recrystallized twice more to give crystals with a slightly yellow color: mp 131–132°; $[\alpha]^{22}D$ +30 ± 2° (*c* 2.88, pyridine); λ_{max}^{KBr} 3.05 (NH), 5.75 ($-CO_2R$), and 6.02 μ (NH); X-ray powder diffraction data, 13.39 (m), 9.72 (s, 2), 7.03 (m), 5.87 (w), 5.25 (vw), 4.74 (m), 4.25 (s, 1), 3.99 (s, 3), 3.66 (m), 3.47 (m), 3.06 (w), and 2.93 (w).

Anal. Calcd for $C_{23}H_{21}N_5O_{14}S$: C, 44.30; H, 3.39; N, 11.28; S, 5.15. Found: C, 44.27; H, 3.85; N, 11.53; S, 5.20.

Ethyl 2-Deoxy-2-(2,4-dinitroanilino)-1-thio-α-D-ribofuranoside (XII).—To a syrupy XI prepared from VIII (9.20 g) by the above procedure was added saturated barium hydroxide solution (100 ml) and the mixture was heated under reflux for 24 hr.[21] Solid carbon dioxide was added and the bulk of the resulting barium carbonate was removed by filtration. The filtrate was concentrated to small volume and the remaining water was removed by repeated evaporation with absolute ethanol. The residue was extracted with ethanol and the extract was concentrated to a syrup. Examination of this syrup by paper chromatography using 5:5:3:1 pyridine–ethyl acetate–water–acetic acid as solvent[22] revealed a single spot with R_f 0.68 which gave a positive indication with silver nitrate–sodium hydroxide and ninhydrin but not with aniline hydrogen phthalate. To the dried syrup (4.73 g) in water (20 ml) was added sodium bicarbonate (1.87 g) and the mixture was stirred. 1-Fluoro-2,4-dinitrobenzene (4.15 g) was added and the transfer was effected with ethanol (20 ml). The mixture was cooled in water and stirred vigorously for 1 hr. The yellow crystalline solid which separated was removed by filtration, washed with water, and dried under reduced pressure over phosphorus pentoxide: yield 6.73 g (80% from VIII), mp 151–155°. Recrystallization from aqueous methanol afforded pure material: yield 6.18 g (73% from VIII); mp 159–160°; $[\alpha]^{22}D$ −64 ± 1° (*c* 1.63 ethanol); λ_{max}^{KBr} 2.80–3.00 (OH), 3.10 (NH), 6.20, 6.30, 6.70 (aryl C=C), 6.58 (NH, NO_2), 7.46 (NO_2), 12.00, and 13.40 μ (substituted phenyl); X-ray powder diffraction data, 13.60 (w), 11.33 (w), 8.42 (s), 6.03 (w), 5.64 (w), 5.22 (s, 3), 4.82 (s, 1,1,1), 4.40 (m), 4.15 (s, 2), 3.82 (m), 3.59 (s, 1,1,1), 3.44 (vw), 3.11 (s, 1,1,1), and 3.02 (vw).

Anal. Calcd for $C_{13}H_{17}N_3O_7S$: C, 43.43; H, 4.88; N, 11.69; S, 8.92. Found: C, 43.53; H, 5.24; N, 12.13; S, 8.89.

(21) F. J. McEvoy, B. R. Baker, and M. J. Weiss, *J. Am. Chem. Soc.*, **82**, 209 (1960).

(22) F. G. Fischer and H. J. Nebel, *Z. Physiol. Chem.*, **302**, 10 (1955).

Configuration of the Glycosidic Linkage of 2-Amino-2-deoxy-D-glucopyranose to D-Glucuronic Acid in Heparin[1]

M. L. WOLFROM, H. TOMOMATSU, AND W. A. SZAREK

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

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Partially acetylated, partially desulfated, carboxyl-reduced heparin was completely desulfated by a second treatment with methanolic hydrogen chloride. The desulfated product was *O*-deacetylated and subjected to periodate oxidation. The glycol units in the D-glucose units were oxidized and from the product 2-amino-2-deoxy-D-glucose hydrochloride was isolated on acid hydrolysis. The periodate-oxidized product was reduced with sodium borohydride and subjected to partial acid hydrolysis. Further oxidation with lead tetraacetate and subsequent sodium borohydride reduction and acetylation led to a crystalline compound which was shown by synthesis to be 2-*O*-(2-acetamido-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol. This α-D anomer was separated from the anomeric mixture synthesized by a Koenigs–Knorr reaction between tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylideneamino)-α-D-glucopyranosyl bromide and *cis*-1,3-*O*-benzylideneglycerol with subsequent acid removal of the benzylidene and *p*-methoxybenzylideneamino groups and acetylation. The pure β-D anomer was synthesized from 2-acetamidotri-*O*-acetyl-2-deoxy-α-glucopyranosyl chloride and *cis*-1,3-*O*-benzylideneglycerol (the product being isolated in two forms shown to be *cis* and *trans* in nature), followed by benzylidene removal and acetylation. These results offer confirmatory evidence for the (1→4) nature of all linkages present in the heparin molecule and demonstrate conclusively that the configuration of the 2-amino-2-deoxy-D-glucose to D-glucuronic acid linkage is α-D.

In previous publications[1,2] from this laboratory it was reported that acid hydrolysis of partially *O*-acetylated, partially desulfated, completely carboxyl-reduced, and *N*-acetylated heparin yielded two crystalline amino sugar containing disaccharides. Methylation studies established that the disaccharides were *O*-α-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-α-D-glucopyranose hydrochloride and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-α-D-glucopyranose hydrochloride. Thus, it was demonstrated that the repeating unit sequence of heparin very probably consists of alternating D-glucuronic acid and 2-amino-2-deoxy-D-glucose units linked glycosidically in an α-D-(1→4) manner.

The α-D stereochemical nature of the interglycosidic linkages was based originally upon indirect data, mainly polarimetric in nature. It was desirable to obtain more direct evidence on this point.

