215–218°; λ_{max} 211.4 mμ (ε 18,400); $\lambda_{max}^{CHCl_3}$ 5.72 (s), 5.86 (s), and 6.13 (w) μ; n.m.r. spectrum at τ 4.06 (multiplet, 1 vinyl proton) and 4.46 (multiplet, 1 vinyl proton).

Anal. Calcd. for $C_{22}H_{26}O_4$: C, 74.55; H, 7.39. Found: C, 74.45; H, 7.21.

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Carboxyl-Reduced Heparin. Monosaccharide Components[1]

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Commercial heparin (sodium salt) was purified through the cetyltrimethylammonium salt to a product readily convertible to the crystalline barium acid salt. The heparin polyelectrolyte molecule resists desulfation and *O*-acetylation. Partial desulfation of the so-purified heparin (sodium salt) with methanolic hydrogen chloride followed by *N*-acetylation allowed further desulfation. Subsequent acetylation converted most of the free hydroxyl groups to acetate esters. The nearly *O*-acetylated product contained two acetamido groups, two (free carboxyl) uronic acid residues, and approximately one ester acid sulfate group per tetrasaccharide unit. Reduction of the uronic acid carboxyl groups in this product with diborane gave the carboxyl-reduced analog with quantitative conversion of the uronic acid moiety to aldose. Acid hydrolysis of the carboxyl-reduced polysaccharide gave 2-amino-2-deoxy-D-glucose (isolated as the crystalline α-D-hydrochloride) and D-glucose (isolated as the crystalline penta-*O*-acetylglucose diethyl dithioacetal and previously as potassium hydrogen D-glucarate) as the only monosaccharide hydrolytic components. This establishment of D-glucose in the hydrolysate characterizes the uronic acid of heparin as D-glucuronic acid.

The polysaccharide heparin has been known for over forty years, and studies in relation to its blood anticoagulant properties have been extensive.[2] Chemical studies on heparin up to 1954 have been reviewed in detail,[3] and later work has been summarized.[4,5] Heparin has proved to be a remarkably intractable polysaccharide for chemical study. It is not amenable to many of the conventional procedures used in polysaccharide structure determination. Much inconclusive and conflicting data exists in the literature regarding the linkage sequence, and even the structural units, of the heparin molecule; a great deal of the supporting evidence presented is based on color reactions and indirect evidence. Work based on inhomogeneous preparations has added considerably to the confusion regarding precise chemical structure.

In this paper and the following papers from this laboratory we have endeavored to base our studies on well-purified material and to draw conclusions on classical proofs of structure, with isolation of crystalline degradation products identified by unequivocal methods.

The starting material we have used was a commercial heparin (sodium salt) of normal anticoagulant activity which we further purified by fractionation from salt solutions of the cetyltrimethylammonium salts of the sulfated polysaccharides present.[6,7] This fractionation was effected with considerable loss (about 50%), but that material with the highest degree of sulfation present in the commercial heparin was so obtained. Undoubtedly the yield could have been raised, but we wished to concentrate our efforts upon this fraction. That the mast cell, wherein heparin originates, contains a mixture[8] of polysaccharides cannot be doubted. We ourselves have described a galactan from this source[9] and dermatan sulfate (β-heparin) also has been found therein.[10]

The purified heparin was readily convertible to the crystalline polymeric barium acid salt first described by Charles and Scott,[11] which has been adequately characterized by chemical and physical methods.[12–14] The infrared spectrum of the purified polysaccharide (sodium salt) was identical with that described by Burson and co-workers[15] for "purified heparin." Our purified preparation contains five sulfate ester groups per tetrasaccharide unit[12,16] and even so undoubtedly contains some free amino groups from which the sulfuric acid residue has been removed by the mild acidity to which the material has been exposed during isolation and purification. It is known that the sulfoamino group of heparin is sensitive to acidity.[12,17,18] In the intact polysaccharide the anticoagulant activity is a function, among other things, of its degree of sulfation,[17] and indeed extrapolation of this activity to complete sulfation of the amino group leads to a predictable maximum activity of 190 I.U./mg. Whether such

(1) Preliminary communication: M. L. Wolfrom, J. R. Vercellotti, and G. H. S. Thomas, *J. Org. Chem.*, **26**, 2160 (1961).

