THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 18, Issue of June 25, pp. 8879–8889, 1988
Printed in U.S.A.

Sulfated *N*-Linked Oligosaccharides in Mammalian Cells

I. COMPLEX-TYPE CHAINS WITH SIALIC ACIDS AND *O*-SULFATE ESTERS*

(Received for publication, October 21, 1987)

Linda Roux‡, Sylvia Holojda‡, Goran Sundblad, Hudson H. Freeze§, and Ajit Varki¶

From the Division of Hematology-Oncology, Department of Medicine and the Cancer Biology Program, Cancer Center, University of California at San Diego, La Jolla, California 92093

The structures of sulfated *N*-linked oligosaccharides have been reported for a few specific proteins. We recently demonstrated that such oligosaccharides occur in many different types of tissue culture cell lines (Freeze, H. H., and Varki, A. (1986) *Biochem. Biophys. Res. Commun.* 140, 967–973). Here we report improved methods to metabolically label cell lines with $^{35}SO_4$ and to release sulfated *N*-linked oligosaccharides with peptide:*N*-glycosidase F as well as the partial structure of some of these novel oligosaccharides. The released $^{35}SO_4$-labeled chains from Chinese hamster ovary (CHO) cells and bovine pulmonary artery endothelial cells (CPAE) were characterized by gel filtration, anion exchange and lectin affinity chromatography, and various enzymatic and chemical treatments. Each cell line contains a class of sulfated oligosaccharide chains bearing from two to six negative charges in varying combinations of *O*-sulfate esters and sialic acids. These molecules represent a significant proportion of both the total $^{35}SO_4$ label and the total anionic *N*-linked oligosaccharides. They are also relatively enriched in a CHO mutant that is deficient in glycosaminoglycan chain synthesis. Lectin affinity chromatography of such molecules from CPAE cells indicates that the majority are sialylated multiantennary complex-type chains. The sulfate esters are exclusively of the primary type. Sequential exoglycosidase digestions, including β-hexosaminidase A treatment at low pH, demonstrate that at least one-third of these sulfate esters are found in the following structure,

$$\begin{array}{l} \qquad\qquad SO_4 \\ \qquad\qquad\quad | \,6 \\ SA\alpha 2\rightarrow(3)6Gal\beta 1\rightarrow 4GlcNAc\beta 1\rightarrow R \end{array}$$

where R is the remainder of the underlying oligosaccharide, and SA is sialic acid.

In addition to these molecules, a more highly charged group of sulfated *N*-linked oligosaccharides sharing structural features with glycosaminoglycans was found in CPAE cells, but not in CHO cells. These are described in the following paper (Sundblad, G., Holojda, S., Roux, L., Varki, A., and Freeze, H. H. (1988) *J. Biol. Chem.* 263, 8890–8896).

Various types of macromolecules are sulfated in mammalian cells. These include lipids (1), steroid sulfates (2), proteins (tyrosine *O*-sulfate) (3), proteoglycans (4), and glycoproteins. The sulfated oligosaccharides of glycoproteins can be bound to the peptide via either *O*-glycosidic (5–7) or *N*-glycosidic linkages. The presence of sulfate in *N*-linked oligosaccharides has been reported in several proteins including viral glycoproteins (8, 9), thyroid plasma membrane proteins (10), basement membrane components (11), the low density lipoprotein receptor (12), ovalbumin (13), pituitary hormones (14, 15), brain glycopeptides (16), and *D. discoideum* lysosomal enzymes (17–19). However, structural details of these sulfated oligosaccharides have been described only in some cases. Freeze and Wolgast (20) characterized sulfated high mannose chains from lysosomal enzymes secreted from *D. discoideum*. Most of the oligosaccharides were enzymatically released and contained both sulfate esters and phosphodiesters. The major sulfated monosaccharide was Man-6-SO_4.[1] Green *et al.* (21) have elucidated the structure of the sulfated *N*-linked oligosaccharides of bovine lutropin, in which one or two βGalNAc-4-SO_4 residues terminate an otherwise typical biantennary chain. In the case of ovalbumin, Man-4-SO_4 was found on hybrid-type oligosaccharides (13). GlcNAc-SO_4 residues have been reported in endothelial cells (22, 23) and in thyroid proteins (10).

Previous work from this laboratory has shown that sulfation of *N*-linked oligosaccharides is more widespread in eukaryotic cell lines than heretofore suspected (24). In this study we have improved the labeling, release, and recovery of such molecules from several mammalian cell lines. The released oligosaccharides consist of two general classes. The first class is described in detail in this report and the second in the next paper (25). A specific protein that carries oligosaccharides of both kinds is described in the following paper (26).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Choice of Cell Lines for Further Study—We used the approach described in the Miniprint Section to label, release,

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‡Postdoctoral trainee supported by Grant HL07107-12.

§ Recipient of an Established Investigatorship of the American Heart Association.

¶ Recipient of Senior Faculty Research Award FRA-295 from the American Cancer Society. To whom correspondence should be addressed.

[1] The abbreviations used are: Man, mannose; CPAE, bovine pulmonary artery endothelial cells; CHO, Chinese hamster ovary cells; CHO 761, Chinese hamster ovary mutant 761 cells; α-MEM, modified Eagle's medium α; SLIM, sulfate label incorporation medium; TM, tunicamycin; PNGaseF, peptide:*N*-glycosidase F; FCS, fetal calf serum; PBS, phosphate-buffered saline; ConA, concanavalin A; RCA-1, *Ricinus communis* agglutinin-1; L-PHA, phytohemagglutinin-L_4; endo, endo-β-*N*-acetylglucosaminidase; SA, sialic acid (type not determined); BSA, bovine serum albumin; AUN, *Arthrobacter ureafaciens* neuraminidase; NDVN, Newcastle disease virus neuraminidase; SDS, sodium dodecyl sulfate.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

and separate sulfated *N*-linked oligosaccharides synthesized by several mammalian cell lines. The specific release of $^{35}SO_4$ by PNGaseF ranged from 6 to 22%. The amount released varied between cell lines, and sometimes between different preparations from the same cell line. Although several cell lines were examined and showed similar findings, most of the results presented here are from Chinese hamster ovary (CHO) cells and bovine pulmonary aortic endothelial (CPAE) cells. The CHO cell line was chosen because of the availability of a mutant, CHO 761. This mutant is unable to synthesize glycosaminoglycan chains because of a deficiency in galactosyltransferase I (28). Since this mutation eliminates the major class of molecules that incorporate $^{35}SO_4$, the labeled mutant cells are a comparatively enriched source of $^{35}SO_4$-labeled sulfated glycoproteins. The endothelial cell line CPAE was chosen because a major portion of the $^{35}SO_4$ label was found in sulfated *N*-linked oligosaccharides.

Release and Fractionation of Labeled Oligosaccharides—The rationale for the release, separation, and fractionation of the sulfated *N*-linked oligosaccharides is described in the Miniprint Section and is outlined schematically in Fig. 3. Monolayer cultures were metabolically labeled under conditions optimal for $^{35}SO_4$ uptake with or without prior labeling with [^{3}H]Man. Labeled cells were lysed in buffer containing SDS and applied to a Sephacryl S-200 gel filtration column run in the presence of SDS. Labeled macromolecules eluting in the void volume region (hereafter called S200VoA) were recovered, treated with PNGaseF, and reapplied to the same gel filtration column. Released oligosaccharides are defined as those which appear in the included volume (S200R). To rule out nonspecific breakdown of macromolecules a control incubation was always carried out under identical conditions in the absence of added enzyme and similarly analyzed by gel filtration.

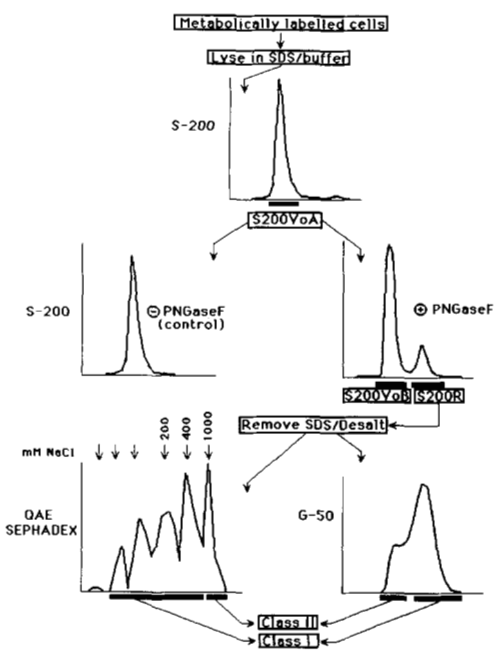


FIG. 3. **Schematic outline of methods for release and isolation of $^{35}SO_4$-labeled *N*-linked oligosaccharides.** Cells were labeled, harvested, lysed in buffer containing SDS, and applied to a Sephacryl S-200 column equilibrated in an SDS-containing buffer. Macromolecules (S200VoA) were recovered as indicated, precipitated with acetone, redissolved, incubated with or without PNGaseF, and chromatographed again on the same S-200 column. Fractions containing the released *N*-linked oligosaccharides (S200R) were precipitated with KCl to remove SDS, desalted, and then applied to either a QAE-Sephadex or a Sephadex G-50 column. The definition of Class I and Class II oligosaccharides based on QAE-Sephadex chromatography is found in the text; the corresponding regions of the Sephadex columns are indicated in the figure. Material appearing in the included volume from Sephadex G-50 contained Class I oligosaccharides and was used for further structural studies. For details see "Experimental Procedures."

Fig. 4 shows some examples of such analyses. S200VoA from the lysate of CPAE cells was a good source of PNGaseF-releasable material (21% $^{35}SO_4$ release, Fig. 4*A*). While the overall incorporation of $^{35}SO_4$ label into the mutant CHO cell line was substantially lower than that for the parent CHO line (28), PNGaseF released a greater proportion of $^{35}SO_4$ cpm from the mutant cells (24%, Fig. 4*C*) than from parent cells (7.5%, Fig. 4*B*). This is consistent with the fact that these mutant cells are genetically deficient in producing *O*-linked glycosaminoglycan chains at normal levels. Fig. 4 also shows the concurrent release of [^{3}H]Man-labeled material from these cell lines. PNGaseF cleaved 84, 62, and 76% of macromolecular ^{3}H from CPAE, CHO, and CHO 761 cells, respectively. In the BW 5147 lymphoma cells, 6% of the $^{35}SO_4$ label and 62% of the ^{3}H label appeared in the included volume after PNGaseF treatment (data not shown).

