Journal of Cellular Biochemistry 50:103–110 (1992)

Effect of Monensin on the Sulfation of Heparan Sulfate Proteoglycan From Endothelial Cells

Lucia O. Sampaio, Carl P. Dietrich, Patricia Colburn, Vincenzo Buonassisi, and Helena B. Nader

Departamento de Bioquímica, Escola Paulista de Medicina, C.P. 20372, CEP 04023, São Paulo, S.P, Brazil

Abstract Monensin is a monovalent metal ionophore that affects the intracellular translocation of secretory proteins at the level of trans-Golgi cisternae. Exposure of endothelial cells to monensin results in the synthesis of heparan sulfate and chondroitin sulfate with a lower degree of sulfation. The inhibition is dose dependent and affects the ratio [^{35}S]-sulfate/[^{3}H]-hexosamine of heparan sulfate from both cells and medium, with no changes in their molecular weight. By the use of several degradative enzymes (heparitinases, glycuronidase, and sulfatases) the fine structure of the heparan sulfate synthesized by control and monensin-treated cells was investigated. The results have shown that among the six heparan sulfate disaccharides there is a specific decrease of the ones bearing a sulfate ester at the 6-position of the glucosamine moiety. All other biosynthetic steps were not affected by monensin. The results are indicative that monensin affects the hexosamine C-6 sulfation, and that this sterification is the last step of the heparan sulfate biosynthesis and should occur at the trans-Golgi compartment.

Key words: glycosaminoglycan-Golgi complex, glycosaminoglycan biosynthesis, 6-sulfation inhibition

Heparan sulfates are a class of sulfated glycosaminoglycans present at the cell surface of mammalian cells in culture and in most vertebrate and invertebrate tissues [Dietrich and Montes de Oca, 1970; Dietrich et al., 1983; Dietrich, 1984; Gallagher et al., 1986; Kraemer, 1971; Nader et al., 1984; Poole, 1986; Rodén, 1980]. They are composed of alternating units of glucosamine and uronic acid (mostly glucuronic) and the hexosamine is either N-acetylated or N-sulfated and/or 6-sulfated [Dietrich et al., 1983; Fransson, 1989; Gallagher and Lyon, 1989].

We have shown that rabbit endothelial cells in culture secrete to the medium an unusual heparan sulfate proteoglycan rich in iduronic acid residues [Nader et al., 1987]. In addition, this structure is not found in heparan sulfates of other cell types of the vessel wall [Nader et al., 1987, 1989].

Recently, we have been searching for compounds capable of interfering with the synthesis and possibly the structure of heparan sulfates. Thus, it was shown that heparin stimulates the synthesis and changes the sulfation pattern of the heparan sulfate from endothelial cells [Nader et al., 1989] and selenate arrests the synthesis of this compound [Dietrich et al., 1988].

Monensin, a monovalent ionophore, has been shown to alter the normal structure of the Golgi complex and appears to slow or to arrest intra-Golgi transport as well as to inhibit trans-Golgi functions such as terminal N-glycosylation, protein processing, and the sulfation of proteoglycans [Farquhar, 1985; Griffiths et al., 1983; Ledger and Tanzer, 1984; Tartakoff, 1982, 1983]. The influence of monensin on chondroitin sulfate and dermatan sulfate proteoglycan biosynthesis has been studied in chondrocytes of different origins [Burditt et al., 1985; Kajiwara and Tanzer, 1981, 1982; Madsen et al., 1983; Nishimoto et al., 1982a,b; Tajiri et al., 1980], rat chondrosarcoma [Mitchell and Hardingham, 1982], human skin fibroblasts [Hoppe et al.,

Abbreviations used: GlcNAc,6S, N-acetylated, 6-sulfated glucosamine; GlcNS, N-sulfated glucosamine; GlcNS,6S, N,6-disulfated glucosamine; iSO_4, inorganic sulfate; [^{35}S]-PAPS, adinosine 3′-phosphate 5′-phosphosulfate; ΔU-GlcNAc, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-acetamido-D-glucose; ΔU-GlcNAc,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-acetamido-D-glucose 6-sulfate; ΔU-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-sulfamino-D-glucose; ΔU-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-sulfamino-D-glucose 6-sulfate; ΔU,2S-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1-4)-2-sulfamino-D-glucose; ΔU,2S-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1-4)-2-sulfamino-D-glucose 6-sulfate.

Received December 5, 1991; accepted May 7, 1992.