(2) J. E. Jorpes, "Heparin," 2nd Ed., Oxford University Press, London, 1946.

(3) A. B. Foster and A. J. Huggard, *Advan. Carbohydrate Chem.*, **10**, 335 (1955).

(4) M. L. Wolfrom, "Heparin and Related Substances," in "Polysaccharides in Biology, Transactions of the Fourth Conference, May 21–23, 1958, Princeton, N. J.," G. F. Springer, Ed., Josiah Macy, Jr., Foundation, New York, N. Y., 1960, p. 115.

(5) R. W. Jeanloz in "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, Ed., Elsevier Publishing Co., Amsterdam, 1963, p. 289.

(6) B. C. Bera, A. B. Foster, and M. Stacey, *J. Chem. Soc.*, 3788 (1955).

(7) J. E. Scott, *Chem. Ind.* (London), 168 (1955); *Methods Biochem. Analy.*, **8**, 146 (1960).

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(9) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, and O. Keller, *Arch. Biochem.*, **14**, 1 (1947); M. L. Wolfrom, G. Sutherland, and M. Schlamowitz, *J. Am. Chem. Soc.*, **74**, 4883 (1952).

(10) R. Marbet and A. Winterstein, *Helv. Chim. Acta*, **34**, 2311 (1951).

(11) A. F. Charles and D. A. Scott, *Biochem. J.*, **30**, 1927 (1936).

(12) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeely, and J. McLean, *J. Am. Chem. Soc.*, **65**, 2077 (1943).

(13) M. L. Wolfrom and F. A. H. Rice, *ibid.*, **69**, 2918 (1947).

(14) M. L. Wolfrom, R. K. Madison, and M. J. Cron, *ibid.*, **74**, 1491 (1952), footnote 20.

(15) S. L. Burson, Jr., M. J. Fahrenbach, L. H. Frommhagan, B. A. Riccardi, R. A. Brown, J. A. Brockman, H. V. Lewry, and E. L. R. Stokstad, *ibid.*, **78**, 5874 (1956).

(16) M. L. Wolfrom, R. Montgomery, J. V. Karabinos, and P. Rathgeb, *ibid.*, **72**, 5796 (1950).

(17) M. L. Wolfrom and W. H. McNeely, *ibid.*, **67**, 748 (1945).

(18) M. L. Wolfrom, T. M. Shen, and C. G. Summers, *ibid.*, **75**, 1519 (1953); R. A. Gibbons and M. L. Wolfrom, *Arch. Biochem. Biophys.*, **98**, 374 (1962).

highly active material is a trisulfate (per disaccharide unit; hexasulfate per tetrasaccharide) is not yet certain, though Jorpes[19,20] tends to favor such a concept.

The high degree of sulfation of the polyelectrolyte heparin profoundly modifies its reactivity and solubility; removal of these sulfate groups is accordingly necessary before the linkages become amenable to investigation. Heparin has been completely desulfated[21] by solution in absolute sulfuric acid followed by acetylation. The product isolated was, however, considerably degraded. A much milder method for the desulfation of sulfated polysaccharides became available when Kantor and Schubert[22] reported that chondroitin 4-sulfate could be desulfated heterogeneously by methanolic hydrogen chloride at room temperature. Soon thereafter this laboratory[1] and a number of others[23-25] found this to be applicable to heparin preparations. The direct treatment with methanolic hydrogen chloride reduced the sulfate content to approximately two groups per tetrasaccharide unit. Further desulfation was possible, after *N*-acetylation, but it was still difficult to desulfate the material completely. This was in accordance with the previous finding of this laboratory that the heparin molecule contains acid-resistant sulfate.[16,26] Complete desulfation could be achieved[27] if the further acetylated product, described subsequently, was desulfated homogeneously with methanolic hydrogen chloride.

