Two Disaccharides from Carboxyl-Reduced Heparin. The Linkage Sequence in Heparin[1]

M. L. WOLFROM, J. R. VERCELLOTTI, AND D. HORTON

Department of Chemistry, The Ohio State University, Columbus 10, Ohio

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Graded acid hydrolysis of partially *O*-acetylated, partially desulfated, completely carboxyl-reduced, and *N*-acetylated heparin, under optimal yield conditions, afforded two crystalline amino sugar-containing disaccharides, isolated from the hydrolysate by preparative paper chromatographic techniques. The unit sequence in each disaccharide was proved by hydrolysis of the corresponding alditols, with isolation of the fragments. Evidence for the linkage positions and configuration was provided by color reactions, electrophoretic data, and infrared spectral and molecular rotatory data on the disaccharides, their *N*-acetyl and alditol derivatives. Methylation analysis, with isolation of crystalline partially methylated hydrolytic fragments, provided unequivocal confirmatory proof for formulation of the disaccharides, as *O*-α-D-glucopyranosyl-(1 → 4)-2-amino-2-deoxy-α-D-glucopyranose hydrochloride (I) and *O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose hydrochloride (II). Juxtaposition of the structures of the two disaccharides demonstrates heparin to possess an α-D-(1 → 4)-linked backbone structure of 2-amino-2-deoxy-D-glucose and D-glucuronic acid units, very probably in an alternating sequence.

As noted in our preceding paper,[2] the elucidation of the structure of heparin has been beset with many difficulties which have now been largely overcome by the preparation, described therein,[2] of a completely carboxyl-reduced heparin. The modified heparin was obtained from a highly purified product which readily formed the crystalline barium acid heparinate of Charles and Scott[3] in high yield. The modified heparin was obtained[2] in the form of a partially desulfated, completely *N*-acetylated, partially *O*-acetylated, completely carboxyl-reduced polymer. This product has been herein subjected to partial acid hydrolysis and the rate of release of disaccharide and monosaccharide units studied by paper chromatographic methods (Table I). The rate of release of total reducing sugar is plotted in Fig. 1 (upper curve). This curve shows a plateau followed by a maximum. The plateau represents the point of maximum disaaccharide formation (Table I), and the maximum represents the initiation of gross monosaccharide destruction by acidity. The qualitative paper chromatographic data of Table I indicate the probable formation, from the *N*-acetylated heparin preparation, of two disaccharides of R_g 0.32 and R_g 0.27 in the solvent systems employed. In addition, a zone, uninvestigated, of R_g 0.13 is present and probably represents a higher oligosaccharide unit. From the *N*-acetylated preparation, the disaccharide of R_g 0.32 was present in the greatest amount. The *N*-acetylated preparation was then largely *N*-deacetylated by hydrazine[4] and the hydrolytic characteristics again determined. The rate of release of reducing sugar (Fig. 1, lower curve) paralleled that of the *N*-acetylated, carboxyl-reduced heparin (upper curve), but the data show that the *N*-deacetylated product was somewhat more resistant to hydrolysis. With the largely *N*-deacetylated preparation, the data of Table I show that the disaccharide of R_g 0.27 is preponderant over the other. Such a differential disaccharide break is in accordance

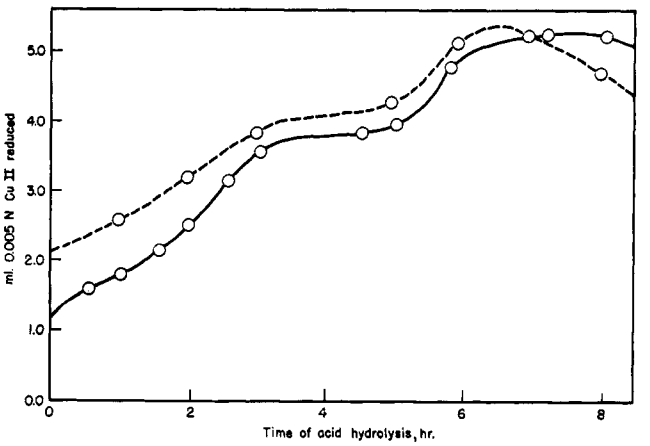

Fig. 1.—Rate of release of reducing sugar (Somogyi[46] method): ----, *N*-acetylated, carboxyl-reduced heparin; ——, *N*-deacetylated, carboxyl-reduced heparin.

with the studies made previously on simpler amino sugars containing the charged RNH_3^+ entity and the initially uncharged acetamido function.[5,6] It is known[5-7] that, for simple pyranosides of the same anomeric configuration, the 2-acetamido-2-deoxy-D-glucopyranosides are hydrolyzed about four times as rapidly as D-glucopyranosides, and about 100 times as rapidly as 2-amino-2-deoxy-D-glucopyranosides. The presence or absence of an *N*-acetyl substituent should, therefore, determine whether the amino sugar–aldose or aldose–amino sugar link is the preferred point of cleavage.

The hydrolytic component with R_g 0.32 was isolated by preparative paper chromatographic techniques and crystallized as the hydrochloride. X-ray powder diffraction pattern attested to the crystallinity of this substance, and chromatographic homogeneity in three solvent systems gave reasonable evidence of purity. The melting range and decomposition point of the compound were well defined. Qualitative tests showed this compound to contain chlorine, amino nitrogen, and the ability to reduce copper in the Benedict test. Hydrolysis with dilute acid gave two components, 2-amino-2-deoxy-D-glucose and D-glucose, in approximately equal amount by qualitative paper chromatographic estimation. The disaccharide showed downward mutarota-

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(2) M. L. Wolfrom, J. R. Vercellotti, and G. H. S. Thomas, *J. Org. Chem.*, **29**, 536 (1964).

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

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

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

(7) E. A. Moelwyn-Hughes, *Trans. Faraday Soc.*, **25**, 81, 503 (1929).

TABLE I

ZONE MAXIMA IN PAPER CHROMATOGRAPHIC ESTIMATION OF HYDROLYTIC PRODUCTS OF HEPARIN MODIFICATIONS

	N-Acetyl derivative		Hydrazine-treated derivative	
R_g (solvent A)[a]	Time to attain max.,[b] hr.	Relative intensity at max.	Time to attain max.,[b] hr.	Relative intensity at max.
1.00[c]	6.0	+++	6.0	+++
0.70[d]	6.0	++++	6.0	++++
0.32[e]	4.5	++	2.5	+
0.27[f]	4.0	+	2.5	++
0.13	3.5	+	1.2	+

[a] Reference 45. [b] Maximum formation. [c] D-Glucose. [d] 2-Amino-2-deoxy-D-glucose hydrochloride. [e] Shown herein to be O-α-D-glucopyranosyl-(1 → 4)-2-amino-2-deoxy-α-D-glucose hydrochloride (I). [f] Shown herein to be O-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1 → 4)-D-glucose hydrochloride (II).

tion (+100 → +81°). The compound contained 3.9% nitrogen, close to the calculated value.

The free amino group in this substance was *N*-acetylated; the crystallinity of the product was shown by its X-ray powder diffraction pattern. This *N*-acetylated product was chromatographically homogeneous in three solvent systems, and it showed downward mutarotation (+89 → +37°). The molecular weight of this acetamido derivative, determined by isothermal distillation,[8] was shown to conform to a disaccharide. Elemental analyses were in agreement with a disaccharide structure composed of 2-acetamido-2-deoxy-D-glucose and D-glucose units.