1985], human melanoma [Bumol and Reisfel, 1982; Bumol et al., 1984; Harper et al., 1986], and rat ovarian granulosa cells [Yanagishita and Hascall, 1985] where it was shown a marked decrease in the incorporation of sulfate into the glycosaminoglycan chains. This undersulfation for chondroitin sulfate proteoglycan was due to a decreased 6-sulfation of the N-acetylgalactosamine moiety [Kajiwara and Tanzer, 1981; Madsen et al., 1983; Nishimoto et al., 1982a,b; Tajiri et al., 1980], whereas for dermatan sulfate proteoglycan it was related to a decrease of the 4-sulfated disaccharide containing iduronic acid residues [Hoppe et al., 1985; Yanagishita and Hascall, 1985]. These studies furnished important clues as to the location of the different biosynthetic steps of chondroitin sulfate and dermatan sulfate in the Golgi apparatus. Thus, the 6-sulfation of chondroitin sulfate and the epimerization of iduronic acid of dermatan sulfate are the late events in the biosynthesis of these compounds and occur at the trans-Golgi apparatus. So, it became of interest to investigate the effect of this ionophore on the synthesis of the heparan sulfate from endothelial cells that bear significant amounts of highly sulfated disaccharides. Of particular significance was the finding that monensin specifically inhibits the C-6 sulfation of the hexosamine moiety of the heparan sulfate chains of endothelial cells in culture.

METHODS

Substrates, Enzymes, and Materials

Heparan sulfate from bovine pancreas was a gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy) and heparin from bovine lung from Dr. L.L. Coleman, UpJohn Co. (Kalamazoo, MI). Chondroitin 4- and 6-sulfates, dermatan sulfate, and chondroitinases AC and ABC were purchased from Seikagaku Kogyo (Tokyo, Japan). Heparinase, heparitinases I and II, disaccharide sulfoesterase, and glycuronidase were prepared from induced *F. heparinum* cells by methods previously described [Dietrich et al., 1973; Nader et al., 1990]. Ethylenediamine (1,2-diaminoethane) and propylenediamine (1,3-diaminopropane) were purchased from Aldrich Co. (Milwaukee, WI) and monensin from Sigma Chemical Co. (St. Louis, MI). D-[1,6-^{3}H(N)]-glucosamine hydrochloride (42.5 Ci/mmol) and carrier free [^{35}S]-sulfuric acid were purchased from New England Nuclear.

Labeling of Cells and Extraction of the Glycosaminoglycans

An endothelial cell line [Buonassisi, 1973; Buonassisi and Venter, 1976] derived from rabbit aorta was used for these studies. Post-confluent cell cultures, grown in 35 mm culture plates (3.5×10^5 cells/plate) were pre-incubated for 2 h with different concentrations of monensin, in Ham's F-12 tissue culture medium supplemented with 10% fetal calf serum. At the end of this period, the culture medium was removed and the cells were reincubated for 5 h with monensin and either 200 μCi of carrier free [^{35}S]-sulfuric acid or 100 μCi of [^{3}H]glucosamine per mL as indicated. At the end of the incubation, the culture medium was removed and the cells were washed twice with F-12 medium. The cells were scraped from the dish and both cells and medium were precipitated in the cold with 2 volumes of ethanol in the presence of 100 μg of carrier heparan sulfate, dermatan sulfate, and chondroitin sulfate. The radioactive glycosaminoglycan free chains were prepared from the cells and culture medium by incubation with 0.1 mg of Superase (protease from Chas Pfizer Co., NY) for 4 h at 60°C in the presence of 0.8 M NaCl, pH 8.0, in a final volume of 200 μL. After incubation, the mixture was heated for 10 min at 100°C (to inactivate the proteolytic enzyme) and the glycosaminoglycans were precipitated with 2 volumes of methanol at −20°C.

Identification and Quantitation of Glycosaminoglycans

The sulfated glycosaminoglycans were identified and quantitated by a combination of agarose gel electrophoresis and enzymatic degradation as previously described [Nader et al., 1987, 1989]. For quantitation, the radioactive bands were scraped off the agarose gels (after fixation, drying, and staining), dissolved in 1 mL of 1.0 M HCl, heated at 100°C for 5 min, and counted in 10 ml of Ready Solv (Beckman Instruments, CA) in a liquid scintillation spectrometer. A typical incubation mixture contained 0.1 U of enzymes, $10–50 \times 10^3$ cpm of sulfated glycosaminoglycans with 50 μg of carrier sulfated glycosaminoglycans, and other additions as indicated in 0.05 M ethylenediamine-acetate buffer, pH 7.0, in a final volume of 30 μL. The incubation mixtures were spotted in Whatman number 1 paper and subjected to chromatography in isobutyric acid: 1.25 M NH_3, 5/3, v/v for 24 h.

The unsaturated products formed from the carrier compounds were detected by short wave UV lamp. The radioactive [^{35}S]-labeled products were located by exposure of the chromatograms to Kodak X-ray film (SB-5) for 3–15 days. For the [^{3}H]-labeled heparan sulfate the degradation products were located using the migration of the carrier. They were quantitated by counting the paper containing the radioactive compounds in 0.5% PPO in toluene in a liquid scintillation spectrometer. When the radioactivity of the disaccharides was measured using [^{35}S]-sulfate isotope, the actual amount of each compound was calculated by dividing the radioactivity incorporated into the individual species by the number of sulfate residues present in the molecule. The different radioactive enzymatic products obtained from the heparan sulfate upon the action of the enzymes were identified by their chromatographic migrations in two different solvents as well as their electrophoretic mobilities [Nader et al., 1987, 1989] using standard disaccharides prepared from heparin and heparan sulfate whose structures have been determined by chemical analyses and nuclear magnetic resonance [Nader et al., 1990; Perlin et al., 1971]. Also the structure of the radioactive disaccharides was further confirmed by degradation with specific sulfatases and glycuronidase as previously described [Dietrich et al., 1973; Nader et al., 1990].

