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Unusual Anionic *N*-Linked Oligosaccharides from Bovine Lung*

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We previously described a diverse family of sulfated anionic *N*-linked oligosaccharides released by peptide: *N*-glycosidase F (PNGaseF) from calf pulmonary artery endothelial (CPAE) cells (Roux, L., Holoyda, S., Sundblad, G., Freeze, H. H., and Varki, A. (1988) *J. Biol. Chem.* 263, 8879–8889). Since a major fraction of the intact lung consists of endothelial cells, we reasoned that bovine lung might be a rich source of similar molecules. Total *N*-linked oligosaccharides from bovine lung acetone powder were released by PNGaseF, labeled by [^{3}H]NaBH$_4$ reduction, and the anionic fractions were studied with a variety of techniques. The sugar chains with lesser negative charge (designated Class I) share several properties of conventional multiantennary complex-type chains. However, unlike the case with CPAE cells, sialic acids account only for a minority of the anionic properties and only a small proportion carry sulfate esters. A variety of different treatments indicate that most of the unexplained negative charge is due to multiple carboxylic acid groups. Resistance to β-glucuronidase and α-iduronidase suggests that these may be previously undescribed modifications of mammalian oligosaccharides. The most highly charged *N*-linked chains (designated Class II) are more similar in general structure to the corresponding ones from CPAE cells, although relatively more abundant. Their high charge is primarily due to chondroitin sulfate, heparin/heparan sulfate, or keratan sulfate glycosaminoglycan chains. Sequential digestion studies suggest that a significant proportion of these molecules have more than one type of glycosaminoglycan chain associated with them. Compositional analysis indicates the presence of xylose residues in Class II, but not Class I molecules. However, unlike the case with conventional glycosaminoglycans, these residues are not at the reducing terminus.

Most previously reported structures of complex-type *N*-linked oligosaccharides are derived from the glycoproteins of blood cells, plasma, or the secretions of cultured mammalian cells. This library of *N*-linked oligosaccharides from an intact mammalian organ (lung) contains a high proportion of novel anionic sugar chains whose structures are different from conventional complex-type sialylated chains and only partially related to those from CPAE cells. Further exploration of the *N*-linked chains of intact mammalian tissues seems warranted.

The anionic character of most known *N*-linked oligosaccharides is due to the presence of sialic acids (1, 2). However, negative charge in such molecules can also be due to phosphate esters (3, 4), sulfate esters (5–12), or, possibly, uronic acids (13–16). Unlike sialylated chains, the other types of anionic molecules are considered rare, being reported only in small amounts and/or only on certain proteins. By metabolic labeling with [^{35}S]sulfate and release with peptide:*N*-glycosidase F (PNGaseF)[1] (17), we previously identified and characterized a diverse family of anionic *N*-linked oligosaccharides in CPAE cells, a calf pulmonary artery endothelial cell line (10, 11). These sugar chains were separated by size and charge into two general classes. "Class I" was composed of molecules bearing various combinations of primary sulfate esters and sialic acids, while "Class II" molecules carried sequences susceptible to cleavage by glycosaminoglycan-degrading enzymes. About half of the negative charge on the sulfated Class I molecules could be attributed to GlcNAc-6-sulfate units at a position subterminal to sialic acid and β-galactose. In the case of Class II molecules, most of the negative charge was susceptible to heparin and chondroitin lyases, suggesting a novel class of "*N*-linked glycosaminoglycans." Collectively, all these sulfated molecules represented ~10% of the total PNGaseF-releasable *N*-linked oligosaccharides from this cell line. Further characterization was hindered by their extreme diversity, as well as by the small quantities of material available (CPAE cells are a primary cell line with limited growth capacity).

Although the mammalian lung has many different cell types, about 40% of its mass is derived from endothelial cells (18, 19). We reasoned that the total bovine lung might therefore be a rich source of molecules similar to those found in CPAE cells. We show here that PNGaseF treatment of an extract of bovine lung acetone powder releases anionic *N*-linked oligosaccharides with some similar properties. However, there were also many striking differences, both in the relative quantity of anionic molecules released and in the fact that the majority of the negative charge is contributed not by sulfate esters, but by carboxylic acids other than sialic acids. We present here the identification, fractionation, and partial characterization of

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[1] The abbreviations used include: PNGaseF, peptide:*N*-glycosidase F; GAG, glycosaminoglycan; HS, heparin/heparan sulfate; CS, chondroitin sulfate; KS, keratan sulfate; CPAE, calf pulmonary artery endothelial cell; HPAEC-PAD, high pH anion exchange chromatography with pulsed amperiometric detection; disodium 2,2′-bicinchoninate, 4,4′-dicarboxy-2,2′-biquinoline; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; NHS, *N*-hydroxysuccinimide; L-PHA, phytohemagglutinin-L$_4$; GC-MS, gas chromatography-mass spectrometry; CH_3I, methyl iodide; MES, 2-(*N*-morpholino)ethanesulfonic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Me_2SO, dimethyl sulfoxide; HPLC, high performance liquid chromatography.

these unusual anionic oligosaccharides. Apart from the novel features mentioned above, we report several additional properties of the *N*-linked glycosaminoglycans. This work also emphasizes that there are very few reported studies of the anionic *N*-linked oligosaccharides from intact mammalian tissues. Indeed, the great majority of previously described anionic *N*-linked oligosaccharides originate from a restricted subset of accessible glycoproteins of the blood plasma and blood cells and of recombinant proteins derived from cultured cells. The assumption that similar molecules will predominate in other mammalian tissues is challenged here.

EXPERIMENTAL PROCEDURES

Materials

Most of the materials used were from Sigma. The following were from the indicated sources: trifluoroacetic acid, EDC, and NHS, Pierce; methyl iodide (CH_3I) and methylamine (CH_3NH_2), Fluka; [^{3}H]$NaBH_4$, ICN; concanavalin A-agarose and L-PHA-agarose, E.Y. Laboratories; *Arthrobacter ureafaciens* sialidase, Calbiochem; heparinase and heparitinase, Seikagaku, Tokyo. Homogeneous *Escherichia coli* alkaline phosphatase was a gift from M. J. Schlesinger, Washington University. Samples of homogeneous human placental β-hexosaminidase A were generously provided by Arnold Miller, University of California, San Diego, Don Mahuran, Hospital for Sick Children, Toronto, and Mario Ratazzi, North Shore University Hospital, Manhasset, NY. Human α-iduronidase was the kind gift of Elizabeth Neufeld, University of California, Los Angeles. Diazomethane in ether was kindly provided by Claudio Schteingart, UCSD.

Isolation/Extraction of Bovine Lung Acetone Powder Oligosaccharides

For every 1 g of bovine lung acetone powder (Sigma), 7 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 0.1 M β-mercaptoethanol) was added. This mixture was homogenized with a Polytron on low speed to solubilize the powder, heated at 37 °C for 30 min, with continued heating overnight at 50 °C. The sample was then boiled for 10 min and ultracentrifuged at ~100,000 × *g* for 40 min. The supernatant was filtered through an 0.8-μm filter with glass wool layered over a prefilter, and aliquots (30 ml, representing ~5% of the column volume) were applied to a Sephacryl S-200 column (100 × 3 cm), eluted with 10 mM Tris-HCl, pH 6.5, 0.2% SDS (fractionation range 250,000–5,000 kDa for globular proteins). The eluent was pumped to achieve a flow rate of 1 ml/min, and 10-ml fractions were collected. For large preparations, it was necessary to do multiple runs to fractionate all of the material. The void area was monitored by A_{280} and pooled, and the proteins precipitated with 9 volumes of ice-cold acetone added slowly to the sample while stirring at 4 °C. The precipitate was pelleted by centrifugation (2000 rpm for 15 min), the acetone was decanted, and the pellet was immediately resuspended in PNGaseF buffer (10 mM Tris-HCl, pH 7.2, 50 mM EDTA, pH 7.5, 0.2% SDS, 1% Nonidet P-40, 20 mM β-mercaptoethanol). The sample was incubated at 37 °C for 30 min with a loosened cap to evaporate excess acetone and split into two fractions: a control (1% of the sample) and the remainder for PNGaseF treatment. PNGaseF, prepared in our laboratory as described (20), was added at a concentration, which was able to completely release *N*-linked oligosaccharides from a similar amount of fetuin, and incubated overnight at 30 °C under a toluene atmosphere. After boiling for 10 min, the samples were reapplied to the S-200 column as above. The released material was included in the column and detected with the reducing sugar assay (see below). These fractions were pooled, and SDS was precipitated by adding 1/100 volume of saturated KCl to the sample followed by refrigeration overnight at 4 °C. The supernatant was collected after spinning at 2000 rpm for 15 min and desalted on either a Sephadex G-25 or a Bio-Gel P-2 column eluted with H_2O.

Reducing Sugar Assay (21, 22)

Aliquots of samples were brought up in a total volume of 300 μl of H_2O. 700 μl of freshly prepared reagent C was added, and the mixture was heated at 80 °C for 30 min. After cooling, the absorbance was read at 560 nm. Reagent C consists of 23 ml of solution A + 1 ml of solution B + 6 ml of ethanol. To prepare solution A, 1.5 g of disodium 2,2′-bicinchoninate is dissolved in 1 liter of water, 71.6 g of anhydrous sodium carbonate is added while stirring, and the volume is brought up to 1.15 liter final with dH_2O. To prepare solution B, 3.7 g of aspartic acid and 5.0 g of anhydrous Na_2CO_3 are dissolved with shaking in 100 ml of water, and then mixed with 1.09 g of copper sulfate dissolved in 40 ml of water.

