[CONTRIBUTION FROM THE TROPICAL PRODUCTS INSTITUTE DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH]

Long-Chain Derivatives of Sugars. I. Some Reactions of *N*-Octadecyl-D-glucosylamine

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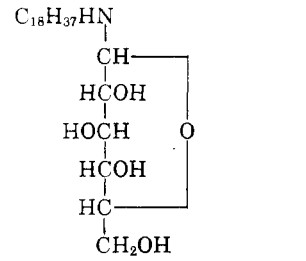
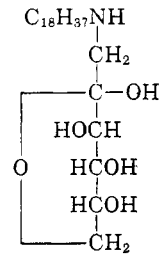
The reaction of *N*-octadecyl-D-glucosylamine (I) with chloroacetic acid in pyridine gives rise to a hydrochloride, probably the iminium salt (V), which is converted by alkali into 1-deoxy-1-*N*-octadecylamino-D-fructose (II), isomeric with I. The formation of II is equivalent to an Amadori rearrangement of I, and these reactions provide evidence on the mechanism of the Amadori rearrangement. Both the isomers I and II lose water on treatment with alkali.

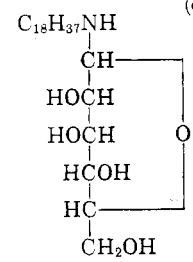
In an attempt to prepare *N*-octadecyl-*N*-D-glucosylglycine, *N*-octadecyl-D-glucosylamine (I) was treated with chloroacetic acid in pyridine. The product, the hydrochloride of a base, was converted by treatment with one equivalent of alkali into a compound isomeric with the glucosylamine I. Since glycosylamines are known to be, in general, subject to rearrangement,[1] it seemed advisable to confirm the structure I assigned[2] to the glucosylamine. The following confirmatory evidence was adduced: The glucosylamine reduced Fehling's solution at an extremely slow rate. On treatment with sodium metaperiodate, a rapid uptake of four moles took place, followed by the slow uptake of a fifth mole.[3] By paper chromatography, using butanol–acetic acid as the developing solvent, the glucosylamine gave two spots, corresponding to glucose (R_f 0.19) and octadecylamine (R_f 0.90).[4] Finally, the infrared spectrum of the glucosylamine did not contain any absorption band between 1460 and 2000 cm.$^{-1}$, confirming that the compound existed as a cyclic form; a number of *N*-arylglucosylamines have also been shown[5] to give no absorption band corresponding to the —C=N— linkage of an open chain form.



I



II
(or ring isomer)



III

The compound produced from the glucosylamine I by successive treatment with chloroacetic acid and alkali was isomeric with the starting material. Although both compounds had similar melting points, there was a distinct depression on admixture. The most likely structure for the new isomer appeared to be 1-deoxy-1-*N*-octadecylamino-D-fructose (II) which would correspond to an Amadori rearrangement[1] of I. This structure was established as follows:

The new isomer was levorotatory (α_D dimethoxyethane −27°), as are most of the deoxyaminofructose compounds produced by Amadori rearrangements.[1] It readily reduced Fehling's solution, and rapidly consumed three moles of sodium metaperiodate, followed by a fourth mole at a very slow rate. It has been reported[6] that the system CH(OH)CH(NHR) is oxidized by periodate only at an extremely slow rate. Unlike the glucosylamine I the isomer moved in paper chromatography as a single substance (R_f 0.88) and gave the color reactions of both an amine (with ninhydrin) and a reducing sugar (with *p*-aminobenzoic acid and

(1) J. E. Hodge, *Advances in Carbohydrate Chem.*, **10**, 169 (1955).

(2) J. G. Erickson, *J. Am. Chem. Soc.*, **77**, 2839 (1955).

(3) Although *N*-acetyl- [C. Niemann and J. T. Hays, *J. Am. Chem. Soc.*, **62**, 2960 (1940)] and *N*-glycylglucosylamine [J. Baddiley, J. G. Buchanan, R. E. Handschumacher, and J. F. Prescott, *J. Chem. Soc.*, 2818 (1956)] have been found to react with two moles of periodate (evidently the oxidation did not involve rupture of the pyranose ring), a number of *N*-arylglucosylamines consumed five moles of periodate [G. A. Howard, G. W. Kenner, B. Lythoe, and A. R. Todd, *J. Chem. Soc.*, 861 (1946)], corresponding to complete degradation of the glucose moiety. M. H. Benn and A. S. Jones [*J. Chem. Soc.*, 3837 (1960)] found that *N*-glucosylurea consumed two moles of periodate at 0°, but above that temperature over-oxidation took place.