Substantially less radioactivity appeared in the included volume in the sham-treated controls (Fig. 4, *D–F*). This indicates that the great majority of the label in the included volume found with CPAE and CHO 761 cells was specifically released by PNGaseF. The results with the sham incubations also indicate that little or no endogenous proteases were active under the conditions used. This is not surprising since the extracts, and subsequently the isolated macromolecules, are extensively boiled in SDS prior to the incubations. The results also indicate that there was no significant loss of $^{35}SO_4$ label from either intact macromolecules or released oligosaccharides, since such free sulfate would have eluted near the fully included volume of these columns.

Effect of Tunicamycin on the Synthesis of Sulfated N-Linked Oligosaccharides—We have thus far defined sulfated *N*-linked oligosaccharides as $^{35}SO_4$-labeled molecules that are specifically released by digestion with PNGaseF. To obtain alternate

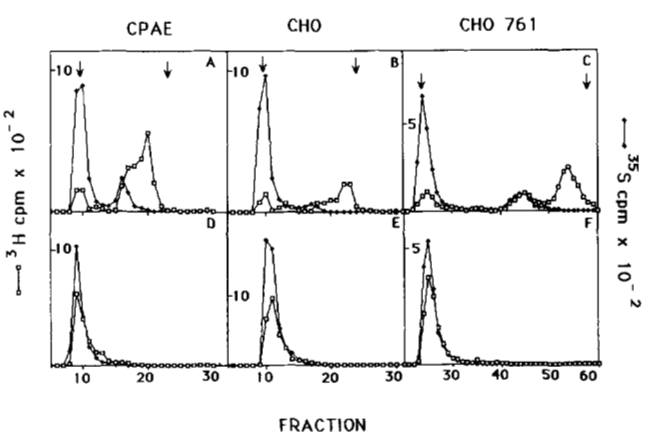


FIG. 4. **Sephacryl S-200 gel filtration analysis of CPAE, CHO, and CHO 761 macromolecules with or without PNGaseF treatment.** SDS lysates of cell lines metabolically labeled with [^{3}H]Man and $^{35}SO_4$ were fractionated by Sephacryl S-200 gel filtration (not shown). Macromolecules eluting in the void volume (S200VoA) were recovered, precipitated with acetone, and divided into two portions, which were incubated with or without PNGaseF. Treated samples (*A–C*) and control samples (*D–F*) were then rechromatographed on Sephacryl S-200, as described under "Experimental Procedures." A large (115 × 0.8 cm) Sephacryl S-200 column was used in the experiments shown in *panels C* and *F*. The elution positions of blue dextran and [^{14}C]fucitol are indicated by *arrows*.

evidence that these chains are bound to the peptide via *N*-glycosidic linkages, the effect of tunicamycin on their synthesis was investigated in CPAE cells. Tunicamycin inhibits the first step in the biosynthesis of *N*-linked oligosaccharides, the formation of GlcNAc-P-P-dolichol (39). Since tunicamycin can also inhibit protein synthesis in some cells, several tunicamycin concentrations were tried and metabolic labeling with [^{3}H]leucine was carried out in addition to labeling with $^{35}SO_4$. When the cells were grown in the presence of 3 μg/ml tunicamycin for 6 h, no significant change (<10%) was observed in the amount of [^{3}H]leucine incorporated into macromolecules, indicating that under these conditions tunicamycin did not greatly affect overall protein synthesis. However, as shown in Table I, tunicamycin treatment at this concentration substantially reduced the percent of $^{35}SO_4$ radioactivity released by PNGaseF (from 14 to 3%). These results provide further evidence that the $^{35}SO_4$ label specifically released by PNGaseF is on *N*-linked oligosaccharides.

Several other conclusions can be drawn from the experiments described in Table I. First, since <2% of [^{3}H]leucine appeared in the included volume after PNGaseF treatment, little or no proteolysis must have occurred. Thus, in addition to the lack of endogenous protease activity originating from the cells, it can be reaffirmed that the homogeneous PNGaseF preparation utilized is free of trace proteases under the conditions used. Second, we also compared $^{35}SO_4$ labeling in the sulfate label incorporation medium for 6 h with that in complete medium with cysteine, methionine, and inorganic sulfate. As expected, the overall incorporation of $^{35}SO_4$ label was less (2-fold) in the latter case. However, as shown in the table, there was no major change in the percent of PNGaseF-released $^{35}SO_4$. Thus, under the conditions used the sulfate-depleted medium did not grossly affect sulfation of these molecules. Of course qualitative changes cannot be ruled out; this issue is dealt with further under "Discussion."

Analysis of Sulfated N-Linked Oligosaccharides by Ion Exchange Chromatography—We next examined the behavior of the $^{35}SO_4$/[^{3}H]Man-labeled oligosaccharides from CHO 761 and CPAE cells on QAE-Sephadex ion exchange columns in a manner analogous to that previously described for phosphorylated *N*-linked oligosaccharides (31, 32). In both cell lines, almost all of the $^{35}SO_4$ bound and eluted with varying concentrations of NaCl (Fig. 5, *A* and *B*). The $^{35}S/^3H$ ratio increased substantially in the more highly charged oligosaccharides. The CPAE cells were enriched in very highly charged material eluting at 400 and 1000 mM NaCl.

The contribution of sialic acid to the total negative charge of these oligosaccharides was investigated by treatment with neuraminidase, followed by QAE-Sephadex analysis (Fig. 5, *C* and *D*). In both cases there is an obvious shift of [^{3}H]Man label toward neutrality or lesser charge. This indicates that much of the ^{3}H-labeled material contains sialic acid residues. However, neuraminidase treatment also caused a significant shift of much of the $^{35}SO_4$ label that had eluted with less than 400 mM NaCl. These $^{35}SO_4$-labeled oligosaccharides must, therefore, contain varying combinations of sialic acids and sulfate esters contributing to their negative charge. However, the CPAE $^{35}SO_4$-labeled oligosaccharides eluting with 400 and 1000 mM NaCl showed only a minimal shift with neuraminidase, indicating that sialic acids do not contribute a major proportion of the negative charge of these molecules.

Thus, the sulfated molecules under study can be operationally defined as belonging to two different classes. In both CPAE and CHO 761 cells there is a group of sulfated sialylated molecules which elute from QAE-Sephadex between 20 and 400 mM NaCl. These are defined as Class I molecules. The second, defined as Class II, includes very highly charged sulfated species which elute above 400 mM NaCl and do not shift significantly after neuraminidase treatment. There is some overlap between the two classes at the 400 mM NaCl elution step. The relative amount of material eluting at 400 mM NaCl also varied between preparations (see Fig. 10 for example). However, in general it can be said that the CHO 761 cells contain predominantly Class I, whereas CPAE cells contain both. The justification for this somewhat arbitrary classification is provided by the findings described in the accompanying paper (25), which indicate that Class II molecules share some structural features with glycosaminoglycans.

In separate experiments, we compared the elution profiles of these molecules from Sephadex G-50 columns. As demonstrated in Fig. 6, the Class I oligosaccharides from CPAE cells

TABLE I

Effect of tunicamycin and different media on the incorporation of [^{3}H]leucine and $^{35}SO_4$ into CPAE macromolecules and PNGaseF-released oligosaccharides

CPAE cells were cultured in replicate dishes in complete modified Eagle's medium α (α-MEM) until nearly confluent. Selected dishes were then incubated with tunicamycin for 0.5 h. Medium from all dishes was then removed and cells labeled with 10 μCi of [^{3}H]leucine and the indicated amounts of $^{35}SO_4$ (Experiment 1, 1.0 mCi; Experiments 2 and 3, 0.1 mCi) in modified Eagle's medium α or sulfate label incorporation medium (SLIM). Tunicamycin treatment was continued in those dishes which had been previously incubated with the drug. Cells were harvested, lysed, subjected to S-200 gel filtration, and the percentage of the isotopes appearing in the S200VoA from each experiment determined. This material (representing macromolecules) was then treated with PNGaseF and reapplied to the same gel filtration column. The percent of total radioactivity released by PNGaseF (S200R) was then determined. Only selected data are presented in this table for the 3 μg/ml tunicamycin concentration. See "Experimental Procedures" for details.

Experiment	Media	Tunicamycin	% of total cpm in			
			S200VoA		S200R	
			^{3}H	^{35}S	^{3}H	^{35}S
1	α-MEM		94	76	2	10
2	SLIM		94	82	1	14
3	SLIM	3 μg/ml	92	86	1	3

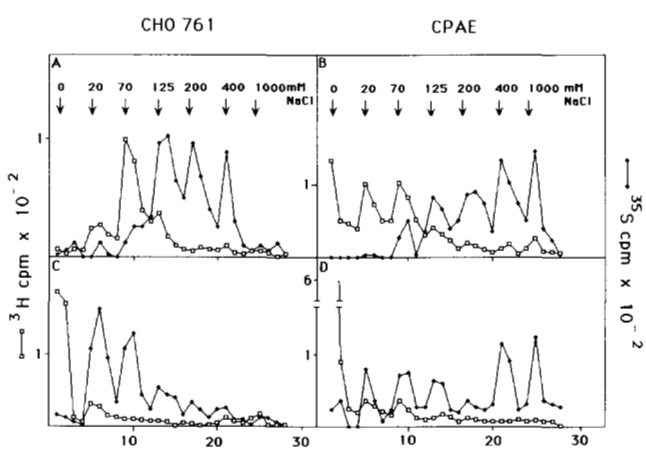


FIG. 5. **QAE-Sephadex anion exchange chromatography of oligosaccharides with and without neuraminidase treatment.** CPAE or CHO 761 cells were metabolically labeled with [^{3}H]Man and $^{35}SO_4$, and oligosaccharides were released and desalted as described under "Experimental Procedures." A portion of the oligosaccharides was incubated with AUN. Untreated and treated samples were then applied to QAE-Sephadex ion exchange columns and eluted with stepwise increases in NaCl concentration, as described under "Experimental Procedures."

