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Sulfated *N*-Linked Oligosaccharides in Mammalian Cells

II. IDENTIFICATION OF GLYCOSAMINOGLYCAN-LIKE CHAINS ATTACHED TO COMPLEX-TYPE GLYCANS*

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In the preceding paper (Roux, L., Holojda, S., Sundblad, G., Freeze, H. H., and Varki, A. (1988) *J. Biol. Chem.* 263, 8879–8889) we described the metabolic labeling and isolation of sulfated *N*-linked oligosaccharides from mammalian cell lines. All cell lines studied contained a class of sulfated sialylated complex-type chains with 2–6 negative charges. In this paper, we show that bovine pulmonary arterial endothelial (CPAE) and human erythroleukemia (K562) cell lines also contain a class of more highly charged sulfated but less sialylated oligosaccharides. These molecules were further characterized by ion exchange chromatography and various enzymatic and chemical treatments. In both cell lines they contained >6 negative charges, but those from K562 were even more highly charged than those from CPAE. Nitrous acid, heparinase, and heparitinase degradation of K562 oligosaccharides released 88, 64, and 78%, respectively, of ^{35}S label. Combined digestion with the two enzymes resulted in 87% release. The corresponding values for CPAE were 48, 25, and 50% (60% for the two enzymes together). Chondroitinase ABC (or AC) digestion of K562 and CPAE oligosaccharides released 10 and 5%, respectively. About 30% of the ^{35}S-labeled oligosaccharides from CPAE were sensitive to endo-β-galactosidase, indicating that poly-*N*-acetyl-lactosamine structures were present on some chains. Highly charged [^3H]mannose-labeled sulfated oligosaccharides from CPAE cells became neutral after treatment with heparinase/heparitinase but were resistant to Pronase, further proving that glycosaminoglycan (GAG)-like chains were directly attached to *N*-linked oligosaccharides. Such neutralized oligosaccharides did not bind to concanavalin A-Sepharose, but some interacted with phytohemagglutinin L_4, indicating that they were bi-, tri-, or tetra-antennary complex-type chains. Thus, K562 and CPAE cells contain different types of GAG chains directly attached to asparagine-linked oligosaccharides.

Such molecules were not found in many other cell lines that synthesize the more typical *O*-linked GAG chains. This suggests that the occurrence of these novel *N*-linked chains is not a random event resulting from accidental initiation of GAG chain synthesis on *N*-linked intermediates in the Golgi apparatus.

Proteoglycans are a group of extremely diverse molecules which are widely distributed in mammalian cells. The heterogeneity is due to different types of repeating disaccharide units, their modification by sulfation and epimerization of specific sugar residues, and their occurrence on a variety of different core proteins (2–5). The linkage region between the glycan and the core protein constitutes yet another source of diversity. Heparin, chondroitin sulfate, and dermatan sulfate chains are found in the commonest linkage type, with xylose *O*-glycosidically linked to serine (2). In heparan sulfate from bovine lung and the proteoglycan from swarm rat chondrosarcoma the xylose residue is phosphorylated at the C2 (6, 7). Three other types of linkages have been described for keratan sulfate. The typical *O*-linked xylose residue is replaced by either *N*-acetylgalactosamine or mannose in keratan sulfate type II of cartilage and brain, respectively (8, 9), or by *N*-acetylglucosamine in *N*-glycosidic linkage as found in corneal keratan sulfate type I (10, 11). The latter is the only currently known example of a glycosaminoglycan (GAG)[1] chain attached to an *N*-linked oligosaccharide.

Several biological roles of the proteoglycans have been described (12). For example, the anticoagulant activity of heparin is known to result from the interaction of a specific pentasaccharide unit with antithrombin III (13, 14). Similarly, fragments of dermatan sulfate with ≥12 residues can bind to and activate heparin cofactor II, another anti-protease that inhibits thrombin action (15). At the cellular level heparan sulfate with anticoagulant activity is preferentially expressed on the surface of endothelial cells (16, 17). A recent intriguing observation is that the gene involved in biological rhythms in *Drosophila* encodes a protein with sequence homology to the proteoglycan core protein (18, 19).

As part of an investigation aimed at characterizing sulfated asparagine-linked oligosaccharides in various mammalian cell lines, we have found a new class of proteoglycans which are *N*-linked to core proteins. Heparin, heparan sulfate, and chondroitin sulfate chains were all identified on *N*-linked oligosaccharides following PNGaseF digestion from both bovine endothelial cells and human erythroleukemia cells.

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[1] The abbreviations used are: GAG, glycosaminoglycan; CPAE, bovine pulmonary artery endothelial cells; K562, human erythroleukemia cells; PNGaseF, peptide:*N*-glycosidase F; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; Man, mannose.

EXPERIMENTAL PROCEDURES

Many of the materials and methods used in this report are described in detail in the Miniprint Section of the preceding paper (1).

Cell Lines—Bovine pulmonary artery endothelial cells (CPAE) were maintained as monolayer cultures in modified Eagle's medium α containing 10% fetal calf serum, 2 mM L-glutamine, 0.05 mg/ml gentamycin sulfate. Human erythroleukemia cells (K562) were maintained in suspension in RPMI 1640 containing 1 mM sodium pyruvate and supplemented as above.

