Reduced Sulfation of Liver Heparan Sulfate in Experimentally Diabetic Rats

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SUMMARY

Analyses by anion exchange chromatography indicate that heparan sulfate proteoglycans isolated from the livers of experimentally diabetic rats have a reduced net negative charge as compared with heparan sulfate proteoglycans from control rats. In contrast, the size of the normal and diabetic proteoglycans are indistinguishable as are the size of individual polysaccharide chains when compared by gel chromatography. The reduced net negative charge of heparan sulfate from diabetic rats is shown to be caused by a reduced number of sulfate groups present in the diabetic heparan sulfate. Results are presented suggesting that one of the biosynthetic enzymes; the N-deacelylase, which tentatively has been identified as a regulatory enzyme affecting the sulfate content of the polysaccharide chains, may be inhibited in the diabetic rat. DIABETES 32:337–342, April 1983.

Heparan sulfate proteoglycans are negatively charged macromolecules that are associated with the surface of a large number of different cell types. The proteoglycans are found either directly bound to the plasma membrane of cells[1–3] or in close vicinity of the cells in basement membranes and related structures.[4,5]

Basement membranes exhibit a filtration capacity, preventing the passage of certain macromolecules.[6] This property is particularly important in the glomerular basement membrane where the filtration results in the retention of proteins in the blood plasma during formation of urine. Fixed negative charges are believed to constitute one kind of filtration barrier in the glomerular basement membrane.[7] Thus, since heparan sulfate contributes to the negative charge,[8] it may have a role in the filtration process. In support of this hypothesis, Kanwar et al. showed that enzymatic removal of heparan sulfate from isolated glomerular basement membrane resulted in an increased leakage of ferritin[9] and serum albumin[10] through the membrane.

Thickening of the basement membranes is a complication of long-standing diabetes.[11] In the kidneys the disorder in basement membrane metabolism leads to a leakage of plasma constituents to the urine. The question of whether changes in heparan sulfate structure and metabolism can be responsible for the impaired filtration has recently been addressed.[12,13] Cohen and Surma have shown that ^{35}S-sulfate incorporation into rat glomerular basement membrane glycosaminoglycans in vitro was decreased in experimental diabetes, while the content of glycosaminoglycan, measured as uronic acid content, was not significantly lowered.[12] Rohrbach et al. showed that less ^{35}S-sulfate was incorporated into the heparan sulfate proteoglycan of a basement membrane producing tumor when the tumor was grown in genetically diabetic mice.[13] These authors could also show that the amount of core protein present in the tumor was reduced.

In this investigation we considered the possibility that there are structural changes in the heparan sulfate proteoglycan produced in diabetic animals. A lower degree of sulfate substitution could explain or contribute to the reported decreased ^{35}S-sulfate incorporation, as pointed out also by Cohen and Surma.[12] Rat liver was chosen as a source of heparan sulfate proteoglycans since proteoglycans isolated from rat liver membranes have previously been characterized in some detail.[14]

The "normal" liver proteoglycan isolated from apparently healthy rats has a molecular weight of about 75,000 and contains four polysaccharide chains (mol wt 14,000) attached to a protein core (mol wt 20,000). Recent work in our laboratory demonstrates the presence of two types of membrane-associated proteoglycan: an intercalated membrane proteoglycan with the core protein rooted in the lipid bilayer,

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and a peripheral membrane proteoglycan with the polysaccharide chains reversibly bound to specific receptors.[3,15]

MATERIALS AND METHODS

Streptozotocin and papain (2 × crystallized) were purchased from Sigma. ^{35}S-sulfate (carrier-free) and ^{3}H-glucosamine (20 Ci/mmol) were obtained from Amersham, Searle. DEAE-Sephacel, Sepharose CL-4B, Sepharose 6B, Sephadex G-50 were from Pharmacia and Bio-Gel P-2 from Bio-Rad. Heparin was obtained from Inolex (Park Forest South, Illinois) and purified as described.[16] Disaccharide standards for gel chromatography and paper electrophoresis were a kind gift from Dr. Ingvar Jacobson at this laboratory. Rats of the Sprague-Dawley strain were obtained from Southern Animal Farm.

To induce diabetes, rats were injected with streptozotocin (45 mg per kg body weight) in 0.05 M citrate, pH 4.2, 2–5 wk prior to the experiments. Control rats were sham injected at the same time. The blood glucose and insulin values were routinely checked every third week and/or immediately before the experiment. Only diabetic rats with glucose values ≥ 500 mg/100 ml plasma and insulin values ≤ 15 μU/ml were used in the study.