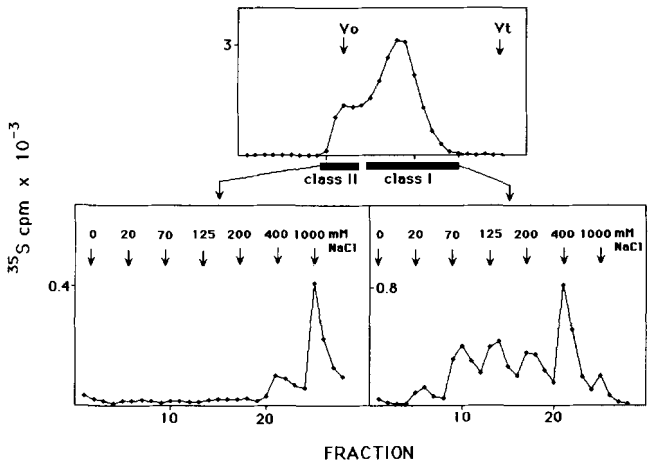


FIG. 6. **Sequential chromatography of CPAE oligosaccharides on Sephadex G-50 and QAE-Sephadex.** Oligosaccharides were prepared from $^{35}SO_4$-labeled CPAE cells, desalted, and chromatographed on a Sephadex G-50 gel filtration column equilibrated with 15 mM Tris/HCl, pH 6.5, as described under "Experimental Procedures." Fractions representing the void volume and those in the included volume were pooled separately, desalted, and subjected to QAE-Sephadex ion exchange chromatography (for details see "Experimental Procedures").

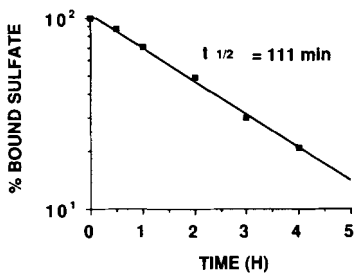


FIG. 7. **Kinetics of acid hydrolysis of $^{35}SO_4$ from CPAE Class I oligosaccharides.** $^{35}SO_4$-Labeled Class I oligosaccharides were hydrolyzed in 0.25 N HCl at 100 °C for designated periods of time and then studied by barium precipitation. The percent of $^{35}SO_4$ radioactivity remaining bound to saccharides after hydrolysis was calculated by dividing the radioactivity remaining barium-soluble by the total soluble radioactivity without acid hydrolysis.

eluted predominantly in the included volume of the column, whereas the more highly charged Class II molecules eluted predominantly in the void volume region. Again, the separation is not complete but sufficient to allow fractionation for further study. The rest of the work reported in this paper describes the properties of Class I molecules which are isolated either by QAE-Sephadex (20–400 mM NaCl elution) or by Sephadex G-50 gel filtration in the absence of SDS (included volume). The characterization of Class II molecules are described in the accompanying paper (25).

Types of Sulfate Esters Present on CPAE Class I Oligosaccharides—Nitrous acid deamination, which specifically cleaves *N*-sulfate esters, did not release any of the $^{35}SO_4$ label from these oligosaccharides. Mild acid hydrolysis sufficient to remove *N*-sulfate esters also did not release any $^{35}SO_4$. However, all of the $^{35}SO_4$ label could be released as free barium-precipitable radioactivity by acid hydrolysis under conditions that release *O*-sulfate esters. Fig. 7 shows the time course for conversion of oligosaccharide-bound sulfate to barium-precipitable sulfate in CPAE Class I sulfated oligosaccharides. The monophasic nature of the kinetic curve as well as the $t_{1/2}$ (111 min) indicate a single class of primary sulfate groups which are in ester linkage to the oxygen of C6 (40). The identification of the sulfated monosaccharide is discussed below.

Distribution of Negative Charges on CPAE Class I Oligosaccharides—The $^{35}SO_4$-labeled Class I molecules should be admixed with anionic molecules carrying various combinations of other negative residues such as sialic acids and phosphate groups. Following neuraminidase treatment of $^{35}SO_4$/[^{3}H] Man-labeled oligosaccharides, 33% of the ^{3}H label remained bound to the QAE-Sephadex columns (Fig. 5). However, since some of this label could be in fucose and since [^{3}H]Man label is not evenly distributed among different types of oligosaccharides, it was difficult to interpret this data further.

To better quantify the relative ratios of the various anionic molecules we, therefore, prepared oligosaccharides metabolically labeled with only $^{35}SO_4$ and introduced a ^{3}H label into the reducing termini of the oligosaccharides by treatment with [^{3}H]$NaBH_4$ (see "Experimental Procedures" for details). Such double-labeled Class I oligosaccharides were then subjected to various treatments such as mild acid, alkaline phosphatase, neuraminidase, dimethyl sulfoxide solvolysis, or a combination of these procedures and applied to QAE-Sephadex columns. Table II shows the effects of these treatments upon the increase of the ^{3}H label in the run-through (neutral) fraction from QAE-Sephadex.

The contribution to total negative charge by each of the various anions can be inferred from the amount of ^{3}H label rendered neutral after removal of that group. Initially, 28% of ^{3}H label from intact untreated oligosaccharides was neutral. After treatment with mild acid (which cleaves sialic acids and "uncovers" phosphodiesters), this percentage increased to 78%. Somewhat less release was obtained with neuraminidase (65% ^{3}H appeared in the neutral region). This could be explained by the presence of neuraminidase-resistant sialic acids (*e.g.* unusual linkages or modifications). Treatment of total oligosaccharides with alkaline phosphatase did not greatly increase ^{3}H in the neutral fraction (28–33%), indicating that approximately 5% of total ^{3}H-labeled oligosaccharides contained phosphomonoesters only. Because phosphate groups may also occur as mild acid-sensitive phosphodiesters with outer GlcNAc (32) or Glc (41) residues, another aliquot was treated with mild acid followed by alkaline phosphatase. The result obtained after these treatments was not different from that observed with mild acid alone (78–79%), indicating that only 1% or less of total ^{3}H-labeled oligosaccharides contain diesterified phosphate groups.

Solvolysis of pyridinium salts of sulfated oligosaccharides in dimethyl sulfoxide has previously been shown to release sulfate residues without affecting the underlying high mannose oligosaccharide structure (18). As shown in Table II, we have found that under the conditions used, this procedure also removes sialic acids. After solvolysis, all of the $^{35}SO_4$ label was released from the oligosaccharides (data not shown) and 90% of total ^{3}H label appeared in the neutral fraction from QAE-Sephadex column, indicating that 62% (90% − 28%) of total ^{3}H-labeled molecules contained sulfate and/or sialic acid. Since the fraction of total oligosaccharides containing sialic acid alone has already been determined to be 50%, the proportion of oligosaccharides bearing sulfate alone is 12% (62% − 50%). The remaining ^{3}H-labeled molecules that still bind to QAE-Sephadex comprise 10% or less of the total ^{3}H-labeled oligosaccharides and remain unidentified.

The proportion of total negative charge contributed by each anion was calculated as described in the legend to Table II. Sialic acid constituted 69% of overall negative charge, based on the result with mild acid. Sulfate accounted for 17% of total negative charge after subtraction of the sialic acid content value from the solvolysis value (86% − 69%). Only 7% of total negative charge was contributed by phosphomonoes-

TABLE II

QAE-Sephadex anion exchange chromatography of PNGaseF-released CPAE oligosaccharides after removal of various anions

$^{35}SO_4$-Labeled oligosaccharides were released from CPAE cells by PNGaseF, reduced with [^{3}H]$NaBH_4$, and desalted as described under "Experimental Procedures." Class I oligosaccharides were then fractionated on QAE-Sephadex with no further treatment or after the chemical or enzymatic treatments indicated. The amount of radioactivity eluting in the run-through (neutral) fractions from QAE-Sephadex was expressed as the percent of total recovered radioactivity. *a*, percent of total [^{3}H]oligosaccharides neutralized = percent of total ^{3}H cpm in run-through from treated sample minus percent of total ^{3}H cpm in run-through from control (untreated) sample (*b*). *c*, percent of total anionic oligosaccharides neutralized = $a/100 - b$.

Treatment	Anion removed	% of total [^{3}H]oligosaccharides		% of anionic oligosaccharides neutralized[c]
		QAE run-through	neutralized[a]	
None		28[b]		0
Mild acid	Sialic acids	78	50	69
Alkaline phosphatase	Phosphomonoesters	33	5	7
Mild acid/alkaline phosphatase	Phosphomonoesters, phosphodiesters, sialic acids	79	51	71
Neuraminidase (AUN)	Sialic acids	65	37	51
Solvolysis	Sulfate esters, sialic acids	90	62	86

ters, and a negligible amount of total negative charges was due to phosphodiesters. These rough estimations do not of course take into account the overlap caused by mixed molecules that contain sialic acids and phosphate groups (described in Ref. 32) nor those with sialic acids and sulfate esters (described above).

We next attempted to fractionate the mixture of $^{35}SO_4$-labeled oligosaccharides by gradient elution from QAE-Sephadex (31) or high pressure liquid chromatography anion exchange columns (36). However, it was not possible to identify and cleanly separate a single major labeled species from this highly complex mixture of sulfated sialylated molecules. We, therefore, chose to continue the structural analysis on the mixture of Class I oligosaccharides.

Lectin Affinity Chromatography of Class I Oligosaccharides—Unreduced CPAE Class I oligosaccharides labeled in $^{35}SO_4$ alone were obtained as shown in Fig. 6, desalted, and analyzed by affinity chromatography on immobilized concanavalin A (ConA), *Ricinus communis* agglutinin-1 (RCA-1), and phytohemagglutinin-L_4 (L-PHA) lectins following established procedures (40).

Very little $^{35}SO_4$-labeled material (1.6%) was specifically bound to and eluted from ConA-Sepharose with either 10 mM α-Me-Glc or 100 mM α-Me-Man (not shown). This indicates that CPAE cells contain few sulfated biantennary or high mannose chains. The failure of endo H to release $^{35}SO_4$ from CPAE macromolecules (data not shown) is also consistent with the lack of sulfated high mannose chains. Of course, sulfate residues could prevent the binding of such underlying oligosaccharides to the lectin or the action of the enzyme.