Enzyme Digestions—$^{35}SO_4$-Labeled samples for analytical experiments were incubated in a volume of 50–100 μl with the following amounts of purified enzymes at 37 °C in the buffers indicated: heparinase and heparitinase (*Flavobacterium heparinum*, Miles Scientific), 0.5 unit in 50 mM Tris-HCl, pH 7.2, containing 2.5 mM $CaCl_2$ for 12 h; chondroitinase ABC and AC (*Proteus vulgaris* and *Arthrobacter aurescens*, Sigma), 0.2 unit in 150 mM Tris acetate, pH 7.5, for 2 h; endo-β-galactosidase (*Escherichia freundii*, gift from Minoru Fukuda, La Jolla Cancer Research Foundation); 8 milliunits in 0.1 M sodium acetate, pH 5.5, for 12 h; neuraminidase (*Arthrobacter ureafaciens*, Behring Diagnostics), 20 milliunits in 0.1 M sodium acetate, pH 6.0, containing 4 mM calcium acetate for 3 h; PNGaseF (*Flavobacterium meningosepticum*, purified as in Ref. 1); 1 milliunit in 20 mM Tris-HCl, pH 7.5, containing 20 mM 2-mercaptoethanol, 50 mM NaEDTA, 1% Nonidet P-40, 0.2% SDS for 12 h; Pronase (*Streptomyces griseus*, Behring Diagnostics); 50 μg in 100 mM Tris-HCl, pH 7.2, containing 20 mM $CaCl_2$ for 3 h at 60 °C.

Nitrous Acid Deamination—This was performed at pH 1.5 as described (20). Briefly, 1 ml of 1 N sulfuric acid was mixed with 1 ml of H_2O containing 114 mg of barium nitrite at 4 °C and centrifuged. 200 μl of the supernatant was added to the sample (in 50 μl of H_2O), kept at room temperature for 10 min, and neutralized. A control was treated with HCl at pH 1.5 and also analyzed.

Analytical separations of enzymatically or chemically degraded samples were performed by chromatography on columns of Sephadex G-50 and QAE-Sephadex as indicated in the figure legends. Lectin affinity chromatography methods are described in the preceding paper (1).

RESULTS

In the preceding paper (1) we described in detail a family of sulfated sialylated *N*-linked oligosaccharide chains that were released by PNGaseF treatment of $^{35}SO_4$-labeled lysates from several mammalian cell lines (these were arbitrarily designated Class I). In addition, a bovine pulmonary arterial endothelial cell line (CPAE) contained another major class of sulfated oligosaccharides that was eluted from QAE-Sephadex by 1 M NaCl. These molecules were excluded on a Sephadex G-50 column, and neuraminidase treatment caused no major shift in the elution pattern from QAE-Sephadex. Based upon these criteria they were designated as Class II. Using the same methodology we have found similar highly anionic oligosaccharides in the K562 human erythroleukemia cell line. Such Class II oligosaccharides from K562 and CPAE are the subject of this study. Several other types of mammalian cell lines were investigated for the presence of these Class II glycans, all with negative results (data not shown). These include: mouse T cell lymphomas (BW 5147, SL-12, and subclones); mouse 3T3 fibroblasts; 3T3 fibroblasts transformed by the v-sis and v-mos oncogenes (NP-6, Rex-2); normal rat and human fibroblasts; human promyelocytic leukemia (HL-60) cells, human hepatoma (HEP-G2) cells, human epidermoid carcinoma (A431) cells, human lung adenocarcinoma (UCP3) cells, and Chinese hamster ovary cell lines CHO K1 (parent) and CHO 761 (mutant deficient in synthesizing the core structure of typical xylose-linked GAG chains I) (21).

Release and Isolation of Class II Oligosaccharides from $^{35}SO_4$-Labeled K562 and CPAE Cells Using PNGaseF—The general rationale for the approach is outlined in Fig. 3 of the preceding paper. Cultures of K562 and CPAE were metabolically labeled with $^{35}SO_4$ and/or [^{3}H]Man and lysed with buffer containing SDS. The cell lysates were fractionated on a Sephacryl S-200 column; the excluded material was recovered and incubated at 37 °C with or without PNGaseF. Fig. 1, *A* and *C*, shows the ^{35}S-elution profiles obtained on subsequent Sephacryl S-200 chromatography of material from K562 and CPAE, respectively, In each case, substantially more of the label was found in the included volume of the enzyme-treated sample than in the control, without added enzyme.