In the present work we elected to proceed with a desulfated, *N*-acetylated heparin preparation still containing essentially one acid-resistant sulfate acid ester group per tetrasaccharide unit. The carboxyl unit in the uronic acid component had then been converted to the methyl ester by the desulfation procedure, and any reducing end groups had been changed to the methyl glycoside. This product was then sufficiently soluble in organic solvents to permit further acetylation with acetic anhydride in a homogeneous pyridine–*N*,*N*-dimethylformamide solution. Such a procedure, but employing formamide,[28] readily and completely acetylates chondroitin 4-sulfate.[29] Surprisingly, acetylation of the modified heparin stopped at a point where 1.6 hydroxyl groups per tetrasaccharide unit remained unacetylated, and repetition of the procedure did not raise the acetyl value. As was found with pectin,[28] the methyl ester present could become deesterified by a transesterification. The acetylated free acid was soluble in water, formamide, *N*,*N*-dimethylformamide, and in mixtures of the last-named with bis(2-methoxyethyl) ether (diglyme), a range of solubilities similar to those shown by peracetylated chondroitin 4-sulfate.[29]

One of the principal difficulties in the elucidation of the structure of heparin arises from the fact that the conditions required for complete hydrolysis destroy the uronic acid component.[30] This results from the inherent acid sensitivity of uronic acids and the difficulty of hydrolyzing glycosidic bonds in sugars containing amino groups, due undoubtedly to the shielding effect exercised by the positively charged monoalkylammonium ion toward protonation of the glycosidic oxygen atom.[31,32] The remarkable reducing agent diborane, introduced into organic chemistry by Brown and Subba Rao,[33] will reduce the carboxyl group to a primary alcohol. This reagent had been utilized for carboxyl reduction of plant acidic polysaccharides by Smith and Stephen[34] and was utilized herein once a suitable derivative possessing diglyme solubility had been obtained. The uronic acid component was thus converted quantitatively to the much more acid-stable aldohexose and the polysaccharide, so modified, was now amenable to complete acid hydrolysis. Sodium borohydride readily reduces the carboxyl function in the methyl ester form of chondroitin (desulfated),[35] but it is much less effective (40% reduction in our hands) for the corresponding derivative of heparin.[36-38]

Complete acid hydrolysis of the carboxyl-reduced heparin modification led to the isolation and characterization of only two components in an approximately equimolar ratio. One of these was 2-amino-2-deoxy-D-glucose, isolated as the crystalline hydrochloride of its α-anomer, a finding corroborative of that of Jorpes and Bergström.[39] It is of interest that comparative X-ray diffraction patterns allow the establishment of anomeric form of known substances without recourse to polarimetric data. The only other component found was glucose, isolated as the crystalline penta-*O*-acetyl glucose diethyl dithioacetal by the procedure of Wolfrom and Karabinos.[40] The amount of this derivative obtained in the experimentation was insufficient for the determination of its optical rotation. However, it previously had been ascertained[41] that potassium acid D-glucarate was obtainable from heparin by oxidative hydrolysis, a finding which limited the uronic acid component to D-glucuronic or L-glucuronic acid. The present result establishes the former and, therefore, the uronic acid component of our heparin preparation was D-glucuronic acid. This accords with the isolation from heparin of a crystalline isopropylidene derivative of

(19) See ref. 2, p. 33.

(20) J. E. Jorpes, "Heparin," Oxford University Press, London, 1939, p. 13.

(21) M. L. Wolfrom and R. Montgomery, *J. Am. Chem. Soc.*, **72**, 2859 (1950).

(22) T. G. Kantor and M. Schubert, *ibid.*, **79**, 152 (1957).

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(24) A. B. Foster, R. Harrison, T. D. Inch, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 2279 (1963).

(25) K. D. Brown, W. A. Rosenthal, and J. R. Helbert, Abstracts of Papers, 140th National Meeting of the American Chemical Society, Chicago, Ill., 1961, p. 8D.

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(27) M. L. Wolfrom, J. R. Vercellotti, H. Tomomatsu, and D. Horton, *Biochem. Biophys. Res. Commun.*, **12**, 8 (1963).

(28) J. F. Carson, Jr., and W. D. Maclay, *J. Am. Chem. Soc.*, **67**, 1015 (1946).

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(30) M. L. Wolfrom and J. V. Karabinos, *J. Am. Chem. Soc.*, **67**, 679 (1945).

(31) R. C. G. Moggridge and A. Neuberger, *J. Chem. Soc.*, 745 (1938).

(32) A. B. Foster, D. Horton, and M. Stacey, *ibid.*, 81 (1957).

(33) H. C. Brown and B. C. Subba Rao, *J. Org. Chem.*, **22**, 1135 (1956).

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(35) M. L. Wolfrom and B. O. Juliano, *J. Am. Chem. Soc.*, **82**, 1673 (1960).