One of the disaccharides, *O*-α-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-α-D-glucopyranose hydrochloride, was synthesized in this laboratory[3] from maltose by reduction of its phenylosazone. This work confirmed the α-D-(1→4)-glycosidic linkage of D-glucuronic acid to 2-amino-2-deoxy-D-glucose in heparin.

In the present publication we wish to report degradative and synthetic evidence which establishes the α-D stereochemical nature of the glycosidic linkage of 2-amino-2-deoxy-D-glucopyranose to D-glucuronic acid. Supporting evidence is also presented for the (1→4) nature of all linkages present in the heparin polymer.

Treatment of partially acetylated, partially desulfated, carboxyl-reduced heparin[4] with methanolic hydrogen chloride gave, after dialysis and freeze drying, a completely desulfated product. *O*-Deacetylation in an ethylene glycol–methanol solution of ammonia yielded a completely desulfated, *O*-deacetylated,

(1) Preliminary communications: (a) M. L. Wolfrom, J. R. Vercellotti, H. Tomomatsu, and D. Horton, *Biochem. Biophys. Res. Commun.*, **12**, 8 (1963); (b) W. A. Szarek, M. L. Wolfrom, and H. Tomomatsu, *Chem. Commun.*, 326 (1965).

(2) M. L. Wolfrom, J. R. Vercellotti, and D. Horton, *J. Org. Chem.*, **27**, 705 (1962); **28**, 278, 279 (1963); **29**, 540 (1964).

(3) M. L. Wolfrom, H. El Khadem, and J. R. Vercellotti, *Chem. Ind.* (London), 545 (1964); *J. Org. Chem.*, **29**, 3284 (1964).

(4) M. L. Wolfrom, J. R. Vercellotti, and G. H. S. Thomas, *ibid.*, **26**, 2160 (1961); **29**, 536 (1964).

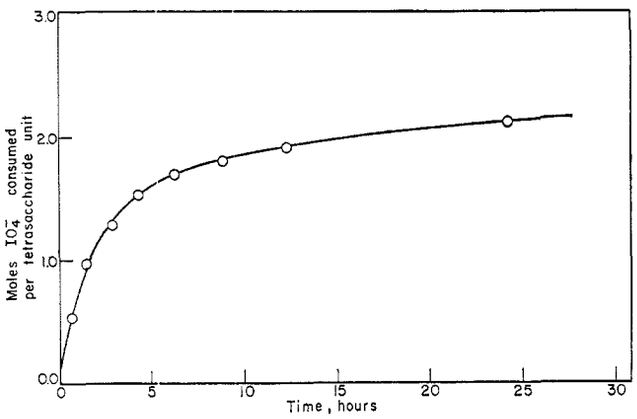


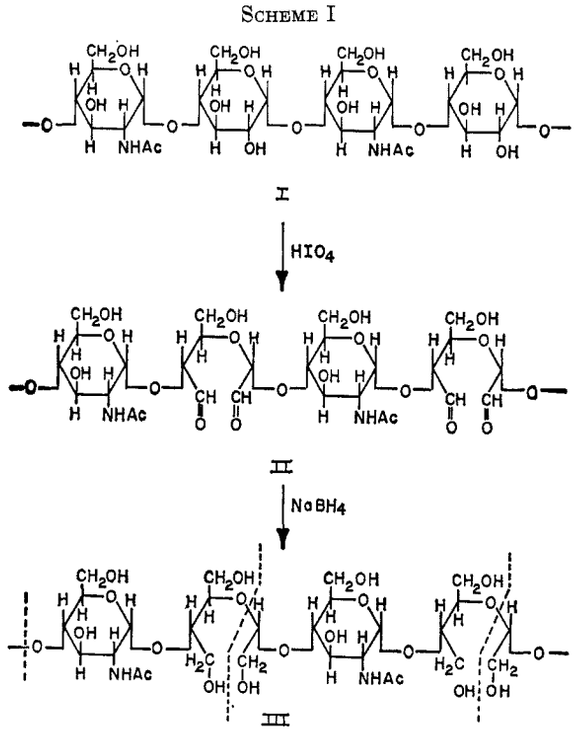
Figure 1.—Periodate consumption by completely desulfated, *O*-deacetylated, *N*-acetylated, carboxyl-reduced heparin (I); 25°.

carboxyl-reduced product (I). The heparin modification I, on periodate oxidation according to the method of Fleury and Lange,[5] consumed 2 moles of periodate per tetrasaccharide repeating unit but did not release any formic acid or formaldehyde (Figure 1). In a preparative experiment, I was oxidized by periodate under the same conditions and the product was isolated, after dialysis and freeze drying, as a white powder (II). The infrared spectrum of II showed strong absorption attributable to aldehydic carbonyl groups. This absorption was absent in the infrared spectrum of I. Vigorous acid hydrolysis of II yielded 2-amino-2-deoxy-D-glucose hydrochloride. This result indicates that the oxidation of I with periodate occurred in the carboxyl-reduced D-glucuronic acid units.

The periodate oxidation data presented above provide further evidence for (1→4)-glycosidic linkages in both the hexosamine–hexuronic acid and the hexuronic acid–hexosamine unit sequences of heparin. This assignment is in accord with other work reported from this laboratory[2,6] and with periodate oxidation studies on the heparin polymer reported by Foster and coworkers[7] and by Durant, Hendrickson, and Montgomery.[8] Moreover, the hexosamine–hexuronic acid linkage assignment is consistent with studies of Hoffman and Meyer[9] on an amorphous hexosamine–hexuronic acid disaccharide, isolated from heparin, and also with methylation studies on heparin modifications by Danishefsky, Eiber, and Williams.[10] Very recently, Danishefsky and Steiner[11] provided further evidence for the presence of alternating (1→4)-linked units of 2-amino-2-deoxy-D-glucose and D-glucuronic acid in heparin by the isolation of amorphous *O*-(2-acetamido-2-deoxy-D-glucopyranosyl)-(1→4)-D-glucopyranuronic acid and amorphous *O*-(D-glucopyranosyluronic acid)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose after partial acid hydrolysis of desulfated, *N*-acetylated heparin. Thus, heparin possesses a linkage structure related to that of amylose and possibly may exist in a helical configuration.[12]

