The Role of Serine in the Linkage of Heparin to Protein*

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The first suggestion of the occurrence of a protein-mucopolysaccharide complex was made by Möller (1) who isolated a crude chondroitin sulfate-protein complex from cartilage. That the carbohydrate was covalently bound to protein was more firmly established by the purification and characterization of chondromucoprotein from bovine nasal septa (2-6). The proposal by Muir (4) that chondroitin 4-sulfate is linked to protein through serine was more conclusively proven by the isolation of glycopeptides (7, 8) which contained only serine. Complexes of other mucopolysaccharides have been described including those of 4-sulfate-protein complex.

In contrast, the presence of several ninhydrin-positive compounds which were assumed to be amino acids could be demonstrated by paper chromatography of heparin hydrolysates (14). Residual amino acids with serine as the dominant component have also been found in heparitin sulfate isolated from human aorta (15) and from tissues of patients with Hurler's syndrome.

This study reports evidence for the presence of bound amino acids, particularly serine, in various commercial heparin preparations. In certain preparations, only serine was found in significant amounts.

Materials

The heparin preparations examined have been obtained from the following commercial sources.

The Wilson Laboratories, Chicago, Illinois, supplied Preparation 1, Lot 110841-14, and Preparation 3, Lot 35-174 B, which were obtained from ox liver capsules. Preparation 1, Lot 110841-14, was obtained from pig intestinal mucosa and was stated to be prepared under mild conditions not involving the use of alkali; Preparation 2, Lot 110977-E, was obtained from Preparation 1 by treatment with alkali at high temperature followed by bleaching at room temperature.

The following samples were obtained from the Upjohn Company, Kalamazoo, Michigan: Preparation 4, Lot 132-RTS-12, an unbleached heparin from lung tissue; Preparation 5, Lot 132B-RTS-12, the bleached product derived from Preparation 4.

The Vitrum AB, Stockholm, Sweden, supplied Preparation 6, Lot 62235, obtained from hog intestinal mucosa; this was further purified as described below, yielding Preparation 6 Cu; Preparation 7, Lot 6002, and Preparation 8, Lot 159323, from lung. Preparations 6 and 8 had been prepared by extraction from the tissues with weak alkali, proteolytic digestion with pancreatic and intestinal enzymes, and fractionation with cetylpyridinium chloride. Preparation 7 had been prepared by methods involving bleaching and alkali treatment at high temperature.

Methods

Analytical Methods—Nitrogen was determined by a micro-Kjeldahl method and glucuronic acid by the method of Dische (16). Hexosamine was determined after hydrolysis in 4 M HCl for 14 hours by a modification of the method of Boas (17) with omission of the resin treatment. Sulfate analyses were performed according to Muir's modification of the method of Dodge and Spencer (4). Anticoagulant activity was determined by the B.P. (18) or the U.S.P. (19) assay methods. Molecular weights were determined viscosimetrically (20) with Lauren's relationship between molecular weight and limiting viscosity number (21). Preparation 3 was also assayed osmotically (20).

Amino acids were estimated quantitatively by means of a Technicon amino acid analyzer and qualitatively by paper electrophoresis with 27-×112-cm strips of Whatman No. 3MM paper and 0.75 M formic acid-1.0 M acetic acid buffer, pH 1.9 (22), at 4000 volts for 5 hours in a Gilson Medical Electronics (Middleton, Wisconsin) high voltage electrophorator, model D. The strips were stained by dipping in a solution of 0.2 g of ninhydrin in 100 ml of 95% acetone to which 2 ml of pyridine had been added. The samples were prepared for chromatography or electrophoresis by hydrolysis in 6 M HCl for 20 hours at 100° and repeated evaporation in a rotary evaporator.

In the course of hydrolysis of proteins, some loss of serine (5 to 15%) is known to occur (23, 24). Control experiments indicated that losses of the same magnitude occurred when serine was added to heparin before hydrolysis. Since the magnitude of the losses was such as to be insignificant for the purposes of this study, no corrections were made.
The glucosamine contents obtained in the course of amino acid analyses of the heparin samples were lower than those determined by the Boas method (compare Tables I and II) and varied between 61% and 91% of the latter, with an average of 73%. Because of these erratic results calculations of serine to hexosamine ratios were based on hexosamine values obtained by the Boas method.

**Purification of Heparin Preparations**—Preparation 6 contained considerable amounts of galactosamine and was further purified by alkaline copper precipitation (26). All operations were carried out at 4°; 400 mg were dissolved in 50 ml of water and 35 ml of Benedict’s reagent and 10 ml of 9 M NaOH were added. After 15 min, the precipitate, which consisted mainly of dermatan sulfate (chondroitin sulfate B), was removed by centrifugation. The supernatant solution was neutralized with NaOH, concentrated to a small volume in a rotary evaporator, and several volumes of ethanol were immediately neutralized with NaOH, concentrated to a small volume in a rotary evaporator, and several volumes of ethanol were added. The precipitated heparin was washed twice with ethanol and once with ether, yielding a product weighing 267 mg (Preparation 6-Cu).

