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[Contribution from the Department of Chemistry of The Ohio State University]

Chondroitin Sulfate Modifications. I. Carboxyl-reduced Chondroitin and Chondrosine

By M. L. Wolfrom and Bienvenido O. Juliano[1]

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Carboxyl-reduced chondroitin (II) was prepared by exhaustive sodium borohydride reduction of chondroitin methyl ester (I) in borate buffer. Partial acid hydrolysis of II, *N*-acetylation and carbon column fractionation of the hydrolyzate facilitated the isolation and characterization of D-glucose, 2-acetamido-2-deoxy-α-D-galactose monohydrate and the sole disaccharide, 3-*O*-β-D-glucopyranosyl-2-acetamido-2-deoxy-α-D-galactopyranose dihydrate (III). Compound III was readily degraded in alkaline solution to D-glucose and a Morgan–Elson reactive sugar, "anhydro-*N*-acetyl-D-galactosamine." Sodium borohydride treatment of III gave the alditol IV, chromatographically identical to the product derived from chondrosine methyl ester hydrochloride. *N*-Acetylhexosaminols were shown to give positive Morgan–Elson reactions. Formula VIII is proposed as the correct structure for the periodate oxidation product of *N*-acetylchondrosinol (VI).

The acid instability of hexuronic acids has been frequently noted.[2] Owing to the difficulty of attaining complete hydrolysis without destruction of the component monosaccharides, an alternative approach used for studying mucopolysaccharide structure has been to convert the hexuronic acid residues to hexose units by reduction of their methyl esters with sodium borohydride.[3–5] Since no configurational changes were involved, deductions from the hydrolysis of reduced material have been applicable to the original polymer.

We report herein the preparation of 96% carboxyl-reduced polymeric chondroitin (desulfated chondroitin sulfate A) (II) and also carboxyl-reduced chondrosine, the sole disaccharide from the acid hydrolysis of II, isolated as the crystalline *N*-acetyl dihydrate derivative III, m.p. 155–157°, $[\alpha]_D$ +47 → +19° (water). Substance II, $[\alpha]_D$ +11° (dimethyl sulfoxide), was derived, in 71% yield, from chondroitin methyl ester[6] (I) by exhaustive sodium borohydride reduction[7]; the first reduction was 66% efficient, the second increased the reduction to 86% and the third to 96%. These results are in striking similarity to the reduction of the methyl ester of desulfated chondroitin sulfate B[5] with sodium borohydride, where 66 and 85% reductions of the L-iduronic acid to L-idose on the first and second reductions of the polymer were attained.

Partial acid hydrolysis of II, *N*-acetylation and carbon column fractionation of the hydrolyzate, facilitated the isolation of D-glucose, 2-acetamido-2-deoxy-α-D-galactose (*N*-acetyl-D-galactosamine) monohydrate and the sole disaccharide, 3-*O*-β-D-glucopyranosyl-2-acetamido-2-deoxy-α-D-galactose dihydrate (III). D-Glucose was characterized as its crystalline β-pentaacetate and the crystalline 2-acetamido-2-deoxy-α-D-galactose monohydrate was further characterized as the crystalline β-pentaacetate.[8] Carboxyl-reduced chondrosine gave positive ninhydrin and Elson–Morgan[9] reactions. Its crystalline *N*-acetyl derivative III gave a positive Morgan–Elson[10] reaction. On acid hydrolysis of III, D-glucose and 2-amino-2-deoxy-D-galactose (D-galactosamine) were detected by paper chromatography. The infrared spectra of II and III were very similar.

The disaccharide III was degraded readily in dilute alkali solution to D-glucose and a Morgan–Elson[10] reactive sugar ($R_{glucose}$ 1.8), "anhydro-*N*-acetyl-D-galactosamine," different from 2-acetamido-2-deoxy-D-galactose ($R_{glucose}$ 1.2). This is by analogy to the reported[11] alkaline degradation of 3-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose to D-galactose and "anhydro-*N*-acetyl-D-glucosamine" ($R_{glucose}$ 1.70), different from 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) ($R_{glucose}$ 1.24). The fact[11] that the 4-*O*-β-D-galactopyranosyl analog did not yield "anhydro-*N*-acetyl-D-glucosamine" and the 6-*O*-β-D-galactopyranosyl compound gave, instead, 6-*O*-β-D-galactopyranosyl- "anhydro-*N*-acetyl-D-glucosamine" with $R_{glucose}$ 1.04, should make alkaline degradation a valuable tool for determining *O*-substitution of *N*-acetylhexosamines. 2-Acetamido-2-deoxy-3-*O*-methyl-D-glucose also provided "anhydro-*N*-acetyl-D-glucosamine," which is of unknown structure. 4-*O*- and 6-*O*-substitution for II can be discounted from the results of the alkaline degradation. Its positive Morgan–Elson[10] test eliminates

