Endothelial cells incorporate $^{35}$SO into a class of glycoproteins which are released from the cell layer into the culture medium. The incorporated $^{35}$SO, was localized on intact oligosaccharide chains which were released from the protein either chemically by hydrazinolysis or enzymatically by a peptide: N-glycosidase activity; thus these $^{35}$S-oligosaccharides are presumable N-glycosidically linked to protein. These $^{35}$S-oligosaccharides were also isolated and analyzed as labeled glycopeptide and found to contain the terminal trisaccharide sequence sialic acid $\rightarrow$ galactose $\rightarrow$ N-acetylglucosamine. After removal of these carbohydrate residues, the remainder of this $^{35}$S-glycopeptide was susceptible to $\alpha$-mannosidase digestion yielding a smaller $^{35}$S-glycopeptide containing GlcNAc-$^{35}$SO$_2$. Monensin ($10^{-6}$ M), a monovalent cation ionophore, inhibited the sulfation ($>$80%) and synthesis ($>$60%) of endothelial cell-sulfated proteoglycans which were released into the culture medium. However, neither the synthesis nor sulfation of cell-released $^{35}$S-glycopeptides was affected at similar monensin concentrations. Higher concentrations of monensin ($>5 \times 10^{-8}$ M) inhibited the incorporation of both $[^3H]$glucosamine and $^{35}$SO into cell-released glycoproteins.

The biosynthesis of sulfated glycoproteins has now been reported to occur in various tissues and cell types. An increasing number of these reports deal with the biosynthesis of glycoproteins where the sulfate moieties have been identified on oligosaccharide side chains known to be N-linked to Asn and whose synthesis is inhibited by tunicamycin (Refs. 1-5, and references therein). Analyses have indicated that sulfated N-linked oligosaccharide structures are diverse. Sulfate moieties have been identified on terminal nonreducing end hexosamine residues (4) and on reducing end GlcNAc residues of nonsialated oligosaccharides (3). Little is known about the function of sulfated carbohydrate residues of glycoprotein oligosaccharides, although they may be important in affecting glycoprotein processing, secretion, turnover, and the circulating half-life of glycoproteins able to reach the vascular flow (6-9). Monensin, a monovalent metal ionophore, has been shown to inhibit the sulfation and to contain sulfated proteoglycans (10). The mechanism for this effect is not well understood, although monovalent cation ionophores have been reported to block the secretion of proteins and glycoproteins (11, 12) presumably by affecting intracellular transport.

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EXPERIMENTAL PROCEDURES

Cell Cultures and Radioisotope Labeling—Human endothelial cell cultures were obtained from umbilical cord veins and were maintained in culture as described previously (5, 19). Cell cultures were labeled in Ham's F-12 nutrient medium containing 5 $\mu$g/ml of albumin, 5 $\mu$g/ml of bovine insulin, and arabinosin (100 units/ml, all from Sigma). Cell cultures were labeled with 25 $\mu$Ci/ml of $[^3H]$glucosamine (20.2 Ci/mmol), 20 $\mu$Ci/ml of $[^3H]$mannoside (16 Ci/mmol), and 250 $\mu$Ci/ml of H$_2$SO$_4$ (43 Ci/mg) (New England Nuclear) for 4-16 h. Cell cultures were preincubated for 4 h in the presence of various concentrations of monensin added in less than 2 $\mu$l of ethanol/ml of medium (Calbiochem-Behring) before labeling and were labeled in the continued presence of monensin.

Glycoprotein Analysis—Labeled glycoproteins were isolated from labeling medium and cell layers as previously described. These materials were treated with 2 $\mu$m diisopropylfluorophosphate for 1 h at 0 °C and were dialyzed against 5% ammonium sulfate and then against water to remove unincorporated label.

Labeled sulfated glycoproteins were separated from labeled proteoglycans by DEAE-cellulose chromatography in the presence of 2 $\mu$m urea as previously described (5). Glycoproteins solubilized in the presence of 20 mM diithiothreitol were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5-12.5% linear gradient slab gels according to the method of Laemmli (20). Radiolabeled materials were detected using fluorography of Enhance (New England Nuclear) or Autorad (National Diagnostics) impregnated gels by exposing X-Omat film (Kodak) to the dried gels at -80 °C for 3-10 days.