The material released from K562 cells by PNGaseF digestion was heterogeneous and distributed over most of the included column volume. Fractions were combined into three pools: PNGaseF-resistant (S200VoB, see Fig. 3 in the preceding paper for the nomenclature); PNGaseF-released Peak 1 (S200R1); and PNGaseF-released Peak 2 (S200R2). Although K562 S200R1 contained a larger proportion of the total ^{35}S-labeled material, the control without added enzyme had a 25% background. Therefore, this material was not studied in detail. The S200R2 released fraction constituted 11% of total ^{35}S radioactivity, contained negligible nonspecific contamination (<0.3%), was more homogeneous that R1, but was apparently of lower average molecular mass and/or charge. When R2 was applied to a Sephadex G-50 column more than 90% of the ^{35}S label appeared in the void volume (Fig. 1*B*). This corresponds to a M_r of at least 6000 as found for a highly negatively charged GAG fragment from endothelial cells (22). In contrast to K562 cells, PNGaseF digestion of ^{35}S-labeled lysate from CPAE cells resulted in one major released peak on Sephacryl S-200 (Fig. 1*C*). Further fractionation of this material on Sephadex G-50 produced two overlapping peaks (Fig. 1*D*). The material eluting in the void volume region was pooled as shown and is described in this paper. The included peak consists of less anionic material which elutes from QAE-Sephadex with 20–400 mM NaCl (Class I oligosaccharides, described in the preceding paper).

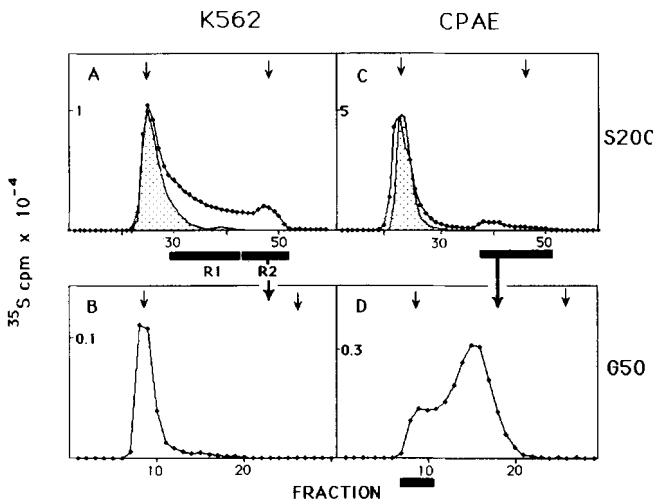

FIG. 1. **Sephacryl S-200 and Sephadex G-50 chromatography of sulfated macromolecules from K562 and CPAE cells with or without PNGaseF treatment.** K562 and CPAE cells were metabolically labeled with $^{35}SO_4$ in sulfate-depleted medium (modified Eagle's medium α) without sulfate, cysteine, and gentamycin sulfate but with 1/50 normal methionine concentration (0.3 μg/ml final) and supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), and sodium selenite (0.172 μg/ml); the labeled cells were washed, lysed (1% SDS, 0.1 M 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5), and fractionated by Sephacryl S-200 gel filtration. Macromolecules eluting in the void volume (S200VoA, not shown) were recovered, precipitated with acetone, and divided into two portions, which were incubated with or without PNGaseF. Treated samples and controls were then rechromatographed on Sephacryl S-200. The elution positions of Blue Dextran and a triantennary chain from orosomucoid (*A* and *C*) and Blue Dextran and [^{3}H]Man (*B* and *D*) are indicated by *arrows*.

Characterization of Class II Oligosaccharides by Ion Exchange Chromatography—The excluded fractions obtained by Sephadex G-50 fractionation of CPAE-released oligosaccharides (Fig. 1*D*) and the R2-released fraction from K562 cells were subjected to QAE-Sephadex chromatography (Fig. 2). In the case of the CPAE oligosaccharides, most of the ^{35}S label required >0.4 M NaCl for elution. The R2-released fraction from K562 was more highly charged, and most of the label was eluted only with 1 M NaCl. After this elution there was still ^{35}S radioactivity bound to the beads: 30% in the case of K562 and 10% for CPAE, further emphasizing the high negative charge of these glycans. No significant change in ^{35}S radioactivity patterns was observed when Class II oligosaccharides from both cell lines were treated with neuraminidase prior to QAE-Sephadex chromatography, suggesting that sialic acids did not contribute a major portion of the negative charge to these molecules. Further attempts at subfractionation of these molecules by HPLC or gradient elution from QAE-Sephadex did not result in useful separations because of their heterogeneity and high negative charge. Therefore, in all subsequent experiments the total S200R2-released fraction from K562 cells and the Sephadex G-50-excluded fraction (Class II) from CPAE cells were used.

As shown below, these anionic oligosaccharides from K562 and CPAE cells share many characteristics with GAGs. DEAE-cellulose chromatography has been traditionally used to characterize the charge of proteoglycans and GAGs. Therefore, the PNGaseF-released molecules under study, as well as their PNGaseF-resistant counterparts, were applied to DEAE-cellulose columns and eluted with a linear gradient of ammonium acetate. The PNGaseF-resistant ^{35}S-labeled fractions (Fig. 1*A*) from K562 produced a relatively simple profile, with the majority eluting with about 0.9 M NH_4 acetate (alkaline borohydride treatment of such resistant material produced a similar DEAE pattern suggesting the presence of typical *O*-linked glycosaminoglycan chains). Part of the PNGaseF-resistant pooled fractions from $^{35}SO_4$-labeled CPAE cells was also subjected to DE52 chromatography as illustrated in Fig. 3, *upper right panel*. A single symmetric peak eluted at about 0.8 M NH_4 acetate.

