The Degradation of Hyaluronic Acid by Methanolysis*

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Received November 26, 1962

Degradation of hyaluronic acid with methanolic hydrochloric acid, followed by total acetylation, afforded a well-crystallized methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside. The same product was obtained by glycosidation, esterification, and total acetylation of the disaccharide *N*-acetylhyalobiuronic acid.

Degradation of hyaluronic acid (compound I), a polysaccharide isolated from human umbilical cord and composed of alternate units of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked at positions 4 and 3, respectively, has been carried out in the past with the use of acid and enzyme. The resulting product, a disaccharide called hyalobiuronic acid (Rapport *et al.*, 1951) or mucosine (Isikawa, 1951), was shown to possess the structure of 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucose (compound II) (Weissmann and Meyer, 1952; 1954). It is, unfortunately, difficult to characterize since it possesses no melting point nor definite rotation (because of mutarotation) and is difficult to crystallize in the presence of impurities. Esterification and acetylation of compound II gave a heptaacetyl derivative (Isikawa, 1951; Weissmann and Meyer, 1952); the optical rotations and the analytical constants of the samples isolated by these investigators did not agree.

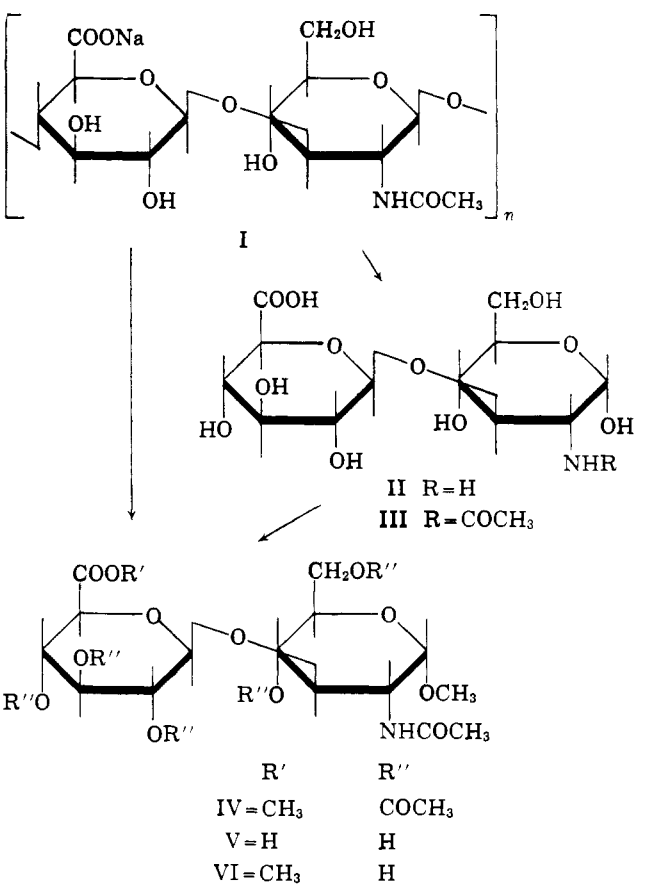
In order to establish the structure of compound II by synthesis, it was desirable to obtain from compound II, or directly from hyaluronic acid, with good yields, a well-crystallized derivative possessing a sharp melting point and a definite rotation. Action of methanolic hydrochloric acid on dry sodium hyaluronate, followed by acetylation, gave a sirupy product, which was purified by chromatography on silicic acid. Crystalline fractions were obtained which gave analytical values corresponding to 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 (compound IV).

Variation of the conditions of hydrolysis did not improve the yields over 18%. The product IV was characterized by formation of methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (compound V) by alkaline hydrolysis, and of the methyl ester VI by further action of diazomethane or methanolic hydrochloric acid on compound V.