In contrast to the behavior of $^{35}SO_4$-labeled CPAE Class I oligosaccharides on ConA-Sepharose, a substantial portion of these molecules was retarded by L-PHA-agarose (Fig. 8, *top panel*). This lectin specifically retards complex-type chains with outer lactosamine units, particularly those containing a mannose substituted at C-2 and C-6 by Galβ1→4GlcNAc (33, 34, 42). Free [^{3}H]Man was included as an internal marker for nonretarded material. The positions of nonretarded and retarded oligosaccharide standards from BW 5147 cells (34) observed in a separate run are indicated by the *arrows*. Forty-three percent of the radioactivity did not bind to L-PHA-agarose (region A); 30% was retarded (region B); and 27% was markedly retarded (region C). Interestingly, the molecules in region C eluted later than even the most strongly retarded BW 5147 standards, suggesting an unusually high affinity of

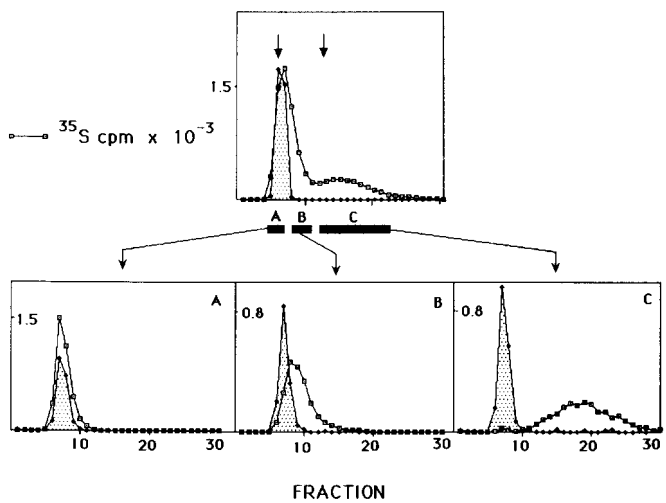


FIG. 8. **L-PHA-agarose affinity chromatography of CPAE Class I oligosaccharides.** $^{35}SO_4$-Labeled oligosaccharides were prepared from CPAE cells, fractionated on Sephadex G-50 in 15 mM Tris/HCl, pH 6.5, and the Class I oligosaccharides retrieved from the included volume as described under "Experimental Procedures" (see also Figs. 3 and 6). Oligosaccharides were mixed with [^{3}H]Man as an internal marker of unbound material and chromatographed on L-PHA-agarose as described under "Experimental Procedures." The column had been previously calibrated (*arrows*) with non-ConA-binding pea lectin-binding [^{3}H]Man-labeled BW 5147 oligosaccharides (34, 35).

region C sulfated oligosaccharides for L-PHA.

The three regions were separately pooled, desalted, mixed with the free [^{3}H]Man marker, and reapplied to the same L-PHA-agarose column. All three pooled regions reproducibly eluted at their original positions (Fig. 8, *A–C*), confirming their respective binding properties. In addition, removal of sialic acids (with mild acid treatment) did not significantly alter binding to L-PHA-agarose (data not shown), as has been reported recently for some reduced oligosaccharides (42). Taken together, these results indicate that at least 57% (regions B and C) of the sulfated CPAE Class I oligosaccharides are complex-type multiantennary chains.

Fig. 9 illustrates the fractionation of sulfated CPAE Class I oligosaccharides on RCA-1 agarose, which binds oligosaccharides and glycopeptides bearing terminal β-galactose residues. The positions of standard IgG glycopeptides (35) containing 0–3 terminal galactose residues are indicated by

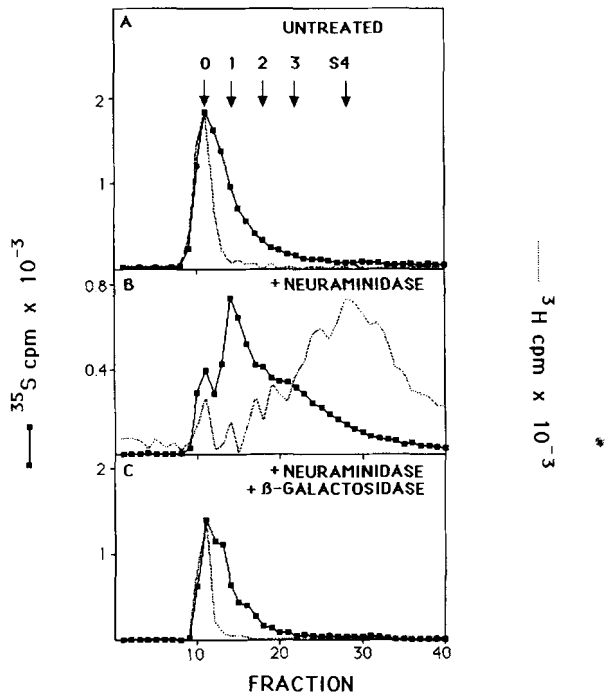


FIG. 9. **RCA-1 agarose affinity chromatography of CPAE Class I oligosaccharides.** Unfractionated $^{35}SO_4$-labeled CPAE Class I oligosaccharides prepared as described in the legend to Fig. 8 were mixed with the [^{3}H]$NaBH_4$-labeled fully sialylated tetra-antennary complex chain from orosomucoid (*S4*) and chromatographed on RCA-1 agarose (see "Experimental Procedures") with or without prior treatment with AUN or AUN plus β-galactosidase. The *arrows* indicate the positions of IgG glycopeptides containing 0–3 terminal galactose residues, as well as the position of desialylated S4 containing 4 terminal galactose residues.

arrows. The fully sialylated tetraantennary chain from orosomucoid labeled at the reducing terminus and designated S4 (36) was included as an internal standard (indicated by *arrow*). Intact ^{3}H-labeled S4 and most of the $^{35}SO_4$-labeled CPAE oligosaccharides are not retarded by RCA-1 agarose. The remainder of the CPAE oligosaccharides (36%) eluted in the region expected for one terminal galactose residue (Fig. 9*A*). However, after treatment with neuraminidase, which exposes subterminal galactose residues, virtually all of the S4 standard and most of the CPAE oligosaccharides (87%) are retarded by the lectin (Fig. 9*B*). The peak of desialylated S4 appears at the position expected for four exposed galactose residues. The CPAE oligosaccharides appear as a heterogeneous mixture of chains with 0–4 terminal galactose residues.

In order to confirm that the sulfated desialylated oligosaccharides bound to RCA-1 agarose via galactose residues, intact S4 and CPAE oligosaccharides were treated with both neuraminidase and β-galactosidase and reapplied to RCA-1 agarose (Fig. 9*C*). The removal of galactose residues by β-galactosidase completely abolished the interaction of S4 and of most of the CPAE oligosaccharides. These results suggest that the Class I CPAE oligosaccharides contain up to three or four antennae, with the majority of outer chain galactose residues being sialylated. It also appears that some of the terminal β-galactose residues remain resistant to β-galactosidase under conditions where the internal standard (desialylated S_4) is completely sensitive.

Taken together, the results from lectin affinity chromatography indicate that sulfated CPAE Class I oligosaccharides are predominantly tri- and tetra-antennary chains that are extensively sialylated and contain subterminal β-galactose residues. Since sulfate esters could be interfering with the binding of lectins to the underlying oligosaccharide, all of these results probably represent the minimal values for such binding.

Determination of Sialic Acid Glycosidic Linkage—In order to determine if sialylation of sulfated Class I oligosaccharides preferentially occurs via α2→3 or α2→6 linkages to β-galactose, oligosaccharides metabolically labeled with [^{3}H]Man and $^{35}SO_4$ were treated with mild acid, *Arthrobacter ureafaciens* neuraminidase (AUN), or Newcastle disease virus neuraminidase (NDVN), and then analyzed by QAE-Sephadex anion exchange chromatography. NDVN specifically cleaves α2→3 linkages (43), whereas AUN cleaves both α2→3 and α2→6 linkages (44). In the experiment illustrated in Fig. 10, AUN is as effective as mild acid in the removal of terminal sialic acids from both [^{3}H]Man- and $^{35}SO_4$-labeled oligosaccharides. In contrast, NDVN was active only toward [^{3}H]Man-labeled oligosaccharides and showed little effect on $^{35}SO_4$-labeled chains, compared to the control sample. If we assume that (i) mild acid cleaves all possible SA→βGal linkages, (ii) that there is no differential effect of sulfate on the two neuraminidases, and (iii) that α2→3 and α2→6 are the only SA linkages present in these oligosaccharides, then we can conclude that sulfated Class I oligosaccharides predominantly contain terminal sialic acid residues in α2→6 linkage to β-galactose. Of course, as has been noted not all the sulfated Class I oligosaccharides are sialylated. Other complexities in interpreting this data are dealt with under "Discussion."