(4) J. L. Barclay, A. B. Foster, and W. G. Overend [*J. Chem. Soc.*, 1541 (1955)] reported that *N*-arylglycosylamines were dissociated to the amine and sugar on attempted chromatography in aqueous media. Several *N*-alkylglycosylamines, including the octyl and decyl compounds, have been shown [E. Mitts and R. M. Hixon, *J. Am. Chem. Soc.*, **66**, 483 (1944)] to be dissociated to the primary amine under acidic conditions.

(5) F. Legay, *Compt. rend.*, **234**, 1612 (1952).

(6) T. Pasternak and H. Pollaczek, *Helv. Chim. Acta*, **24**, 1190 (1941); R. Adams and M. Gianturno, *J. Am. Chem. Soc.*, **78**, 1920 (1956).

oxalic acid). Similar results have been obtained with the fructosyl derivatives of amino acids.[7] There were no absorption bands between 1460 and 2000 cm.$^{-1}$ in the infrared spectrum of the new isomer, and no maximum was observed in the ultraviolet spectrum, showing that the reducing group in the carbohydrate exists as a hemiacetal and not in the carbonyl form.

The Amadori rearrangement of nonaromatic glycosylamines has been effected by their treatment with compounds containing an active methylene group[8,9] or with acetic acid,[1] but no solid product could be isolated when the glucosylamine I was treated with malonic or acetic acids.

If structure II is correct, then the same compound should be produced from *N*-octadecyl-D-mannosylamine (III). The latter substance was readily produced by reaction of mannose with octadecylamine in aqueous isopropyl alcohol at room temperature,[2] and it was converted into a hydrochloride (see below) by treatment with chloroacetic acid in pyridine. Neutralization of the hydrochloride then yielded the compound II identical with that prepared from I.

Glycosylamines and their Amadori rearrangement products may be differentiated by means of a color test with *o*-dinitrobenzene,[10] in which the rearranged products give a purple color in 1 minute whereas the glycosylamines give no color within 15 minutes. The glucosylamine I gave a purple color in 5–6 minutes, and the isomer II in 1 minute.

The hydrochloride obtained from I by the action of chloroacetic acid in pyridine had the elementary analysis, active hydrogen equivalent (Zerevitinov), and acid equivalent corresponding to the hydrochloride of a compound $C_{24}H_{49}NO_5$, *i.e.*, either I or an isomer. It did not have any discrete melting point, gradually becoming wax-like between 70° and 150° and decomposing at the latter temperature. It was readily soluble in hot water, readily reduced Fehling's solution, and had α_D ethanol −12°. The ultraviolet spectrum had λ_{max} 261 mμ (ϵ 94) and the infrared spectrum contained a band at 1630 cm.$^{-1}$ and a weak absorption band at 1575 cm.$^{-1}$; the latter band corresponds to an —NH— absorption, as there is an overtone at 3140 cm.$^{-1}$ The consumption of sodium periodate and of periodic acid corresponded to a reaction with four moles of periodate. In the *o*-dinitrobenzene color test, a purple color was produced within one minute. As already stated, the hydrochloride was converted to II by treatment with alkali; precipitation of the latter occurred immediately when one equivalent of alkali was added to an alcoholic solution of the hydrochloride.

When one equivalent of sulfuric acid or sodium sulfate was added to an aqueous solution of the hydrochloride, the corresponding sulfate was precipitated. This salt also readily reduced Fehling's solution, and its infrared spectrum contained a band at 1610 cm.$^{-1}$ The periodate consumption was 4 moles. This sulfate was also converted to II by treatment with one equivalent of alkali.

It seemed certain, therefore, that the hydrochloride was the quaternary salt of one of the equivalent forms of the glucosylamine I, and that in its formation the chloroacetic acid functions merely as a source of hydrogen chloride.[11,12] This was confirmed by the isolation of the same material from treatment of the glucosylamine I with 4.5 moles of hydrogen chloride in pyridine (equivalent to the quantity of chloroacetic acid previously used).

