THE LINKAGE SEQUENCE IN HEPARIN*

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The component units and their linkage sequence in heparin have been under investigation in this laboratory (Wolfrom, Vercellotti, and Thomas, 1961; Wolfrom, Vercellotti, and Horton, 1962; Wolfrom, Vercellotti, and Horton, 1963a and 1963b). Partial desulfation, N-acetylation, and diborane carboxyl-reduction of heparin gave a product (Wolfrom, Vercellotti, and Thomas, 1961) which was amenable to graded hydrolysis, and the isolation of crystalline O-α-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-α-D-glucopyranose hydrochloride (I) (Wolfrom, Vercellotti, and Horton, 1962b) and its characterization by methylation (Wolfrom, Vercellotti, and Horton, 1963a) established the α-D-(1→4) D-glucuronic acid→ 2-amino-2-deoxy-D-glucose sequence in heparin.

A second crystalline disaccharide, present to a lesser extent in the hydrolyzate from carboxyl-reduced heparin (Wolfrom, Vercellotti, and Horton, 1963b) was tentatively identified, by color reactions, as O-2-amino-2-deoxy-α-D-glucopyranosyl-(1→4)-α-D-glucopyranose hydrochloride (II). This structure has now been firmly established by methylation. The crystalline N-acetyl derivative of II (163 mg.) was twice methylated with methyl

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iodide and barium oxide-barium hydroxide in N,N-dimethylformamide (Kuhn and Trischmann, 1963), and the product N-acetylated (Roseman and Ludowieg, 1954) to yield the permethylated N-acetylated disaccharide (III) (105 mg., 38%) which showed no hydroxyl absorption in the infrared and was chromatographically homogeneous (R_f 0.68) by thin-layer chromatography (all by silica gel, ascending, 3:2 benzene-methanol). The sirup (III) (81 mg.) was hydrolyzed 5 hr. at 90° with N hydrochloric acid. Acid removal followed by isolative thin-layer chromatography (0.5 mm. thickness) yielded zones of R_f 0.76 (A, 8 mg.) and 0.61 (B, 7 mg.), chromatographically homogeneous in two other solvent systems. The material in zone A crystallized as needles from ethanol-ether-petroleum ether; yield 8 mg., m.p. 120-124°, X-ray powder diffraction pattern identical with that of authentic 2,3,6-tri-O-methyl-α-D-glucose (IV, Charlton, Haworth, and Peat, 1926). The material in zone B crystallized from the same solvent as small granules; yield 2 mg., m.p. 211-214° (dec.), X-ray powder diffraction pattern identical with that of authentic 2-amino-2-deoxy-3,4,6-tri-O-methyl-β-D-glucose hydrochloride (V, Cutler, Haworth, and Peat, 1937).

Table I lists molecular rotatory data for the N-acetyl derivatives of the two disaccharides I and II, together with rotatory data for the corresponding alditols, in comparison with values for other D-glucose disaccharides and their alditols. The molecular rotations of the α-D-linked disaccharide alditols lie between +200° and +350°, while those of the β-D-linked disaccharide alditols lie between -100° and +30°. The alditols of N-acetylated disaccharides I and II have $[M]_D$ values of +285° and +209° respectively. These data afford very strong evidence in favor of an α-D linkage in both disaccharides.

An enzymic synthesis has been reported (Selinger and Schramm, 1961) to yield a disaccharide with the structure II and possessing a specific rotation

Table I

Comparison of $[M]_D$ of Disaccharides and Disaccharide Alditols

Compound	Linkage	$[M]_D$ (Equil.)	Derived Alditol $[M]_D$
Maltose	α-D-(1→4)	+450*	+352*
Isomaltose	α-D-(1→6)	+357	+308
O-2-Acetamido-2-deoxy-α-D-gluco-pyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucose**	α-D-(1→6)	+530	+328
Disaccharide I		+308	+285
Disaccharide II		+209	+205
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside		+308	
D-Glucose		+95	+4
2-Acetamido-2-deoxy-D-glucose		+88	-24
Cellobiose	β-D-(1→4)	+124	+30
Gentiobiose	β-D-(1→6)	+30	-82
O-2-Acetamido-2-deoxy-β-D-gluco-pyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucose**	β-D-(1→6)	+27	-94
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside		-104	