Identification of the Sulfated Monosaccharide—Intact $^{35}SO_4$-labeled Class I oligosaccharides were treated with various combinations of mild acid, β-galactosidase, and β-hex-

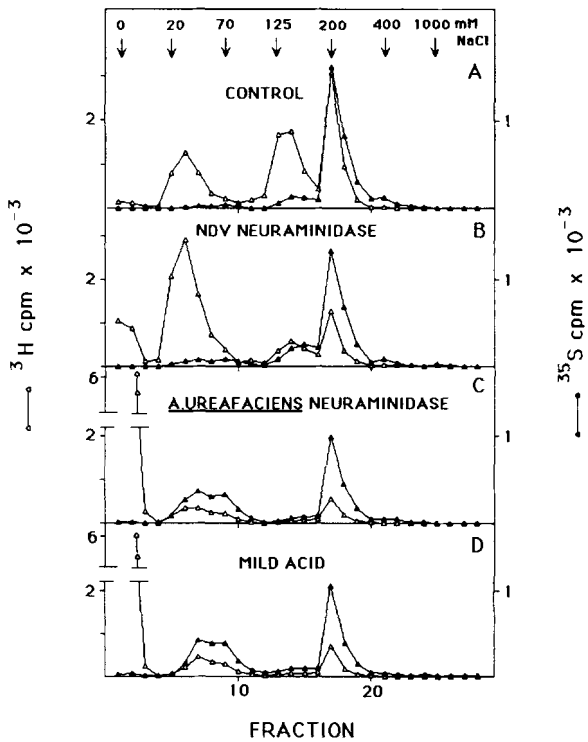
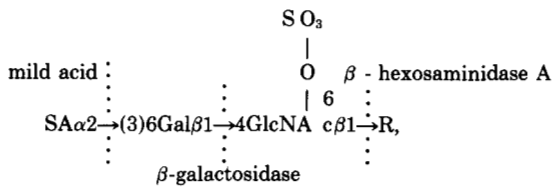


FIG. 10. **Effect of treatment with neuraminidases or mild acid on QAE-Sephadex anion exchange chromatography of double-labeled CPAE Class I oligosaccharides.** Class I oligosaccharides from [^{3}H]Man- and $^{35}SO_4$-labeled CPAE cells were depleted of SDS, desalted, and dried, and aliquots were treated with mild acid, Newcastle disease virus (*NDV*) neuraminidase, or *A. ureafaciens* neuraminidase as described under "Experimental Procedures." Samples were then subjected to QAE-Sephadex anion exchange chromatography.

osaminidase A. Mild acid was used to ensure complete release of sialic acids. No free sulfate was released under these conditions (data not shown). The β-hexosaminidase A was used at low pH, since it has been reported to cleave intact GlcNAc-6-SO_4 residues from oligosaccharides under these conditions (45). Treated or control samples were then analyzed by gel filtration on Sephadex G-25 in 0.1 M acetic acid.

Fig. 11 shows chromatograms from some of these experiments. Untreated intact sulfated oligosaccharides (Fig. 11*A*) elute in the void volume, indicated by the *first arrow*. Sequential treatment of intact oligosaccharides with mild acid, β-galactosidase, and β-hexosaminidase A at low pH (Fig. 11*B*) generates an included peak which elutes at the position of authentic glucitol-6-SO_4 (*second arrow*). This peak must represent GlcNAc-6-SO_4, since little $^{35}SO_4$ radioactivity is detected in the comparable region without β-hexosaminidase A. The complete results from this series of experiments are shown in Table III. It can be seen that specific release of sulfated monosaccharide requires treatment with mild acid, β-galactosidase, and β-hexosaminidase A. This release represents more than one-third of total $^{35}SO_4$ label. The results indicate that it is necessary to first remove terminal sialic acid and then β-galactose in order for GlcNAc-6-SO_4 to become accessible to β-hexosaminidase A.

These results are consistent with the following structure for some of the antennae on these oligosaccharides.



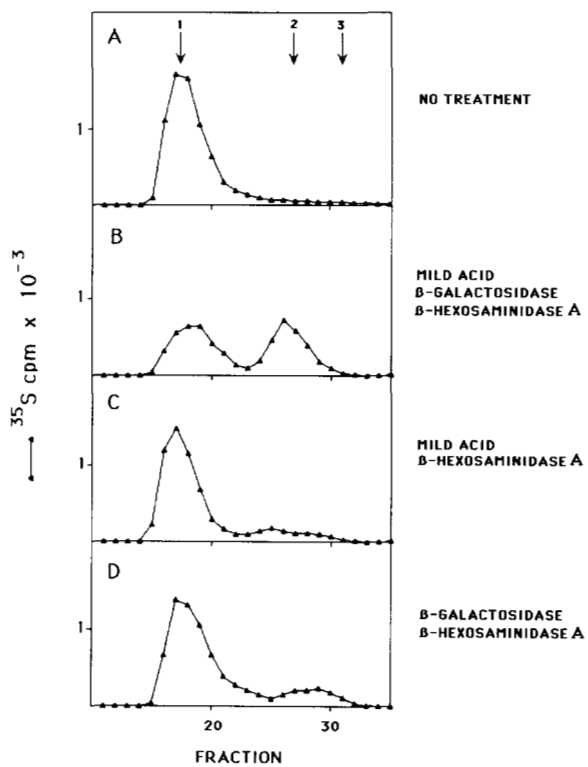


FIG. 11. **Effect of treatment of CPAE Class I oligosaccharides with mild acid, β-galactosidase, and β-hexosaminidase.** Intact $^{35}SO_4$-labeled oligosaccharides, which had been depleted of SDS and desalted, were treated with various combinations of mild acid, β-galactosidase, and β-hexosaminidase at low pH (see "Experimental Procedures" for details) or with none of these reagents (control). Samples were then subjected to gel filtration on Sephadex G-25 in 0.1 M acetic acid. *Arrows* indicate the elution positions of: *1*, intact $^{35}SO_4$- and [^{3}H]Man-labeled CPAE Class I oligosaccharides; *2*, [^{3}H]glucitol-6-SO_4; and *3*, [^{3}H]Man. Complete results from this series of experiments are shown in Table III.

TABLE III

Effect of sequential mild acid, β-galactosidase, and β-hexosaminidase A treatments on Sephadex G-25 gel filtration of CPAE Class I oligosaccharides

Intact $^{35}SO_4$-labeled Class I oligosaccharides, which had been depleted of SDS and desalted, were treated with various combinations of sequential mild acid, β-galactosidase, and β-hexosaminidase A at low pH (see "Experimental Procedures" for details). Samples were then subjected to gel filtration on Sephadex G-25 in 0.1 M acetic acid. The percentage of radioactivity appearing in the included volume at the elution position of [^{3}H]glucitol-6-SO_4 is indicated as percent released. The true negative control (*a*) was not sham-incubated. All other samples which lacked one or more of the treatments were sham-incubated.

Treatment			% released
Mild acid	β-Galactosidase	β-Hexosaminidase A (at low pH)	
−	−	−	6[a]
−	+	−	9
−	−	+	8
−	+	+	10
+	−	−	7
+	+	−	6
+	−	+	9
+	+	+	44

Digestion with Other Enzymes—No low molecular weight labeled material was released by heparinase, heparitinase, or chondroitinase ABC, indicating the absence of heparin, chondroitin, or dermatan sulfate chains (data not shown). Endo-β-galactosidase also did not release low molecular weight material, indicating that extended type 2 chains (Galβ1-4GlcNAc)$_n$ with occasional GlcNAc-6-SO_4 residues were not present.

DISCUSSION

We have used an approach that allows the release and isolation of *radiochemically* pure $^{35}SO_4$-labeled *N*-linked oligosaccharides from cells. However, it is important to discuss artifacts that could provide alternate explanations for the apparently released label. The study of *N*- or *O*-linked oligosaccharide-bound sulfate in cells by $^{35}SO_4$ labeling is complicated by the overwhelming excess of incorporation of the label into glycosaminoglycans and the presence of $^{35}SO_4$ label in proteins in the form of sulfotyrosine. The following criteria verify that the sulfated molecules we are studying are *N*-linked oligosaccharides and not fragments of $^{35}SO_4$-labeled glycosaminoglycans or proteolytic products of $^{35}SO_4$-labeled proteins. (*a*) We have used homogeneous protease-free PNGaseF, which specifically cleaves the GlcNAc-Asn bond of *N*-linked oligosaccharides (30), under conditions where maximal release of [^{3}H]Man label (which is predominantly in *N*-linked oligosaccharides) occurs. (*b*) Endogenous proteases from the cellular material should have been inactivated by the repeated boiling in SDS but could theoretically still be present in trace amounts during the incubation. Alternatively, nonspecific chemical breakdown of ^{35}S-labeled glycosaminoglycan chains could also occur during the incubation. To deal with these possibilities, identical sham incubations (without

PNGaseF) were performed in every case. These showed little (1–2% or less) of the radioactivity eluting in the region where released material would be expected to appear. However, in cell lines from which a small fraction (6% or less) of the $^{35}SO_4$ label is released by PNGaseF, such trace contaminants could represent a significant proportion of the "released material." We, therefore, confined our detailed studies to cell lines in which >10% of the $^{35}SO_4$ label was specifically released by PNGaseF. (*c*) If a trace of undetected protease is still present in the PNGaseF preparation itself, nonspecific release could occur during the enzyme incubation which would not be seen in the control. However, the absence of significant degradation of whole cell [^{3}H]leucine label in the presence of PNGaseF indicates that this cannot be occurring. (*d*) Tunicamycin, a drug which inhibits *N*-linked glycosylation, greatly reduced the amount of $^{35}SO_4$ incorporated in these molecules, under conditions where there was no significant inhibition of protein synthesis (Table I). (*e*) Esko *et al.* (28) have described mutant lines of CHO cells which do not make sulfated glycosaminoglycan chains due to defects in the synthesis of the core region of the chains. In one of these mutants (CHO 761), we found that while $^{35}SO_4$ incorporation was markedly reduced, the proportion of $^{35}SO_4$ label released as *N*-linked oligosaccharides was substantially increased compared to that of the parent. It is instructive to note that in the parent CHO cell line, the signal to noise ratio (specific release over nonspecific background) is such that it is difficult to confidently identify the sulfated *N*-linked oligosaccharides over the predominant background of $^{35}SO_4$-labeled glycosaminoglycan chains. (*f*) The majority of the sulfated oligosaccharides we studied showed several other characteristics of complex-type *N*-linked oligosaccharides such as the presence of terminal sialic acid residues on subterminal β-galactoside residues and specific retardation by the L-PHA lectin. Taken together, these data confirm that the $^{35}SO_4$-labeled molecules that we have studied are indeed *N*-linked oligosaccharides and are not nonspecific breakdown products of other $^{35}SO_4$-labeled macromolecules.

During the course of these investigations of sulfated oligosaccharides, we have also improved our previous methods (24) for the labeling and isolation of these molecules. The use of homogeneous PNGaseF rather than a mixture of two enzymes (PNGaseF/endo F) is desirable because this glycosidase has a wider substrate specificity than endo F and because it cleaves at the GlcNAc/Asn bond rather than within the chitobiosyl linkage (30). We have also found that gel filtration with Sephacryl S-200 rather than Sephadex G-50 allows the display of released species not seen with the latter column. For unknown reasons, the released oligosaccharides also tend to appear in two major peaks on the S-200 column, with the first containing almost all of the anionic oligosaccharides grouped together. This allows the convenient isolation of this group of molecules, which can then be fractionated by other means. The structural integrity of the sulfated oligosaccharides was also monitored throughout their isolation and characterization. None of the procedures generated free sulfate, as determined by the absence of ^{35}S label in appropriate regions of the gel filtration columns and the absence of barium-precipitable $^{35}SO_4$ radioactivity.

