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[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE EDWARD DANIELS FAULKNER ARTHRITIS CLINIC, PRESBYTERIAN HOSPITAL]

The Hexosaminidic Linkage of Hyaluronic Acid[1]

SHIGEHIRO HIRANO AND PHILIP HOFFMAN

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A methylation study of the tetrasaccharide from hyaluronic acid confirms that the 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) moiety is linked (1 ⟶ 4) to the glucuronic acid.

Consideration of the available evidence has led to the general acceptance of hyaluronic acid and the chondroitin sulfates as linear polysaccharides with alternating uronic acid and acetylhexosamine units. Extensive investigations of the products of enzymatic degradation have failed to reveal branching or variations in the glycosidic sequence of these polysaccharides.

The characterization of the crystalline disaccharide which has been obtained in high yield from hyaluronic acid as 3-*O*-β-D-glucopyranosyluronic acid-(2-amino-2-deoxy-D-glucopyranose) has established the (1→3) glucuronidic linkage in hyaluronic acid.[2]

The hexosaminidic linkages in hyaluronic acid and the chondroitin sulfates are considered to be (1→4) due to the ease with which bacterial hexosaminidases act upon them by an elimination process to form Δ^{4-5} uronides.[3] It would be difficult to conceive of the formation of a Δ^{4-5} uronide by the elimination of a hexosaminide with any other linkage.

However, in view of the uniqueness of this elimination reaction by glycosidic cleaving enzymes and the lack of analogy to a well established mechanism, it was considered desirable to confirm the hexosaminidic linkage by other, more universally acceptable evidence, preferably from the use of classical methods such as methylation studies.

As previous methylation studies on hyaluronic acid did not establish the hexosaminidic linkage and indicated substantial experimental difficulties,[4] it seemed more feasible to attempt the methylation of the tetrasaccharide I which is obtained in high yield from hyaluronic acid by the action of testicular hyaluronidase. The ready cleavage by bacterial hexosaminidase of the one hexosaminidic linkage in I by an elimination process[5] serves to confirm that this is the same hexosaminidic linkage which is cleaved in the polymer.

Earlier methylation studies[6] were performed on the trisaccharide obtained from I by the action of liver β-glucuronidase, as this removed the nonreducing end glucuronic acid which did not contain a hexosaminidic linkage. However, it was difficult to obtain β-glucuronidase free of β-hexosaminidase activity so that the disaccharide lacking a hexosaminidic linkage often replaced the trisaccharide as the major digestion product. As it was found that both I and trisaccharide were permethylated readily, later work was carried out on I.

The difficulty of hydrolysing the glucuronidic linkage in hyaluronic acid and its oligosaccharides due to the presence of the carboxyl group would also prevail among the permethylated oligosaccharides. Reduction of the carboxyl group *via* its methyl ester to a hydroxymethyl group would remove the resistance to hydrolysis and yield methylated glucose derivatives for which authentic samples for comparison were available. When lithium aluminum hydride was used as reducing agent on the methylated trisaccharide, the acetamido group was reduced to an ethylamine which served to protect the glycosidic linkage from hydrolytic cleavage and resulted in low yields of methylated monosaccharide products. The report[7] that lithium borohydride reduced methyl ester groups but did not affect acetamido groups was verified on methylated I in the present study and high yields of methylated monosaccharides were obtained from reduced,

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(1) This work was supported in part by grants to Professor Karl Meyer from the National Science Foundation and the U. S. Public Health Service.

(2) B. Weissmann and K. Meyer, *J. Am. Chem. Soc.*, **76**, 1753 (1954).

(3) P. Hoffman, A. Linker, V. Lippman, and K. Meyer, *J. Biol. Chem.*, **235**, 3066 (1960).

(4) R. W. Jeanloz, *Helv. Chim. Acta*, **35**, 262 (1952).

(5) A. Linker, K. Meyer, and P. Hoffman, *J. Biol. Chem.*, **219**, 13 (1956).

