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

The Structure of Hyalobiuronic Acid and of Hyaluronic Acid from Umbilical Cord[1,2]

BY BERNARD WEISSMANN AND KARL MEYER

RECEIVED OCTOBER 26, 1953

Hyalobiuronic acid, a glucuronido glucosamine earlier isolated from hydrolysates of hyaluronic acid from umbilical cord, has been converted to its heptaacetyl methyl ester and its N-acetyl derivative. Esterification of the disaccharide, oxidation of the glucosamine residue to glucosaminic acid, and reduction of the uronic ester residue to a glucose residue yields a crystalline glucosidoglucosaminic acid. This is oxidatively deaminated to give a glucosidoarabinose, isolated as its crystalline heptaacetate, identical with the heptaacetate VII obtained by Zemplén degradation of laminaribiose (VIII). Hyalobiuronic acid is thus 3-O-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucose (I). That its N-acetyl derivative II is the basic repeating unit of hyaluronic acid linked linearly in the polymer, probably by 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl) linkages (Fig. 2), follows from earlier hydrolytic and enzymatic experiments, and from periodate oxidation data in the literature. A modification of the hydroxamic acid test suitable for sugar esters is described.

Hyaluronic acid is a polysaccharide composed of equivalent quantities of D-glucuronic acid, acetic acid and D-glucosamine. It was first isolated from vitreous humor of bovine eyes,[3] subsequently from other sources including synovial fluid, skin, tumor tissue, umbilical cord, cock's comb, and from groups A and C hemolytic streptococci.[4] Both in itself, and as a representative of the hexosamine-containing polysaccharides classed as mucopolysaccharides which find wide distribution in the animal kingdom, this substance is of substantial biological and medical importance.[4,5] While it has not been established whether hyaluronic acids from diverse sources are identical, identification of the sugar components, analysis, optical rotation and enzymatic specificities indicate similarity of at least many of them. In this, as in most other work on hyaluronic acid, umbilical cord has been chosen as the most convenient source.

The structure of this mucopolysaccharide has not yet been elucidated. The number of reports on this subject appearing in recent years[6-10] testifies to interest in the substance, and also suggests its resistance to treatment by the classical structural methods of sugar chemistry. The components of hyaluronic acid, N-acetyl-D-glucosamine and D-glucuronic acid, cannot, as concluded by Kaye and Stacey from methylation studies,[6] form segregated "cores." They are undoubtedly present, rather, in an alternating arrangement; the repeating unit of the polymer is accordingly a disaccharide, or multiple thereof. This view[4] receives support from experiments showing that the oligosaccharides produced by enzymatic hydrolysis all have equivalent uronic acid and hexosamine content,[11] and especially from the isolation in good yield of an aldobiuronic acid by hydrolysis of the polysaccharide.[12] The N-acetyl derivative II (see below) of this compound is isolated from some enzymatic reactions involving hyaluronic acid.[11b,13] The aldobiuronic acid represents the basic repeating unit of hyaluronic acid, and contains the predominating if not sole uronidic linkage present in it. Information about the structural features of this compound therefore apply also to structure of the polymer.

The aldobiuronic acid, designated hyalobiuronic acid, is a crystalline substance characterized as a glucuronidoglucosamine, which is isolated in better than 60% yield after mineral acid hydrolysis of the oligosaccharides produced by action of testicular hyaluronidase on umbilical cord hyaluronic acid, and also by direct acid hydrolysis of the polymer.[12] In this report there is described the conversion of this aldobiuronic acid to derivatives suitable for its further characterization and for comparison with certain enzymatic products, and the unequivocal proof of its structure as 3-O-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucose (I) by degradation to a disaccharide of simpler functionality whose structure is more readily ascertained.

The pertinent reactions are indicated in Fig. 1. Ketene selectively acetylates the amino group[14] of the aldobiuronic acid (I). The resulting amorphous N-acetyl derivative II, apparently pure after removal of starting material by a cation exchange resin (70–80% yield), is readily esterified by cold, very dilute methanolic hydrogen chloride.[15] Cold acetylation of the resulting ester gives methyl α(?)-heptaacetylhyalobiuronate (III). This derivative is prepared more directly (66% yield) by esterification of the aldobiuronic acid with cold methanolic hydrogen chloride, and cold acetylation of the resulting amorphous methyl ester hydrochloride IV. As previously mentioned,[12b] acid hydrolysis of the pure heptaacetyl methyl ester III yields aldobiuronic acid having the same optical rotation and X-ray powder pattern as the original. This completes the characterization of the aldobiuronic acid, whose lack of characteristic melting behavior and

(1) Preliminary communication: B. Weissmann and K. Meyer, THIS JOURNAL, **74**, 4729 (1952).

(2) This work was supported by grants from the National Institutes of Health and the Helen Hay Whitney Foundation.

(3) K. Meyer and J. Palmer, *J. Biol. Chem.*, **107**, 629 (1934).