(1) National Science Foundation Predoctoral Fellow, 1957–1958, under Grant NSF-G4494 to The Ohio State University; C. F. Kettering Research Foundation Fellow, 1958–1959.

(2) R. L. Whistler, A. R. Martin and M. Harris, *J. Research Natl. Bur. Standards*, **24**, 13 (1940); E. Stutz and H. Deuel, *Helv. Chim. Acta*, **41**, 1722 (1958).

(3) B. Weissmann and K. Meyer, This Journal, **76**, 4729 (1952); **76**, 1753 (1954).

(4) E. A. Davidson and K. Meyer, *ibid.*, **76**, 5686 (1954).

(5) R. W. Jeanloz and P. J. Stoffyn, *Federation Proc.*, **17**, 1078 (1958).

(6) T. G. Kantor and M. Schubert, This Journal, **79**, 152 (1957).

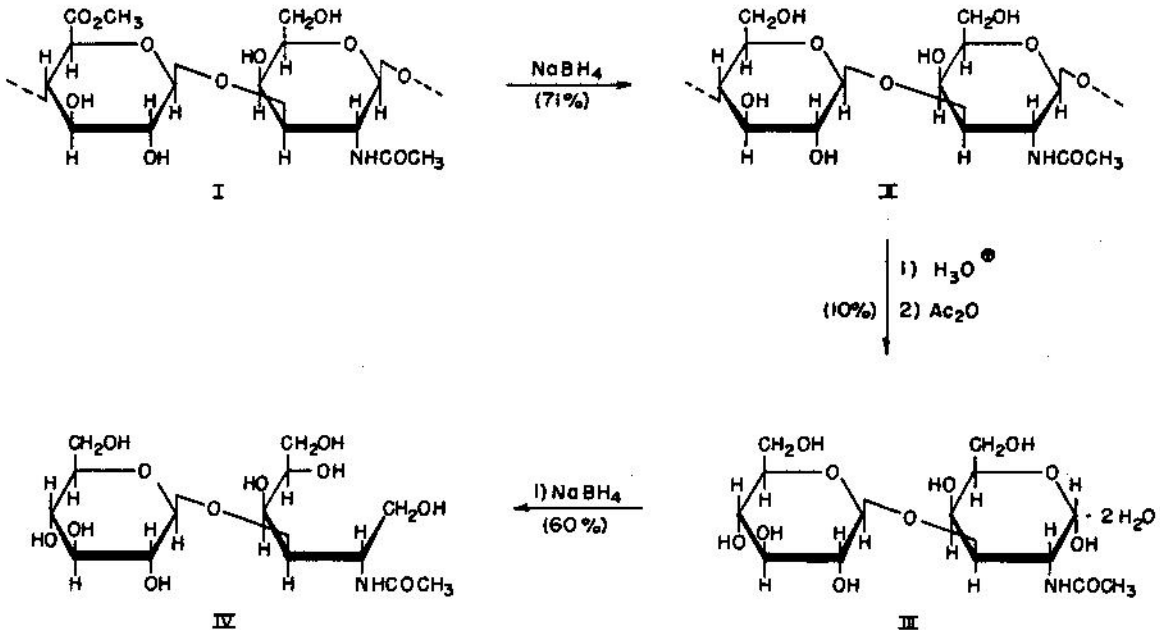
(7) Harriet L. Frush and H. S. Isbell, *ibid.*, **73**, 2844 (1956).

(8) M. Stacey, *J. Chem. Soc.*, 272 (1944).

(9) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).

(10) W. T. J. Morgan and L. A. Elson, *ibid.*, **28**, 988 (1934).