The ultraviolet and infrared spectra of the hydrochloride show that it possesses a double bond system. This necessarily excludes the possibility that the hydrochloride is the salt of either the glucosylamine I or its isomer II. Two alternative structures, both containing a double bond, would be the salts, V and VIII, derived from the open chain forms of I and II, respectively. The former, V, derived from the Schiff base IV, would be an iminium salt, a grouping which is known to exist in the salts (X) obtained from tetrahydropyridines (IX) and the corresponding quinolines.[13]

The salt V would be expected to yield II on treatment with base, for the removal of a proton could occur either from the nitrogen atom (giving again the Schiff base IV) or from the "allylic" carbon atom (giving the enol VI corresponding to the fructose derivative II). The more stable of the isomers I or II would therefore be formed, and the existence of the Amadori rearrangement suggests that II is more stable than I.

The ultraviolet absorption of the hydrochloride (λ_{max} 261 mμ, ϵ 94) resembles that of a carbonyl group, and is compatible with structures V and VIII.[14]

(7) Dr. H. S. Burton, private communication.

(8) J. E. Hodge and C. E. Rist, *J. Am. Chem. Soc.*, **75**, 316 (1953).

(9) J. E. Hodge and C. E. Rist, *J. Am. Chem. Soc.*, **74**, 1494 (1952).

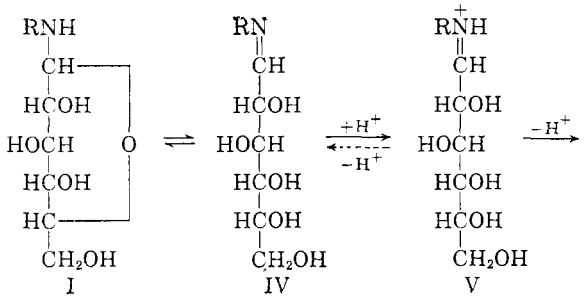
(10) W. R. Fearon and E. Kawerau, *Biochem. J.*, **37**, 326 (1943).

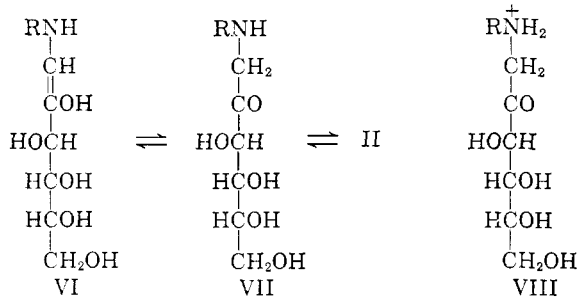
(11) Evaporation of a pyridine solution of chloroacetic acid gave a crystalline salt, whose aqueous solution contains free chloride ions. This must be, at least in part, *N*-carboxymethylpyridinium chloride, ($C_5H_5\overset{+}{N}CH_2COOH$) Cl^-, which would be equivalent to the zwitterion $C_5H_5\overset{+}{N}CH_2CO_2^-$ and free hydrogen chloride.

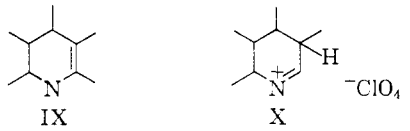
(12) L. Rosen, and J. W. Woods, and W. Pigman, *Chem. Ber.*, **90**, 1038 (1957), found that *N*-*p*-tolylglucosylamine underwent the Amadori rearrangement in almost quantitative yield on treatment with one equivalent of hydrogen chloride in pyridine.

(13) N. J. Leonard and F. P. Hauck, Jr., *J. Am. Chem. Soc.*, **74**, 5279 (1957) and previous papers.

(14) A. E. Gillam and E. S. Stern, *An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry*, Arnold, London, 2nd Ed., 1957. The system $C=\overset{+}{N}$ is isoelectronic with the carbonyl group.