Isolation of heparan sulfate proteoglycans. Intercalated and peripheral membrane heparan sulfate proteoglycans were purified from isolated rat liver plasma membranes as previously described.[15] Rats were injected with 1 mCi of ^{35}S-sulfate or ^{3}H-glucosamine 2 h before they were killed. The livers were dissected out and homogenized, and plasma membranes were isolated from the nuclear fraction using the method of Ray.[17] The buffers used in the isolation of the membranes were supplemented with the protease inhibitors benzamidine (1 mM) and phenylmethylsulfonyl fluoride (0.1 mM) as were the buffers used in the purification of the proteoglycans. Peripheral heparan sulfate proteoglycans were released from their "receptors" by incubating the membranes obtained from two rats in 25 ml of 0.05 M Tris-HCl buffer pH 8.0 containing 0.35 M NaCl and 0.l mg/ml of heparin. After centrifugation, the membrane pellet was solubilized in the same buffer supplemented with 1% Triton X-100. After a second centrifugation the supernatant was applied to a DEAE-Sephacel column. Prior to elution the pH was lowered by washing the column with 0.05 M acetate buffer pH 4.0 containing 0.35 M NaCl. All buffers used in the further purification of the intercalated proteoglycan were supplemented with 0.1% Triton X-100. The column was eluted in 0.05 M acetate buffer pH 4.0 with a gradient ranging from 0.35 to 1.5 M NaCl. Heparan sulfate proteoglycans eluted as a homogenous peak in the gradient. The heparan sulfate purified by this procedure did not contain any delectable ^{3}H- or ^{35}S-labeled contaminant.[15] Unless otherwise stated, the proteoglycans were further purified by gel chromatography on Sepharose 4B-CL columns. The proteoglycans were thereby separated from a low-molecular-weight heparan sulfate polysaccharide, which has been shown to be a degradation product of the proteoglycans (L. Kjellén, H. Pertoft, Å. Oldberg, and M. Höök, manuscript in preparation).

Isolation of polysaccharide chains. For isolation of ^{35}S- or ^{3}H-labeled heparan sulfate chains, peripheral and intercalated membrane proteoglycans were co-purified by solubilizing the isolated plasma membranes in buffers containing both heparin and Triton X-100, followed by DEAE-ion exchange chromatography and gel chromatography as described above. According to previous results,[15] about one-third of the labeled proteoglycans in this preparation were originally peripheral and two-thirds were intercalated membrane proteoglycans. The polysaccharide chains were released from the core proteins by either alkali treatment (0.05 M NaOH for 48 h at room temperature) or papain digestion carried out as previously described.[18]

Nitrous acid deamination of heparan sulfate was performed at pH 1.5 under conditions where N-sulfated glucosamine residues are cleaved with concomitant loss of sulfamino groups.[19] Radioactivity was determined in a Packard scintillation counter using Scintiverse as emulsifier.

RESULTS

CHARACTERISTICS OF NORMAL AND DIABETIC HEPARAN SULFATE

Polyanionic properties. Heparan sulfate proteoglycans from control and diabetic rats were compared by anion exchange chromatography. ^{3}H-labeled proteoglycans, isolated from control rats, were mixed with ^{35}S-labeled proteoglycans isolated from diabetic rats and applied to a column of DEAE-Sephacel eluted with a shallow NaCl gradient. Intercalated heparan ^{35}S-sulfate proteoglycans produced in diabetic rats eluted somewhat ahead of the normal intercalated proteoglycans (Figure 1A). A similar difference in elution positions was observed between peripheral membrane heparan sulfate proteoglycans purified from control and diabetic rats (data not shown). This difference in elution positions was reproducible and found also, although to a lesser degree, between isolated heparan sulfate chains (Figure 1C). On the other hand, ^{35}S- and ^{3}H-labeled proteoglycans obtained from separate control rats co-eluted as a single peak when chromatographed on the same column (Figure 1B). Thus, it appears likely that the partial separation of normal and diabetic heparan sulfate proteoglycans by anion exchange chromatography is the consequence of structural differences between the molecules.