DEAE chromatography of the PNGaseF-released radioactivity in each case showed a different pattern. Fig. 3, *lower left panel*, shows the profile obtained with K562 R2; a small amount (13%) eluted with the starting buffer, 0.25 M NH_4 acetate, while the majority eluted at about 0.7 M NaCl. In contrast, most of the total released CPAE oligosaccharides were eluted with 0.25 M NH_4 acetate (Class I) and the remaining 18% at about 0.4 M (Class II). These results show that the majority of K562 oligosaccharides are more highly charged than those from CPAE and that in each case these molecules are different from the more typical GAGs found in the resistant fraction. The anionic character of these Class II glycans and their apparent lack of sialic acids suggested to us that they might contain GAG-like chains.

Class II Oligosaccharides Contain Different Types of Glycosaminoglycan Chains—We, therefore, subjected Class II glycans from K562 and CPAE cells to various enzymatic and chemical treatments that degrade glycosaminoglycan chains. The results are illustrated in Figs. 4 and 5 and summarized in Table I. Chondroitinase ABC released 10 and 5% of the ^{35}S label from K562 and CPAE glycans, respectively, as analyzed by Sephadex G-50 chromatography. The same values were obtained with chondroitinase AC, indicating the presence of chondroitin sulfate rather than dermatan sulfate chains (23). About 30% of the ^{35}S label in oligosaccharides from CPAE was sensitive to endo-β-galactosidase, whereas such treatment did not affect K562 glycans. However, a shift in the ^{35}S profile was observed (rather than a clearly released peak) suggesting the presence of undersulfated keratan sulfate chains. Alternatively, since this enzyme hydrolyzes polylactosamine structures without sulfate residues, it is possible that such glycan chains exist as part of some of the *N*-linked sulfated oligosaccharides but do not themselves carry the sulfate esters. Fig. 5 shows the results obtained from experiments designed to establish whether heparin and/or heparan sulfate like glycan chains are present in Class II oligosaccharides. Nitrous acid degradation (20) released 88 and 48% of the ^{35}S label from K562 and CPAE glycans, respectively, showing that these oligosaccharides contain a

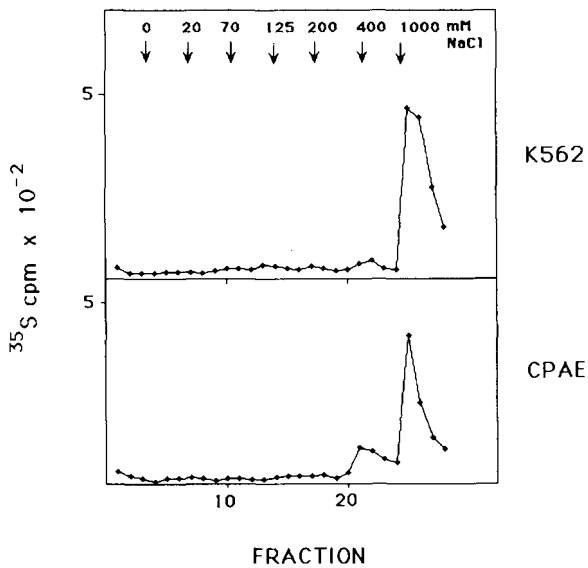

FIG. 2. **QAE-Sephadex anion exchange chromatography of sulfated oligosaccharides.** A portion of the total released oligosaccharides from $^{35}SO_4$-labeled K562 and the Sephadex G-50 fractionated Class II oligosaccharides from CPAE (see Fig. 1*D*, *barred* fractions) were applied to QAE-Sephadex ion exchange columns and eluted with step increases in NaCl concentration in 2 mM Tris base. No change in elution patterns was obtained by *A. urefaciens* neuraminidase treatment of the respective samples (data not shown).

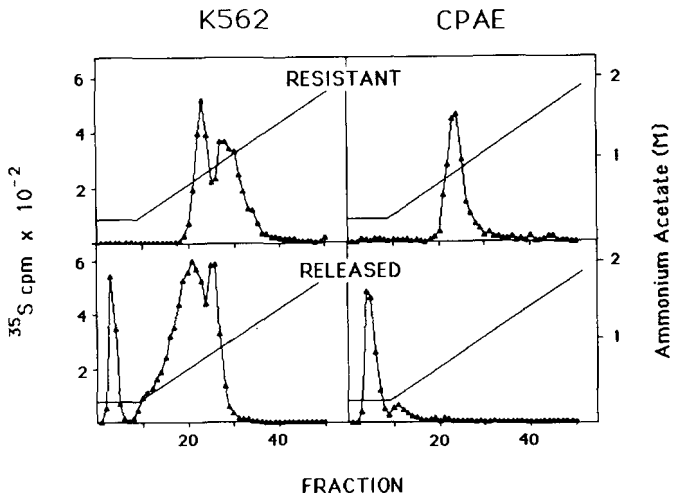

FIG. 3. **DEAE-cellulose chromatography of Class II oligosaccharides and PNGaseF-resistant molecules.** PNGaseF-released and resistant material from $^{35}SO_4$-labeled K562 and CPAE cells (cf. Fig. 1) were fractionated on DE52 (Whatman) columns (2 ml) equilibrated with 0.25 M NH_4 acetate and eluted with a linear gradient of NH_4 acetate to 2 M. 1-ml fractions were collected, and a sample was used for liquid scintillation counting.