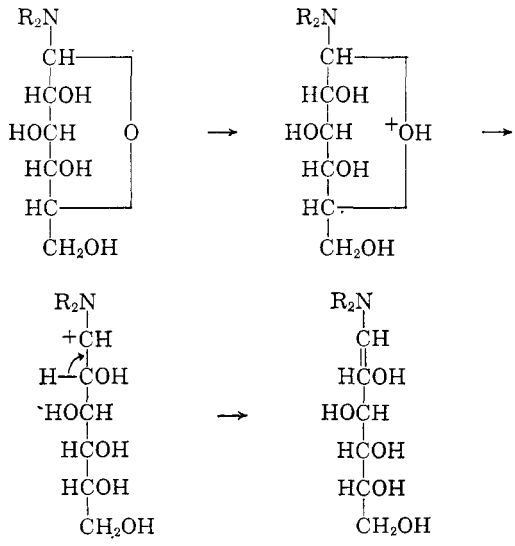




If the hydrochloride possessed structure VIII, it would be expected to show keto carbonyl absorption,[15] *i.e.*, at 1705–1725 cm.$^{-1}$ The infrared spectrum of the hydrochloride (ν_{max} 1630 cm.$^{-1}$) is therefore scarcely compatible with structure VIII. With regard to the alternative structure V, the infrared bands of the hydrochloride and the derived sulfate (1610 cm.$^{-1}$) occurs at lower frequencies than has been observed for the iminium salts X (1670–1700 cm.$^{-1}$),[13] but this discrepancy is not unexpected as the systems are not closely similar. In fact, in view of the values quoted by Bellamy[16] the absorption at 1630 cm.$^{-1}$ does not seem incompatible with structure V, so that this structure is tentatively assigned to the hydrochloride.

Several workers[17,18] have recently reported that the Amadori rearrangement of glycosylamines or their benzylidene derivatives can be effected by treatment with oxalic acid. The products were initially isolated as the oxalates, which were considered to be the salts (as VIII) of the keto form on the basis of an infrared band at 1725 cm.$^{-1}$ We have been unable to isolate any recognizable product from treatment of either the glucosylamine I or the mannosylamine III with oxalic acid. However, the fructose derivative II was converted by oxalic acid into a salt whose infrared spectrum showed medium intensity bands at 1730 and 1715 cm.$^{-1}$ and weak bands at 1646 and 1634 cm.$^{-1}$ On the basis of the previous work,[17,18] this oxalate would appear to be the salt of the keto form VIII. It must, however, be noted that Micheel and his coworkers[17] and also Zaugg[18] did not quote any absorption bands for the oxalate group; the spectrum of the oxalate from II did not show the strong bands reported[15] for oxalate salts (1550–1610 cm.$^{-1}$) but oxalic acid absorbs at 1690 and 1710 cm.$^{-1}$ It may be, therefore, that the absorptions observed between 1715 and 1730 cm.$^{-1}$ in all these compounds could be due to the oxalate group, in which case the salts would be presumed to exist in the cyclic form, or possibly as the iminium salts V. The oxalate from II showed no maximum in the ultraviolet.

The various mechanisms which have been proposed for the Amadori rearrangement have been discussed in detail by Hodge.[1] One of the suggested mechanisms[19] is in fact that represented by formulas I, IV, V, VI, VII, II above. An alternative mechanism, proposed by Gottschalk,[20] involves addition of a proton to the ring oxygen atom, and proceeds through a carbonium ion:



The isolation of a stable salt from the glucosylamine I (irrespective of the accuracy of the as-

(15) L. J. Bellamy, *The Infrared Spectra of Complex Molecules*, Methuen, London, 2nd ed., 1958.

(16) Bellamy (ref. 15) concludes that "Provided that the C=N link is not conjugated and carries no charge on the nitrogen atom, the stretching frequency for unstrained compounds of all types may be expected at 1680–1650 cm.$^{-1}$, with somewhat lower values in the solid state . . . in $C=\overset{+}{N}$ compounds the frequencies are raised a little above the normal." (All spectra in the present work were determined in Nujol mull).

(17) F. Micheel and A. Frowein, *Chem. Ber.*, **90**, 1599 (1957); F. Micheel and G. Hagemann, *Chem. Ber.*, **93**, 2381 (1960).

(18) H. E. Zaugg, *J. Org. Chem.*, **26**, 603 (1961).

(19) R. Kuhn and F. Weygand, *Ber.*, **70**, 769 (1937); F. Weygand, *Ber.*, **73**, 1259 (1940).