(11) R. Kuhn, Adeline Gauhe and H. H. Baer, *Chem. Ber.*, **87**, 289 1138 (1954).



the (1 → 4)-linkage since 4-*O*-substituted *N*-acetylhexosamines were unreactive to the reagent.[11-13]

Sodium borohydride reduction of III readily gave 3-*O*-β-D-glucopyranosyl-2-acetamido-2-deoxy-D-galactitol (IV), as the sirupy main product, which was chromatographically identical ($R_{glucose}$ 0.76), in three developer systems, to IV[4] derived from chondrosine methyl ester hydrochloride. The glycitol IV was still Morgan–Elson[10] reactive and gave (by paper chromatography) D-glucose as the only reducing sugar on acid hydrolysis. Compounds III and IV have similar $R_{glucose}$ values. 2-Acetamido-2-deoxy-D-galactose and the corresponding alditol have been shown to also have similar R_f values,[14] whereas similar R_f values have been reported for 2-acetamido-2-deoxy-D-galactose in various solvent systems.[15] Substance IV was resistant to alkaline degradation under conditions which degraded III. Its trace impurities of D-glucitol and "anhydro-*N*-acetyl-D-galactosaminol" may be accounted for by the degradation of III in the alkaline borohydride medium prior to reduction to IV, and subsequent reduction of these products.

The isolation of only one disaccharide, III, from II implies that the stability of the β-D-glucuronidic linkage[16] alone cannot account for the selective nature of the acid hydrolysis of chondroitin sulfate A to chondrosine[4] since conversion of this to a β-D-glucosidic linkage also gave the related disaccharide III and none for the alternative sequence. On the basis of the established component sequence for chondrosine, after Davidson and Meyer,[4] the periodate oxidation data of Wolfrom and coworkers,[17] based upon the sequence utilized by Levene,[18] is hereby reinterpreted. The diamide glycitol (VI) from the *O*-deacetylation of the *N*-acetylchondrosinol heptaacetate methyl ester (V) of Levene,[18] on periodate oxidation, underwent formaldehyde and formic acid scission in the reduced portion to yield initially the intermediate VII. Formula VIII, as suggested by Toro-Feliciano,[19] is proposed for the crystalline oxidation product isolated.[17] The formation of such a substituted dioxane ring (VIII) should be favored and such a ring has been well substantiated in the difructose dianhydrides.[20] Its presence is in harmony with the reported[17] one mole further uptake [IX] of oxidant. Scaled molecular models show that VIII could be formed readily. Color tests showed that the hexuronic acid was intact in V, thus supporting the established[4] component sequence for chondrosine.

That the β-D-glucopyranosidic linkage in II is more stable than the 2-acetamido-2-deoxy-β-D-galactopyranosidic linkage is consistent with comparative kinetic data[21] showing that the rate of glucosidic hydrolysis of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside was 9–18 times that of methyl β-D-glucopyranoside.[22] Since desulfation of chondroitin sulfuric acid proceeds faster than glycosidic cleavage in acid solution,[6] the absence of sulfate groups in II does not affect the problem. Hydrolytic studies of II showed the presence of *N*-acetylated sugars, III and 2-acetamido-2-deoxy-D-galactose, during the first two hours of reaction, denoting that glycosidic cleavage was faster

(12) R. W. Jeanloz and Monique Trémiège, *Federation Proc.*, **15**, 282 (1956).

(13) S. A. Barker, A. B. Foster, M. Stacey and J. M. Webber, *J. Chem. Soc.*, 2218 (1958).

(14) W. R. C. Crimmin, *ibid.*, 2838 (1957).

(15) S. Roseman and J. Ludowieg, THIS JOURNAL, **76**, 301 (1954).

(16) R. L. Whistler and G. N. Richards, THIS JOURNAL, **80**, 4880 (1958).

(17) M. L. Wolfrom, R. K. Madison and M. J. Cron, *ibid.*, **74**, 1491 (1952).

