Biosynthesis of Heparan Sulfate in Rat Liver
CHARACTERIZATION OF POLYSACCHARIDES OBTAINED WITH INTACT CELLS AND WITH A CELL-FREE SYSTEM*

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A rat liver microsomal fraction was incubated with UDP-[14C]glucuronic acid and UDP-β-acetylgalactosamine. Chromatography of the resulting labeled polysaccharide on DEAE-cellulose yielded two distinct components, a fully N-acetylated and a partially N-deacetylated polysaccharide, respectively. Addition of 3′-phosphoadenylylsulfate to a microsomal fraction containing such preformed, nonsulfated polysaccharide resulted in the conversion of the partially N-deacetylated component into sulfated products. These products were partially separated, by ion exchange chromatography, into a less retarded, essentially N-sulfated fraction, and a more retarded, N- and O-sulfated fraction. In the two fractions, [14C]iduronic acid comprised 35 and 65%, respectively, of the total labeled hexuronic acid. In either fraction about 25% of the glucosamine residues were N-acetylated. The isolated polysaccharides were highly similar to compounds previously implicated as intermediates in the biosynthesis of heparin (see Jacobsson, L., and Lindahl, U. (1980) J. Biol. Chem. 255, 5094–5100).

Incubation of isolated rat liver cells with [14C]glucosamine yielded labeled heparan sulfate (see Oldberg, Å., Höök, M., Obrink, B., Pertoft, H., and Rubin, K. (1977) Biochem. J. 164, 75–81). This material displayed less extensive polymer modification (40 to 50% of the glucosamine residues remained N-acetylated) than did the product of cell-free biosynthesis. A microsomal fraction prepared from the same batch of liver cells produced polysaccharides that were indistinguishable from those obtained with the microsomes from whole liver. These results suggest that the mechanism of heparan sulfate biosynthesis is similar to that of heparin biosynthesis. However, in the cell-free system, the regulation of the polymer-modification process is defective, resulting in the formation of an extensively modified, heparin-like polysaccharide. The regulatory mechanism apparently depends on the integrity of the intact cell.

Heparan sulfate proteoglycan are produced by most cells grown in vitro. The molecule appears to be preferentially located in the micro-environment of cells, either directly as

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1The abbreviations used are: N-sulfate, sulfamino group; O-sulfate, ester sulfate group; PAPS, 3′-phosphoadenylylsulfate; PS, polysaccharide.

2Recent studies indicated that the antithrombin-binding region of the heparin molecule contains a 3-O-sulfated glucosamine residue (11). The 3-sulfate group is assumed (but not yet proven) to be essential to the interaction of the heparin molecule with antithrombin, and thus, essential to anticoagulant activity. The biosynthesis of the 3-sulfate group has not been investigated; nor is it known whether it occurs only in heparin or also in heparan sulfate.
involved in each reaction. This incomplete polymer modification is characteristically reflected in the structural microheterogeneity of the final product.

While the biosynthesis of heparan sulfate has been documented in various cultured cells, little is known of its organization at the subcellular level. Levy et al. (12) observed the formation of an N- and O-sulfated polysaccharide on incubating a microsomal fraction from pig aorta with UDP-glucuronic acid, UDP-N-acetylgalactosamine and PAPS. They also noted that N-sulfation appeared to precede O-sulfation. In the present investigation, the biosynthesis of heparan sulfate was studied in more detail, using a cell-free rat liver system.

The results indicate striking similarities with heparin biosynthesis and point to novel aspects regarding the regulation of polymer modification.

**EXPERIMENTAL PROCEDURES**

**Materials**—The glycosaminoglycans used as reference compounds were as described (10). UDP-[3H]glucuronic acid was prepared from d-[1-3H]glucose (250-360 mCi/mmol, New England Nuclear) as described (13). d-[1-14C]Glucosamine hydrochloride (68 mCi/mmol) was purchased from the Radiochemical Center, Amersham, Bucks, U. K. Unlabeled UDP-glucuronic acid and UDP-N-acetylglucosamine were obtained from Sigma. Chondroitinase ABC was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. PAPS was prepared as reported previously (13). Hexuronolysin = 2,5-anhydro-[1-14C]mannitol disaccharides with or without O-sulfate groups in various positions were prepared as described (14).