(20) A. Gottschalk, *Biochem. J.*, **52**, 455 (1952).

signment of structure V) provides support for a mechanism which involves quaternary ammonium compounds, such as that proposed by Kuhn and Weygand.[19]

The iminium salt form (as V) has also been postulated as an intermediate in the mutarotation of glycosylamines.[21]

Attempts were made to prepare the imine hydrochloride (V) from the fructose derivative (II). The latter was recovered unchanged from treatment with one or two equivalents of hydrogen chloride in pyridine. On the other hand reaction with chloroacetic acid in pyridine gave material having a low chlorine content and which rapidly consumed five moles of periodate. It behaved similarly to II and V on paper chromatography, and was reconverted to II on neutralization. This material must contain a hydrochloride, and although it appears to differ from V, it may nevertheless be a mixture of II and V.

When the glucosylamine I or its isomer II was treated with one equivalent of sodium hydroxide, a substance (A) was obtained whose analysis and periodate consumption (1 mole) suggested that the substance was formed by the loss of two moles of water from I or II.[22]

The intermediate in the preparation of the fructose derivative II from the mannosylamine III contained less than one mole of chlorine. It readily reduced Fehling's solution, and on paper chromatography it behaved like II and V, giving a single spot (R_f 0.90) which gave the color reactions of both an amine and a reducing sugar. The periodate consumption differed from that of V, only 3.5 moles being used up. This material presumably differs from the hydrochloride from I only in the configuration at C-2, but in view of the low chlorine content, no direct comparison with V was possible.

In the *o*-dinitrobenzene color test, the mannosylamine III gave no color within 20 hours at room temperature, although an immediate color was produced on warming. The derived hydrochloride differed from V in that it only gave a strong color after five minutes.

EXPERIMENTAL

"Light petroleum" refers to the fraction b.p. 60–80°. Ultraviolet spectra were determined in ethanol, and infrared spectra in Nujol mull. The analytical figures for oxygen refer to direct determinations. All analytical samples were dried at 25°/0.01 mm. for 5 hr.

Physical state of the substances. The substances described were not crystalline in the accepted sense, but were amorphous, somewhat waxy solids. The term "recrystallization" is used only to describe the effective process.

Paper chromatography. All chromatograms of the ascending type used, except where otherwise stated, butanol–acetic acid–water (4:1:5) (upper phase) as the developing solvent. All papers were run in duplicate, one paper being sprayed with ninhydrin and the other with *p*-aminobenzoic acid and oxalic acid.

Periodate oxidation. All the compounds described gave an immediate precipitate on treatment with sodium metaperiodate or periodic acid. The measurements therefore refer to individual experiments (in duplicate) and not to aliquot samples. The oxidation experiments were carried out in aqueous solution except that when the compound was insufficiently soluble in water, ethanol (for the sulfate derived from V) or redistilled 1:2-dimethoxyethane (for II and compound A) was used as solvent.

N-Octadecyl-D-glucosylamine (I), prepared by Erickson's method[2], crystallized from ethanol having m.p. 104–106°. Erickson gives m.p. 104–105°.

Anal. Calcd. for $C_{24}H_{49}NO_5$: C, 66.78; H, 11.44. Found: C, 67.23; H, 11.60.

Paper chromatograms were run using as developing solvents butanol–acetic acid, ethyl acetate–pyridine, and aqueous isopropanol. In each case, two separate spots were obtained, exactly corresponding to glucose and octadecylamine. With ethyl acetate–pyridine and isopropanol, there was marked streaking (towards the front) of the glucose spot, presumably due to gradual dissociation of the glucosylamine in these solvents.

The periodate consumption of the glucosylamine was as follows:

	30 Min.	24 Hr.	48 Hr.
Sodium metaperiodate consumed, moles/mole	3.79	4.44	4.51
Formic acid liberated, moles/mole	1.83	2.71	—
Periodic acid consumed, moles/mole	3.66	4.22	4.25

Treatment of the glucosylamine with benzoyl chloride afforded the tetrabenzoate, m.p. 116–118° (from light petroleum).

Anal. Calcd. for $C_{52}H_{65}NO_9$: C, 73.64; H, 7.73. Found: C, 74.24; H, 7.58.

