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The Synthesis of the Repeating Unit of Hyaluronic Acid*

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The synthesis of the heptaacetyl-methyl ester of 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose, the repeating unit of hyaluronic acid, is described. This compound was obtained by condensation of (methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate) bromide with methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, followed by removal of the benzylidene group, *O*-acetylation, and finally acetolysis. The intermediate *N*-acetyl–methyl ester–methyl α-glycoside of the disaccharide, characterized as a de-*O*-acetylated uronamide, was transformed into methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyl)-α-D-glucopyranoside by lithium borohydride reduction. This compound was also obtained by condensation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, followed by de-*O*-acetylation and removal of the benzylidene group, and it was characterized as the fully acetylated derivative. All intermediate derivatives were obtained in crystalline form.

The isolation of a disaccharide from the acid hydrolysate of hyaluronic acid was reported simultaneously by Rapport *et al.* (1951) (hyalobiuronic acid) and Isikawa (1951) (mucosine). The structure of this disaccharide was established as 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucose (compound X) by Weissmann and Meyer (1952, 1954), who degraded it into 2-*O*-(β-D-glucopyranosyl)-α-D-arabinose, subsequently synthesized from laminaribiose. Since the presence of the 3-*O*-β-D- (or α-L-) glycuronosylhexosamine linkage has been established in four glycuronoglycosaminans isolated up to the present time from animal connective tissues, its chemical synthesis was of interest. In addition to being further evidence for the chemical structure proposed for the isolated disaccharide X, and for hyaluronic acid, the polysaccharide from which it is derived, this synthesis makes possible the preparation of derivatives of compound X, thus helping to elucidate the biological pathways for the biosynthesis of heteropolysaccharides.

It has been shown (Jeanloz and Jeanloz, 1964) that the fully acetylated methyl α-glycoside-methyl ester VII of compound X is the most convenient derivative for the identification of the disaccharide obtained by acid degradation of hyaluronic acid.

The first attempt to synthesize the derivative VII was based on condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl bromide (compound I) (see Carter, 1949) with methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (compound II) (Flowers and Jeanloz, 1963) in the presence of mercuric cyanide. After removal of the *O*-acetyl groups, the 4,6-*O*-benzylidene derivative III of the disaccharide was isolated in 36% yield following purification by chromatography on silica gel. It had a constant melting point, but failed to give a correct elemental analysis. Debenzylidenation and purification by chromatography on a mixture of charcoal and Celite gave, however, methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyl)-α-D-glucopyranoside (compound IV), showing a correct analysis, in an over-all yield of 35% calculated from the starting material II. This material was further characterized as a crystalline heptaacetyl derivative V.

As attempts to oxidize the primary alcoholic group of the D-glucose moiety of compound IV to a carboxylic group in the presence of platinum oxide were unsuccessful, the direct condensation of a derivative of D-glucuronic acid with compound II was investigated. When (methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate) bromide (compound VI) (Bollenback *et al.*, 1955) in excess was condensed with methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (compound II) under the same conditions used for the condensation of the glucosyl bromide I, a disaccharide IX was obtained in 54% yield. Removal of the benzylidene group, followed by acetylation, gave the hexaacetyl derivative VII, identical with the product obtained by degradation of hyaluronic acid or by synthesis from hyalobiuronic acid (mucosine) (Jeanloz and Jeanloz, 1964). The disaccharide IX was further characterized by a crystalline amide VIII. Acetolysis of compound VII gave the heptaacetyl derivative XI of the methyl ester of compound X. The value of the melting point observed for the derivative XI does not agree with the one observed on a similar derivative obtained from the natural disaccharide (Isikawa, 1951; Weissmann and Meyer, 1952, 1954), but this discrepancy probably results from differences in the content of solvent of crystallization in the crystals. Treatment of VII with lithium borohydride afforded a product identical with methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyl)-α-D-glucopyranoside (compound IV) described. The observed optical rotations and infrared spectra of the disaccharides IV and VII agree with a β-configuration of the interglycosidic linkage; this finding is in agreement with the mode of synthesis.