(4) K. Meyer, *Physiol. Revs.*, **27**, 335 (1947).

(5) *Ann. N. Y. Acad. Sci.*, **52**, 843-1196 (1950).

(6) M. A. G. Kaye and M. Stacey, *Biochem. J.*, **48**, 249 (1951).

(7) (a) R. W. Jeanloz and E. Forchielli, *J. Biol. Chem.*, **190**, 537 (1951); (b) **188**, 361 (1951); (c) R. W. Jeanloz, *ibid.*, **197**, 141 (1952); (d) *Helv. Chim. Acta*, **35**, 362 (1952).

(8) K. H. Meyer, J. Fellig and E. H. Fischer, *ibid.*, **34**, 939 (1951).

(9) G. Blix, *Acta Chem. Scand.*, **5**, 981 (1951).

(10) (a) Masamune, Z. Yosizawa and T. Isikawa, *Tohoku, J. Exp. Med.*, **55**, 166 (1952); (b) T. Isikawa, *ibid.*, **53**, 217 (1951); **54**, 197 (1951).

(11) (a) M. M. Rapport, K. Meyer and A. Linker, THIS JOURNAL, **73**, 2416 (1951); (b) B. Weissmann, K. Meyer, P. Sampson and A. Linker, *J. Biol. Chem.*, in press.

(12) (a) M. M. Rapport, B. Weissmann, A. Linker and K. Meyer, *Nature*, **168**, 996 (1951); (b) B. Weissmann, M. M. Rapport, A. Linker and K. Meyer, *J. Biol. Chem.*, **205**, 205 (1953).

(13) A. Linker, K. Meyer and B. Weissmann, unpublished experiments.

(14) M. Bergmann and F. Stern, German Patent 453,577 (1927); *Chem. Zentr.*, **99**, I, 2663 (1928).

(15) E. F. Jansen and R. Jang, THIS JOURNAL, **68**, 1475 (1946).

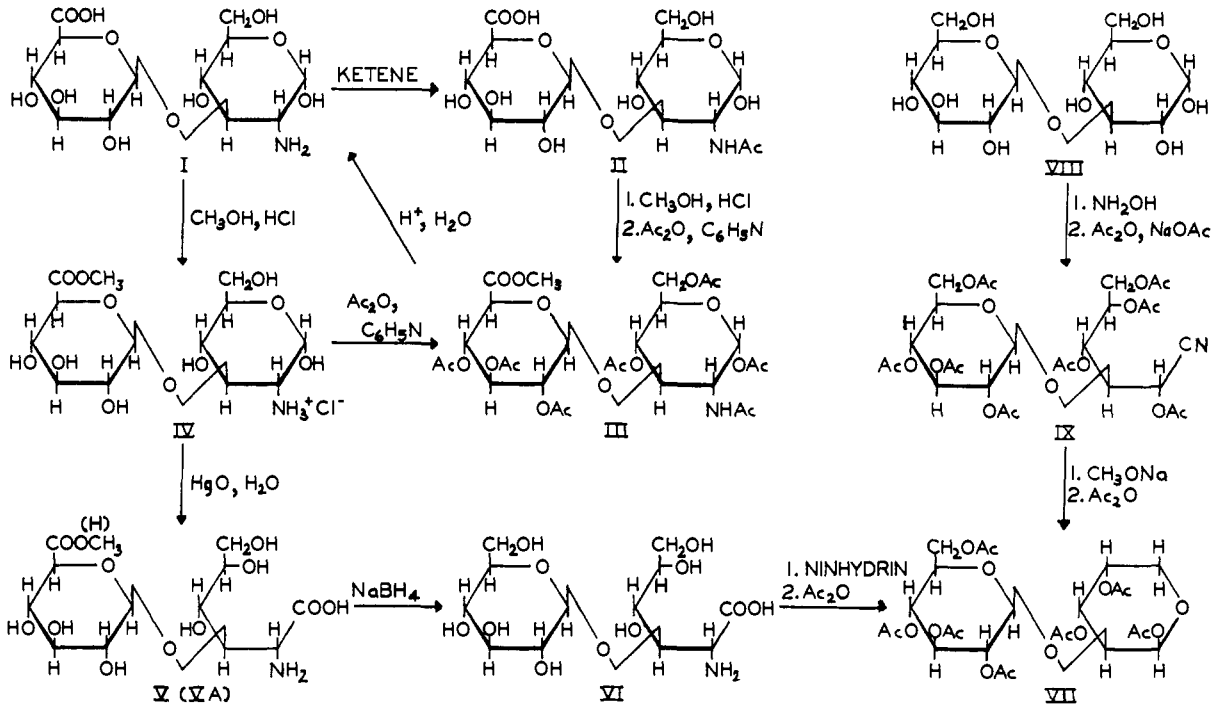

Fig. 1.—Demonstration of structure of hyalobiuronic acid.

low solubility otherwise make demonstration of purity difficult. The degradative study below, first carried out with a crude preparation, has been repeated without significant difference in results with a sample regenerated from the heptaacetyl methyl ester.