Analytical Procedures

Cell protein was determined as follows: after incubation, the dishes containing the cells were washed 3 times with 2 mL of serum-free F-12 medium and the cells dispersed in 500 μL of a 25 mM solution of Tris-HCl buffer, pH 7.8, containing 3.5 M urea. Protein was determined in 10 μL aliquots by the BioRad assay [Spector, 1978]. Agarose gel electrophoresis in 3 different buffers and polyacrylamide gel electrophoresis were performed as previously described [Bianchini et al., 1980; Dietrich and Nader, 1974]. Paper electrophoresis of the degradation products was performed in Whatman number 3MM paper in 0.25 M NH_4HCO_3 buffer, pH 8.5, and 0.3 M pyridine-acetic acid buffer, pH 4.5, at a constant voltage of 7 V/cm. Gel filtration chromatography was performed in Sepharose CL-6B column (1 × 110 cm) previously equilibrated with 0.5 M acetic acid. The compounds were eluted from the column in 1 mL fractions with 0.5 M acetic acid and analyzed for radioactivity.

RESULTS

Sulfated Glycosaminoglycans Synthesized by Endothelial Cells in Culture

The incorporation of [^{35}S]-inorganic sulfate into sulfated glycosaminoglycans synthesized by endothelial cells is shown in Table I. Heparan sulfate is the major sulfated glycosaminoglycan found both in cell (90%) and medium (80%). It is also shown that the heparan sulfate/chondroitin sulfate ratio was constant between 4 and 24 h of sulfate incorporation. The heparan sulfate that is secreted into the medium was extensively studied in previous works and it was shown to be composed of significant amounts of heparin-like structures [Nader et al., 1987, 1989]. Figure 1 shows the degradation of the heparan sulfates purified from both cells and medium by heparitinases. It is clear that the heparan sulfate from the cells also shows the same basic pattern of sulfated disaccharides with significant amounts of the trisulfated disaccharide (ΔU,2S-GlcNS, 6S), a typical heparin unit. These products showed the same chromatographic migration in different solvents as the standard disaccharides prepared from heparin (bovine lung) and heparan sulfate (bovine pancreas). Also their

TABLE I. [^{35}S]-Sulfated Glycosaminoglycans Synthesized by Endothelial Cells in Culture*

Sulfated glycosaminoglycan	Cells		Medium	
	4 h	24 h	4 h	24 h
Heparan sulfate	277.6 ± 5.9 (90%)	845.3 ± 38.4 (93%)	36.9 ± 1.2 (81%)	272.5 ± 20.1 (83%)
Chondroitin sulfate	31.4 ± 1.1 (10%)	65.2 ± 5.3 (7%)	8.4 ± 0.4 (19%)	54.2 ± 3.7 (17%)

*Cpm × 10^3/plate. The values represent the average of 5 different experiments, expressed as mean ± standard error. Different sulfated glycosaminoglycans were separated by agarose gel electrophoresis, and the amount of radioactivity incorporated into the individual species was quantitated as indicated in Methods.

