Biosynthesis of Heparin

AVAILABILITY OF GLUCOSAMINYL 3-O-SULFATION SITES

Marion Kusche, Giangiacomo Torri‡, Benito Casu‡, and Ulf Lindahl§

From the Department of Veterinary Medical Chemistry, The Swedish University of Agricultural Sciences, The Biomedical Center, S-751 23 Uppsala, Sweden and the IG. Ronzoni Institute for Chemical and Biochemical Research, Via G. Colombo 81, 20133 Milan, Italy

Heparin preparations isolated from pig intestinal mucosa and from hovine lung were fractionated with regard to affinity for antithrombin. The resulting fractions, with high (HA) or low (LA) affinity for the proteinase inhibitor, were analyzed by 35S NMR or by identification of di- and tetrasaccharides obtained through deaminative cleavage with nitrous acid. Structural differences between corresponding HA and LA fractions were essentially restricted to minor constituents, in particular 3-O-sulfated glucosamine units that occurred (1 or 2 residues/chains) in all HA preparations but were scarce or absent in LA heparin. The HA fractions also consistently showed higher contents of nonsulfated iduronic acid and, to a lesser extent, N-acetylated glucosamine units than the LA fractions. The two tetrasaccharide sequences, -IdoA-GlcNac(6-OSO3)-GlcA-GlcNSO3- and -IdoA-GlcNac(6-OSO3)-GlcA-GlcNSO3(6-OSO3)-, recently implicated as part of the acceptor site for glucosaminyl 3-O-sulfate groups (Kusche, M., Råkström, G., Riesenfeld, J., Petitou, M., Choay, J., and Lindahl, U. (1988) J. Biol. Chem. 263, 15474-15484), were identified in mucosal LA heparin; it was calculated that the preparation contained approximately one potential acceptor site/polysaccharide chain. Yet this material did not yield any labeled HA components on incubation with adenosine 3′-phosphate 5′-phospho-[35S]sulfate in the presence of glucosaminyl 3-O-sulfotransferase, solubilized from a mouse mastocytoma microsomal fraction. The failure to incorporate any 3-O-sulfate groups could conceivably be explained by the occurrence of a D-glucuronic acid rather than L-iduronic acid unit linked at the reducing ends of the above tetrasaccharide sequences. Alternatively, 3-O-sulfation may be restricted by other, as yet unidentified, inhibitory structural elements that are preferentially expressed in polysaccharide sequences selected for the generation of LA heparin.

The key step in the blood anticoagulant action of the sulfated glycosaminoglycan heparin is binding of the anticoagulant proteinase inhibitor antithrombin (AT) to the polysaccharide chain (Björk and Lindahl, 1982). Thus bound, AT inactivates the enzymes of the coagulation process much more efficiently than does the free inhibitor. Functional binding of AT to the heparin chain requires the presence of a specific pentasaccharide sequence, the structure/function relationships of which have been explored in detail (Thunberg et al., 1980, 1982; Riesenfeld et al., 1981; Choay et al., 1981, 1983; Lindahl et al., 1980, 1983, 1984; Aher et al., 1985, 1987; Petitou et al., 1988). The distinguishing structural feature of the AT-binding region is a 3-O-sulfated glucosamine residue (Fig. 1) that is either absent or very rare elsewhere in the heparin molecule (Lindahl et al., 1980).

In commercial heparin preparations, only a fraction, usually 30-40% of the molecules contains the AT-binding sequence and has high affinity for AT (HA heparin). The majority of the chains lack the 3-O-sulfate group and thus show low affinity for the inhibitor (LA heparin) and only weak anticoagulant activity (see Björk and Lindahl, 1982). Apart from the 3-O-sulfate group, no apparent qualitative structural difference between HA and LA heparin has been found. However, it has been reported that HA heparin contains more N-acetylated glucosamine, D-glucuronic acid, and nonsulfated L-iduronic acid than does LA heparin (Rosenberg et al., 1978; Rosenberg and Lam, 1979; Lindahl et al., 1979, 1980; Radoff and Danishefsky, 1985). Reports by Radoff and Danishefsky (1984) and by Rosenfeld and Danishefsky (1988), proposing that the AT-binding sequence is preferentially located toward the nonreducing end of the HA heparin chain, were not confirmed by Oscarsson et al. (1989), who favored rather a random distribution of the binding region. It may be noted that an AT-binding region similar or identical to that of heparin has also been found in heparan sulfate (Lane et al., 1986; Mareum et al., 1986; Pfeifer et al., 1987).

