal brain) irrespective of the age of the individual and the histopathology of the cortex specimen. No significant difference was noted between HS obtained from control and from AD tissue. By contrast, the composition of HS isolated from brain differed significantly from that of HS preparations derived from other human organs.

INTRODUCTION

The sulphated glycosaminoglycans (GAGs) usually occur in the tissues as proteoglycans, in which the GAG chains are covalently bound to a core protein (for a review, see Kjellén and Lindahl, 1991). Heparan sulphate (HS) proteoglycans, abundant on cell surfaces and in the extracellular matrices of most tissues, have attracted particular attention due to the propensity of HS chains for binding to proteins. HS has thus been implicated in a variety of interactions with proteins of manifest or alleged functional importance. The physiological or pathophysiological significance of such interactions have been the subject of much speculation.

While the aetiology of Alzheimer's disease (AD) remains unclear, its neuropathological features are fairly well defined. Amyloid deposits are associated with the typical lesions, i.e. senile plaques and neurofibrillary tangles, and are also found in the vascular walls. The predominant constituent of senile plaques has been identified as the 40–43 amino acid residue β-amyloid protein (also referred to as Aβ; Husby et al., 1993). The β-protein is the proteolytic-cleavage product of a larger amyloid precursor protein (APP; 365–770 amino acids in various isoforms, corresponding to alternatively spliced exons) (for reviews see Glenner, 1988; Kosik, 1992).

Numerous reports have indicated the occurrence of GAGs/proteoglycans in AD lesions (Snow et al., 1988; Snow and Wight, 1989; Perry et al., 1991; Kisilevsky, 1992; Su et al., 1992). Immunohistochemical evidence particularly implicates the HS proteoglycan 'perlecan' (Snow et al., 1994a,b). Curiously, the core proteins of both HS (Schubert et al., 1988) (however, see Gowda et al., 1989) and, more recently, chondroitin sulphate (CS) (Shioi et al., 1992) proteoglycans were claimed to be strongly related or identical to the APP itself. The ubiquitous distribution of HS proteoglycans (Herndon and Lander, 1990; Margolis and Margolis, 1993) and their functional involvement with important processes such as neurite outgrowth (Raulo et al., 1994) and Schwann cell proliferation (Ratner et al., 1988) have further focused attention on this type of proteoglycan in relation to AD. A 'consensus sequence' for heparin/HS binding (Cardin and Weintraub, 1989) has been identified corresponding to residues 12–17 of the Aβ peptide, and interaction with the sulphate substituents of GAG chains is believed to influence the aggregation behaviour of the peptide (Fraser et al., 1992). Indeed, it has been speculated that altered binding properties due to deranged sulphation of HS may be a primary cause of the disease (Zebrower and Kieras, 1993). However, little is known regarding the structure of HS GAG from human brain, let alone the correlation to AD. The present study was initiated in order to obtain such information.

MATERIALS AND METHODS

Materials

Patients with Alzheimer's disease [designated 3 (80 years), 5 (80 years), 6 (81 years) and 11 (75 years)] were selected by clinical diagnosis and the typical neuropathological findings. The following criteria were used for histopathological diagnosis: numerous senile plaques with a central core of amyloid, Alzheimer neurofibrillary degeneration and deposition of amyloid in cerebral vessel walls. Brain tissue was also obtained from three control subjects [2 (76 years), 4 (26 years), 13 (59 years)] without dementia and without any histopathological signs of AD, as well as from a newborn. The tissue specimens were recovered at autopsy 2–4 days post-mortem. Cerebral cortex was separated from leptomeninges and its vessels through minute dissection, yielding ~ 5 g of cortical tissue from each of four regions (frontal, parietal, occipital and temporal lobes) of the right hemisphere. The material was immediately deposited at -20 °C. Material from the left hemisphere was fixed in 4% buffered formaldehyde solution, embedded in paraffin, cut and stained with Mayer's haematoxylin-eosin and alkaline Congo Red.

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; CS, chondroitin sulphate; DS, dermatan sulphate; GAG, glycosaminoglycan; GlcA, D-glucuronic acid; GlcN, 2-amino-2-deoxy-D-glucose (D-glucosamine); HA, hyaluronan; HS, heparan sulphate; IdoA, L-iduronic acid; aMan$_R$, 2,5-anhydro-D-mannitol.

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Sections with the latter stain were studied in polarized light. Specimens of liver and kidney tissue were obtained from patient 13.

The following enzymes were obtained from the commercial sources indicated: papain (twice crystallized suspension, 16–40 units/mg protein; Sigma); Pronase (type XXV from *Streptomyces griseus*; Sigma); endonuclease (Benzonase; Benzon Pharma A/S); chondroitinase ABC (chondroitin lyase ABC; Sigma), heparitinase I (from *Flavobacterium heparinum*; Seikagaku). [^{3}H]Acetic anhydride (500 mCi/mmol) and NaB^3H_4 (24–28 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, U.K.).

A sample of bovine kidney HS, given by Dr. K. Sugahara (Kobe Pharmaceutical University, Kobe, Japan), was radiolabelled by N-deacetylation/N-[^{14}C]acetylation, essentially as described below (N-deacetylation by hydrazinolysis was extended to 2 h), yielding a product with a specific radioactivity of $\sim$ 50000 c.p.m. of ^{14}C/μg. HS isolated from human aorta (pooled specimens from many individuals; processed according to Iverius, 1971) was provided by W. Murphy, University of Monash, Melbourne, Australia. Unlabelled standards of hyaluronan, CS and heparin were as described by Enerbäck et al. (1985). *Escherichia coli* K5 capsular polysaccharide was given by K. Jann, Max-Planck-Institut für Immunbiologie, Freiburg, Germany.