Reduction of the anomeric carbon atom with borohydride gave a sirupy, chromatographically homogeneous alditol derivative with a specific rotation of +75°. Hydrolysis yielded two equal components, by qualitative paper chromatography, corresponding to 2-amino-2-deoxy-D-glucitol hydrochloride and D-glucose. The 2-amino-2-deoxy-D-glucitol hydrochloride was isolated crystalline, and its X-ray powder diffraction pattern was shown to be identical with that of an authentic sample. This establishes that the amino sugar comprises the reducing end of the disaccharide.

The exact nature of the glycosidic linkage in the D-glucose-2-amino-2-deoxy-D-glucose disaccharide was investigated in several ways. 2-Amino-2-deoxyhexoses admit analytically quantitative colorimetric determination as the free amino sugar (Elson–Morgan determination)[9,10] and as the *N*-acetyl derivative (Morgan–Elson determination).[11,12] A review[13] which discusses these reactions and their use in structure determination has been published.

The Elson–Morgan determination involves formation of colorless chromogens by the alkaline condensation of 2,4-pentanedione with the aldehyde and amino functions of 2-amino-2-deoxyaldohexoses, and subsequent elimination of substituents to form pyrrole derivatives,[14] which react with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) to produce strongly colored final products, which are determined spectrophotometrically or colorimetrically. The color intensity developed by 2-amino-2-deoxy-D-glucose in the determination is slightly depressed by methylation at C-6, while methylation at C-4 depresses color formation by approximately one-half.[6] Methylation at C-3 causes a shift in the absorption band, with enhancement of color intensity at the absorption maximum (Table II[15]).[6] The disaccharide under discussion, which bears an amino sugar as the reducing end, gave approximately one-half of the color intensity given by an equivalent amount of 2-amino-2-deoxy-D-glucose, without shift in the color absorption maximum, indicating probable substitution of the amino sugar in the 4-position and, therefore, a (1 → 4) linkage in the disaccharide.

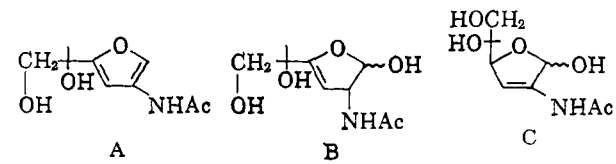
TABLE II

RELATIVE COLOR INTENSITIES OF MONOMETHYL ETHERS OF 2-AMINO-2-DEOXY-D-GLUCOSE IN THE ELSON–MORGAN DETERMINATION[a]

Compound	Relative optical density at 515 mμ
2-Amino-2-deoxy-D-glucose hydrochloride	1.00
2-Amino-2-deoxy-3-O-methyl-D-glucose hydrochloride	0.66[b]
2-Amino-2-deoxy-4-O-methyl-D-glucose hydrochloride	0.58
2-Amino-2-deoxy-6-O-methyl-D-glucose hydrochloride	0.80

[a] See ref. 6 and 15. [b] Maximal relative optical density 1.37 at 503 mμ (ref. 15).

The Morgan–Elson determination involves treatment of 2-acetamido-2-deoxyaldohexose derivatives with dilute alkali at 100°, followed by addition of acidic *p*-dimethylaminobenzaldehyde. A detailed study of the reaction has been made by Kuhn and associates[16–19] and reviewed by Foster and Horton.[13] In the first stage, chromogens are formed, by elimination from the furanose ring form, with the probable structures[19] A, B, and C, which interact with the Ehrlich reagent in the second stage to produce red-violet-colored products. Color production in the determination is little affected



by substitution of the acetamido sugar at C-6, but an alkali-stable substituent at C-4 prevents formation of the furanose ring and thus inhibits color production.[19]

(8) C. E. Childs, *Anal. Chem.*, **26**, 1963 (1954).
(9) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).
(10) R. Belcher, A. J. Nutten, and C. M. Sambrook, *Analyst*, **79**, 201 (1954).
(11) W. T. J. Morgan and L. A. Elson, *Biochem. J.*, **28**, 988 (1934).
(12) D. Aminoff, W. T. J. Morgan, and W. M. Watkins, *ibid.*, **51**, 379 (1952).
(13) A. B. Foster and D. Horton, *Advan. Carbohydrate Chem.*, **14**, 213 (1959).
(14) J. W. Cornforth and M. E. Firth, *J. Chem. Soc.*, 1091 (1958).
(15) D. Horton, Ph.D. dissertation, University of Birmingham, Birmingham, England, 1957.
(16) R. Kuhn and W. Kirschenlohr, *Ber.*, **87**, 384 (1954).
(17) R. Kuhn, A. Gauhe, and H. H. Baer, *ibid.*, **87**, 289, 1138 (1954).
(18) R. Kuhn and A. Gauhe, *ibid.*, **87**, 1553 (1954).
(19) R. Kuhn and G. Krüger, *ibid.*, **89**, 1473 (1956); **90**, 264 (1957).

A substituent at C-3 facilitates elimination and chromogen formation and, thereby, leads to enhanced color production.[17,18] The *N*-acetyl derivative of the disaccharide in question gave no detectable color in the Morgan–Elson analytical determination,[12] an observation which clearly supports assignment of a (1 → 4) glycosidic linkage.

The Elson–Morgan determination on the borohydride-reduced, hydrolyzed disaccharide gave no detectable color. This provides further evidence for location of the amino sugar at the reducing end of the disaccharide, since amino sugar alditols are known to give no reaction in the Elson–Morgan determination.[20]

Electrophoretic mobility of polyhydroxy compounds in a molybdate buffer depends on the formation of tridentate complexes with the molybdate anion in a solution sufficiently acidic to permit dissociation.[21,22] In the case of disaccharide alditols, the availability of three hydroxyl groups on the alditol residue, suitably oriented for molybdate complexing, is dependent on alditol configuration and on the position of substitution by the glycosyl residue[21,22]; these factors consequently influence the electrophoretic mobility of the substance. Table III summarizes data of Bourne

TABLE III

IONOPHORETIC MOBILITY OF VARIOUS ALDITOL DERIVATIVES IN BUFFERED (pH 5) MOLYBDATE SOLUTION[21]

Compound	Linkage	Relative mobility
D-Glucitol		1.0
Sophoritol	β-D-(1 → 2)	0.9
Nigeritol	α-D-(1 → 3)	0.0
Laminaribiitol	β-D-(1 → 3)	0.0
Maltitol	α-D-(1 → 4)	0.4
Cellobiitol	β-D-(1 → 4)	0.4
Isomaltitol	α-D-(1 → 6)	0.8
Gentiobiitol	β-D-(1 → 6)	0.8
Melibiitol	α-D-(1 → 6)	0.8

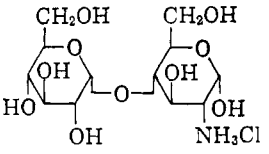
and co-workers[21] on various substituted D-glucitol derivatives and demonstrates the clear-cut differences in electrophoretic mobility according to position of substitution. 2-Acetamido-2-deoxy-D-glucitol showed an electrophoretic mobility[23] of approximately 0.9, closely similar to that of D-glucitol (1.0), while the *N*-acetylated alditol of the disaccharide showed a mobility of 0.38, a value in the same mobility range as other (1 → 4)-linked disaccharide alditols (Table III).