The periodate oxidation product II could be reduced readily with sodium borohydride to yield a fine, white powder (III) which did not exhibit any aldehyde absorption in its infrared spectrum. When III was treated with dilute sulfuric acid the optical rotation became constant in about 4 hr. In a preparative experiment, III was treated with dilute sulfuric acid for 10 hr. Paper chromatography of the hydrolysate revealed the presence of three components which migrated more slowly than 2-amino-2-deoxy-D-glucose. One of these had almost zero mobility on paper, in the solvent system employed and was not investigated. The other two components had R_{GN} (GN = 2-amino-2-deoxy-D-glucose) 0.45 (fraction A) and 0.11 (fraction B). These were separated by preparative paper chromatography and isolated as white, amorphous powders which gave positive ninhydrin reactions and whose infrared spectra did not show any carbonyl absorption. Thus the two substances possessed free amino groups. The formation from III of units containing free amino groups is in accordance with studies of Moggridge and Neuberger[13] and of Foster, Horton, and Stacey[14] on the acidic hydrolysis of derivatives of 2-amino-2-deoxy-D-glucose. Initial hydrolysis of the *N*-acetyl groups in III would yield a derivative in which the 2-amino-2-deoxy-D-glucosidic linkage would be strongly resistant to acidic hydrolysis. This behavior is attributable to the electrostatic shielding effect of the positively charged monoalkylammonium ion formed in the reaction medium against protonation of the neighboring glycosidic oxygen atom.[13,14] The probable initial hydrolytic cleavage of III is indicated by the broken lines shown in Scheme I. Such a postulated scission would be in accord with the established finding

SCHEME I



(5) P. E. Fleury and J. Lange, *J. Pharm. Chim.*, **17**, 107, 196 (1933).
(6) M. L. Wolfrom, J. R. Vercellotti, and D. Horton, *J. Org. Chem.*, **29**, 547 (1964).
(7) A. B. Foster, R. Harrison, T. D. Inch, M. Stacey, and J. M. Webber, *Biochem. J.*, **80**, 12P (1961); *J. Chem. Soc.*, 2279 (1963).
(8) G. J. Durant, H. R. Hendrickson, and R. Montgomery, *Arch. Biochem. Biophys.*, **99**, 418 (1962).
(9) P. Hoffman and K. Meyer, *Federation Proc.*, **21**, 170, 1064 (1962).
(10) I. Danishefsky, H. B. Eiber, and A. H. Williams, *ibid.*, **22**, 539 (1963); *J. Biol. Chem.*, **238**, 2895 (1963).
(11) I. Danishefsky and H. Steiner, *Biochim. Biophys. Acta*, **101**, 37 (1965).
(12) A. L. Stone, *Federation Proc.*, **23**, 282 (1964).
(13) R. C. Moggridge and A. Neuberger, *J. Chem. Soc.*, 745 (1938).
(14) A. B. Foster, D. Horton, and M. Stacey, *ibid.*, 81 (1957).

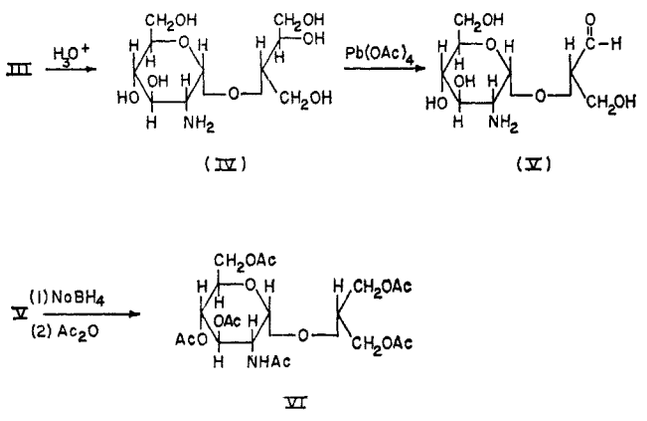
of Smith and co-workers[15] that such polysaccharide reduced aldehyde derivatives are hydrolyzed very easily. It is of interest to note that 2-acetamido-2-deoxy-D-glucose as well as 2-amino-2-deoxy-D-glucose were present in the hydrolysate. These results indicate that some cleavage of glycosidic bonds occurred first to give 2-acetamido-2-deoxy-D-glucose which was further hydrolyzed to yield the free amino sugar.

Fractions A and B were then subjected to lead tetraacetate oxidation according to the general technique of Perlin.[16]

The oxidation rate curve for fraction A is presented in Figure 2. The curve shows the initial consumption of 1 mole of lead tetraacetate followed by a much slower uptake of a second mole. This is in harmony with the formulation of fraction A as IV, in which the glycol unit in the amino sugar portion would be expected to be oxidized more slowly than that in the erythritol portion. In a preparative experiment, the oxidation was interrupted immediately after 1 mole of lead tetraacetate had been consumed. Reduction of the product with sodium borohydride with subsequent acetylation gave crystalline VI, the synthesis of which will be noted later. Similar oxidation and borohydride reduction of fraction B also yielded crystalline 2-*O*-(2-*O*-acetamido-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VI). Lack of material prevented adequate characterization of fractions A and B, yet both yielded crystalline VI.

In the present work the anomeric configuration of 2-*O*-(2-acetamido-tri-*O*-acetyl-2-deoxy-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VI) obtained by the degradative route outlined in Scheme II, and hence the configuration of the 2-amino-2-deoxy-D-glucopyranose to D-glucuronic acid linkage in heparin, was established by comparison of the naturally derived product VI with its two anomeric forms obtained by structurally definitive syntheses.