Heparin is synthesized in connective tissue-type mast cells as a proteoglycan that consists of multiple polysaccharide chains joined by a polypeptide sequence of alternating serine and glycine units (Yurt et al., 1977; Robinson et al., 1978; Lidholt et al., 1980; Kjellén et al., 1989). The polymer initially formed from the appropriate UDP-sugars has a simple structure of repeating -GlcA-GlcNac- disaccharide units. In the

1 The abbreviations used are: AT, antithrombin; HA and LA saccharides, heparin-related molecules with high and low affinity for AT, respectively; HexA, unspecified hexuronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcNac, 2-deoxy-2-acetamido-D-glucose (N-acetyl-D-glucosamine); PAPS, adenosine 5′-phosphate 5′-phosphosulfate; aMans, 2,5-anhydro-D-mannitol formed by reduction of 2,5-anhydro-D-mannose residues with NaBH₄; N-sulfate group: OSO₃, O-sulfate, ester sulfate group (the locations of O-sulfate groups are indicated in parentheses); HPLC, high pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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FIG. 1. Structure of the antithrombin-binding region in heparin. The binding region is contained within the pentasaccharide sequence (units 2–6) enclosed by brackets. Structural variants are indicated by -R' (-COCH₃ or -SO₃H) or -R'' (-H or SO₃H). In addition, a d-glucuronic acid residue may replace the l-iduronic acid unit 7 (outside the actual AT-binding region; not shown). For additional information, see the text.

presence of PAPS, this polysaccharide is carried through a series of modification reactions which is initiated by N-deacetylation and N-sulfation of glucosamine units, proceeds through C-5 epimerization of d-glucuronic to l-iduronic acid units, and is concluded by incorporation of O-sulfate groups at various positions (for reviews, see Lindahl et al., 1986; Lindahl and Kjellén, 1987; Lindahl, 1989). The structural heterogeneity typical of heparin (and heparan sulfate) reflects the fact that these reactions are generally incomplete, a variable proportion of the disaccharide units remaining at lower levels of modification. For the formation of defined saccharide sequences, the selection of target units in each reaction must be stringently regulated. Although the mechanisms behind such regulation are only partly understood, they clearly depend to a large extent on the substrate specificities of the corresponding enzymes.

The formation of the AT-binding region highlights the problems of regulation in glycosaminoglycan biosynthesis. A previous study focused on the introduction of the specific marker group for this region, i.e. the glucosaminyl 3-O-sulfate residue (Kusche et al., 1988). It was found that 3-O-sulfation concludes the polymer modification process and is essentially restricted to saccharide sequences that already exhibit all other structural features required for high affinity binding to AT. These findings raised some specific questions regarding those polysaccharide chains that escape 3-O-sulfation and thus will be ultimately classified as LA heparin. Do these molecules lack the appropriate acceptor sequence required for substrate recognition by the 3-O-sulfotransferase, or do they simply not encounter the enzyme? These problems have been addressed in the present study, which involves a detailed structural comparison between heparin preparations with high and low affinity for AT as well as direct attempts to test LA heparin as a potential acceptor molecule in the glucosaminyl 3-O-sulfotransferase reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**

Two heparin preparations were derived from pig intestinal mucosa. Preparation A (lot 22407-01) was obtained from Abbott Laboratories (N. Chicago, IL). Preparation B (stage 14) was obtained from Inoex Pharmaceutical Division (Park Forest South, IL). This material was purified by repeated precipitation with cetavlyprydinium chloride from 1.2 M NaCl, essentially as described (Lindahl et al., 1985). The product was 3-H-labeled by acetylating free amino groups with [3H]acetic anhydride (Hnek et al., 1989). In addition, a preparation (lot 1750; FW) of heparin derived from bovine lung was obtained from Upjohn Co. The various preparations were fractionated by affinity chromatography on antithrombin Sepharose, essentially as described (Laurent et al., 1978). The procedure (continuous salt gradient elution) was scaled up to accommodate samples of 300 mg, using a column of 600-ml bed volume. Polysaccharides in the effluents which were either not adsorbed to the affinity matrix or emerged at a low salt concentration (≤0.7 M NaCl) distinctly separated from the HA material were combined into one LA pool. The LA fractions were rerun once or twice on the same column to ensure the complete absence of any residual HA material; usually no significant amounts of HA material could be detected (by carbazole analysis) after the initial separation. The HA fractions thus obtained amounted to 49, 48, and 42% of pig mucosal heparin preparations A and B and of the bovine lung heparin, respectively.