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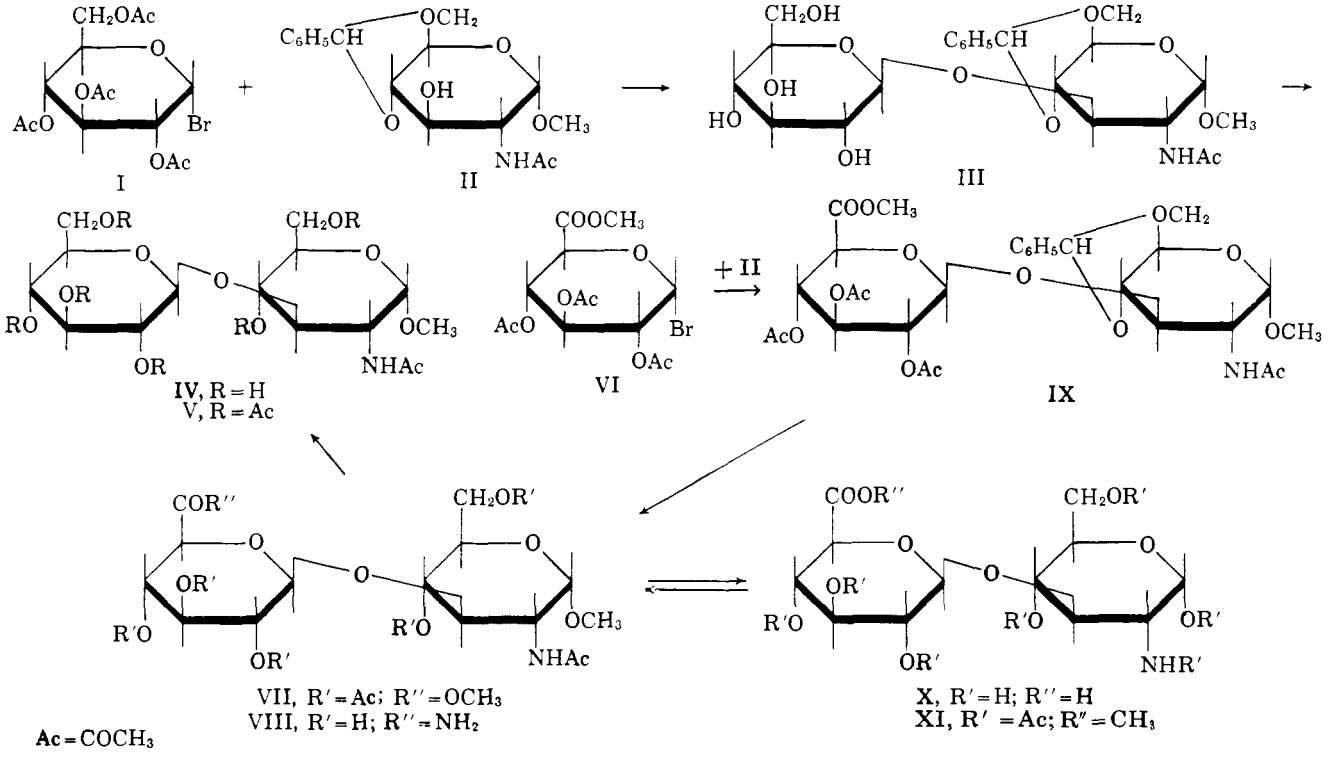
The synthesis of the disaccharide 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (hyalobiuronic acid, mucosine), using different intermediates and modes of condensation, has been carried out independently by Takanashi *et al.* (1962) and was reported simultaneously with the preliminary communication of the present work (Jeanloz and Flowers, 1962).



EXPERIMENTAL[1]

Methyl 2-Acetamido-4,6-O-benzylidene-2-deoxy-3-O-(β-D-glucopyranosyl)-α-D-glucopyranoside (III).—A mixture of 4.96 g of tetra-*O*-acetyl-α-D-glucopyranosyl bromide (compound I), 3.82 g of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (compound II), and 3.36 g of mercuric cyanide in 180 ml of nitromethane and 75 ml of benzene was stirred for 24 hours at room temperature. The resulting solution was diluted with benzene, then washed several times with a cold sodium bicarbonate solution and with water, dried over sodium sulfate, and evaporated. The residue (7.9 g) was deacetylated catalytically with 100 ml of a solution of 0.1 N sodium methoxide in methanol overnight at room temperature. The solution was evaporated and the residue was extracted with 250 ml of a mixture of acetone and ethyl acetate 4:1. The resulting solution was chromatographed on silica gel. A crystalline fraction, eluted by acetone, was recrystallized from dioxane containing 1% aqueous ammonia and gave 2.0 g of needles, mp 265–267° (36%). Two further recrystallizations raised the mp to 272–274°, $[\alpha]_D^{23}$ +26° (in 95% ethanol, *c* 0.84).