The elution position of a glycosaminoglycan on anion exchange chromatography depends not only on the charge but also on the size of the molecule. Therefore the early elution position of the diabetic proteoglycan can be caused by a lower number of polysaccharide chains per proteoglycan molecule and/or shorter polysaccharide chains in addition to a reduced net negative charge of the molecule.

Size. To compare the size of the proteoglycans ^{3}H-labeled proteoglycans from normal rat liver were co-chromatographed with ^{35}S-labeled diabetic proteoglycans on a column of Sepharose 4B-CL. Peripheral membrane proteoglycans (Figure 2A) as well as intercalated membrane proteoglycans (Figure 2B) from the diabetic rats had similar size distributions as the corresponding proteoglycans isolated from control rats. The second peak in Figure 2A represents heparan sulfate oligosaccharides from lysosomes where they undergo degradation. This material is released from the membrane fraction on incubation with heparin (L. Kjellén, H. Pertoft, Å. Oldberg, and M. Höök; manuscript in preparation). The sizes of the polysaccharide chains of normal and diabetic proteoglycans were compared by gel chromatography on a column of Sepharose 6B. The chains had

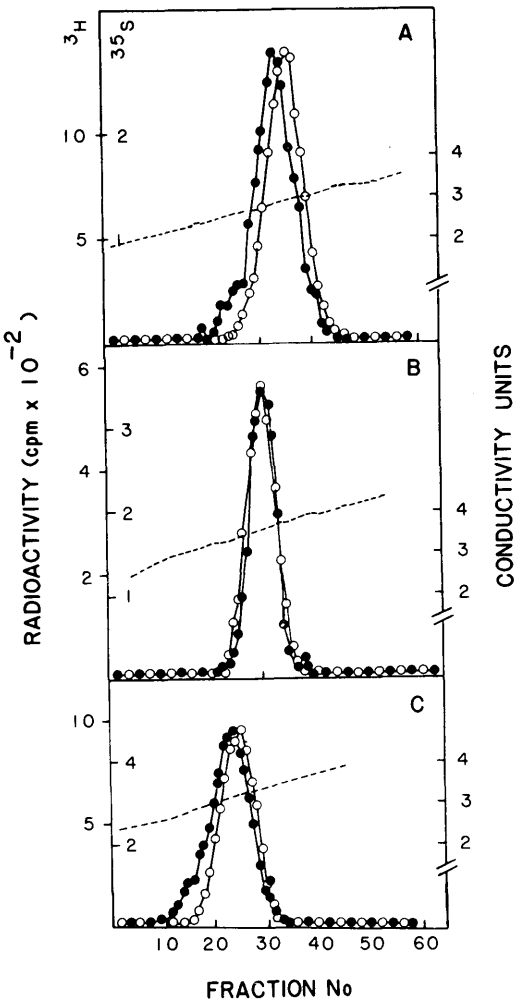

FIGURE 1. Chromatography on DEAE-Sephacel of heparan sulfate from control and diabetic rats. Heparan sulfate samples to be analyzed were applied to a column (1 × 3 cm) of DEAE-Sephacel equilibrated in 0.2 M NaCl 0.05 M acetate buffer, pH. 4.0. The column was then washed with 0.5 M NaCl in 0.05 M acetate, pH 4.0, and eluted at room temperature with a linear gradient (total volume 150 ml) from 0.5 to 1.0 M NaCl in acetate buffer, pH 4.0. For analyses of membrane intercalated proteoglycans, the buffers were supplemented with 0.1% Triton X-100. The flow rate was 8 ml/h and fractions of about 3 ml were collected. Each fraction was analyzed for ^{3}H- and ^{35}S-radioactivity and conductivity. The following samples were analyzed: (A) intercalated membrane proteoglycans from rat livers of control (o, ^{3}H-labeled) and diabetic (•, ^{35}S-labeled) rats; (B) intercalated membrane proteoglycans purified from the livers of two different control rats (o, ^{3}H-labeled), (•, ^{35}S-labeled); (C) heparan sulfate chains obtained after papain digestion of proteoglycans isolated from control (•, ^{3}H-labeled) and diabetic (•, ^{35}S-labeled) rats.

