THE LINKAGE OF HEPARIN TO PROTEIN*

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The presence of residual amino acids in heparin isolated under mild conditions (Lindahl et al., 1964) suggests that heparin may exist in the native state as a covalently-linked complex with protein. In certain heparin preparations, serine was the only amino acid found in significant amounts. Since chondroitin 4-sulfate is linked to protein through serine (Muir, 1958; Rodén et al., 1963; Gregory et al., 1964), these findings indicate that both polysaccharides are bound to protein in a similar manner. That heparin (Lindahl and Rodén, 1964), as well as glycopeptides isolated from the chondroitin 4-sulfate-protein complex (Gregory et al., 1964), contains galactose and xylose, indicates that these sugars may be involved in the carbohydrate-protein linkage. In order to gain more information concerning the nature of this linkage, attempts were made to obtain carbohydrate-serine compounds from heparin by mild acid hydrolysis. This communication describes the results of these experiments.

Isolation of carbohydrate-serine compounds. Crude heparin (lot #125233-14 A1, Wilson Laboratories, Chicago, Illinois), demonstrating an anticoagulant activity of 148 U.S.P. units/mg, was purified by fractionation with cetylpyridinium chloride, essentially according to the method of Scott et al.(1957). Material, twice precipitated from 1.4 M NaCl, was used for the experiment. Analyses (% of dry weight): hexosamine 23.2, uronic acid 40.6, nitrogen 2.0, sulfur 9.4, anticoagulant activity 171 units/mg. Serine (36.2 μmoles/g dry weight) was the only amino acid present in significant amounts. The galactosamine content was less than 0.5% of the total hexosamine.

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A solution of 5.0 g of purified heparin in 500 ml of water was passed through a 4.5 X 26 cm column of Dowex 50W-X8, H^+, 200-400 mesh, at 4°. After concentration to 500 ml in a rotary evaporator, the solution, which had a pH of 1.55, was heated at 100° for 3 hours. The hydrolysate, diluted to a final volume of 2.5 liters, was adjusted to pH 3.0 with Dowex 3, CO_3^{--}. The solution was then passed through a 4.5 X 40 cm column of Dowex 1-X2, Cl^-, 200-400 mesh, which was subsequently washed with 1 liter of water in the cold. The combined effluents (3.5 liters) were concentrated to 2 ml; the pH was repeatedly adjusted to approximately 4 with Dowex 3, CO_3^{--}. The final solution, which will henceforth be referred to as the Dowex 1-treated hydrolysate, exhibited a pH of approximately 3. It contained a total of 10 mg of uronic acid, which represented less than 1% of the uronic acid content of the starting material.

A 5 µl sample of the Dowex 1-treated hydrolysate was applied to a 48 X 59 cm paper (Schleicher and Schuell, No. 589 green ribbon, acid washed) and subjected to high voltage electrophoresis[1] at 4600 V (105 V/cm) for 1 hour in 0.15 M formic acid - 1.6 M acetic acid, pH 2.0. The paper was stained by dipping in a ninhydrin reagent containing pyridine (Efron, 1960) to reveal the pattern given in Fig. 1.

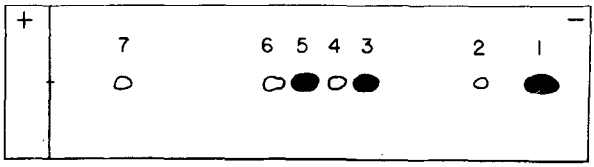

Fig. 1. High voltage electrophoresis of heparin hydrolysate after treatment with Dowex 1.

Three major spots appeared and, in addition, a number of minor spots, all of which are not indicated in the figure. Spot 1, the leading of the three major spots, had the mobility of serine and was not further investigated. The two other major components, 3 and 5, were isolated by preparative paper electrophoresis under the conditions described above, with the modification that the current was maintained for 1 hour 25 minutes. Seven papers were used for the fractionation of 1.75 ml of the Dowex 1-treated hydrolysate. After elution with water

[1] High voltage electrophorator, model D, Gilson Medical Electronics, Middleton, Wisconsin.

in the cold, the eluates were concentrated to 30 ml and stored at -20°.

Characterization of carbohydrate-serine compounds. Fig. 2 illustrates the chromatographic behaviour of the unfractionated Dowex 1-treated hydrolysate on Dowex 50-X8 (Chromobeads type A), using a

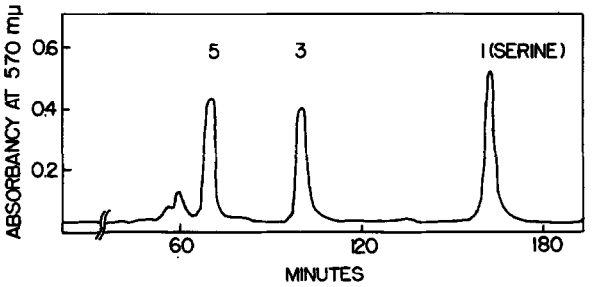

Fig. 2. Chromatography on Dowex 50 of heparin hydrolysate after treatment with Dowex 1.

Technicon automatic amino acid analyzer. When the isolated fractions 3 and 5 were applied to the column, they emerged as single peaks in the indicated positions.

On paper chromatography (Whatman No. 1, butanol-ethanol-water 10:3:5) compounds 3 and 5 appeared as single ninhydrin-positive spots with R_{serine} values of 0.73 and 0.34, respectively.