Methods

Isolation of polysaccharides

The isolation of GAGs from tissue specimens was performed according to a modification of the procedures of Singh and Bachhawat (1968) and of Oohira et al. (1986). Tissues were cut into small pieces that were homogenized in a Potter–Elvehjem homogenizer in 10 vol. of ice-cold acetone. The homogenate was centrifuged at 2000 ***g*** for 10 min and the resulting supernatant was discarded. The pellet was suspended in 5 vol. (relating to the initial amount of tissue) of ice-cold chloroform/methanol (2:1 by vol.) and was then rehomogenized (in a Kontes tissue grinder) and centrifuged. The extraction with chloroform/methanol was performed five times altogether. The defatted material was finally washed with cold ethyl ether and dried overnight. The residue was weighed to obtain dry weight of defatted material (400–500 mg from 5 g of cerebral cortex).

Defatted tissue (up to 1 g) was digested with 5 mg of Pronase in 25 ml of 0.1 M Tris/HCl/2 mM $CaCl_2$/3 % ethanol (pH 8.0). After 12 h of incubation at 50 °C, the digest was heated at 100 °C for 10 min. The sample was cooled and adjusted to pH 5.5 with 1 M acetic acid along with further additions to give final concs. of 10 mM EDTA, 10 mM cysteine/HCl and 2 M NaCl. Papain (5 mg) was added and the sample was digested at 60 °C for 18 h. After heat-inactivation (10 min at 100 °C) and centrifugation the residue was washed twice with 15 ml of 0.05 M sodium acetate/2 M NaCl (pH 5.5) and the washes were combined with the supernatant. The final solution was dialysed first against water and then against 0.05 M Tris/HCl, pH 7.2, before ion-exchange chromatography. The final insoluble residue was washed twice with ethanol, then with ether, and was finally dried. To solubilize any residual GAG in this material, 100 mg of the dried sample was extracted at 4 °C with 1 ml of 0.5 M NaOH. After 24 h the sample was neutralized, diluted to 5 ml with 0.05 M Tris/HCl, pH 7.2, and centrifuged. The supernatant was fractionated by anion-exchange chromatography as described below (Procedure B).

The samples obtained after proteolytic digestion were further processed according to two alternative procedures. In Procedure A the samples were applied to columns (1 × 5 cm) of DEAE-Sephacel (Pharmacia) equilibrated with 0.05 M Tris/HCl, pH 7.2, which were then washed with 5 bed vol. of the same buffer followed by 5 bed vol. of 0.05 M sodium acetate, pH 4.0. The columns were then eluted using a linear gradient from 0.05 M to 1.5 M LiCl in the acetate buffer (75 ml total elution volume). Effluent fractions were analysed for hexuronic acid (see below) and were combined into two pools corresponding to non-sulphated (essentially hyaluronan, HA) and sulphated GAGs. The sulphated fraction was desalted by dialysis against water and was then lyophilized. For degradation of nucleic acids and galactosaminoglycans, samples (containing up to 0.5 mg of GAG) were dissolved in 0.5 ml of 0.05 M Tris/HCl/1 mM $MgCl_2$/3 mM sodium acetate (pH 8.0) containing 0.1 units of chondroitinase ABC and 125 units of Benzon endonuclease (Benzonase), and the mixtures were incubated at 37 °C for 15 h. After heating at 100 °C for 2 min the samples were applied to a column (1 × 57 cm) of Sephadex G-50 (superfine) and were subsequently eluted with 0.2 M NH_4HCO_3. Effluent fractions of $\sim$ 2 ml were collected and analysed for hexuronic acid. Fractions of excluded material were combined and were desalted by freeze-drying. In Procedure B, anion-exchange chromatography was initiated by washing the DEAE-Sephacel columns with pH 7.2 and pH 4.0 buffers, as described for Procedure A. An additional washing step with 0.15 M LiCl in 0.05 M sodium acetate, pH 4.0, was followed by elution with 2 M LiCl in the same buffer. The high-salt fractions were recovered and desalted, followed by Benzonase digestion as described above. The final product thus contained HA and galactosaminoglycans in addition to HS.

N-[^{3}H]Acetyl-labelling of polysaccharides

Chondroitinase-resistant polysaccharide fractions were N-[^{3}H]acetyl-labelled, in essence according to the procedure of Höök et al. (1982). Samples (corresponding to 10–20 μg of hexuronic acid) were partially N-deacetylated by treatment for 30 min at 96 °C with 500 μl of hydrazine, containing 30 % (v/v) water and 1 % (w/v) hydrazine sulphate (Guo and Conrad, 1989). The products were repeatedly evaporated to dryness after addition of toluene, and the polysaccharide was then reisolated either by gel chromatography on a column (1 × 90 cm) of Sephadex G-15 in 10 % aqueous ethanol or by extensive dialysis against water. After freeze-drying, the partially N-deacetylated material was dissolved in 100 μl of 0.05 M Na_2CO_3, containing 10 % (v/v) methanol. A total of 4 mCi of [^{3}H]acetic anhydride was added intermittently over 1 h, while the pH was kept at 7.0 by additions of 10 % methanol saturated with Na_2CO_3, and was followed by 5 consecutive additions of 10 μl of unlabelled acetic anhydride during an additional 1 h period. The mixture was applied to a column (1 × 12 cm) of Sephadex G-15, eluted with 0.5 M sodium acetate/1 M NaCl. The labelled, excluded material was pooled and dialysed against two changes of 0.5 M sodium acetate/1 M NaCl followed by several changes of water. The product was freeze-dried to dryness [specific activity (0.5–1) × 10^6 c.p.m. ^{3}H/μg hexuronic acid; however, see heterogeneity of product in the Results section].