^{3}H Labeling of PNGaseF Released Bovine Lung Oligosaccharides (23, 24)

Purified desalted *N*-linked oligosaccharides released by PNGaseF were dried, and 200 μl of 0.2 M sodium borate, pH 9.8, was added (25). [^{3}H]Sodium borohydride (1–5 mCi/oligosaccharides derived from 1 g of bovine lung acetone powder) were each dissolved in 0.2 M sodium borate, pH 9.8, mixed in a fume hood, and the reduction was allowed to proceed for 1–3 h at room temperature. An excess of unlabeled sodium borohydride (10-fold) over the amount of expected oligosaccharides was added, and the reduction was continued for 1 h at room temperature. The reaction was quenched either by acidification using glacial acetic acid and repeated drying with acidified methanol or by the addition of a 10-fold molar ratio of acetone over the non-radiolabeled sodium borohydride which was left at room temperature for an additional hour. The sample treated by either procedure was further separated from unreacted radioactivity by direct application to a disposable 17-ml Bio-Gel P-2 column and desalted in H_2O, collecting only the void volume area.

QAE Fractionation of Released Oligosaccharides (4, 10, 11)

QAE-Sephadex columns (0.8 ml) were washed with 10 column volumes of 2 mM Tris base. The samples were loaded in 2 mM Tris base and washed with 7 × 750 μl of 2 mM Tris base. Oligosaccharides were eluted in a batchwise manner with increasing concentrations of sodium chloride (20, 70, 125, 200, 400, and 1000 mM NaCl, each in 2 mM Tris base). Each step consisted of 4 or 5 fractions, with each fraction composed of 2 × 750 μl additions of buffer.

Enzyme Reactions

A. ureafaciens sialidase digestions were performed by adjusting the sample to 50 mM sodium acetate, pH 5.5 (final), with 5 milliunits of *A. ureafaciens* sialidase in a 10-μl reaction volume, and incubating at 37 °C for 2 h. Alkaline phosphatase digestions were performed by adjusting the sample to 200 mM Tris-HCl, pH 8.0 (9 μl final volume) with 0.6 milliunit of enzyme, incubating at 37 °C for 30 min. GAG degrading enzyme digestions were performed using a common buffer, consisting of 10 mM Tris-HCl, pH 7.2, 2.5 mM $CaCl_2$ (final) in a 10-μl volume, using 3 μl of each enzyme, and incubating at 37 °C overnight (15–18 h): heparinase (1 unit/μl), heparitinase (1 unit/μl), chondroitinase AC (1 unit/50 μl), chondroitinase ABC (1 unit/50 μl), or keratanase (1 unit/50 μl). Human placental β-*N*-acetylhexosaminidase A incubations were carried out in 100 mM sodium formate, pH 3.5, at 37 °C under a toluene atmosphere with 0.5 unit of enzyme per 200 μl final reaction volume. All these enzyme reactions were inactivated by heating the samples at 100 °C for 10 min. β-Glucuronidase (13 milliunits) and α-iduronidase (48 milliunits) treatments of oligosaccharides were carried out in 40 μl of 80 mM sodium citrate, pH 4.6, overnight under a toluene atmosphere. For some incubations, these reactions were carried out in the presence of jack bean β-*N*-acetylhexosaminidase (53 milliunits), bovine testicular β-galactosidase (2 milliunits), and coffee bean α-galactosidase (5 milliunits). All commercial enzymes were tested for activity under the conditions used.

Acid Hydrolysis Conditions

To selectively remove sialic acids and cleave phosphodiester bonds, samples were heated in either 10 mM HCl at 100 °C for 30 min (4) or 2 M acetic acid at 80 °C for 3 h (26). Strong acid hydrolysis, needed to break all neutral and amino sugar glycosidic bonds, was carried out with 2 M trifluoroacetic acid at 100 °C for 4 h (27).

1,2-Diamino-4,5-methylenedioxybenzene Derivatization and Analysis (28, 29)

1,2-Diamino-4,5-methylenedioxybenzene derivatization and analysis of sialic acids was done exactly as described previously.

High Voltage Paper Electrophoresis (30)

Samples were hydrolyzed with 6 M HCl for 4 h at 100 °C, then spotted on a Whatman No. 3 paper. The entire paper was moistened with 0.06 M sodium borate, pH 6.5, placed between two sheets of Mylar, and the ends of the paper were immersed in the same buffer. Constant voltage was applied at 40 V/cm for 2.5 h with continuous cooling of the system. Detection was accomplished by cutting 1-cm strips, soaking them in water overnight, and counting in a Beckman scintillation counter.

HPAEC-PAD Monosaccharide Analysis (27, 31)

The samples were strong acid-hydrolyzed as described above, and monosaccharides were analyzed using a CarboPac PA1 column (250 × 4 mm; Dionex Corp.) eluted isocratically at 1 ml/min with 18 mM NaOH. The post-column concentration of NaOH was increased by helium pressure-driven on-line addition of 1 M NaOH at a rate of 0.3–0.4 ml/min. Detection was accomplished using a PAD-I cell (Dionex Corp.) with the settings of: $E_1 = 0.15$, $E_2 = 0.7$, $E_3 = -0.3$, $T_1 = 9$, $T_2 = 2$, $T_3 = 6$, response time = 1, range = 1, output = 300 nA. The elution position of each monosaccharide standard was checked separately, and standard runs were always carried out at the beginning and end of each set of samples. Occasional inversion in the order of elution of the pairs Gal/$GlcNH_2$ or Xyl/Man were sometimes noted and seem to be the related to the use of a particular column and the specific elution conditions used.

Anion Exchange HPLC Analysis of Oligosaccharides

Samples were applied to a TSK-DEAE-2SW (250 × 4.6 mm) column from TosoHaas, which was then eluted with the following gradient: 0–30 min, 0–300 mM NaCl; 30–35 min, hold at 300 mM NaCl; 35–65 min, 300–700 mM NaCl; 65–70 min, hold at 700 mM NaCl; 70–120 min, 700–0 mM NaCl, at a constant flow rate of 0.6 ml/min. Detection was done with either a Radiomatic Flow-One-Beta on-line radioactive detector or by collecting fractions and monitoring radioactivity.

Rhodizonate Assay for Sulfate (32)

This procedure was done as described previously. Briefly, the sample was brought up in 100 μl of H_2O. 600 μl of a barium buffer was added, followed by 300 μl of rhodizonate reagent. The sample was vortexed, and complex formation was allowed to occur for 10 min at room temperature. The absorbance was then read at 520 nm.

Solvolysis (9, 33)

The samples were converted to their pyridinium form by passage over a Dowex 50 column (hydrogen form). The run-through and water wash were collected on ice into a 15-ml glass conical tube containing 4 drops of pyridine and lyophilized to dryness. A 0.2-ml aliquot of Me_2SO containing 10% methanol was added to each sample and heated at 80 °C for 2 h. The sample was lyophilized, brought up in 2 mM Tris base, and reapplied to a QAE-Sephadex column for analysis of loss of negative charge.

Lectin Affinity Chromatography (34)

Concanavalin A—A 1-ml column of concanavalin A-agarose was poured and equilibrated with 10 ml of TBS-NaN_3 (0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3). The sample was loaded in 750 μl of TBS-NaN_3. Elution of bound material was as follows: 30 ml of TBS-NaN_3, 30 ml of 10 mM methyl-α-D-glucopyranoside in TBS-NaN_3, 30 ml of 100 mM methyl-α-D-mannopyranoside in TBS-NaN_3, prewarmed to 60 °C.

L-PHA—A 1-ml column of L-PHA-agarose was poured and equilibrated with 10 ml of phosphate-buffered saline-NaN_3. The samples were loaded and run in phosphate-buffered saline-NaN_3; sample loading volumes were 200 μl. Fractions of 0.4 ml were collected and counted analytically.

Linkage Analysis by Permethylation (35, 36)

The samples were permethylated as described by Hakomori. Briefly, to the dried samples, 100 μl of anhydrous Me_2SO was added and the vials were flushed with N_2. Dissolution was accomplished by sonication for 5 min intervals over a period of 4 h. 100 μl of methylsulfinyl carbanion was added, N_2 flushed into the vials and sonicated every 15 min for 1 h. 100 μl of methyl iodide (CH_3I) was added dropwise on ice and the sample was sonicated every 15 min for 2 h. 400 μl of methylsulfinyl carbanion was added, and excess of base was ascertained by testing an aliquot with triphenylmethane. The reaction mixture was kept overnight at room temperature, and CH_3I addition was repeated as before. An equal volume of chloroform was added to the sample as well as 10 ml of H_2O, and the entire sample was dialyzed against H_2O and lyophilized. The sample was subjected to acetolysis/hydrolysis as described (35, 36). 0.3 ml of 0.5 N H_2SO_4 in 90% acetic acid was added, and the samples were heated at 80 °C for 6 h. After cooling, the samples were neutralized by addition of 0.5 N NaOH and dried with N_2 and exposure to P_2O_5 overnight. Hydrolyzed samples were dissolved in 10 mM NaOH (pH 9.0) and reduced with $NaBD_4$ overnight at room temperature. Excess $NaBD_4$ was destroyed with glacial acetic acid, and the methyl borates were removed by repeated evaporation with acidified methanol (5 times). Samples were dried over P_2O_5 in a desiccator. Acetylation was done with acetic anhydride (0.3 ml) at 100 °C for 3 h. After repetitive extraction with toluene, the partially methylated, partially acetylated alditols were recovered by partition in $CHCl_3/H_2O$. Samples were dissolved in acetone (10 μl), 1 μl was injected onto a DB-5 (J & W Scientific) capillary column (25 mm × 30 m) with the following temperature program: start at 50 °C, held for 2 min; at 20 °C/min, from 50 °C to 150 °C, and at 4 °C/min, from 150 °C to 250 °C, and maintaining the final temperature for an additional 5 min. A Finnigan MAT 4500 gas chromatograph-mass spectrometer with a computerized data system was used in the EI mode (ionizing potential 70 eV) for detection.

Masking of Carboxylic Acids by Carbodimide Activation and Coupling to Methylamine

QAE-fractionated ^{3}H-oligosaccharides were desalted by gel filtration using a Bio-Gel P-2 column eluted with water, desialylated by mild acid treatment with 10 mM HCl for 30 min at 100 °C, and lyophilized. The samples or [^{3}H]sialyl-(α2,6)-lactose (standard with carboxylic acid residues) were dissolved in 5 μl of 50 mM MES buffer, followed by addition of 10 μl of 1 M methylamime. A stock activation solution of EDC/NHS was freshly prepared by the addition of 100 μl of H_2O to an Eppendorf tube containing 10 mg of EDC and 5 mg of NHS and vortexed. 5 μl of the EDC/NHS solution was immediately added to the samples, vortexed, and incubated at 37 °C. After 1 h, another 5 μl of fresh EDC/NHS solution was added and the incubation continued for another hour. Control samples were treated identically except that the EDC/NHS solution was replaced with water. The samples were diluted to 4 ml with 2 mM Tris base, loaded onto a QAE-column in 2 × 2 ml fractions, and step-eluted with sodium chloride as described above, and radioactivity was monitored.

