Investigations on the Chemistry of Heparin

VI. POSITION OF THE SULFATE ESTER GROUPS*

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SUMMARY

The position of the O-sulfates in heparin was investigated by methylation procedures. Heparin was converted to the acetamido derivative and permethylated with diethyl sulfate in aqueous alkali. Other methylation methods in organic solvents were also used. The carboxyl groups in the uronic acid moieties were then reduced and the resulting permethylated polysaccharide was hydrolyzed. The products were identified either as crystalline derivatives or by chromatography and specific color reactions. These were characterized as 3-O-methyl-α-glucose, 2,3-di-O-methyl-α-glucose, 3-O-methyl-α-glucosamine, glucosamine, and 3,6-di-O-methylglucosamine. On the basis of the amounts isolated, it is concluded that about one-third of the glucuronic acid moieties in heparin are sulfated on carbon atom 2 whereas the rest are not sulfated. Most of the glucosamine in heparin contains sulfate ester groups on carbon 6. Glucosamine units which are not sulfated and 3,6-di-O-sulfoglucosamine units are also present in small proportions. It is proposed that the degree of sulfation of the individual units in the heparin polymer is not uniform and that it may vary in different parts of the chain.

The structure of heparin has been the subject of numerous investigations for several decades (2). It is established that heparin is a mucopolyasaccharide (glycosaminoglycan) in which the sugar units are α-glucosamine (2-amino-2-deoxy-α-glucose) and glucuronic acid. In addition to the latter, heparin apparently contains an undetermined amount of iduronic acid (3-6). The glycosidic linkages are to carbon atom 4 of the neighboring sugar (7, 8), and the polymer consists principally of alternating units of glucosamine and glucuronic acid (1, 9). The nitrogen in the glucosamine is linked to sulfate as a sulfoamino group. Additional sulfates are linked to oxygens as O-sulfates.

The purpose of the present investigation was to determine the position of the O-sulfate groups. The approach was to de-N-sulfate and N-acetylate heparin (10), methylate the free hydroxyl groups in the polymer, reduce the glucuronic acid units to glucose (11), and identify the individual sugars obtained after hydrolysis. Since the carbon atoms involved in the glycosidic linkages are known, it could be assumed that any other nonmethylated position in the isolated sugars was originally the site of an O-sulfate linkage.

MATERIALS AND METHODS

Commercial heparin (Abbott) was purified through the cetyltrimethylammonium salt (12) and finally converted to the sodium salt with ethanolic sodium iodide (13). The analytical data on this material were N, 2.08%; S, 12.88%; hexosamine, 23.1%.

We are grateful to Dr. Roger Jeanloz for gifts of methyl 3-O-methyl-α-glucosaminide and of 3-O-methyl-, 6-O-methyl-, and 3,4-di-O-methyl-α-glucosamine hydrochlorides. The late Dr. Fred Smith supplied us with samples of 3-O-methyl-, 3,6-di-O-methyl-, and 2,3,6-tri-O-methyl-α-glucose. Additional 3-O-methyl-α-glucose and methyl 2,3-di-O-methyl-α-n-glucopyranoside were commercial preparations.

The Elson-Morgan reaction for hexosamine was performed by the Roas modification (14), and reducing sugar was assayed by the Nelson method (15). Uronic acid was determined by the carbasole (16) and orcinol (17) procedures. The Morgan-Elson reaction on hexosamines involving preliminary N-acetylation was carried out by the procedure of Roseman and Dafner (18). The phenol-sulfuric acid (19) method was used to detect sugars in column effluents.

Both analytical and preparative paper chromatography were performed with Whatman No. 1 filter paper. The following solvent systems (v/v) were used: A, butan-1-ol-acetic acid-water, 4:1:5; B, butan-1-ol-ethanol-water, 4:1:1; and C, pyridine-ethyl acetate-acetic acid-water, 5:5:1:3. Components were located with ammoniacal silver nitrate, aniline hydrogen phthalate, ninhydrin, and glucose oxidase (20).

PROCEDURE AND RESULTS

Conversion of Heparin to Acetamido Derivative

Heparin (4 g) was N-desulfated with 0.04 N HCl and N-acetylated according to procedures described previously (10). This yielded 2.8 g of the N-desulfated, N-acetylated heparin

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derivative. Analysis: N, 2.12%; S, 7.12%; acetyl, 7.2%. The molar ratio of nitrogen to sulfur is thus 2:3.