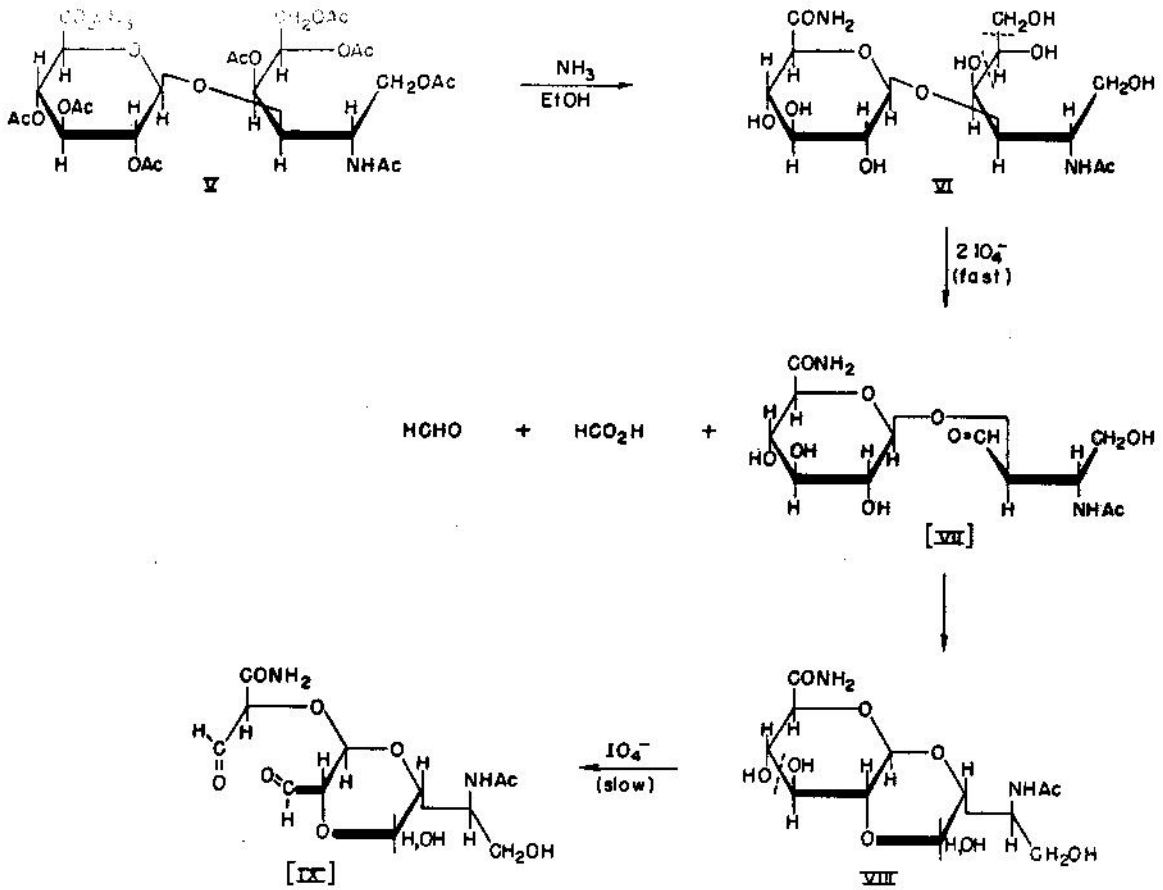
(18) P. A. Levene, *J. Biol. Chem.*, **140**, 267 (1941); P. A. Levene and F. B. LaForge, *ibid.*, **15**, 69 (1913).

(19) E. D. Toro-Feliciano, M.Sc. Thesis, The Ohio State University, 1957.

(20) Emma J. McDonald, *Advances in Carbohydrate Chem.*, **2**, 253 (1946).

(21) A. B. Foster, D. Horton and M. Stacey, *J. Chem. Soc.*, 81 (1957); see also R. C. G. Moggridge and A. Neuberger, *ibid.*, 745 (1938).

(22) E. A. Moelwyn-Hughes, *Trans. Faraday Soc.*, **25**, 503 (1929).



than *N*-deacetylation. This phenomenon has also been noted for chondroitin sulfate A.[4]

This approach to acid mucopolysaccharide structural studies by reduction of the uronic acid moiety and subsequent graded hydrolysis should find application in the study of related polymers, particularly heparin. Prior *N*-acetylation of the hydrolyzate was found to be essential to effective carbon column fractionation, as has been the case with the homologous chitosaccharide hydrochlorides.[18] The neutral nature of the *N*-acetyl derivatives allows more latitude in their isolation. The alkali-sensitivity of III precludes the use of basic developers for its isolation.

N-Acetylhexosaminols were found to be new exceptions to the Morgan–Elson[10] test for *N*-acetylhexosamines. Besides IV and "anhydro-*N*-acetyl-D-galactosaminol,"[11] 2-acetamido-2-deoxy-D-glucitol[23] (*N*-acetyl-D-glucosaminol) also gave positive tests. Since some *N*-acetylhexosaminides were reported[11,24] also to be reactive and 4-*O*-substituted *N*-acetylhexosamines are unreactive,[10-13] the Morgan–Elson[10] test must be interpreted with care.