Compositional analysis of HS

Deamination of polysaccharide, resulting in cleavage at N-sulphated 2-amino-2-deoxy-D-glucose (GlcN) units, followed by reduction of the products with NaB^3H_4, was used to determine the composition of HS preparations (Bienkowski and Conrad, 1985; Pejler et al., 1987). Samples containing $\sim$ 5 μg of hexuronic acid were evaporated to dryness and dissolved in 100 μl of HNO_2 reagent (pH 1.5) (Shively and Conrad, 1976). After 10 min at

Table 1 Glycosaminoglycan preparations from cerebral cortex

Preparation*	Age of patient (years)	Glycosaminoglycans (μg hexuronic acid/g)†		
		HA	CS/DS	HS
2pC	76	340	200	80
3pAD	80	460	360	90
4pC	26	290	280	90
5pAD	80	700	530	140
6pAD	81	370	770	110

* The numbers refer to different patients. C, control (i.e. no sign of AD). All samples shown in the Table were derived from parietal cortex.

† The amounts of the various GAGs were determined as described in the Methods section and are expressed as μg of hexuronic acid per gram of dry, defatted cortex.

room temperature the reaction was interrupted by the addition of 20 μl of 2 M Na_2CO_3 and the sample was reduced with 0.5 mCi of NaB^3H_4. After an additional 15 h at room temperature the pH of the sample was adjusted to $\sim$ 4 with 4 M acetic acid (in a fume-hood) and then to $\sim$ 7 with 4 M NaOH. Alternatively, the polysaccharide samples were initially treated with 5 μmol of unlabelled $NaBH_4$ before the deaminative cleavage (which was again followed by reduction with NaB^3H_4), thus ensuring that the incorporation of ^3H could be ascribed to the specific cleavage process. The more elaborate dual-reduction procedure was applied to selected samples of HS that contained potentially reducible impurities, such as material solubilized by alkali treatment from protease-resistant tissue residues. The ^3H-labelled di- and oligo-saccharides were first separated in one pool from smaller labelled compounds on a column (1 × 170 cm) of Sephadex G-15 in 0.2 M NH_4HCO_3. This pool was subsequently fractionated into di-, tetra- and larger oligo-saccharides on a somewhat longer column (1 × 200 cm) under similar conditions. Pooled fractions were desalted by freeze-drying. The disaccharides were separated further by anion-exchange chromatography on a Partisil-10 SAX column (Whatman), connected to a model Flo-One HS radioactive-flow detector, as described by Pejler et al. (1987).

Additional analytical methods

High-voltage paper electrophoresis of ^3H-labelled saccharides was conducted on Whatman 3MM paper in 1.6 M formic acid (pH 1.7) at 40 V/cm. The paper strips were cut into 1 cm segments that were extracted with 1 ml of water for 30 min, and the ^3H was determined by scintillation counting.

Hexuronic acid was quantified by the carbazole method (Bitter and Muir, 1962), with glucuronolactone as standard. Radioactivity was measured by liquid scintillation, using either a Beckman LS 6000IC liquid scintillation spectrometer or the radioactive-flow detector indicated above.

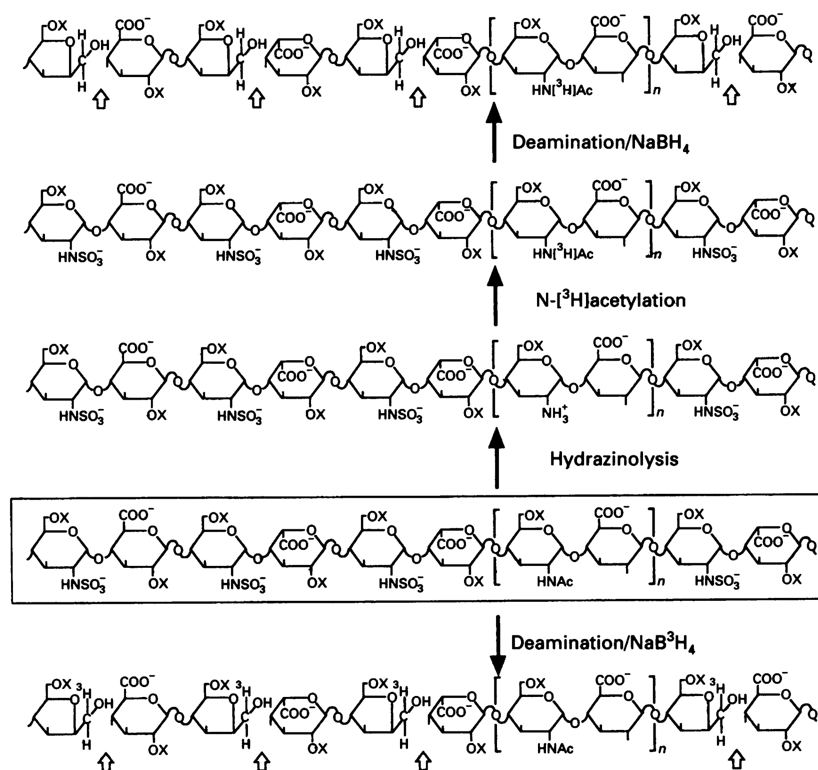

Figure 1 Scheme of radiolabelling procedures used in the structural analysis of HS

Different procedures were used to preferentially label the N-acetylated or the *N*-sulphated regions of the HS chain. For N-[^{3}H]acetylation, HS samples (native sample framed) were partially N-deacetylated by hydrazinolysis and were then treated with [^{3}H]acetic anhydride. The labelled product was treated with HNO_2 to induce deaminative cleavage of the chain at the sites of N-sulphated GlcN residues; the N-[^{3}H]acetylated GlcN units were recovered in labelled oligosaccharides the size of which varied with the number of consecutive N-acetylated disaccharide units. Alternatively, direct deaminative cleavage of the polysaccharide followed by reduction with NaB^3H_4 led to incorporation of ^{3}H-label at the sites of N-sulphated GlcN residues, yielding labelled disaccharides from sequences of consecutive N-sulphated disaccharide units, and larger oligosaccharides from regions containing HNO_2-resistant, N-acetylated disaccharide units. For further details see the text.