Methylation of N-Acetylated Heparin

Dimethyl Sulfate and Aqueous Alkali—A 2.5-g portion of the preparation described above was dissolved in 225 ml of water, and 15 ml of carbon tetrachloride were added to the solution. The subsequent reaction was performed at 0–5° under a nitrogen atmosphere. Dimethyl sulfate (18 ml) and 30% sodium hydroxide (27 ml) were added over a period of 5½ hours. The solution was stirred overnight and the addition of dimethyl sulfate (11 ml) and NaOH (20 ml) was continued over 8 hours during the next day. The procedure was repeated for 3 days with an additional 13 ml of dimethyl sulfate and 18 ml of NaOH each day. The mixture was then neutralized to pH 7 with sulfuric acid and concentrated, at 45°, to 200 ml.

The polysaccharide was separated from sodium sulfate by passage through Sephadex G-25 (90 x 2 cm) and elution with water. Aliquots (10 ml) of the concentrated solution were applied, and 5-ml fractions were collected. Each tube was checked for carbohydrate with phenol-sulfuric acid and for inorganic nitrate, and those fractions containing only carbohydrate were combined. Analyses of the product showed 4.97% methoxyl, 2.31% nitrogen, and 7.62% sulfur.

When the sodium sulfate was removed by dialysis instead of the Sephadex procedure the recovery of polysaccharide was somewhat less than 50%. Analysis of the nondialyzable material showed 4.97% methoxyl, 2.31% nitrogen, and 7.62% sulfur.

The partially methylated material purified by the Sephadex method was remethylated by the procedure described above, with the use of 50 ml of dimethyl sulfate and 65 ml of sodium hydroxide solution. After desalting with Sephadex, 2 g of product were obtained. Analysis: OCH₃, 6.0%; N, 2.22%; S, 7.6%.

A third methylation with 45 ml of dimethyl sulfate and 60 ml of 30% NaOH over 2 days yielded 1.7 g of material. Analysis: OCH₃, 10.75%; N, 2.13%; S, 7.0%.

The methylation was repeated on the above product with 40 ml of dimethyl sulfate and 55 ml of aqueous NaOH over a period of 2 days, giving 1.4 g of product (Preparation MH-4). The yield after the four methylations was 62% of theoretical starting with acetamido heparin, and 57% with respect to heparin.

Tetrasaccharide: C₃H₁₇O₃N₃S₃Na₃(OCH₃)₄

Calculated: N 2.38, S 8.16, OCH₃ 13.16
Found: N 2.4, S 7.28, OCH₃ 13.0

This was the optimum amount of methoxyl which could be introduced by the dimethyl sulfate-aqueous alkali procedure. Although the analytical data indicated apparently complete methylation, additional etherification by other procedures was attempted.

Dimethyl Sulfate and Barium Oxide in Dimethyl Formamide (21)—MII-4 (25 mg) dissolved in 20 ml of dimethyl formamide was flushed with nitrogen and stirred for 3 days with 100 mg of barium hydroxide, 100 mg of barium oxide, and 3 ml of dimethyl sulfate. The mixture was filtered, concentrated under vacuum to 10 ml, and mixed with 20 ml of chloroform. The gel-like precipitate which appeared was recovered by centrifugation, washed with chloroform, and extracted exhaustively with water. The chloroform fraction contained minimal amounts of polysaccharide. The aqueous extract was passed through Sephadex G-25 and the fractions containing polysaccharide were combined and lyophilized. This yielded 6 mg of product with 12.6% methoxyl.

Methyl Iodide and Sodium Hydride in Dimethyl Sulfoxide (22)—The permethylated material (Preparation MH-4) (50 mg) was dissolved in 2 ml of anhydrous dimethyl sulfoxide and 0.3 ml of methyl iodide. To this mixture was added 0.8 ml of dimethyl sulfoxide in which 15 mg sodium hydride had been dissolved. The reaction was allowed to proceed for 4 hours, whereupon another 25 mg of sodium hydride in 1 ml of dimethyl sulfoxide and 0.4 ml of methyl iodide were added. After 1 day of stirring, the reaction mixture was poured into 3 volumes of cold ethanol. The precipitated material was collected by centrifugation, washed with water to remove excess reagent, and dried in a vacuum desiccator over phosphorus pentoxide.