The amorphous methyl ester hydrochloride IV is heated with an aqueous suspension of mercuric oxide,[16] and the products of the oxidation, presumably containing V, are reduced with sodium borohydride.[17] A crystalline glucosidoglucosaminic acid VI (20% yield from I) is isolated by ion exchange chromatography. A by-product, the glucuronidoglucosaminic acid Va, apparently results from hydrolysis of the ester grouping prior to reduction, and can also be prepared by direct oxidation of the aldobiuronic acid (poor yield). Glucosaminic acid can be oxidatively deaminated more efficiently with ninhydrin (yield over 66%) than with chloramine-T, previously used.[18] With ninhydrin, VI gives a glucosidoarabinose, isolated as the heptaacetyl derivative VII (30% yield). The small amount of this compound obtained makes further degradative reactions impractical, necessitating preparation of appropriate comparison compounds. Assuming that the aminosugar occurs as its pyranose form in hyaluronic acid and that the uronidic linkage has the β-configuration (rotation of polymer *ca.* $-70°$) there are only three possibilities. One of these is eliminated by comparison of VII with the heptaacetylglucosidoarabinose derivatives prepared by Zemplén from cellobiose.[19]

Application of the Zemplén degradation[19] to the disaccharide laminaribiose (VIII) goes smoothly. The intermediate crystalline octaacetyllaminaribionitrile (IX), on treatment with sodium methoxide gives a glucosidoarabinose (not isolated), the heptaacetyl derivative VII of which is identical with the one from hyaluronic acid. Laminaribiose, a disaccharide first isolated fron hydrolyzates of laminarin, a polysaccharide from the sea-weed *Laminaria cloustoni*,[20] has been prepared synthetically,[21] and its structure definitively established[21b] as 3-β-D-glucopyranosyl-D-glucose (VIII). Degradation of the aldobiuronic acid and of this disaccharide of known structure to the same glucosidoarabinose establishes uniquely not only the position of the uronidic linkage in the former, but also its configuration, and the identity and ring size of the uronic acid. These structural features must also be present in hyaluronic acid.

Kaye and Stacey[16] encountered difficulties in attempting full methylation of hyaluronic acid without concomitant degradation, and isolated a crystalline methylated pyranose derivative of glucuronic acid in only trace amounts. Careful study of the periodate oxidation under controlled conditions of four glucosamine derivatives has yielded findings approximately consistent with rational stoichiometric interpretation for only one of these.[7b] Structures have nevertheless been assigned based on periodate oxidation studies of: (1) the polymer and a partially deacetylated, poorly characterized product obtained by action of 5 *M* sodium hydroxide at 80°[7a]; (2) the polymer and methanolysis products of the methylated material[8,9,cf.7d]; and (3) the polymer and a disaccharide derivative.[10a] On the basis of the negligible periodate consumption

(16) H. Pringsheim and G. Ruschmann, *Ber.*, **48**, 680 (1915).

(17) M. L. Wolfrom, H. B. Wood and K. Anno, American Chemical Society, 119th Meeting Abstracts, 1951.

(18) R. M. Herbst, *J. Biol. Chem.*, **119**, 85 (1937).

(19) G. Zemplén, *Ber.*, **59**, 1254 (1926).

(20) V. C. Barry, *Sci. Proc. Roy. Dublin Soc.*, **22**, 422 (1939); J. J. Connell, E. L. Hirst and E. G. V. Percival, *J. Chem. Soc.*, 3494 (1950).

(21) (a) K. Freudenberg and K. v. Oertzen, *Ann.*, **574**, 37 (1951); (b) P. Bächli and E. G. Percival, *J. Chem. Soc.*, 1243 (1952).

by the polymer,[7a,8,9,10a] all workers have concluded that if the acetylglucosamine is present as the pyranose form, it must be linked to the uronic acid residue in the 3-position, if it be further assumed that the latter also has a pyranose structure. The uronidic linkage has variously been represented as 1,3-,[7a] 1,4-,[8,10a] 1,3- *and* 1,4-[9] pyranoside or 1,3-furanoside.[10a] Some crystalline disaccharide derivatives have been reported by Isikawa[10b] (highest yield from the polymer, 3%). One of these is a heptaaceyl methyl ester, whose constants, however, differ significantly from those of III. Very mild methylation recently has been applied[7c] to hyaluronic acid, yielding a product degradation of which, according to a preliminary announcement,[7d] gives a crystalline methylated disaccharide.