RESULTS

Isolation of polysaccharides

Samples of HS were isolated from cerebral cortex of patients with clinical diagnosis of AD and characteristic neuropathology, and from control subjects. Defatted cortex was subjected to proteolytic digestion and the digests were fractionated by anion-exchange chromatography (Procedure A, see Methods section). Carbazole-positive material emerged in two major peaks of about equal size, the less retarded of which appeared at the same elution position as HA (results not shown). Fractions appearing after the HA peak were pooled, desalted and digested with chondroitinase ABC and nuclease. A resistant HS fraction was separated from the resultant CS or dermatan sulphate (DS) oligosaccharides by gel chromatography (results not shown). The yields of the various GAGs, as assessed from these relatively crude analyses, showed appreciable variability between samples (Table 1). While a larger number of observations would be required to establish any correlation between GAG contents and the disease state, the data are compatible with slightly increased overall polysaccharide contents in afflicted tissue. The yield of HS averaged ~ 100 μg of hexuronic acid per gram of dry defatted cortex, the AD samples tending to give somewhat higher values (see also Jenkins and Bachelard, 1988). The interpretation of these results is complicated by the fact that the tissue specimens varied with regard to the contents of protease-resistant material (results not shown), which in one case accounted for as much as 26% of the dry, defatted starting material. Most samples contained ~ 10% of protease-insoluble material. Cortex of neonatal brain contained < 2% of protease-insoluble material and an exceptionally high content of total GAG [1900 μg of hexuronic acid per gram, determined according to Procedure B (see Methods section) and thus accounting for HA as well as sulphated GAGs].

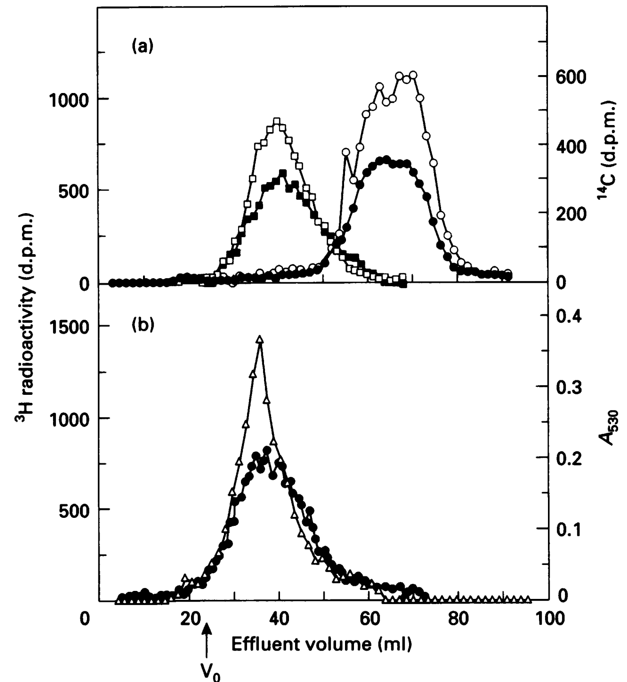

Figure 3 Gel chromatography of N-[^{3}H]acetyl-labelled HS and of its deamination products

N-[^{3}H]acetyl-labelled HS, isolated by anion-exchange chromatography (Figure 2b), was analysed on a column (1 × 100 cm) of Sephadex G-100, eluted with 1 M NaCl and effluent fractions were analysed for radioactivity or for hexuronic acid using the carbazole method. (**a**) [^{3}H]HS from sample 5fAD (□, ○), together with N-[^{14}C]acetyl-labelled standard kidney HS (■, ●), before (□, ■) and after (○, ●) treatment with HNO_2 (pH 1.5 procedure); and (**b**) intact [^{3}H]HS from sample 2pC (●), together with unlabelled pig mucosal heparin (M_r ~ 12000) (△, carbazole analysis). For further details see the text.

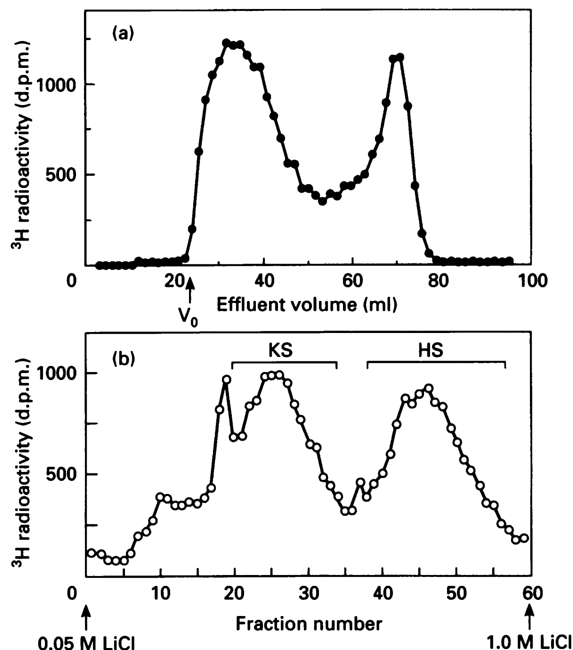

Figure 2 Characterization of chondroitinase-resistant GAGs

Cerebral GAGs resistant to digestion with chondroitinase ABC were partially N-deacetylated and re-N-[^{3}H]acetylated as described in the Methods section (see also Figure 1). Products were (**a**) analysed for susceptibility to bacterial heparitinase I; and (**b**) separated by anion-exchange chromatography. (**a**) Sample 2pC (~ 50000 c.p.m.) was incubated at 43 °C for 9 h with 5 m-units of heparitinase I in 150 μl of 50 mM Hepes/1 mM $CaCl_2$ (pH 7.0). The product was analysed by gel chromatography on a column (1 × 100 cm) of Sephadex G-100, eluted with 1 M NaCl. (**b**) N-[^{3}H]Acetyl-labelled sample 5fAD was fractionated by anion-exchange chromatography on a column (1 × 6 cm) of DEAE-Sephacel, equilibrated with 0.05 M LiCl in 0.05 M acetate buffer, pH 4.0. The column was eluted using a linear gradient (total volume, 100 ml) extending from 0.05 M to 1.0 M LiCl in the same buffer. Effluent fractions of 1.8 ml were analysed for radioactivity, combined as indicated by the horizontal brackets and dialysed against water. Further characterization of fractions KS and HS was performed as described in the text.