Anal. Calcd. for $C_{22}H_{31}NO_{11}$: C, 54.43; H, 6.44; N, 2.89. Found: C, 53.31; H, 6.52; N, 2.22.

Methyl 2-Acetamido-2-deoxy-3-O-(β-D-glucopyranosyl)-α-D-glucopyranoside (IV). From Compound III.—A solution of 96 mg of compound III in 1 ml of 60% acetic acid was heated at 100° for 30 minutes. After evaporation the residue was crystallized from a mixture of alcohol and acetone to give 75 mg (95%) of needles, mp 252–253°, $[\alpha]_D^{27}$ +43° (in water, *c* 0.90); $[\alpha]_D^{27}$ +54° (in 95% ethanol, *c* 2.15).

Directly from Compound II Without Isolation of Compound III.—The crude residue obtained from condensation of 0.98 g of compound II was deacetylated catalytically with sodium methoxide and then hydrolyzed with 60% acetic acid as described above. The residue was dissolved in 100 ml of water and the solution was chromatographed on a column (170 × 22 mm) containing a mixture of Darco G-60 and Celite 535 in proportions 1:1. Elution with 300 ml of water removed inorganic salts and monosaccharides. Gradient elution with a mixture of water and ethanol (with a concentration of ethanol up to about 5%) afforded a number of fractions which gave, after acid hydrolysis, a positive reaction in the anthrone test and in the Elson and Morgan test. These fractions, combined and evaporated, gave 0.42 g (35%) of crystalline residue. Recrystallization from a mixture of ethanol and acetone gave 0.29 g of needles, mp 251–253°, $[\alpha]_D^{27}$ + 44° (in water, *c* 1.00).

Anal. Calcd. for $C_{15}H_{27}NO_{11}$: C, 45.34; H, 6.85; N, 3.52; OCH_3, 7.81. Found: C, 45.88; H, 7.13; N, 3.59; OCH_3, 8.07.

Methyl 2-Acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,-4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside (V).—A solution of 50 mg of compound IV in 0.5 ml of acetic anhydride and 0.5 ml of pyridine was left overnight at room temperature. The solution was then poured into ice water, and the precipitate was separated by filtration, giving 60 mg (73%) of needles, mp 235–236°. Recrystallization from a mixture of acetone and hexane raised the mp to 236–237°, $[\alpha]_D^{28}$ +24° (in chloroform, *c* 1.11).

Anal. Calcd. for $C_{27}H_{39}NO_{17}$: C, 49.92; H, 6.05. Found: C, 50.02; H, 6.08.

Methyl 2-Acetamido-4,6-O-benzylidene-2-deoxy-3-O-(methyl 2,3,4,-tri-O-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (IX).—A mixture of 0.40 g of (methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate) bromide (compound VI), 0.323 g of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (compound II), and 0.28 g mercuric cyanide in 20 ml of nitromethane and 10 ml of benzene was stirred at

[1] See Jeanloz and Jeanloz (1964).

40° for 24 hours. A further quantity of compound VI (0.30 g) and of mercuric cyanide (0.21 g) was added, and the reaction was allowed to proceed a further 24 hours. An excess of benzene was added, and the solution was washed several times with a cold sodium bicarbonate solution and with water, dried over sodium sulfate, and evaporated. The residue (1.075 g) was dissolved in a mixture of benzene and ether 1:1 and chromatographed on silica gel. A crystalline fraction, eluted with a mixture of ether and ethyl acetate 1:1, was recrystallized from a mixture of ether and acetone to give 0.34 g of long needles, mp 228–230° (54%). Recrystallization from the same solvents raised the mp to 230–232°, $[\alpha]_D^{28}$ + 16° (in chloroform, *c* 1.21).

Anal. Calcd. for $C_{29}H_{37}NO_{15}$: C, 54.46; H, 5.83; N, 2.19. Found: C, 54.02; H, 5.93; N, 2.34.