Methyl Esterification of Carboxylic Acids with Diazomethane

Samples were converted to their H^+ form by passage over a Dowex AG 50W column (H^+ form), lyophilized in Reacti-Vials, and 100 μl of dry Me_2SO or methanol was added. The vials were flushed with argon and capped, and the samples were dissolved by vortexing and cooled on ice. Approximately 100 μl of cold diazomethane (dissolved in ether) was transferred into the vials via a cannula and vortexed. This procedure was repeated twice, until a persistent yellow color was seen. The samples were then incubated at room temperature for 2 h. Diazomethane and ether were removed by a stream of argon gas, and the samples were dried in a Speed Vac evaporator. QAE fractionation was then performed as described above, but in 2 mM sodium cacodylate, pH 5.8, instead of Tris base (to avoid base hydrolysis of the methyl esters). An aliquot of the methyl esterified (neutralized) fraction that ran through the QAE-column was treated with NaOH (final 20 mM, 1 ml) to regenerate the carboxylic acids. After incubation at room temperature for 4 h, the base-treated samples were neutralized with HCl. Controls were similarly treated with 20 mM NaCl. Both samples were desalted by overnight dialysis with 500 molecular weight cut-off tubing and then refractionated on QAE-columns.

Methyl Esterification of Carboxylic Acids with Methyl Iodide in Me_2SO or Methanol

Samples were converted to their Na^+ salt form by passage over Dowex AG 50W (Na^+ form) and dried by vacuum centrifugation. 100-μl of dry Me_2SO and 20 μl of methyl iodide were added to the samples, which were vortexed and incubated at room temperature for ~4 h with occasional vortexing. QAE fractionation was performed as described above in 2 mM sodium cacodylate, pH 5.8 (instead of Tris base), and monitored for radioactivity. Control samples were treated similarly, but the methyl iodide was deactivated by heating in water before addition to the samples.

Methyl Esterification of Carboxylic Acids by Methyl Iodide Treatment on a DEAE-column

Carboxylic acid-containing samples were methyl-esterified with methyl iodide after binding to a DEAE-column as described previously (37) with the following modifications: (i) the column and washes were scaled down by a factor of 4 and (ii) the methyl esterification on the column was carried out somewhat longer and repeated 4 times. The latter modification from the original protocol (which was designed for methyl esterification of sialic acids) was found necessary to adequately neutralize a glucuronic acid standard. Briefly, the samples in methanol were loaded onto 1-ml DEAE-Sephadex A-25 columns equilibrated in methanol and washed with dry methanol. The carboxylic acids were methyl esterified by the addition of 0.4 ml of Me_2SO/CH_3I (5:1) which was allowed to flow into the top of the gel. The flow was then stopped leaving ~2 mm of solvent height above the bed surface, and the column was incubated for 10 min at room temperature. This procedure was repeated 4 times. Carboxylic acid-containing samples were neutralized

by this on-column treatment and were washed out with methanol. Any remaining negatively charged glycans were then eluted with 1 M pyridinium acetate, pH 5.5.

Conversion of Methyl Esters to Methyl Amides by Methylamine Treatment

Methyl-esterified samples eluted from the DEAE-column as above were converted to their respective methylamides as described previously (37), with minor modifications. Briefly, methanol and Me_2SO were removed from the samples by a stream of nitrogen gas and lyophilization, respectively. The samples were redissolved in 200 μl of dry methanol, incubated with 100 μl of 25% methylamine in methanol for 20 min at room temperature, and dried by vacuum centrifugation.

Regeneration of Charged Carboxylic Acids from Methyl Esters by Saponification

Saponification of methyl-esterified samples prepared by methyl iodide treatment on a DEAE-column was performed to regenerate carboxylic acids and to monitor the extent of conversion to methyl amides by base resistance. 100 mM NaOH (50 μl) was added to the samples and incubated for 2 h at 50 °C. The base was then neutralized by addition of an equal volume of 100 mM HCl. 50 μl of 100 mM NaCl were added to control samples. The base-treated or control samples were dissolved in 2 ml of methanol, loaded onto an 0.8-ml DEAE-column, and washed with 4 × 2 ml fractions of methanol. Charged glycans were eluted with 4 × 2 ml fractions of 1 M pyridine acetate, pH 5.5.

RESULTS

Release, Isolation, and Radioactive Labeling of N-Linked Oligosaccharides from Bovine Lung Acetone Powder—As a convenient and concentrated source of glycoproteins from bovine lung, we used a commercially available acetone powder. To specifically release and purify *N*-linked oligosaccharides, we used the same approach (see "Experimental Procedures") as previously employed for *N*-linked chains from CPAE cells (10, 11). Briefly, the acetone powder was homogenized into a lysis buffer containing SDS and heated to solubilize glycoproteins. The soluble material was prefractionated on a Sephacryl S-200 column (run in a buffer containing SDS) to eliminate any small to medium-sized molecules, *e.g.* nucleotides, salts, and degraded cellular products. The material eluting in the void volume of this column (which includes all large molecules as well as most glycoproteins that bind SDS and become incorporated into SDS micelles) was treated with PNGaseF and refractionated on the same Sephacryl S-200 column. The elution profile was monitored using a reducing sugar assay, which also reacts weakly (about 100-fold less) with proteins. As shown in Fig. 1, PNGaseF treatment results in the appearance of a new peak in the partially included area that is not observed in a control incubation done in the absence of enzyme. The PNGaseF-released material was pooled as shown in Fig. 1, avoiding any overlap with fractions where undigested material eluted (as shown in Fig. 1, this precaution prevents inclusion of some of the largest released molecules, which were not studied further). Since PNGaseF-released free oligosaccharides have a reducing end, we next introduced tritium label into the sugar chains by [^{3}H]$NaBH_4$ reduction, as described under "Experimental Procedures." Over the last 5 years, we have made more than 10 such preparations and characterized each to varying extents. While some batch-to-batch variation has been noted, the general properties of all of the released molecules were similar. The following results are representative examples derived mostly from one of the preparations.

A Large Percentage of the N-Linked Oligosaccharides from Bovine Lung Are Highly Anionic—To purify and partially fractionate the oligosaccharides by negative charge, we applied the PNGaseF-released ^{3}H-labeled molecules to a QAE-Sephadex column and eluted them by stepwise increases in sodium chloride concentration, as described previously (10, 11). The salt concentrations chosen for elution were based on prior experience with anionic *N*-linked oligosaccharides. In general, *N*-linked oligosaccharides with a single negative charge are expected to elute with 20 mM NaCl, those with two charges with 70 mM NaCl, and those with three to four negative charges with 125 mM NaCl (at higher concentrations of salt, the resolution is less clear, but molecules eluting with 200 mM and 400 mM NaCl are expected to have 4 or more negative charges). As shown in Fig. 2, about 80% of the radioactivity bound to the column and eluted with a profile roughly similar to that seen previously with CPAE oligosaccharides (10). The CPAE molecules were labeled with [^{35}S]sulfate, whereas the bovine lung oligosaccharides had tritium label introduced uniformly in the reducing end of each molecule. Thus, the ratios of the different fractions cannot be compared directly. Regardless, there was a surprisingly high percentage of moderate to highly anionic molecules in the lung preparation (51% of the negatively charged oligosaccharides eluting with 200 mM NaCl or higher, representing molecules expected to have four or more negative charges). Most highly anionic oligosaccharides reported to date (other than those with polysialic acid) are of the glycosaminoglycan (GAG) type, *e.g.* the heparin/heparan sulfates (HS), chondroitin sulfates (CS), and keratan sulfates (KS). With the exception of KS, these oligosaccharides are generally thought to be linked to proteins via *O*-xylosyl linkage and therefore should not be released by PNGaseF (38, 39). However, in our previous study, a small fraction (<1%) of the PNGaseF-released oligosaccharides from CPAE cells were shown to carry GAG chains, either HS or CS. In the present preparation from bovine lung, about 13% of the anionic molecules elute with 1 M NaCl, suggesting that a larger fraction might carry GAG-type structures.

The Anionic Oligosaccharides from Bovine Lung Fall into Two Major Classes—Treatment of the oligosaccharide mixture with sialidases or with GAG-degrading enzymes gave shifts in the profile of negative charge seen by batch elution on QAE-Sephadex (data not shown). As an alternate approach to subfractionating these oligosaccharides, we used a TSK-DEAE-2SW HPLC anion exchange column eluted with a salt gradient. As seen in Fig. 3, the less charged molecules were partially

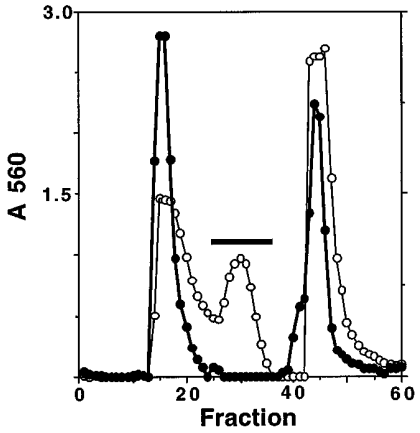


FIG. 1. **Release and isolation of *N*-linked oligosaccharides from bovine lung.** Bovine lung acetone powder was solubilized in an SDS lysis buffer, and the extract was fractionated on a Sephacryl S-200 gel filtration column as described under "Experimental Procedures," collecting material that eluted in the void volume (as monitored by absorbance at 280 nm, data not shown). This material was pooled, and aliquots were incubated with (○——○) or without (●——●) PNGaseF and reapplied to the same S-200 column. Newly created reducing ends were monitored with a reducing sugar assay as described under "Experimental Procedures" (A_{560}). This modified bicinchoninate assay gives a weak reactivity with proteins and also reacts with β-mercaptoethanol, likely explaining the peaks seen in the void and totally included areas, respectively. The *closed bar* indicates the fractions where the released oligosaccharides were detected and pooled for further studies. This figure is representative of over 10 such profiles obtained with various preparations.