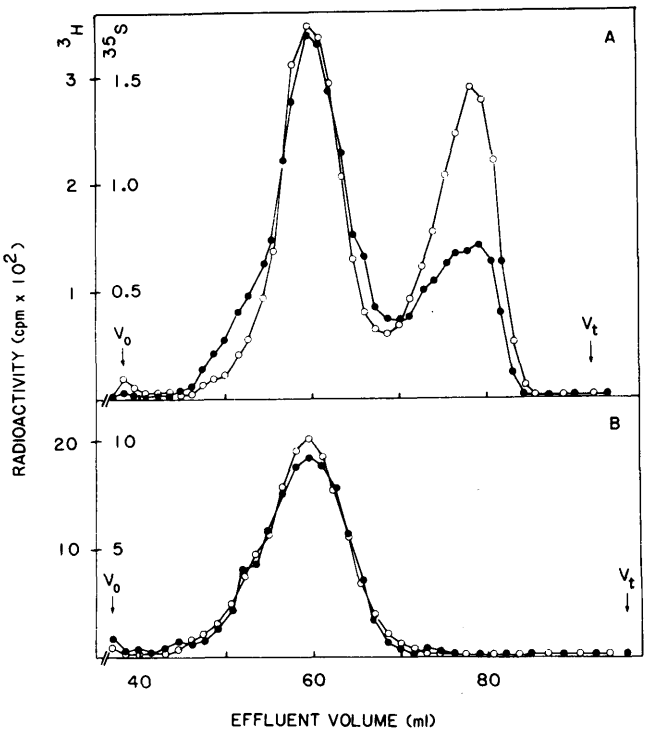

FIGURE 2. Gel chromatography on Sepharose (CL-4B of heparan sulfate proteoglycans from control and diabetic rats. Heparan sulfate proteoglycans were purified from livers of control and diabetic rats injected with [^{3}H]glucosamine and [^{35}S]sulfate, respectively. Prior to gel chromatography (see MATERIALS AND METHODS), samples of ^{3}H-labeled proteoglycans from control rats were mixed with ^{35}S-labeled proteoglycans from diabetic rats and applied to a column (95 × 1 cm) of Sepharose CL-4B eluted with 0.1% SDS, 0.05 M Tris HCl buffer pH 8.0. Fractions of about 1.3 ml were collected and analyzed for ^{3}H- (o) and ^{35}S-radioactivity (•). Blue dextran and DNP-alanine were used to indicate the void and total volume of the column, respectively. Samples analyzed were: (A) peripheral heparan sulfate proteoglycans, and (B) intercalated heparan sulfate proteoglycans.

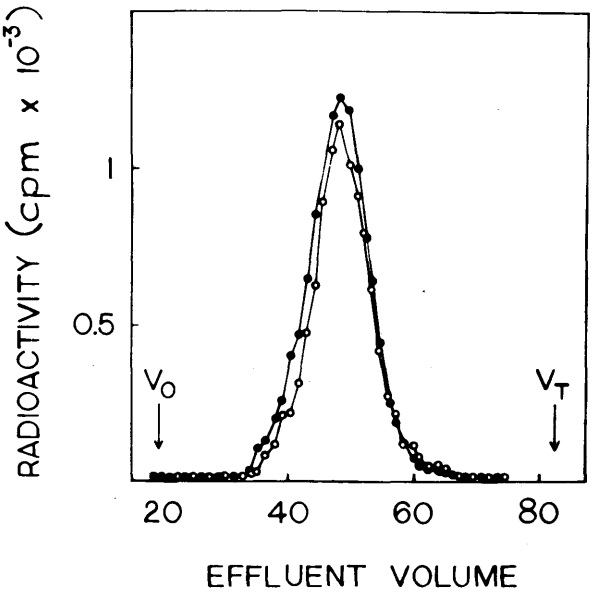

FIGURE 3. Gel chromatography on Sepharose 6B of heparan sulfate chains from control and diabetic rats. Polysaccharide chains obtained after alkali treatment of ^{3}H- and ^{35}S-labeled proteoglycans purified from control and diabetic rats, respectively, were applied to a column of Sepharose 6B eluted with 1 M NaCl. Fractions of about 1.2 ml were collected and analyzed for ^{3}H- (o) and ^{35}S-radioactivity (•).

previously been released from the core proteins by treatment of the proteoglycans with 0.5 M NaOH. As shown in Figure 3 the ^{35}S-labeled chains released from the diabetic proteoglycan were eluted at the same position as the ^{3}H-labeled chains from the normal proteoglycan.