We also found that labeling in cysteine-free sulfate-free low methionine medium has the advantage of increasing the total amount of radiolabeled material. However, it did not adversely affect the level of protein synthesis nor the proportion of the sulfate label which was released by the PNGaseF digestion during a 6-h labeling period. Recently, several workers have shown that lowering the exogenous sulfate concentration may alter the composition and the degree of sulfation of some macromolecules (46, 47). Thus, we cannot rule out *qualitative* alterations in the types of sulfated oligosaccharides produced under the different labeling conditions.

The identity of the $^{35}SO_4$ radioactivity remaining in void volume material after treatment with PNGaseF (S200VoB) is unknown but should include glycosaminoglycans, sulfolipids, sulfated *O*-linked oligosaccharides, or tyrosine-sulfate residues of proteins. However, it is also possible that they include sulfated *N*-linked oligosaccharides of unknown structure that are resistant to PNGaseF or are so large that they still run in this region after release from proteins.

Our structural studies of the sulfated CPAE Class I oligosaccharides indicate that these molecules bear *O*-sulfate monoesters on otherwise typical complex-type oligosaccharide chains with sialic acids at the nonreducing termini. The exact number of negative charges cannot be ascertained in each case, because it may not be appropriate to extrapolate data on QAE-Sephadex elution previously obtained with phosphorylated high mannose-type chains (32) to these larger complex-type molecules. However, taken together, the results indicate that these molecules may carry from two to six negative charges that can be contributed to by any combination of sialic acids and sulfate esters.

Another structural feature that these chains share with typical complex-type *N*-linked chains is the ability to interact with certain lectins. Since Galβ1-3GlcNAc residues bind very poorly to RCA-1-agarose,[3] the lectin affinity studies indicate that most, if not all, of the galactose residues are in β1-4 linkage. The L-PHA binding indicates the presence of the Galβ1-4GlcNAcβ-Man sequence and the likelihood of the Galβ1-4GlcNAcβ1-6Man sequence and/or bissecting GlcNAc residues on the β-linked mannose (33, 34, 42). Both the terminal (sialic acid) and subterminal (β-galactose) monosaccharides typical of complex chains are present on these molecules. Treatment with neuraminidases suggests that while the total oligosaccharides contain sialic acids in both α2→3 and α2→6 linkages, the sulfated chains are highly enriched in α2→6-linked residues. However, these data are inconsistent with the recent report of Green and Baenziger (42) that L-PHA-agarose cannot bind oligosaccharides completely substituted with α2→6-linked sialic acids. It is possible that their data obtained with *reduced* oligosaccharides cannot be extrapolated to the *unreduced* oligosaccharides studied here. An alternate explanation is that the sulfate esters selectively inhibit NDVN and not AUN. A third possibility is that there are in fact several terminal β-galactoside residues which mediate binding to L-PHA, but whose binding to RCA-1 is selectively affected by sulfate esters.

Identification of the sulfated monosaccharide(s) was based on the following empirical and theoretical considerations. The acid hydrolysis kinetics indicated that the monosaccharide(s) was sulfated on a primary hydroxyl group. SA and Gal were unlikely candidates because they were susceptible to conventional enzymatic cleavage. In those tri- or tetra-antennary chains which bind to L-PHA-agarose, only one α-linked Man would have C6 free for sulfation; however, each oligosaccharide must accommodate from one to four sulfate esters. Thus, GlcNAc-6-SO_4 residues seemed the most probable sulfated monosaccharide. The sequential removal of SA, Gal, and GlcNAc-6-SO_4 confirmed this hypothesis for at least a third of the sulfate esters. Since the β-galactosidase appears to be inhibited to some extent by neighboring sulfate residues the estimated percentage of sulfate in GlcNAc-6-SO_4 is a minimal one. The possibility that sulfate is also esterified to C6 of an

[3] R. Cummings, personal communication.

α-mannose or to C6 of core GlcNAc residues cannot be excluded at this time.

Previous structural analysis of phosphorylated oligosaccharides was greatly aided by the availability of a nonspecific alkaline phosphatase (31, 32). Unfortunately a corresponding *nonspecific* oligosaccharide sulfatase is not available. On the other hand, sulfate esters will block the activity of most exoglycosidases. We have taken advantage of the fact that β-hexosaminidase A (at low pH) is an exception to this rule (45). We did observe (data not shown) that different batches of β-hexosaminidase A were variable in this activity. This is not surprising, since it has been shown recently that the active site for cleavage of β-GlcNAc residues may be different from that which cleaves β-GlcNAc-6-SO_4 residues (48).

Since both β-galactosidase and β-hexosaminidase A were present together in the digestions, we cannot completely rule out the presence of repeating Galβ1-4GlcNAc-6-SO_4 units found in undersulfated keratan sulfate chains. However, the number of such sulfated repeating units cannot exceed the number of sulfate esters (up to 4) present on each oligosaccharide. On the other hand, the presence of an occasional GlcNAc-6-SO_4 residue on an extended type 2 chain (Galβ1-4GlcNAc)$_n$ is also unlikely, since endo-β-galactosidase did not release low molecular weight ^{35}S-labeled material. Thus, while these molecules share some structural features with keratan sulfate type I (4), they are distinct in being much shorter, in having terminal sialic acid residues on almost every antenna, and in being at least partly attached to tri- or tetra-antennary oligosaccharides. Hiefetz and others (23) reported the occurrence of sulfated GlcNAc residues in the core region of certain *N*-linked glycopeptides in endothelial cells. Although we do not know if this type of sulfation is also present in the oligosaccharides reported here, sulfated core GlcNAc residues alone could obviously not account for 4 or more sulfate esters on a single oligosaccharide.

Anionic modifications of *N*-linked oligosaccharides, such as sialylation, phosphorylation, and sulfation, are of interest because it is possible that they, and not the underlying oligosaccharide structures, confer biological specificity or function to some *N*-linked carbohydrates. An example of this is the Man-6-PO_4 residue of lysosomal enzymes which is responsible for the targeting of these glycoproteins to lysosomes (49–51). It is interesting to note that these biologically critical molecules represent a much smaller percentage of the total oligosaccharides in the endothelial cells than do the sulfated *N*-linked oligosaccharides. Thus, the sulfated molecules described in this and the accompanying papers (25, 26) are good candidates for similar functional studies. Ultimately it is necessary to obtain larger amounts of these molecules, fractionate the individual species, and determine their structure by physical methods such as fast atom bombardment-mass spectrometry and NMR. Such detailed analysis of the structure of these oligosaccharides and the identification of the proteins to which they are bound will be the subjects of our future studies.

Acknowledgments—We acknowledge the help of Tien Vu and Sandra Diaz with some of the experiments, and the excellent secretarial assistance of Keia Simpson and Buck Williamson.

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SUPPLEMENTARY MATERIAL TO

SULFATED N-LINKED OLIGOSACCHARIDES IN MAMMALIAN CELLS I : COMPLEX-TYPE CHAINS WITH SIALIC ACIDS AND O-SULFATE ESTERS

Linda Roux, Sylvia Holojda, Goran Sundblad,

Hudson H. Freeze and Ajit Varki*

Materials. Modified Eagle's Medium Alpha (α-MEM) and gentamycin sulfate were obtained from the UCSD Core Tissue Culture facility. Fetal Calf Serum (FCS) was purchased from Gemini Scientific. Sulfate-free medium containing various concentrations of methionine and cysteine was prepared from a Selectamine kit (Gibco). L-Glutamine was purchased from Irvine Scientific. Insulin, transferrin, sodium selenite, Sephadex G-25-80 and QAE-Sephadex were obtained from Sigma. Sephadex G-50, Sephacryl S-200, and Con A-Sepharose were purchased from Pharmacia. Ricinus communis agglutinin-1 (RCA-1)-agarose and phytohemagglutinin-L4 (L-PHA)-agarose were purchased from EY Labs. Tunicamycin was obtained from Lilly Laboratories. All other chemicals used were reagent grade.

Enzymes. Peptide:N-Glycosidase F (PNGaseF) was purified from Flavobacterium meningosepticum (see below). Arthrobacter ureafaciens neuraminidase was purchased from Calbiochem. Newcastle Disease Virus neuraminidase was prepared as previously described (27). Homogeneous E. coli alkaline phosphatase was a gift from M. Schlesinger, Washington University. Jack bean ß-galactosidase was a gift from Walter Gregory and Stuart Kornfeld, Washington University, St. Louis. Samples of homogeneous human placental ß-hexosaminidase A were generously provided by Arnold Miller, University of California at San Diego, Don Mahuran, Hospital for Sick Children, Toronto, and Mario Ratazzi, North Shore University Hospital, Manhasset, N.Y. Endo-ß-N-Acetyl-glucosaminidase H (endo H) was purchased from Miles.

Radioisotopes. Carrier-free $^{35}SO_4$ (43 Ci/mg S) was purchased from ICN Biomedicals, Inc. [2-^{3}H]mannose (15Ci/mmol) and [^{3}H]$NaBH_4$ (5Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. [^{3}H]leucine (1Ci/mmol) was purchased from Amersham.

Cell Lines. Bovine pulmonary artery endothelium cells (CPAE) were obtained from the American Type Culture Collection (ATCC CCL 209). Low alpha passage (< 20) cultures were maintained in monolayer culture. Modified Eagle's Medium (α-MEM) supplemented with 10% fetal calf serum, 2mM L-Glutamine, and 0.05mg/ml gentamycin sulfate (complete α-MEM). The Chinese Hamster Ovary (CHO) and mutant (CHO 761) cell lines (kindly provided by J. Esko, University of Alabama at Birmingham) were also maintained as monolayers in complete α-MEM. The latter cell line lacks the ability to initiate glycosaminoglycan chain synthesis (28). BW 5147 mouse lymphoma cells were maintained in suspension culture in complete α-MEM.