Purification of HS

To obtain structural information of the HS present in normal versus diseased cortex the alleged HS fractions were radiolabelled by N-deacetylation (through hydrazinolysis) followed by re-N-[^{3}H]acetylation (see Methods section and Figure 1). Unexpectedly, only part of this material was susceptible to digestion with bacterial heparitinase, a major labelled macromolecule remaining virtually unaffected by the enzyme (Figure 2a). The occurrence in the 'HS' fraction of two distinct ^{3}H-labelled polysaccharide species, as suggested by this finding, was verified by anion-exchange chromatography on DEAE-Sephacel, using a shallow salt-gradient, which yielded two major, almost completely separated peaks of ^{3}H-labelled material (Figure 2b). The more retarded of these components (designated HS in Figure 2b), derived from either AD or control tissue, emerged slightly before and partly overlapping with standard CS (results not shown). Isolation of this component by preparative

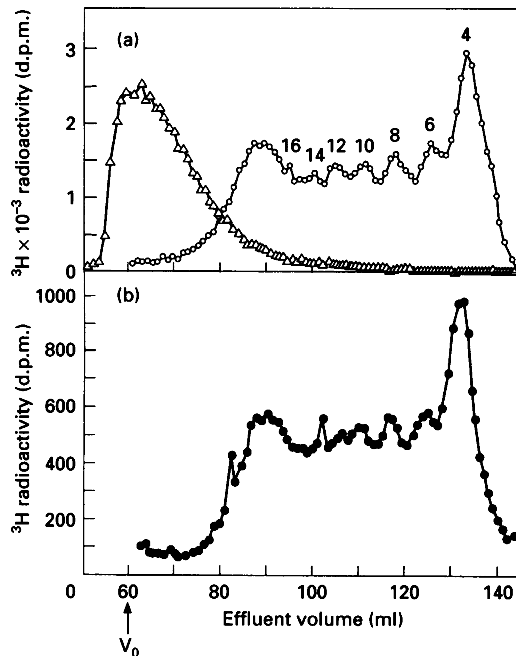

Figure 4 Size-distribution of N-acetylated regions in cerebral HS

Samples of N-[^{3}H]acetyl-labelled HS (corresponding to fraction HS in Figure 2b) from (**a**) preparation 3pAD; and (**b**) preparation 4pC, were deaminated with HNO_2 (pH 1.5 procedure) and the products were subjected to gel chromatography on a column (1 × 200 cm) of Sephadex G-50 (superfine) eluted with 1 M NaCl (○, ●); (**a**) also shows a chromatogram of sample 3pAD before deamination (△). The figures above the oligosaccharide peaks in (**a**) indicate the number of monosaccharide units per molecule. This assignment was based on calibration of the column with oligosaccharides, obtained from *E. coli* K5 capsular polysaccharide by partial N-deacetylation (hydrazinolysis as described in the Methods section, for 15 min) followed by deamination at pH 3.9 (Shively and Conrad, 1976). The resulting even-numbered oligosaccharides were detected by the carbazole reaction (results not shown).

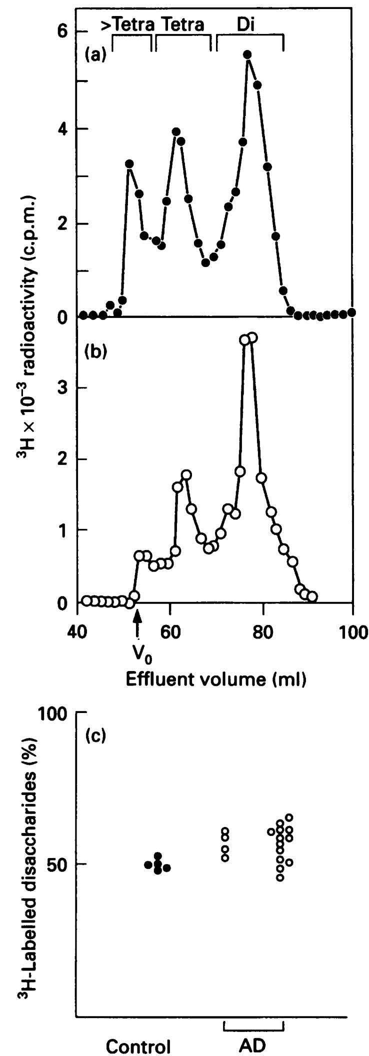

Figure 5 Gel chromatography of deamination products from cerebral HS

The HS constituents of samples (**a**) 2pC and (**b**) 6oAD, were degraded by HNO_2/NaB^3H_4 treatment (see Figure 1) and the resultant ^{3}H-labelled oligosaccharides were separated by gel chromatography on Sephadex G-15, as described in the Methods section. Effluent fractions were pooled as indicated by the horizontal brackets in (**a**) and were analysed further as described in the text. (**c**) Ratios of ^{3}H-labelled disaccharides/total saccharides obtained by HNO_2/NaB^3H_4-treatment of HS from control or AD specimens. The disaccharide peaks of chromatograms similar to those shown in (**a**) and (**b**) were related to the corresponding overall elution profiles. One of the control samples is derived from neonatal brain. The AD samples were from 13 analyses of separate HS preparations (shown in the right-hand column) from four different patients (3, 5, 6, 11). The data in the middle column include only one sample from each patient and refer in each case to cortex samples showing the highest density of senile plaques.