(6) K. Meyer, *Harvey Lectures*, **51**, 88 (1957).

(7) A. C. Chibnall and M. W. Rees, *Biochem. J.*, **68**, 105 (1958).

methylated I. I was converted to the methylated and reduced product, IV, according to the following scheme:

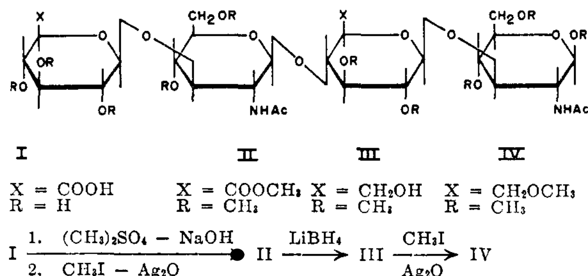

Figure 1

Methanolysis and hydrolysis of IV to monosaccharides was followed by separation of the neutral sugars from the positively charged hexosamine derivatives on a Dowex 50 (H^+) column. The neutral monosaccharides were identified as 2,3,4,6-tetra-*O*-methyl-D-glucose which originated from the nonreducing end glucuronic acid and 2,3,6-tri-*O*-methyl-α-D-glucose originating from the nonterminal glucuronic acid and characterizing the hexosaminidic linkage as (1→4). As already mentioned, bacterial hyaluronidases attack the hexosaminidic linkage in hyaluronic acid and in I similarly and previously cited evidence demonstrates that testicular hyaluronidase, which is known to be a transglycosylative enzyme,[8] does not alter the hexosaminidic linkage in I from that in the polymer.[9] Hence, in view of the high yields of I with testicular hyaluronidase and of unsaturated disaccharide with bacterial hyaluronidase, the (1→4) linkage may be considered the major if not the only hexosaminidic linkage in hyaluronic acid.

Since an independent method has established the (1→4) hexosaminidic linkage in hyaluronic acid verifying the conclusions drawn from a consideration of the production of unsaturated products by bacterial enzymes, the same considerations of the strikingly similar unsaturated products from the chondroitin sulfates should be more readily acceptable as evidence for (1→4) hexosaminidic linkages in the chondroitin sulfates.

Elution of the Dowex 50 (H^+) column with acid to obtain the methylated hexosamines yielded two products in about equal amounts; one was the expected 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose characterized as the 2-acetamido derivative and its methyl glucoside and the other, which was nonreducing, was the methyl 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucopyranoside, also characterized as its 2-acetamido derivative. The two products are due to the two different pathways of hydrolysis described by Stacey *et al.*[10] for methyl 2-acylamino-2-deoxy-D-glucopyranosides.

(8) B. Weissmann, *J. Biol. Chem.*, **216**, 783 (1955).

(9) P. Hoffman, K. Meyer, and A. Linker, *J. Biol. Chem.*, **219**, 417 (1954).

EXPERIMENTAL

Melting points were obtained on a microscope hot stage and are uncorrected.

Infrared spectra were obtained with potassium bromide pellets in a Perkin-Elmer Model 21 spectrophotometer with sodium chloride prism.

Paper chromatograms of the methylated monosaccharides were developed by the descending flow of *n*-butyl alcohol-ethanol-water (4:1:1) on Whatman No. 1 paper except where otherwise noted. Ninhydrin and aniline trichloroacetate spray reagents were utilized for the detection of the sugars.

Tetrasaccharide from hyaluronic acid. The tetrasaccharide was obtained from umbilical cord hyaluronic acid after exhaustive digestion with testicular hyaluronidase and separation on an ion exchange column as described previously for mixed hyaluronic acid–chondroitin sulfate digests.[9]

The amorphous product was analytically and chromatographically identical with the crystalline tetrasaccharide previously reported.[11]