Metabolic Labelling of Cells. Cultures were metabolically labelled with $^{35}SO_4$ alone or with $^{35}SO_4$ plus [^{3}H]mannose or [^{3}H]leucine for various lengths of time in the following media: 1) complete α-MEM; 2) Sulfate-free complete α-MEM; 3) α-MEM without sulfate, cysteine, or gentamycin sulfate but with 1/50th normal methionine concentration (0.3ug/ml final), and supplemented with insulin (10ug/ml), transferrin (10ug/ml), and sodium selenite (.172ug/ml). Medium 3 is designated Sulfate Label Incorporation Medium (SLIM). Growth medium was removed and cells were rinsed with sterile phosphate buffered saline (PBS). SLIM or other media containing 0.67 - 0.83mCi/ml $^{35}SO_4$ was then added. In experiments in which cultures were to be double-labelled with $^{35}SO_4$ and [^{3}H]mannose, subconfluent cells were first metabolically labelled with 0.03mCi/ml [2-^{3}H]mannose in complete α-MEM for 48h. Monolayers were then incubated with SLIM containing $^{35}SO_4$ as described above.

Tunicamycin Treatment. CPAE cells were grown in replicate dishes in complete α-MEM until nearly confluent. Selected dishes were incubated with various concentrations of tunicamycin (TM) for 0.5h. Media was then removed from all dishes and replaced with complete α-MEM or SLIM containing 0.1 or 1.0mCi $^{35}SO_4$ and 10uCi [^{3}H]leucine, for 6 h at 37°C. TM treatment was continued in those dishes which had been preincubated with the drug.

Harvesting and Lysis of Cells. Labelling medium was removed from cells, the monolayers rinsed with PBS, scraped in PBS with a rubber policeman, and centrifuged at 1000 rpm for 10min at 4°C in an IEC HN-SII centrifuge. Cell pellets were resuspended in a small volume of PBS, transferred to Eppendorf tubes and centrifuged at 10,000 x g for 2min. The PBS was decanted and cells extracted by boiling for 10min in a small volume of lysis buffer (50mM Tris-HCl pH7.5, 0.1M 2-mercaptoethanol, 1% SDS).

Gel Filtration. The cell lysate was cooled, centrifuged at 10,000 x g, and the resulting supernate applied to an S-200 or G-50 gel filtration column. Radioactivity eluting from the column was monitored and fractions representing the void volume (S200VoA in Figure 3) collected, concentrated by acetone precipitation (see "Other Procedures") and re-dissolved in lysis buffer as above. Portions of this extract were incubated with or without PNGaseF (see below) and reapplied to the same gel filtration column. Cell lysates, enzyme-treated and non-treated samples were fractionated on a 0.7 x 50 cm column of Sephadex G-50 or Sephacryl S-200 equilibrated with 10mM Tris-HCl pH6.5, 0.2% SDS (column buffer). Fractions of 700ul were collected using an LKB Microperpex peristaltic pump at a flow rate of 7ml/h.

Selected samples of released oligosaccharides collected from S-200 columns were also studied by gel filtration on a 0.7 x 50cm column of Sephadex G-50 equilibrated with 15mM Tris-HCl pH6.5. Some samples treated with mild acid or glycosidases (see below) were studied by gel filtration on a 0.7 x 50cm Sephadex G-25 column equilibrated with 0.1M acetic acid. Desalting of samples was carried out on a 0.7 x 50cm Sephadex G-25 column in water.

Simplified Assay for PNGaseF. To easily monitor the purification of PNGaseF, we developed a simple, rapid assay for its activity. Intact fetuin was [^{3}H]-labelled in sialic acids by periodate/[^{3}H]$NaBH_4$ treatment (29), denatured by heating in 2% SDS at 100°C for 5min, and used as a substrate. The enzyme reaction contained [^{3}H]fetuin (containing $19x10^{-7}$ mmol sialic acid, 0.1% SDS, 50mM EDTA, 20mM Tris-HCl pH7.5, 20mM 2-mercaptoethanol, 1% NP40 plus enzyme in a final volume of 20ul. Incubation was carried out at 37°C for 10min. The reaction was terminated by transferring samples to ice and then adding 10ul 10mg/ml BSA followed by 200ul cold 2% phosphotungstic acid/ 10% trichloroacetic acid. Samples were then centrifuged at 10,000 x g for 5min, and 200ul of the released oligosaccharides in the supernate counted. One milliunit is defined as the amount of enzyme required to convert 1 nmol SA to acid-soluble form in one minute. (Because 25% of the tritiated sialic acids on fetuin are on O-linked chains, a maximum of 75% of the radioactive sialic acid can be released by PNGaseF. Under conditions where <15% of the label was released, the assay was linear with time and added enzyme).

Purification of PNGaseF. Homogeneous Peptide:N-Glycosidase F (35.5Kd) was purified from the culture supernate of Flavobacterium meningosepticum exactly as described by Tarentino et al (30) except that the final sulfopropyl-Sephadex C-25 column was omitted. This preparation is free of contamination by endo-β-N-Acetyl-glucosaminidaseF (32Kd) (as judged by SDS-PAGE and by paper chromatographic assay using dansylated ovalbumin asparginyl-glycopeptides as substrate). The preparation is also devoid of detectable protease activity against deglycosylated ovalbumin, or against total cell [^{3}H]leucine-labelled extracts (see Table 1).

Release of Oligosaccharides by PNGaseF. Acetone-precipitated pellets of material isolated from the void volume of S-200 or G-50 columns were solubilized by boiling in a small volume of lysis buffer (see "Harvesting and Lysis of Cells") for 5-10min, cooled, and centrifuged at 10,000 x g for 5min. 100ul of supernate was mixed with 300ul H_2O, 50ul 0.5M EDTA, 50ul 10x PNGaseF buffer (0.2M Tris-HCl pH 7.5, 0.2M 2-mercaptoethanol, 10% NP40). This enzyme reaction mixture could be scaled up, depending on the volume of the acetone pellet for a final concentration of 0.2% SDS and 2% NP-40. The major portion of the reaction mixture was incubated with 1mU PNGaseF 14 - 16 hours at 37°C, while the remainder was incubated identically without enzyme. Samples were then boiled 5-10min to solubilize any proteins precipitated by deglycosylation, cooled, additional PNGaseF (1mU) added and incubated for 2-6h. Samples were boiled again, centrifuged at 10,000 x g for 5min, and the supernates applied to the gel filtration column.

Removal of SDS and Salt from Released Oligosaccharides. Fractions containing PNGaseF-released material (e.g. S200R in Figure 3) were pooled and SDS removed by precipitation with 1/100th volume saturated KCl at 4°C overnight (31). The solution was centrifuged at 2000 rpm for15min at 4°C, the supernate saved and the precipitate resuspended in a small volume of cold H_2O and centrifuged again. The two supernates were combined and dried by lyophyllization, resuspended in water and desalted.

Ion Exchange Chromatography of Oligosaccharides. A disposable Econocolumn containing 0.8ml QAE-Sephadex was equilibrated with 2mM Tris base, and samples dissolved in 750ul of this buffer were applied to the column. 1.5ml fractions were collected by gravity flow. Unbound material was allowed to pass through the column, and any remaining unbound material was collected by elution with 2mM Tris. Bound material was then eluted with step increases of NaCl in 2mM Tris base, using 6ml for each concentration (0.75ml per addition). Under these conditions, high mannose oligosaccharides containing 1, 2, 3, and 4 charges are eluted with 20, 70, 100, and 140mM NaCl, respectively (32).

An abbreviated version of this QAE-Sephadex chromatography was carried out on oligosaccharides reduced with [^{3}H]$NaBH_4$ (see below). After reduction samples were applied to QAE-Sephadex and the unbound fractions collected as described above. Bound material was then eluted in one step with 6ml of 400mM NaCl in 2mM Tris base.

Reduction and Labelling of Released Oligosaccharides with [^{3}H]$NaBH_4$ Some samples which were metabolically labelled with only $^{35}SO_4$ were reduced with [^{3}H]$NaBH_4$ prior to ion exchange chromatography. After removal of SDS by KCl precipitation (see above) released oligosaccharides were desalted, dried on a Buchler shaker-evaporator, and resuspended in 100-200ul 0.2M NaBorate pH 9.8. One mCi of dried [^{3}H]$NaBH_4$ was taken up in 100ul of the above buffer, and incubated with the sample for 2-3h at room temperature. Excess unlabelled $NaBH_4$ (1-5mg) was then added to the sample and the incubation continued at room temperature for 1h. The sample was then adjusted to pH 4 with glacial acetic acid. 1ml MeOH was added and sample was taken to dryness. The following procedure was then repeated three times: the dried sample was taken up in 1ml MeOH plus 1 drop glacial acetic acid and dried again. The final dried sample was dissolved in 15mM Tris-HCl pH 6.5 for fractionation on a 0.7 x 50cm Sephadex G-50 column equilibrated in this buffer. Under these conditions the sulfated oligosaccharides described in this study appeared in the included volume (not shown). The included material containing Class 1 oligosaccharides was recovered, diluted to less than 2mM chloride with 2mM Tris base and subjected to QAE-Sephadex ion exchange chromatography (see above).

Enzymatic and Chemical Treatments Prior to Analytical QAE-Sephadex Chromatography.
Arthrobacter ureafaciens neuraminidase (AUN). The dried sample was brought up in 20-100ul containing 100mM NaAcetate pH 6.0, 4mM CaAcetate, and 5 - 25mU of AUN. Samples were incubated at 37°C for 2h, taken up in 5ml 2mM Tris base and subjected to ion exchange chromatography (see above).
Newcastle Disease Virus neuraminidase. Dried samples were treated identically in 100mM NaCacodylate pH 6.5.
Alkaline phosphatase. The dried sample was taken up in 20ul 100mM Tris-HCl pH 8 plus 0.4U homogeneous *E. coli* alkaline phosphatase. Some of the sample was also treated with mild acid for 30 min (see below) before alkaline phosphatase treatment in order to remove potential phosphodiester groups. Reactions proceeded at 37°C for 0.5h. Samples were taken up in 2mM Tris base, and applied to QAE-Sephadex.
Solvolysis was carried out as described in (18).