With regard to the structure of polymeric hyaluronic acid, it has been inferred from its stability to acid hydrolysis and other considerations[4] that the acetyl is present as an N-acetylglucosamine residue and this is amply confirmed by isolation of N-acetylglucosamine[13] and N-acetylhyalobiuronic acid[11b,13] from enzymatic digests. In all the periodate oxidation studies, negligible uptake of oxidant by the polymer has been noted. On the assumption that this negative evidence demonstrates absence of vicinal hydroxyl groups, it is possible in conjunction with the data now reported to make further structural deductions. Taken with the 1,3-uronidic linkage now found, this assumption leads to a pyranose ring structure for the acetylglucosamine residue, and the pyranose ring structure of the uronic acid residue leads to a 1,3-glucosaminidic linkage. The rotation of the polymer, $[\alpha]_D$ −70°, suggests that this linkage has the β-configuration. Regularity of the size distribution of the oligosaccharides isolated from enzymatic digests[11b] points clearly to an unbranched or nearly unbranched polymer. These deductions are summarized in the structure for hyaluronic acid: 3-O-β-D-glucopyranuronosyl-(2-acetamido-2-deoxy-3-O-β-D-glucopyranosyl-3-O-β-D-glucopyranuronosyl)$_{n-1}$-2-acetamido-2-deoxy-D-glucose (Fig. 2).

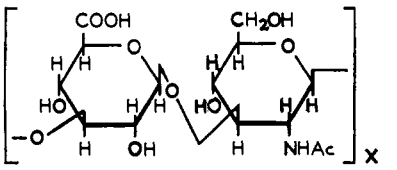

Fig. 2.—Hyaluronic acid.

Experimental[22]

3-O-(Methyl 2,3,4-Tri-O-acetyl-β-D-glucopyranosyluronate)-2-acetamido-2-deoxy-1,4,6-tri-O-acetyl-α(?)-D-glucopyranose (III) *via* the Methyl Ester Hydrochloride.—Hyalobiuronic acid (preparation I[12b]), 1.07 g., stirred at room temperature with 60 ml. of dry methanol 0.075 *M* in hydrogen chloride, dissolved in about 12 hr. After 24 hr., the solvent was distilled *in vacuo* below 10°, and the residual mush was dehydrated by additions of abs. ethanol and distillation. The residual colorless amorphous **3-O-(methyl β-D-glucopyranosyluronate)-2-amino-2-deoxy-D-glucose hydrochloride (IV)**, dried briefly at room temperature and 0.1 mm., weighed 1.27 g. (theory 1.22 g.). The hydroxamic acid reaction indicated 97% esterification. No changes in reducing value, ninhydrin-nitrogen or uronic acid had occurred.

To the chilled solid, 5 ml. each of pyridine and acetic anhydride were added. Little solid remained after shaking 20 min. at 0°. After 2 hr. at room temperature, excess reagent was removed (70°, 0.1 mm.). The residual glass gave colorless crystals from abs. ethanol, pure after washing, of the **heptaacetyl derivative (III)**, 1.40 g. (66%). The air-dried crystals melted at 120° to a stiff sirup, $[\alpha]^{24}D$ +24.5° (*c* 2, chloroform). They contained one mole of ethanol of crystallization, not quite completely lost at 110° in one hour, as shown by loss of weight and methoxyl determinations. The compound was very soluble in chloroform, soluble in cold methanol or hot ethanol, sparingly soluble in cold ethanol or hot benzene, insoluble in water or ether.

Anal. Calcd. for $C_{27}H_{37}O_{18}N + C_2H_6O$: N, 1.97; CH_3O, 8.75; loss on drying, 6.5. Found(crystals): N, 2.08; CH_3O, 8.97; loss, 5.1. Calcd. for $C_{27}H_{37}O_{18}N$: C, 48.9; H, 5.62; N, 2.11; CH_3O, 4.68; CH_3CO, 45.4; mol. wt., 663.5. Found (dried substance): C, 48.5; H, 5.77; N, 2.10; CH_3O, 6.05; CH_3CO, 45.9; mol. wt. (camphor), 668.

With ether, the mother liquor deposited a number of impure solid fractions of rotation as low as $[\alpha]^{25}D$ −1°. The substance isolated pure was therefore probably the α-anomer. (In this case, Hudson's rule predicts $[\alpha]^{25}D$ −26° for the β-anomer.)

3-O-(Methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-2-acetamido-2-deoxy-1,4,6-tri-O-acetyl-α(?)-D-glucopyranose (III) *via* N-Acetylhyalobiuronic Acid (II).—To a rapidly stirred suspension of 1.00 g. of hyalobiuronic acid in 5 ml. of water, 2.85 meq. of 1 *M* sodium hydroxide was added dropwise. When solution was nearly complete, ketene was passed in with continued stirring. The *p*H was 9 after 5 min., 4.5 after 30 min. The reaction mixture was filtered, passed through a small Dowex 50-H[23] column, decolorized with carbon, diluted to 100 ml., lyophilized, redissolved, relyophilized and dried *in vacuo* over sodium hydroxide and phosphorus pentoxide. The colorless amorphous **N-acetylhyalobiuronic acid (II)** weighed 0.88 g., $[\alpha]^{24}D$ −32° (*c* 2, water), *pK'* 3.3. It contained 49.2% uronica cid (gasometric), 48.6% (colorimetric, carbazole), and 41% hexosamine. Its reducing value was 65% as glucose (ferricyanide), 8.2 μeq. hypoiodite/mg. (Macleod and Robison). In the Elson and Morgan reaction without prior hydrolysis it gave 87% of the color given by an equal weight of N-acetylglucosamine. Ninhydrin and hydroxamic acid color tests were negative.