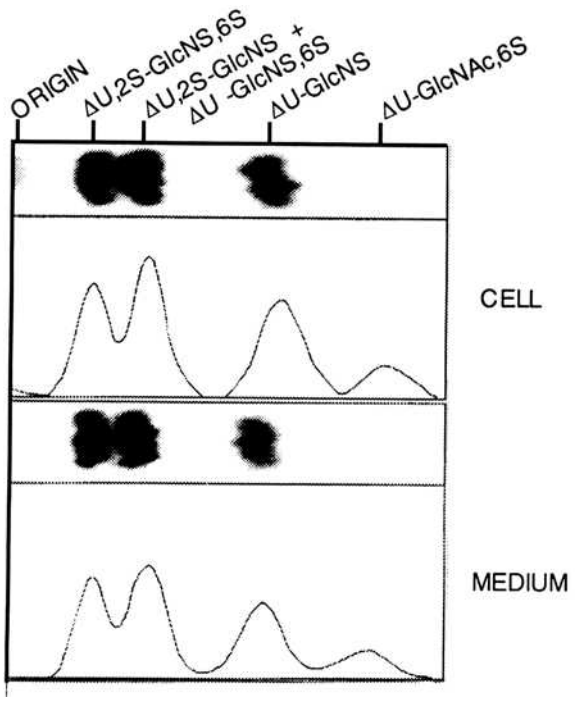

Fig. 1. Degradation products formed from [^{35}S]-heparan sulfate by action of heparitinases I and II. About 30,000 cpm of [^{35}S]-heparan sulfates from cells and medium in the presence of 100 μg of carrier heparan sulfate were incubated with 0.1 units of heparitinases I and II in 0.05 M ethylenediamine acetate buffer (pH 7.0) in a final volume of 30 μl for 4 h at 30°C. After incubation the mixtures were applied to Whatman number 1 paper and chromatographed for 32 h with isobutyric acid/1.25 M NH3,5:3.6 (v/v) as descending solvent. A radioautogram as well as the respective densitometric profile were then prepared from the chromatogram with X-ray film. Note: Heparan sulfates are totally degraded by the combined action of heparitinases I and II by an elimination reaction producing unsaturated disaccharides. Heparitinase I acts upon glucosaminido-glucuronic acid linkages producing mostly N-acetylated and N-sulfated disaccharides. Heparitinase II shows a broader specificity producing mainly N-acetylated 6-sulfated disaccharide and N,6 disulfated disaccharide. For more information on the specificities of these enzymes see Nader et al. [1990].

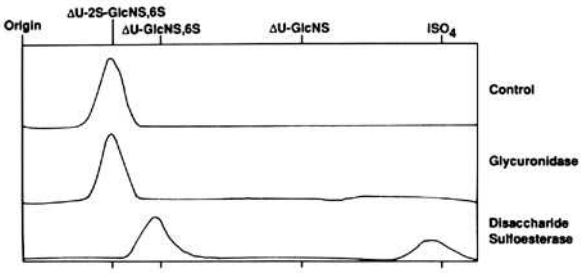

Fig. 2. Enzymatic degradation of the [^{35}S]-trisulfated disaccharide by disaccharide sulfoesterase and glycuronidase. About 10,000 cpm of the [^{35}S]-trisulfated disaccharide (ΔU,2S-GlcNS,6S) obtained from endothelial cells heparan sulfate after the combined action of heparitinases were incubated with 0.1 units of either glycuronidase or disaccharide sulfoesterase in 0.05 M ethylenediamine acetate buffer (pH 7.0) in a final volume of 20 μl for 4 h at 30°C. After incubation the mixtures were applied to Whatman number 1 paper and the products visualized and screened as described in Fig. 1.

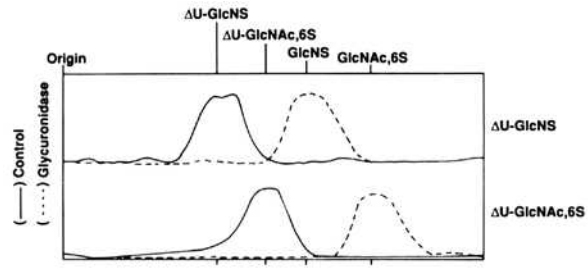

Fig. 3. Enzymatic degradation of the [^{35}S]-monosulfated disaccharides by glycuronidase. About 5,000 cpm of each of the [^{35}S]-monosulfated disaccharides (ΔU-GlcNS and ΔU-GlcNAc,6S) obtained from endothelial cells heparan sulfate after the combined action of heparitinases were incubated with 0.1 units of glycuronidase as described in Fig. 2.

electrophoretic mobilities are compatible with the net charge of the disaccharides.

The identity of each of these products was further confirmed by degradation with specific glycuronidase and sulfatases. The product migrating as trisulfated disaccharide (ΔU,2S-GlcNS,6S) was resistant to the action of glycuronidase and degraded by an enzyme (disaccharide sulfoesterase) which specifically removes a sulfate from the C-2 position of the unsaturated uronic acid moiety, yielding as products inorganic sulfate and a compound with the migration of disulfated disaccharide (Fig. 2). This last compound was totally degraded by glycuronidase forming glucosamine N,6-disulfate (not shown). On the other hand the compounds migrating as unsaturated 6-sulfated-N-acetylated disaccharide (ΔU-GlcNAc,6S) as well as the one migrating as N-sulfated disaccharide (ΔU-GlcNS) were resistant to the action of sulfoesterase and totally degraded by glycuronidase releasing N-acetylated, 6-sulfated glucosamine and N-sulfated glucosamine, respectively (Fig. 3). The band migrating as disulfated disaccharide was partially degraded by glycuronidase as well as by disaccharide sulfoesterase, indicating the presence of two different species of disaccharides, one containing a sulfate at the C-2 position of the uronic acid moiety (ΔU,2S-GlcNS) and another which is constituted of glucosamine N,6-disulfate (ΔU-GlcNS,6S) (Fig. 4). Table II summarizes the relative amounts of the different disaccharides of the heparan sulfates from both cells and medium. It is clear that the same

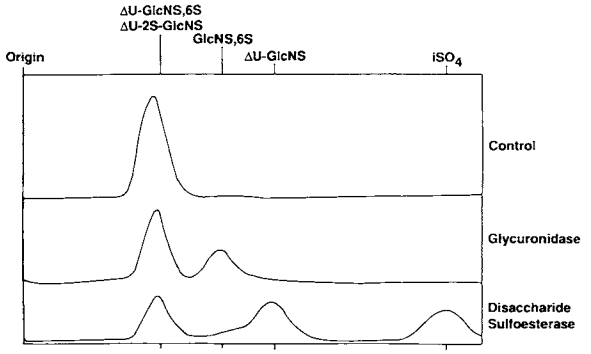

Fig. 4. Enzymatic degradation of the [^{35}S]-disulfated disaccharides by glycuronidase and disaccharide sulfoesterase. About 10,000 cpm of each of the [^{35}S]-disulfated disaccharide fraction (ΔU-GlcNS,6S/ΔU,2S-GlcNS) obtained from endothelial cells heparan sulfate after the combined action of heparitinases were incubated with 0.1 units of glycuronidase or disaccharide sulfoesterase as described in Fig. 2.