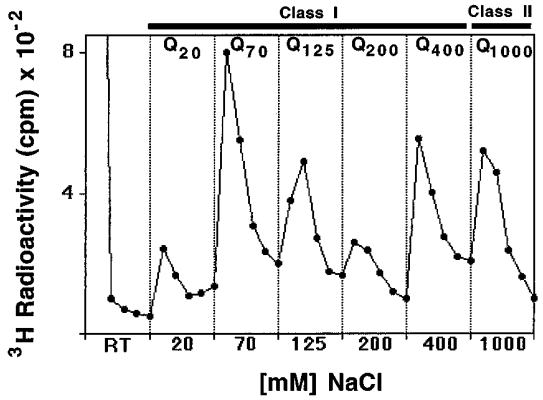


FIG. 2. **Fractionation of *N*-linked oligosaccharides by negative charge.** PNGaseF-released oligosaccharides were reduced with [^{3}H]$NaBH_4$ and separated from unreacted radioactivity by gel filtration on either a Sephadex G-25 or Bio-Gel P-2 column, as described under "Experimental Procedures." The labeled oligosaccharides were applied to a QAE-Sephadex anion exchange column, which was washed and eluted batchwise by increasing amounts of sodium chloride. This figure illustrates a profile representative of over 10 such preparations. The fractions which comprise the Class I and Class II oligosaccharides are indicated. *RT* denotes material which ran through the column and accounts for ~20% of total labeled material.

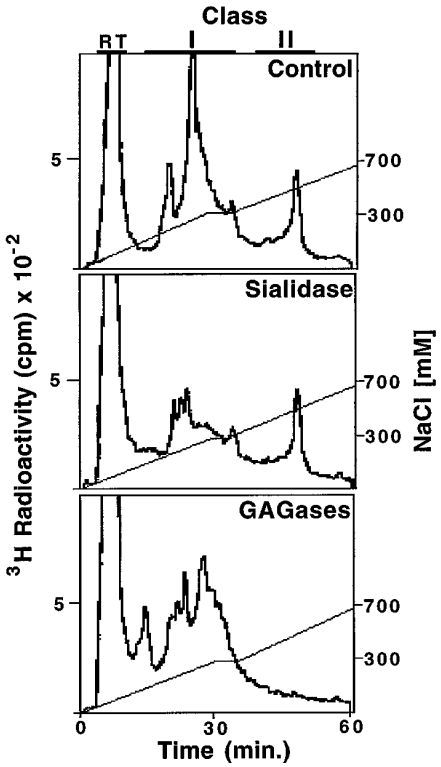


FIG. 3. **Effects of sialidase and GAG-degrading enzymes on anionic properties of bovine lung *N*-linked oligosaccharides.** The total mixture of PNGaseF-released ^{3}H-labeled oligosaccharides was applied to a TSK-DEAE-2SW anionic HPLC column and eluted with a gradient of sodium chloride. *A* shows the profile obtained without any treatment. In *B*, the mixture was digested with *A. ureafaciens* sialidase, and in *C* with a combination of the following GAG-degrading enzymes: heparinase, heparitinase, chondroitinase ABC, chondroitinase AC, and keratanase. *RT*, material running through the column.

susceptible to digestion with *A. ureafaciens* sialidase (apparently similar to Class I oligosaccharides from CPAE cells). The highly charged oligosaccharides were resistant to this treatment, but were completely digested to less negatively charged fragments by a combination of GAG-degrading enzymes (similar to Class II oligosaccharides from CPAE cells). Thus, on initial

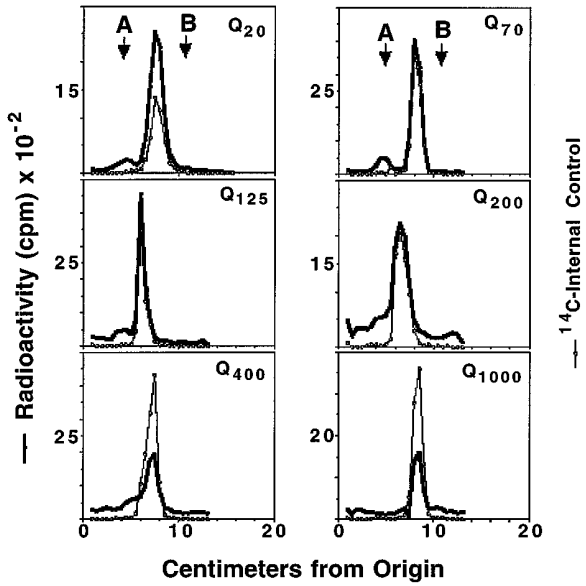


FIG. 4. **High voltage paper electrophoresis of reducing end monosaccharides.** Each fraction from the QAE-Sephadex column (see Fig. 2) was mixed with a [^{14}C]GlcNAcitol internal standard, acid-hydrolyzed to monosaccharide constituents, spotted on Whatman No. 3 paper, and subjected to electrophoresis in 0.06 M borate at 40 V/cm for 2.5 h, as described under "Experimental Procedures." One-cm strips were cut, and radioactivity was determined by liquid scintillation counting. GalNAcitol and xylitol standards ran at the positions indicated by the *arrows A* and *B*, respectively (data not shown).

evaluation, the bovine lung oligosaccharides seem to fall into the same general classes as the structures found in the CPAE cells.

The complete structural characterization of these oligosaccharides would first require their separation into distinct species. However, as might be expected for a whole tissue library, both classes of oligosaccharides are extremely complex mixtures that have defied many attempts at purification into defined species. The methods tried included a variety of anionic exchange systems including QAE-columns with gradient separations, DEAE- and TSK-DEAE-2SW HPLC columns eluted with either sodium acetate or sodium chloride gradients, FPLC Mono Q chromatography with either sodium chloride or sodium sulfate gradients, and a Varian AX-5 HPLC anion exchange column eluted with phosphate gradients (data not shown). Gel filtration sizing with Bio-Gel P-4, P-6, or Sephadex G-50 run in water, Bio-Gel P-60, P-100, and P-300 run in 200 mM Tris-HCl, pH 6.5, with 0.2% SDS, Bio-Gel P-100 run in 2 M sodium acetate, and Superose-12 FPLC size fractionation have also been tried. All of these methods gave broad peaks, but none gave adequate separation of the numerous individual species contained within the mixture. We therefore obtained composite structural information on the mixtures of the Class I and Class II oligosaccharides obtained by Class II Sephadex or TSK-DEAE HPLC fractionation, as shown in Figs. 2 and 3, respectively.

The Label Introduced by NaB[^{3}H]$_4$ Is in N-[^{3}H]Acetylglucosaminitol—Although these molecules were specifically released from bovine lung acetone powder using PNGaseF, an enzyme whose specificity is restricted to most known *N*-linked sugars, we wished to confirm directly that the ^{3}H-labeled oligosaccharides had the expected *N*-[^{3}H]acetylglucosaminitol (GlcNAcitol) at their reducing end. Strong acid hydrolysis conditions were used to break all the glycosidic linkages, and the resulting ^{3}H-labeled monosaccharides were analyzed by high voltage paper electrophoresis. As shown in Fig. 4, most of the label from the Class I fractions migrated with an authentic [^{14}C]GlcNAcitol internal standard and displayed similar recovery (55–78%) when compared with authentic ^{3}H-labeled *N*-

TABLE I

Molar ratios of monosaccharides in bovine lung oligosaccharides

The QAE-fractions were combined as indicated (maintaining their original ratios), subjected to acid hydrolysis, and analyzed by HPAEC-PAD as described under "Experimental Procedures." The ratios presented show the range of values obtained in analyses of three different oligosaccharide preparations and are corrected for recovery of standard amounts of the test monosaccharides subjected to the same hydrolysis conditions. All values are presented relative to the 3 mannose residues expected in the core of typically complex-type *N*-linked oligosaccharides. ND, not detected.

Monosaccharide	QAE-fractions (NaCl concentration)		
	Group A Q_{20} + Q_{70}	Group B Q_{125} + Q_{200} + Q_{400}	Group C Q_{1000}[a]
Man	3	3	3
$GlcNH_2$	5–6	12–16	12–31
Gal	6–7	13–23	11–59
Fuc	0.6–1	0.6–1	0–1
Xyl	ND	ND	3–3.7
$GalNH_2$	0.1–0.9	2–4	8–95
Glc[b]	0.4–0.5	4–16	10–31

[a] The compositional analysis of the Q_{1000} fraction gave widely variable results in different assays, perhaps because of the difficulty in obtaining complete acid hydrolysis of GAG chains.

[b] Glucose content may represent environmental contamination.

TABLE II

Linkage analysis of monosaccharides in bovine lung oligosaccharide

The QAE-fractions were combined as indicated in Table I (maintaining their original ratios), subjected to permethylation, and analyzed by GLC-MS as described under "Experimental Procedures." As indicated in the text, only the Group A oligosaccharides gave complete methylation, and the resulting data are presented in this table. All values are presented relative to the 3,6-substituted mannose residue expected in the core of all typically complex-type *N*-linked oligosaccharides.