Anal. Calcd. for $C_{14}H_{23}O_{12}N$: N, 3.52; CH_3CO, 10.8; neut. equiv., 397.3. Found: N, 3.08; CH_3CO, 11.2; neut. equiv., 407.8.

In one run, prisms slowly deposited from water–methanol–acetone; these appeared to contain solvent of crystallization not lost at 60° (neut. equiv., 472).

When a solution of 0.42 g. of II in 20 ml. of dry methanol 0.02 *M* in hydrogen chloride was allowed to stand at 5° for 2 days, the decrease in acidity indicated 98% esterification, and no loss in reducing power was detected. The solvent was removed below room temperature after neutralization with a little pyridine. The residual amorphous ester, acetylated as above described for IV, gave 0.39 g. of crystalline **heptaacetyl methyl ester (III)**, pure after recrystallization, identical with that above. Here, too, the mother liquor contained materials of lower optical rotation.

Regeneration of Hyalobiuronic Acid (I) from the Heptaacetyl Derivative (III).—The heptaacetyl derivative, 1.00 g., boiled with 20 ml. of 0.5 *M* sulfuric acid, rapidly dissolved. In three hours, 90 ml. of dilute acetic acid was distilled, the solution volume being maintained at 15–20 ml. by addition of water. The residual solution was cooled, cautiously brought to *p*H 5 with barium hydroxide, filtered, and concentrated *in vacuo;* it gave 0.33 g. (66%) of long prisms, $[\alpha]^{27}D$ −23° (*c* 2, 1 *M* HCl), preparation IV.[12b]

3-O-β-D-glucopyranosyl-2-amino-2-deoxy-D-gluconic Acid (VI).—Freshly precipitated yellow mercuric oxide (4.0 millimole) was added to an aqueous solution (10 ml.) of methyl ester hydrochloride IV prepared from 300 mg. of the hyalobiuronic acid preparation just described. The sus-

(22) Microanalyses by Elek Microanalytical Laboratories, Los Angeles, and by Micro-Tech Analytical Laboratories, Skokie, Ill. We wish to thank Dr. Harold Markowitz for the methoxyl determinations, and Mr. Alfred Linker for the gasometric uronic acid determinations. Melting points are corrected.

(23) Sulfonated polystyrene resin, Dow Chemical Co., Midland, Mich.

pension was stirred 30 min. at 99° and centrifuged hot. The supernatant solution and hot water washings were heated to boiling, treated with hydrogen sulfide, filtered and concentrated *in vacuo*. The residual sirup crystallized from aqueous ethanol, giving rosettes of needles, 94 mg., apparently the methyl ester V, contaminated with some free acid (Va), as indicated by color tests: reducing value as glucose, 1% (ferricyanide); ninhydrin-N, 4.0%; uronic acid, 50% (carbazole); hydroxamic acid test, 1.7 meq. ester/g.; theory for V: N, 3.6%; uronic acid, 50%; ester, 2.6 meq./g. Further purification was not attempted.

To a solution of 90 mg. of the product from above in 2 ml. of water, a solution of 60 mg. of sodium borohydride in 1 ml. of 0.05 *M* sodium bicarbonate was added over 5 min. with stirring. After 1 hr. at room temperature, acetic acid was added to *p*H 6.5. The carbazole and anthrone color tests indicated 52% conversion to the corresponding glucose derivative. To minimize interaction of the product with borate ion, 0.50 g. of sorbitol was added. On the next day, the solution was added to a small column (0.5 $cm.^2$ × 10 cm.) of Dowex 50-H, 100 mesh, which was then washed with water (2 ml.) and developed with 0.002 *M* acetic acid. Two ninhydrin-positive peaks appeared. Material in peak A, eluate volume 10–40 ml., gave a positive test (carbazole) for uronic acid, while material in peak B, eluate volume 35–80 ml., contained glucose (anthrone test). Concentration of peak B material (eluate volume 40–80 ml.), and crystallization of the residue from aqueous ethanol gave well-formed needles of the **glucosidoglucosaminic acid (VI)**, 42 mg. (15% yield from I), $[\alpha]^{29}D$ −32° (*c* 0.9, water). The analytical sample was produced by recrystallization of a specimen similarly prepared in 20% yield from crude I (preparation I[12b]). The compound charred without melting. It was slightly soluble in cold, soluble in hot water, insoluble in methanol or ethanol. An air-dried sample showed $[\alpha]^{30}D$ −34° (*c* 0.9, water). Colorimetry showed no uronic acid (carbazole) or reducing power (ferricyanide), 4.0% ninhydrin-nitrogen and 46% glucose (anthrone). The product was dried at 110° for analysis.