(36) G. H. S. Thomas and J. R. Vercellotti, unpublished experimental results from this laboratory.

(37) J. A. Cifonelli and A. Dorfman, *Biochem. Biophys. Res. Commun.* **4**, 328 (1961).

(38) I. Danishefsky, H. B. Eiber, and E. Langholtz, *J. Biol. Chem.*, **237**, 1413 (1962).

(39) J. E. Jorpes and S. Bergström, *Z. Physiol. Chem.*, **244**, 253 (1936).

(40) M. L. Wolfrom and J. V. Karabinos, *J. Am. Chem. Soc.*, **67**, 520 (1945).

(41) M. L. Wolfrom and F. A. H. Rice, *ibid.*, **68**, 532 (1946); the experimental work in this paper has been verified by the late Dr. A. Thompson of this laboratory.

D-glucuronolactone by Foster and associates.[42] After, or nearly simultaneously with our publication,[1] the finding of D-glucose in the hydrolysate of a partially reduced (sodium borohydride) heparin preparation was reported by Danishefsky and associates[38] (β-D-glucopyranose pentaacetate) and by Cifonelli and Dorfman[37] (by D-glucose oxidase). It is true that heparin does not show a typical Dische (concentrated sulfuric acid and carbazole) glucuronic acid color test and indeed exhibits a color with concentrated sulfuric acid and anthrone similar to that given by a hexulosonic acid.[25,43,44] However, the present isolative degradation demonstrates unequivocally that D-glucuronic acid is the uronic acid component of heparin. Current reports of the presence of L-iduronic acid in heparin preparations[45] were not confirmed with the purified and completely reduced preparations utilized by us.

Experimental[46]

Purification of Heparin through the Cetyltrimethylammonium Salt.—A modification of the method of Korn[47] was used to purify the commercial (Upjohn, sodium salt) heparin samples employed; 126 I.U./mg.; $[\alpha]^{29}D$ +44° (*c* 1.0, water).

Anal. Calcd. for $C_{24}H_{31}O_{35}N_2S_5Na_7$: C, 23.45; H, 2.52; N, 2.28; S, 13.04. Found: C, 23.03; H, 2.86; N, 2.35; S, 12.62.

To an amount of 10 g. of this material dissolved in water was added, with stirring, 50 ml. of 20% (w./v.) cetyltrimethylammonium bromide (Eastman Organic Chemicals, Rochester, N. Y.). The yellowish gray precipitate formed was isolated by centrifugation, the supernatant decanted, and the precipitate washed in the centrifuge tube five times with 100-ml. portions of water. To the supernatant was added another 50 ml. of 20% (w./v.) cetyltrimethylammonium bromide, and the precipitate formed was removed by centrifugation and washed as described above. The combined wet precipitates were dried in a desiccator under reduced pressure until the surface water was removed. The lumps were then pulverized and the final drying was effected in a vacuum desiccator over phosphorus pentaoxide; yield 28.3 g. of crude cetyltrimethylammonium heparinate. This 28.3 g. of quaternary ammonium heparin complex was dissolved in 600 ml. of warm ethanol and reprecipitated by dropping the cooled ethanol solution into 2 l. of cooled ether. After centrifuging and washing the precipitate three times with ether, the white amorphous powder was dried in a vacuum desiccator; yield 22.6 g. The complex (22.6 g.) was then dissolved in 4 *M* sodium chloride (900 ml.) and stirred for 1 hr. Dilution of the solution to 1 *M* sodium chloride with 2700 ml. of water precipitated a white flocculent solid. To this solid was added, under stirring, 25 g. of Celite. The Celite and heparin complex was isolated by centrifugation and treated with 900 ml. of 4 *M* sodium chloride. A further amount of 20 ml. of 20% (w./v.) cetyltrimethylammonium bromide was added, and the solution was again made 1 *M* in sodium chloride. The reprecipitation procedure was thrice repeated. The final precipitate was washed in the centrifuge with three 100-ml. portions of water to remove excess cetyltrimethylammonium chloride. The precipitate was dissolved in 800 ml. of 4 *M* sodium chloride, centrifuged, and the supernatant dropped slowly into ethanol (5500 ml.). After centrifuging the solids from the alcohol solution and washing with 100 ml. of ethanol, the precipitate was dissolved in water (100 ml.) and dialyzed for 3 days against running distilled water. Upon concentration and freeze-drying, a white powder was isolated; yield 4.5 g. (45%); $[\alpha]^{25}D$ +46° (*c* 0.73, water); λ_{max}^{KBr} (μ) 2.85 (OH), 6.25 (carboxyl C=O), 7.0, 8.15 (SO_4 ester), 9.75, 10.15, and 11.35.