TABLE II. [^{35}S]-Sulfated Disaccharide Units Present in Heparan Sulfate From Endothelial Cells*

Disaccharides	Cells (%)	Medium (%)
ΔU,2S-GlcNS,6S	18 (± 1.5)	14 (± 1.0)
ΔU,2S-GlcNS	16 (± 1.0)	20 (± 1.2)
ΔU-GlcNS,6S	6 (± 1.0)	7 (± 0.8)
ΔU-GlcNS	46 (± 2.0)	43 (± 1.0)
ΔU-GlcNAc,6S	14 (± 0.9)	16 (± 0.7)

*The unsaturated disaccharides were separated by paper chromatography and the amount of radioactivity incorporated into the individual species was quantitated as indicated under Methods. The values represent the average of 4 different experiments, expressed as mean ± standard error.

type of disaccharide units are present in both heparan sulfates.

Analyses of the chondroitin sulfate indicate the presence of both 6-sulfated disaccharide (60%) and 4-sulfated disaccharide (40%). Furthermore no differences were observed in the degradation pattern obtained by chondroitinases AC and ABC, demonstrating that the chondroitin sulfate synthesized by endothelial cells contains only glucuronic acid residues (results not shown).

Effect of Monensin on the Synthesis of [^{35}S]-Sulfate or [^{3}H]-Glucosamine Labeled Glycosaminoglycans

Endothelial cells in culture were labeled with [^{35}S]-sulfate or [^{3}H]-glucosamine in the presence of different concentrations of monensin.

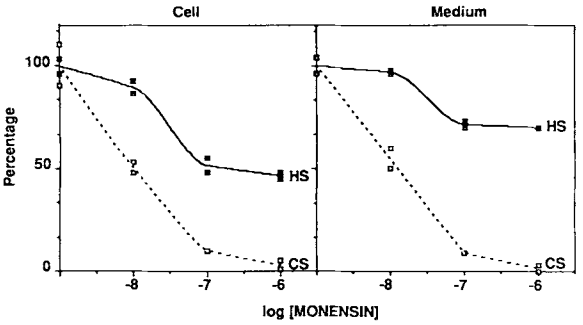

Fig. 5. Effect of monensin on the incorporation of [^{35}S]-sulfate into glycosaminoglycans from cells and medium. The endothelial cell cultures were incubated with different molar concentrations of monensin (as shown in the figure) and the [^{35}S]-heparan sulfate (■—■) and [^{35}S]-chondroitin sulfate (□—□) synthesized were analyzed as described in Methods. The data are expressed as percentage of heparan sulfate synthesized in the absence of monensin.

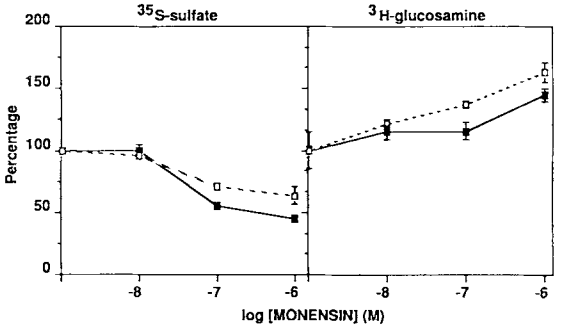

Fig. 6. Effect of monensin on the incorporation of [^{35}S]-sulfate and [^{3}H]-glucosamine into heparan sulfates from cells (■—■) and medium (□—□). The values were obtained as described in Methods and represent the mean ± standard error of 4 different experiments.

Sulfate incorporation into chondroitin sulfate and heparan sulfate was inhibited for both cells and medium in a dose-dependent manner (Fig. 5). Curiously there is a marked difference in the observed effect for heparan sulfate and chondroitin sulfate. Monensin at 10^{-8} M inhibits around 50% of the sulfation of chondroitin sulfate from cell and medium, with practically no effect on the sulfation of heparan sulfate. The incorporation of sulfate into heparan sulfate is affected only at higher concentrations of the drug ($\geq 10^{-7}$ M). The effect of monensin on [^{3}H]-glucosamine incorporation into glycosaminoglycans was also investigated. Figure 6 compares the effect of monensin on the [^{35}S]-sulfate and [^{3}H]-glucosamine incorporation on the heparan sulfates from cells and medium. Whereas sulfate incorporation was markedly de-

creased, the [^{3}H]-glucosamine incorporation was increased up to 50% at high monensin concentration. This increase was not related to cell division since the number of cells was constant in all experimental conditions. Similar findings were also observed by other authors using different cell lines and studying chondroitin/dermatan sulfate synthesis [Tajiri et al., 1980; Yanagishita and Hascall, 1985] and will be discussed later.