Monosaccharide	Methyl positions	Linkage	Molar ratio
Man	2,4-	-6 Man 1--3	1.0
	3,4-	-6 Man 1--2	1.2
	3,6-	-4 Man 1--2	2.0
	2,3,4-	-6 Man 1-	6.0
GlcNAc	*N*,3,4,6-	Terminal	6.0
	N,3,6-	-4 GlcNAc1-	33.8
Fuc	2,3,4-	Terminal	1.0
Gal	2,3,4,6-	Terminal	41.0
	2,4,6-	-3 Gal 1-	4.1
	2,3,4-	-6 Gal 1-	2.0

linked oligosaccharides from bovine fetuin prepared and analyzed in the same manner (~50% recovery in the [^{3}H]GlcNAcitol peak, data not shown). The Class II fraction (Q_{1000})[2] showed a somewhat lower percentage of recovery of label in [^{3}H]GlcNAcitol (39%). However, very little radioactivity co-migrates with either *N*-acetylgalactosaminitol or xylitol, the two alditols expected to be derived from the reducing terminus of common *O*-linked oligosaccharides. The small quantities of label migrating in the position of GalNAcitol in some of the Class II fractions could potentially represent contamination by *O*-linked glycopeptides which could undergo partial β-elimination during the tritiation/reduction reaction. As shown below, some unlabeled GalNAc was also found on compositional analysis. However, these were different fractions from those which contained the small quantities of labeled GalNAcitol .

Monosaccharide Compositional Analyses—The individual QAE-derived fractions were acid-hydrolyzed to their monosaccharide constituents, and their composition was analyzed with an HPAEC-PAD system as described under "Experimental Procedures." Monosaccharides expected in *N*-linked oligosaccharides were found in all the fractions, but the quantities of material in some fractions were inadequate for accurate analyses (data not shown). Since subsequent studies (presented in Table IV and discussed below) suggested that most of the negative charge in the Q_{20} and Q_{70} fractions was due to sialic acids, while the Q_{125}, Q_{200}, and Q_{400} fractions had other negative charges as well, we grouped together the fractions as shown in Table I for further studies of monosaccharide composition. When normalized to the predicted 3 mannose residues in the core region of complex-type *N*-linked oligosaccharides, a high percentage of both galactose and glucosamine in many of the pools suggests the presence of polylactosamine repeat units (Table I). Since glucosamine residues are also present in the core of typical *N*-linked oligosaccharides, all Gal residues cannot be accounted for by lactosamines. At least some of the excess is probably accounted for by terminal α-galactose residues, which appear to cap many of the chains (determined by α-galactosidase treatment, data not shown). The fucose present in all fractions could represent core or outer chain fucosylation. The substantial amounts of $GalNH_2$ in the Class II molecules is explained by the content of CS chains (see below), but smaller amounts are also present in the moderately charged group. The presence of significant amounts of xylose in the Class II material was unexpected and is explored further below. Under the elution conditions used, uronic acids could not be detected.

Both the specificity of the original PNGaseF release (17) and the demonstration of [^{3}H]glucosaminitol at the core indicate that both Class I and Class II molecules consist predominantly of complex-type *N*-linked oligosaccharides with a chitobiose core and extended outer chains. Since the two classes of oligosaccharides have otherwise very distinct properties, their further characterization is presented separately below.

Linkage Analysis of the Class I Oligosaccharides—To examine the type of linkages present, the oligosaccharides were permethylated and analyzed by GC-MS. Class II molecules proved to be very difficult to dissolve in Me_2SO, even after the addition of 4-methylmorpholine *N*-oxide, and gave no useful results. The Class I molecules were grouped together as in Table I, "Group A" (eluted with 20 or 70 mM NaCl) and "Group B" (eluted with 125, 200, or 400 mM NaCl). A summary of the type of linkages found for each monosaccharide in the Group A oligosaccharide mixture (Q_{20} + Q_{70}) is presented in Table II, and the deduced structural are elements summarized in Fig. 5. These data indicate that the lesser negatively charged Class I structures contain many tri- and tetraantennary structures, repeating lactosamine units, the expected subterminal Gal units (substituted at either positions 3 or 6), and significant amounts of terminal galactose or *N*-acetylglucosamine residues. Both the excess of terminal Gal units and the presence of the unusual 6-substituted Man residues were not pursued further, because of the complexity of the mixture. Group B molecules were severely undermethylated, again due to poor solubility in Me_2SO. However, the detection of 3,4,6-methylated mannose suggested the presence of biantennary structures, and lactosamine repeating units were indicated by the presence of high proportions of 2,4,6-methylated galactose and *N*,3,6-GlcNAcMe (data not shown).

Lectin Affinity Chromatography of Class I Oligosaccharides—As with the Class I material from CPAE cells, many Class I oligosaccharides from bovine lung ran through a con-

[2] The fractions derived from the initial QAE-Sephadex fractionation (see Fig. 2 for an example) are designated according to the concentration of NaCl required for elution, *e.g.* the fraction eluted with 20 mM NaCl is denoted Q_{20}.

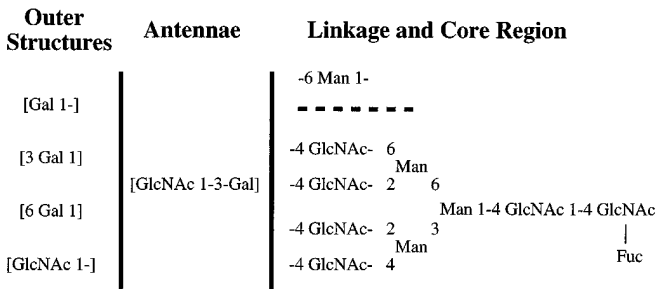


FIG. 5. **GC-MS analysis of combined Q_{20} and Q_{70} fractions.** The QAE-fractions were pooled as indicated, subjected to permethylation, and partially methylated, partially acetylated alditol acetates were separated using a fused silica capillary column, with monitoring by electron impact mass spectrometry as indicated under "Experimental Procedures." This figure illustrates a composite structure derived from these data (see text and Table II for further details). Note that the structures [3 Gal 1-] and [6 Gal 1] are presumed to be capped with sialic acids or terminal α-galactose residues.

canavalin A column (see Table III for some examples), indicating that they are complex-type chains (34). A significant fraction of these molecules were retarded on an L-PHA affinity column (see Table III), indicating the presence of the following minimum structure (34):

Galβ1,4GlcNAc β1,6
Manα1,6
Galβ1,4GlcNAc β1,2 Manβ1,4
Galβ1,4GlcNAc β1,2Manα1,3

STRUCTURE 1

These results further indicate that the Class I oligosaccharides contain mainly branched complex type oligosaccharides. However, because many chains had unknown anionic substituents that might unpredictably affect binding, we did not use other lectin columns for structural analysis. For the same reason, we also did not study the interactions of the very highly charged Class II molecules with lectins.

While Sialic Acids and Sulfates Are Present on the Class I Oligosaccharides, Another Anionic Group Accounts for the Majority of the Negative Charge—Negative charge on *N*-linked oligosaccharides may be due to sialic acids, sulfate esters, phosphate esters, or, less commonly, uronic acids. Sialidase treatment caused a decrease in negative charge of many of the Class I molecules, but did not completely neutralize the majority of them (Table IV). Mild acid hydrolysis under conditions which selectively remove sialic acids gave similar results, indicating the absence of sialidase-resistant sialic acids (data not shown and Table IV). HPLC analysis showed that the acid-released sialic acids were mainly *N*-acetylneuraminic acid (Neu5Ac), with small amounts of *N*-glycolylneuraminic acid (Neu5Gc) (data not shown). We found no evidence for phosphomonoesters or phoshodiester groups, using combinations of sialidase, mild acid, and alkaline phosphatase treatments (data not shown).

We previously showed that about one-third of the $^{35}SO_4$-labeled Class I oligosaccharides from CPAE cells contained the terminal structure: Siaα2-(3)6Galβ1–4GlcNAc(6$^{35}SO_4$)β1-(10). This was demonstrated by release of ^{35}S label after sequential removal of Sia and β-Gal residues, followed by digestion with human placental β-hexosaminidase A incubated at pH 3.5, which gives specific removal of the exposed terminal GlcNAc(6SO_4) (40). We subsequently noted that some *N*-linked structures from CPAE cells also carry terminal α-linked galactose residues (41). Based on these observations, we carried out a series of experiments using combinations of mild acid treatment, jack bean, or bovine testicular β-galactosidases, coffee bean α-galactosidase, and human placental β-hexosaminidase A (pH 3.5) to look for the presence of either of the following terminal structures on the bovine lung anionic oligosaccharides: Siaα2-(3)6 Galβ1-(3)4GlcNAc(6SO_4)β1- or Galα1–3 Galβ1–4 GlcNAc(6SO_4)β1-. A small loss of negative charge was seen with some of these treatments, but the great majority of the anionic charge remained unchanged (data not shown) indicating that there are very few subterminal GlcNAc(6SO_4) residues present. Thus, most of the non-sialic acid-dependent negative charge is different from that found in the CPAE cells or any other cultured cells analyzed to date.

Chemical analysis (32) or solvolysis (9, 33) was used to determine total sulfate groups regardless of location. As expected for GAG-type structures (see below), the Class II fractions had a sulfate content easily detectable with the rhodizonate assay: 140 nmol of sulfate per nmol of reducing end sugar (data not shown). With this assay (3 nmol detection limit), only trace amounts of sulfate were detected in the Q_{400} fraction, and none was detectable in the lesser-charged fractions. For the latter, we therefore used solvolysis to remove the sulfate esters and then reapplied the samples to QAE-columns to check for loss of negative charge (data not shown). While we did see some minor shifts with this treatment, the great majority of the negative charge persisted (data not shown), suggesting that these are not due to sulfate esters. Thus, unlike Class I oligosaccharides

TABLE III
Examples of interaction of oligosaccharides with concanavalin A and L-PHA lectin columns

For the concanavalin A column, the complex-type biantennary oligosaccharides, which elute with α-methyl glucoside (αMG), and the high mannose oligosaccharides, which elute with α-methyl mannoside (αMM), are tabulated separately. The L-PHA interaction, based on the retardation of the oligosaccharides on the affinity column, is presented as the percentage of label which elutes after the void volume. As a positive control, [2−^{3}H]mannose-labeled oligosaccharides from BW5147 lymphoma cells (51) were used.