The ninhydrin-positive components of fractions 3 and 5 were characterized by chromatography on Dowex 50 after hydrolysis at 100° in either 1 M HCl for 3 hours or 6 M HCl for 20 hours. Each hydrolysate showed one single peak in the position of serine. The identity of this material with serine was further confirmed by paper chromatography in butanol-acetic acid-water (12:3:5).

Analysis of the carbohydrate moieties of compounds 3 and 5 was carried out in the following manner: One ml samples were mixed with 0.33 ml of 4 M HCl and hydrolyzed in sealed tubes at 100° for 3 hours. After cooling, the pH of the hydrolysates was adjusted to 5.0 with Dowex 3, CO_3^{--}, and the solutions were passed through 1 X 15 cm columns of Dowex 50W-X8, H^+, 20-50 mesh. The effluents were evaporated to dryness and the residue dissolved in 20 μl of water, of which 10 μl were applied to a Whatman No. 1 paper. Papers were developed with butanol-ethanol-water (10:3:5) for 35 hours or butanol-pyridine-water (6:4:3) for 30 hours and stained with aniline hydrogen phthalate (Partridge, 1949). Compound 3 gave only one spot, which stained pink and had the R_f value of xylose. Compound 5 showed an

identical spot and, in addition, a brown spot with the R_f value of galactose.

With both solvent systems used, xylose was well separated from ribose and arabinose, but less completely from lyxose. The hydrolysates were therefore also examined by paper electrophoresis in borate buffer. The samples were applied to an 18 X 40 cm paper (Schleicher and Schuell, No. 589 green ribbon, acid washed), which had been soaked in 0.05 M borax solution and equilibrated in a LKB electrophoresis apparatus for 1 hour. After electrophoresis at 300 V for 3 hours, the paper was dried and stained with aniline hydrogen phthalate. Lyxose was completely separated from the unknown pentose, which had a mobility identical to that of xylose.

For quantitative analyses, 1.0 ml samples were hydrolyzed as described above. After neutralization with Dowex 3, CO_3^{--}, the solutions were concentrated to dryness and dissolved in 1.0 ml of water. The serine content of a 0.2 ml sample was determined as described above. Pentose analyses were carried out with the orcinol method (Brown, 1946), and galactose was estimated by the galactose dehydrogenase method of Doudoroff (1962). Reducing activity of the hydrolysates was determined with the Park-Johnson method (Park and Johnson, 1949). The results are given in Table 1. Compound 3 contained equimolar

TABLE 1

ANALYSES OF COMPOUNDS 3 AND 5 AFTER HYDROLYSIS IN 1 M HCl FOR 3 HOURS[a]

Compound	Serine	Xylose	Galactose	Serine: Xylose: Galactose[b]	Reducing power[c]
	µmoles/ml	µmoles/ml	µmoles/ml		µmoles/ml
#3	0.49	0.51	0.04	1.00:1.04	0.51
#5	0.52	0.54	0.52	1.00:1.04:1.00	0.79

a) Results are expressed as µmoles per ml of the eluates from preparative paper electrophoresis. The eluates had a total volume of 30 ml.
b) Molar ratios with serine as 1.00.
c) Determined by the Park-Johnson method with glucose as a standard.

amounts of serine and xylose, and compound 5 equimolar amounts of serine, xylose, and galactose. Before hydrolysis, neither of the two

compounds showed any reducing activity. Hydrolysis of substance 3 liberated reducing groups corresponding exactly to its xylose content. The hydrolysate of compound 5 exhibited a reducing activity which was somewhat less than expected on the basis of its sugar content.

Analyses for hexosamine were negative. When the presence of uronic acid in the unhydrolyzed materials was examined by the carbazole reaction (Dische, 1947), a slight brownish discoloration was observed. This was probably due to impurities from the paper. The calculated uronic acid content was less than 10% of the xylose content.

Discussion. The isolation of two carbohydrate-serine compounds from heparin has been described. One substance was composed of serine and xylose, and the other of serine, xylose and galactose. No additional sugars could be detected by paper chromatography. Also, the reducing power of the hydrolysates was in essential agreement with what could be expected from the components known to be present. Both compounds were non-reducing before hydrolysis. As a consequence, substance 3 must consist of xylose glycosidically linked to serine. Since it seems unlikely that more than one type of serine-heparin linkage exists, it is most probable that compound 5 has the structure galactosyl xylosyl serine.

The electrophoretic properties of the carbohydrate-serine compounds permit further conclusions as to the mode of linkage to serine. The substances migrated as cations at pH 2, but behaved as neutral molecules at pH 6 in pyridine acetate buffer. These observations indicate that both the amino group and the carboxyl group of serine are free, which leaves a linkage to the hydroxyl group as the only reasonable alternative.

The possibility that the isolated carbohydrate-serine compounds originate in glycoprotein contaminants rather than in heparin itself can not be conclusively ruled out at the present time. It seems likely, however, that the repeated precipitation with cetylpyridinium chloride from 1.4 M NaCl should have removed any contaminating glycoprotein.

Of the serine present in the starting material, the hydrolysis procedure used liberated 17% as free serine, 13% as compound 3, and 13% as compound 5. These figures indicate that the linkage to xylose represents a major mode of linkage of serine in heparin.

In preliminary experiments xylosyl serine and galactosyl xylosyl serine have also been obtained from glycopeptides isolated from the chondroitin 4-sulfate-protein complex. These findings furnish further

evidence that chondroitin-4-sulfate and heparin are linked to protein in the same manner.

In conclusion, the isolation of xylosyl serine and galactosyl xylosyl serine strongly suggests that galactose and xylose are both involved in the linkage of heparin to protein. A glycosidic linkage between xylose and the hydroxyl group of serine appears to constitute the carbohydrate-protein linkage.

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