Glycopeptide Preparation and Analysis—Glycopeptides were prepared by pronase digestion as previously described (2) and were analyzed by Bio-Gel P-4 (1 x 210 cm) column chromatography in 0.2 M ammonium acetate. Glycopeptides and oligosaccharides were digested with neuraminidase (Clostridium perfringens, Sigma), $\beta$-galactosidase (Escherichia coli, Aspergillus niger, jack bean, Sigma, Charonia lampas, Miles), $\beta$-N-acetylglucosaminidase (human placenta, jack bean, Sigma), $\alpha$-mannosidase (jack bean, Sigma), endo-$\beta$-galactosidase (Escherichia freundii, Miles), and endo-$\beta$-N-acetylglucosaminidase D and H (Miles) as previously described (21, 22).

Oligosaccharide Preparation—Glycoproteins were digested with a peptide-N-glycosidase-active fraction prepared from almond $\beta$-glucosidase (23, 24). During a 5-h incubation (7, 8) this enzyme released...
about 20% of the presumably Asn-linked [3H]mannose-labeled or
35SO4-labeled oligosaccharides, which were recovered as trichloroacetic acid-soluble materials. These oligosaccharides were then analyzed by P-4 chromatography. Oligosaccharides were prepared from glycoproteins by anhydrous hydrazinolysis at 100 °C for 3 h as described (25-27). The liberated oligosaccharides were N-acetylated (28) and were subjected to Bio-Gel P-6 chromatography to remove small molecular weight materials before analysis by P-4 chromatography.

RESULTS

Endothelial Sulfated Glycoproteins—In order to study the regulation of endothelial cell-sulfated glycoproteins which are released into the culture medium, cells were incubated with 35SO4 for various times. The labeled glycoconjugates in the medium and cell layer were isolated, and the labeled sulfated glycoproteins were separated from labeled proteoglycans by DEAE-cellulose chromatography as described under “Experimental Procedures.” As seen in Fig. 1, there is an increase in the incorporation of 35SO4 into both the glycoprotein (Fig. 1A) and proteoglycan (Fig. 1B) materials. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these sulfated glycoproteins (Fig. 2) demonstrated the presence of several 35SO4-labeled proteins with a major component with an apparent molecular weight of about 70,000. Under these labeling and harvesting conditions greater than 80% of the total 35S-glycoproteins was found in the culture medium after 24 h. Therefore, cells were routinely labeled under these conditions to study the sulfated glycoproteins which were released by the cells into the medium.

Glycopeptide Analysis of Sulfated Glycoproteins—Cells were incubated with [3H]glucosamine and 35SO4 for 24 h. [3H]Hexosamine and 35SO4-labeled glycoproteins from the culture medium were partially purified on DEAE-cellulose and then were digested with pronase, as previously described (2). The resulting 3H/35S glycopeptides, which were previously reported (5) to be composed of mild alkali-stable tunicamycin-sensitive sulfated oligosaccharide chains were analyzed by Bio-Gel P-4 chromatography as shown in Fig. 3. The partially purified 3H/35S glycopeptides (Fig. 3A) were subjected to mild acid hydrolysis (0.01 N HCl, 1 h, 75 °C) to remove bound sialic acid and then were rechromatographed (Fig. 3B). The release of free [3H]sialic acid (but no 35SO4) was accompanied by a decrease in size in the 3H/35S glycopeptides. Treatment of the 3H/35S-asialoglycopeptides with β-galactosidase plus β-N-acetylglucosaminidase (Fig. 3C), but not with endo-β-galactosidase or β-N-acetylglucosaminidase alone (not shown), resulted in the release of [3H]GlcNAc residues and smaller size 3H/35S-glycopeptides. Further digestions of these glycopeptides with α-mannosidase (Fig. 3D) resulted in a 3H/35S product that eluted slightly ahead of the position of a mannosyl-(GlcNAc)2-peptide prepared from ovalbumin. Treatment of this “inner core” glycopeptide with additional α-mannosidase or N-acetylglucosaminidase digestions, or with placental hexosaminidase A, which has been shown to release terminal GlcNAc-6-SO4 residues (29), did not result in further glycopeptide degradation. Controlled acid digestion (5) released GlcNAc-SO4 from these products.