Experimental

Chondroitin Methyl Ester (I).—Commercial sodium chondroitin sulfate[25] (50 g.) was purified by treatment with Magnesol–Celite,[26] and converted to the potassium salt by ethanol fractionation from dilute potassium chloride solution, according to the procedure of Malawista and Schubert,[27] differing only in the cation employed; yield 34.9 g. (66%) of white potassium chondroitin sulfate A, $[\alpha]^{18}D$ −20° (*c* 2.36, water), reported[4] −25°, identical in infrared spectra to that reported[28] for chondroitin sulfate A in the region 700 to 1,000 cm.$^{-1}$. This (30.0 g.) was converted to chondroitin methyl ester after the method of Kantor and Schubert[6]; yield 18.0 g. (82%) of white ester, $[\alpha]^{25}D$ −15° (*c* 1.21, water), reported[6] −15°.

Anal. Calcd. for $C_{13}H_{20}NO_9(CO_2CH_3)$: S, absent; OCH_3, 7.89; uronic acid, 49.4. Found: S, none; OCH_3, 7.64; uronic acid,[29] 40.1 (cor. 50.1).

Infrared spectral analysis showed a sharp ester absorption at 1,740 cm.$^{-1}$ and the absence of sulfate bands in the region 700 to 1,000 cm.$^{-1}$.

96% Carboxyl-reduced Chondroitin (II).—Reduction of the chondroitin methyl ester followed the general procedure of Frush and Isbell[7] in borate buffer. An amount of 17.6 g. of chondroitin methyl ester in 300 ml. of 0.4 *M* boric acid solution was treated dropwise at 0°, under stirring, with a fresh solution of 6.4 g. of sodium borohydride in 500 ml. of water (5°) during a period of 50 min. Stirring was continued for 45 min. more and the *p*H of the reaction mixture was adjusted to 9 with dilute sodium hydroxide. After keeping overnight at 5°, the solution was neutralized with formic acid and dialyzed against distilled water for 3 days. The dialyzate was filtered through asbestos, concentrated and lyophilized. The white product obtained was sparingly water soluble.

Anal. Calcd. for $C_{13}H_{20}NO_9(CH_2OH)_{0.66}(CO_2Na)_{0.34}$: uronic acid, 17.5. Found: uronic acid, 13.7 (cor.[29] 17.1).

(23) Prepared in this Laboratory by Dr. K. Onodera through sodium borohydride reduction of 2-acetamido-2-deoxy-D-glucose.

(24) R. Kuhn, Adeline Gauhe and H. H. Baer, *Chem. Ber.*, **86**, 827 (1953).

(25) Wilson Laboratories, Inc., Chicago, Ill.

(26) M. L. Wolfrom and K. Onodera, THIS JOURNAL, **79** 4737 (1957).

(27) Ina Malawista and M. Schubert, *J. Biol. Chem.*, **230**, 535 (1958).

(28) M. B. Mathews, *Nature*, **181**, 421 (1958); P. Hoffman, A. Linker and K. Meyer, *Biochim. et Biophys. Acta*, **30**, 184 (1958).

(29) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947). These values are *ca.* 20% low, probably due to uronic acid destruction on acid hydrolysis. Analyses will be so corrected.

This partially reduced chondroitin was esterified with methanolic hydrogen chloride (0.06 *N*),[6] reduced with sodium borohydride in borate buffer as above, and rid of inorganic impurities by dialysis; yield 14.3 g. of white powder, sparingly soluble in water.

Anal. Calcd. for $C_{13}H_{20}NO_9(CH_2OH)_{0.86}(CO_2Na)_{0.14}$: uronic acid, 7.34. Found: uronic acid, 5.6 (cor.[29] 7.0).

A third esterification and reduction was made on this material and the product was processed and dialyzed, concentrated to a thick white paste and lyophilized; yield 13.04 g. (71% from the original chondroitin methyl ester) of a very sparingly water-soluble powder (dried at 78°, 5 mm.), $[\alpha]^{19}D$ +11° (*c* 0.46, dimethyl sulfoxide).