The colorimetric and electrophoretic data all strongly support the (1 → 4)-linked structure for the disaccharide. To confirm this unequivocally on a crystalline basis, the *N*-acetyl derivative was methylated and hydrolyzed. Though methylation was incomplete and perhaps some *O*-methyl ether was cleaved during hydrolysis, the crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose and 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose (as its crystalline *N*-acetyl derivative) were isolated. The definitive characterization of these compounds was made through physical constants as recorded in the Experimental section, X-ray powder diffraction pattern comparisons with authentic samples being the most definitive of the physical methods used.

The foregoing data firmly establish the D-glucopyranosyl-(1 → 4)-2-amino-2-deoxy-D-glucose linkage for the disaccharide of R_g 0.32. An α-D-glycosidic linkage was assigned on the basis of the high positive molecular rotation of the alditol derivative of the disaccharide. Table IV[24–31] lists the molecular rotations of analogous α(and β)-D-linked disaccharides and the corresponding alditols, with the D-*gluco* configuration in each moiety. The difference between the α-D-linked and β-D-linked disaccharides is clearly evident, particularly in the case of the alditols, since, in the latter, the rotatory contribution of the D-glucitol moiety is small; the molecular rotations of α-D-linked disaccharide alditols lie between $[M]_D$ +30,000 and +35,000° while those of the β-D analogs lie in the range −10,000 to −3000°. The *N*-acetylalditol derivative of our disaccharide has $[M]_D$ +28,500° which indicates an α-D interglycosidic link. Molecular rotatory data of disaccharide alditols have been used in other instances[27] for disaccharide structure assignments.

An α-D anomeric configuration of the reducing portion of the disaccharide was assigned on the basis of downward mutarotation as well as on the presence of an infrared absorption maximum at about 11.6 μ in both the free amino compound and the *N*-acetyl derivative,[32] indicative of an equatorial hydrogen atom on one or both anomeric carbon atoms.

The structure assigned to the disaccharide of R_g 0.32 (Table I) is, therefore, that shown.



I

A previous report of a compound purported to have the structure I has been made by Selinger and Schramm.[33] Hoffman and Meyer[34] called attention to the fact that Selinger and Schramm's substance was reported to have a considerably higher positive optical rotation ($[\alpha]_D$ +147° at equilibrium) than the disaccharide ($[\alpha]_D$ +81° at equilibrium) described above. Selinger and Schramm had presented no elemental analytical data, proof of crystallinity, or rigorous proof of structure. A sample of the original preparation of this substance, received from Dr. Schramm, was found by us to be amorphous, inhomogeneous, and contaminated with sodium chloride. The optical rotation of this enzymically synthesized disaccharide must be redetermined on a well-purified sample.

(20) D. Horton, J. R. Vercellotti, and M. L. Wolfrom, *Biochem. Biophys. Acta*, **50**, 358 (1961).

(21) E. J. Bourne, D. H. Hutson, and H. Weigel, *Chem. Ind.* (London), 1047 (1959); *J. Chem. Soc.*, 4252 (1960); 35 (1961).

(22) H. Weigel, *Advan. Carbohydrate Chem.*, **18**, 61 (1963).

(23) This work was suggested by Dr. David H. Hutson while working in this laboratory. Dr. Hutson did the preliminary electrophoresis work on 2-acetamido-2-deoxy-D-glucitol.

(24) M. L. Wolfrom and T. S. Gardner, *J. Am. Chem. Soc.*, **62**, 2553 (1940).

(25) P. Karrer and J. Büchi, *Helv. Chim. Acta*, **20**, 86 (1937).

(26) M. L. Wolfrom, A. Thompson, A. N. O'Neill, and T. T. Galkowski, *J. Am. Chem. Soc.*, **74**, 1062 (1952).

(27) A. B. Foster and D. Horton, *J. Chem. Soc.*, 1890 (1958).

(28) R. Kuhn, F. Zilliken, and A. Gauhe, *Ber.*, **86**, 466 (1953).

(29) M. L. Wolfrom and D. L. Fields, *Tappi*, **41**, 204 (1958).

(30) P. Karrer and J. Meyer, *Helv. Chim. Acta*, **20**, 626 (1937).

(31) M. L. Wolfrom and T. S. Gardner, *J. Am. Chem. Soc.*, **65**, 750 (1943).

(32) S. A. Barker, E. J. Bourne, and D. H. Whiffen in "Methods of Biochemical Analysis," Vol. III, D. Glick, Ed., Interscience Publishers, Inc., New York, N. Y., 1956, p. 213.

(33) Z. Selinger and M. Schramm, *J. Biol. Chem.*, **236**, 2183 (1961).

(34) P. Hoffman and K. Meyer, *Federation Proc.*, **21**, 1064 (1962).

TABLE IV

COMPARISON OF [M]D VALUES OF DISACCHARIDES AND DISACCHARIDE ALDITOLS

Parent compound	Linkage	Aldose[a] [M]D, deg.[b]	Alditol [M]D, deg.[b]	Reference
Maltose	α-D-(1 → 4)	+45,000	+35,200	24
			+31,100	25
Isomaltose	α-D-(1 → 6)	+35,700	+30,800	26
O-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-glucose	α-D-(1 → 6)	+53,000	+32,800	27
Disaccharide[c] of R_g 0.32		+30,800	+28,500	
Disaccharide[c] of R_g 0.27		+20,900	+20,500	
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside		+30,800		28
D-Glucose		+ 9,500	+360	
2-Acetamido-2-deoxy-D-glucose		+ 8,800	− 2,400	30
Cellobiose	β-D-(1 → 4)	+12,400	− 3,000	29
Gentiobiose	β-D-(1 → 6)	+ 3,000	− 8,200	31
O-2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-glucose	β-D-(1 → 6)	+ 2,700	− 9,400	27
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside		−10,400		28

[a] Equilibrium value. [b] In water. [c] See Table I.

Initial characterization of the disaccharide of R_g 0.27 (Table I) was carried out in the manner discussed above for *O*-α-D-glucopyranosyl-(1 → 4)-2-amino-2-deoxy-D-glucose hydrochloride (I). The collected physical data (see Experimental section) indicated this to be a reducing amino sugar disaccharide, with a high positive optical rotation (downward mutarotation), composed of glucose and 2-amino-2-deoxyglucose. Borohydride reduction of the *N*-acetyl derivative, followed by hydrolysis and paper chromatographic resolution, gave crystalline 2-amino-2-deoxy-D-glucose (hydrochloride) in almost quantitative yield. This showed that the sequence of units is 2-amino-2-deoxy-D-glucose → D-glucose.

Application of the Elson–Morgan and Morgan–Elson analytical determinations to derivatives of this second disaccharide, of R_g 0.27, permitted a tentative glycosidic linkage assignment. Glycoside derivatives of 2-amino-2-deoxy sugars cannot undergo base-catalyzed condensation with 2,4-pentanedione to give pyrrole derivatives, and in consequence do not give a color in the Elson–Morgan determination, unless the glycoside itself undergoes cleavage to the free amino sugar under basic conditions. In the case of a 2-amino-2-deoxy-D-glucosyl → D-glucose disaccharide, the extent of this cleavage, with liberation of 2-amino-2-deoxy-D-glucose, is dependent on the position of linkage to the D-glucose residue.