Permethylated reduced tetrasaccharide (IV). The tetrasaccharide (1.70 g.) was dissolved in 20 ml. of water and 10 ml. of carbon tetrachloride was added. During the following procedure the mixture was maintained under nitrogen, in an ice bath, with constant stirring. Dimethyl sulfate (30 ml.) was slowly added, then 45 ml. of 40% sodium hydroxide was added dropwise during 5 hr. After maintaining the mixture overnight a further 62 ml. of dimethyl sulfate was added during 2 hr. followed by 100 ml. of 40% sodium hydroxide over a 5-hr. period. The next morning the addition of 62 ml. of dimethyl sulfate was repeated and after being maintained at 0° for another night the mixture was allowed to warm to room temperature and stirred for an additional 12 hr. The mixture was then cooled in an ice bath, acidified to *p*H 2.5 with 40% sulfuric acid, and extracted six times with 50-ml. portions of chloroform. The combined extracts were washed with 50 ml. of water, dried over sodium sulfate, filtered, and evaporated, yielding 1.61 g. of a dried sirup.

The sirup was dissolved in 3 ml. of absolute methanol, 25 ml. of methyl iodide, and 3 g. of freshly prepared silver oxide were added and the mixture shaken at room temperature for 27 hr. It was then filtered, concentrated to a sirup, and dried. An infrared spectrum of the sirup showed strong methyl ester absorption at 1730 $cm.^{-1}$

The sirup was dissolved in 8 ml. of an 0.94*M* solution of lithium borohydride in tetrahydrofuran which was maintained at room temperature for 20 hr. It was then cooled in an ice bath and 20 ml. of methanol-water (1:1) was added, followed by dry Dowex 50 (H^+) to adjust the *p*H to 8. After 2 hr. at room temperature, more Dowex 50 (H^+) was added to *p*H 5 and after an additional 12 hr. the mixture was filtered and then evaporated to dryness; methanol-benzene (2:1) was added and evaporated several times to remove borate. The yield of dried sirup was 1.45 g. An infrared spectrum verified the reduction of the methyl ester by the absence of absorption at 1730 $cm.^{-1}$

The product was treated twice more with methyl iodide and silver oxide, being maintained at reflux temperature for 11 hr. during the second treatment. The isolated sirup was dissolved in 50 ml. of chloroform, filtered, concentrated to a sirup, and dried. The yield was 1.25 g.

Anal. Calcd. for $C_{23}H_{34}O_9N_2(CH_2O)_{12}$: OCH_3, 40.6%. Found: OCH_3, 40.7.

An infrared spectrum of the product showed the virtual absence of OH absorption at 3200–3300 $cm.^{-1}$

Hydrolysis of IV *to monosaccharides.* The product IV (1.25 g.) was refluxed for 3 hr. in 50 ml. of a solution of 2%

(10) A. B. Foster, D. Horton, and M. Stacey, *J. Chem. Soc.*, 81 (1957).

(11) B. Weissmann, K. Meyer, P. Sampson, and A. Linker, *J. Biol. Chem.*, **208**, 417 (1954).

hydrochloric acid in methanol. Silver carbonate was added, the mixture was filtered, and the filtrate was concentrated to a sirup; yield, 1.05 g.

Examination of the sirup by paper chromatography revealed four monosaccharide components with R_f values of 0.80, 0.72, 0.49, and 0.30; the 0.49 component reacted negatively with reducing spray reagents and was faintly positive with ninhydrin spray reagent, and the 0.30 component reacted positively to both spray reagents indicative of a 2-amino-2-deoxyhexose.

Separation of the methylated monosaccharides. The monosaccharide mixture (1.05 g.) was dissolved in 100 ml. of water, and the solution was passed through a Dowex 50 (H^+) column (10 × 2.5 cm.) in order to separate the neutral sugars from the glucosamine (2-amino-2-deoxy-D-glucose) derivatives. The column was washed with 800 ml. of water and the combined effluents after evaporation yielded 0.51 g. of sirup containing tetra-*O*-methyl-D-glucose (R_f 0.80) and tri-*O*-methyl-D-glucose (R_f 0.72). The fractions were separated on sheets of Whatman 3MM filter paper using methyl ethyl ketone saturated with water as the developing solvent. The fractions were eluted from the appropriate sections with water and concentrated to sirups yielding 0.23 g. of the tetra-*O*-methyl; 0.18 g. of the tri-*O*-methyl; and 0.06 g. of a mixture of the tetra- and tri-*O*-methyl-D-glucose.