ion-exchange chromatography afforded a product that was susceptible to digestion with bacterial heparitinase (results not shown) and to degradation with nitrous acid (pH 1.5 procedure), as evidenced by gel chromatography of intact and deaminated HS (mixed with N-[^{14}C]acetyl-labelled bovine kidney HS standard) (Figure 3a). A gel chromatogram of undegraded HS, purified from control brain (Figure 3b), did not differ significantly from that of AD HS (Figure 3a). The peak elution position of the cerebral HS preparations coincided approximately with that of commercial heparin (M_r ~ 12000) from pig intestinal mucosa (Figure 3b). These findings indicate that the HS species derived from normal and from AD brain were of roughly similar charge, density and molecular size.

The less retarded fraction (designated KS in Figure 2b) was found to be degradable by *Pseudomonas* 1,4,-β-D-galactanohydrolase (keratanase), suggesting that it is a sulphated poly-N-acetyllactosamine (low-sulphated keratan sulphate) (results not shown). The KS fraction was not analysed further.

Structural analysis of HS

Information regarding the distribution of N-acetyl groups in purified HS was obtained by gel chromatography of oligosaccharides, generated by deaminative cleavage of N-[^{3}H]acetyl-labelled polysaccharide at the sites of (isolated or consecutive) N-sulphated disaccharide units (see Figure 1). While the intact polysaccharides were partly excluded from Sephadex G-50 (Figure 4a) the deamination products yielded distinct patterns of labelled oligosaccharides that extended from a predominant tetrasaccharide peak over a series of even-numbered, essentially equi-abundant 6- to 16-saccharides, to a slight accumulation of larger, 18- to ~ 24-mers. The chromatograms relating to AD (Figure 4a) and control (Figure 4b) HS were virtually indistinguishable. From the relative size of the tetrasaccharide peaks it may be calculated that ~ 30% of the total N-acetyl groups occur as solitary units, surrounded by N-sulphated structures, the remainder forming N-acetylated block sequences composed of from 2 to ~ 12 consecutive N-acetylated disaccharide units.

Detailed information regarding the distribution of sulphate

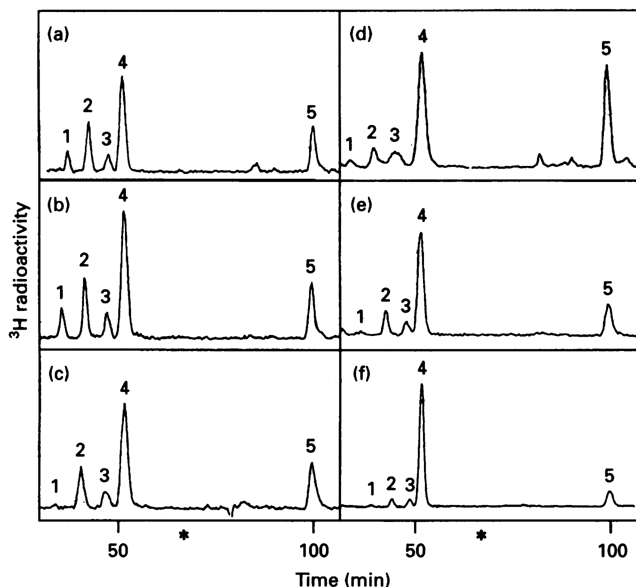

Figure 6 Anion-exchange h.p.l.c. of ^{3}H-labelled disaccharides obtained by deaminative cleavage of human HS samples followed by NaB^3H_4-treatment

The chromatograms shown relate to HS samples derived from (**a**) adult cerebral cortex specimen 13fC; (**b**) adult cerebral cortex specimen 11pAD; (**c**) neonatal cerebral cortex; (**d**) kidney; (**e**) liver; and (**f**) aorta. The numbers indicate the elution positions of the following O-sulphated disaccharide standards (the early portions of the chromatograms, containing non-sulphated disaccharides, are not included): (1) GlcA(2-OSO_3)-$aMan_R$; (2) GlcA-$aMan_R$(6-OSO_3); (3) IdoA-$aMan_R$(6-OSO_3); (4) IdoA(2-OSO_3)-$aMan_R$; (5) IdoA(2-OSO_3)-$aMan_R$(6-OSO_3). The asterisk (*) indicates the switch from 0.026 M to 0.154 M KH_2PO_4 elution buffer.

groups was obtained by analysis of ^{3}H-labelled disaccharides and larger oligosaccharides generated by HNO_2/NaB^3H_4-treatment of unlabelled HS fractions (see Methods section and Figure 1). The HS preparations yielded labelled disaccharides and larger oligosaccharides from (-GlcNSO_3)-*HexA-GlcNSO$_3$*- and (-GlcNSO_3)-*HexA-[GlcNAc-GlcA]$_{\geqslant 1}$-GlcNSO$_3$*- sequences, respectively, in the intact polysaccharides (see Figure 1). The proportions of these components varied somewhat, as illustrated for two samples in Figures 5(a) and 5(b). Compiling these data for all cerebral HS preparations analysed, revealed a broader range of N-sulphate:N-acetyl molar ratios for the AD as compared with control polysaccharides, as reflected by the proportions of disaccharide deamination products (Figure 5c). This finding suggests an over-representation of more highly N-sulphated HS in AD brain.