Anal. Calcd. for $C_{12}H_{23}O_{11}N$: C, 40.4; H, 6.49; N, 3.92; mol. wt., 357.3. Found: C, 40.2; H, 6.37; N, 3.89; neut. equiv., 355 (formol titration).

3-O-(β-D-Glucopyranosyluronic acid)-2-amino-2-deoxy-D-gluconic Acid (Va).—Concentration of the material in peak A (eluate volume 15–35 ml.) from the Dowex 50 column of the preceding section gave a residue which crystallized as colorless needles, 31 mg., from aqueous ethanol. (Less had been obtained in an earlier run.) From its origin, solubility behavior and color tests the substance was undoubtedly identical with compound Va, which for comparison was produced in 28% yield by direct mercuric oxide oxidation of hyalobiuronic acid. Both specimens were almost insoluble in cold, sparingly soluble in hot water, insoluble in methanol or ethanol, readily soluble in sodium bicarbonate solution. Colorimetry showed 3.5% ninhydrin-nitrogen, 52% uronic acid (carbazole) and no glucose (anthrone) or reducing sugar (ferricyanide). The second preparation was analyzed. The substance, dried at 110°, apparently retained one mole of water of crystallization.

Anal. Calcd. for $C_{12}H_{21}O_{12}N + H_2O$: C, 37.0; H, 5.98; N, 3.59. Found: C, 37.1; H, 6.16; N, 3.63.

Ninhydrin Degradation of the Glucosidoglucosaminic Acid (VI).—A solution of 41 mg. of VI (derived from crude I, preparation I[12b]) and 27 mg. of ninhydrin in 2 ml. of water was heated 30 min. at 99°, cooled and filtered. The filtrate and water washings after passage through a small Dowex 50-H[23] column were extracted several times with large volumes of chloroform and butanol. Lyophilization of the aqueous layer gave an amorphous solid. This had a reducing value of 22% as glucose (ferricyanide), contained 45% pentose (orcinol) and 61% glucose (anthrone). The presumed glucosidoarabinose was heated on the steam-bath for 60 min. with 70 mg. of sodium acetate and 1 ml. of acetic anhydride, cooled, treated with water and let stand overnight in the refrigerator. Neutralization with sodium bicarbonate, chloroform extraction, and concentration of the extract gave 50 mg. of an amber glass. Crystallization from abs. ethanol gave colorless needles, recrystallized to give 14 mg. of **2-O-β-D-glucopyranosyl-D-arabinose heptaacetate (VII)**, m.p. 198–199° (microblock), no depression when mixed with the same compound from degradation of laminaribiose (see below), $[\alpha]^{23}D$ −47° (*c* 0.7, chloroform). The rotation sample was recrystallized for analysis. Colorimetry showed 23.2% arabinose (orcinol, calcd. 24.7), 29.8% glucose (anthrone, calcd. 29.7).

Anal. Calcd. for $C_{25}H_{34}O_{17}$: C, 49.6; H, 5.65. Found: C, 49.9; H, 6.00.

An identical product (m.p. and mixed m.p. 198–199°) was obtained similarly from the specimen of VI derived from the preparation of I which had been purified through its acetate.

A limited study of the oxidative deamination of glucosaminic acid, used as a model, showed that at 99° and concentration of 0.1 *M*, 1.4 moles of ninhydrin and 30–60 min. heating time were satisfactory. Colorimetry showed 93% conversion to arabinose. After removal as above of excess ninhydrin and its reduction products, D-arabinose was crystallized in 40% yield, its diphenylhydrazone in 66% yield.

Octaacetylaminaribionitrile (IX).—Laminaribiose (VIII) was synthesized according to Bächli and Percival,[21b,24] $[\alpha]^{30}D$ +25.5° → +17.5° (*c* 2, water); m.p. 202–205° (slow heating, yellowing from 180°), m.p. 212–214° dec. (heated 6°/min., in bath at 188°); reported[22b] $[\alpha]^{16}D$ +24.9° (20 min.) → +18.6, m.p. 204–206°. To a solution of 1.71 g. in 7 ml. of water, heated on the steam-bath, there was added rapidly 12 ml. of a 1.0 *M* hydroxylamine solution, prepared in dry ethanol–methanol (1:1) from sodium methoxide and excess hydroxylamine hydrochloride. After being refluxed for one hour, the solution, filtered, was concentrated to dryness *in vacuo*, and the residue was dehydrated by repeated addition of abs. ethanol and concentration. Without purification, the sirupy oxime, 15 ml. of acetic anhydride and 3 g. of sodium acetate were heated at 110° for 40 min. with shaking. The chilled brown mixture, shaken with ice and water (50 g.), dissolved completely and slowly crystallized in the refrigerator. Recrystallization from ethanol (Norite) gave the **nitrile** as fine single needles, m.p. 140–141°, $[\alpha]^{30}D$ +3° (*c* 2, chloroform), yield 1.59 g. The compound was soluble in hot ethanol or cold chloroform, sparingly soluble in cold ethanol, almost insoluble in ether. Brief treatment of a sample with methanolic sodium methoxide gave a solution in which cyanide ion was detected by the prussian blue test.