The Dowex 50 (H^+) column was eluted with 800 ml. of *N* sulfuric acid and the eluent was neutralized with barium carbonate, filtered, and concentrated *in vacuo* yielding 0.47 g. of a sirup containing 2-amino-2-deoxy-di-*O*-methyl-D-glucose (R_f 0.30) and methyl 2-amino-2-deoxy-di-*O*-methyl-D-glucopyranoside (R_f 0.49). The fractions were separated on sheets of Schleicher and Schuell No. 470 filter paper, using the n-butyl alcohol–ethanol-water (4:1:1) as the developing solvent, yielding 0.18 g. of the di-*O*-methyl-D-glucosamine fraction, 0.19 g. of the methyl di-*O*-methyl-D-glucosaminide, and 0.05 g. of a mixed fraction containing both sugars.

2,3,6-Tri-O-methyl-α-D-glycopyranose. The sirup (0.18 g., R_f 0.72) was dissolved in about 5 ml. of anhydrous ether, filtered, the residue being washed with 5 ml. of ether, and the ether solution was concentrated to a sirup. The sirup was converted to a crystalline mass after 2 days in a vacuum dessicator, which yielded 120 mg. of needles after washing with a small amount of cold ether, and after three recrystallizations from ether had m.p. 109–112°; $[\alpha]_D^{24}$ +78 → +67° (*c* 0.8, water). *Anal.* Calcd. for $C_9H_{18}O_6$: OCH_3, 41.9. Found: OCH_3, 41.4. The mixed m.p. with authentic 2,3,6-tri-*O*-methyl-α-D-glucose[12] was 106–112° and the mixed m.p. with the other possible tri-*O*-methyl derivative, 2,4,6-tri-*O*-methyl-α-D-glucose, was depressed to 80–92°. The infrared spectrum of the isolated material was identical with that of authentic 2,3,6-tri-*O*-methyl-α-D-glucose and different from that of authentic 2,4,6-tri-*O*-methyl-α-D-glucose.

2,3,4,6-Tetra-O-methyl-N-phenyl-D-glucosylamine. The sirup (0.23 g., R_f 0.80) was refluxed with 0.7 ml. of aniline and 10 ml. of anhydrous ethanol for 3 hr. The reaction mixture was decolorized with carbon and the filtrate was evaporated to dryness, dissolved in 10 ml. of ether, filtered, and concentrated to a small volume. Petroleum ether (b.p. 30–60°) was added to incipient turbidity and, after being kept in the refrigerator overnight, 210 mg. of crystals were obtained. Three recrystallizations from ether–petroleum ether yielded long needles, m.p. and mixed m.p. with authentic 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucopyranosylamine[13] was 135–137°. *Anal.* Calcd. for $C_{16}H_{25}O_5N$: OCH_3, 39.9. Found: OCH_3, 39.6. The infrared spectrum of the isolated crystals was identical with that of the authentic sample.

2-Acetamido-2-deoxy-4,6-di-O-methyl-α-D-glucopyranose. The fraction (0.18 g., R_f 0.30) did not crystallize as the hydrochloride from methanolic hydrochloric acid and the product was mixed with 2.7 ml. of sodium methoxide solution (prepared by adding 10 mg. of sodium metal to each ml. of absolute methanol). The mixture was filtered, washed, and 0.065 ml. of acetic anhydride was added to the filtrate which was then kept in the refrigerator for 1 day. The product was evaporated to dryness, taken up in 20 ml. of water, and passed through a short Dowex 50 (H^+) column which was washed with 100 ml. of water. The combined effluents were evaporated to dryness. The product was dissolved in absolute methanol, decolorized with carbon, and the filtrate was evaporated slowly over calcium chloride in a dessicator to yield, after washing with cold methanol-ether (1:1), 79 mg. of needles. They were recrystallized from methanol-ether; m.p. 220–222°; $[\alpha]_D^{19}$ + 97° (equil.; *c* 0.2, methanol).[14]

Anal. Calcd. for $C_{10}H_{19}O_6N$: OCH_3 24.9. Found: OCH_3, 25.5. The crystals yielded no color in the Morgan and Elson reaction[15] indicating that the 4- position was substituted. The infrared spectrum of the crystals was identical with that of authentic 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranose.