Oligosaccharide Characterization—The above results as well as previous analyses (5) do not absolutely preclude the possibility that the isolated 3H/35S-glycopeptide might consist of a nonsulfated Asn-linked carbohydrate unit attached via a pronase-insensitive peptide to a sulfated carbohydrate unit which was either hydroxylsine linked or otherwise stable to β-elimination reactions (30). Therefore, partially purified 3H/35S-glycopeptides were subjected to two different procedures to release intact Asn-linked oligosaccharides from the polypeptide backbone of the glycoprotein. 3H/35S-Oligosaccharides were released from 3H/35S-glycopeptides isolated from combined medium and cellular fractions by anhydrous hydrolysis and then N-acetylated and analyzed by P-4 chromatography as shown in Fig. 4A. The majority of these peptide-free 3H/35S-oligosaccharides appear to be about the same size (Fractions 50-60) or smaller than (Fractions 60-80) the isolated 3H/35S-glycopeptides (Fig. 3A) prepared by pronase digestion. The smaller size 3H/35S-oligosaccharides (Fig. 4A, Fractions 60-80) may be due to some partial degradation.

**Fig. 1.** Incorporation of 35SO4 into endothelial cell glycoconjugates. Endothelial cells were continuously incubated with 35SO4, and at the indicated times the incorporation into glycoproteins (GP) (A) and into proteoglycans (PG) (B) for both the labeling medium (A- - - A) and cell layers (● - ●) was determined.
Sulfated Glycoproteins

An enzymatic treatment was also used to release peptide-free oligosaccharides from proteins. As shown in Fig. 5, almond peptide: N-glycosidase activity released $^3$H/$^{35}$S-oligosaccharides (Fig. 5A) or $[^3]$Hmannose oligosaccharides (Fig. 5B) similar in size to either pronase (Fig. 5C) or hydrazine (Fig. 4) generated $^3$H/$^{35}$S-oligosaccharide materials. These results (Fig. 3-5) strongly indicate that complex-type N-Asn-linked oligosaccharides containing GlcNAc-S04 on the “inner core” residues are synthesized by endothelial cells in culture.

**Effects of Monensin**—Monensin has been reported to inhibit sulfation of secreted proteoglycans in chondrocyte cultures (10). Thus, monensin appears to be a useful probe to study the synthesis and sulfation of both proteoglycans and N-linked glycoproteins in human endothelial cell cultures. Cells were labeled with $[^3]$Hglucosamine and $^{35}$S04 in the presence or absence of various concentrations of monensin. As shown in Fig. 6, monensin caused a marked inhibition of $^{35}$S04 incorporation into both medium (Fig. 6A) and cell-associated (Fig. 6B) glycoconjugates. At increasing concentrations of monensin, a less dramatic inhibition of $[^3]$Hglucosamine incorporation into total glycoconjugates was also observed. The labeled glycoconjugates isolated from the medium and cell layers of monensin-treated cultures were chromatographed on DEAE-cellulose (chloride form) and were eluted with a linear gradient of 60-40-20.

![Bio-Gel P-4 gel filtration chromatography of $^3$H/$^{35}$S-glycopeptides.](image)

During hydrazinolysis, in order to show that some of these sulfated oligosaccharides contain terminal sialic acid residues, the $^3$H/$^{35}$S-oligosaccharides were digested with neuraminidase. The resulting asialo-oligosaccharides were subjected to DEAE-cellulose (acetate form) chromatography. Neutral asialo-oligosaccharides would not be expected to bind, whereas sulfated asialo-oligosaccharides would bind to DEAE-cellulose. The bound $^3$H/$^{35}$S materials were batch eluted with 0.2 M ammonium acetate. All of the $^{35}$S materials did bind to DEAE-cellulose, as did 10% of the total digested $^3$H materials. These anionic products were further analyzed by P-4 chromatography. As shown in Fig. 4B, the majority of the resultant $^3$H/$^{35}$S-asialo-oligosaccharides were now smaller in size after neuraminidase treatment (Fig. 4, A versus B). In addition, 40% of these $^3$H anionic products was found to be neuraminidase-released sialic acid (Fig. 4B, Fractions 95-110).

**Fig. 3.** Bio-Gel P-4 gel filtration chromatography of $^3$H/$^{35}$S-glycopeptides. $[^3]$HGlucosamine (O--O) and $^{35}$S04 (●—●) labeled glycopeptides were prepared and chromatographed on a column of Bio-Gel P-4 as described under “Experimental Procedures” (A). The labeled glycopeptides (●—●) in each case were recovered and treated sequentially with 0.01 N HCl (B), β-galactosidase + β-N-acetylglucosaminidase (C), and α-mannosidase (D). The positions of elution of heparan sulfate (Vo), α-mannosidase-treated ovalbumin glycopeptide (T), chitobiotol (C), sialic acid (SA), and glucose (G) are indicated.