Anal. Calcd. for $C_{28}H_{37}O_{18}N$: C, 49.8; H, 5.52; N, 2.07. Found: C, 50.2; H, 5.51; N, 2.10.

2-O-β-D-Glucopyranosyl-D-arabinose Heptaacetate (VII).—To a solution of 1.47 g. of the nitrile IX, chilled by ice–salt, in 5 ml. of dry chloroform, 6 ml. of a 1 *M* methanolic sodium methoxide solution was added rapidly. The mixture was shaken intermittently (precipitate present) for 30 min. and 10 ml. of water was added in the cold. The aqueous layer was acidified with acetic acid, treated with silver acetate, and excess silver was removed with sodium chloride. The filtrate, decolorized with carbon, was lyophilized. To the residual glass were added 10 ml. of acetic anhydride and 2 g. of sodium acetate; the mixture was heated at 99° for 30 min., brought to boiling for 2 min., then cooled. Treatment with ice and water (35 g.) gave a clear amber solution from which colorless needles soon separated. These, filtered after chilling for 2 hr. and washed with dilute acetic acid, weighed 0.26 g., m.p. 199–200° (microblock, constant), 200.5–201° (capillary), $[\alpha]^{30}D$ −46° (*c* 2, chloroform).

Anal. Calcd. for $C_{25}H_{34}O_{17}$: C, 49.6; H, 5.65; CH_3CO, 49.7. Found: C, 49.2; H, 5.62; CH_3CO, 47.8.

Chloroform extraction of the neutralized mother liquor gave a sirup from which only small further amounts of this compound were recovered.

Colorimetry.—The paucity of material frequently made colorimetric methods extremely valuable in following changes of functional groups. Some of the methods have been referred to elsewhere,[12] including the Dische carbazole reaction for measuring bound uronic acid. Bound glucose was measured by the anthrone reaction, using conditions similar to those recently described.[25] Bound arabinose was measured by the Mejbaum modification of the orcinol reaction.[26] Experiments with pairs selected from the saccharides: arabinose, glucose, glucurone, glucosamine, N-acetylglucosamine and glucosaminic acid, and with other appropriate

(24) We are indebted to Prof. E. L. Hirst for seed material.

(25) W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, **50**, 298 (1952).

(26) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

models of known structure, showed that these three methods each measured only one sugar, and interference produced by equimolar amounts of the others was less than 10%. The Harding and MacLean modification of the ninhydrin reaction was used[27]; this measures amino nitrogen alpha to carboxyl or carbonyl but not other amines or ammonia.

The hydroxamic acid reaction, under conditions previously described, *e.g.*, ref. 28 gave low (30–40%) and variable color yields with sugar esters; as modified here (decreased *p*H, increased hydroxylamine concentration) it gave molar yields for model sugar esters and a lactone[29] which were concordant within 10% and were about 90% those of ethyl acetate or acetylcholine. In the modified procedure, 0.20 ml. of solution containing 0.3–3 μeq. of ester was mixed with 0.4 ml. of reagent freshly prepared from equal volumes of 8.0 *M* hydroxylamine hydrochloride solution and 1.0 *M* glycine in 8.5 *M* sodium hydroxide solution. After 2–3 hr. at room temperature, 2.50 ml. of 1.00 *M* hydrochloric acid and 6.0 ml. of 0.1 *M* ferric chloride in 0.01 *M* hydrochloric acid were added, and the optical density at 540 mμ was measured at once.

(27) V. J. Harding and R. M. MacLean, *J. Biol. Chem.*, **24**, 503 (1916).

(28) S. Hestrin, *ibid.*, **180**, 249 (1949).

(29) We wish to thank Dr. Harold Markowitz for a specimen of α-methylgalacturonide methyl ester, used as a standard, and Mr. Eugene Davidson for preparation of methyl D-galacturonate and chondrosin methyl ester hydrochloride, as well as performance of some of the colorimetry. Other models were D-glucuronolactone, methyl α-D-glucuronate tetraacetate and β-D-glucose pentaacetate.

NEW YORK 32, NEW YORK

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, U. S. PUBLIC HEALTH SERVICE, DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE]

The Acetolysis of Some Carbohydrate Benzyl Ethers

BY R. ALLERTON[1] AND HEWITT G. FLETCHER, JR.