Based on the original speculations of Isbell and the excellent experimental work of Kenner and associates,[35] it is now well-established that reducing disaccharides can undergo a type of enediol-activated β-elimination in which the reducing component is split off as a saccharinic acid. This reaction takes place most readily when the substitution is on C-3 of the reducing unit, very much less readily when on C-4, and essentially not at all when C-2 or C-6 is substituted. Applied to the case of the 2-amino-2-deoxyhexose → hexose disaccharides, the ease of elimination of the reducing hexose, with its concomitant freeing of the amino sugar for reaction with 2,4-pentanedione, will determine the amount of color production in the Elson–Morgan assay. The Morgan–Elson determination, on the *N*-acetylated disaccharide, similarly will be subject to release of 2-acetamido-2-deoxy-D-glucose by the alkaline degradation mechanism. A tabulation of predictable results is given in Table V.[16-19,34,36,37]

TABLE V

PREDICTABLE[a-d] COLORIMETRIC RESULTS IN THE ELSON–MORGAN AND MORGAN–ELSON DETERMINATIONS FOR AMINO SUGAR DISACCHARIDES WITH UNIT SEQUENCE 2-AMINO-2-DEOXY-GLUCOSE → GLUCOSE

Glycosidic linkage	Color intensity with respect to 2-amino-2-deoxy-D-glucose: Elson–Morgan	Color intensity with respect to 2-acetamido-2-deoxy-D-glucose: Morgan–Elson
(1 → 2)	Negligible	Negligible[a]
(1 → 3)	Approximately equal	Approximately equal[b]
(1 → 4)	Low (5–25%)	Low (5–25%)[c]
(1 → 6)	Negligible	Negligible[d]

[a] See ref. 36. [b] See ref. 16–19. [c] See ref. 34 and 37. [d] See ref. 16.

In the case of the disaccharide under investigation, the Elson–Morgan determination on the free amino derivative gave 8% of the color given by an equivalent amount of 2-amino-2-deoxy-D-glucose. The Morgan–Elson determination gave 19% of the color given by an equivalent amount of 2-acetamido-2-deoxy-D-glucose. Electrophoresis in molybdate buffer[21] gave an $M_{glucitol}$ value of 0.33 (see Table III). All three of these determinations are indicative of a (1 → 4) glycosidic linkage.

Further confirmation of the linkage was made after methylation of the *N*-acetyl derivative, hydrolysis, and resolution of the hydrolytic components by isolative thin layer chromatography. The crystalline hydrolytic fragments were chromatographically homogeneous in three solvent systems. The derivatives were characterized through their melting point, chromatographic identity with authentic specimens, and X-ray powder diffraction patterns, as 2,3,6-tri-*O*-methyl-α-D-glucose and 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-β-D-glucose hydrochloride, indicating that the glycosidic linkage in the methylated disaccharide was (1 → 4).

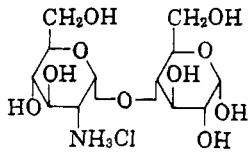
The high positive optical rotation of all the derivatives, and in particular the molecular rotation of the *N*-acetylated alditol ([M]D +20,500°, Table IV), indicated an α-D glycosidic linkage. The free amino

(35) See R. L. Whistler and J. N. BeMiller, *Advan. Carbohydrate Chem.*, **13**, 289 (1958).

(36) R. Kuhn, H. H. Baer, and A. Gauhe, *Ann.*, **611**, 242 (1958).

(37) A. Linker, K. Meyer, and P. Hoffman, *J. Biol. Chem.*, **235**, 924 (1960).

compound and the *N*-acetyl derivative both showed downward mutarotation indicating an α-D configuration of the hydroxyl on the anomeric carbon atom of the reducing portion. Infrared spectral data supported this assignment. On the basis of these findings structure II has been assigned to the second disaccharide hydrolytic fragment of the carboxyl-reduced heparin.

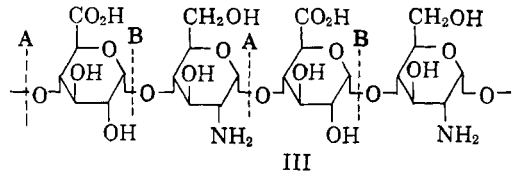


II

To the best of our knowledge this disaccharide has not been isolated, synthesized, or characterized on a rigorous basis previously. These results are in agreement with investigations on an amorphous disaccharide isolated from unreduced heparin by Hoffman and Meyer[34] and considered to be a 2-amino-2-deoxyhexose-(1 → 4)-hexuronic acid, on the basis of colorimetric data, optical rotatory behavior, ability to form a flavazole derivative, and disaccharide reducing end determination through borohydride reduction. Such a disaccharide, at the time designated heparosin, had been isolated previously[38] (through its cupric salt) in this laboratory, as an amorphous monosulfate. Indirect evidence for the structure, *O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucuronic acid was furnished, but, as the substance could not be put on a crystalline basis, it was not considered to be a useful degradation product. The retention of one sulfate group by the disaccharide is, however, of interest.

Hoffman and Meyer[34] also isolated another amorphous disaccharide, in a minor proportion, from unreduced heparin; its *N*-acetyl derivative was Morgan–Elson negative, and reducing end determination supported by colorimetric data suggested the structure to be *O*-D-glucuronosyl-(1 → 4)-2-amino-2-deoxy-D-glucose.

Noncrystalline fragments from heparin hydrolyzates have been variously described[39-41] as (1 → 3)-, (1 → 4)-, and (1 → 6)-linked disaccharides, on the basis of nondefinitive evidence, but without, in most cases, accurate quantitative comparisons with suitable reference substances, or consideration of the nature of the characterizing reactions. There can be no question that the two crystalline disaccharides, isolated herein from carboxyl-reduced heparin, are definitively characterized, are true polysaccharide components, and are not acid reversion artifacts. The linkage sequence in heparin is thus α-D-(1 → 4) throughout. Assuming alternating linkages, the structure of desulfated heparin would then be III as shown. Structure III is a modified amylose, and, like amylose, it may tend to assume a helical chain configuration which may well account for some of the steric difficulties encountered on subjecting this polymer to chemical manipulations.



III

N-Acetylation favors the A–A hydrolytic break in carboxyl-reduced III, whereas the amine salt would favor the B–B break, in accordance with our findings (Table I).

Structure III is not entirely in accord with our previous linkage report[38] in that an α-D-(1 → 3)-hexuronic acid to 2-amino-2-deoxy-D-glucose linkage was favored at that time. This had been based mainly on an interpretation of periodate oxidation data, obtained in this laboratory, through extrapolation of very steep periodate consumption rate curves with amorphous materials. The present findings supersede these.

Methylation studies on a heparin modification, reported in 1963 by Danishefsky and co-workers,[42] and periodate oxidation studies on the heparin polymer, by Foster, Stacey, and associates[43] as well as by Montgomery and co-workers,[44] are in essential agreement with the linkage sequence proposed in this work.