SCHEME II



Treatment of 2-acetamidotri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[17] with *cis*-1,3-*O*-benzylideneglycerol[18,19] in the presence of mercuric cyanide[20]

(15) Personal communication from the late F. Smith.

(16) A. S. Perlin, *Advan. Carbohydrate Chem.*, **14**, 9 (1959).

(17) D. Horton and M. L. Wolfrom, *J. Org. Chem.*, **27**, 1794 (1962); D. Horton, *Org. Syn.*, in press.

(18) H. S. Hill, M. S. Whelen, and H. Hibbert, *J. Am. Chem. Soc.*, **50**, 2235 (1928).

(19) N. Baggett, J. S. Brimacombe, A. B. Foster, M. Stacey, and D. H. Whiffin, *J. Chem. Soc.*, 2574 (1960).

(20) B. Helferich and K. F. Wedemeyer, *Ann.*, **563**, 139 (1949); B. Helferich and K. Weis, *Ber.*, **89**, 314 (1956).

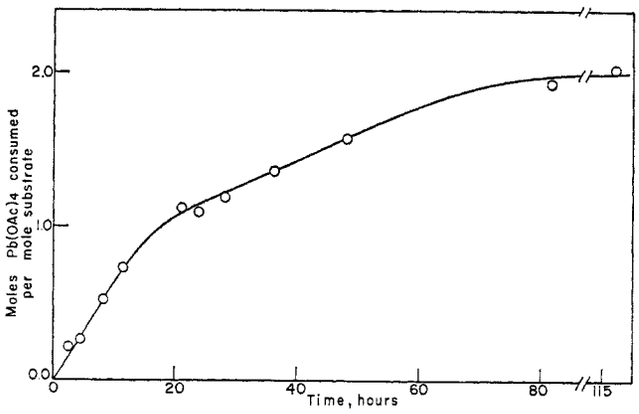
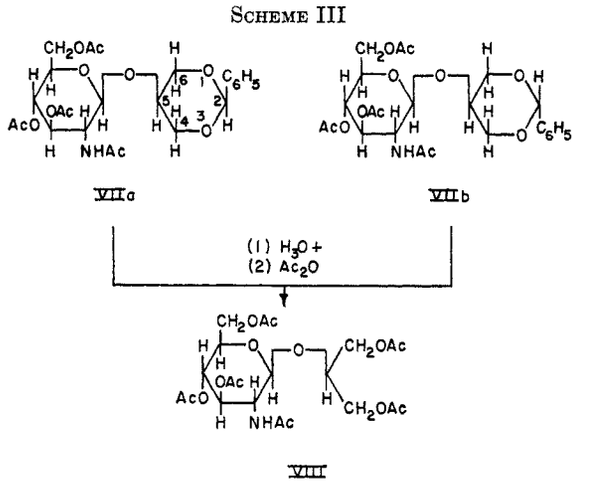


Figure 2.—Lead tetraacetate consumption by fraction A (calcd as IV); 25°.

in anhydrous benzene gave a crystalline material whose elemental analysis was consistent with the molecular formula for 2-*O*-(2-acetamidotri-*O*-acetyl-2-deoxy-D-glucopyranosyl)-1,3-*O*-benzylideneglycerol. This material existed in two forms which were separated by preparative thin layer chromatography. They possessed different melting points but like rotations and were isomeric by analysis. The two compounds have been formulated[1b] as *cis*- and *trans*-2-*O*-(2-acetamido-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-*O*-benzylideneglycerol (VIIa and VIIb), where the *cis* and *trans* refer to the hydrogens on carbons 2 and 5 on the 1,3-dioxane ring. It was established that the *trans* form was the more stable. The basis for the *cis* and *trans* assignments made has been discussed previously.[1b] Partial acid-catalyzed hydrolysis of the two stereoisomers followed by treatment of the product with acetic anhydride in pyridine gave in each case crystalline 2-*O*-(2-acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VIII) (see Scheme III).

SCHEME III



The assignment of the β-D configuration to compound VIII and the α-D configuration to compound VI has been made on the basis of Hudson's rules of isorotation,[21] which are known to be applicable to such acetylated derivatives.[22] The specific rotations in chloroform of the crystalline compounds VI and VIII

(21) C. S. Hudson, *J. Am. Chem. Soc.*, **31**, 66 (1909); *Advan. Carbohydrate Chem.*, **3**, 1 (1948).

(22) D. Horton, *J. Org. Chem.*, **29**, 1776 (1964).

are +59 ± 2° and −7°, respectively. It is to be noted that the glycerol aglycon is optically inactive.

The synthetic α-D anomer VI was obtained as follows. Both 2-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)glycerol and 2-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)glycerol, the latter detected chromatographically but not isolated, were formed by a Koenigs–Knorr reaction from tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylideneamino)-α-D-glucopyranosyl bromide[23] and 1,3-*O*-benzylideneglycerol, with subsequent acid-catalyzed hydrolysis of the benzylidene and *p*-methoxybenzylideneamino groups. The condensation was performed under the conditions reported by Hardy[24] for the reaction of tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylideneamino)-α-D-glucopyranosyl bromide with 1,3-di-*O*-benzylglycerol. The mixture of anomers was peracetylated, and the product was submitted to thin layer chromatography. One of the components migrated at the same rate as the 2-*O*-(2-acetamido-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VIII) synthesized from 2-acetamido-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride, as described previously, and the other migrated at the same rate as the naturally derived product VI. The latter component was isolated in crystalline form and was shown to be identical with VI but different from VIII by comparison of optical rotations, melting points, infrared and nuclear magnetic resonance (nmr) spectra, and X-ray powder diffraction patterns.

Thus, the assignment of the α-D configuration to compound VI in the present work establishes this configuration for the glycosidic linkage of 2-amino-2-deoxy-D-glucopyranose to D-glucuronic acid in heparin. There remains the definitive placement of all five sulfate groups per tetrasaccharide repeating unit before the complete organic structure for heparin can be written.