The HexA-GlcNAc-GlcA-[1-^{3}H]aMan_R (aMan_R = 2,5-anhydro-D-mannitol formed by reduction of terminal 2,5-aMan residues, deamination products of *N*-sulphated glucosamine units in the intact saccharide) tetrasaccharides were analysed with regard to O-sulphate contents by high-voltage paper electrophoresis at pH 1.7 (see Methods section; electropherograms not shown). No difference was noted between AD and control HS. For example, the relative proportions of non-sulphated, mono-O-, di-O- and tri-O-sulphated tetrasaccharides derived from 3pAD HS were 31, 50, 15 and 5%, whereas the corresponding values for 4pC HS were 35, 47, 14 and 4% respectively. [Samples of HS from cerebral cortex are designated by a running number, the origin of the sample (f, frontal; p, parietal; o, occipital, t, temporal region) and an indication of AD or control (C) status.]

The labelled HexA-[1-^{3}H]aMan_R disaccharides were separated further by anion-exchange h.p.l.c. (Figure 6) into non-sulphated species (not determined), four different mono-O-sulphated disaccharides (the O-sulphate groups are indicated in parentheses) [GlcA(2-OSO_3)-aMan_R, GlcA-aMan_R(6-OSO_3), IdoA-aMan_R(6-OSO_3), IdoA(2-OSO_3)-aMan_R] and a di-O-sulphated disaccharide (IdoA = L-iduronic acid). The di-O-sulphated component derived from cerebral HS was identified as IdoA(2-OSO_3)-aMan_R(6-OSO_3), since IdoA-aMan_R(6-OSO_3) and IdoA(2-OSO_3)-aMan_R were the only mono-O-sulphated disaccharide products found in significant amounts after mild acid treatment [0.2 M trifluoroacetic acid, 100 °C, 30 min; results not shown (see Kusche et al., 1988)]. All samples derived from adult brain HS (altogether 13 separate preparations; Table 2) yielded strikingly similar patterns, with predominant peaks of GlcA-

Table 2 O-Sulphated disaccharides produced on deamination of human HS preparations

		Disaccharide deamination products* (%)				
Source of HS	Age of patient (years)	GlcA(2-OSO_3)-aMan_R	GlcA-aMan_R(6-OSO_3)	IdoA-aMan_R(6-OSO_3)	IdoA(2-OSO_3)-aMan_R	IdoA(2-OSO_3)-aMan_R(6-OSO_3)†
Cerebral cortex						
AD ($n = 13$)‡	75–81	8.8 ± .1.5	20 ± 1.9	5.8 ± 1.2	48 ± 2.4	18 ± 2.9
Protease insoluble§ ($n = 1$)	75	11	20	8.6	45	16
Control adult‖ ($n = 3$)	26–76	8.0 ± 2.3	19 ± 0.6	5.7 ± 1.1	46 ± 1.4	21 ± 1.1
Neonatal ($n = 1$)	< 1	ND¶	16	8.3	53	23
Aorta ($n = 1$)	(Pooled tissue samples)	1.0	4.5	4.3	77	13
Liver ($n = 1$)	59	ND	12	7.6	62	19
Kidney ($n = 1$)	59	2.6	6.2	8.5	48	35

* Percentage (± S.D. as applicable) of total-O-sulphated HexA-[1-^{3}H]aMan_R disaccharides obtained after HNO_2/NaB^3H_4 (or $NaBH_4/HNO_2/NaB^3H_4$ treatment; see Methods section).

† The di-O-sulphated disaccharide was identified by a procedure involving partial hydrolytic O-desulphation as described in the text. However, it has been shown that the isomeric disaccharide, IdoA(2-OSO_3)-aMan_R(3-OSO_3), has the same retention time in h.p.l.c. (Edge and Spiro, 1990), and the generation of this disaccharide species by some of the samples (not cerebral HS) cannot be excluded.

‡ Means ± S.D. The samples were derived from patients/cortical regions 3pAD, 5fAD, 5pAD, 5oAD, 5tAD, 6fAD, 6pAD, 6oAD, 6tAD, 11fAD, 11pAD, 11oAD, 11tAD.

§ HS preparation solubilized from protease-resistant residue of sample 11AD (material combined from different cortical regions) by alkali treatment (see Methods section).

‖ Control HS samples were from patients/cortical regions 2pC, 4pC, 13fC.

¶ ND, not detected.

Table 3 O-Sulphated disaccharides produced on deamination of HS preparations from different regions of AD cerebral cortex

Source of HS	Disaccharide deamination products*				
	$GlcA(2\text{-}OSO_3)\text{-}aMan_R$	$GlcA\text{-}aMan_R(6\text{-}OSO_3)$	$IdoA\text{-}aMan_R(6\text{-}OSO_3)$	$IdoA(2\text{-}OSO_3)\text{-}aMan_R$	$IdoA(2\text{-}OSO_3)\text{-}aMan_R(6\text{-}OSO_3)$
Frontal cortex	9.9 (8.0–12)	20 (17–23)	6.1 (4.3–7.7)	48 (47–49)	16 (13–19)
Parietal cortex	8.7 (8.4–9.4)	19 (17–22)	5.4 (4.2–7.2)	47 (44–48)	20 (18–21)
Occipital cortex	8.9 (8.4–9.5)	20 (20–21)	5.9 (5.6–7.4)	49 (48–51)	15 (14–17)
Temporal cortex	8.6 (6.9–11)	19 (17–22)	5.9 (4.7–6.9)	48 (45–53)	18 (15–22)

* Percentage of total O-sulphated $HexA\text{-}[1\text{-}^3H]aMan_R$ disaccharides obtained after HNO_2–NaB^3H_4 treatment. The HS samples were derived from the different regions of cerebral cortex indicated, from three different AD patients (5, 6, 11). Each disaccharide thus is determined three times for each cortical region; the results are expressed as means and, in parentheses, maximal and minimal values.