The concentration of [^{35}S]-PAPS in both control and monensin-treated cultures was measured and no differences were observed (results not shown). Thus, the decrease in sulfation was not due to a decrease in the PAPS pool.

Effect of Monensin on the Charge and Size of Heparan Sulfate

The [^{35}S]-heparan sulfates synthesized by the cells at different concentrations of monensin were analyzed regarding charge density and molecular weight. Figure 7 shows that the heparan sulfate synthesized in the presence of monensin has a slower electrophoretic mobility in agarose gel, pH 2.0, when compared to the one from control cells. This behavior reflects a decrease in the net charge of the polymer due to the amount of sulfate residues in the molecule. On the other hand heparan sulfate synthesized in the absence or presence of different concentrations of monensin displays the same profile on a Sepharose CL-6B column (Kav = 0.41), indicating that the drug is not affecting the heparan sulfate chain elongation (Fig. 8).

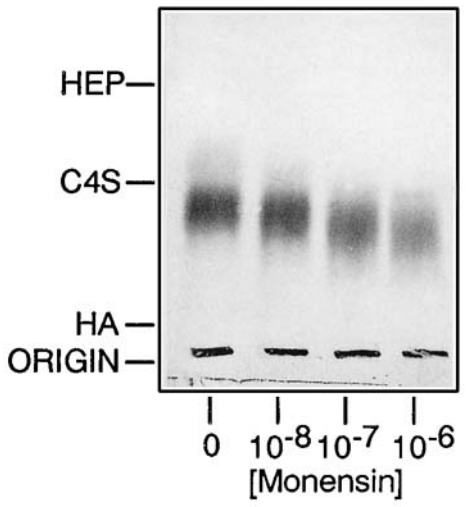

Fig. 7. Electrophoretic behavior of [^{35}S]-heparan sulfates synthesized in the absence as well as in the presence of different concentrations of monensin. About 5,000 cpm of [^{35}S]-heparan sulfates from the cells were applied to the agarose gel and subjected to electrophoresis in 0.05 M of KCl/HCl buffer (pH 2.0) for 30 min at 120 V. After fixation and staining, an autoradiogram was prepared by exposing the gel to X-ray film. C4S, chondroitin 4-sulfate; HA, hyaluronic acid; HEP, heparin.

Effect of Monensin on the Structure of Heparan Sulfate

[^{35}S]-heparan sulfate synthesized by endothelial cells at different concentrations of monensin was incubated with heparitinases I and II, and the radioactive products visualized and quantitated after paper chromatography and radioautography. A decrease of ΔU,2S-GlcNS,6S and ΔU-GlcNAc,6S is observed with increasing concentrations of monensin. Table III shows that the relative decrease of the 6-sulfated disaccharides is accompanied by a relative increase of the N-sulfated and uronic acid sulfated ones in the presence of increasing concentrations of monensin. Thus the decrease in sulfation is due to a decrease in the relative amount of disaccharides bearing a sulfate at C-6 position of the glucosamine moiety—that is, ΔU,2S-GlcNS,6S (A), ΔU-GlcNS,6S (B_1), and ΔU-GlcNAc,6S (D).

Effect of Monensin on the Structure of Chondroitin Sulfate

As shown in Figure 5, monensin decreases the sulfation of chondroitin sulfate from endothelial cells. Analyses of the degradation products of the chondroitin sulfate, synthesized in the absence and presence of monensin, formed by the action of chondroitinase AC, show that the ratio of 6-sulfated disaccharide to 4-sulfated disaccharide (ΔU-GalNAc,6S/ΔU-GalNAc,4S) decreases

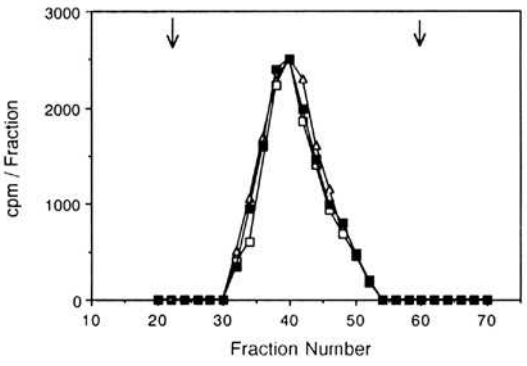

Fig. 8. Gel filtration in Sepharose CL-6B of [^{35}S]-heparan sulfates synthesized in the absence as well as in the presence of different molar concentrations of monensin. About 20,000 cpm of [^{35}S]-heparan sulfates from the cell fraction were applied to a column of Sepharose CL-6B in the conditions described in Methods. Control (Δ—Δ); 10^{-8} M of monensin (□—□); 10^{-7} M of monensin (■—■).