Reaction of I *with chloroacetic acid:* A solution of 100 g. of the glucosylamine I and 100 g. of chloroacetic acid in 1 l. of dry pyridine was set aside overnight. The mixture, from which some pyridine chloroacetate had separated, was partitioned between 2 l. of water and 2 l. of 4:1 ethyl acetate–butanol. The organic layer was washed twice with water, counterwashings between carried out, and then dried (sodium sulfate) and evaporated. The semisolid residue was taken up in hot ethyl acetate–ethanol. On cooling 77 g. of the product was separated. Further recrystallization from the same solvent mixture, or from benzene–light petroleum, afforded the hydrochloride, probably V, a pale fawn solid which became wax-like at 60–70° and decomposed at *ca.* 150°; α_D ethanol −12° λ_{max}^{Nujol} 1630 and 1575 cm.$^{-1}$; there were no other maxima between 1460 and 2000 cm.$^{-1}$

(21) R. Kuhn and L. Birkhofer, *Ber.*, **71**, 1535 (1938); G. P. Ellis and J. Honeyman, *Advances in Carbohydrate Chem.*, 10, 95 (1955).

(22) *N*-Arylglycosylamines are relatively stable to alkali (ref. 1), but Hodge and Rist (ref. 9) reported that *N*-D-glucosyl-, galactosyl-, and mannosylpiperidine did not reduce dichlorophenolindophenol in 0.1*N* sodium hydroxide. These glycosylamines may have been converted to materials analogous to the above.

(23) As isolated, V contained up to 1% of ash, which presumably results from the drying with sodium sulfate; this drying was essential for the isolation of the product, for if this step were omitted, the extraction liquid set to an intractable rigid gel on attempted evaporation. The analytical figures are corrected for the ash content. Most crude batches of V contained a slight trace of sulfate.

Anal. Calcd. for $C_{24}H_{50}NO_5Cl$: C, 61.58; H, 10.77; N, 2.99; Cl, 7.58; O, 17.09; acid equivalent, 467; Zerevitinov equiv., 93. Found[23]: C, 61.25; H, 11.10; N, 2.96; Cl, 7.16; O, 17.08; acid equiv., 455; Zerevitinov equiv., 96.

This hydrochloride was readily soluble in hot water, but a 1% solution set to a rigid gel on cooling.

Periodate consumption:

	30 Min.	24 Hr.	48 Hr.
Sodium metaperiodate consumed, moles/mole	3.12	3.89	4.17
Formic acid liberated, moles/mole	2.21	2.44	—
Periodic acid consumed, moles/mole	3.24	3.84	—

The hydrochloride was chromatographed using butanol–acetic acid, ethyl acetate–pyridine, and isopropyl alcohol as developing solvents. In each case a single spot (R_f 0.88, 0.90, and 0.95 respectively) was observed, which gave the the color reactions of both an amine and a reducing sugar.

When 4.3 g. (0.01 mole) of the glucosylamine I was treated with 107 ml. (0.045 mole) of 0.373*N* hydrogen chloride in dry pyridine and the product isolated as described above, there was obtained 3.0 g. of material in all respects identical with the hydrochloride V.

On one occasion, the reaction of I with chloroacetic acid gave a product which closely resembled V, but whose analysis (C, 63.24; H, 11.43; N, 3.16; Cl, 7.22; O, 12.35) suggested that partial dehydration had occurred. Nevertheless, the fructose derivative II was obtained on neutralization. We are unable to account for the formation of this material.

When a solution of 2 g. of the hydrochloride V in 50 ml. of water was cooled until it became opaque (*ca.* 35°) and then mixed with 10 ml. of 2*N* sulfuric acid, the corresponding sulfate was precipitated. After recrystallization from ethanol, this material melted to a wax between 70° and 150°. It readily reduced Fehling's solution, and consumed 3.39 moles of sodium periodate in 30 min. and 3.93 moles in 24 hr

Anal. Calcd. for $2C_{24}H_{49}NO_5 \cdot H_2SO_4$: C, 59.97; H, 10.49; SO_4, 10.00. Found: C, 59.85; H, 10.18; SO_4, 9.26.

This sulfate was also obtained by treatment of V with aqueous sodium sulfate.