Experimental[45]

Determination of Hydrolytic Characteristics of Partially Desulfated, Partially Acetylated, Carboxyl-Reduced Heparin.—A 1% solution of partially desulfated, partially acetylated, carboxyl-reduced heparin (sodium salt)[2] in 1.5 *N* hydrochloric acid was heated at 90°. Duplicate 1-ml. aliquots of the hydrolysate were pipeted from the solution into test tubes, diluted to 5 ml. (total volume), and neutralized with an equivalent amount of sodium bicarbonate solution; chromatography spots (0.5 ml.) were applied to Whatman No. 1 paper for each sample. The reducing sugar concentration in the remainder of the solution was determined by the method of Somogyi[46] (Fig. 1). Paper chromatograms of the hydrolyzate were developed with solvent A. The number of zones as well as their relative intensities varied with the time at which the samples were withdrawn from the hydrolysate. Principal zones were as shown in Table I.

From the foregoing data it was decided that optimal hydrolytic conditions for release of the moiety with R_g 0.32 could be approached by hydrolyzing a 1% solution of carboxyl-reduced heparin in 1.5 *N* hydrochloric acid for 4.5 hr. at 90°.

Determination of Hydrolytic Characteristics of Hydrazine-Treated, Partially Desulfated, Partially Acetylated, Carboxyl-Reduced Heparin.—Partially acetylated, partially desulfated, carboxyl-reduced heparin[2] (5.0 g.) was placed in a small flask,

(38) M. L. Wolfrom, R. Montgomery, J. V. Karabinos, and P. Rathgeb, *J. Am. Chem. Soc.*, **72**, 5796 (1950).

(39) J. Kallos, Abstracts of Papers, 130th National Meeting of the American Chemical Society, Atlantic City, N. J., 1956, p. 22D.

(40) I. Danishefsky, H. B. Eiber, and E. Langholtz, *Biochem. Biophys. Res. Commun.*, **3**, 571 (1960).

(41) J. A. Cifonelli and A. Dorfman, *J. Biol. Chem.*, **231**, 11 (1958); **235**, 3283 (1960); *Biochem. Biophys. Res. Commun.*, **4**, 328 (1961).

(42) I. Danishefsky, H. B. Eiber, and A. Williams, *Federation Proc.*, **22**, 539 (1963); *J. Biol. Chem.*, **238**, 2895 (1963).

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

(44) G. J. Durant, H. R. Hendrickson, and R. Montgomery, *Arch. Biochem. Biophys.*, **99**, 418 (1962).

(45) Paper chromatography was carried out using the descending technique with the upper 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. Procter, and J. S. Harrison, *Nature* (London), **166**, 444 (1950)] or with ninhydrin (0.2% in ethanol). R_g refers to 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 powder diffraction pattern data refer to interplanar spacing in Å. with Cu Kα radiation. Relative intensities were estimated visually: s, strong; m, medium; w, weak; v, very. The strongest lines are numbered in order (1, strongest); double numbers indicate approximately equal intensities. Infrared spectra were determined on a Perkin-Elmer Infracord infrared spectrophotometer with pellets pressed from a finely ground mixture of the sample with dried analytical reagent grade potassium bromide.

(46) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

dissolved in hydrazine (95%, 2 ml.), and the mixture heated on a water bath at 90° for 10 hr.[4] The excess hydrazine was neutralized with an equivalent amount of dilute hydrochloric acid, and the solution was dialyzed against running water for 2 days. After concentration the product was freeze-dried; the yield was 1.6 g.

Anal. Calcd. for $C_{12}H_{22}O_9NCl$: N, 3.9. Found: N, 4.17.

The product (1.25 g.) recovered from the hydrazine treatment of the partially desulfated, partially acetylated, carboxyl-reduced heparin was dissolved in 1.5 *N* hydrochloric acid (125 ml.) and heated on a water bath at 90°. Chromatography and the Somogyi reducing sugar determination[46] were carried out as above for the *N*-acetyl derivative hydrolysate (Fig. 1 and Table I).

From the foregoing data it was determined that optimal hydrolytic conditions for release of the moiety with R_g 0.27 could be approached by hydrolyzing a 1% solution of hydrazine-treated, carboxyl-reduced heparin in 1.5 *N* hydrochloric acid for 2.5 hr. at 90°.

O-α-D-Glucopyranosyl-(1 → 4)-2-amino-2-deoxy-α-D-glucopyranose Hydrochloride (I).—Partially desulfated, partially acetylated, carboxyl-reduced heparin (2.54 g.) was refluxed for 4.5 hr. in 1.5 *N* hydrochloric acid (254 ml.), neutralized, and the hydrolysate solution evaporated. The sirup (1.8 g.) was resolved on nine sheets of Whatman No. 3 paper using solvent A as the eluting agent. In order to provide a more even solvent flow, 10 × 46 cm. strips of Whatman No. 1 paper were sewn to the ends of 46 × 57 cm. sheets of Whatman No. 3 paper.[47] 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 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 spray. Elution of the zone with R_g 0.32 yielded a sirup which crystallized from the elution solvent and gave granules on recrystallization from ethanol–ether; yield 252 mg. (13.2% based on deacetylated polymer); m.p. 180–185° dec.; $[\alpha]^{25}D$ +110 → +81° (2.5 hr., equil., *c* 2.8, water); $\lambda_{max}^{KBr}(\mu)$ 3.10 (OH), 3.45 (NH), 6.15 ($-NH_3Cl$), 11.65 (equatorial H on anomeric carbon atoms); X-ray powder diffraction data[45]: 7.87 (s) (2,2), 4.30 (vs) (1), 3.15 (m), 3.07 (s) (2,2), 2.87 (s) (3,3), 2.77 (vw), 2.67 (m), 2.62 (vw), 2.48 (m), 2.19 (m), 2.07 (s) (3,3), 1.9 (m), 1.8 (m).

Anal. Calcd. for $C_{12}H_{24}ClNO_{10}$: N, 3.72. Found: N, 3.90.

Hydrolysis of this compound with 2 *N* hydrochloric acid gave approximately equal quantities of glucose and 2-amino-2-deoxyglucose hydrochloride by visual comparison on paper chromatography. Calculated as a discaccharide of glucose and 2-amino-2-deoxyglucose hydrochloride, this substance gave 47% the color given by an equivalent amount of 2-amino-2-deoxy-D-glucose hydrochloride in the Elson–Morgan determination.[10]

O-α-D-Glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose.—*O*-α-D-Glucopyranosyl-(1 → 4)-2-amino-2-deoxy-α-D-glucose hydrochloride (144 mg.) was dissolved in water (25 ml.) and methanol (5 ml.) containing Dowex-1 ion-exchange resin (10 ml., CO_3^{-2} form).[48] The stirred solution was cooled to 0–5°, and acetic anhydride (2 ml.) was added. Stirring was maintained for 1.5 hr. at this temperature. The ion-exchange resin was then removed by filtration and washed with water. The combined filtrate and washings were passed through a column (20 × 100 mm.) of Amberlite IR-120 ion-exchange resin (H^+ form), the column washed thrice with water (20 ml.), and the combined effluents concentrated to a sirup, which crystallized in small needles from ethanol–ether; yield 109 mg. (74%); m.p. 144.5–146° (preliminary softening, 138°); $[\alpha]^{19}D$ +87° (extrapolated) → +39° (2.5 hr., equil., *c* 0.9, water); $\lambda_{max}^{KBr}(\mu)$ 2.97 (OH), 6.09, 6.46 (NHAc), 11.83 (equatorial H on anomeric carbon atoms); X-ray powder diffraction data[45]: 3.77 (m) (3), 2.76 (vs) (1), 2.29 (s) (2), 1.99 (w), 1.91 (m), 1.80 (w), 1.48 (m), 1.35 (m).