An α-methylglucoside of a polysaccharide with the structure GlcNSO₃(6-OSO₃)₄:GlcA-GlcNSO₃(6-OSO₃)₃:IdoA(2-OSO₃):GlcNSO₃(6-OSO₃)₅:IdoA(2-OSO₃) or -R''(-H or SO₃H), i.e. a sequence corresponding to the antithrombin-binding region except for the lack of a 3-O-sulfate group at unit 4 (see Fig. 1), was kindly given to us by Dr. M. Petitou of the Institut Choay, Paris, France. Labeled standard di- and tetrasaccharides of the types IexA-[1-3]Manα₁R and IexA-GlcNac-GlcA-[1-3] Manα₁R, respectively, with O-sulfate groups in various positions, were also derived (Pejler et al., 1987). Inorganic Na₂SO₄ (carrier-free) was purchased from Amersham Corp. and was used in the preparation of [35S]PAPS as reported (Jacobsson and Lindahl, 1980).

Sephadex G-15, bovine liver β-d-glucuronidase (contaminated with some α-l-iduronidase), and human antithrombin covalently coupled to Sepharose 4B were obtained as described (Kusche et al., 1988). A microsomal fraction was prepared from a transplantable mouse mastocytoma (Furth et al., 1975) according to Jacobsson et al. (1979a).

**Methods**

Hexuronic acid was determined by the carbazole reaction (Bitter and Muir, 1962) and radioactivity either in a Beckman model LS 3800 liquid scintillation spectrometer or by a flow detector connected to an HPLC apparatus as described (Pejler et al., 1987).

**Analysis of Heparin Deamination Products**—The composition of heparin preparations was investigated by separation and quantification of 3-H-labeled fragments obtained by deaminative cleavage of the polysaccharides with nitrous acid followed by reduction of the products with NaBH₄. The procedure employed (Shively and Conrad, 1970; Thunberg et al., 1982; Bienkowski and Conrad, 1985; Pejler et al., 1987) was as follows. Samples (300 µg) of polysaccharides (dissolved in 20 µl of H₂O) were mixed with 200 µl of HNO₃ reagent (pH 1.5), and the reaction was allowed to proceed at room temperature for 10 min. Following the addition of 75 µl of 1 M Na₂CO₃, the products were reduced with 5 mCi of NaBH₄ (Amersham; specific activity, 15 Ci/mmol). The mixtures were left at room temperature for ~15 h and were then adjusted (in a fume hood) first to pH ~4 with 4 M acetic acid and then to pH ~7 with 4 M NaOH. Labeled saccharides were isolated by gel chromatography on a column (1 x 170 cm) of Sephadex G-15 in 0.2 M NH₄HCO₃ and were then separated into di- and tetrasaccharides on a column (1 × 190 cm) of Sephadex G-25 (superfine), also in 0.2 M NH₄HCO₃. The pooled fractions were desalted by lyophilization.

Labeled disaccharides were separated further by ion-exchange HPLC on a 0.46 × 25-cm Partisil-10 SAX column (Whatman) that was eluted at a rate of 1 ml/min using step gradients with increasing concentrations of aqueous K₂HPO₄. The column was connected to a model Fio-One d/CR radioactive flow detector (Radiomatic Instruments & Chemical Co. Inc., Tampa, FL) equipped with a 2.5-ml cell, using Flo-Scan III (Radiomatic) as scintillation medium (5 ml/min). The radioactivity was integrated continuously and recorded on a strip chart. The retention times of labeled components were related to those of disaccharide standards (see Fig. 3). The identification of disaccharides was ascertained by rerunning samples after digestion with β-glucuronidase (Jacobsson et al., 1979b).