Experimental Section

Completely Desulfated, Partially Acetylated, Carboxyl-Reduced Heparin.—Partially acetylated, partially desulfated, carboxyl-reduced heparin[4] (3.6 g) was dissolved in 0.15 *N* methanolic hydrogen chloride (200 ml), and the solution was shaken at room temperature for 2 days. The solution was then dialyzed for 2 days against running, distilled water, and the dialyzate was concentrated to a small volume and freeze dried; yield 2.5 g. The product gave a negative test for sulfate.

***O*-Deacetylation of Completely Desulfated, Partially Acetylated, Carboxyl-Reduced Heparin.**—A solution of completely desulfated, partially acetylated, carboxyl-reduced heparin (4.2 g) in ethylene glycol (820 ml) and methanol (820 ml) was nearly saturated at 0° with ammonia and allowed to stand overnight at 0°. Ammonia was removed under reduced pressure, and the solution was dialyzed against running distilled water for 2 days. The dialyzate was concentrated to a small volume and freeze dried; yield 2.3 g; $[\alpha]^{25}D$ +59.5° (*c* 2.54, water); λ_{max}^{KBr} 3.10 (OH), 6.14, 6.60 ($NHCOCH_3$) 7.20, 7.70, 9.00, 9.90, and 11.70 μ; negative ninhydrin test.

Anal. Calcd for $C_{14}H_{23}NO_{10}$: C, 46.08; H, 6.35; N, 3.84. Found: C, 45.90; H, 6.03; N, 4.4; S, 0.0; OAc, 0.0.

Periodate Oxidation of Completely Desulfated, *O*-Deacetylated, *N*-Acetylated, Carboxyl-Reduced Heparin (I).—The heparin modification I (0.1015 g) was dissolved in water (45 ml), aqueous periodic acid (50 ml, 0.01 *M*) was added, and the total volume was brought to 100 ml with water. A blank experiment was carried out at the same time. The oxidation was effected at room temperature in the dark. Periodate consumption was determined by the method of Fleury and Lange;[5] the results are plotted in Figure 1. Formic acid yield was determined by acid titration, but none had been liberated after 25 hr. No formaldehyde was detected by the chromotropic acid method.[25] In a preparative experiment, I (220 mg) was treated with periodic acid as described above. After 48 hr the solution was dialyzed for 4 days against running distilled water. The dialyzate was concentrated to a small volume and freeze dried to a white powder (II); yield 200 mg (89%); $[\alpha]^{21}D$ +223° (*c* 2.33, water); λ_{max}^{KBr} 3.10 (OH), 5.90 (CHO), 6.15, 6.55 ($NHCOCH_3$), 7.38, 9.05, 9.75, and 11.35 μ; positive Benedict reduction.

2-Amino-2-deoxy-D-glucose from II.—A portion (20 mg) of the periodate oxidation product isolated above was treated for 4 hr on a boiling water bath with 2 *N* hydrochloric acid (5 ml). The hydrolysate was decolorized with carbon and concentrated to drynesss several times with 1-propanol to give a colorless syrup. Paper chromatography with the top layer of a 4:1:5 (v/v) 1-butanol–ethanol–water system revealed the presence of one component with a mobility corresponding to 2-amino-2-deoxy-D-glucose hydrochloride. The syrup was dissolved in a small volume of water, and acetone was added to incipient turbidity. The solution was allowed to stand overnight in a refrigerator. Needle-like crystals were formed whose X-ray powder diffraction pattern was identical with that of authentic 2-amino-2-deoxy-α-D-glucose hydrochloride; yield 8 mg.

Reduction of the Periodate Oxidation Product II.—The periodate oxidation product II (3.90 g) was dissolved in water (390 ml), mixed with an equal volume of an aqueous solution of sodium borohydride (0.26 *M*), and stirred for 20 min at room temperature. The pH of the solution was adjusted to 4.5 by the addition of acetate buffer (pH 4.3). The solution was dialyzed for 5 days against running distilled water, and the dialyzate was concentrated and freeze dried to yield a fine white powder (III); yield 3.9 g (99%); $[\alpha]^{23}D$ +118° (*c* 0.6, water); λ_{max}^{KBr} 3.10 (OH), 6.10, 6.60 ($NHCOCH_3$), 7.40, 9.10, 9.80, and 11.55 μ, CHO absent; negative Benedict reduction.

Hydrolysis of the Reduced Periodate Oxidation Product III.—The reduced periodate oxidation product III (15.0 mg) was dissolved in 0.1 *N* sulfuric acid (25 ml) and heated on a water bath at 75–80°. Aliquots were withdrawn at intervals and their optical rotations were measured. The rotation became constant at +89° in about 4 hr. The aliquots also were examined on paper chromatograms run in 4.0:1.2:1.2 (v/v) 1-butanol–ethanol–water. After 10 hr with 0.1 *N* sulfuric acid, silver nitrate–sodium hydroxide spray reagent[26] revealed the presence of one component with almost zero mobility and components at R_{GN} (GN = 2-amino-2-deoxy-D-glucose) 0.11, 0.45, 1.00, 1.25, 1.75, 2.22 (2-acetamido-2-deoxy-D-glucose), 2.61, and 3.50.

In a preparative experiment, the reduced periodate oxidation product III (1.0 g) was dissolved in 0.1 *N* sulfuric acid (100 ml) and heated on a water bath at 75–80° for 10 hr. The solution was then neutralized with barium carbonate, and the precipitate was removed by centrifugation. The supernatant was passed through a glass cloth filter pad, and the inorganic precipitate was washed several times with water. The combined filtrates were concentrated to a small volume. This solution was applied to 46 × 57 cm sheets of Whatman No. 3 paper to the ends of which had been sewn 10 × 46 cm strips of Whatman No. 1 paper. The chromatograms were developed with 4.0:1.2:1.2 (v/v) 1-butanol–ethanol–water for 13 days. The zones with R_{GN} 0.11 and 0.45 were located, excised, and extracted with the above solvent. Each of the extracts was concentrated to a volume of approximately 40 ml. The white, amorphous material that precipitated was removed by centrifugation and washed with alcohol and then with ether. Further material was obtained by the addition of ether to the supernatants; yield from combined zones with R_{GN} 0.11 (fraction B), 220 mg; mp 195–200° dec; $[\alpha]^{23}D$ +103° (*c* 0.84, water); yield from combined zones with R_{GN} 0.45 (fraction A), 120 mg; mp 145–150° dec; $[\alpha]^{23}D$ +112° (*c* 0.67, water). Both zone materials gave a positive ninhydrin reaction and were nonreducing toward Benedict solution. Their infrared spectra showed no carbonyl absorption.