RECEIVED NOVEMBER 4, 1953

The acetolysis of the benzyl ethers of **1,3**:4,6-di-*O*-methylene-D-mannitol, 2,4:3,5-di-*O*-methylene-L-iditol, 1,6-anhydro-β-D-glucopyranose and 1,4:3,6-dianhydro-D-mannitol has been studied in mixtures of acetic acid, acetic anhydride and sulfuric acid. In each case the benzyl group was readily cleaved. The results appear to indicate that primary–secondary methylene groups are cleaved more readily than secondary benzyl groups while secondary–secondary methylene groups are more stable toward acetolysis than primary benzyl groups. The acetolysis of 1,4:3,6-dianhydro-2,5-di-*O*-benzyl-D-mannitol is a slow and complex reaction which gave an amorphous sulfur-bearing material. Treatment of this latter with lithium aluminum hydride afforded **1**,4-anhydro-D-mannitol in low yield.

Burton and Praill[2] have recently shown that a variety of aryl benzyl ethers may be acetolyzed with acetic anhydride in the presence of perchloric or sulfuric acid and, since the benzyloxy group is widely used in the synthesis of carbohydrate derivatives, it becomes of interest to enquire, first, whether the benzyloxy group in carbohydrate derivatives may be removed by acetolysis and, second, if this proves to be true, how readily such a removal takes place in comparison with some other substituents which are frequently cleaved from carbohydrate moieties by acetolysis.

Although more stable than their isopropylidene and benzylidene analogs methylene bridges between primary and secondary carbons are known to acetolyze with ease[3] and therefore the first substance tested was 2,5-di-*O*-dibenzyl-1,3:4,6-di-*O*-methylene-D-mannitol, a substance which could be obtained in pure but amorphous form from the readily accessible, crystalline 1,3:4,6-di-*O*-methylene-D-mannitol.[4,5] When this benzyl ether was dissolved in a mixture of acetic anhydride and glacial acetic acid containing about 0.09% (v./v.) of sulfuric acid, a very rapid dextromutarotation took place followed by a slower levomutarotation. When the reaction was halted at the inflection point and the product deacetylated with methanolic hydrogen chloride,[6] a crystalline di-*O*-benzylhexitol was obtained in 18% yield. Its structure as 2,5-di-*O*-benzyl-D-mannitol was confirmed by the fact that it consumed but one mole of periodate. The same substance was obtained in 11% yield by the action of hydrochloric acid in aqueous ethanol on 2,5-di-*O*-benzyl-1,3:4,6-di-*O*-methylene-D-mannitol. It was characterized further through its tetra-*p*-nitrobenzoate and tetratosylate.

When 2,5-di-*O*-benzyl-1,3:4,6-di-*O*-methylene-D-mannitol was dissolved in an acetolysis mixture containing more sulfuric acid (2.4% v./v.) and the reaction allowed to go to completion, the product, after deacetylation with methanolic hydrogen chloride, was D-mannitol, the benzyl groups as well as the methylene groups having been cleaved. It is apparent, then, that in this particular substance the primary–secondary methylene bridges are cleaved with somewhat greater ease than the benzyl groups under these conditions.

Secondary–secondary methylene bridges are much more resistant to acetolysis than are primary–secondary ones.[7] How does the acetolysis of a benzyl group compare with that of a secondary–secondary methylene group? To answer this

(1) Chemical Foundation Fellow, 1952–1953.

(2) H. Burton and P. F. G. Praill, *J. Chem. Soc.*, 522 (1951).

(3) A. T. Ness, R. M. Hann and C. S. Hudson, THIS JOURNAL, **65**, 2215 (1943).

(4) W. T. Haskins, R. M. Hann and C. S. Hudson, *ibid.*, **65**, 67 (1943).

(5) H. G. Fletcher, Jr., and H. W. Diehl, *ibid.*, **74**, 3797 (1952).

(6) It is presumed that the initial product here is 3,4-di-*O*-acetoxymethyl-1,6-di-*O*-acetyl-2,5-di-*O*-benzyl-D-mannitol. Fletcher and Diehl (ref. 5) found methanolic hydrogen chloride a useful reagent for the removal of acetyl and acetoxymethyl groups.

(7) The acetolysis of secondary–secondary methylene groups is not unknown. R. M. Hann, J. K. Wolfe and C. S. Hudson [THIS JOURNAL, **66**, 1898 (1944)] found that the 3,5-methylene group (but not the 2,4-methylene group) of 2,4:3,5-di-*O*-methylene-D-glucitol could be acetolyzed, scission taking place at position 5. It should be remembered, however, that in contrast to the usual stability of secondary–secondary methylene groups to acetolysis, they may, in some cases at least, be removed by *hydrolysis*. M. L. Wolfrom, B. W. Lew and R. M. Goepp, Jr. [*ibid.*, **68**, 1443 (1946)] were able to effect the complete hydrolysis of 1,3:2,4:5,6-tri-*O*-methylene-D-glucitol and other methylenated glycitols by heating in aqueous acid with phloroglucinol as a formaldehyde acceptor. The acetolysis of 1,3:2,5-4,6-tri-*O*-methylene-D-mannitol which contains the rare C_5O_2 dioxepane ring produces 2,5-*O*-methylene-D-mannitol while hot aqueous hydrochloric acid gives 1,3:4,6-di-*O*-methylene-D-mannitol as well as D-mannitol itself (ref. 5).