**Fig. 4.** Gel filtration of $^3$H/$^{35}$S-oligosaccharides after treatment with hydrazine. $^3$H/$^{35}$S-oligosaccharides, released from $^3$H/$^{35}$S-glycoproteins by hydrazinolysis, were analyzed by P-4 chromatography before (A) and after (B) neuraminidase treatment as described in the text. The positions of elution of heparan sulfate (Vo), sialic acid (SA), and $H_2SO_4$ (SO) are indicated.
Sulfated Glycoproteins

Fig. 5. P-4 gel filtration of labeled oligosaccharides enzymatically released. [3H]Glucosamine (\(\text{O}--\text{O}\)) and \(^{35}\text{S}\)glycoproteins (●—●) labeled glycoproteins (A) and [3H]mannose-labeled (\(\text{O}--\text{O}\)) glycoproteins (B) were digested with almond peptide: N-glycosidase activity and the released trichloroacetic acid-soluble oligosaccharides were analyzed by P-4 chromatography. C, the elution positions of \(^{3}H^{35}S\) glycopeptides from pronase digestions; chitobiitol (C), sialic acid (SA), and mannose (M) are indicated.

Fig. 6. Effects of monensin on the synthesis and secretion of endothelial cell glycoconjugates. Glycoconjugates were labeled with [\(^{3}H\)glicosamine (\(\text{O}--\text{O}\)) and \(^{35}\text{S}\)glycoproteins (●—●) and isolated as described under “Experimental Procedures.” A, glycoconjugates secreted or released into the medium; B, cell-associated glycoconjugates.

Fig. 7. DEAE-cellulose chromatography of \(^{3}H/{^{35}}S\)-glycoconjugates isolated from cell cultures. The [\(^{3}H\)glucosamine (\(\text{O}--\text{O}\)) and \(^{35}\text{S}\)glycoproteins (●—●) labeled glycoconjugates isolated from the medium (A and B) or cell layer (C and D) were chromatographed on DEAE-cellulose (chloride form) in 0.05 M Tris buffer, pH 8.0, containing 2 M urea. The labeled glycoproteins (GP) were eluted with a linear gradient of 0-0.3 M NaCl, and additional labeled proteoglycans (PG) were eluted with 1 M NaCl. Glycoconjugates are from cells labeled in the absence (A and C) or presence (B and D) of \(5 \times 10^{-7}\) M monensin.

Fig. 8. Effects of monensin on the secretion or release of labeled glycoproteins and proteoglycans. [\(^{3}H\)glicosamine (\(\text{O}--\text{O}\), ●—●) and \(^{35}\text{S}\)glycoproteins (\(\text{O}--\text{O}\), ●—●) and proteoglycans (△—△) from the culture medium were partially purified by DEAE-cellulose chromatography, and the values for monensin-treated cultures are expressed as percentages of cultures labeled in the absence of monensin.
The effects of several concentrations of monensin on the synthesis of glycoproteins and proteoglycans are shown in Fig. 8. At $10^{-8}$ M monensin, greater than 80% of proteoglycan sulfation and synthesis is inhibited while no inhibition of glycoprotein sulfation and synthesis was detected. The incorporation of $^{35}$S, into glycoproteins is markedly less sensitive to the effects of monensin over a large range of monensin concentrations. Monensin can thus be used to inhibit proteoglycan sulfation effectively at concentrations which will not inhibit the synthesis of sulfated glycoproteins.

Cells were labeled with $^{35}$S in the presence or absence of $10^{-8}$ M monensin. The combined medium and cell layer $^{35}$S-glycoproteins were partially purified by DEAE-cellulose chromatography and were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 9, $10^{-8}$ M monensin (Fig. 9, lane 2) caused little change in the migration or distribution among the major $^{35}$S-glycoprotein products at a drug concentration which inhibited greater than 80% of secreted proteoglycan sulfation. In order to see if the oligosaccharide chains of sulfated glycoproteins are altered in the presence of monensin, cells were incubated with $[^3H]$glucosamine and $^{35}$S in the presence or absence of monensin. The labeled glycoproteins in the culture medium were isolated, partially purified by DEAE-cellulose chromatography, and digested with pronase. As shown in Fig. 10, monensin acted to produce smaller size glycopeptides. Many of the $[^3H]$glucosamine-labeled glycopeptides appear markedly smaller (Fig. 10C), although the $^{35}$S-labeled materials ($K_m = 0.08, 0.13, 0.17; A-C$, respectively) appear to be no smaller than would be expected for asialoglycopeptides ($K_m = 0.23$, Fig. 3B) with many $^{35}$-glycopeptides (Fig. 10C) being comparable to $^{35}$S-glycopeptides synthesized in the absence of monensin.