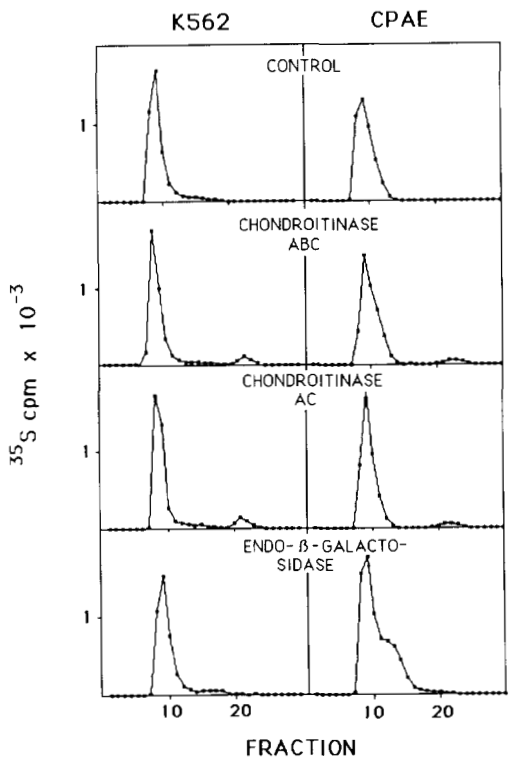

FIG. 4. **Sephadex G-50 fractionation of Class II oligosaccharides treated with glycosaminoglycan-degrading enzymes.** PNGaseF-released [^{35}S]sulfate-labeled oligosaccharides from K562 and CPAE cells were treated with the enzymes indicated and separated on Sephadex G-50 columns in 10 mM Tris-HCl, pH 6.5, containing 0.2% SDS. Blue Dextran and [^{3}H]Man were used as markers in each run to allow comparison.

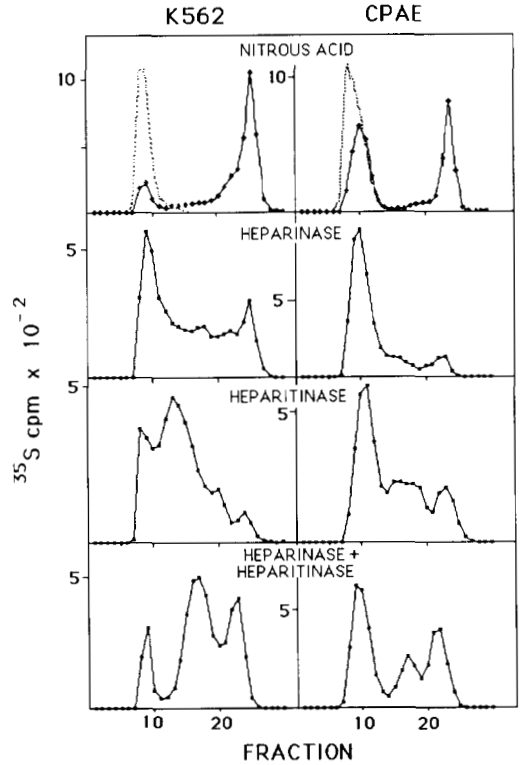

FIG. 5. **Sephadex G-50 fractionation of Class II oligosaccharides treated with nitrous acid and glycosaminoglycan-degrading enzymes.** PNGaseF-released [^{35}S]sulfate-labeled oligosaccharides from K562 and CPAE cells were treated with nitrous acid and enzymes as indicated and separated on Sephadex G-50. Blue Dextran and [^{3}H]Man were used as markers in each run to allow comparison. *Dotted lines* indicate controls.

large proportion of the label in *N*-sulfate groups. The released label eluted mostly in the position expected for free sulfate. However, some label also ran immediately adjacent to the main peak, suggesting that small oligosaccharides carrying *O*-sulfate residues might also be released by the deamination procedure. These findings are typical for heparin or heparan sulfate chains (24). Digestion of K562 oligosaccharides with heparinase and heparitinase released 64 and 78% of ^{35}S label, respectively, and the simultaneous action of both enzymes released 87%. The corresponding values for CPAE oligosaccharides were 25, and 50, and 60% for the two enzymes combined. These results indicate that Class II oligosaccharides from both K562 and CPAE cells contain heparin, heparan sulfate, and probably small amounts of chondroitin sulfate chains, similar to those found in proteoglycans of the *O*-linkage type. Table I summarizes the percent of ^{35}S label released from K562 and CPAE oligosaccharides by the above treatments. The amounts of ^{35}S label released by a combination of heparinase and heparitinase digestion are consistent with those obtained with nitrous acid deamination for both cell lines. The results furthermore indicate that some oligosaccharide chains are degraded by both enzymes. The sum of total ^{35}S label released by all enzymes was 97 and 95% from K562 and CPAE cells, respectively, showing that most of the label can be accounted for as sulfated GAG-like chains on this class of *N*-linked oligosaccharides.