Liver cells were isolated from male Sprague-Dawley rats by perfusion of the livers in situ with a collagenase solution (15). The resulting cell suspension was washed in a Ca2+- and Mg2+-containing buffer as described (16). More than 90% of the cells obtained by this procedure attach to a Petri dish coated with fibronectin (17) where they acquire a morphology characteristic of hepatocytes (18). Furthermore, at least 90% of the cells attach to a culture dish coated with asialooucerolipasin3, indicating the presence of the hepatocyte-specific receptor involved in the clearance of asialoglycoproteins from the plasma (19). Microscopic inspection of the cells after staining with toluidine blue (20) failed to show any metachromatically staining mast cells (5,000 cells counted).

A microsomal fraction was prepared from whole rat liver as follows. Two livers were finely divided with a pair of scissors in 100 ml of 0.25 M sucrose, containing 2 mM Tris-HCl, pH 7.4, and were homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000 × g for 20 min, and the supernatant was then centrifuged at 20,000 × g, at 4°C for 90 min in a microfuge. A microsomal fraction was collected by centrifugation of the 20,000 × g supernatant at 100,000 × g for 2 h. The pellet was washed once by suspension in 50 ml of the buffered sucrose, followed by recentrifugation at 200,000 × g for 2 h. The resuspended pellet was suspended in buffered sucrose. For the preparation of microsomal fraction from isolated hepatocytes, cells from four livers were suspended in 350 ml of 1 mM NaHCO3, containing 0.5 mM CaCl2, and left for 90 min in an ice bath. The lysed cells were then collected in a small volume by centrifugation at 2,000 × g for 3 min, and were homogenized in a 50-ml Dounce homogenizer with 25 strokes with a B pestle. The homogenate was mixed with the remainder of the supernatant from the centrifugation, and a microsomal fraction (sedimenting between 20,000 × g and 100,000 × g) was collected by centrifugation as described above.

**Analytical Methods**—The methods used to determine uronic acid, protein, radioactivity, and ratios of d-[1-14C]glucuronic acid to L-[14C] iduronic acid were as indicated in a previous report (13). High voltage paper electrophoresis was performed on Whatman 3MM paper in 1.6 M formic acid, pH 1.7, at 40 volts/cm. Ion exchange chromatography of polysaccharides on DEAE-cellulose (Whatman DE52) was carried out as described earlier (13; see also the legend to Fig. 1). The N-substituent pattern of heparin-related polysaccharides was analyzed by selective cleavage with nitrous acid followed by gel chromatography of the products. Polysaccharides were treated with nitrous acid at pH 1.5 or at pH 3.9, for deamination of N-sulfated or N-substituted glucosamine residues, respectively. The resulting deamination products were analyzed by ion exchange chromatography on DEAE-cellulose.

**RESULTS**

**Polysaccharide Produced by Microsomal Fraction from Whole Liver**—Incubation of rat liver microsomal fraction with UDP-[14C]glucuronic acid and UDP-N-acetylglucosamine, under the conditions described under “Experimental Procedures,” resulted in the formation of 10 to 20 × 103 cpm of [14C]-labeled polysaccharide/ml of incubation mixture and per h. The amount of labeled product increased with increasing concentration of UDP-N-acetylglucosamine within the range tested (up to 2.5 mM). About 40% of the labeled material was degraded to oligosaccharides on digestion with chondroitinase ABC. Ion exchange chromatography on DEAE-cellulose separated the chondroitinase-resistant [14C]polysaccharide into two distinct, major peaks, designated MI and M1 in Fig. 1A. Fraction MII co-eluted with standard hyaluronic acid whereas fraction M1 was less retarded. The chromatographic behavior of these compounds was similar to that of the fully N-acetylated and partially N-deacetylated heparin precursor polysaccharides, PS-NAc and PS-NH2, respectively, previously characterized in a mastocytoma microsomal system (9, 25).

Continued incubation of the microsomal fraction, containing such nonsulfated polysaccharide, in the presence of PAPS, resulted in the disappearance of component M1 and the appearance of a new component M2.