$aMan_R(6\text{-}OSO_3)$, $IdoA(2\text{-}OSO_3)\text{-}aMan_R$ and $IdoA(2\text{-}OSO_3)\text{-}aMan_R(6\text{-}OSO_3)$; the relative proportions of the various disaccharide species being almost identical from one sample to another (shown for two samples only in Figures 6a and 6b). The disaccharide patterns relating to AD or control tissue were virtually indistinguishable (Figures 6a and 6b; Table 2). Moreover, no significant difference in composition was observed between HS samples isolated from different regions of the brain (frontal, parietal, occipital, or temporal cortex) (Figures 6a and 6b; Table 3), notwithstanding the marked topical variability of the histopathological AD lesions. Interestingly, HS from neonatal cerebral cortex yielded the same pattern of deamination products as the other samples, except that $GlcA(2\text{-}OSO_3)\text{-}aMan_R$ was missing (Figure 6c; Table 2).

The AD polysaccharide preparations studied were derived from whole, unfractionated cerebral cortex with a variable load of senile plagues, but not specifically from amyloid deposits. Also, cortex from predilection sites, such as the hippocampal region, could not be selectively recovered on dissection of the specimens. It could therefore be argued that a specific subpopulation of HS, selectively associated with the AD lesion, might have escaped detection. Microscopic examination of the material remaining insoluble after proteolytic digestion of defatted AD cortex showed the presence of some residual amyloid fibrils. Alkaline extraction of such material (see Methods section) yielded GAG fractions corresponding to total hexuronic acid contents ranging from ~ 10 to $\sim 60\ \mu g$ per g of dry, defatted starting material. However, the labelled disaccharides obtained on analysis of this polysaccharide by the $NaBH_4/HNO_2/NaB^3H_4$ procedure did not differ significantly from those obtained from the proteolytically solubilized HS fractions (Table 2).

Contrary to the disaccharides generated by deamination of the multiple HS preparations from brain, the corresponding components derived from human kidney (Figure 6d), liver (Figure 6e) and aorta (Figure 6f) HS yielded markedly different h.p.l.c. profiles. While all chromatograms, similar to those of the brain-derived HS disaccharides, showed a predominant $IdoA(2\text{-}OSO_3)\text{-}aMan_R$ peak the relative amounts of the various disaccharide components diverged between each one of the samples, including the 'average' brain pattern (Figure 6; Table 2).

DISCUSSION

Proteoglycans exert their biological functions primarily by interacting, through their GAG moieties, with other macromolecules, mostly proteins (Jackson et al., 1991; Kjellén and Lindahl, 1991). Such interactions, generally ionic in nature, depend on the distribution of negative charges along the polysaccharide chains and can be highly specific, as illustrated by the antithrombin-binding region in heparin and HS (see Kjellén and Lindahl, 1991, for references). In other instances binding is less selective and involves cooperative electrostatic interaction rather than defined binding regions. HS, which is produced by virtually every type of cell investigated, displays the highest level of structural complexity of all GAGs, presumably reflecting its diverse functional requirements involving binding of different protein ligands. HS is produced by an elaborate biosynthetic machinery, the regulation of which, as required to generate different saccharide sequences, is only partly understood (Lindahl, 1989; Lidholt and Lindahl, 1992). Conversely, surprisingly little is known concerning the topological variability of HS structures in the various organs and tissues of the body.

HS (or heparin) has been shown to interact with a number of proteins of established or potential functional importance to the nervous system. Such ligands include β-amyloid peptide (Brunden et al., 1993), N-CAM (Cole et al., 1986), acetylcholinesterase (Brandan et al., 1985), basic fibroblast growth factor (Rapraeger et al., 1991), laminin (Martin and Timpl, 1987), protease nexin 1 (Farrell and Cunningham, 1987), heparin-binding growth-associated molecule (Raulo et al., 1994) and amphoterin (Parkkinen et al., 1993). The structural requirements for these interactions, in terms of saccharide sequence, have not been established. We are therefore intrigued by the striking compositional uniformity of HS preparations isolated from human cerebral cortex. This characteristic appears to be independent of the origin of the cortex specimen and, for adults, the age of the patient; the neonatal brain shows a conspicuous lack of one particular disaccharide unit. By contrast, the composition of the cerebral HS deviates markedly from those of HSs from other human organs, as shown in particular on analysis of disaccharide deamination products. Other recent studies likewise point to distinctive structural properties of HS isolated from a particular organ (Lyon et al., 1994). It is possible that the data for brain HS reflect a composite pattern, contributed by several different polysaccharide species, in which case the various HS species would have to occur in remarkably constant ratios. The identification of proteins capable of specific (or preferential) interaction with HS from cerebral cortex would obviously be of prime importance.

Is HS proteoglycan implicated in the pathogenesis of AD? Of particular interest is the report of Wagner et al. (1989) pointing to dramatically lowered amounts of protease-nexin 1 in afflicted regions of brains from AD patients as compared with controls. HS proteoglycan has been ascribed an important role in regulating the activity of this proteinase inhibitor (Farrell and Cunningham, 1987). Our results do not exclude that this or other

effects of HS may be perturbed in AD. Functional abberations might be considered in relation to the slight elevation in N-sulphation noted for some of the AD cerebral HS preparations in the present study. However, the findings do not support the proposal of a primary structural change in the HS molecule, caused by altered control of O-sulphotransferases in HS biosynthesis (Zebrower and Kieras, 1993), as a causative factor in AD. On the other hand, it seems likely that overproduction of the amyloid β-protein, for whatever reason, may lead to the formation of β-protein–HS complexes, with secondary effects on vital HS functions. It should be important therefore to study in some detail the mode of interaction between the β-protein and the particular HS species occurring in human brain, and to define the functional consequences of such interaction.

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