2-*O*-(2-Acetamido-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VI). a. From Fraction A.—Fraction A (0.0427 g) was dissolved in glacial acetic acid (25 ml), a 0.05 *M* solution of lead tetraacetate in acetic acid (10 ml, 5.0×10^{-4} mole) was added, and the mixture was allowed to stand at room

(23) L. Zervas and S. Konstas, *Ber.*, **93**, 435 (1960).
(24) F. E. Hardy, *J. Chem. Soc.*, 375 (1965).
(25) D. A. MacFadyen, *J. Biol. Chem.*, **158**, 107 (1945).
(26) W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature*, **166**, 444 (1950).

temperature with occasional shaking. At intervals, 2-ml aliquots were withdrawn and the consumption of lead tetraacetate was determined by the addition of 0.12 *N* potassium iodide (2 ml) to each aliquot and titration with 0.05 *N* sodium thiosulfate. The results are plotted in Figure 2.

In a preparative experiment, fraction A (394 mg) was dissolved in glacial acetic acid (391 ml), a 0.05 *M* solution of lead tetraacetate in acetic acid (82.2 ml, 4.1×10^{-3} mole) was added, and the mixture was kept at room temperature for 24 hr with occasional shaking. A 0.0185 *M* solution of anhydrous oxalic acid in acetic acid (199 ml) was then added. The precipitate was removed by centrifugation. The supernatant was filtered, and the filtrate was concentrated under reduced pressure to a syrup. This material was dissolved in water (21 ml), mixed with a 1% aqueous solution of sodium borohydride (21 ml), and stirred at room temperature for 20 min. An acetate buffer solution of pH 4.3 (21 ml) was then added with stirring. The insoluble material was removed by filtration, and the filtrate was concentrated under reduced pressure to a syrup. This syrup was treated with acetic anhydride (10 ml) in pyridine (10 ml), and the solution was maintained at room temperature for 18 hr. The acetylated derivative was isolated in the usual manner and purified by preparative thin layer chromatography on silica gel G with ethyl acetate development. This material was dissolved in ethanol, and petroleum ether (bp 30–60°) was added to incipient turbidity. Nucleation with a synthetic sample (see below) of 2-*O*-(2-acetamidotri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VI) resulted in the formation of needlelike crystals: mp 122.0–123.5°; $[\alpha]^{22}D$ +57° (*c* 1.72, chloroform); λ_{max}^{KBr} 5.75 ($OCOCH_3$), 6.04, 6.55 ($NHCOCH_3$), 8.10 ($OCOCH_3$), and 9.58 μ; nmr data,[27] τ 8.05 (three protons, $NCOCH_3$), 7.98 (six protons, $OCOCH_3$), 7.94 (nine protons, $OCOCH_3$), and 3.90 (one-proton broad doublet, NH); X-ray powder diffraction data,[28] 12.99 (m), 8.76 (vs, 2), 7.56 (vs, 1), 6.51 (m), 5.68 (m), 5.25 (m), 4.80 (vw), 4.59 (vw), 4.42 (s), 4.21 (s), 3.97 (s), 3.83 (w), 3.72 (vs, 3), 3.36 (vw), 3.24 (vw), 3.14 (vw), 2.05 (vw), 2.96 (vw), 2.86 (vw), and 2.66 (vw).

Anal. Calcd for $C_{21}H_{31}NO_{13}$: C, 49.89; H, 6.17; N, 2.77. Found: C, 49.81; H, 6.41; N, 2.66.

b. From Fraction B.—Fraction B (206 mg) was reduced with sodium borohydride, then acetylated as described above, and the crystalline product VI was isolated in the same manner; yield 25 mg. This substance was found to be identical with that obtained in method a by melting point, infrared and nmr spectra, rotation, and X-ray powder diffraction pattern.

***cis* and *trans* Forms of 2-*O*-(2-Acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-*O*-benzylideneglycerol (VIIa and VIIb).**—To a solution of 2-acetamidotri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[17] (5.1 g) in dry benzene (63.8 ml) were added mercuric cyanide (4.02 g) and *cis*-1,3-*O*-benzylideneglycerol[18,19] (6.50 g), and the mixture was shaken mechanically at room temperature for 2.5 days. Chloroform (100 ml) was then added, and the solution was extracted several times with water. The chloroform solution was concentrated under reduced pressure to yield a syrup which crystallized from ethanol–petroleum ether (bp 30–60°); yield 1.5 g (20.4%); mp 197–203°; $[\alpha]^{21}D$ −10° (*c* 1.97, chloroform); λ_{max}^{KBr} 5.75 ($OCOCH_3$), 6.04, 6.55 ($NHCOCH_3$), 8.10 ($OCOCH_3$), 9.58, 13.20, and 14.35 (aromatic ring) μ; X-ray powder diffraction data,[28] 15.2 (vw), 13.4 (vw), 12.1 (vw), 10.4 (w), 9.20 (m), 8.34 (m), 7.78 (w), 6.80 (vw), 6.09 (vw), 5.48 (vw), 5.04 (s, 1), 4.70 (w), 4.39 (s, 2), 4.04 (w), 3.75 (s, 3), 3.64 (w), 3.41 (w), 3.20 (w).

Anal. Calcd for $C_{24}H_{31}NO_{11}$: C, 56.55; H, 6.15; N, 2.76. Found: C, 56.76; H, 6.23; N, 2.82.