	% oligosaccharides bound to		
	Concanavalin A		L-PHA
	αMG	αMM	
Positive control	43%	46%	26%
70 mM NaCl	11%	2%	18%
125 mM NaCl	3%	1%	17%
400 mM NaCl	2%	1%	3%

TABLE IV
Effects of various treatments on the negative charge of Class I oligosaccharides

Each QAE-fraction was treated with sialidase to specifically remove sialic acids and then refractionated on a QAE-column to determine the amount (percentage) of negative charge removed from the original sample (as detected by the ability of the sample to elute from the column with lower amounts of sodium chloride). The column "% total anionic molecules" indicates the percentage of anionically charged oligosaccharides that each QAE-fraction represented with the initial fractionation. The column labeled "Sialic acid" indicates the percentage of that QAE-fraction which was completely neutralized by sialidase treatment. The column labeled "Unidentified negative charge" indicates the percentage of that fraction which did not shift at all with sialidase treatment. The column labeled "Sialic acid + unidentified charge" represents the percentage of the fraction which shifted to a lower negative charge (eluted with less sodium chloride), but was not completely neutralized. NA, not applicable, sensitive to GAG-degrading enzymes.

QAE-fraction	% total anionic molecules	Sialic acids only	Sialic acids + unidentified charge(s)	Unidentified negative charge(s) only
			% *fraction*	
Q_{20}	9	82	0	18
Q_{70}	24	74	15	11
Q_{125}	16	18	55	27
Q_{200}	10	0	25	75
Q_{400}	19	0	26	74
Q_{1000}	22	0	NA	NA

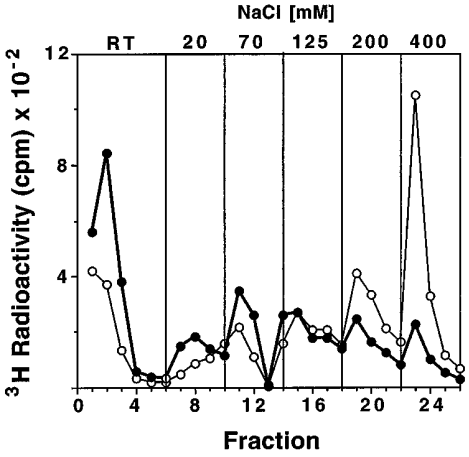


FIG. 6. **Example of decrease in negative charge caused by neutralization of carboxyl groups.** The individual QAE-Sephadex-derived fractions were first treated with mild acid to remove any sialic acids present. The remaining oligosaccharide structures were then incubated with a mixture of EDC/NHS (*closed circles*) or water (*open circles*) in the presence of methylamine as described under "Experimental Procedures." Samples were then diluted 160-fold in 2 mM Tris base, reapplied to a QAE-Sephadex column, and re-eluted batchwise with increasing sodium chloride concentrations, as in Fig. 2. Shown is an example of this treatment using the original Q_{400} fraction. Varying degrees of partial neutralization were seen for all the other QAE-fractions (data not shown).

from CPAE cells, sulfate esters do not account for much of the negative charge.

Much of the Negative Charge in Class I Molecules Is Due to Carboxylic Acids—The fraction that remains acidic after removal of sialic acids represents ~65% of the anionic oligosaccharides and ~49% of the total oligosaccharides released by PNGaseF. Since sulfate and phosphate esters do not explain this negative charge, the most likely candidates were uronic acids. A direct search for uronic acids by compositional analysis after acid hydrolysis was not possible because the amounts of material available were limited, and because uronic acids are notoriously difficult to release and recover after acid hydrolysis. Furthermore, trace contamination by Class II molecules (known to contain high proportions of uronic acids in HS and CS chains, see below) could confound the compositional analysis of the higher charged subsets of Class I chains. We therefore sought alternate approaches to look for the presence of carboxylate groups. First, we chose to specifically neutralize carboxylic acids by activation with a water-soluble carbodiimide in the presence of NHS. Any resulting NHS esters were subsequently reacted with an excess of methylamine, which should give a neutral methylamide.

$$\sim COOH \xrightarrow{EDC/NHS} \sim COO\text{–}NHS \xrightarrow{CH_3NH_2} \sim CO\text{–}NH\text{–}CH_3$$

REACTION 1

After establishing general conditions to neutralize 52 ± 10% of the negative charge of the carboxyl groups on a sialyllactose standard (see "Experimental Procedures" for details), we applied this procedure to the individual Class I fractions after first treating with mild acid to remove all sialic acids. One example of the results (from the treatment of the Q_{400} fraction) is shown in Fig. 6. It can be seen that substantial shifts in negative charge were obtained as a result of either the complete or partial neutralization of these oligosaccharide structures. Since the efficiency of this treatment is incomplete, the results give an underestimate of the amount of negative charge due to carboxyl groups. All of the other fractions also showed such partial reduction in negative charge (data not shown). Thus, the majority of the negative charge in the Class I oligosaccharides is not due to sialic acids and may be accounted for by carboxylate substituents (presumably uronic acids). However, the negative charge on Class I molecules was found to be completely resistant to the action of either β-glucuronidase or α-iduronidase, with or without the addition of β-galactosidase, β-hexosaminidase, and α-galactosidase (data not shown). Thus, it is unlikely that the putative carboxylates are part of uronic acid residues that are present at terminal positions or at subterminal positions covered by Gal or GlcNAc residues. Alternatively, such residues might render other adjacent monosaccharides resistant to the action of the glycosidases used.

Reversible Methyl Esterification of the Carboxylate Groups with Diazomethane or Methyl Iodide—Carboxylate groups can be methyl-esterified by treatment with diazomethane (reaction proceeds best on the acid form) or by CH_3I (reaction proceeds best on a salt form).

$$\sim COO^- H^+ \xrightarrow{H_2=N=N} \sim CO\text{–}OCH_3$$

$$\sim COO^- Na^+ \xrightarrow{CH_3I/Me_2SO} \sim CO\text{–}OCH_3$$

REACTION 2

A mixture of Class I molecules that continued to carry negative charge after mild acid hydrolysis (desialylation) was subjected to these treatments, as described under "Experimental Procedures." In each case, [^{14}C]GlcA or [^{3}H]Neu5Ac was used as standards containing carboxylic acid residues. The maximum efficiency of esterification of the above standards (as detected by loss of binding to a QAE-Sephadex column, data not shown) were 75% and 94%, respectively (for diazomethane treatment) and 25% and 41%, respectively (for methyl iodide treatment). Under these treatment conditions, a substantial loss of negative charge was also seen in the Class I molecules (as determined by QAE-Sephadex, see Table V). To further check for the presence of methyl esterification, an aliquot of the molecules neutralized by diazomethane treatment was isolated and subjected to saponification. This resulted in restoration of the negative charge to ~50% of the molecules (data not shown). This is as expected for methyl esters of carboxylic acids.

Methyl Esterification of Carboxylate Groups with Methyl Iodide on a DEAE-column and Subsequent Conversion to Methylamides—The results presented above provide three different lines of evidence for the presence of carboxylate groups on the Class I molecules. However, in each case, the neutralization of negative charge was only partial. Since the carboxylic acid standards also underwent partial neutralization, it can be extrapolated that the majority of the non-sialic acid-negative charge is due to these additional carboxylate residues. However, it is also possible that other unknown groups might be responsible for the remaining negative charges. To explore this further, we used a recently described method to obtain near-complete methyl esterification of sialic acid carboxylate residues on oligosaccharides which are bound to a DEAE-column, by treatment with methyl iodide *in situ* (37). As described under "Experimental Procedures," this method also has the advantage that the methyl-esterified molecules will wash off the column only if they have been completely neutralized. Thus, any molecules that are eluted from the column upon methyl iodide treatment must have all of their original negative charge entirely accounted for by carboxylate groups. When this treatment was optimized for a glucoronic acid standard and applied to a mixture of desialylated anionic Class I molecules, 60% of the radioactivity was eluted from the column upon treatment with methyl iodide, in comparison with elution

TABLE V

Evidence for the presence of carboxylic acids in Class I oligosaccharides

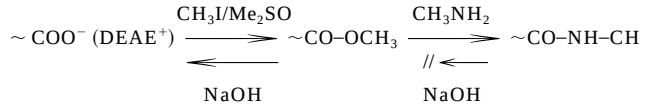
Class I oligosaccharides that bound to a QAE-column and eluted directly with 300 mM NaCl were first treated with mild acid to remove sialic acids, the acid evaporated, and then treated with methyl iodide in Me_2SO or methanol, or diazomethane in ether/methanol to methyl esterify any remaining carboxylic acids, as described under "Experimental Procedures." The samples were then reapplied to QAE-Sephadex columns, and fractions were eluted with increasing steps of sodium chloride. The table summarizes the percentage of total radioactivity recovered in each individual QAE-fraction.

Treatment	RT[a]	20 mM	70 mM	125 mM	200 mM	400 mM	1 M
				% total radioactivity			
Mild acid only	11	36	19	12	13	7	2
CH_3I (Me_2SO)	33	23	12	17	5	9	1
CH_3I (MeOH)	33	28	11	20	5	2	1
Diazomethane	39	26	12	8	8	4	2

[a] RT, material running through the column.

of 85% of a GlcA standard (see Fig. 7). Subsequent elution of the Class I molecules with salt released 37% of the radioactivity, confirming that most of the non-sialic acid-negative charge was eliminated by methyl esterification.

To ensure that the elution from the column was not due to any nonspecific effect of methyl iodide, we restudied the behavior of these "neutralized" molecules on DEAE-Sephadex. Indeed, the great majority of these molecules now ran through the column (data not shown), and binding could be substantially restored by base treatment (see Table VI, stronger base hydrolysis was not attempted since the effects of base on the oligosaccharides themselves could potentially confound the results). To further demonstrate the presence of methyl ester groups, we converted them to stable methyl amide groups by treatment with methylamine (37).



REACTION 3

As shown in Table VI, this treatment provided substantial protection from the base treatment. Appropriate positive and negative controls gave the expected results (see Table VI and data not shown). Taken together, these results indicate that the great majority of the non-sialic acid-negative charge of the Class I molecules is due to these methyl-esterifiable carboxylate groups. Overall, this as yet unidentified modification accounts for about half of all the negative charge on Class I chains.