Anal. Calcd. for $C_{24}H_{31}O_{35}N_2S_5Na_7$: C, 23.45; H, 2.52; N, 2.28; S, 13.04. Found: C, 23.48; H, 2.79; N, 2.31; S, 13.01.

This material was convertible to the crystalline barium acid heparinate by the procedure of Wolfrom and associates[12] with the modification that all the solutions were maintained at room temperature or below and periods of standing were minimized; yield 71.3%. When this material was reconverted to the sodium salt[12] (above-noted precautions followed), the product was identical in rotation and infrared spectrum with the above-described sodium salt prepared through the cetyltrimethylammonium salt alone. The infrared spectrum obtained was in agreement with that recorded by Burson and co-workers[15] for "purified heparin."

Partially Desulfated, *N*-Acetylated Heparin Methyl Ester.—The sodium heparinate, purified as described above, was desulfated to the monosulfate stage by the general procedure of Kantor and Schubert[22] for the complete desulfation of chondroitin sulfate and essentially as described by Danishefsky, Eiber, and Carr[23] for their heparin preparation. An amount of 3.0 g. of the sodium heparinate was shaken for 20 hr. with 0.15 *N* dry methanolic hydrogen chloride (200 ml.). The mixture was centrifuged, the supernatant discarded, and the precipitate treated with another portion of 0.15 *N* methanolic hydrogen chloride (200 ml.) for 2 days. The insoluble, partially desulfated heparin methyl ester was collected by centrifuging, washing twice with methanol (50 ml.) and once with ether in the centrifuge tube, and air-drying; yield 2.1 g.

Anal. Found: S, 5.01.

The partially desulfated heparin methyl ester was *N*-acetylated by the general procedure of Roseman and Ludowieg.[48] An amount of 2.1 g. was dissolved in water (150 ml.) to which was added methanol (15 ml.) and Dowex-1 resin (90 ml., $-CO_3^{-2}$ form). This solution was cooled to 0–5°, under stirring, and acetic anhydride (10 ml.) added. After stirring for 3 hr. the resin was filtered and washed several times on the filter with water. The filtrate was dialyzed 3 days against running distilled water, and the dialyzate was concentrated to a small volume and freeze-dried; yield 2.0 g.; λ_{max}^{KBr} (μ) 6.04, 6.54 ($-NHCOCH_3$), and 5.78 ($-CO_2CH_3$).

Anal. Found: S, 5.14; H_3CCO, 5.3.

The methanolysis with 0.15 *N* methanolic hydrogen chloride was repeated, for 1 day, and the product was isolated in the same manner; yield 1.8 g. (90%); $[\alpha]^{25}D$ +66° (*c* 0.8, water).

Anal. Calcd. for $C_{22}H_{28.9}O_{15.3}(NHCOCH_3)_2(CO_2CH_3)_2(OSO_2Na)_{1.1}$: S, 3.98. Found: S, 3.8.

In subsequent experiments it was found that a nearly desulfated product could be obtained by repeating the methanolysis on the *N*-acetylated product several times; $[\alpha]^{25}D$ +66° (*c* 0.5, water).

Anal. Found: C, 39.55; H, 5.39; N, 3.21; S, 0.2; Na (sulfate ash), 0.1.

Partially Desulfated, Partially Acetylated Heparin, Sodium Salt.—The partially desulfated *N*-acetylated methyl ester of heparin (1.0 g.) was dissolved in water and freeze-dried to give a fluffy white powder. This powder was placed in *N*,*N*-dimethylformamide (50 ml.), to which was added 16 ml. of freshly distilled acetic anhydride and 8 ml. of pyridine.[29] The heterogeneous mixture was shaken overnight, and the solid material became solubilized. More pyridine (8 ml.) and acetic anhydride (16 ml.) was then added. After shaking overnight again, the reaction mixture was poured into 250 ml. of 0.001 *N* sodium bicarbonate, dialyzed against running distilled water for 2 days, and freeze-dried; yield 0.9 g.; $[\alpha]^{24}D$ +15° (*c* 1, water); nonreducing to Benedict solution. This product was soluble in water, form-

(42) A. B. Foster, A. H. Olaveson, M. Stacey, and J. M. Webber, *Chem. Ind.* (London), 143 (1961).