3 M. Höök, K. Rubin, and B. Öbrink, unpublished observation.
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**Fig. 1.** Chromatography on DEAE-cellulose of 14C-labeled rat liver microsomal polysaccharide, isolated before (A), or after (B) the addition of PAPS to the incubation mixtures (see "Experimental Procedures"). The isolated polysaccharides were digested with chondroitinase ABC, and were then applied to columns (1 × 5 cm) of DEAE-cellulose. The columns were washed with 5 bed volumes of 0.05 M LiCl in 0.05 M acetate buffer, pH 4.0, and were then eluted with a linear salt gradient (started at fraction 1) extending from 0.05 to 1.5 M LiCl in the same acetate buffer. Each gradient vessel contained 75 ml of salt solution; fractions of 5 ml were collected and analyzed for radioactivity. The arrows at the bottom of the figure indicate the peak elution positions of standard hyaluronic acid (HA), chondroitin sulfate (CS), and heparin (HEP). Effluent fractions were pooled as indicated by the vertical lines and were then desalted by dialysis. Most of the chondroitinase-susceptible material emerged with the wash fraction (not shown). The labeled components emerging in fractions 1 to 5, presumably incompletely chondroitinase-digested material, were not further investigated.

**Fig. 2A**

**Fig. 2B**

The analogy between the microsomal liver polysaccharides and the mastocytoma heparin-precursor polysaccharides was confirmed by structural characterization of the former species. Fraction MII resisted deaminative cleavage under any conditions tested (Fig. 2B) and was thus fully N-acetylated. Fraction MII was partly depolymerized by nitrous acid at pH 3.9 (Fig. 2A) but was unaffected at pH 1.5, indicating N-unsubstituted glucosamine residues. In contrast, fractions MIII (Fig. 2C) and MIV (Fig. 2D) were susceptible to deamination at pH 1.5 but not at pH 3.9, as expected for N-sulfated polysaccharides. Since fraction MI obviously represents a precursor of fractions MIII and MIV, it is concluded that the formation of N-sulfated polysaccharides in the liver system may proceed via a partially N-deacetylated intermediate species. The analogy with heparin biosynthesis (22, 25) is further emphasized by the observation that conversion of MII into MIV and MIV is accompanied by additional N-deacetylation, as evident from the shift toward more low molecular deamination products (Fig. 2). Moreover, electrophoretic analysis of deamination products showed considerable amounts of O-sulfate groups in fraction MIV, about 80% of the material migrating toward the anode at pH 1.7 (Fig. 3B). The major sulfated component appeared to be monosulfated heparosyl → 2,5-anhydromannose disaccharide; no disulfated disaccharide was seen. The much smaller amounts of O-sulfated components obtained from fraction MII (Fig. 3A) probably derives from contaminating MIV material; note that the separation between MIII and MIV in Fig. 1B is incomplete. [3H]Iduronic acid comprised 35 and 65% of the total labeled hexuronic acid in fractions MIII and MIV, respectively. These results further underline the similarity between fraction MIII and the heparin-precursor polysaccharide PS-NSO₃⁻, which contains N- but no O-sulfate groups and small amounts of iduronic acid. The transformation of PS-NSO₃⁻ into PS-N/O-SO₃⁻ involves O-sulfation and further C₅-epimerization of D-glucuronic acid into L-iduronic acid units (9, 13, 25).

**Polysaccharide Produced by Isolated Liver Cells—Hepar sulfate produced by cultured rat liver cells (hepatocytes) was characterized in a previous study (23). It differed from fraction MIV, i.e., the end product of the cell-free biosynthetic process, by its lower iduronic acid content (40% of the total uronic acid) and, in particular, by its N-substitution pattern, which indicated a blockwise distribution of N-acetylated glucosamine residues, typical for heparan sulfate. In contrast to the extended sequences of consecutive N-acetylated disaccharide units displayed by this polysaccharide (see Fig. 3 in Ref. 23), the N-acetyl groups in fraction MIV occurred largely as disulfated disaccharide was seen. The much smaller amounts of O-sulfated disaccharide units in the product of cell-free biosynthesis is reflected by the intact cells. On the other hand, it could be argued that cells other than hepatocytes, e.g., mast cells, might have contributed to the microsomal fraction prepared from whole liver, thus affecting the pattern of polymer modification. Such cells would not necessarily be represented in a population of isolated liver cells. In order to eliminate this objection, a microsomal fraction was isolated from the same preparation of liver cells as was used in whole cell biosynthesis experiments.