TABLE III. Relative Amounts of [^{35}S]-Sulfated Disaccharide Units Present in Heparan Sulfate Synthesized by Endothelial Cells Treated With Monensin*

Disaccharides	Control	Monensin 10^{-8} M	Monensin 10^{-7} M	Monensin 10^{-6} M
ΔU,2S-GlcNS,6S (A)	17.7 (± 1.5)	14.7 (± 0.7)	7.0 (± 0.6)	4.3 (± 0.7)
ΔU-GlcNS,6S (B_1)	6.5 (± 1.0)	5.0 (± 0.6)	3.0 (± 0.2)	1.6 (± 0.2)
ΔU,2S-GlcNS (B_2)	15.8 (± 1.0)	20.5 (± 0.6)	25.7 (± 0.2)	26.2 (± 0.2)
ΔU-GlcNS (C)	46.0 (± 2.0)	51.0 (± 1.0)	56.3 (± 4.0)	63.7 (± 1.5)
ΔU-GlcNAc,6S (D)	14.3 (± 0.9)	8.7 (± 0.7)	4.7 (± 0.7)	4.3 (± 0.7)

*The unsaturated disaccharides were separated by paper chromatography and the amount of radioactivity incorporated into the individual species was quantitated as indicated under Methods. The values represent the average of 4 different experiments, expressed as mean ± standard error.

from 1.5 in control to 0.4 at 10^{-7} M of monensin (results not shown).

DISCUSSION

Disruption of normal Golgi structures by monensin has been extensively studied by several authors [Farquhar, 1985; Griffiths et al., 1983; Tartakoff, 1982, 1983]. The present results show that in endothelial cells in culture, monensin inhibits sulfate incorporation into macromolecules in a dose-dependent manner. Inhibition of sulfation of chondroitin sulfate occurs at lower monensin concentration (10^{-8} M) than the inhibition of heparan sulfate (10^{-7} M). This is suggestive that the respective sulfotransferases are located at different Golgi sites.

Endothelial cells in culture synthesize and secrete to the medium an iduronic acid-rich heparan sulfate proteoglycan. Structural analyses of the medium and cell heparan sulfate clearly indicated that both are composed of 6 different disaccharide units and that among the sulfated disaccharides, the ones containing 2-sulfated iduronic acid residues correspond to about 35%. The effect of monensin upon the structure of the heparan sulfate was mainly due to a decrease in the overall sulfate incorporation. The polymer synthesized in the presence of the drug showed a decrease in the net negative charge without differences in the average molecular weight.

Based upon the specificity of action of heparitinases, glycuronidase, and disaccharide sulfoesterase, we were able to show that monensin interferes with the 6-sulfation of the glucosamine. The results have shown a decrease in the disaccharides bearing 6-sulfated hexosamines, with an increase of the respective non-sulfated ones.

Besides interfering with the 6-sulfation of the hexosamine, monensin also stimulates the incorporation of [^{3}H]-glucosamine into the polymer chains. Since there were no detectable changes in the molecular weight of the heparan sulfate and no increase of the number of cells in culture, one would initially suggest that monensin was stimulating the synthesis of a higher number of heparan sulfate chains. If this were the case, as judged by the results shown in Figure 6, at 10^{-6} M monensin concentration, we should have decreased by 4 times the overall sulfation of the heparan. Nevertheless the 6-sulfated residues account for only 25% of the heparan sulfate molecule. Thus, other explanations must be sought for this apparent discrepancy. Indeed, preliminary experiments have shown that the ratio of [^{3}H]-glucosamine/[^{35}S]-sulfate of the disaccharide ΔU-GlcNS (which contains only one sulfate per hexosamine) was increased by 2 times in the presence of 10^{-6} M of monensin. Similar results were also observed by Yanagishita and Hascall [1985] for the dermatan sulfate synthesized by rat ovarian granulosa cells. The authors suggested that this effect could be related to a decrease by monensin of the endogenous pool of UDP-N-acetyl hexosamine, leading to an increase in the incorporation of the ^{3}[H]-labeled precursor. This could also be the explanation for the apparent stimulation in endothelial cells of hexosamine incorporation in the presence of monensin.

Besides the action of monensin on the 6-O-sulfation all other steps involved in the biosynthesis of heparin/heparan sulfate have not been affected. The structural analyses of the different disaccharide units present in the polymer synthesized in the presence and absence of monensin have shown no differences in the relative amounts of N-acetylated and N-sulfated glucosamine units, as well as D-glucuronic acid and 2-O-sulfated iduronic acid moieties. These results suggest that the 6-sulfation is not a prerequisite to other steps of the biosynthesis of heparan sulfate and is thus probably the last event of this pathway. This is in agreement to the proposed role of monensin as a membrane-disruptive agent of the distal (trans) Golgi ele-

ments. The present results are also in accordance with the biosynthetic pathway of heparin proposed by Lindahl. The author, based upon the substrate specificity of the enzymes, has postulated that the heparin polymer is synthesized through a series of modification reactions where N-deacetylation, N-sulfation, and C-2 epimerization precedes the 6-O-sulfation [Lindahl, 1989].