The Class II Molecules Carry Different Types of GAG Chains and More Than One Type May Be Present on a Single Oligosaccharide—Class II molecules were first separated from the less negatively charged Class I molecules by QAE-Sephadex chromatography, eluting with 1 M NaCl (see Fig. 2). Sizing analysis showed these molecules to be generally large, but heterogeneous in size, ranging from some that are excluded on a Bio-Gel P-100 column (exclusion limit for globular proteins, ~100 kDa), to some that are partly included on a Bio-Gel P-60 column (exclusion limit for globular proteins, ~60 kDa). However, no distinct peak separations were obtained by these methods (data not shown). As shown in Fig. 8, treatment of the mixture with individual GAG-degrading enzymes produced a shift in the elution profile on a TSK-DEAE-HPLC anion exchange column, indicating the presence of KS, CS, and HS chains. However, the products of the individual enzyme treatments still carried a considerable amount of negative charge (Fig. 8). Totaling the percentage shift produced by the individual GAG-degrading enzyme treatments gives a value of ~140%. This may be an underestimate of the total loss of charge, since some highly charged oligosaccharides may not have shifted sufficiently relative to the control profile to be

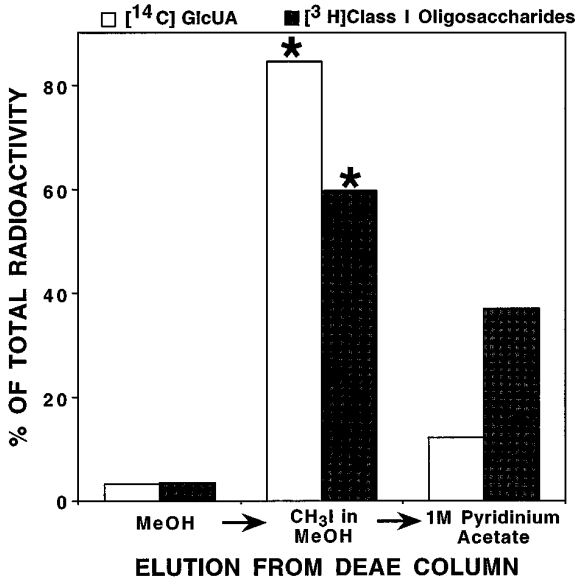


FIG. 7. **Neutralization of carboxyl groups by methyl iodide treatment on a DEAE-column.** Class I molecules were treated with mild acid to remove sialic acids, and the material remaining anionic was reisolated on a QAE-Sephadex column by direct elution with 300 mM NaCl. After desalting, the molecules were applied to a DEAE-Sephadex A-25 column in methanol and eluted with CH_3I/methanol and then 1 M pyridinium acetate as described under "Experimental Procedures." The samples eluted with CH_3I (indicated by the *asterisks*) were dried and studied further as described in Table VI. As a positive control, [^{14}C]GlcUA was treated similarly.

detected. Regardless, the results suggested that more than one GAG chain might be associated with each oligosaccharide reducing end. To explore this possibility, sequential digestions with the GAG-degrading enzymes were performed. A variety of such experiments was performed, and one example of the results is shown in Fig. 9. Digestion of a highly anionic fraction first isolated from the TSK-DEAE-2SW column with chondroitinase ABC showed a partial loss of negative charge when reapplied to the same column (Fig. 9, *upper panel*). Digestion of the resulting peaks with heparitinase and heparinase gave a shift, and subsequent treatment with keratanase gave a further loss of negative charge (see example in Fig. 9). The other peaks in each case were also subjected to sequential digestions and gave similar results, only one example is shown here. Such experiments suggest that many of these molecules contain two types of GAG chains, and a small percentage may even contain all three types of GAG chains. Although these data are indirect, they suggest the existence of *N*-linked oligosaccharides with multiple GAG chains, each perhaps extending from different antennae of a complex-type *N*-linked molecule. A less likely possibility would be the existence of copolymers between the different types of GAG chains, which has never been reported

TABLE VI

Further evidence for carboxylic acids in desialylated Class I oligosaccharides

A mixture of Class I oligosaccharides was first treated with mild acid to remove sialic acids. All molecules with persisting negative charge were then directly recovered from a QAE-Sephadex column by eluting with 300 mM NaCl. After desalting, an aliquot of these anionic molecules or a [^{14}C]GlcA standard was applied to a DEAE-Sephadex A-25 column, which was washed in methanol, and then treated with methyl iodide in methanol as described under "Experimental Procedures" (Step I treatment). The molecules eluted from the column by this treatment (presumed to be neutralized by methyl esterification, see *asterisk* in Fig. 7) were treated with or without methylamine (Step 2 treatment) to convert the methyl esters to stable methylamides, and then treated with or without base (Step 3 treatment) under conditions that should partially saponify most methyl esters, as described under "Experimental Procedures." The samples were then reapplied to DEAE-Sephadex A-25 columns in methanol and eluted with 1 M pyridine acetate. Samples that were *not* methyl-esterified were also treated similarly with methylamine and base and reapplied as a control. The table summarizes the percentage of run-through or bound material seen in the DEAE-fractions after each set of sequential treatments.

Sample	Sequential treatments			% total radioactivity	
	CH_3I elution from DEAE (Step 1)	Methylamine (Step 2)	Base (Step 3)	DEAE-run-through	DEAE-bound
[^{14}C]GlcA	−	+	+	7	93
Class I	−	+	+	15	85
[^{14}C]GlcA	+	+	−	85	15
Class I	+	+	−	73	27
[^{14}C]GlcA	+	−	+	13	87
Class I	+	−	+	28	72
[^{14}C]GlcA	+	+	+	41	59
Class I	+	+	+	51	49

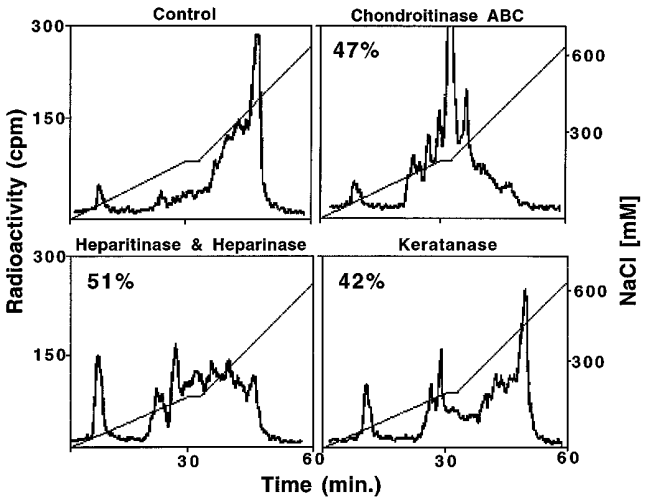


FIG. 8. **Susceptibility of Class II molecules to GAG-degrading enzymes.** Class II molecules were isolated by QAE-Sephadex chromatography (as in Fig. 2) and desalted, and aliquots either were sham-incubated or treated with the GAG-degrading enzymes as indicated. After digestion, they were analyzed for loss of negative charge by reapplication to a TSK-DEAE-2SW HPLC column. The *number shown in the upper left hand corner* of each panel indicates the percent of oligosaccharides which shifted to a lower negative charge in each case (calculated from the change in the non-overlapping areas of the profiles from the control and treated samples).

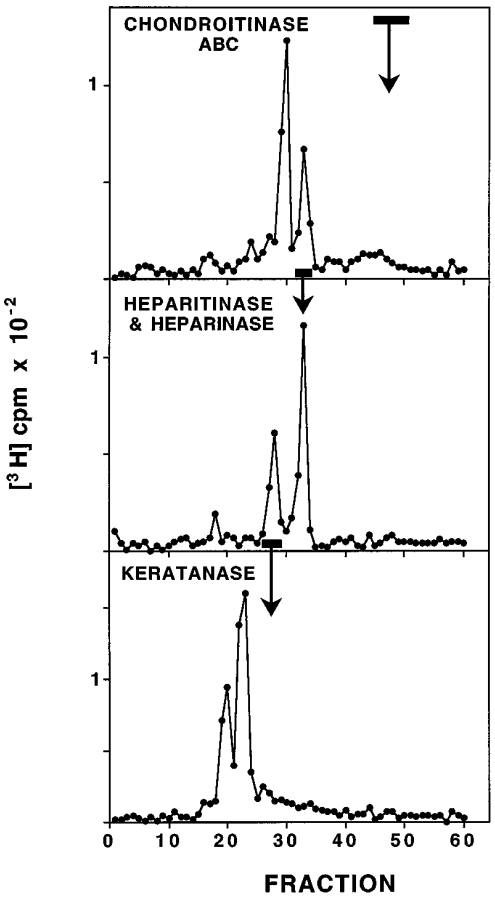


FIG. 9. **Sequential treatments with GAG-degrading enzymes.** A preparative run of undigested Class II molecules was done on a TSK-DEAE-2SW HPLC column to isolate the most negatively charged species. Such material was subjected to sequential digestions by GAG-degrading enzymes, with intermediate refractionation on the same HPLC column. The initial run is not shown, but see Fig. 7 for a representation of the profile. In each panel, the *solid bar* indicates the elution position of the peak from the previous run which was subjected to the enzyme digestion indicated.

before in the literature.