Before analysis of tetrasaccharides, "anomalous" deamination products containing an internal 2-deoxy-2-C-formyl-β-D-glucopyranosyl unit (Lindberg et al., 1975; Shively and Conrad, 1976) were eliminated by mild acid treatment (25 mM H₂SO₄ at 80 °C for 30 min); control experiments with tetrasaccharide standards showed no detectable O-desulfation under these conditions. Resistant tetra-
The tetrasaccharide was reisolated by gel chromatography on Sephadex G-15, as described above. The remaining labeled tetrasaccharides were analyzed by chromatography on the same Partisil-10 SAX column that was used for the disaccharides (6 ml of scintillation medium/min), the KH$_2$PO$_4$ concentrations employed (see Fig. 2) allowing the separation of distinct groups of mono-, di-, and trisulfated species. The elution procedure was designed such that the two tetrasaccharides derived from the antithrombin-binding region, the di-sulfated IdoA-GlcNAc-(6-OSO$_4$)-GlcA-Man$_9$ and the tri-O-sulfated IdoA-GlcNAc-(6-OSO$_4$)-GlcA-Man$_9$ (3,6-di-OSO$_4$), both appeared as single peaks. These components could be identified directly by use of the corresponding standards (components a and b, respectively, in Fig. 7A in Thunberg et al., 1982) but nevertheless were, along with the other tetrasaccharide fractions, subjected to more detailed structural analysis, essentially according to methods described by Thunberg et al. (1982), Shaklee and Conrad (1984), and Bienkowski and Conrad (1985). In brief, the following procedure was employed. Fractions of H-labeled tetrasaccharides were collected by preparative HPLC on the same column. The sulfate contents of the various tetrasaccharide species were established by high voltage paper electrophoresis at pH 1.7 (see Thunberg et al., 1982). The reducing terminal half of each fraction was released as HexA-[1-$^3$H]Man$_9$, disaccharide by deamination at pH 9.0 following deacetylation of the internal N-acetylglucosamine unit by hydrazinolysis. The released labeled disaccharide was isolated by gel chromatography on Sephadex G-15 before analysis by ion-exchange HPLC as described above. For identification of the nonreducing terminal disaccharide units, tetrasaccharides were initially reduced with unlabeled rather than with H-labeled NaBH$_4$ (see Peijer et al., 1987) and were then fractionated by ion-exchange HPLC (analyzed by the carboxylate reaction) before hydrazinolysis. Labeled HexA-[1-$^3$H]Man$_9$, disaccharides corresponding to the nonreducing terminal half of the tetrasaccharides were generated by treating the N-deacetylated saccharides with HNO$_3$ at pH 3.3 followed by reduction of the deamination products with NaBH$_4$. These disaccharides were isolated by gel chromatography as above and were subsequently identified by analytical ion-exchange HPLC. The combined results of these analyses and those of the reducing terminal disaccharide units allowed the structural assignment and quantitative determination of all tetrasaccharides formed in significant amounts on deamination of the HA and LA heparin preparations.

$^{13}$C NMR Analysis of Heparin - $^{13}$C NMR spectra were obtained at 75 MHz with a Bruker CXP-300 spectrometer at 23 °C. Solutions (-10%, w/v) were prepared by direct dissolution in D$_2$O (99.7%). Reported chemical shifts were measured using the CH$_3$ signal of GlcNAc residues of heparin (24.0 ppm) as internal reference. The areas of relevant signals were determined by direct instrumental integration and normalized to 100% for total hexuronic and total glucosamine, essentially as described by Casu et al. (1983). Under the experimental conditions used (acquisition time, 0.215 s; relaxation delay, 1 s), no selective saturation of signals was observed.