In one set of experiments trimethylxylonium fluoroborate (25) was used as the methylating agent. One gram of trimethylxylonium fluoroborate was dissolved in 100 ml of nitromethane-methylene chloride (1:1, v/v) and added to a solution of 1 g of polysaccharide-quaternary ammonium complex in 100 ml of methylene chloride. The precipitate which appeared after a short while was collected and washed with nitromethane and methylene chloride. Since a considerable proportion of the uronic acid groups was esterified in the process, the product had to be saponified before definitive methoxyl determinations could be made. The material was dissolved in 0.1 N sodium hydroxide and stirred for 2 days at 5°. Subsequent precipitation with ethanol yielded 60 mg of polysaccharide with 4.3% methoxyl.

Comparable results were also obtained when the cetetyl methylammonium complex of heparin dissolved in methylene chloride was methylated with diazomethane and boron trifluoride (26). The number of methyl ether groups introduced in one
step was equivalent to somewhat more than one per tetrasaccharide. In another procedure the cetyltrimethylammonium complex of N-acetylated heparin dissolved in methylene chloride was methylated with methyl iodide and cetyltrimethylammonium hydroxide. This procedure yielded material with 4.6% methoxyl. It was thus apparent that although these methods may have applications in the methylation of polysaccharides, they did not yield highly methylated products in one step.

**Reduction of Carboxyl Groups of Uranic Acid Moieties**

The procedure involved esterification of the carboxyl groups with methanolic hydrogen chloride followed by reduction of the resulting carbomethoxyl groups with sodium borohydride (11). The desulfation which occurs in the process does not affect the final conclusions, since methylation was completed at this stage. 

Permethylated, N-acetylated heparin (Preparation M1-4) (240 mg) was dissolved in 100 ml of 0.07% anhydrous methanolic hydrogen chloride and the solution was stirred at room temperature for a day. The solvent was evaporated under reduced pressure and residual HCl was removed by codistillation with additional methanol. The solid residue was dissolved in 15 ml of water and cooled in an ice-water bath. A solution of 180 mg of sodium borohydride in 25 ml of 0.1 M borate buffer, pH 9.1, was added slowly and the reaction was allowed to proceed for 3 hours. The reaction mixture was adjusted to pH 5, and the solution was passed through a column of Dowex 50. The eluate and subsequent water washes were combined and evaporated to dryness. Boric acid was separated from the polysaccharide residue by codistillation with methanol. This procedure resulted in reduction of only 30% of the uranic acid residues. After the esterification and reduction process had been repeated three times, 70% of the original uronic acid moieties were reduced. The amount obtained was 125 mg, representing 78% of the theoretical yield obtainable from M1-4.

**Characterization of Methylated Components**

*Hydrolysis and Fractionation*—Preliminary experiments on the rate of hydrolysis of the reduced, methylated, N-acetylated heparin in 1 N H2SO4 at 100° showed that the release of reducing sugar started to level off after 4 hours and became extremely slow after 8 hours. Two experiments were therefore performed: one with 115 mg for 4 hours and another with 175 mg for 7 hours. After each hydrolysis the solution was neutralized with Dowex 1 carbonate, concentrated to a small volume, and passed through a column of Dowex 50 (hydrogen form). The eluate and subsequent wash emerging from the resin contained the neutral sugars (Fraction N). Subsequent elution with 0.3 N HCl yielded the hexosamine fraction (Fraction H). Hydrogen chloride was removed from Fraction H by codistillation with ethanol.

Paper chromatography revealed that both fractions contained several components. These were separated by preparative paper chromatography with Solvent A. Fraction N yielded two components: N-1 (Rf 0.30) and N-2 (Rf 0.55). Fraction H contained three components: H-1 (Rf 0.13), H-2 (Rf 0.17), and H-3 (Rf 0.38). The components in both fractions stained with ammonical silver nitrate and aniline hydrogen phthalate; those in Fraction H also stained with ninhydrin. Both hydrolysis experiments yielded the same components; however, there were some differences in the relative proportions in the products obtained (Table I).