*1-Deoxy-1-N-octadecylamino-*D*-fructose* (II). (i) To a solution of 10 g. (0.021 mole) of V in 250 ml. of ethanol was added 0.8 g. of sodium hydroxide in 10 ml. of water. The precipitated solid was collected and recrystallized from ethanol to give 6 g. of the almost colorless *1-deoxy-1-N-octadecylamino-*D*-fructose* (II) m.p. 104–107° mixed m.p. with I 97–102°; α_D dimethoxyethane −27°. The ultraviolet spectrum showed a plateau, λ 255–320 mμ, ε 29, and the infrared spectrum, which differed markedly from that of I, did not contain any band between 1460 and 2000 cm.$^{-1}$

Anal. Calcd. for $C_{24}H_{49}NO_5$: C, 66.78; H, 11.44; O, 18.53. Found: C, 67.73; H, 11.73; O, 19.08.

The periodate consumption was as follows:

	30 min.	24 hr.	96 hr.
Sodium metaperiodate consumed, moles/mole	3.00	3.27	3.51
Formic acid liberated, moles/mole	1.77	2.15	—

The compound was recovered unchanged from treatment with hydroxylamine in anhydrous ethanol.

(ii) A solution of 2.5 g. (0.0058 mole) of the glucosylamine I in 25 ml. of dry pyridine was mixed with 21 ml. of 0.28 *N* hydrogen chloride in pyridine (0.0058 mole). Next day, 200 ml. of water and 200 ml. of 4:1 ethyl acetate–butanol were added, and the organic layer was separated, washed with water, and dried (sodium sulfate). On standing overnight, there separated 0.6 g. of II, m.p. and mixed m.p. 104–107° (Found: C, 67.62; H, 11.58).

The compound II gave a sirupy benzoate, and no product could be isolated from attempted acetylation.

*1-Deoxy-1-N-octadecylamino-*D*-fructose oxalate.* A solution of 1.3 g. of oxalic acid in 10 ml. of ethanol was added to 4.3 g. of II in 100 ml. of ethanol and the precipitated solid was collected and recrystallized from ethanol: the oxalate (hydrated) had m.p. 135–150° dec., ν_{max}^{Nujol} 1730 (m), 1715 (m), 1646 (w), and 1631 cm.$^{-1}$ (w). It readily reduced Fehling's solution, and consumed 4.01 moles of sodium periodate in 30 min., 4.85 moles in 24 hr.

Anal. Calcd. for $C_{26}H_{49}NO_5 \cdot C_2H_2O_4 \cdot H_2O$: C 57.88; H, 9.80; acid equiv., 270. Found: C, 58.54; H, 9.84; acid equiv., 260.

*N-Octadecyl-*D*-mannosylamine* (III). Solutions of 8.0 g. of octadecylamine in 75 ml. of isopropyl alcohol and of 5.4 g. of mannose in 30 ml. of water were mixed and allowed to stand for 2 days at room temperature. The precipitated solid was collected and recrystallized from ethanol to give 12.2 g. of the mannosylamine III, m.p. 100–102°. When chromatographed, spots were obtained corresponding to mannose (R_f 0.22) and octadecylamine (R_f 0.90). The consumption of periodate was 3.89 moles in 30 min. and 4.59 moles in 24 hr.

Anal. Calcd. for $C_{24}H_{49}NO_5$: C, 66.78; H, 11.44. Found: C, 65.92; H, 11.60.

Preparation of II *from the mannosylamine* III. Ten grams of the mannosylamine and 10 g. of chloroacetic acid were dissolved in 100 ml. of pyridine and the solution was set aside overnight. The mixture was poured into water and the product was extracted with ethyl acetate–butanol. Recrystallization from ethyl acetate yielded a product (6.6 g.) melting between 57° and 130° (Found: C, 60.21; H, 10.04; Cl, 3.35). Paper chromatography gave a single spot (R_f 0.90) which gave the color reactions of both an amine and a reducing sugar. Periodate consumption (Calcd. for M = 467): 3.14 moles in 30 min., 3.51 in 24 hr., 3.59 in 48 hr.

This material, dissolved in ethanol, was neutralized by the addition of the calculated quantity of 0.1*N* sodium hydroxide solution. The product was identical (m.p. and mixed m.p. and infrared spectrum) with II above.