**Intact Cells—**Isolated liver cells were incubated with [14C] glucosamine for 16 h. The resulting labeled polysaccharide was isolated and digested with chondroitinase ABC in order to eliminate any hyaluronate or galactosaminoglycan present. Chromatography of the chondroitinase-resistant material on DEAE-cellulose revealed a component that was eluted largely after chondroitin 4-sulfate but before heparan sulfate (Fig. 4). The result of these studies was that this conclusion applies also to the incorporation of O-sulfate groups. The reason for the lack of di-O-sulfated disaccharide units in the product of cell-free biosynthesis is unknown.

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5 Comparison of the results shown in Fig. 3B with those in Figs. 3 and 4 of Ref. 23 indicates that this conclusion applies also to the incorporation of O-sulfate groups. The reason for the lack of di-O-sulfated disaccharide units in the product of cell-free biosynthesis is unknown.
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Fig. 3. Paper electrophoresis of products obtained on deamination of fraction MIII (A); and fraction MIV (B). Each sample (about 5,000 cpm) was treated with 50 μl of deamination reagent (pH 1.5) at room temperature for 10 min. The reactions were terminated by the addition of 5 μl of 2 M NaOH, and the mixtures were applied to the electrophoresis paper. The standards shown below the tracings are: I, nonsulfated hexuronosyl → anhydro[3H]mannitol; II, mono-O-sulfated hexuronosyl → anhydro[3H]mannitol; III, dis-O-sulfated Iduronosyl → anhydro[3H]mannitol.

Fig. 2. Gel chromatography on Sephadex G-25 of the microsomal polysaccharide fractions MI (A); MII (B); MIII (C); and MIV (D) following treatment with nitrous acid. About 5,000 cpm of each fraction were deaminated according to the combined pH 1.5–pH 3.9 procedure; the gel chromatography was performed as described under "Experimental Procedures." The fraction MII used was obtained from the incubation performed in the presence of PAPS (Fig. 1). Deamination of fraction MI at pH 3.9 only, yielded the same elution pattern as that shown in A, whereas deamination at pH 1.5 was without effect. Deamination of fractions MII and MIV at pH 1.5 only, yielded the same elution patterns as those shown in C and D, respectively, whereas deamination at pH 3.9 was without effect. The number above each peak indicates the number of monosaccharide units/molecule of the corresponding oligosaccharide. These numbers were assigned on the assumption that each two consecutive species differ by a disaccharide unit; the identity of the most retarded component, a disaccharide, was ascertained by co-chromatography with mono-O-sulfated hexuronosyl → anhydro[3H]mannitol. For explanation of the vertical lines, see Table I.

Fig. 4. Chromatography on DEAE-cellulose of [3H]-labeled polysaccharide obtained by incubating rat liver cells in vitro with [3H]-glucosamine (see "Experimental Procedures"). The chromatography was carried out as described in the legend to Fig. 1, except for the use of a slightly steeper salt gradient. Effluent fractions were pooled, as indicated by the vertical lines, and were then desalted by dialysis.

Consecutive sequence (Table I). The polysaccharide produced by the cultured liver cells thus displayed a distribution of N-acetyl groups typical of heparan sulfate, in agreement with previous results (23), but in contrast to the polysaccharide produced by the microsomal fraction derived from whole liver.

Microsomal Fraction—The microsomal fraction prepared from isolated liver cells was less efficient in producing labeled polysaccharide than was the corresponding material obtained from whole liver; the yield amounted to about 5 × 10⁸ cpm of [3H]polysaccharide/h and per ml of incubation mixture. However, apart from the difference in yield, the two preparations of microsomal polysaccharide were indistinguishable, both as
mental error in this method may be considerable, and the results, the legend to Fig. 2.

A calculation from the gel chromatograms (Figs. 2 and 5) of deamination products, as described under "Experimental Procedures." The oligosaccharide composition of each sample was estimated from the areas under the appropriate peaks (calculated after projecting the vertical lines on the curves to the base-line). Obviously, the experimental error in this method may be considerable, and the results, although satisfactory for the purposes of the present study, should be regarded as approximations.

Synthesized by microsomal fraction prepared from whole liver.

Synthesized by microsomal fraction prepared from isolated liver cells.

Synthesized by intact liver cells.

Fig. 5. Gel chromatography on Sephadex G-25 of the cell-derived polysaccharide fraction CI (A), and fraction CII (O—O) (B), following treatment with nitrous acid. Superimposed in B is an analogous chromatogram ( •••••••) pertaining to fraction MIV of polysaccharide produced by microsomal fraction prepared from isolated liver cells (see the text). For additional experimental details and explanation of the numbers above the peaks, see the legend to Fig. 2.