**Table I**

<table>
<thead>
<tr>
<th>Component and identification</th>
<th>Recovery</th>
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<tbody>
<tr>
<td></td>
<td>4-hr hydrol. ylds</td>
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<tr>
<td>N-1: 3-O-methyl-D-glucose</td>
<td>4.1</td>
</tr>
<tr>
<td>N-2: 2,3-di-O-methyl-D-glucose</td>
<td>9.8</td>
</tr>
<tr>
<td>H-1: D-glucosamine</td>
<td>5.7</td>
</tr>
<tr>
<td>H-2: 3-O-methyl-D-glucosamine</td>
<td>16.5</td>
</tr>
<tr>
<td>H-3: 3,6-di-O-methyl-D-glucosamine</td>
<td>2.4</td>
</tr>
</tbody>
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* Paper chromatography of the 7-hour hydrolysate also revealed a trace amount of glucose. This is probably due either to de-methylation or to a minimal amount of uronic acid which was not etherified.

Component N-1—This product was chromatographically indistinguishable from 3-O-methyl-D-glucose in Solvents A and B. The specific rotation, [α]25 +55.6° (c, 0.3 in water) was similar to that reported previously for this compound, i.e. +55° (27). De-O-methylation was effected with boron trichloride (28). One milligram was dissolved in a mixture of 1.5 ml of methylene chloride and 1 g of boron trichloride cooled in an acetone-solid carbon dioxide bath. The mixture was kept at the low temperature for 30 min and then allowed to attain room temperature overnight. After the remaining boron trichloride and methylene chloride were removed, methanol was added to the residue and flash evaporated until no chloride could be detected. The residue was dissolved in a minimal amount of water and chromatographed on paper with the three solvents. The de-O-methylated sugar was identified as glucose by its mobility and staining with glucose oxidase.

A portion of component N-1 (2.5 mg) was dissolved in 0.07 ml of anhydrous pyridine and allowed to stand for a day at room temperature. After cooling in an ice bath, 0.07 ml of benzoyl chloride was added and the reaction was allowed to proceed for a day. Water (0.01 ml) was then added and the mixture was dissolved in 10 ml of benzene. The benzene layer was washed twice with 2 N HCl, twice with 2 N NaOH, and finally with water. After treatment with anhydrous sodium sulfate the solution was concentrated under reduced pressure and the residue was crystallized from methanol. This yielded 3 mg of 3-O-methyl-1,2,4,6-tetra-O-benzoyl-β-D-glucopyranoside, m.p. 199-200°. The tetrabenzoate prepared from authentic 3-O-methyl-D-glucose melted at 199-200°, and a mixture of the two preparations did not result in any depression of the melting point.

Component N-2—The mobility of this material on paper chromatography with Solvents A and B was the same as that of 2,3-di-O-methyl-D-glucose. The specific rotation was +48.4° (c, 0.3 in acetone) as compared with the reported value of +48° (29). De-O-methylation of this material yielded only glucose. The experimental method and identification procedure were as described for Component N-1. A portion of Component N-2 was converted to the N-phenylglycosylamine derivative. Eight milligrams were dissolved in 2 ml of ethanol, 0.1 ml of freshly distilled aniline, and 0.1 ml of 0.001 N H2SO4. The mixture was
which indicates substitution on carbon atom 3.

It gave a positive Morgan-Elson reaction after N-acetylation, which would not be the case for 3,4-di-O-methylglucosamine. The ratio of $A_{440}$ to $A_{410}$ was 0.83, which indicates substitution on carbon atom 3.

Component H-2—This fraction has the same $R_F$ as 2-amino-2-deoxy-3-O-methyl-d-glucopyranoside (glucosamine) in Solvents A, B, and C. It stained with silver nitrate, aniline hydrogen phthalate, ninhydrin, and the Elson-Morgan reagent. Degradation with ninhydrin followed by paper chromatography (30) yielded arabinose. It was also shown to be glucosamine by the optical density at 530 nm in the Elson-Morgan reaction and by the ratio of $A_{540}$ to $A_{510}$ (32).

Component H-3—This was identified as 3,6-di-O-methyl-d-glucosamine on the basis of the following findings. Paper chromatography in Solvents A and B showed it to have mobilities similar to those of di-O-methyl glucosamine. De-O-methylation yielded glucosamine. It gave a positive Morgan-Elson reaction after N-acetylation, which would not be the case for 3,4-di-O-methylglucosamine (32). The ratio of $A_{440}$ to $A_{410}$ was 0.83, which indicates substitution on carbon atom 3.