Incubation of Saccharides with Mastocytoma Microsomal Fractions — HA or LA heparin fractions as well as the synthetic pentasaccharide related to the antithrombin-binding region (see “Materials”) were tested as sulfate acceptors in O-sulfotransferase reactions utilizing a mastocytoma microsomal enzyme preparation. Reaction mixtures contained, in a total volume of 0.5 ml, 0.05 M Hepes (pH 7.4), 100 mM NaCl, 10 mM MnCl$_2$, 10 mM MgCl$_2$, 5 mM CaCl$_2$, 3.5 mM NaF, and 1% (v/v) Triton X 100. After 2 h at 37 °C, 0.5 mg of carrier heparin was added, and the incubation mixtures were heated at 100 °C for 5 min. After mixing of the samples with 0.5 ml of 2 M Na$_2$SO$_4$, denatured protein was removed by centrifugation, and $^{13}$S-labeled saccharides were isolated by gel chromatography on a column (1 × 100 cm) of Sephadex G-15 equilibrated with 0.2 M NH$_4$Cl. At these conditions, HA heparin was resolved from the labeled polysaccharide and pentasaccharide appeared in the excluded volume of the column, well separated from residual PAPS and inorganic $^{35}$S sulfate. The excluded fractions were recovered and desalted by lyophilization. The $^{35}$S-labeled saccharide fractions were analyzed by affinity chromatography on antithrombin-Sepharose as described by Thunberg et al. (1982), using a 3-ml column of the affinity matrix and continuous salt gradient elution.
Fig. 2. Anion-exchange HPLC of [3H]-labeled tetrasaccharides. Samples of HA fraction (panel A) and LA fraction (panel B) of mucosal heparin (preparation A) were subjected to HNO3 (pH 1.0)/NaBH4 treatment, and the resulting labeled products were separated by gel chromatography (see "Methods"). The tetrasaccharides were subjected to mild acid treatment, and samples (-250,000 cpm) of the residual material were analyzed on a Partisil-10 SAX column as described under "Methods." The elution patterns of the standard tetrasaccharides GlcA-GlcNAc(6-OSO3)-GlcA-aMan4(3,6-di-OSO3) (arrow 10) and IdoA-GlcNAc(6-OSO3)-GlcA-aMan4(3,6-di-OSO3) (arrow 11) are indicated. In preparative separations of unlabeled reduced tetrasaccharides, effluent fractions were analyzed for hexuronic acid by the carbazole reaction; the resulting elution patterns (not shown) were essentially similar to those of the corresponding [3H]-labeled samples (see the text). The horizontal brackets indicate how fractions derived from mucosal heparin preparation B and from beef lung heparin (spectra not shown; results summarized in Table II). Some variability is noted with regard to the signals attributed to the binding site for antithrombin. For instance, they are somewhat stronger in the mucosal heparin preparation A than in most other preparations of unfractionated heparin5 (cf. Gatti et al., 1979; Choay et al., 1980), in accord with the unusually high proportion of HA fraction in this material (see "Materials"). Further, whereas a weak peak in the C-2 region of 3-0-sulfated glucosamine residues (at about 58 ppm) is still observable in the spectrum of the LA fractions derived from mucosal heparin preparation A (Fig. 4), there is no evidence of these signals in the LA fraction of preparation B. This is clearly illustrated by the partial spectra relating to preparation B, shown in Fig. 5. This latter figure also shows that the C-2 signal of the 3-O-sulfated glucosamine residue has a low field, still unassigned, component (indicated by an arrow in the figure), which is much less prominent for the HA fraction of preparation A but is well evident in other HA heparin fractions.3

The relative contents of the variously substituted monosaccharide units, as determined by 13C NMR (values in parentheses), are compared in Table II with the corresponding unfractionated heparins values calculated from Table I). The HPLC and the NMR data are generally in good agreement for the prevalent residues such as 2-O-sulfated iduronic acid and 3,6-di-O-sulfated glucosamine units as well as the corresponding unfractionated heparins were also characterized by 13C NMR spectroscopy. The spectra shown in Fig. 4, obtained with mucosal heparin preparation A, consist of a set of major signals due to the prevalent trisulfated disaccharide units and of weak signals attributable to minor residues (see Gatti et al., 1979). Peaks associated with the binding site for antithrombin (especially the C-2 signal of 3- or 3,6-di-O-sulfated, N-sulfated glucosamine units, marked with an asterisk in the spectrum of unfractionated heparin (Casu et al., 1981; Meyer et al., 1981)) are seen in the sample of unfractionated heparin but are, as expected, stronger and better defined in the HA fraction. Also the weak C-1 signal upfield to the major GlcNOSO3 signal and indicated by the arrow in the HA spectrum becomes clearly stronger in the HA fraction. The signal was tentatively attributed (Braud et al., 1985) to 3-O-sulfated GlcNOSO3 residues. These two signals as well as the C-1 signal of nonsulfated iduronic acid are much weaker or barely observable in the spectrum of the LA fraction. Similar trends were observed for the corresponding fractions derived from mucosal heparin preparation B and from beef lung heparin (spectra not shown; results summarized in Table II).

3 B. Casu and G. Torri, unpublished data.
The analyses described above confirmed in showing that the HA and LA fractions of the various heparin preparations differed primarily with regard to the presence or absence of 3-O-sulfated glucosamine units. We therefore decided to test whether HA heparin may indeed serve as a substrate in the HPLC (HNO₂/NaBH₄) technique which recognizes only the latter type of residues (which will be recovered in labeled tetrasaccharides). Moreover, since the instrumental integration of the area of weak signals located close to strong ones (as in the case of the C-2 signal upfield to the major C-2 signal of N-sulfated glucosamine) usually overestimates the minor component, the contents of total 3-O-sulfated glucosamine are probably more accurately represented by the HPLC data. It also should be noted that the still unassigned minor signal of 3-O-sulfated glucosamine contributes up to 30% of the area measured for estimating total 3-O-sulfated glucosamine. Should this minor peak turn out not to be associated with 3-O-sulfated glucosamine units, the appropriate correction would bring the NMR data closer to the HPLC values. NMR studies are in progress to resolve and assign this and other minor heparin signals.