The polysaccharide chains of the proteoglycans isolated from diabetic rats thus have the same average length as the polysaccharide chains of the normal proteoglycan. As the proteoglycans are of similar size it appears reasonable to assume that they contain the same number of polysaccharide chains. The difference in elution position of the two proteoglycans on anion exchange chromatography is therefore

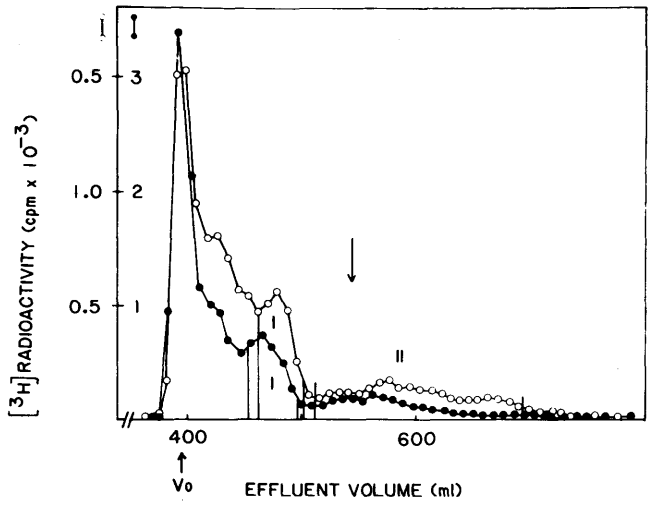

FIGURE 4. Gel chromatography on Bio-Gel P-2 of nitrous acid degraded ^{3}H-labeled heparan sulfate from control and diabetic rats. Heparan sulfate chains, isolated from papain digested ^{3}H-labeled heparan sulfate proteoglycans were degraded by nitrous acid treatment. The deamination products of normal heparan sulfate chains (o) and heparan sulfate chains obtained from diabetic rats (•) were separately applied to a column (~ 1100 ml) of Biogel P2 eluted with 0.2 M ammonium bicarbonate. Fractions of about 9 ml were collected and analyzed for radioactivity. The arrow indicates the elution position of a disulfated uronosylanhydromannose standard. Fractions containing tetrasaccharides (pool I) and disaccharides and smaller material (pool II), respectively, were combined as indicated by the vertical bars.

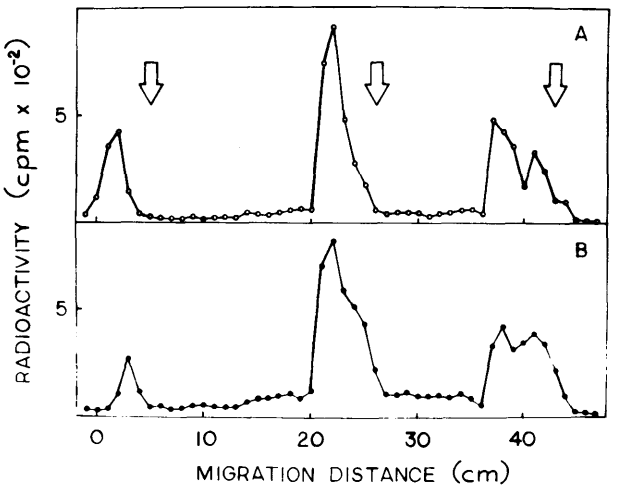

FIGURE 5. Paper electrophoresis of heparan sulfate disaccharides isolated after nitrous acid deamination. Pool II containing disaccharides generated from normal (A) and diabetic (B) heparan sulfate were evaporated, dissolved in water and spotted on Whatman 3 mm paper and subjected to electrophoresis in 0.1 M formic acid, pH 1.7. Subsequently the paper was dried and cut into 1-cm strips, which were transferred to scintillation vials and assayed for radioactivity. The arrows indicate the migration distances of standards; from left, uronosyl-anhydromannitol, mono-sulfated uronosyl-anhydromannitol, and disulfated uronosyl-anhydromannitol.

most likely due to a reduced net negative charge in the polysaccharide chains of the diabetic proteoglycan.

SULFATE SUBSTITUTION OF NORMAL AND DIABETIC HEPARAN SULFATE

A heparan sulfate chain is composed of alternating units of uronic acid (D-glucuronic or L-iduronic acid) and glucosamine. The carboxyl group of the uronic acid residue is negatively charged under physiological conditions. Sulfate groups occur attached to the carbohydrate skeleton in ester linkage to carbon atom 2 of the iduronic acid unit and carbon atom 6 of the glucosamine residue as well as in amide linkage to the hexosamine unit. Since the density of uronic acid carboxyl groups is fixed, the observed difference in polyanionic properties between normal and diabetic heparan sulfate most likely reflects a difference in their sulfate contents.