Preparation 1 was fractionated essentially as described by Scott, Gardell, and Nilsson (26). To a solution of 1 g of Preparation 1 in 500 ml of 1.4 M NaCl, 300 ml of a 1% solution of cetylpyridinium chloride in 1.4 M NaCl were added. After the mixture was kept overnight in a water bath at 40°, the precipitate was separated by centrifugation at 10,000 × g at room temperature for 25 min, and the supernatant solution was retained for further fractionation as described below. The precipitate was dissolved in 200 ml of 3.0 M NaCl, and the solution diluted to 1.4 M. After standing in a water bath at 40° for 3 hours, the cetylpyridinium complex was collected by centrifugation, dissolved in 50 ml of 3.0 M NaCl, and 200 ml of ethanol were added. The heparin so obtained was dissolved in 30 ml of water and reprecipitated with 4 volumes of ethanol. This precipitation procedure was repeated twice. After drying with ethanol and ether, the product (Preparation 1-CP I) weighed 487 mg. Further fractions were obtained from the supernatant by dilution with water. The precipitate formed at 1.2 and 0.6 M NaCl solutions were separated by cetylpyridinium chloride fractionation, the results indicating that serine is covalently linked to heparin. The cetylpyridinium chloride fractions obtained from Preparation 1 (1-CP I to 1-CP III) contained 1 residue of serine per 20 repeating disaccharide units of heparin and only trace amounts of other amino acids. The presence of serine was revealed also by paper electrophoresis and by paper chromatography. Since mixtures of serine and heparin were easily separated by cetylpyridinium chloride fractionation, the results indicate that serine is covalently linked to heparin. The cetylpyridinium chloride fractions obtained from Preparation 1 (1-CP I to 1-CP III) contained 1 residue of serine per 53, 31, and 26 repeating units, respectively. The weight average molecular weights of the three fractions were 12,200, 11,900, and 9,600, corresponding to 20, 19, and 16 repeating disaccharide units (molecular weight, 615, calculated for the sodium salt of a disaccharide with 2.5 molecules of sulfate). A higher serine content, 1 residue per 22 repeating units, was found in Preparation 3. This sample had a number average molecular weight of 9,600, corresponding to 16 repeating units. However, Preparation 3 contained considerable amounts of other amino acids. Preparation 2 which had been obtained from Preparation 1 by treatment with alkali at high temperature and bleaching at room temperature, contained only small amounts of amino acids, indicating that such treatment causes cleavage of the protein-carbohydrate linkage. Preparation 6, which had been prepared under relatively mild conditions, showed essentially the same amino acid pattern as Preparation 1 with a preponderance of serine and only small amounts of other amino acids. Since Preparation 5 contained galactosamine which amounted to 14% of the total hexosamine, it was purified further with alkaline copper precipitation (25) in the cold. This procedure removed 98% of the galactosamine without loss of serine (Preparation 6-Cu).

**Lung Heparin**—Preparation 4 contained a series of amino acids, serine and glycine being the most abundant. Fractionation with cetylpyridinium chloride caused a relative enrichment of serine primarily in Fraction 4-CP III. The amount of serine in the three precipitate fractions, 4-CP I to 4-CP III, was 1 residue per 83, 56, and 42 repeating units, respectively. The supernatant material (4-CP IV) showed increased concentrations of glycine and galactosamine and a decreased amount of serine. It should be pointed out that this fraction had no anticoagulant activity and a low sulfur content and was therefore not heparin. Preparation 5, which was derived from Preparation 4 by a bleaching process, contained only traces of amino acids. Analysis of Preparation 7, an alkali-treated and bleached lung heparin, gave essentially the same results. Preparation 8, the isolation of which involved extraction of tissue with dilute alkali, similarly contained only small amounts of amino acids.

In instances where amino acids other than serine were present, glycine, glutamic and aspartic acids, proline, alanine, and threonine were present. The analytical data for the various heparin preparations are shown in Table I. The values agree with data reported by others although certain of the samples had lower sulfur contents than generally reported. The anticoagulant activities varied over a wide range. Preparations 1-CP I and 1-CP II had the highest activities, 252 and 281 B.P. units per mg, respectively. There was a considerable discrepancy between the B.P. and U.S.P. assays; when assayed with the U.S.P. method the above two fractions gave values of 214 and 199 units per mg, respectively.

**Amino Acid Composition**

Qualitative analysis by paper chromatography and paper electrophoresis showed that the majority of the heparin preparations examined contained amino acids. Serine was the major amino acid and in some preparations the only one found in significant amounts.