Xylose Present in the Class II Molecules Is Not at the Reducing End—Most known glycosaminoglycans are linked to the protein in an *O*-xylose linkage. Since the molecules studied here were released with PNGaseF (17), xylose should not be at the reducing terminus. This was supported by the results of high voltage paper electrophoresis following acid hydrolysis (Fig. 4). However, compositional analysis (Table I) indicated that xylose was present in the Class II fraction, raising the possibility of an artifactual contamination with conventional GAG chains. To rule out the presence of xylose at the reducing end of these molecules, we analyzed acid hydrolysates of non-reduced or reduced Class II oligosaccharides by a HPAEC-PAD system under conditions where all monosaccharide alditols would run through the column, while nonreduced monosaccharides would be retained and separated. With prior reduction, only the reducing end monosaccharide would be converted to the alditol form and therefore would run through the column. As seen in Fig. 10, a xylose standard was completely converted to xylitol by reduction (consequently running through the column), validating the method. In contrast, when Class II mole-

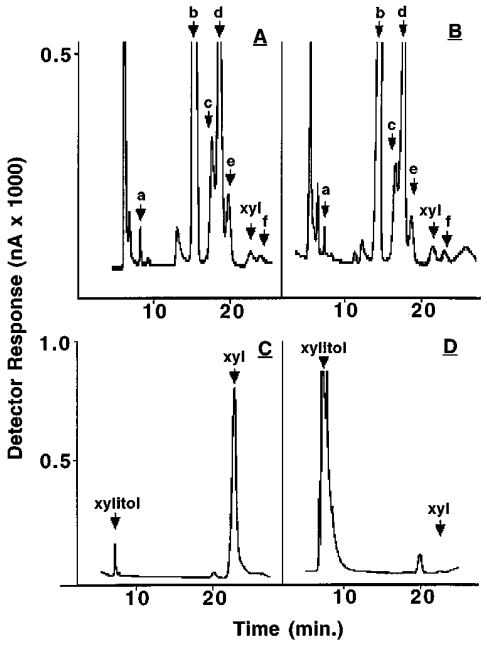


FIG. 10. **The xylose present in Class II molecules is not at the reducing terminus.** One batch of bovine lung oligosaccharides released by PNGaseF was subjected to "blind" fractionation without introducing a tritium label into the reducing end, *i.e.* the reducing end was not converted to an alditol. The Class II molecules from this preparation were acid-hydrolyzed and analyzed for monosaccharide composition by the HPAEC-PAD system (*a*, fucose; *b*, galactosamine; *c*, galactose; *d*, glucosamine; *e*, glucose; *f*, mannose; *xyl*, xylose). *A* shows that a small peak of xylose is detected in nonreduced Class II molecules. *B* shows that when the Class II molecules are reduced with $NaBH_4$ prior to acid hydrolysis, the xylose peak persists. *C* and *D* show profiles of a xylose standard run with (*D*) and without (*C*) prior reduction with $NaBH_4$.

cules were reduced before acid hydrolysis, the xylose peak was retained (Fig. 10), indicating that the xylose is not at the reducing terminus but somewhere within the oligosaccharide structure.

DISCUSSION

We have demonstrated here that bovine lung contains a heterogeneous population of unusual *N*-linked anionic oligosaccharides. The discovery of these oligosaccharides arose from our earlier finding that a calf pulmonary artery endothelial cell (CPAE) synthesized a complex population of anionic *N*-linked oligosaccharides which contained both sialic acids and sulfate groups (10, 11). Hoping to obtain larger amounts of similar structures for detailed analysis, we studied intact lung in which ~40% of the mass is composed of endothelial cells (18, 19). Using a commercially available bovine lung acetone powder and relying on the specificity of the enzyme PNGaseF (17), we have found an equally complex array of anionic *N*-linked oligosaccharides, only some of which are similar to those from the CPAE cell line. Initial fractionation of these oligosaccharides on an anion exchange column shows two distinct classes of structures. Class I chains included the less negatively charged molecules that were partially susceptible to sialidase. However, in striking contrast to the CPAE cell molecules, the remaining non-sialic acid-negative charge in this class of chains was not primarily due to sulfate esters. Rather, most if not all of the negative charge is due to carboxylic acids. The more negatively charged Class II oligosaccharides were similar to those in the CPAE cells, being sensitive to the GAG-degrading enzymes including heparin lyases, chondroitin lyases, and keratanases. However, they seemed to be present in much greater abundance, representing >10% of the total oligosaccharides released by PNGaseF. This confirms the existence of the unusual *N*-linked GAG structures we had previously detected in CPAE cells. We also show another unusual feature of these molecules, that multiple GAG chains may be present on a single core structure.

Most structural analyses of oligosaccharides are performed on purified glycoproteins. The great majority of these have originated from plasma glycoproteins, blood cell membranes, or from cells grown in tissue culture. A reasonable assumption is that *N*-linked oligosaccharides of intact tissues and organs will be similar, with a dominance of sialylated complex-type chains. However, this has not been proven by direct analyses. The present work shows that in a library of total oligosaccharides from bovine lung, the dominant *N*-linked chains are in fact not the expected sialylated ones, but rather a complex family of unusual structures bearing many unexpected anionic charges. In this regard, it is worth noting that the only other such reported attempt at making a library of total *N*-linked oligosaccharides from an intact mammalian tissue is the work of Wing *et al.* (42), who have created a "library" of hydrazine-released *N*-linked oligosaccharides which also contain many unexplained anionic structures. It is reasonable to speculate that the high level expression of these novel molecules in intact tissues might be the consequence of differentiating stimuli that are found only in the *in vivo* situation and perhaps lost or diminished in *in vitro* culture. Regardless of the reason, it may be worthwhile to explore such libraries from other tissues.

A disadvantage of studying a library of oligosaccharides is the complexity of the resulting mixture, which can pose major separation problems to obtain individual structures. In our case, we have tried a variety of techniques to isolate a single pure species which could be fully characterized; however, all such approaches have so far resulted only in dividing the molecules into mixtures of unresolvable oligosaccharides with similar size or charge. Combinations of techniques have also been so far unsuccessful in obtaining pure species for study. We have therefore chosen to group the molecules into several fractions of similar charge but variable size and gathered preliminary data on the structures present in these mixtures.

Although all oligosaccharides studied were specifically released by PNGaseF, we confirmed their *N*-linked nature by showing that they contained the expected *N*-acetylglucosamine residue as the reducing end monosaccharide (a small amount of *N*-acetylgalactosaminitol may be explained by minor contamination with *O*-linked glycopeptides). The Class I oligosaccharides with small numbers of negative charge contain typical multiantennary *N*-linked core structures with sialic acids and no phosphate esters. With the more highly charged Class I structures, sialic acids contribute only a minority of the negative charge, and sulfate esters are even less common. Most if not all of the negative charge seems to be due to residual carboxylic acids that persist after chemical removal of sialic acids. We presume that these carboxylic acids are associated with uronic acids and, as such, present unusual *N*-linked structures. There are two prior lines of indirect evidence for the presence of uronic acids on *N*-linked oligosaccharides. First, monoclonal antibodies known to recognize sulfated glucuronosyl groups on glycolipids are known to cross-react with *N*-linked glycoproteins (13, 14, 16). Secondly, Kawasaki and colleagues (15) have provided evidence for two distinct glucuronosyltransferases in brain tissues: one which transfers to glycolipids, and the other which transfers to *N*-linked glycoproteins. While the most obvious explanation for the carboxylates in these Class I molecules would be the presence of uronic acids, the residual negative charge on desialylated molecules is completely resistant to the action of either β-glucuronidase and

α-iduronidase, with or without the addition of β-galactosidase, β-hexosaminidase, and α-galactosidase. Thus, it is unlikely that the putative carboxylates are part of uronic acid residues present at terminal positions, or at subterminal positions covered by Gal or GlcNAc residues. Of course, we cannot rule out the possibility that uronic acid residues are present at more internal positions and/or are blocking the action of the other glycosidases.

The Class II oligosaccharides detected in this study seem to be similar to the corresponding ones previously found in the CPAE cells and thus may be derived from lung endothelial cells. Although KS chains have been detected in association with *N*-linked structures in certain tissues (43, 44), HS and CS chains have been thought to be linked to proteins only via an *O*-xylosyl linkage. In our previous study (11), we showed that the CPAE cells did indeed contain HS and CS chains releasable by PNGaseF.[3] Here, we have extended this observation in bovine lung by demonstrating a reducing end *N*-acetylglucosamine and raising the possibility of multiple GAG chains on a single *N*-linked chain. Interestingly, although xylose was detected in the compositional analysis of these oligosaccharides, it is not at the reducing end, but resides somewhere within the structure. Previous reports have described the addition of a β1–2-linked xylose to the core β1–4 linked mannose residue in the *N*-linked oligosaccharides of horseradish peroxidase (45), laccase excreted by sycamore (46), miraculin from miracle fruit berries (47), and *Drosophila* brain (48). Thus, one can speculate that this xylose residue might convert the trimannosyl chitobiose core structure to a substrate for the conventional enzymatic extension of glycosaminoglycan chains. Another possibility is that the addition of glucuronosyl residues to the outer antennae of *N*-linked oligosaccharides might serve to prime GAG chain biosynthesis. Finally, since the action of PNGaseF requires only the presence of a chitobiose unit, one can speculate that the core region of Class II molecules might arise from a biosynthetic pathway distinct from that of classical *N*-linked chains. In this regard, it is interesting to note that earlier work on the biosynthesis of oligosaccharide dolichols reported the possibility of a chitobiose unit being transferred from GlcNAcβ1–4GlcNAc-P-P-dolichol to proteins *in vitro* (49). Also, we have recently reported unusual xylosides primed by 4-methylumbelliferyl-β-xyloside in melanoma cells, in which GlcA residues can be directly linked to Xyl; some of these molecules carry multiple Xyl residues, suggesting the possibility of branching (50).

To summarize, we have isolated a heterogeneous and complex population of anionically charged *N*-linked oligosaccharides from bovine lung. These sugar chains can be generally separated into two groups with unusual characteristics. The majority of the negative charge from the less negatively charged group (Class I) arises from carboxylic acids which are not associated with sialic acids and presumably may be uronic acids. These molecules are clearly distinct from the sulfated/sialylated "Class I" molecules previously reported in the pulmonary artery endothelial cells. The most highly charged structures (Class II) contain multiple glycosaminoglycan chains associated with a single *N*-linked core and have an unusual internal xylose residue.

These data also serve to emphasize the fact that the great majority of previously described *N*-linked oligosaccharides originate from a restricted subset of easily accessible glycoproteins of the blood plasma and blood cells or of recombinant proteins derived from cultured cell lines. The assumption that similar patterns will occur in other mammalian tissues is challenged by this study.

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[3] We previously reported similar chains in a human erythroleukemia cell line K562 (11). However, the cell line originally studied was lost, and we were subsequently unable to find such chains in the original K562 line obtained from the American Type Culture Collection. We are uncertain of the reasons for this discrepancy. Regardless, the presence of these chains in CPAE cells has been observed repeatedly since the original publication.

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