**Enzymatic Sulfation of Heparin Fractions**

The analyses described above confirmed in showing that the HA and LA fractions of the various heparin preparations differed primarily with regard to the presence or absence of 3-O-sulfated glucosamine units. We therefore decided to test whether HA heparin may indeed serve as a substrate in the glucosaminyl 3-O-sulfotransferase reaction, thereby being converted into HA heparin. The enzyme source in these experiments was a detergent-solubilized mouse mastocytoma microsomal fraction, which was found previously to catalyze the 3-O-sulfation of appropriate oligosaccharide substrates (Kusche et al., 1988). Incubation of the microsomal enzymes with [³⁵S]PAPS, as described under “Methods,” in the absence of any exogenous sulfate acceptor, yielded labeled polysaccharide due to enzymatic sulfation of endogenous acceptor precursors.

### TABLE I

| Heparin fraction | GlcA(2-OSO₃)-aManR | GlcA(aMan₆(6-OSO₃)) | IdoA(aMan₆(3-OSO₃)) | IdoA(2-OSO₃)-aManR | GlcA(aMan₆(6-OSO₃)) | IdoA(aMan₆(3-6,di-OSO₃)) | GlcA(aMan₆(5,6-di-OSO₃)) |
|------------------|---------------------|---------------------|---------------------|---------------------|---------------------|------------------------|------------------------|
| Mucosa HA        | 0.9                 | 9.0                 | 2.9                 | 7.4                 | ND                  | 55                     | 2.4                    |
| Mucosa LA        | 1.2                 | 8.2                 | 2.0                 | 11.0                | ND                  | 61                     | 1.0                    |
| Lung HA          | 1.0                 | 5.5                 | 3.7                 | 5.7                 | ND                  | 68                     | 5.0                    |
| Lung LA          | 1.3                 | 4.4                 | 2.1                 | 6.9                 | ND                  | 79                     | ND                     |

| Nonsulfated tetrasaccharide | IdoA(2-OSO₃)-GlcNAc-GlcA-aManR | IdoA-GlcNAc-GlcA-aMan₆(6-OSO₃) | IdoA-GlcNAc(6-OSO₃)-GlcA-aMan₆(6-OSO₃) | IdoA-GlcNAc(6-O SO₃)-GlcA-aMan₆(3-OSO₃) | IdoA-GlcNAc(6-O SO₃)-GlcA-aMan₆(3,6-di-OSO₃) | IdoA-GlcNAc(6-O SO₃)-GlcA-aMan₆(5,6-di-OSO₃) |
|-----------------------------|---------------------------------|-------------------------------------|----------------------------------------|--------------------------------------------|------------------------------------------------|---------------------------------------------|
| Mucosa HA                   | 0.6                            | 1.4                                 | 1.9                                    | 2.0                                        | 2.5                                           | 3.2                                      |
| Mucosa LA                   | 0.9                            | 4.4                                 | 2.5                                    | 3.3                                        | 3.5                                           | ND                                       |
| Lung HA*                    | 1.1                            | 0.9                                 | 3.0                                    | 1.9                                        | 0.6                                           | 3.2                                      |
| Lung LA*                    | 3.9                            | 0.5                                 | 3.0                                    | 2.0                                        | ND                                            | ND                                       |

*ND, none detected.

*The tetrasaccharides derived from the lung heparin fractions were not individually identified; structural assignments and quantification were based on ion-exchange HPLC elution patterns (not shown) only. Values within brackets represent incompletely separated components, not individually identified (see the corresponding patterns for tetrasaccharides derived from mucosal heparin in Fig. 2).
The immediate product of heparin biosynthesis is a multichain proteoglycan with extended polysaccharide chains, $M_\text{r}$ 60–100 × 10^3 (Lindahl et al., 1986; Lindahl and Kjellen, 1987; Lindahl, 1989). Within hours after completed formation, this proteoglycan undergoes intracellular degradation, the polysaccharide chains being cleaved by an endoglucuronidase into fragments of $M_\text{r}$, 5–30 × 10^3 (Jacobsson and Lindahl, 1987). Such fragments constitute the bulk of commercial heparin preparations. Analysis of heparin proteoglycan isolated from rat skin showed that the antithrombin-binding sequences are not randomly distributed but are accumulated in a restricted fraction of the polysaccharide chains (Jacobsson et al., 1986; Horner et al., 1988). Chain fragments containing the binding sequence will be designated HA heparin. Which factors determine whether a heparin sequence will become 3-O-sulfated, and thus ultimately recovered in HA heparin, or escape 3-O-sulfation and remain an LA component?