Thin layer chromatography of the crystalline product on silica gel G with ethyl acetate development revealed two components. Separation by preparative thin layer chromatography afforded approximately equal amounts of crystalline *cis*- and *trans*-2-*O*-(2-acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-*O*-benzylideneglycerol (VIIa and VIIb).[1b] The *cis* compound VIIa has R_f 0.43 (thin layer chromatography as above); mp 191°; $[\alpha]^{33}D$ −8° (*c* 2.06, chloroform); λ_{max}^{KBr} 5.75 ($OCOCH_3$), 6.04, 6.55 ($NHCOCH_3$), 8.10 ($OCOCH_3$), 9.58, 13.20, and 14.35 (aromatic ring) μ; nmr data,[27] τ 8.19 (three protons, $NCOCH_3$), 7.98 (six protons, $OCOCH_3$), 7.94 (three protons, $OCOCH_3$), 4.48 (one proton, Ph–CH), 3.35 (one-proton broad doublet, NH); X-ray powder diffraction data,[28] 9.40 (w), 8.52 (m), 7.59 (m), 7.19 (w), 4.86 (s, 1), 4.25 (w), 3.88 (s, 2), 3.68 (m), 3.32 (s, 3), 3.18 (w).

Anal. Calcd for $C_{24}H_{31}NO_{11}$: C, 56.55; H, 6.15; N, 2.76. Found: C, 56.63; H, 6.14; N, 3.10.

The *trans* compound VIIb has R_f 0.60; mp 211–212°; $[\alpha]^{33}D$ −9° (*c* 2.12, chloroform); λ_{max}^{KBr} 5.75 ($OCOCH_3$), 6.04, 6.55 ($NHCOCH_3$), 8.10 ($OCOCH_3$), 9.58, 13.20, and 14.35 (aromatic ring) μ; nmr data,[27] τ 8.05 (three protons, $NCOCH_3$), 7.98 (six protons, $OCOCH_3$), 7.94 (three protons, $OCOCH_3$), 4.61 (one proton, Ph–CH), 3.69 (one-proton broad doublet, NH); X-ray powder diffraction pattern data,[28] 12.3 (w), 10.8 (s, 3,3), 10.0 (vw), 8.21 (s, 3,3), 6.60 (m), 6.38 (w), 6.12 (s, 2), 4.89 (m), 4.56 (w), 4.25 (vs, 1), 4.07 (vw), 3.86 (w), 3.60 (w), 3.46 (vw), 3.30 (w), and 3.15 (vw).

Anal. Calcd for $C_{24}H_{31}NO_{11}$: C, 56.55; H, 6.15; N, 2.76. Found: C, 56.57; H, 6.46; N, 2.81.

Refluxing a benzene–chloroform (9:1 v/v) solution of VIIa for 7 hr resulted in its partial conversion into VIIb, as demonstrated by thin layer chromatography. Refluxing a toluene–chloroform (9:1 v/v) solution of VIIb did not cause any formation of VIIa.

2-*O*-(2-Acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VIII).—The crystalline mixture of stereoisomers isolated above (1.0 g) was dissolved in a 3% solution (30 ml) of hydrochloric acid in 70% aqueous ethanol and allowed to stand at room temperature for 15 min. The hydrolysate was concentrated to dryness several times with 1-propanol. The residual material was treated with acetic anhydride in pyridine in the usual manner. The acetylated derivative VIII crystallized from ethanol–petroleum ether (bp 30–60°); yield 0.62 g (63%); mp 150–152°; $[\alpha]^{33}D$ −7° (*c* 1.65, chloroform); λ_{max}^{KBr} 5.75 ($OCOCH_3$), 6.04, 6.40 ($NHCOCH_3$), 8.10 ($OCOCH_3$), 8.57, and 9.60 μ, aromatic absent; nmr data,[27] τ 8.05 (three protons, $NCOCH_3$), 7.98 (six protons, $OCOCH_3$), 7.94 (nine protons, $OCOCH_3$), 3.68 (one-proton broad doublet, NH); X-ray powder diffraction data,[28] 12.3 (vs, 3), 10.7 (vs, 1), 8.65 (vs), 7.46 (s), 7.06 (m), 5.82 (vw), 5.46 (vw), 5.36 (vw), 4.95 (vw), 4.72 (vs, 2), 4.40 (s), 4.12 (s), 3.92 (vw), 3.76 (w), 3.50 (m), and 3.38 (vw).

Anal. Calcd for $C_{21}H_{31}NO_{13}$: C, 49.89; H, 6.17; N, 2.77. Found: C, 50.33; H, 6.59; N, 2.71.

2-*O*-(2-Acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VIII) was obtained also when compounds VIIa and VIIb were treated individually as described above.

Synthesis of 2-*O*-(2-Acetamidotri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VI).—To a solution of *cis*-1,3-*O*-benzylideneglycerol[18,19] (3.6 g) in dry benzene (40 ml) were added Drierite[29] (20 g) and silver carbonate (7.2 g), and the mixture was shaken mechanically for 15 hr in the dark. A solution of tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylideneamino)-α-D-glucopyranosyl bromide[23] (2.5 g) in dry benzene (24 ml) was introduced during 30 min with constant shaking in the dark. The shaking with occasional release of carbon dioxide was continued for 24 hr. More silver carbonate (2 g) and glycosyl bromide (2.5 g) in benzene (24 ml) were added, and the mixture was shaken for 54 hr. The mixture was then centrifuged and the supernatant and benzene washings were extracted several times with water. The benzene solution was dried (sodium sulfate) and concentrated to dryness. Methanol (300 ml), saturated with ammonia at 0°, was added, and the solution was maintained overnight and then concentrated to dryness. The residue was heated for 40 min on a water bath with 0.13 *N* sulfuric acid (200 ml) and cooled, and 1 *N* sulfuric acid (10 ml) was added. The solution was extracted several times with chloroform and neutralized with barium carbonate. The precipitate was removed by centrifugation, and the supernatant was concentrated to a syrup; yield 3.2 g. A portion (0.96 g) of this material was dissolved in a little water and applied to a column of Dowex-1 (OH^-) resin (60 ml). Both 2-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)glycerol and 2-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)glycerol[24] were eluted together with water.