Lectin Analyses of the N-Linked Glycan Core of Class II Oligosaccharides—To further study the nature of the underlying *N*-linked oligosaccharide core of Class II molecules, they were subjected to affinity chromatography on different lectins conjugated to agarose. No binding of K562 and CPAE Class II glycans was observed using concanavalin A and phytohemagglutinin L_4, under conditions where control oligosaccharides were bound (see preceding paper). A small proportion of the label from both cell lines (<10%) bound to *Ricinus communis* agglutinin I (specific for terminal β-Gal residues) after neuraminidase treatment. However, only the oligosaccharides not bound to *R. communis* agglutinin I could be degraded by combined heparinase/heparitinase digestion (data not shown). This finding suggests that unlike the Class I structures described in the preceding paper, Class II molecules do not contain terminal sialic acid residues substituting β-linked galactose residues. Rather, the preparations of Class II seem to contain small quantities of Class I-like material.

TABLE I

Degradation of PNGaseF-released class II oligosaccharides from $^{35}SO_4$-labeled K562 and CPAE cells analyzed by Sephadex G-50 chromatography

Values calculated from results in Figs. 4 and 5.

Treatment	%^{35}S label released	
	K562	CPAE
Nitrous acid	88	48
Heparinase	64	25
Heparitinase	78	50
Heparinase + heparitinase	87	60
Chondroitinase ABC	9	5
Chondroitinase AC	10	5
Endo-β-galactosidase	0	30[a]

[a] Change in size of oligosaccharides only; no release of low molecular weight material.

[^{3}H]Man-labeled Class II Oligosaccharides Become Neutral after Heparinase/Heparitinase Digestion but Are Resistant to Pronase—Despite the substrate specificity of highly purified

PNGaseF used and the fact that control incubations without enzyme did not release Class II oligosaccharides, it could be argued that these molecules only appeared to "released" and were in fact still bound to a peptide backbone due to some unknown artifact. We, therefore, labeled CPAE cells with [^{3}H]Man, which is incorporated only as [^{3}H]Man and [^{3}H] fucose (25), the former being primarily in *N*-linked oligosaccharides. From this labeled material, we released and isolated ^{3}H-labeled Class II oligosaccharides using the identical approach that was used for the $^{35}SO_4$-labeled material. More than 80% of the total [^{3}H]Man was released by PNGaseF treatment (not shown). The S-200 column region that corresponded to sulfated oligosaccharides (20%) was desalted and applied to QAE-Sephadex. The ^{3}H label that eluted with 1 M NaCl was isolated and represented 0.6% of the released [^{3}H] Man label. This material was desalted and shown to consist of more than 90% [^{3}H]Man (<10% fucose) by HPLC analysis (26). Such negatively charged oligosaccharides were treated with different enzymes and reapplied to QAE-Sephadex. As can be seen from Fig. 6 some of this material eluted at lower salt concentrations after neuraminidase digestion (*second panel*), indicating that some of the [^{3}H]Man-containing oligosaccharides were sialylated. However, combined heparinase/heparitinase treatment degraded most of the oligosaccharides (*third panel*) resulting in a significant amount (35%) of neutral label. This indicates that GAG-like structures were the major contributors to the negative charge of most of these [^{3}H]Man-labeled molecules.

It could still be argued that this [^{3}H]Man-labeled material actually represented neutral oligosaccharides that were linked to a peptide which also carried a heparin/heparan-like chain without [^{3}H]Man in an independent linkage. If this were true, protease digestion should cause the molecules to shift to a lower charge (unless charge or glycosylation density totally precluded Pronase action). Therefore, we treated the labeled molecules with Pronase. The *last panel* of Fig. 6 shows that this causes no shift of negative charge compared to the untreated material (*top panel*). This provides further evidence that glycosaminoglycan chains were directly attached to the [^{3}H]Man-labeled asparagine-linked glycans.

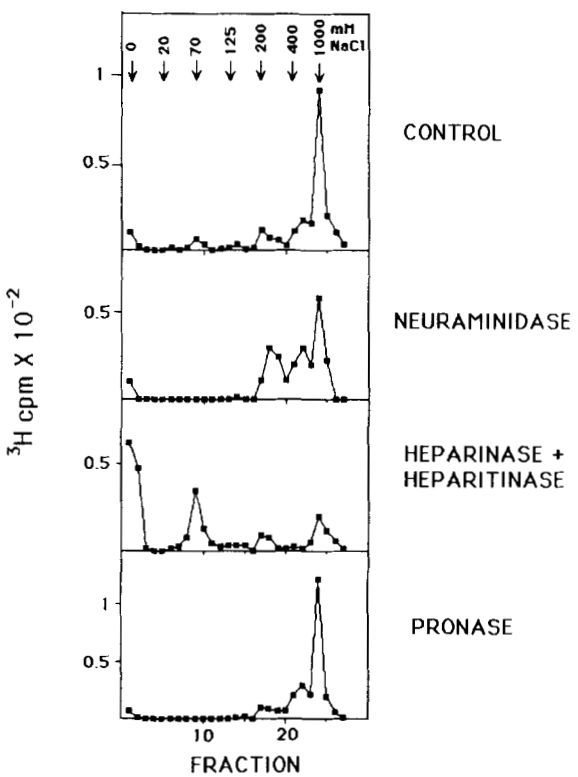

FIG. 6. **QAE-Sephadex analyses of [^{3}H]Man-labeled Class II oligosaccharides from CPAE cells after different enzyme digestions.** PNGaseF-released oligosaccharides were fractionated on QAE-Sephadex. The [^{3}H]Man-labeled 1 M NaCl-eluted material (Class II oligosaccharides (cf. Fig. 2)) was isolated, desalted, treated with the indicated enzymes, and reapplied to QAE-Sephadex.