Methyl 2-Acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (VII).—A solution of 80 mg of compound IX in 8 ml of 60% acetic acid was heated at 100° for 15 minutes. Concentration and azeotropic distillation with toluene afforded a dry residue which was acetylated with 0.4 ml of acetic anhydride in 0.4 ml of pyridine. After concentration, the residue was crystallized from a mixture of acetone and ether to give 60 mg of needles (72%), mp 238–240°. Recrystallization from the same solvents raised the mp to 238–239° $[\alpha]_D^{23}$ +30° (in chloroform, *c* 0.98). This product was identical with the product prepared from natural sources (Jeanloz and Jeanloz, 1964) with regard to melting point, optical rotation, and infrared spectrum.

Anal. Calcd. for $C_{36}H_{37}NO_{17}$: C, 49.13; H, 5.87; OCH_3, 9.77. Found: C, 49.27; H, 5.96; OCH_3, 9.91.

Methyl 2-Acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronamide)-α-D-glucopyranoside (VIII).—After treatment of 68 mg of compound IX with 60% acetic acid to hydrolyze the benzylidene group, the residue was treated overnight at room temperature with 0.5 ml of methanol, which had been saturated with ammonia at 0°. Evaporation of the solution and recrystallization of the residue from a mixture of ethanol and ether gave 30 mg of needles (65%), mp 263–264°. Recrystallization from methanol raised the mp to 264–265°, $[\alpha]_D^{23}$ + 25° (in ethanol, *c* 0.87).

Anal. Calcd. for $C_{15}H_{26}N_2O_{11}$: C, 43.90; H, 6.38; N, 6.82. Found: C, 43.89; H, 6.39; N, 6.89.

2-Acetamido-1,4,6-tri-O-acetyl-2-deoxy-3-O-(methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranose (XI).—A solution of 100 mg of compound VII in a mixture of 1 ml of acetic anhydride, 0.5 ml of acetic acid, and 0.025 ml of sulfuric acid was left for 7 hours at room temperature with exclusion of moisture. The colorless solution was poured into ice water and extracted with chloroform. The organic layer was washed thoroughly with cold water, dried, and evaporated. The residue was dissolved in 0.5 ml of methanol and an excess of diazomethane in ether solution was added to ensure reesterification of any methyl ester cleaved by the acetolysis procedure. The product obtained on evaporation (100 mg) was dissolved in ethylene dichloride and purified by chromatography on silica gel. Ethyl acetate eluted fractions (89 mg, 85%), which were crystallized from absolute alcohol in prisms (60 mg, mp 190–192° after sintering at 124°). Recrystallization from a mixture of methanol and ether gave prisms, mp 215–217°, unaffected by further crystallization; $[\alpha]_D^{23}$ +20° (in chloroform, *c* 0.98). The analytical results for the material dried for 3 hours at 80° *in vacuo* indicate the presence of one molecule of methanol of crystallization.[2]

Anal. Calcd. for $C_{27}H_{37}NO_{18}\cdot CH_3OH$: C, 48.34, H, 5.89; OCH_3, 9.20. Found: C, 48.54; H, 5.62; OCH_3, 9.02.

Methyl 2-Acetamido-2-deoxy-3-O-(β-D-glucopyranosyl)-α-D-glucopyranoside (IV). From Compound VII.—A solution of 90 mg of compound VII in 10 ml of dry tetrahydrofuran was added dropwise to a suspension of 200 mg of lithium borohydride in 5 ml dry tetrahydrofuran stirred at room temperature. After being left 24 hours at room temperature, the solution was treated with 0.5 ml of ice water and 10 ml of dilute (10%) acetic acid. It was then passed through a column of Dowex 50 × 8 (H^+ form) to remove lithium ions. The effluent was concentrated, and repeated additions of absolute alcohol removed the boric acid. The residue was finally crystallized from methanol, giving 30 mg (54%) needles, mp 252–254°, which showed no depression of the mp in admixture with the product IV described previously; $[\alpha]_D^{24}$ +57° (in 95% ethanol, *c* 1.37).

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[2] Isikawa (1951) reported mp 130° and $[\alpha]_D$ +14.8° (in chloroform, *c* 3.042) for a product containing three moles of water of crystallization; Weissmann and Meyer (1952, 1954) reported mp 120° and $[\alpha]^{24}_D$ +24.5° (in chloroform, *c* 2) for a product containing one mole of ethanol of crystallization.