The content of N-sulfated glucosamine residues in normal and diabetic heparan sulfate was compared by subjecting ^{3}H-labeled polysaccharides to nitrous acid deamination under conditions where N-sulfated glucosaminidic linkages are selectively cleaved. The extent of polysaccharide degradation thus reflects the content of sulfamino groups in the intact polymers. Chromatography of nitrous acid treated polymers on a column of Bio-Gel P-2 (Figure 4) revealed a more extensive degradation of the normal heparan sulfate than of the polysaccharide produced in diabetic rats. Thus, heparan sulfate produced in the livers of normal rats contains a higher density of N-linked sulfate groups compared to liver heparan sulfate obtained from diabetic rats.

The content of ester-linked sulfate groups was determined by paper electrophoresis at pH 1.7 of heparan sulfate oligosaccharides isolated by gel chromatography after nitrous acid deamination (Figures 5 and 6). At this pH, sulfate groups are the only charged residues, and since sulfamino groups are lost during deamination, the migration rates of the oligosaccharides reflect their content of ester-linked sulfate groups. Since previous studies[28] have indicated that ester-linked sulfate groups are preferentially located in N-sulfated regions of the polysaccharide, these analysis were restricted to di- and tetra-saccharides.

Electrophoresis of disaccharides from normal and diabetic heparan sulfate resulted in essentially idential patterns (compare Figures 5A and B). The migration of the three major

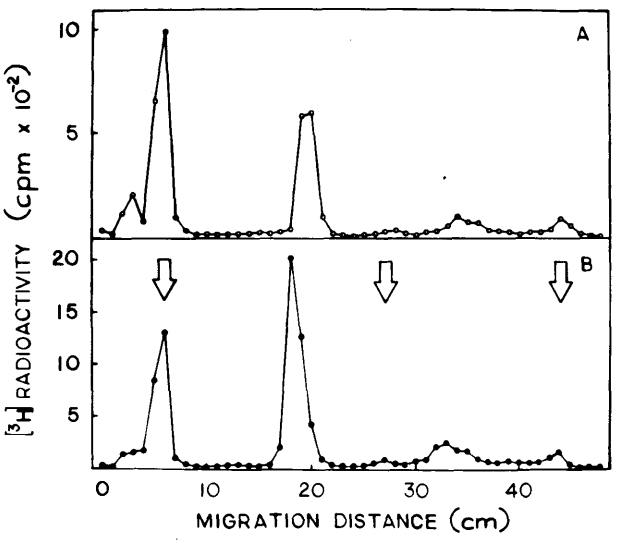

FIGURE 6. Paper electrophoresis of heparan sulfate tetrasaccharides isolated after nitrous acid deamination. Pool I containing tetrasaccharides generated from normal (A) and diabetic (B) heparan sulfate were subjected to high voltage paper electrophoresis. Experimental details were as described in the legend to Figure 5.

peaks of labeled material* corresponded to those of non-sulfated, monosulfated, and disulfated disaccharides, respectively.[20]

Electrophoresis of the tetrasaccharides resulted in the separation of two major peaks (Figure 6), presumably containing no or one sulfate residue per molecule.[21] Two minor components of higher sulfate content could also be detected. The proportion of tetrasaccharides containing sulfate groups was somewhat larger in the tetrasaccharide fraction obtained from diabetic heparan sulfate than in the tetrasaccharides from normal heparan sulfate. However, this difference has in repeated experiments not been reproducible.

Taken together, the analysis of sulfate substituents show that heparan sulfate originating from the diabetic rats has a reduced content of sulfamino groups compared with that isolated from control rats, which is demonstrated by a reduced degradation of the diabetic polysaccharide by nitrous acid treatment. However, N-sulfated regions in the polysaccharides that on nitrous acid deamination are cleaved to di- and tetra-saccharides have essentially the same contents of O-sulfate groups regardless of whether the heparan sulfate originates from diabetic or control rats.