As mentioned above, untreated $^{35}SO_4$-labeled Class II oligosaccharides did not bind to some lectins that usually interact with complex-type glycans. It is possible, however, that the sulfate esters preclude lectin binding by steric hinderance or charge. Therefore, the neutral [^{3}H]Man-labeled oligosaccharides isolated following heparinase/heparitinase digestion (Fig. 6, *third panel*) were subjected to lectin affinity chromatography. All of the ^{3}H label passed through a column of concanavalin A-Sepharose. This material was then applied to a phytohemagglutinin L_4 column. In this case, 40% of the oligosaccharides interacted with the lectin (data not shown). Based on the carbohydrate-binding specificities of these lectins (27–29) these findings indicate that some of the GAG chains are attached to complex-type bi-, tri-, and/or tetraantennary glycans containing Galβ1-4GlcNAcβMan sequence.

DISCUSSION

Taken together the results indicate that a small, but significant, part of the total $^{35}SO_4$ label of K562 and CPAE cells is present in *N*-linked GAG chains (about 10 and 2%, respectively). These molecules contain heparin and heparan sulfate (and possibly also chondroitin sulfate) chains covalently attached to complex-type *N*-linked oligosaccharides. Specific release by PNGaseF is the primary criterion for determining this linkage. In the preceding paper, we have discussed in detail the evidence against each of the possible artifacts that could provide alternate explanations for this release (1). This discussion will not be repeated here except to state the conclusion, that each of the artifacts can be ruled out. In this study we have also isolated [^{3}H]Man-labeled oligosaccharides shown to carry heparin/heparan-like chains, providing independent confirmation for the existence of these unusual molecules.

Table II shows a comparison of general properties of Class I and Class II sulfated oligosaccharides, as summarized from this and the preceding paper. It is acknowledged that the biochemical distinction between these two classes is somewhat arbitrary; the major criterion for defining Class II is the presence of *N*-sulfate esters and heparinase/heparitinase-sensitive chains. However, the following paper (30) describes a single cell surface glycoprotein that contains sulfated *N*-linked oligosaccharide structures with characteristics of both classes.

The known lectin binding specificities, the specificity of the heparin/heparan sulfate-degrading enzymes, and the fact that neutral oligosaccharides were obtained by such enzymatic treatment suggest that most of the GAG chains are linked to antennae of complex-type glycans that do not carry sialic acid. Further detailed studies of these unusual molecules will be required to establish the exact nature of the linkage region to the GAG chains. One possibility is that terminal galactose or *N*-acetylglucosamine residues serve as the starting point for the GAG chain elongation. Another possibility is that the GAG chains are linked to a β-linked xylose attached to the β-linked mannose residue. However, while such a xylose residue has been identified in both plant and animal proteins (31,

TABLE II

Comparison of class I and class II sulfated oligosaccharides released by PNGase F from cell lines

The sulfated oligosaccharides are divided into two general classes based on the various properties indicated. Limited overlap may exist between the two classes.

Properties	Class I	Class II
Sephadex G-50 behavior on gel filtration	Included	Excluded
Negative charge		
Overall charge	Low	High
Primary *O*-sulfate esters	Yes	Yes
N-sulfate esters	No	Yes
Contribution to charge	Minor	Major
Sialic acids	Yes	No
Contribution to change	Major	None
Core structure		
High mannose[a]	No	No
Biantennary[a]	No	No
Tri- or tetra-antennary[b]	Yes	Yes
Side chains		
Penultimate β-galactose residues unmasked by neuraminidase	Yes	No
Polylactosamine chains	No	Yes
Chondroitin sulfate	No	Yes[c]
Heparin/heparan sulfate	No	Yes

[a] Based on concanavalin A affinity chromatography studies and may be invalid since sulfation can preclude lectin binding.

[b] Based on leukoagglutinin (L4-PHA) affinity chromatography studies.

[c] Minor component.

32), it has not yet been described in mammalian *N*-linked oligosaccharides.

The biosynthetic relationships between these molecules and the more typical *N*-linked and xylose-linked oligosaccharides need to be explored. It could be argued that the synthesis of these novel glycans is a random occurrence due to accidental initiation of glycosaminoglycan chain synthesis on incomplete *N*-linked oligosaccharides in the Golgi complex. Against this possibility is the finding that only a limited number of mammalian cell lines were found to contain these glycans (2 out of 22 studied). The negative cells included fibroblasts and Chinese hamster ovary cells that incorporate large quantities of $^{35}SO_4$ label into the more typical *O*-linked GAGs. Furthermore, a Chinese hamster ovary cell line that is defective in the synthesis of the xylose-linked core of *O*-linked GAG chains (but contains all other enzymes necessary for chain elongation and sulfation) did not make these *N*-linked GAG-like structures. On the other hand, it seems likely that the modification reactions of the chains themselves are mediated by the same enzymes in the Golgi complex responsible for generating the more conventional *O*-linked GAG chains.