Anal. Calcd. for $C_{13}H_{20}NO_9(CH_2OH)_{0.98}(CO_2Na)_{0.04}\cdot 2H_2O$: C, 41.74; H, 6.73; N, 3.48; $COCH_3$, 10.69; uronic acid, 1.93; ash (as oxide), 0.38. Found: C, 41.75; H, 6.65; N, 3.77; $COCH_3$,[30] 11.2; uronic acid, 1.3 (cor.[29] 1.6); ash, 0.60. Paper chromatographic analysis confirmed the presence of D-glucose. Infrared absorption spectral examination revealed the absence of the carboxylate absorption band at 1,612 cm.$^{-1}$ and the appearance of a band at 889 cm.$^{-1}$, absent in chondroitin sulfate A, and characteristic of β-D-glucopyranose.[31]

The hexuronic assay was corrected, according to Dische,[29] for the D-glucose present.

Hydrolytic Studies on II.—A preliminary study showed that a hydrolysis time of 2.25 to 3 hr. (100°, 1% soln. in *N* sulfuric acid) was required for an optimum yield of ninhydrin and Elson–Morgan reactive carboxyl-reduced chondrosine from I, by paper chromatographic analysis on Whatman No. 1 filter paper with 1-butanol, pyridine and water (3:2:1.5 by vol.) developer and both (separately) the Elson–Morgan[9,32] and alkaline silver nitrate[33] indicators. Substance II dissolved in *N* sulfuric acid after 1 hr. of refluxing. Morgan–Elson[10] reactive zones, 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine) ($R_{glucose}$ 1.2) and III ($R_{glucose}$ 0.74) were noted during the first 2 hr. of hydrolysis. An amount of 4.00 g. of II was refluxed for 2.25 hr. in 125 ml. (*c* 3.2) of *N* sulfuric acid and the cooled hydrolyzate was neutralized with solid barium carbonate. After the filtration of inorganic residue, the filtrate was made acidic with 10 ml. of *N* hydrochloric acid prior to *N*-acetylation.[15] A solution of the concentrated yellow hydrolyzate in water (75 ml.) was treated at 0° with 7.5 ml. of methanol, 90 ml. (settled vol.) of Dowex 1[34] (carbonate form) and 2 ml. of acetic anhydride and stirred for 90 min. at 0–5°. The reaction mixture was filtered and the filtrate and washings were passed through a column (180 × 12 mm., diam.) of Dowex 50[34] (H^+ form) to remove any non-acetylated amino sugar. Paper chromatographic analysis at this stage showed the absence of 2-amino-2-deoxy-D-galactose, D-glucuronic acid and *N*-acetylchondrosine and the presence only of distinct zones for D-glucose, 2-acetamido-2-deoxy-D-galactose and III. The *N*-acetylated hydrolyzate was fractionated on a carbon[35] (Nuchar C unground[36]) column (210 × 44 mm., diam.) previously washed with 2 liters of water. After placing the sample on the column, the chromatogram was developed with water (9 liters), 2% ethanol (4.5 liters), 3% ethanol (1.8 liters), 5% ethanol (3.2 liters) and 6% ethanol (4 liters). By paper chromatographic analysis it was shown that D-glucose only was present in the first liter of the water effluent but was mixed with 2-acetamido-2-deoxy-D-galactose in the rest of the water effluent; pure 2-acetamido-2-deoxy-D-galactose was eluted with 2% ethanol, but was contaminated with II in the 3% ethanol eluate; and the 5% and 6% eluates contained pure II. The fractions corresponding to the above pure hydrolyzates were concentrated, turbidity was removed by filtration through a fritted-glass filter and trace impurities were removed by passing through a column (60 × 13 mm., diam.) of mixed-bed resin (Amberlite MB-3[37]) and the effluent and washings were evaporated to dryness under reduced pressure.