**Intestinal Muscosa Heparin** Table II shows the amino acid compositions of the heparin preparations used in this study. Preparation 1 is of particular interest since it contained 1 residue of serine per 30 repeating disaccharide units of heparin and only trace amounts of other amino acids. The presence of serine was revealed also by paper electrophoresis and by paper chromatography. Since mixtures of serine and heparin were easily separated by cetylpyridinium chloride fractionation, the results indicate that serine is covalently linked to heparin. The cetylpyridinium chloride fractions obtained from Preparation 1 (1-CP I to 1-CP III) contained 1 residue of serine per 53, 31, and 26 repeating units, respectively. The weight average molecular weights of the three fractions were 12,200, 11,900, and 9,600, corresponding to 20, 19, and 16 repeating disaccharide units (molecular weight, 615, calculated for the sodium salt of a disaccharide with 2.5 molecules of sulfate). A higher serine content, 1 residue per 22 repeating units, was found in Preparation 3. This sample had a number average molecular weight of 9,600, corresponding to 16 repeating units. However, Preparation 3 contained considerable amounts of other amino acids. Preparation 2 which had been obtained from Preparation 1 by treatment with alkali at high temperature and bleaching at room temperature, contained only small amounts of amino acids, indicating that such treatment causes cleavage of the protein-carbohydrate linkage. Preparation 6, which had been prepared under relatively mild conditions, showed essentially the same amino acid pattern as Preparation 1 with a preponderance of serine and only small amounts of other amino acids. Since Preparation 5 contained galactosamine which amounted to 14% of the total hexosamine, it was purified further with alkaline copper precipitation (25) in the cold. This procedure removed 98% of the galactosamine without loss of serine (Preparation 6-Cu).
TABLE I

Analyses of heparin preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Nitrogen (%)</th>
<th>Hexosamine (%)</th>
<th>Uronic acid (%)</th>
<th>Sulfur (%)</th>
<th>Ratio of sulfur to hexosamine</th>
<th>Anticoagulant activity</th>
<th>Molecular weight</th>
<th>Intein viscosity</th>
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<tr>
<td>I-CP I</td>
<td>2.3</td>
<td>26.6</td>
<td>45.4</td>
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<td>1.85</td>
<td>165</td>
<td>U.S.P.</td>
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a Values corrected for moisture content, determined in an Abderhalden drying apparatus by heating to 65°C with phosphorus pentoxide under reduced pressure for 5 hours.

b Molar ratios with hexosamine as 1.00.

c Calculated from viscosimetric data.

d Calculated from osmometric data.

TABLE II

Amino acid analyses of heparin preparations

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<th>Preparation</th>
<th>GlcN</th>
<th>GalN</th>
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<th>Glu</th>
<th>Pro</th>
<th>Gly</th>
<th>Ala</th>
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<th>Leu</th>
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<th>Phe</th>
<th>Lys</th>
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</table>

- Calculated on a dry weight basis.
- Trace amounts are indicated by Tr.

Preparations 1, 3, 4, 6, and 8 were reported to have been prepared under mild conditions, while Preparations 2, 5, and 7 had been subjected to harsher treatments.

nine were found in amounts decreasing in this order. Other amino acids generally occurred in much smaller amounts.

DISCUSSION

The present work has established that heparin prepared by mild methods, including the use of dilute alkali, contains residual amino acids. Harsher treatment involving alkali at elevated temperatures and bleaching agents removes virtually all amino acids. Certain of the heparin preparations possessed unusually high anticoagulant activities, particularly Fraction 1-CP I, which demonstrated the highest activity for pig intestinal mucosa preparations thus far described. The presence of amino acids in highly purified heparin indicates that they are covalently linked to heparin. These findings reopen the question of the existence of a native heparin-protein complex.

It is obvious that in all preparations serine was the predomi-
The carbohydrate complex with protein and that the carbohydrate-attachment of protein to polysaccharide. 

It is suggested that heparin occurs in the native state as a complex with protein and that the carbohydrate-attachment of protein to polysaccharide. 

A striking resemblance is found between the amino acid pattern of heparin and that of chondroitin 4-sulfate, prepared with the use of proteolytic enzymes (4, 8). These observations suggest that in the protein complexes of both polysaccharides there may be similarities in the amino acid sequence at the point of attachment of protein to polysaccharide.

A number of commercial heparin preparations have been examined for the presence of amino acids. Heparin which had been prepared by use of mild methods essentially based on treatment with proteolytic enzymes, contained residual amino acids with serine as the main component. In some preparations serine was the only amino acid found in significant amounts. Samples prepared under more drastic conditions, including alkali extraction and bleaching at elevated temperatures, contained only traces of amino acids. It is suggested that heparin occurs in the native state as a complex with protein and that the carbohydrate-protein linkage is similar to that of the chondroitin 4-sulfate-protein complex.

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SUMMARY

A number of commercial heparin preparations have been examined for the presence of amino acids. Heparin which had been prepared by use of mild methods essentially based on treatment with proteolytic enzymes, contained residual amino acids with serine as the main component. In some preparations serine was the only amino acid found in significant amounts. Samples prepared under more drastic conditions, including alkali extraction and bleaching at elevated temperatures, contained only traces of amino acids. It is suggested that heparin occurs in the native state as a complex with protein and that the carbohydrate-protein linkage is similar to that of the chondroitin 4-sulfate-protein complex.

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