(43) J. R. Helbert and K. D. Brown, Abstracts of Papers, 139th National Meeting of the American Chemical Society, St. Louis, Mo., 1961, p. 13D.

(44) *J. R. Helbert and K. D. Brown, Anal. Chem.*, **33**, *1610* (1961).

(45) J. A. Cifonelli and A. Dorfman, *Biochem. Biophys. Res. Commun.* **7**, 41 (1962).

(46) Paper chromatography was carried out using the descending technique with the top layer of a 4:1:5 1-butanol–ethanol–water system (solvent A), 5:5:3:1 pyridine–ethyl acetate–water–acetic acid [solvent B according to F. G. Fischer and H. J. Nebel, *Z. Physiol. Chem.*, **302**, 10 (1955)], and 9:2:2 ethyl acetate–acetic acid–water (solvent C). Zones were located by the silver nitrate–sodium hydroxide procedure [W. E. Trevelyan, D. P. Proctor, and J. S. Harrison, *Nature* (London), **166**, 444 (1950)] as well as by ninhydrin (0.2% in ethanol). R_g is mobility relative to that of glucose. Melting points were determined on a Hershberg type apparatus (A. Thompson and M. L. Wolfrom, in "Methods in Carbohydrate Chemistry," Vol. I, R. L. Whistler and M. L. Wolfrom, Ed., Academic Press, Inc., New York, N. Y., 1961, p. 517).

X-ray diffraction pattern data refer to interplanar spacing in Å. with Cu K_α radiation. Relative intensities were estimated visually: s, strong; m, medium; w, weak; v, very. The first three strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities. All acetyl determinations were done by the method of A. Chaney and M. L. Wolfrom, *Anal. Chem.*, **28**, 1614 (1956). Combustion analyses on the sodium salts were made by the Huffman Microanalytical Laboratories, Wheatridge, Colo.

(47) E. Korn, *J. Biol. Chem.*, **234**, 1325 (1959).

(48) S. Roseman and J. Ludowieg, *J. Am. Chem. Soc.*, **76**, 301 (1954).

amide, *N*,*N*-dimethylformamide, 2-methoxyethanol, and bis(2-methoxyethyl) ether (diglyme)–*N*,*N*-dimethylformamide (4:1).

Anal. Calcd. for $C_{22}H_{24}O_4(NHCOCH_3)_2(CO_2Na)_2(OCOCH_3)_{4.3}(OH)_{1.6}(OSO_2Na)_{1.1}$: C, 39.58; H, 4.31; CH_3CO, 24.98; Na, 6.4. Found: C, 39.28; H, 4.55; CH_3CO, 25.14; Na (sulfate ash), 5.7; OCH_3, trace.

The sodium bicarbonate treatment was omitted when the sodium salt was not desired.

The acetylation procedure was repeated on this product with no significant change in acetyl content.

Methoxyl content[49] varied in subsequent experiments from trace amounts to 2%, indicating the probable presence of some methyl ester.

Diborane Reduction of Partially Desulfated, Partially Acetylated Heparin.—A water solution of partially desulfated, partially acetylated heparin (1.6 g., sodium salt) was passed through a column of Amberlite IR-120 resin (H^+, 100 ml.), concentrated to a small volume, and freeze-dried; yield 1.5 g. This free-carboxyl form of the polysaccharide (1.5 g., 2.6 mequiv.) was dissolved in *N*,*N*-dimethylformamide (40 ml.) contained in a 500-ml., three-necked, round-bottomed flask equipped with ground glass joints. Bis(2-methoxyethyl) ether (160 ml.) was then added slowly with stirring. Sodium borohydride (1.66 g., 43.6 mmoles) was added to this solution and the reaction mixture stirred with a magnetic stirring bar under a stream of dried (sulfuric acid) nitrogen. By means of a dropping funnel, freshly distilled boron trifluoride etherate (8.74 g., 66 mmoles) in bis(2-methoxyethyl) ether (20 ml.) was introduced over 3 hr. into the reaction mixture and stirring was maintained overnight. The reaction mixture, containing a slurry of inorganic salts, was poured with stirring into 0.1 *N* sodium bicarbonate (150 ml.) and crushed ice (200 ml.). The washings were dialyzed for 3 days and the concentrated dialysate was freeze-dried; yield 1.06 g.; $[\alpha]^{25}D$ +53° (*c* 1, water).