The present results have also shown that the structure of chondroitin sulfate from endothelial cells was affected by monensin, leading to a decrease in the 6-sulfation of the galactosamine moiety. These data are in accordance with the ones reported in the literature for chondroitin sulfate from different cell types.

In conclusion, the 6-O sulfation of the hexosamine residues seems to be the common last step in the biosynthesis of heparin, heparan sulfate, and chondroitin sulfate and occurs in the trans-Golgi compartment.

ACKNOWLEDGMENTS

This research was aided by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), FINEP (Financiadora de Estudos e Projetos), Brazil, and NIH HL38208 (USA).

REFERENCES

Bianchini P, Nader HB, Takahashi HK, Osima B, Straus AH, Dietrich CP: J Chromatogr 196:455–462, 1980.

Bumol TF, Reisfel RA: Proc Natl Acad Sci USA 79:1245–1249, 1982.

Bumol TF, Walker LE, Reisfel RA: J Biol Chem 259:12733–12741, 1984.

Buonassisi V: Exp Cell Res 76:363–368, 1973.

Buonassisi V, Venter JC: Proc Natl Acad Sci USA 73:1612–1616, 1976.

Burditt LJ, Ratcliffe A, Freyer PR, Hardingham TE: Biochim Biophys Acta 844:247–255, 1985.

Dietrich CP, Braz J Med Biol Res 17:5–15, 1984.

Dietrich CP, Montes de Oca M: Proc Soc Exp Biol Med 134:955–962, 1970.

Dietrich CP, Nader HB: Biochim Biophys Acta 343:34–44, 1974.

Dietrich CP, Nader HB, Buonassisi V, Colburn P: FASEB J 2:56–59, 1988.

Dietrich CP, Nader HB, Straus AH: Biochem Biophys Res Commun 111:865–871, 1983.

Dietrich CP, Silva ME, Michelacci YM: J Biol Chem 250: 6841–6846, 1973.

Farquhar MG: Annu Rev Cell Biol 1:447–488, 1985.

Fransson, LÅ: In Lane DA, Lindahl U (eds): "Heparin. Chemical and Biological Properties Clinical Applications." London: Edward Arnold, 1989, pp 115–133.

Gallagher JT, Lyon M: In Lane DA, Lindahl U (eds): "Heparin. Chemical and Biological Properties Clinical Applications." London: Edward Arnold, 1989, pp 135–158.

Gallagher JT, Lyon M, Steward WP: Biochem J 236:313–325, 1986.

Griffiths G, Quinn P, Warren G: J Cell Biol 96:835–850, 1983.

Harper JR, Quaranta V, Reisfeld RA: J Biol Chem 261:3600–3606, 1986.

Hoppe W, Glössl J, Kresse H: Eur J Biochem 152:91–97, 1985.

Kajiwara T, Tanzer ML: FEBS Lett 134:43–46, 1981.

Kajiwara T, Tanzer ML: FEBS Lett 149:17–21, 1982.

Kraemer PM: Biochemistry 10:1437–1445, 1971.

Ledger PW, Tanzer ML: Trends Biochem Sci 9:313–314, 1984.

Lindahl U: In Lane DA, Lindahl U (eds): "Heparin. Chemical and Biological Properties Clinical Applications." London: Edward Arnold, 1989, pp 159–189.

Madsen K, Holmströms, Ostroski K: Exp Cell Res 148:493–501, 1983.

Mitchell D, Hardingham T: Biochem J 202:249–254, 1982.

Nader HB, Buonassisi V, Colburn P, Dietrich CP: J Cell Physiol 140:305–310, 1989.

Nader HB, Dietrich CP, Buonassisi V, Colburn P: Proc Natl Acad Sci USA 84:3565–3569, 1987.

Nader HB, Ferreira, TMPC, Paiva JF, Medeiros MGL, Jerônimo SMB, Paiva VMP, Dietrich CP: J Biol Chem 259:1431–1435, 1984.

Nader HB, Porcinatto MA, Tersariol ILS, Pinhal MA, Oliveira FW, Moraes CT, Dietrich CP: J Biol Chem 265: 16807–16813, 1990.

Nishimoto SK, Kajiwara T, Ledger PW, Tanzer ML: J Biol Chem 257:11712–11716, 1982a.

Nishimoto SK, Kajiwara T, Tanzer ML: J Biol Chem 257: 10558–10561, 1982b.

Perlin AS, Mackie DM, Dietrich CP: Carbohydr Res 18:185–194, 1971.

Poole, AR: Biochem J 236:1–14, 1986.

Rodén L: In Lennarz WJ (ed): "The Biochemistry of Glycoproteins and Proteoglycans." New York: Plenum Press, 1980, pp 267–371.

Spector T: Anal Biochem 86:142–146, 1978.

Tajiri K, Uchida N, Tanzer ML: J Biol Chem 255:6036–6039, 1980.

Tartakoff AM: Trends Biol Sci 7:174–176, 1982.

Tartakoff AM: Cell 32:1026–1028, 1983.

Yanagishita M, Hascall VC: J Biol Chem 260:5445–5455, 1985.