Recent studies on the biosynthesis of heparin in a cell-free mastocytoma system have yielded some information concerning the relation between 3-O-sulfation and other polymer modification reactions. Experiments using an N/O-desulfated, re-N-sulfated HA octasaccharide (isolated after partial deaminative cleavage of heparin) as [35S]sulfate acceptor thus indicated that the distinctive structural feature of HA heparin, i.e., the glucosaminyl 3-O-sulfate group, is introduced at a late stage of the modification process (Kusche et al., 1988; see also the Introduction). A labeled HA product was obtained which accounted for only ~2% of the total incorporated O-sulfate but for >90% of the glucosaminyl 3-O-sulfate groups.

The 3-O-sulfate groups appeared at the appropriate position for the antithrombin-binding region (unit 4 of the structure shown in Fig. 1), additional O-sulfate groups occurring at C-6 of units 2 and 6. The essential lack of 3-O-sulfate groups in fractions of lower antithrombin affinity suggested that the 3-O-sulfation reaction concludes the formation of the antithrombin-binding region and hence occurs only when all other structural requirements for binding have been fulfilled. These requirements include in particular the 6-O-sulfate group on unit 2 and the two $N$-sulfate groups on units 4 and 6. The $N$-substituent on unit 2, on the other hand, may be either acetyl or sulfate. Moreover, the $\beta$-gluco and $l$-ido configurations of units 3 and 5, respectively, are essential, as shown in recent experiments using GlcNSO$_2$-HexA-GlcNSO$_2$-HexA-GlcNSO$_2$ pentasaccharides, with various permutations of the hexuronic acid.
The values show the proportions of disaccharide units (in percent of total) that contain a particular monosaccharide residue and are based on identification of di- and tetrasaccharides obtained by HNO₃ (pH 1.5)/NaBH₄ treatment (see Table I) as well as on ¹³C NMR analysis (values in parentheses). The values for mucosal heparin that are not in parentheses (i.e., data based on deamination analysis) refer to preparation A, whereas those in parentheses (NMR data) refer to both preparation A (left column) and preparation B (right column). In the calculations it was assumed that the nonsulfated tetrasaccharide fraction obtained after deamination (Table I) was an equimolar mixture of GlcA-GlcNAc-GlcA-Mana and IdoA-GlcNAc-GlcA-Mana. It should be noted that certain glucosamine units (for instance, 6-O-sulfated, N-acetylated residues) will appear twice in the table.

|                     | Mucosal heparin | Lung heparin* |
|---------------------|-----------------|---------------|
|                     | HA              | LA            | HA              | LA            |
|                     | mol %/disaccharide unit |               | mol %/disaccharide unit |               |
| Glucuronic acid, total | 23 (23; 20)      | 19 (24; 24) | 16 (13)         | 8.4 (8)       |
| Iduronic acid       |                 |               |                 |
| Nonsulfated         | 11 (11; 12)     | 5.4 (2.8; 7) | 6.7 (8)         | 4.2 (4)       |
| 2-O-Sulfated        | 65 (62; 51)     | 78 (74; 61)  | 75 (79)         | 89 (88)       |
| Glucosamine         |                 |               |                 |
| N-Acetylated        | 11 (16; 17)     | 8.1 (12; 13) | 4.4 (≤3)        | 2.7 (ND)†     |
| 6-O-Sulfated        | 86 (86; 87)     | 80 (81; 81)  | 89 (89)         | 87 (91)       |
| 3-O-Sulfated        | 8.1 (14; 13)    | 1.0 (≤4; ≤2) | 6.9 (12)        | ND (ND)       |
| 6-OH                | 14 (14; 13)     | 9R (19; 19)  | 9.0 (11)        | 9.9 (9)       |

*The values for the lung heparin fractions, based on analysis of deamination products, were calculated assuming that the unspecified nonsulfated, monosulfated, and disulfated tetrasaccharides shown in Table I were distributed similar to the corresponding components from the mucosal heparin fractions.

†ND, none detected.

remain LA type might lack a functional acceptor site for 3-O-sulfation. The predominant acceptor sequence (in mucosal heparin), as predicted from the results summarized above, would have the structure (-IdoA)-GlcNAc(6-OSO₃)-GlcA-GlcNSO₃(6-OSO₃)-IdoA(2-OSO₃)-GlcNSO₃(2-OX)- (corresponding to units 1-6 in Fig. 1), where X stands for either -H or SO₃-. The target for 3-O-sulfation is the internal glucosamine unit, which may be either unsubstituted or sulfated at C-6. The N-acetylated unit 2 may be replaced by N-sulfated glucosamine and then appears to be preceded by a 2-O-sulfated rather than unsubstituted iduronic acid unit (Lindahl et al., 1994); this latter arrangement is seen especially in lung heparin.