Hyalobiuronic acid (compound II), which had been obtained by acid hydrolysis of hyaluronic acid, was *N*-acetylated. Treatment of the resulting product III with methanolic hydrochloric acid gave the methyl glycoside and methyl ester V. This product V contained about 10% of the β-anomer as established by comparison with the synthetic product (Flowers and Jeanloz, 1964). Preparation of the methyl glycosides of 2-acetamido-2-deoxy-D-glucose by action of methanolic hydrochloric acid is known to give predominantly the α-anomer (Kuhn *et al.*, 1953) with a small amount (15–20%) of the β-anomer. Removal of the β-anomer from the crude product V was carried out more conveniently on the acetylated derivative IV, obtained by acetylation with acetic anhydride in pyridine solution. This product IV was identical to the product obtained directly from hyaluronic acid by methanolysis and acetylation.

It is of interest that the degradation of hyaluronic acid with mineral acid in methanol solution splits the same glucosaminidic linkage as the one preferentially broken by mineral acid or by most of the hyaluronidases in water solution. The yield in disaccharide from the methanolysis is higher than the one from the hydrolysis, but lower than when similar conditions of degradation are applied to the methylated polysaccharide (R. W. Jeanloz, unpublished data). These differences in yield may be due to the ease of isolation of the various derivatives of the disaccharide.

The structure of the disaccharide IV obtained by degradation of hyaluronic acid has been confirmed by



* This is publication XXXIII of the series *Amino Sugars* and No. 347 of The Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston 14, Mass. This investigation has been supported by a research grant (A-3564-C-1) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service. A preliminary note has been published (Jeanloz and Flowers, 1962).

its synthesis from D-glucosamine and D-glucuronic acid (Flowers and Jeanloz, 1964).

Experimental

Melting points were taken on a hot stage, equipped with a microscope, and correspond to "corrected melting point." Rotations were determined in semimicro or micro (for amounts smaller than 3 mg) tubes with lengths of 100 or 200 mm, using a Rudolph photoelectric polarimeter attachment, Model 200; the chloroform used was A.R. grade and contained approximately 0.75% ethanol. Infrared spectra were determined on a Perkin-Elmer spectrophotometer, Model 237. Chromatograms were made with the flowing method on "Silica Gel Davison," from the Davison Co., Baltimore 3, Md. (grade 950; 60–200 mesh) used without pretreatment. When deactivation by contact with moist air occurred, reactivation was obtained by heating to 170–200° (manufacturer's instructions). The sequence of eluants was hexane, benzene or dichloroethane, ether, ethyl acetate, acetone, and methanol individually or in binary mixtures. The proportion of weight of substance to be adsorbed to weight of adsorbent was 1 to 50–100. The proportion of weight of substance in grams to volume of fraction of eluant in ml was 1–100. The ratio of diameter to length of the column was 1–20. Evaporations were carried out *in vacuo*, with an outside bath temperature kept below 45°. Amounts of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were done by Dr. M. Manser, Zürich, Switzerland.

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 (IV) from Sodium Hyaluronate (I).—A suspension of 0.68 g of sodium hyaluronate from human umbilical cords, which had been dried overnight at 110° in high vacuum over phosphorus pentoxide, in 50 ml of 3 N hydrogen chloride in methanol, was refluxed under moisture protection for 24 hours. After cooling, the precipitated sodium chloride was filtered off and the clear yellow solution was concentrated. The remaining hydrogen chloride was removed by addition of excess silver carbonate. After filtration, the solution was treated with hydrogen sulfide, filtered, and concentrated. The residual sirup was dissolved in 5 ml of pyridine and 3 ml of acetic anhydride was added. After 24 hours the excess of acetic anhydride was decomposed by addition of methanol under cooling and the solvents were evaporated. The remaining traces of pyridine were eliminated by additions of toluene followed by evaporation. The residual sirup was dissolved in dichloroethane and chromatographed on silica gel. After elution of a first sirupy fraction with ethylene dichloride containing increasing concentrations of ether, a crystalline fraction (402 mg) was obtained by elution with ethyl acetate and a mixture of ethyl acetate and acetone 99:1.

Crystallization of this fraction from a mixture of acetone and ether gave 193 mg (18%) of prismatic needles, mp 236–238°, $[\alpha]_D^{26}$ + 30° (in chloroform, *c* 0.68).