β-D-Glucopyranose Pentaacetate.—The dry D-glucose fraction (0.41 g.) was heated with 1 g. of anhydrous sodium acetate and 7 ml. of acetic anhydride until the reaction proceeded spontaneously. The reaction mixture became homogeneous and was reboiled twice, cooled slightly and poured into 5 vol. of ice-water and stirred for 5 hr. at room temperature. The solution was extracted with four 15-ml. portions of chloroform and the combined extracts were evaporated to dryness. The residue was dissolved in anhydrous diethyl ether, filtered through a fritted-glass filter and crystallized by the addition of petroleum ether (b.p. 30–60°) to incipient cloudiness; yield 0.31 g. (35%) of white microneedles, m.p. 133.5–134°, $[\alpha]^{22}D$ +2° (*c* 0.9, chloroform); X-ray powder diffraction data[38]: 12.4[39]m[40] 9.38vs(1), 5.61s(3), 5.21vw, 4.91m, 4.66w, 4.47s(2), 4.30m, 3.75vw, 3.53m, 3.41vw, 3.25vw, 3.10vw, 2.56vw, 2.44vw, 2.35vw, 2.20vw, 2.12vw, 1.82vw.

Anal. Calcd. for $C_{16}H_{22}O_{11}$: C, 49.23; H, 5.68. Found: C, 49.22; H, 5.78.

2-Acetamido-2-deoxy-α-D-galactose Monohydrate.—The pure 2-acetamido-2-deoxy-D-galactose fraction (0.24 g.) was crystallized by the addition of a small amount of ethanol to the dry sirup to incipient turbidity and standing for a few hours at 5°. Recrystallization was effected in the same manner; yield 0.18 g. of white crystals, m.p. 118–120° (preliminary softening), $[\alpha]^{25}D$ +84° (*c* 1.04, water, final, downward mutarotation); X-ray powder diffraction data: 10.5[39]s[40](1,1), 7.80m, 7.18w, 5.16m(3), 4.64m, 4.40m, 4.19s(1,1), 3.90w, 3.63vw, 3.19vw, 2.17vw. Stacey[8] cites 120–122° and +80° for this substance.

Anal. Calcd. for $C_8H_{15}NO_6\cdot H_2O$: C, 40.16; H, 7.16; N, 5.86. Found: C, 40.30; H, 7.37; N, 5.81.

2-Acetamido-tetra-*O*-acetyl-2-deoxy-β-D-galactopyranose.—The procedure was essentially that of Stacey.[8] An amount of 90 mg. of 2-acetamido-2-deoxy-α-D-galactose monohydrate described above was suspended in 1.2 ml. of acetic anhydride and shaken with powdered, fused zinc chloride (30 mg.) for 24 hr. The reaction mixture was poured into 4 vol. of ice-water and the suspension was carefully neutralized by the addition of solid sodium carbonate. The mixture was made slightly alkaline with dilute sodium hydroxide and extracted 6 times with chloroform (10-ml. portions). The combined extracts were dried over anhydrous sodium sulfate overnight, filtered and the filtrate and washings concentrated under reduced pressure until crystallization ensued. Then the mixture was diluted with ethanol and kept at 5°; yield 40 mg. (37%) of white crystals, m.p. 235°, $[\alpha]^{25}D$ +8° (*c* 0.4, chloroform); X-ray powder diffraction data: 8.10[39]w[40] 7.44s(2,2), 6.28m, 5.10vw, 4.00s(1), 3.84w, 3.55vw, 3.31s(2,2), 3.03vw, 2.34m. Stacey[8] cites 235° and +7° for this substance.

Anal. Calcd. for $C_{16}H_{23}NO_{10}$: C, 49.35; H, 5.95; N, 3.60. Found: C, 49.25; H, 5.85; N, 3.70.

3-*O*-β-D-Glucopyranosyl-2-acetamido-2-deoxy-α-D-galactose Dihydrate (III).—Crystallization of the pure III fractions (1.0 g.) involved the addition of a small volume of ethanol to the dry sirup and keeping at 5°. Recrystallization was effected in the same manner; yield 0.40 g. (10% from II) of white microneedles, m.p. 155–157° (preliminary softening), $[\alpha]^{25}D$ +47 (extrapolated) → +19° (final, *c* 1.07, water); X-ray powder diffraction data: 14.3[39]w,[40] 13.0vs(1), 10.4s(2,2), 7.36vw, 6.56vw, 4.91vw, 4.59s, 4.36m, 4.14s(2,2), 4.01m, 3.88m, 3.60m, 3.33w, 2.89w, 2.79vw, 2.71vw, 2.51vw, 2.28vw, 2.17vvw, 2.14vvw, 1.90vw, 1.87vvw.

Anal. Calcd. for $C_{14}H_{23}NO_{11}\cdot 2H_2O$: C, 40.09; H, 6.97; N, 3.34; H_2O, 8.59. Found: C, 40.09; H, 7.09; N, 3.27; H_2O, 8.32; Morgan–Elson[10] test, (+).