The present analysis of heparin preparations from various sources, using either HPLC of deamination products or NMR spectroscopy, indicated that the overall composition of HA and LA fractions was very similar; apart from the glucosaminyl 3-O-sulfate marker group, only minor quantitative differences were detected (Table II). LA heparin isolated from intestinal mucosa (preparation A) was selected for a more detailed structural analysis, aimed at identifying potential 3-O-sulfate acceptor sites. Special attention was given to the tetrasaccharides obtained after deamination, since these components would contain the N-acetylated glucosamine unit 2 and adjacent sugar residues, in all units 1-4 of the acceptor sequences. Two tetrasaccharides, IdoA-GlcNAc(6-OSO₃)-GlcA-Mana and IdoA-GlcNAc(6-OSO₃)-GlcA-AlphaManα, implicated as markers for the 3-O-sulfate acceptor site (Kusche et al., 1988), were found in appreciable amounts (Table I) together accounting for approximately 0.8 potential acceptor site/polysaccharide chain. Since at least one-third of the antithrombin-binding regions in mucosal HA heparin contain N-sulfated rather than N-acetylated glucosamine at position 2 (Lindahl et al., 1994), the occurrence of 3-O-sulfate acceptor sites that are undetectable by the present approach appears highly probable. It thus seemed plausible that the majority of the LA chains might contain a target site for the glucosaminyl 3-O-sulfotransferase.

This assumption was tested directly in attempts to use LA heparin as an exogenous acceptor for a mastocytoma micro-

Fig. 5. C-2 signals of HA and LA fractions of mucosal heparin preparation B. The C-2 signals obtained for the HA (solid line) and LA (broken line) fractions show the major (asterisk) and the low field (arrow) components of the C-2 signal of GlcNSO₃(3,6-dioSO₃ δ + GlcNSO₃(3,6-OSO₃ 6δ) in the HA spectrum and the essential absence of both signals in the LA spectrum.

acid units, as O-sulfate acceptors. The role of other O-sulfate groups appears to be less decisive, since 3-O-sulfation can apparently take place in the presence as well as in the absence of O-sulfate groups at C-6 of the target unit 4 and at C-2 of the adjacent unit 5 (Kusche et al., 1988). The importance of 6-O-sulfation at unit 6 is unclear.

The lack of 3-O-sulfate groups in a fraction of heparin molecules could conceivably reflect the topographical organization of the biosynthetic apparatus, both the enzymes and the proteoglycan substrate involved in polymer modification being membrane bound (Lindahl et al., 1986). The location of the 3-O-sulfotransferase molecules could be such as to preclude the interaction of the enzyme with some of the polysaccharide chains. On the other hand, chains predestined to
NaCl in the above Tris buffer. Fractions of ~2.2 ml were collected and analyzed for radioactivity. The arrows indicate the beginning of the salt gradients.

Figure 6. Affinity chromatography on antithrombin-Sepharose of 3H-labeled saccharides. Enzymatically 3H-labeled saccharides (generally ~15 × 10^6 cpm, most of which was due to sulfation of the endogenous acceptor in the microsomal preparation) were isolated by gel chromatography as described under “Methods,” and samples of 500,000 cpm were mixed with 500,000 cpm of 3H-labeled standard pig mucosal heparin and applied to a column (~3 ml) of antithrombin-Sepharose equilibrated with 0.05 M NaCl, 0.05 M Tris-HCl (pH 7.4). A, elution pattern of 3H-labeled standard heparin (similar patterns of 3H-labeled HA and LA components were observed in all chromatographies B–E; data not shown); B, saccharides generated in the absence of any exogenous sulfate acceptor; C–E, saccharides generated in the presence of C mucosal heparin preparation A, HA fraction (200 µg); D, mucosal heparin preparation A, LA fraction (200 µg); similar results were obtained with 50 µg of LA heparin; data not shown); E, synthetic pentasaccharide (50 µg). The column was eluted with a linear salt gradient (---) extending from 0.05 to 3.0 M NaCl in the above Tris buffer. Fractions of ~2.2 ml were collected and analyzed for radioactivity. The arrows indicate the beginning of the salt gradients.

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