(27) The nmr spectra reported herein were determined with a Varian A-60 nmr spectrometer using approximately 10% by weight solutions in deuteriochloroform with tetramethylsilane as internal reference. The probe temperature was approximately 30°.

(28) X-Ray powder diffraction pattern data refer to interplanar spacing in angstroms 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.

(29) A product of the W. A. Hammond Drierite Co., Xenia, Ohio.

The mixture of anomers was treated with acetic anhydride in pyridine in the usual manner. Two components were revealed by thin layer chromatography on silica gel G with ethyl acetate development. The slower moving component migrated at the same rate (R_f 0.47) as 2-*O*-(2-acetamidotri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3-di-*O*-acetylglycerol (VIII), and the other component migrated at the same rate (R_f 0.53) as the naturally derived product VI. The latter component was isolated by preparative thin layer chromatography performed in the same manner and obtained in crystalline form from ethanol–petroleum ether (bp 30–60°); mp 123.5°, undepressed on admixture with crystalline, naturally derived VI; $[\alpha]^{20}D$ +60° (*c* 1.99, chloroform); infrared and nmr spectra and X-ray powder diffraction pattern identical with those of crystalline, naturally derived VI.

Anal. Calcd for $C_{21}H_{31}NO_{13}$: C, 49.89; H, 6.17; N, 2.77. Found: C, 50.07; H, 5.93; N, 2.86.

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The Configuration and Formation of Percival Dianhydroosazone[1a]

H. EL KHADEM AND M. M. A. ABDEL RAHMAN

Chemistry Department, Faculty of Science, Alexandria University, Alexandria, Egypt, United Arab Republic

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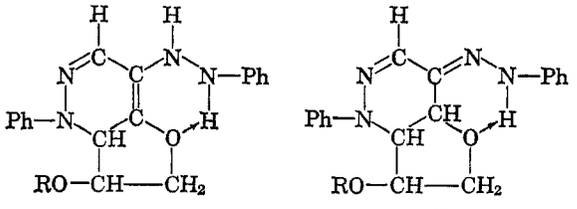
The nuclear magnetic resonance spectrum of Percival dianhydroosazone acetate agreed with structure III which possesses one imino proton and a *trans* arrangement between H_c and H_d and between H_d and H_e. Since the configuration of H_e is known, it was possible to determine the configuration of H_c and H_d and show that Percival osazone was a dianhydro-D-*lyxo* derivative. Dianhydro-D-*lyxo*-hexulose *o*-, *m*-, and *p*-tolylosazones and *p*-methoxyphenylosazone were also prepared from tetra-*O*-acetyl-D-*arabino*-hexulose arylosazones, but *N*-acetylated osazones and acetates of 3,6-anhydroosazones failed to give dianhydro derivatives.

Nuclear magnetic resonance spectroscopy has recently[1-4] been used to establish the structure of osazones. We have now used this technique to prove the structure of Percival dianhydroosazone and to establish its configuration.

Percival dianhydroosazone[5] is formed by deacetylation of hexose phenylosazone tetraacetates with sodium hydroxide in aqueous acetone. It exists in two enantiomorphic forms depending upon the D and L configuration of the starting osazone. Thus, saccharides having different configuration on C-3 and C-4, such as D-glucose, D-galactose, and D-gulose, give the same dianhydro compound, and its enantiomer is obtained from the L sugars. It is therefore believed[5] that an inversion of configuration at C-3 and C-4 takes place in certain cases to assume a stable configuration and that only the asymmetric carbon atom at C-5 in the osazone retains its configuration during anhydride formation.

Percival dianhydroosazone gives a monoacetate and a monomethyl ether and upon treatment with nitrous acid loses only one hydrazone residue, suggesting that the other hydrazone residue is involved in anhydroring formation. This, and the fact that the anhydroosazone fails to give the formazan reaction, led Henseke[6] to suggest the tautomeric structures Ia and IIa for this anhydroosazone.

It is obvious that the nmr spectrum of structure I would reveal two imino protons, whereas that of structure II would reveal only one imino proton, thus affording a simple means of distinguishing between the



I II

a, R = H
b, R = CH_3CO

two tautomeric forms. Our nmr study of Percival dianhydroosazone acetate (Figure 1) showed only one imino proton at δ 9.53 ppm, thus favoring tautomeric form II. The rest of the nmr spectrum was also in agreement with the carbon–hydrogen skeleton of IIb. The doublet at δ 5.08 ppm was assigned to the methine proton of C-3 of the osazone precursor, which is linked to the etheric oxygen; the coupling constant of this proton to the proton whose resonance appeared at δ 4.65 ppm and which was assigned to the C-4 proton of the osazone precursor was relatively large (J = 8 cps). This large coupling was explained by an axial–axial *trans* relationship between the C-3 and C-4 protons. The latter proton, appearing at δ 4.65 ppm, was split by a large coupling of J = 8 cps by the *trans* proton of C-3, and with a moderately large coupling of J = 6 cps by the proton at about δ 5.65 ppm, assigned to the methine proton of C-5 bearing the *O*-acetyl group. The moderate size coupling of J = 6 cps was also ascribed to a *trans* arrangement of the protons of C-4 and C-5, but probably quasi-axial–axial. The proton of C-5, appearing at δ 5.65 ppm, was split with a coupling constant of J = 6 cps by the *trans* proton of C-4 and the *trans* proton of the C-6 methene, and a small coupling of J = 3 cps by the *cis* proton at C-6. The geminal protons of the methene group at C-6 of the osazone precursor appeared at δ 4.3 and 3.7 ppm, both being split by a large geminal coupling of J = 11 cps. The *trans* proton at δ 4.3 ppm was split by the

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