Anal. Calcd. for $C_{24}H_{28}O_8(NHCOCH_3)_2(OCOCH_3)_{2.5}(OH)_{6.6}(OSO_2Na)_{0.9}$: C, 43.4; H, 5.5; N, 3.1; S, 3.2; CH_3CO, 21.12; Na, 2.26. Found: C, 43.7; H, 5.7; N, 3.6; S, 3.5; CH_3CO, 20.8; Na (sulfate ash), 1.6; uronic acid,[50] trace; OCH_3, trace.

Total Hydrolysis of Partially Desulfated, Partially Acetylated, Carboxyl-Reduced Heparin.—The product from the preceding reaction (3.1 g.) was hydrolyzed in 4 *N* hydrochloric acid (310 ml.) for 7 hr. at reflux. The solution was neutralized with silver carbonate, filtered, and concentrated to a small volume. The precipitate which formed was filtered; the filtrate was treated with hydrogen sulfide, filtered again, and evaporated several times to dryness with 1-propanol to give a sirup; yield 1.2 g. Chromatography with solvent A revealed the reducing sugar components of R_g 1.00 and 0.72 to be the principal substances present, the latter being ninhydrin-positive; accompanying minor zones had low mobilities. Resolution of the sirup was effected by preparative paper chromatography. In order to provide a more even solvent flow, 10 × 46 cm. strips of Whatman No. 1 paper were sewn[51] to the ends of 46 × 57 cm. sheets of Whatman No. 3 paper. After applying neutralized hydrolysate to the thick paper (200 mg. per sheet on a 0.5-cm. band 3 cm. below the stitching), the strip of Whatman No. 1 paper was placed in the empty irrigating trough and solvent A poured in. Zones corresponding to the R_g values cited above were located by cutting 1-cm. strips from the center and edges of the sheet and spraying with silver nitrate–sodium hydroxide.

Elution of the material in the zone of R_g 0.72 was effected with solvent A, and the resultant sirup obtained on solvent removal was crystallized from water–acetone to give 2-amino-2-deoxy-α-D-glucose hydrochloride; yield 0.41 g. (34%) based on weight of hydrolysate; $[\alpha]^{24}D$ +72° (*c* 0.44, water, final); X-ray powder diffraction pattern[52] identical with that of an authentic specimen.

The sirupy material in the zone of R_g 1.00 was isolated in the same manner; yield 0.301 g. (25% based on weight of hydrolysate). It was found to be chromatographically indistinguishable from glucose in solvents A, B, and C. A 40-mg. sample of this sirup was dissolved in concentrated hydrochloric acid (0.5 ml., 12 *N*), cooled in an ice bath, and ethanethiol (0.5 ml.) added.[40] The mixture was shaken for 1 hr. and then neutralized in the cold with concentrated ammonium hydroxide. The salt residue was dried by codistillation at 40° with ethanol under reduced pressure, the process being repeated several times. The dried residue was treated with a 2:1 mixture of acetic anhydride and pyridine (3 ml.). After standing overnight at room temperature, the solution was poured into water (10 ml.) and extracted twice with 10-ml. portions of chloroform. The chloroform extract was washed four times with 10-ml. portions of a saturated sodium bicarbonate solution and finally with water. The sirup obtained on evaporation of the solvent was crystallized from methanol by the gradual addition of water; yield 10 mg. (16%); m.p. 45.8–47°, unchanged on admixture with an authentic sample of penta-*O*-acetyl-D-glucose diethyl dithioacetal[53]; X-ray powder diffraction pattern identical with that of an authentic sample[46]: 9.023 (vs) (1), 7.654 (m), 7.158 (s) (3), 6.992 (m), 6.036 (m), 5.367 (w), 4.178 (s) (2), 3.721 (m), 3.556 (w), 3.309 (m), 3.089 (w), 2.916 (m), 2.710 (vw), 2.576 (w), 2.300 (vw).

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