![Fig. 9. Fluorograph of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of $^{35}$S-glycoproteins. $^{35}$S-Glycoproteins from cells labeled in the absence (lane 1) or presence (lane 2) of $10^{-8}$ M monensin were partially purified by DEAE-cellulose chromatography and subjected to analysis as described under “Experimental Procedures” in the presence of dithiothreitol. The relative migrations ($M_r$) of molecular weight standards are indicated.](image)

![Fig. 10. Gel filtration of glycopeptides from monensin-treated cell cultures. $[^3H]$glucosamine (---) and $^{35}$S labeled glycoproteins from cells labeled in the absence (A) or presence of monensin (B, $10^{-8}$ M; C, $5 \times 10^{-7}$ M) were pronase digested, and the resultant glycopeptides were analyzed by P-4 chromatography. Glycopeptides analyzed in C were isolated from cells labeled with a $[^3H]^{35}$S ratio different from those in A and B. Position of elution of chitobiitol (C) is indicated.](image)

**DISCUSSION**

Human vascular endothelial cells in culture release into the medium several glycoproteins which appear to contain N-glycosidically linked sulfated oligosaccharide side chains as well as sulfated proteoglycans. Monensin, a monovalent cation ionophore, differentially affected the synthesis and sulfation of endothelial cell glycoproteins and proteoglycans. Over a wide range of concentrations ($10^{-9}$ to $10^{-6}$ M) monensin inhibited the incorporation of $^{35}$S to a greater extent than it inhibited the incorporation of $[^3H]$glucosamine into proteoglycans (or glycosaminoglycans). These results imply that monensin caused the secretion of undersulfated proteoglycan species, as had been observed by Tajiri et al. (10). However, since no previous reports had dealt with the effects of ionophores upon glycoprotein sulfation, it was interesting to observe that the incorporation of both $[^3H]$glucosamine and $^{35}$S into secreted (or released) glycoproteins was not affected at monensin concentrations which markedly inhibited proteoglycan sulfation. While higher monensin concentrations ($>10^{-7}$ M) did inhibit the synthesis of sulfated glycoproteins, sulfation was not inhibited to a greater extent than oligosaccharide chain formation. It is possible that monensin differentially affects the sulfation of these two glycoconjugate products for several reasons. The sites of sulfation for glycoprotein and proteoglycan carbohydrate chains may be in two distinct intracellular
Standing the role that sulfated oligosaccharides play in cellular function. Brewer for technical assistance and S. Dougherty for the skilled isolation of rat kidney and lung tissues.

Many reports of O-linked mucin-type sulfated glycoproteins have been reported as developmentally regulated components of prolactin granules and entactin. Thus, endothelial cells which have a distinct apical and basal cell surface may have distinct pathways for transport of glycoproteins, although this concept is not well understood.

Whether this might be related to differential processing sites in Golgi sites (31, 32) in relation to secretory vesicle production. Whether this might be related to differential processing sites of N- or O-linked side chains is also not known. Another explanation is that the release of sulfated glycoconjugates may be asymmetrically distributed at the cell surface. The release of basolateral sulfated components might be expected to respond differently to changes in membrane potentials (33) than to processes at the apical surface (15). In this regard it is interesting to note that the synthesis of influenza virus, containing a sulfated glycoprotein component (34), is not affected by monensin in cells displaying an apical-basal polarity in culture (15). Thus, endothelial cells which have a distinct apical and basal cell surface may have distinct pathways for transport of glycoproteins, although this concept is not well understood.

Many studies have recently reported on the incorporation of $^{35}$S into diverse classes of mammalian glycoproteins including fibronectin (36, 37) and entactin (38). Sulfated glycopeptides have been found in melanocytes (39), rat kidney tissues (40), human kidney tumor cells (41), and brain tissues (42). Sulfated glycoproteins have been described as components of secretory granules in pituitary glands (27, 43) and in prolactin granules (43). Sulfated glycoproteins have also been reported as developmentally regulated components of Volvox (44), rabbit uterus (45), human fetal lung (46), chick liver and lung (28), and sea urchin embryos (2). There are also many reports of O-linked mucin-type sulfated glycoproteins (47-49). Thus, monensin may be useful in studying the sulfation of diverse classes of glycoproteins and may aid in understanding the role that sulfated oligosaccharides play in cellular function.

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