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-α-D-glucopyranoside. In order to form the methyl glycoside for verification, 20 mg. of the crystalline 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranose was refluxed with 20 mg. of dried Dowex 50 (H^+) in 5 ml. of absolute methanol for 2.5 hr. The mixture was filtered and the filtrate was concentrated to a sirup. Crystallization and recrystallization was effected from ethanol, ether, and petroleum ether; m.p. 205–206°. The mixed m.p. with authentic methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranoside[16] was 205–206°. The infrared spectrum of the derivative was identical with that of authentic methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranoside and different from those of the 3,4- and the 3,6-di-*O*-methyl derivatives.

The fraction (0.19 g., R_f 0.49) which had been retained on the Dowex 50 (H^+) column indicating the presence of a free amino group and which had no reducing value indicating, when the R_f value was also considered, that it was the methyl glycoside of a methylated monosaccharide, was converted to an acetamido derivative with acetic anhydride in absolute methanol as described above for the 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose. A yield of 131 mg. of crystals was obtained which were recrystallized from absolute ethanol–ether; m.p. 198–199° (204–205° when taken by Dr. R. W. Jeanloz); mixed m.p. with authentic methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranoside was 199–200°.[16] The infrared spectra of the isolated crystals and the authentic sample were identical and the Morgan and Elson reaction yielded no color value verifying the 4-substitution. Acid hydrolysis of the acetamido derivative with *N* hydrochloric acid at 100° for 15 hr. gave products with R_f values of 0.30 and 0.49, identical with the values obtained from the authentic samples of 2-amino-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranose and its methylglucoside, respectively.

Acknowledgment. We are indebted to Dr. R. Kuhn for authentic 2,3,6-tri-*O*-methyl-α-D-glucopyranose; to Dr. F. Smith for 2,4,6-tri-*O*-methyl-α-D-glucopyranose; to Dr. E. Chargaff for 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucopyranosylamine; and we are doubly indebted to Dr. R. W. Jeanloz

(12) Reported for 2,3,6-tri-*O*-methyl-α-D-glucopyranose: m.p. 112–113°; $[\alpha]_D^{22}$ +89.5° ⟶ +69° (water). See R. Kuhn and R. Brossmer, *Angew. Chem.*, **70**, 25 (1958).

(13) Reported m.p. 135°. See J. C. Irvine and A. M. Moodie, *J. Chem. Soc.*, **93**, 95 (1908).

(14) Reported m.p. 227–228°; $[\alpha]_D$ +88° ⟶ +68° (water). See R. W. Jeanloz, *Adv. in Carbohydrate Chem.*, **13**, 189 (1958).

(15) D. Aminoff, W. T. J. Morgan, and W. M. Watkins, *Biochem. J.*, **51**, 379 (1952).

(16) Reported m.p. 199–200°; $[\alpha]_D$ +150° (*c* 0.77, methanol). See R. W. Jeanloz, *J. Am. Chem. Soc.*, **76**, 555 (1954).

for authentic samples and for obtaining mixed melting points and infrared spectra for comparison of 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranose and the methyl α-D-glucopyranosides of the 3,4-, 3,6- and 4,6-di-*O*-methyl derivatives.

We also wish to express our appreciation to Dr. K. Meyer for his cooperation and sage counsel during the course of this work.