Anal. Calcd. for $C_{14}H_{25}NO_{11}$: C, 43.86; H, 6.52; N, 3.65; mol. wt., 380. Found: C, 43.98; H, 6.27; N, 3.44; mol. wt.,[8] 364.

This *N*-acetyl derivative gave no color in the Morgan–Elson[12] analytical determination. The compound was chromatographically homogeneous in solvents A, B, and C (R_g 0.46 in solvent A).[45]

O-α-D-Glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucitol.—*O*-α-D-Glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose (74 mg.) was dissolved in 0.05 *M* boric acid (20 ml.) containing Amberlite IR-120 ion-exchange resin (4 ml., H^+ form)[49] and the mixture cooled to 0° under stirring. Aqueous sodium borohydride (10 ml., 0.3 *M*) was dropped carefully into the mixture over a 5-min. period. After stirring for 0.5 hr., the mixture was again treated with the sodium borohydride solution (10 ml.), stirred another 0.5 hr., adjusted to pH 9 with dilute sodium hydroxide solution, and left at 5° overnight. The solution was brought to pH 5 with acetic acid and evaporatively codistilled with methanol (10-ml. portions) eight times. The sirup was taken up in water (10 ml.), acidified with acetic acid, passed through a column (20 × 100 mm.) of Amberlite IR-120 ion-exchange resin (H^+ form), and the column washed with several portions (20 ml.) of water. The combined effluents were evaporated to a sirup and the sirup codistilled six times with methanol (10-ml. portions). An aqueous solution of the sirup (10 ml.) was passed through a column (20 × 100 mm.) of Dowex-1 ion-exchange resin (CO_3^{-2} form), which was then washed with water three times (20-ml. portions). After concentrating the combined effluents and codistilling with portions of methanol twice more, the sirup, chromatographically homogeneous in solvent A (R_g 0.30), was nonreducing to Benedict solution; yield 24.5 mg. (32.7%), $[\alpha]^{19}D$ +75° (*c* 0.45, water), $M_{glucitol}$ 0.38 on electrophoresis in molybdate buffer.[21]

After hydrolysis of the reduced disaccharide (24 mg.) with 2 *N* hydrochloric acid at 100° for 2 hr. and removal of the acid by 1-propanol codistillation, paper chromatography revealed a reducing component which behaved as glucose and a nonreducing zone with R_g 0.65 (solvent A), corresponding to 2-amino-2-deoxy-D-glucitol hydrochloride.

The hydrolysate revealed no free amino sugar in the Elson–Morgan analytical determination.[10]

The sirup was taken up in ethanol, and ethyl ether added until faint turbidity appeared. After standing at 5° overnight, white crystals formed (8 mg.), which gave an X-ray powder diffraction pattern identical with that of authentic 2-amino-2-deoxy-D-glucitol hydrochloride[30]; X-ray powder diffraction data[45]: 7.55 (s) (2), 5.72 (s) (2,2), 5.36 (w), 4.34 (vs) (1), 4.19 (w), 3.75 (m) (3), 3.15 (vw), 2.96 (vw), 2.65 (w), 2.49 (w), 2.34 (vw), 2.22 (vw), 2.12 (vw), 2.02 (vw).

Methylation of O-α-D-Glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose.—*O*-α-D-Glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose (25 mg.) was dissolved in *N*,*N*-dimethylformamide (15 ml.) previously cooled to 0°. To the cooled solution was added, under stirring, barium oxide (0.6 g.), barium hydroxide octahydrate (17 mg.), and methyl iodide (0.7 ml.).[50] The stirred solution was maintained under nitrogen at 0° for 18 hr. when it was brought to room temperature for an additional 48 hr. Chloroform (20 ml.) was added, the solution cooled, filtered, and the insoluble salts washed on the filter paper with chloroform. The reddish orange chloroform solution was washed twice with water (15-ml. portions), once with sodium thiosulfate (15 ml., 0.05 *N*), and once again with water. The water extracts were shaken with chloroform (20 ml.) and the combined chloroform extracts dried over sodium sulfate. The chloroform was removed on a rotary evaporator leaving a brown sirup; yield 30 mg.

Hydrolysis of the partially methylated sirup in 2 *N* hydrochloric acid for 2 hr. at 100°, removal of acid by codistillation with 1-propanol, and chromatography (solvent B) revealed three ninhydrin-positive components, corresponding to 2-amino-2-deoxyglucose hydrochloride (1.00), and $R_{2\text{-amino-2-deoxyglucose hydrochloride}}$ 1.72 and 3.15.

The sirup was dissolved in a small volume of water and passed through a column (100 × 20 mm.) of Amberlite IR-120 ion-exchange resin (H^+ form) which was washed with water until the effluent was neutral. The effluent and washings were concentrated to a sirup, which was codistilled several times with 1-propanol. The column was eluted with 2 volumes of 1 *N* hydrochloric acid (20 ml. each), followed by water until the effluent was neutral. The acidic portion was concentrated to a small volume and codistilled several times with 1-propanol.

The sirup from the neutral portion was dissolved in ethanol to which ether–petroleum ether (b.p. 30–60°) was added. Needle-like crystals appeared upon standing overnight; yield 3 mg.; m.p.

(47) The authors wish to acknowledge Dr. I. R. Siddiqui for suggesting this method.

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

(49) H. L. Frush and H. S. Isbell, *ibid.*, **78**, 2844 (1956).

(50) R. Kuhn and A. Gauhe, *Ber.*, **95**, 518 (1962).

83–86°; X-ray powder diffraction pattern was identical with that of 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose.[51]

Upon concentrating the 1-propanol solution of the acidic eluate, crystals formed (5 mg.), were isolated, and gave an X-ray powder diffraction pattern identical with 2-amino-2-deoxy-α-D-glucopyranose hydrochloride.[52]

The 1-propanol-soluble portion of the acidic eluate was evaporated to a sirup and *N*-acetylated as described above for *O*-α-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranose. Upon concentration of the *N*-acetylation mixture, the resulting sirup was taken up in ethanol to which was added ether–petroleum ether. Upon standing overnight, needlelike crystals formed; yield 6 mg.; m.p. 232–233°; $[\alpha]^{19}D$ +77 → +37 ± 5° (6 hr., final, water); X-ray powder diffraction data[45]: 10.92 (s) (2), 8.45 (w), 5.58 (m) (3), 4.38 (vs) (1), 4.15 (w), 3.78 (vw), 3.47 (vw), 3.07 (vw), 2.73 (m) (3,3), 2.22 (m).

For 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α-D-glucose Jeanloz[53] reports m.p. 232–233° and $[\alpha]^{25}D$ +90 (15 min.) → +35 ±5° (24 hr., *c* 0.29, water). Kuhn and Gauhe[50] give $[\alpha]^{22}D$ +36° (5 hr., *c* 0.8, water).