DISCUSSION

Little is known about the biosynthesis of heparan sulfate. However, the main enzymatic steps involved in the biosynthesis of heparin, a structurally related polysaccharide, have been elucidated in cell-free experiments (for a review see ref. 21). As the two polysaccharides contain the same structural components it appears likely that the biosynthetic procedures are closely related.[22]

In heparin biosynthesis, a polymer consisting of repeating N-acetylglucosamine and glucuronic acid residues is initially formed by a stepwise transfer of sugar units from corresponding UDP derivatives. Subsequently, a series of polymer modification reactions takes place. First, a N-deacetylase removes some of the acetyl groups from the N-acetylglucosamine residues exposing primary amino groups that then are accessible to N-sulfation catalyzed by an N-sulfo-transferase. Glucuronic acid is then epimerized to iduronic acid followed by O-sulfation of C-2 of the iduronic acid and of C-6 of the glucosamine residues.

The polymer modification reactions are all incomplete (with N-sulfation as a possible exception) leaving some sugar units unmodified. The extent of polymer modification varies from one tissue to another. As a result, a considerable structural variation in heparan sulfates has been noted. The mechanisms regulating polymer modification are largely unknown. A regulatory role for the N-deacetylase has been suggested,[23] not only because deacetylation is a prerequisite for N-sulfation, but also because the later polymer modification reactions (e.g., uronic acid epimerization and O-sulfation) preferentially occur in the vicinity of N-sulfated glucosamine units due to the substrate specificities of the enzymes involved.[22,24]

In the present communication, we report that rat liver heparan sulfate proteoglycans produced in experimentally diabetic rats have a reduced sulfate content. Less N-sulfate groups are found in the polysaccharide chains, suggesting that the activity of the N-deacetylase and/or the N-sulfotransferase† is inhibited in the diabetic rat.

In contrast, the O-sulfotransferases seem to work normally since the amount of ester-sulfate groups in di- and tetrasaccharides obtained after nitrous acid treatment of normal and diabetic heparan sulfate are essentially the same. Therefore, no difference in the ratio of N- to O-sulfate could be noted in heparan sulfate isolated from control and diabetic rats, respectively. Since the amount of N-sulfate groups is reduced in the diabetic heparan sulfate the overall content of O-sulfate groups is also lower in diabetics compared with normal heparan sulfate, magnifying the effect of the impaired N-sulfation.

The present study deals with the biosynthesis of heparan sulfate in liver. Although the sulfate content of the polysaccharide may vary greatly from one tissue to another, the same set of enzymes are presumably involved in the synthesis of heparan sulfate in all cells of the body. Consequently, a reduction in sulfate content may be a common feature of heparan sulfate in different tissues of the diabetic animal.

The physiologic role of cell surface heparan sulfate is poorly understood. Preliminary data has led to speculations about its possible role in different types of cellular interaction phenomena (e.g., adhesion and control of proliferation and differentiation.[25–27] Reduced sulfation of heparan sulfate has been shown to affect the affinity of the cell surface receptor for the polysaccharide.[3] Whether a reduced sulfate content of cell surface heparan sulfate could be correlated with any of the pathologic changes seen in diabetes remains to be investigated.

It is also possible that the anchoring of heparan sulfate in the basement membrane involves an interaction between heparan sulfate polysaccharide chains and other basement membrane components and that the strength of this binding is related to the charge density of the polysaccharides. Along this line it has been found that the apparent affinity of heparan sulfate for fibronectin is reduced in polysaccharides with lower sulfate contents (J. Robinson and M. Hook, manuscript in preparation). In the diabetic glomerular basement membrane, an under-sulfated heparan sulfate could therefore not only reduce the negative charges of those molecules incorporated into the basement membrane but also result in a lower number of molecules being incorporated into the tissue.

ACKNOWLEDGMENTS

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*The peak of highest mobility was partly separated into two fractions. Due to lack of material, these fractions were not further analyzed. However, previous analyses of similar material have resulted in the tentative identification of a monosulfated monosaccharide in addition to the disulfated disaccharide.[28]

†N-unsubstituted glucosamine units are selectively cleaved in a nitrous acid deamination procedure carried out at pH 3.8.[29] Analysis of the deamination products obtained under these conditions indicates that normal and diabetic heparan sulfate contains 2–10 unsubstituted amino groups per chain. However, different results were obtained in different experiments and a reproducible difference between normal and diabetic heparan sulfate could not be demonstrated. Furthermore, it is unclear whether N-unsubstituted glucosamine groups are actually present in heparan sulfate in the tissue or whether the primary amino groups are a consequence of a loss of sulfamino groups during the isolation procedure.

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