Enzymatic Treatments Prior to Lectin Affinity Chromatography.
Neuraminidase. Samples were dried, dissolved in 50ul solution containing 100mM NaAcetate pH6.0, 4mM CaAcetate, 15mU *Arthrobacter urefaciens* neuraminidase (AUN), and incubated at 37°C for 2h.

Neuraminidase and ß-galactosidase. Dried samples were dissolved in 50ul solution containing 100mM NaAcetate pH 5.0, 15mU AUN, 2U jack bean ß-galactosidase, and incubated at 37°C overnight in a toluene atmosphere. At the end of the incubations, samples were heated at 100°C for 2min, cooled, and centrifuged at 10,000 x g for 2min. The supernates were mixed with 600ul buffer and applied to RCA-I-Agarose (see below).

Other Enzymatic Treatments.
Endo-ß-N-acetyl-glucosaminidase H. 11ul of material eluting in the void volume (S200VoA) was adjusted to a final volume of 114ul containing 44mM citrate-phosphate buffer pH5.5 and 3ul (3mU) endo H or 3ul of the above buffer (control). Samples were incubated overnight at 37°C in a toluene atmosphere. Incubation was terminated by boiling, followed by cooling and centrifuging at 10,000 x g for 2min. The supernates were mixed with 500ul S-200 column buffer and subjected to gel filtration.
ß Hexosaminidase A. $^{35}SO_4$-labelled CPAE Class I oligosaccharides were adjusted to 10mM HCl and heated at 100°C for 30min. Treated or untreated (control) samples were then lyophyllized, adjusted to 100ul 50mM NaFormate pH 4.5 with or without 3U ß-galactosidase (previously dialyzed against the above buffer), and incubated at 37°C for 24h in a toluene atmosphere. 15ul from each sample was then adjusted to 200ul NaFormate pH 3.6 by the addition of 185ul 100mM NaFormate pH 3.5. 10ul of the above buffer (control) or ß-hexosaminidase A was then added and samples incubated at 37°C for 20h in a toluene atmosphere. Reactions were terminated by boiling for 2min, cooling, and centrifuging at 10,000 x g for 2min. Supernates were mixed with an equal volume of 0.2M acetic acid and then subjected to gel filtration on a Sephadex G-25 column equilibrated with 0.1M acetic acid (see above).

Lectin Affinity Chromatography of PNGaseF-released $^{35}SO_4$-labelled Oligosaccharides. Samples were fractionated on Con A-Sepharose, RCA-1-Agarose, and L-PHA-Agarose according to the methods of Merkle and Cummings (33), with the following modifications: RCA-1-Agarose chromatography was performed at 4°C, the lactose elution step was omitted, and the dimensions of the L-PHA-Agarose column were 0.7 x 13cm. Some samples were treated with mild acid to remove sialic acids before application to L-PHA-Agarose. Columns were calibrated with standards prior to fractionation of sample oligosaccharides. Con A-Sepharose and L-PHA-Agarose were calibrated with [^{3}H]mannose-labelled oligosaccharides or glycopeptides from BW 5147 cells (34). RCA-1-Agarose was calibrated with IgG glycopeptides (35) and with the fully sialylated tetraantennary chain from orosomucoid, kindly provided by Jacques Baenziger, Washington University (36).

Other Procedures.
Acetone precipitation was carried out exactly as previously described (31).
Mild acid treatment for cleavage of phosphodiesters and removal of sialic acids was carried out in 10mM HCl at 100°C for 30 min. (32). Kinetics of acid hydrolysis of sulfate esters was studied as described (20).
Liquid scintillation counting. Samples were adjusted to 400ul with H_2O, mixed with 4ml Liquiscint, and counted in a Beckman LA 6800 liquid scintillation counter. ^{35}S spillover was determined by dual-channel counting using a standard that was similarly quenched. In some figures from double-label experiments the actual values for ^3H or ^{35}S were scaled up or down for the purpose of clarity.
Barium precipitation of free $^{35}SO_4$ was carried out exactly as previously described (20).

RESULTS

Gel Filtration for Separation of Released Oligosaccharides. We have previously used Sephadex G-50 gel filtration columns run in an SDS-containing buffer to first isolate labelled macromolecules and to then display the oligosaccharides released by endoglycosidases (24,32). A potential disadvantage of this approach is that very large oligosaccharides that are released might still remain in the void volume of such columns. Figure 1 shows the gel filtration analysis of macromolecules from $^{35}SO_4$-labelled CPAE cells that were incubated in the presence or absence of PNGaseF. Using Sephadex G-50 columns (1A and 1C), a rather small amount of released oligosaccharides (9% of total recovered cpm) were found in the included volume (fractions 12-20). However, fractionation of the same two samples on a Sephacryl S-200 column of the same dimensions (Figures 1B and 1D), revealed a much greater proportion of released material (20% of total recovered $^{35}SO_4$). Thus, very large and/or highly charged oligosaccharides are released by PNGaseF, but still run close to the void volume region of a G-50 column. For this reason, S-200 columns were used in all subsequent experiments described in this paper.

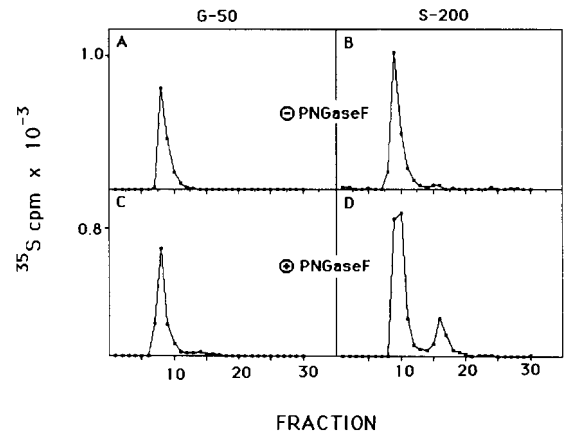

Figure 1. Gel Filtration of CPAE Macromolecules With and Without Prior PNGaseF Treatment. An SDS lysate of $^{35}SO_4$-labelled CPAE cells was fractionated on either Sephadex G-50 or Sephacryl S-200. Fractions from the void volume region of each column were pooled, acetone-precipitated, and redissolved in lysis buffer. Equal aliquots were incubated in the presence or absence of PNGaseF, and then fractionated on the same columns. For other details see "Experimental Procedures".

Optimization of $^{35}SO_4$ Incorporation into Cellular Macromolecules. Since most $^{35}SO_4$-labelled macromolecules in any cell are in proteoglycans, the sulfated N-linked oligosaccharides should be relatively minor components of the total label incorporated (28). Thus, it would be useful to optimize the incorporation of the $^{35}SO_4$ label into macromolecules. Mammalian cells are capable of utilizing inorganic sulfate, by converting it into PAPS, which donates sulfate esters to acceptors such as oligosaccharides and tyrosine residues (4). However, unlike bacteria, mammalian cells cannot reduce inorganic sulfate to form sulfur-containing amino acids such as methionine and cysteine (37). On the other hand, it has recently been emphasized that a major portion of the inorganic sulfate pool in such cells can arise from the breakdown of sulfur-containing amino acids, rather than the uptake of sulfate from the medium (38). Thus, while sulfate-free medium can be used to improve labelling with $^{35}SO_4$, the label is still considerably diluted by the endogenous production of free sulfate. We therefore compared metabolic labelling with $^{35}SO_4$ in complete α-MEM with labelling in media containing altered concentrations of unlabelled sulfate and/or its amino acid precursors. The labelled cells were then extracted in lysis buffer and the lysates fractionated on S-200 gel filtration columns. Figure 2 shows examples of the results of such experiments. For CHO and BW 5147, removal of unlabelled SO_4 causes an increase in both the relative and absolute amount of radioactivity appearing in the void volume region (hereafter called S200VoA), where macromolecules are eluted. Removing sulfate and cysteine and decreasing methionine (to 1/50th its usual concentration) further increased incorporation of $^{35}SO_4$ into S200VoA. In this medium, called Sulfate Label Incorporation Medium (SLIM), the final concentration of inorganic sulfate using carrier-free label was calculated to be 0.4 - 0.5 μM. However, the incorporation of [^{3}H]leucine into protein over a 6h period was not affected by removal of these sulfur-containing amino acids (data not shown). As described later, these conditions also did not cause any major change in the incorporation of $^{35}SO_4$ into N-linked oligosaccharides.

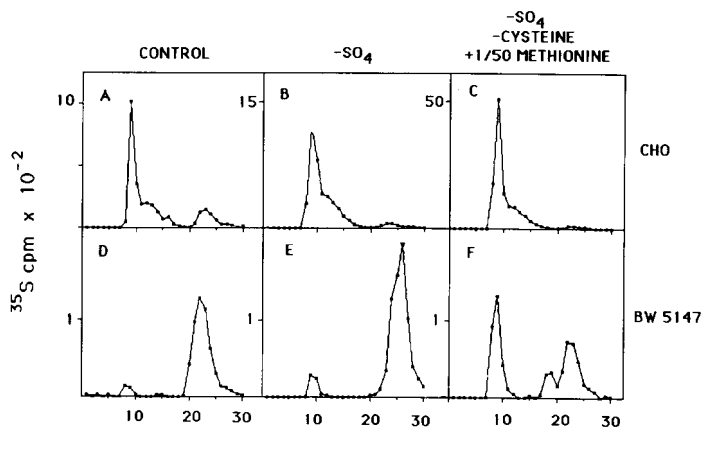

Figure 2. Gel Filtration Analysis of the Effect of Labelling Medium Composition on Incorporation of $^{35}SO_4$ into Macromolecules. CHO cells (A, B, C) or BW 5147 cells (D, E, F) were metabolically labelled with 0.25mCi $^{35}SO_4$ for 6 hours in complete α-MEM (A, D); α-MEM without inorganic sulfate (B, E); or medium without sulfate or cysteine and with 1/50 methionine ("SLIM") (C, F). Cells were harvested, lysed in SDS buffer, and subjected to Sephacryl S-200 gel filtration analysis, as described in "Experimental Procedures".