The occurrence of *N*-linked complex or high mannose-type oligosaccharides (without GAG chains) on the core protein backbone of proteoglycans is well established (5, 33). Keratan sulfate type I as found in cornea (10, 11), and recently also in glycoproteins of thymocytes (34) and baby hamster kidney cells (35), is to our knowledge the only previously described GAG chain directly attached to a complex-type asparagine-linked oligosaccharide. We do not know whether the novel *N*-linked glycans reported here are attached to the same proteoglycan core proteins that carry the more typical xylose-linked chains or to completely separate glycoproteins. There are, however, several observations in the literature suggesting the attachment of heparin/heparan-like chains to typical glycoproteins. Heparan sulfate chains have been reported to be covalently bound to fibronectin of mouse teratocarcinoma stem cells (36) and to rabbit thrombomodulin (37). However, in both these cases, the nature of the linkage region was not investigated. Other relevant observations include the demonstration of glucuronic acid in human thyroglobulin (38), *N*-glycosidically linked nitrous acid-sensitive sulfate esters in calf thyroid plasma membrane glycoproteins (39), and small covalently bound GAGs in membrane glycoproteins from baby hamster kidney cells (40).

There are several reasons why these novel structures might not have been found before. First, they seem to be present in a limited number of mammalian cell lines. Second, while their physical properties might cause them to co-purify with the total proteoglycans of a cell or tissue, they represent only a minor proportion of the glycosaminoglycan chains. Third, the relative stability of the asparagine linkage to the mild base treatment usually used to release *O*-linked chains might have caused them to remain behind after such treatment as a minor "resistant" fraction which was ignored. In this study, these unusual molecules were discovered because of specific release by PNGaseF combined with sequential gel filtration on Sephacryl S-200. It should also be pointed out that the failure of mild base to reduce the apparent M_r of a proteoglycan-like molecule is frequently taken as evidence that they are present as free GAG chains and not as intact proteoglycans. Our findings indicate that mild base-resistant *N*-linked GAG chains could also explain such results.

In the preceding paper (1), we have also discussed the possibility that the sulfate-free medium used in these studies could have caused qualitative (but not major quantitative) changes in the nature of the sulfated oligosaccharides (41–43). Ultimately, it will be necessary to study the synthesis of these unusual molecules under different types of culture conditions to ascertain if variations in structure do occur.

K562 is a human erythroleukemia cell line which grows in suspension. It is well characterized, particularly with respect to induction of differentiation by various agents and the presence of several cell surface markers (44). We have shown here that the great majority of $^{35}SO_4$-labeled PNGaseF-releasable oligosaccharides in these cells are of the Class II type (>80%). It would be of interest to examine the distribution of the two classes of *N*-linked glycans and their correlation with certain cell surface markers during induced differentiation of this cell line by various agents.

In the CPAE endothelial cell line, about 15% of the PNGaseF-released sulfated oligosaccharides are of the Class II type. Rosenberg and others (16) have shown that a very small proportion of the total heparan sulfate chains in some types of endothelial cells have anticoagulant heparin-like properties. It will be of interest to see if the specific sugar sequences mediating such activity are selectively expressed in these unusual *N*-linked GAG chains.

Heparan sulfate has been localized to different cellular compartments in numerous cell types and tissues and apparently plays a crucial role in many physiological processes (2, 45, 46), in particular those associated with blood coagulation, *e.g.* thrombosis, angiogenesis, and arteriosclerosis (46–51). A number of heparin-binding growth factors have also been identified (53, 54). A novel nuclear-localized heparan sulfate has also been described in rat hepatocytes which is proposed to have a role in growth control (55). A similar proteoglycan is apparently involved in sea urchin development (56). The proportion of *N*-linked heparan chains we have found to the total heparan chains of the cells is small. However, it is comparable to the proportion of phosphorylated oligosaccharides of lysosomal enzymes when compared to the total cell oligosaccharides. In spite of the rarity of the latter structures, they are known to mediate one of the few specific functions of carbohydrates (57). Thus, it is possible that these *N*-linked

chains could have specific functions that are distinct from those mediated by the more conventional xylose-linked molecules.

In this study, we have released and "isolated" these sulfated oligosaccharides only by the criterion of radiochemical purity. Ultimately, it is necessary to isolate larger quantities of the same oligosaccharides, fractionate them to biochemical purity, and to ascertain the details of their structure by direct chemical methods. Such information will also provide further clues as to the mechanisms that are involved in their biosynthesis. In addition to such studies, our current efforts are directed toward an understanding of the possible biological role of these *N*-linked glycosaminoglycans in the differentiation of erythroid cells and the control of blood coagulation on the surface of endothelial cells.

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