Table I

Amounts and distribution of N-acetylated glucosamine residues in polysaccharides synthesized in whole cell or in cell-free systems.

| Polysaccharide fraction | N-acetylated glucosamine units | Isolated units | Two consecutive units | Three consecutive units | >Three consecutive units |
|-------------------------|-------------------------------|----------------|-----------------------|------------------------|-------------------------|
| MI'                     | 60                            | 30             | 25                    | 15                     | 30                      |
| MIV'                    | 100                           |                |                       |                        |                         |
| MIIV'                   | 30                            | 50             | 20                    | 10                     | 15                      |
| MIV'                    | 25                            | 55             | 20                    | 10                     | 15                      |
| MI'                     | 25                            | 50             | 20                    | 10                     | 15                      |
| CI'                     | 50                            | 25             | 15                    | 10                     | 50                      |
| CII'                    | 40                            | 30             | 15                    | 15                     | 40                      |

*Per cent of total glucosamine units, determined as described under "Experimental Procedures."

**Calculated from the gel chromatograms (Figs. 2 and 5) of deamination products, as described under "Experimental Procedures." The oligosaccharide composition of each sample was estimated from the areas under the appropriate peaks (calculated after projecting the vertical lines on the curves to the base-line). Obviously, the experimental error in this method may be considerable, and the results, although satisfactory for the purposes of the present study, should be regarded as approximations.

Synthesized by microsomal fraction prepared from whole liver.

Synthesized by microsomal fraction prepared from isolated liver cells.

Synthesized by intact liver cells.

The corresponding enzymes, including a N-acetylglucosaminyl N-deacetylase, a uronsyl CS-epimerase, and N- and O-sulfotransferases have been demonstrated in a rat liver microsomal fraction, using specific assay procedures developed for the determination of mastocytoma enzymes (involved in heparin biosynthesis; I. Jacobsson and J. Riesenfeld, unpublished results).

**DISCUSSION**

Previous studies have demonstrated that isolated rat hepatocytes produce a glycosaminoglycan that, by all current criteria, is identified as a high sulfated heparan sulfate (23, 27). These results were confirmed in the present investigation. It seemed reasonable to assume that the microsomal fraction prepared from these cells contained the enzymes utilized, by the intact cells, in producing heparan sulfate. Cell-free incubations of such microsomal fraction yielded polymeric intermediates similar to those formed during heparin biosynthesis, using a mouse mastocytoma microsomal fraction (9, 25). The polymer-modification reactions involved in the biosynthesis of heparan sulfate are apparently very similar to those leading to the formation of heparin. In fact, the isolated intermediates were more heparin-like than anticipated; the cell-free product (fraction MIV) had been subjected to more extensive polymer modification than had the heparan sulfate manufactured by the intact cells.

These results suggest that the regulation of the polymer-modification process depends on the integrity of the intact cell. In the cell-free system, a restrictive control function has been lost, leading to the formation of a more extensively modified product. The mechanism of regulation and its relation to other cellular functions are unknown. However, studies on the substrate specificities of the various polymer-modifying enzymes involved in heparin biosynthesis have suggested that the initial reaction, the deacetylation of N-acetylglucosamine residues, has a key regulatory role, since it determines the maximal possible extent of all subsequent reactions (8-10, 13).

In accord with this postulate, the polymer obtained from the cell-free system contained not only less N-acetyl (and hence more N-sulfate) groups but also more O-sulfate groups (see Footnote 5) and iduronic acid units. Recent observations indicated that the level of N-deacetylation in heparin biosynthesis may be modulated by a complex interaction between the N-deacetylase and N-sulfotransferase enzymes (22). The concentrations of cofactors (or inhibitors) to these enzymes, or of PAPS, potentially of importance to the interaction, may be spatially arranged to heparin biosynthesis in the membranes of the endoplasmic reticulum. It seems likely that the organization of the biosynthetic apparatus may be deregulated during the preparation of the microsomal fraction.
The blood anticoagulant activity of heparin was recently shown to be critically dependent on the fine structure of the polysaccharide molecule (11, 28–30). It seems probable that similar conditions may apply to the interaction between heparan sulfate proteoglycans and other macromolecules at the cell surface or in the pericellular space. These aspects underline the functional significance of the regulatory mechanisms associated with polymer modification in the biosynthesis of heparin-like polysaccharides.

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