(30) A. Chaney and M. L. Wolfrom, *Anal. Chem.*, **26**, 1614 (1956).

(31) S. A. Barker, E. J. Bourne and D. H. Whiffen, *Methods of Biochem. Anal.*, **3**, 213 (1956).

(32) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(33) W. E. Trevelyan, D. P. Proctor and J. S. Harrison, *Nature*, **166**, 444 (1950).

(34) A product of The Dow Chemical Co., Midland, Mich.

(35) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(36) A product of The West Virginia Pulp and Paper Co., Chicago, Ill.

(37) A product of Rohm and Haas Co., Resinous Products Division, Philadelphia, Pa.

(38) Identical with those of authentic β-D-glucopyranose pentaacetate. These measurements replace those of M. L. Wolfrom and H. B. Wood, THIS JOURNAL, **71**, 3175 (1949).

(39) Interplanar spacing, Å., CuK_α radiation.

(40) Relative intensity, estimated visually; s, strong; m, medium, w, weak; v, very. First three strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities.

Its infrared spectrum was strikingly similar to that of II, except for an 807 cm.$^{-1}$ peak in III, absent in II. The 886 cm.$^{-1}$ band may be due to the C_1–H absorption of β-D-glucopyranose.[31] Hydrolysis of III (5 mg.) in 3 ml. of *N* sulfuric acid and subsequent paper chromatography of the hydrolyzate showed D-glucose and 2-amino-2-deoxy-D-galactose (D-galactosamine).

To determine the alkali stability of III, an amount of 5 mg. of III was treated with 1 ml. of 0.04 *N* sodium carbonate for 2 hr. at room temperature and the mixture was analyzed paper chromatographically. Aside from III, D-glucose and a Morgan–Elson[10] reactive sugar ($R_{glucose}$ 1.8), "anhydro-*N*-acetyl-D-galactosamine,"[11] different from 2-acetamido-2-deoxy-D-galactose ($R_{glucose}$ 1.2), were detected.

An amount of 100 mg. of III in 5 ml. of 50% methanol was added in portions, with stirring, to a solution of 40 mg. of sodium borohydride in 5 ml. of 0.1 *M* borate buffer (*p*H 8) at 0°. The mixture was stirred at 0° for 2 hr., an additional hr. at room temperature and acidified to *p*H 5 with acetic acid and passed through a column (100 × 13 mm., diam.) of mixed-bed resin (Amberlite MB-3[37]). A hygroscopic sirup was obtained after carbon[38] (Nuchar C ungrounded[39]) column purification and solvent removal under reduced pressure; yield 60 mg. This product was found to be chromatographically identical, with three developers, to sirupy 3-*O*-β-D-glucopyranosyl-2-acetamido-2-deoxy-D-galactitol (IV) prepared from authentic chondrosine according to Davidson and Meyer.[4] The principal and non-reducing spot ($R_{glucose}$ 0.76) was unreactive to aniline hydrogen phthalate,[41] but was reactive to the alkaline silver nitrate[33] and (purple) to the Elson–Morgan[9,32] indicators. Traces of D-glucitol ($R_{glucose}$ 1.0) and Morgan–Elson[10] reactive "anhydro-*N*-acetyl-D-galactosaminol," ($R_{glucose}$ 1.8) also were detected, with alkaline silver nitrate,[33] in both preparations.

Besides IV and "anhydro-*N*-acetyl-D-galactosaminol,"[11] 2-acetamido-2-deoxy-D-glucitol[28] (*N*-acetyl-D-glucosaminol) also gave the characteristic purple color of the Morgan–Elson[10] reaction with the reagent.[32]

Characterization of 3-*O*-(Methyl Tri-*O*-acetyl-β-D-glucopyranosyluronate)-2-acetamido-tetra-*O*-acetyl-2-deoxy-D-galactitol (V).[42]—Substance V[17,18] gave a positive uronic acid assay[29] and a negative Elson–Morgan[9,43] reaction.

(41) S. M. Partridge, *Nature*, **164**, 443 (1949).

(42) Experimental work by Mr. J. N. Schumacher.

(43) J. W. Palmer, Elizabeth M. Smyth and K. Meyer, *J. Biol. Chem.*, **119**, 491 (1937).

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