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[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

The Acid–Base-Catalyzed Conversion of Aldohexose into 5-(Hydroxymethyl)-2-furfural[2]

M. L. MEDNICK

Received September 8, 1961

Either glucose or starch, in water containing weak acids and weak bases, is converted at 150–250° into 5-(hydroxymethyl)-2-furfural (X) in maximum yields considerably higher than those obtained with acid catalysts alone. A reaction pathway is proposed involving the isomerization of glucose to fructose followed by dehydration.

The acid-catalyzed conversion of carbohydrates to 5-(hydroxymethyl)-2-furfural (HMF) has long been known to produce comparatively high yields from ketohexose but relatively insignificant yields from aldohexose.[3] In the more recent literature, Montgomery and Wiggins[4] obtained 6.7% of HMF by heating glucose in water with a hydrogen atmosphere at 162–167°. Under these same conditions, 21.6% of HMF was produced from sucrose. The highest reported yield of HMF by direct conversion of glucose of 16.6%.[5] McKibbins obtained this yield by heating a dilute aqueous sulfuric acid solution at 250° for a short time. In a similar manner, but using levulinic acid as catalyst, Garber, Cranford, and Jones obtained 31 to 38% HMF from sucrose.[6] They described the unchanged residue as mainly glucose. Haworth and Jones[7] equilibrated glucose, primarily with mannose and fructose, by pretreatment with aqueous sodium hydroxide. The solution was then neutralized with hydrochloric acid, acidified with oxalic acid, and heated. The best yield of HMF obtained was 17%. Using calcium hydroxide for pretreatment, they passed unchanged residue through two additional cycles of this system for a total yield of 28.5%.

The history of this process led us to the tentative conclusion that the fructofuranose structure (VI, Fig. 1) is a possible intermediate on the reaction route between glucose and HMF. The keto-enol isomerization is acid–base-catalyzed.[8] We, therefore, tested the hypothesis that suitable combinations of acidic and basic catalyst constituents might accelerate its formation and, accordingly, the over-all rate of glucose conversion to HMF. It was found that any chosen combination of weak acid and weak base, under suitable conditions of concentration, time, and temperature, improved maximum HMF yield over that obtained in a control experiment using phosphoric acid alone as catalyst. Table I summarizes a comparison series of such experiments conducted in the 170–190° range. All examples show improved yield over the control except those employing ammonia and hydrochloric, the strongest acid used. In this temperature range, the best yield (23%) was obtained with ammonia and phosphoric acid in such proportions as to produce an initial *p*H of approximately 4. When the process was carried out at somewhat higher temperatures (200–230°), the yield, using ammonia and phosphoric acid, fell off to 12%, and there was increased rate of humin formation. That this was probably due to reactions involving the active hydrogens of ammonia was indicated by the fact that, under similar conditions, increased yields were obtained using either trimethylamine or triethylamine and phosphoric acid (27–36%).

In addition to the factors noted, the examples

(1) One of the laboratories of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented at the 140th National Meeting of the American Chemical Society, Chicago, Illinois, September 3-8, 1961.

(3) G. Dull, *Chemiker Ztg.*, **19**, 216 (1895); J. Kiermayer, *Chemiker Ztg.*, **19**, 1003 (1895); J. A. Middendorp, *Rec. trav. chim.*, **38**, 1 (1919); W. Alberda Van Eckenstein and J. J. Blanksma, *Ber.*, **43**, 2355 (1910); F. H. Newth, *Adv. Carbohydrate Chem.*, **6**, 83 (1951).

(4) W. R. Montgomery and L. F. Wiggins, *J. Soc. Chem. Ind. (London)*, **66**, 31 (1947).

(5) S. W. McKibbins, Doctoral dissertation, University of Wisconsin, 1958.

(6) J. D. Garber, Cranford and R. E. Jones, U. S. Patent **2,929,823**, March 22, 1960.

(7) W. N. Haworth and W. G. M. Jones, *J. Chem. Soc.*, 667 (1944).

(8) J. C. Speck, *Adv. Carbohydrate Chem.*, **13**, 79 (1958).