The compound was homogeneous by paper chromatography in solvent A ($R_{2\text{-acetamido-2-deoxy-D-glucose}}$ 2.06), and solvent B ($R_{2\text{-acetamido-2-deoxy-D-glucose}}$ 1.41) as well as by thin layer chromatography in 7:3 benzene–methanol[54] ($R_{2\text{-acetamido-2-deoxy-D-glucose}}$ 2.62). The substance was identical by X-ray powder diffraction data, melting point, mixture melting point, specific rotation, and chromatographic behavior, in the systems recorded before, with an authentic sample (of 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α-D-glucose) kindly furnished by Professor R. Kuhn and with a sample of the same compound prepared from methylated chitin.[55]

O-2-Amino-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose Hydrochloride (II).—The zone, R_g 0.27, present in the hydrolyzate of partially desulfated, carboxyl-reduced heparin was isolated by the preparative paper chromatography procedure described before. The material contained in it was eluted from its zone strip in the same manner and was crystallized from ethanol–ether; yield 45 mg. (2.5%); m.p. 175–181° dec.; $[\alpha]^{19}D$ +100 (extrapolated) → +54° (1.5 hr., final, *c* 0.55, water); λ_{max}^{KB} (μ) 2.96 (OH), 3.40 (NH), 6.20 (NH_3Cl), 11.65 (equatorial H at anomeric carbon atoms); X-ray powder diffraction data[45]: 9.32 (w), 7.64 (w), 6.05 (w), 5.50 (w), 4.82 (vw), 4.28 (m), 3.89 (s) (2), 3.46 (w), 3.03 (m) (3), 2.79 (vs) (1), 2.66 (m) (3,3), 1.96 (w), 1.86 (w), 1.74 (vw), 1.71 (vw).

The substance gave a positive Benedict test and ninhydrin reaction and was chromatographically homogeneous in solvents A, B, and C (R_g 0.34 in solvent C). In the Elson–Morgan analytical determination[10] 8% of the color produced by an equimolar amount of 2-amino-2-deoxy-D-glucose hydrochloride was observed.

Anal. Calcd. for $C_{12}H_{24}ClNO_{10}$: N, 3.72. Found: N, 4.09.

Hydrazine-treated, carboxyl-reduced heparin (0.292 g.) was hydrolyzed under the conditions described for optimal yield of disacharide with R_g 0.27. Three ninhydrin positive zones were detected after neutralization on paper chromatography, in solvent A, with R_g 0.72 (2-amino-2-deoxyglucose hydrochloride), R_g 0.32, and R_g 0.27. Upon isolation by the paper chromatographic technique described before, elution of the zone with R_g 0.27 gave a crystalline product (35 mg., 12% based on deacetylated polymer) exhibiting physical constants identical with those of the *O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose hydrochloride isolated previously.

O-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose.—*N*-Acetylation of *O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose hydrochloride (50 mg.) by the method previously described[48] for *O*-α-D-glucopyranosyl-(1 → 4)-2-amino-2-deoxy-α-D-glucopyranose hydrochloride gave a crystalline product from ethanol–ether; yield 35 mg. (70%); m.p. 124–125° (preliminary softening 118°); $[\alpha]^{20}D$ +129 (extrapolated) → +75 ± 10° (2.5 hr., final, *c* 0.2, water); λ_{max}^{KBr} (μ) 3.00 (OH), 6.10, 6.40 ($NHCOCH_3$), 11.55 (equatorial H at anomeric carbon atoms); X-ray powder diffraction data[45]: 8.67 (s) (2), 4.23 (m), 3.99 (m), 2.86 (vs) (1), 2.58 (s) (3), 2.18 (w), 2.06 (w).

This compound was reducing to Benedict solution, gave no reaction with ninhydrin, was chromatographically homogeneous in solvents A (R_g 0.47), B, and C, and gave 19% of the color observed with an equivalent amount of 2-acetamido-2-deoxy-D-glucose in the Morgan–Elson analytical determination.[12]

Anal. Calcd. for $C_{14}H_{25}O_{11}N \cdot 3H_2O$: C, 38.97; H, 7.19; N, 3.24; H_2O, 12.53; mol. wt., 431. Found: C, 39.16; H, 6.89; N, 3.11; H_2O, 12.98; mol. wt.,[8] 394.

O-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 3)-L-gulitol.—*O*-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose (35 mg.) was reduced with sodium borohydride by the method described for *O*-α-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose; yield 21.9 mg. (63%) of a nonreducing (Benedict test) sirup, chromatographically homogeneous in solvents A, B, and C (R_g 0.49, solvent B); $[\alpha]^{19}D$ +55° (*c* 0.42, 50% aqueous ethanol); $M_{glucitol}$ 0.33 (molybdate buffer ionophoresis).[21]

Hydrolysis of the reduced disaccharide (19 mg.) with 2 *N* hydrochloric acid at 100° for 2 hr. and removal of the acid by 1-propanol codistillation and paper chromatography, revealed a reducing component which behaved as 2-amino-2-deoxy-D-glucose hydrochloride and a nonreducing zone with R_g 1.05 (solvent A), corresponding to D-glucitol. The hydrolysate sirup was taken up in ethanol and ether added until just turbid. After being allowed to stand at 5° overnight, crystals formed; yield 7 mg. (73%); X-ray powder diffraction pattern was identical with that of authentic 2-amino-2-deoxy-D-glucose hydrochloride.[52]

Methylation of O-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose.—*O*-2-Acetamido-2-deoxy-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose (162 mg.) was dissolved in *N*,*N*-dimethylformamide (10 ml.) cooled to 0°. To the cooled solution was added, under stirring, barium oxide (1.8 g.), barium hydroxide octahydrate (47 mg.), and methyl iodide (1.8 ml.).[50] The solution was maintained under nitrogen at 0° for 18 hr. when it was brought to room temperature for an additional 48 hr. Chloroform (20 ml.) was added, the solution cooled, filtered, and the insoluble salts washed on the filter paper with chloroform. The chloroform was evaporated under vacuum and the *N*,*N*-dimethylformamide codistilled with benzene until a sirup remained. The sirup showed three zones on thin layer chromatography[54] with benzene–methanol (7:3) developer.

After *N*-acetylation according to the Roseman and Ludowieg technique[48] a sirup was recovered, which was evaporated to dryness under reduced pressure over phosphorus pentaoxide. The partially methylated, *N*-acetylated sirup was dissolved in *N*,*N*-dimethylformamide (10 ml.) and cooled to 0°. To the cooled solution was added, under stirring, barium oxide (2 g.), barium hydroxide octahydrate (1 g.), and methyl iodide (3 ml.).[56] The stirred solution was maintained under nitrogen at 0° for 18 hr., when it was brought to room temperature for an additional 48 hr. Chloroform (50 ml.) was added, the solution cooled, filtered, and the insoluble salts washed on the filter with chloroform. The yellow chloroform solution (120 ml.) was extracted thrice with water (40-ml. portions), once with 0.05 *N* sodium thiosulfate solution (40 ml.), and again with water (40 ml.). The combined aqueous washings were then extracted once with chloroform (40 ml.) and the combined chloroform extracts dried over anhydrous sodium sulfate (30 g.). After filtering the sodium sulfate, the chloroform was evaporated and the sirup dried under vacuum over phosphorus pentaoxide; yield 105 mg. (38.5%); R_f 0.68 on thin layer chromatography[54] using benzene–methanol (7:3) developer.