** Foster and Horton, 1958

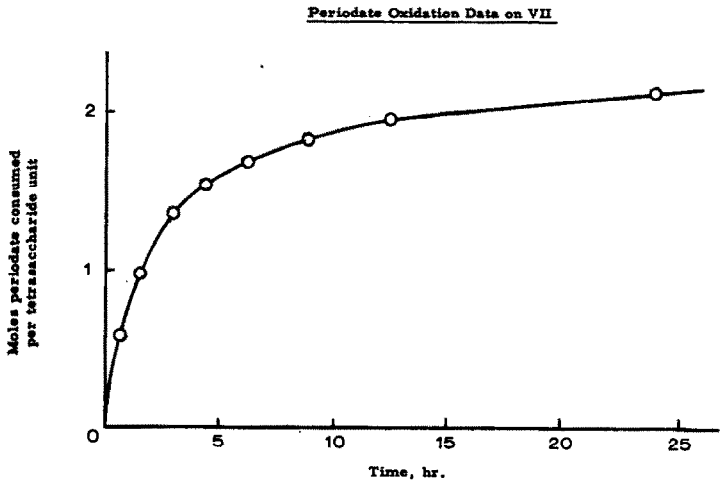

Figure 1.

not in agreement with our value (see Hoffman and Meyer, 1962b). A sample supplied by Dr. Schramm was found to be amorphous and inhomogeneous.

The structures determined for I and II would indicate an α-D-(1→4)-linked backbone sequence in heparin, with the D-glucuronic acid and 2-amino-2-deoxy-D-glucose units probably in an alternating arrangement. Further support for this structure was obtained by periodate oxidation studies on desulfated, carboxyl-reduced heparin. Partially acetylated, partially desulfated, carboxyl-reduced heparin (3.6 g.) was shaken with 0.15 N methanolic hydrogen chloride for two days. Dialysis and freeze-drying gave a completely desulfated product (VI); yield 2.5 g. O-Deacetylation of VI in formamide-ammonia gave a completely desulfated, O-deacetylated product (VII); $[\alpha]^{21}D$ +59.5° (c 2.54, water), ninhydrin (-).

Anal. Calcd. for $C_{12}H_{20}O_9N(COCH_3)$: C, 46.1; H, 6.35; N, 3.84. Found: C, 45.9; H, 6.03; N, 4.4; S, 0.0; O-$(COCH_3)$ (Wolfrom and Thompson, 1962a), 0.0.

Periodate oxidation was performed on VII according to the procedure of Fleury and Lange (1933), and the data of this oxidation are recorded in Figure I. Two moles of periodate were consumed per tetrasaccharide repeating unit with no formic acid nor formaldehyde being released.

In another experiment, VII (0.22 g.) was oxidized by periodate under the same conditions and isolated after dialysis and freeze-drying as a white powder (VIII); yield 0.195 g. (89%), $[\alpha]^{21}D$ +223° (c 2.33, water). This material (VIII) exhibited strong infrared absorption with $\lambda_{max(\mu)}^{KBr}$ 5.9 (C = O), not present in the infrared spectrum of VII.

Anal. Calcd. for $C_{12}H_{18}O_7N(COCH_3)$: C, 46.3; H, 5.82. Found: C, 45.7; H, 5.63.

Hydrolysis of VIII (10 mg.) in 2N hydrochloric acid (5 ml.) for 4 hr., removal of acid, decolorization with carbon, and evaporation of the solution to a small volume gave a sirup which upon paper chromatography in two solvent systems exhibited one zone with mobility corresponding to 2-amino-2-deoxy-D-glucose hydrochloride. On addition of acetone to the hydrolyzate crystals formed which were identified by X-ray powder diffraction data as 2-amino-2-deoxy-D-glucose hydrochloride (IX); yield 3.3 mg. (56%).

From the foregoing data on the structure of II, previous reports on I, as well as periodate oxidation data presented in this paper, heparin is shown to be glycosidically linked α-D-(1→4) in both the hexosamine-hexuronic acid and the hexuronic acid-hexosamine unit sequences. The hexosamine-hexuronic acid linkage assignment is in agreement with studies on a hexosamine-hexuronic acid disaccharide (Hoffman and Meyer, 1962a and 1962b), and also with methylation studies on heparin modifications (Danishefsky, Eiber, and Williams, 1963). Periodate oxidation studies on the heparin polymer by Foster, Stacey, and associates (1961, 1963) and by Durant, Hendrickson, and Montgomery (1962) are in agreement with the (1→4) linkages proposed herein. Heparin therefore possesses a linkage structure related to that of amylose and may exist in a helical configuration. Preliminary experiments indicate that the desulfated, carboxyl-reduced heparin is hydrolyzable by β-amylase, a procedure which should provide an excellent route to hydrolytic products.

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