**DISCUSSION**

The sulfoamino sulfates in heparin were first removed and the amino groups were acetylated. Structural studies were carried out on the acetamido derivative of heparin in order to obviate difficulties which could arise from N-desulfation and N-methylation if heparin were methylelated directly. Preliminary experiments on the effect of alkali on the sulfate ester linkages showed that there was no significant amount of cleavage under conditions of the permethylation procedure. After four extended methylations of the acetamido heparin, the product contained 13% methoxyl, corresponding to an average of five methoxyl groups per two glucosaminylglucuronic acid units. Since the sulfur analysis for N-desulfated heparin indicated three O-sulfate groups per tetrasaccharide (10), only five hydroxyl groups are available for etherification. The number of methoxyl groups introduced in the present studies is thus the calculated maximum. Further methylation with other reagents did not increase the methoxyl content, and incurred considerable losses in yield.

Since a substantial proportion of uronic acid is degraded under conditions required for total hydrolysis of heparin (10), the carboxyl groups in these residues were first reduced to primary hydroxyl groups. This was accomplished by esterification with methanolic hydrogen chloride followed by treatment with sodium borohydride. Reduction by this procedure is incomplete, owing to concomitant de-esterification in the alkaline medium. The process, therefore, had to be repeated several times. In the final product over 70% of the uronic acid residues were reduced.

Hydrolysis of the permethylated, reduced acetamido heparin for 4 and 7 hours yielded the same monosaccharide derivatives. The products obtained and the amounts isolated are shown in Table I. The values should be considered as minimal, since significant losses are incurred in the isolation process. The yield is about 85 to 40% of the methylated polysaccharide. The total amount of methylated glucose derivatives is considerably less than that of the hexosamines. This is due partly to the fact that only 70% of the uronic acid was reduced. In addition, the percentage of any uronic acid that is iduronic is not accounted for in the total balance.

The isolation of 3-O-methylglucose and 2,3-di-O-methylglucose in a ratio of about 1:2 leads to the conclusion that approximately one-third of the glucuronate units contain sulfate ester groups on carbon atom 2 and the other glucuronate units contain no sulfate (Fig. 1, Units A and C, respectively). The presence of O-sulfates on some of the uronic acid units was also proposed by Foster et al. (35) from the results of periodate oxidation studies.

The data shown in Table I indicate that over 75% of the glucosamine units are sulfated on carbon atom 6 (Fig. 1, Unit B).
The isolation of unsubstituted glucosamine suggested that 3, 6-di-0-sulphoglucosamine units are also present (Fig. 1, Unit D). The possibility that the free glucosamine in the hydrolysate cannot be excluded, although this seems improbable in view of the relative amount present and the number of methoxyl groups introduced. Analyses with Fisher-Hirshfelder models show that two O-sulphate groups can be accommodated on some of the glucosamine units. The presence of sulphate ester groups on carbon atom 6 of glucosamine has also been indicated from methylation (36) and periodate oxidation (35, 37) analyses.

The third hexosamine component in the hydrolysate (H-3), which is present in a comparatively small amount, was identified by chromatography and characteristic color reactions as 3, 6-di-0-methylglucosamine. This would arise from a hexosamine moiety which contained no O-sulphate groups (F in Fig. 1).

An over-all structure of the principal units in heparin based on present findings and previous work is shown in Fig. 1. The formula is not meant to suggest a specific repeating unit, but only to show the positions of the different sulphate groups. The individual units are not sulphated to the same degree throughout the heparin polymer. Carbon atom 6 is sulphated in the major number of the glucosamine units, yet a small amount of the hexosamine apparently contains no O-sulphate. In addition, certain portions of the glucosamine units are di-O-sulphated. Similarly, most of the glucuronic acid moieties are unsubstituted, whereas about 35% contain sulphate ester groups on carbon atom 2. Whether there is a definite regularity in sulphate ester distribution within the heparin polymer or whether there are areas which are more dense in sulphate groups remains to be established. It is quite conceivable that heparin exhibits the “molecular heterogeneity" with respect to sulphate ester groups discussed in the review by Schiller on other mucopolysaccharides (38). Such a possibility could occur if the sulphation step in the biosynthesis of heparin follows the formation of the polysaccharide.

REFERENCES