The sirup (81 mg.), partially insoluble in *N* hydrochloric acid (10 ml.), was solubilized with tetrahydrofuran (10 ml.) and the solution heated at 90° for 6 hr. The acidic hydrolyzate was evaporated, codistilled several times with 1-propanol to remove the last traces of hydrochloric acid, and examined by thin layer chromatography[54] with benzene–methanol (6:4) developer. The chromatogram revealed three zones on spraying with sulfuric acid: R_f 0.81, 0.76 (2,3,6-tri-*O*-methyl-α-D-glucose), and 0.61 (2-amino-2-deoxy-3,4,6-tri-*O*-methyl-β-D-glucose hydrochloride). Isolative thin layer chromatography on 19.5 × 19.5 cm. plates[54] with a 0.5-mm. thickness of silica gel G (benzene–methanol, 6:4, developer) gave, on elution of the zones R_f 0.76 and 0.61 with methanol and concentration, sirups in yields of 8 mg. (20%) and 7 mg. (17.5%), respectively.

(51) M. L. Wolfrom, A. Thompson, and A. M. Brownstein, *J. Am. Chem. Soc.*, **80**, 2015 (1958).

(52) I. Werner, *Mikrochemie ver. Mikrochim. Acta*, **39**, 133 (1952).

(53) R. W. Jeanloz, *J. Org. Chem.*, **26**, 905 (1961).

(54) Silica gel G (E. Merck, Darmstadt, West Germany) activated at 100°, with ascending solvent. The zones were located by spraying with 1% potassium permanganate in 10% aqueous sodium hydroxide.

(55) M. L. Wolfrom, J. R. Vercellotti, and D. Horton, *J. Org. Chem.*, **29**, 547 (1964).

(56) R. Kuhn and H. Trischmann, *Ber.*, **96**, 284 (1963).

The sirup from zone R_f 0.76 was chromatographically homogeneous on Whatman No. 1 paper in solvent A (R_f 0.94) and solvent B (R_f 0.84) with mobility corresponding to the R_f values of 2,3,6-tri-*O*-methyl-D-glucose in each solvent. The sirup was taken up in a small amount of ethanol, and ether and petroleum ether added until incipient turbidity. After the sirup was cooled overnight, crystals (small needles) formed which were isolated; yield 3 mg. (7.5% of sirup applied to plates; 37% of sirup isolated); m.p. 120–124° (lit.[57] m.p. 118–119°); X-ray powder diffraction data, identical with that of an authentic sample of 2,3,6-tri-*O*-methyl-α-D-glucose[45]: 11.65 (s) (2), 10.10 (s) (2,2), 7.61 (m), 5.76 (m), 5.09 (w), 4.61 (m) (3), 4.25 (vs) (1), 4.05 (m), 3.86 (m), 3.67 (w), 3.41 (w), 3.26 (w), 2.97 (w), 2.81 (w), 2.73 (w), 2.47 (w), 2.38 (w), 2.33 (w), 2.24 (w).

The sirup from the zone of R_f 0.61 was chromatographically homogeneous on Whatman No. 1 paper in solvent A (R_f 0.707) and solvent B (R_f 0.67) with mobility corresponding to the R_f values of 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose hydrochloride in each solvent. The sirup was taken up in a small amount of ethanol, and ether and petroleum ether were added until incipient turbidity. After the solution was cooled overnight, small crystalline granules formed which were isolated; yield 2 mg. (5% of sirup applied to plates; 28% of sirup isolated); m.p. 211–214° dec.[58]; X-ray powder diffraction data identical with that of authentic sample of 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-β-D-glucose hydrochloride[45]: 9.88 (s) (3), 7.04 (m), 6.11 (s) (2), 5.20 (w), 4.45 (w), 4.14 (w), 3.85 (m), 3.63 (w), 3.38 (vs) (1), 2.96 (w), 2.63 (m).

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(57) W. Charlton, W. N. Haworth, and S. Peat, *J. Chem. Soc.*, 89 (1926).

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Methylation Studies on Carboxyl-Reduced Heparin. 2-Amino-2-deoxy-3,6-di-*O*-methyl-α-D-glucopyranose from the Methylation of Chitosan[1]

M. L. WOLFROM, J. R. VERCELLOTTI, AND D. HORTON

Department of Chemistry, The Ohio State University, Columbus 10, Ohio

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Permethylation and acid hydrolysis of partially acetylated, completely desulfated, carboxyl-reduced heparin gave, by thin layer chromatographic resolution, crystalline 2,3,6-tri-*O*-methyl-β-D-glucose and 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose (isolated as the crystalline α-D, *N*-acetyl derivative); this provides further evidence for regular (1 → 4) linkages in heparin. *O*-Methylation of chitosan to a high degree of substitution has been achieved, and acid hydrolysis of the product provides a convenient source of 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose and its crystalline *N*-acetyl derivative, key reference compounds in the structural work on heparin.

Structural studies on heparin by the methylation procedure are beset with difficulties arising from limited solubility, and hindrance to substitution.[2] In the present study, these difficulties were avoided by performing the methylation procedure on a heparin modification which was completely desulfated, carboxyl-reduced, and partially acetylated.[3] It was possible, with this material, to achieve almost complete *O*-methylation, and hydrolysis of the product provided crystalline, partially methylated monosaccharide fragments, whose structures provided further definitive evidence for the presence of (1 → 4) linkages in the heparin macromolecule.

The completely desulfated, carboxyl-reduced, partially acetylated heparin,[3] which was soluble in *N*,*N*-dimethylformamide, was twice methylated in this medium with dimethyl sulfate in the presence of barium oxide–barium hydroxide, by the procedure of Kuhn and Trischmann,[4] to give a nondialyzable product which contained 78% of the theoretical methoxyl content. Dialysis stages were employed during each methylation to remove low-molecular-weight degradation products; it was considered important to retain only nondialyzable, methylated high polymer, at the expense of yield, in the procedure. Further methylation, in *N*,*N*-dimethylformamide, with methyl iodide in the presence of barium oxide–barium hydroxide,[4] gave a product with methoxyl content 97% of the theoretical value for complete *O*-methylation when determined after *N*-acetylation.[5] Acid hydrolysis of the methylated polymer was initiated in 90% aqueous formic acid, in which the material was soluble, and the partially hydrolyzed, water-soluble product was then hydrolyzed completely in 1 *N* hydrochloric acid. The hydrolysate was N-acetylated,[5] and was then found by thin layer chromatography to consist of two principal components, R_f 0.41 and 0.59 in the system employed. Isolative thin layer chromatography gave the material in the two zones as chromatographically homogeneous sirups in good yield. The sirup from the slower moving zone crystallized, to give 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α-D-glucose, identical by X-ray powder diffraction pattern, melting point, and thin layer chromatographic techniques with an authentic sample.[6,7] The sirup from the faster moving zone crystallized from the anomeric mixture to give 2,3,6-tri-*O*-methyl-β-D-glucose, identical by X-ray powder diffraction pattern, melting

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