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Note

A new method for covalent coupling of heparin and other glycosaminoglycans to substances containing primary amino groups

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Heparin contains (1→4)-linked 2-amino-2-deoxy-α-D-glucopyranosyl, α-L-idopyranosyluronic acid, and a small proportion of β-D-glucopyranosyluronic acid residues. The hexosamine and hexuronic acid residues are linked alternately and are partially *O*-sulfated. Most of the 2-amino-2-deoxy-D-glucosyl residues are *N*-sulfated, the remainder being *N*-acetylated[1]. Heparin exerts its main blood-anticoagulant activity by binding, and thereby potentiating the inhibitory effect of, the plasma protein antithrombin (AT)[2]. Only about one-third of the molecules in a heparin preparation bind with high affinity to AT[3–5]. These molecules contain a specific AT-binding pentasaccharide-sequence[6].

Several attempts have been made to bind heparin covalently to solid supports in order to prepare blood-compatible surfaces and chromatographic gels for affinity chromatography[7]. Some procedures partly or completely inactivate the immobilised heparin, presumably because of coupling, and thereby inactivation, of the active sequence.

Compounds containing aldehyde groups react with primary amines to give labile Schiff-bases that can be converted into stable secondary amines by reduction. Synthetic glycoproteins have been prepared by treating proteins with different reducing-disaccharides in the presence of sodium cyanoborohydride[8,9]. The yields are limited, because the aldehyde groups are largely present as cyclic hemiacetals and Schiff-base formation is not extensive. Oligo-and poly-saccharides are expected to give even lower yields on coupling by reductive amination.

Nitrous acid converts 2-amino-2-deoxy-D-glucopyranosyl residues into 2,5-anhydro-D-mannose residues, the aglycon being released[10]. Heparin was partially depolymerised by deaminative cleavage with nitrous acid[11]. Fragments of low molecular weight were removed by dialysis. The resulting heparin preparation (*NH*) contained fragments having 2,5-anhydro-D-mannose residues as reducing terminal units. The aldehyde groups of these units were not engaged in intramolecular hemiacetal formation. The ^{13}C-n.m.r. spectrum of *NH* contained a sig-

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nal for the hydrated aldehyde group at δ 90.4, whereas the signals for the anomeric carbons of the other residues appeared in the region[12] δ 97–105. Integration of these signals showed that *NH* contained an average of 30 sugar residues, corresponding to a molecular weight of ~10,000.

Covalent coupling of *NH* to Sepharose was performed in two steps: (*a*) the gel was converted into 6-aminohexyl-Sepharose[13,14], and (*b*) *NH* was coupled to the product by reductive amination in aqueous solution at pH 7.0, using sodium cyanoborohydride[15]. The coupling yield was determined by measuring the sulfur content of the derivatised gel. The reducing terminal units in approximately half of the *NH* molecules consist of 2,5-anhydro-D-mannose residues and, under the conditions used, 25% of the *NH* preparation was covalently attached to the gel. The chromatographic properties of the resulting gel were not significantly altered[14], and the binding capacity of the gel for AT was the same as, or better than, that of commercial heparin–Sepharose[7].

NH was also coupled to curdlan, a (1→3)-linked unbranched β-D-glucan[16], essentially in the same way. The conjugate was isolated by gel chromatography and the coupling yield was determined as above.

Heparin fragments produced by partial, deaminative cleavage can be fractionated on AT–Sepharose[6]. With these active heparin-fragments, it should be possible to prepare more-active conjugates. The coupling of heparin to proteins (human serum albumin and AT) is being studied.

Other polysaccharides containing 2-amino-2-deoxy-gluco- or -galacto-pyranosyl residues can be covalently linked, by the same procedure, to substances containing primary amino groups.

EXPERIMENTAL

General. — Concentrations were carried out under reduced pressure at bath temperatures not exceeding 40°. The ^{13}C-n.m.r. spectra were recorded at 15.14 MHz and 80° with a Jeol FX 90 Q instrument. The samples were dissolved in D_2O, and external tetramethylsilane was used as reference. Optical rotations were determined with a Perkin–Elmer 141 instrument.

Heparin was obtained from KabiVitrum (Stockholm, Sweden). Sephadex G 150 and Sepharose CL 4 B were obtained from Pharmacia (Uppsala, Sweden), and curdlan was a gift from Takeda Chemical Industries (Osaka, Japan).

Nitrous acid-degraded heparin (NH). — A solution of heparin (7.6 g) in water (400 mL) was adjusted to pH 2.5 with 0.1M hydrochloric acid. A solution of sodium nitrite (60 mg) in water (10 mL) was added dropwise, and the reaction mixture kept at room temperature. After 2 h, the pH was adjusted to 6.5–7 with 0.1M sodium hydroxide. The solution was concentrated to ~100 mL, dialysed against distilled water, and freeze-dried, to give *NH* (3.0 g), $[\alpha]_{578}^{20}$ +28.5° (*c* 1, water) (Found: C, 23.8; H, 3.8; N, 2.1; S, 9.9%).

Coupling of NH *to 6-aminohexyl-Sepharose.* — Suction-dried Sepharose CL

4B (75 g) was oxidised with 0.2M aqueous bromine (115 mL) and washed with water (2 L). 50% Aqueous acetic acid (~75 mL) was added to 1,6-hexanediamine (25 g) at 0° until the pH of the solution was 6.5. The oxidised gel was added together with $NaBH_3CN$ (700 mg), and the reaction mixture was processed, washed, and analysed as described previously[13]. The aminohexyl content of the dry gel was 7% (by weight).

To a solution of *NH* (500 mg) in 0.2M phosphate buffer (pH 7, 10 mL) were added suction-dried 6-aminohexyl-Sepharose (10 g) and $NaBH_3CN$ (50 mg), and the reaction mixture was swirled for 24 h. The resulting *NH*-Sepharose was transferred to a column, and washed sequentially with 0.5% aqueous acetic acid (500 mL), distilled water (400 mL), M sodium chloride (400 mL), and water (400 mL). The sulfur content of a freeze-dried sample of the gel was 2.2%, as determined by elemental analysis, corresponding to a 20% content of heparin (by weight).

Coupling of NH *to curdlan.* — Curdlan (400 mg) was treated with 85% aqueous formic acid (10 mL) at 100° for 1 h. The mixture was evaporated to dryness, and the residue was stirred with distilled water (100 mL). The undissolved material (300 mg) was collected, and oxidised[13] with 0.1M aqueous bromine (30 mL) at pH 7. The oxidised curdlan was treated with 1,6-hexanediamine (1.25 g) and $NaBH_3CN$ (120 mg) in acetic acid as described above, dialysed against distilled water, and freeze-dried. The resulting 6-aminohexylcurdlan (180 mg) contained 2% of aminohexyl units (by weight), as determined by elemental analysis.

6-Aminohexylcurdlan (50 mg) was treated with *NH* (50 mg) and $NaBH_3CN$ (10 mg) in 0.2M phosphate buffer (10 mL) at pH 7, as described above. The resulting mixture was subjected to chromatography on a column (100 × 2.6 cm) of Sephadex G 150 equilibrated in 0.1M phosphate buffer at pH 7. One fraction between the void and the total volume was collected, dialysed against distilled water, and freeze-dried. The resulting *NH*-curdlan (55 mg) contained 55% of heparin (by weight), as determined by elemental analysis (Found: C, 19.0; H, 3.9; N, 1.1; S, 5.7%).

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