Anal. Calcd. for $C_{26}H_{37}O_{17}N$: C, 49.13; H, 5.87; N, 2.20; OCH_3, 9.77. Found: C, 49.19; H, 5.95; N, 2.35; OCH_3, 10.36.

Methyl 2-Acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (V).—To a solution of 74 mg of compound IV in 1.5 ml of methanol was added 0.3 ml of 1 N barium methylate at 0°. After standing overnight, the solution was evaporated to dryness and the residue was dissolved in 5 ml of water. The solution was filtered through a column of Dowex 50 in the acid form and evaporated. The crystalline residue was recrystallized from a mixture of methanol, acetone, and ether, giving 49 mg (98%) of small needles, mp 207–210° (dec), containing one molecule of water; $[\alpha]_D^{24}$ + 32° (in methanol, *c* 0.73 for the anhydrous product).

Anal. Calcd. for $C_{15}H_{25}O_{12}N \cdot H_2O$: C, 41.96; H, 6.34. Found: C, 41.94; H, 6.40.

Methyl 2-Acetamido-2-deoxy-3-O-(methyl β-D-glucopyranosyluronate)-α-D-glucopyranoside (VI). From Compound V.—A solution of 30 mg of anhydrous V in 1 ml of 0.2 N hydrogen chloride in methanol was left at room temperature for 24 hours. It was neutralized by an excess of silver carbonate and filtered over a double layer of Celite and Darco G-60. The residue left after evaporation of the solvent was recrystallized from a mixture of methanol, acetone, and ether, and gave 28 mg (90%) of fine needles, mp 225–227°. A second crystallization raised the mp to 228–230°, $[\alpha]_D^{26}$ + 28° (in methanol, *c* 0.56). The melting point showed no depression in admixture with the product described below.

From N-Acetylhyalobiuronic Acid, Compound III.—A solution of 140 mg of dry *N*-acetylhyalobiuronic acid —2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose—(Weissmann and Meyer, 1954) in 3 ml of 1.5 N hydrochloric acid in dry methanol was refluxed for 1.5 hours. After being cooled, the solution was neutralized with an excess of silver carbonate and filtered. The filtrate was treated with hydrogen sulfide and filtered over a double layer of Celite and Darco G-60, giving, after evaporation, 144 mg of sirup, $[\alpha]_D^{27}$ +23° (in methanol, *c* 1.77). It started to crystallize after a few months in the ice-box. It was dissolved in benzene and purified by chromatography on silica gel. Elution with a mixture of acetone and methanol 19:1 gave 101 mg of crystalline fractions, which afforded, after recrystallization from a mixture of methanol and acetone, 75 mg (51%) of small prisms in clusters, mp 223–225°, $[\alpha]_D^{23}$ + 16° (in methanol, *c* 1.09).

Anal. Calcd. for $C_{16}H_{27}O_{12}N$: C, 45.17; H, 6.16; OCH_3, 14.59. Found: C, 45.42; H, 6.53; OCH_3, 14.32. As shown by the optical rotation, the product contained about 10% of the β-anomer.

Acetylation of 30 mg of the above product with 0.2 ml of acetic anhydride and 0.3 ml of pyridine in the usual manner gave a crude product which was dissolved in a mixture of ether and ethyl acetate 1:1 and purified by chromatography on silica gel. Elution with ethyl acetate and a mixture of ethyl acetate and acetone 99:1 afforded, after recrystallization from a mixture of acetone and ether, 35 mg (78%) of pure compound IV, mp 237–239°, $[\alpha]_D^{26}$ + 28° (in chloroform, *c* 0.42). This product showed no depression of the melting point in admixture with the product described above.

Acknowledgment

The authors wish to thank Dr. Karl Meyer for the kind gift of *N*-acetylhyalobiuronic acid.

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The Synthesis of the Repeating Unit of Hyaluronic Acid*

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Received July 19, 1963

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.

* This is publication XXXVIII of the series *Amino Sugars* and No. 348 of The Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston 14, Mass. This investigation has been supported by research grants from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service (A-3564-6-2) and the National Science Foundation (9-2312). A preliminary note has been published (Jeanloz and Flowers, 1962).

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