Reaction of II *with chloroacetic acid.* Treatment of the fructose derivative II (4 g.) with chloroacetic acid as described for I above, yielded a product (3.0 g.) melting with decomposition between 65° and 140°. (Found: C, 62.07; H, 9.55; Cl, 1.71). This material consumed 4.82 moles of periodate in 30 min., 5.34 in 24 hr., and 5.35 in 48 hr. (Calcd. for M = 467); when chromatographed, it behaved like II and V above. On neutralization, it was reconverted to II.

Reaction of I *and* II *with alkali.* Addition of 2 g. of sodium hydroxide (0.05 mole) in 10 ml. of water to 21.5 g. (0.05 mole) of I in 300 ml. of ethanol gave 10 g. of a *compound*, m.p. 66–68°, $\lambda_{max}^{ethanol}$ 312 mμ, ε 1,380; ν_{max}^{Nujol} 1663 and 1604 cm.$^{-1}$ This substance was also obtained from similar treatment of II.

Anal. Calcd. for $C_{24}H_{45}NO_4$ (I-2H_2O): C, 72.86; H, 11.47; N, 3.54.

Found: C, 71.90; H, 12.30; N, 4.08.

This compound did not reduce Fehling's solution, and it took up 0.84 mole of sodium periodate in 30 min., 1.05 moles in 24 hr. On attempted chromatography, no color reaction was observed with ninhydrin or with *p*-aminobenzoic acid and oxalic acid.

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The Hexosaminidic Linkage of Hyaluronic Acid[1]

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A methylation study of the tetrasaccharide from hyaluronic acid confirms that the 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) moiety is linked (1 ⟶ 4) to the glucuronic acid.

Consideration of the available evidence has led to the general acceptance of hyaluronic acid and the chondroitin sulfates as linear polysaccharides with alternating uronic acid and acetylhexosamine units. Extensive investigations of the products of enzymatic degradation have failed to reveal branching or variations in the glycosidic sequence of these polysaccharides.

The characterization of the crystalline disaccharide which has been obtained in high yield from hyaluronic acid as 3-*O*-β-D-glucopyranosyluronic acid-(2-amino-2-deoxy-D-glucopyranose) has established the (1→3) glucuronidic linkage in hyaluronic acid.[2]

The hexosaminidic linkages in hyaluronic acid and the chondroitin sulfates are considered to be (1→4) due to the ease with which bacterial hexosaminidases act upon them by an elimination process to form Δ^{4-5} uronides.[3] It would be difficult to conceive of the formation of a Δ^{4-5} uronide by the elimination of a hexosaminide with any other linkage.

However, in view of the uniqueness of this elimination reaction by glycosidic cleaving enzymes and the lack of analogy to a well established mechanism, it was considered desirable to confirm the hexosaminidic linkage by other, more universally acceptable evidence, preferably from the use of classical methods such as methylation studies.

As previous methylation studies on hyaluronic acid did not establish the hexosaminidic linkage and indicated substantial experimental difficulties,[4] it seemed more feasible to attempt the methylation of the tetrasaccharide I which is obtained in high yield from hyaluronic acid by the action of testicular hyaluronidase. The ready cleavage by bacterial hexosaminidase of the one hexosaminidic linkage in I by an elimination process[5] serves to confirm that this is the same hexosaminidic linkage which is cleaved in the polymer.

Earlier methylation studies[6] were performed on the trisaccharide obtained from I by the action of liver β-glucuronidase, as this removed the nonreducing end glucuronic acid which did not contain a hexosaminidic linkage. However, it was difficult to obtain β-glucuronidase free of β-hexosaminidase activity so that the disaccharide lacking a hexosaminidic linkage often replaced the trisaccharide as the major digestion product. As it was found that both I and trisaccharide were permethylated readily, later work was carried out on I.

The difficulty of hydrolysing the glucuronidic linkage in hyaluronic acid and its oligosaccharides due to the presence of the carboxyl group would also prevail among the permethylated oligosaccharides. Reduction of the carboxyl group *via* its methyl ester to a hydroxymethyl group would remove the resistance to hydrolysis and yield methylated glucose derivatives for which authentic samples for comparison were available. When lithium aluminum hydride was used as reducing agent on the methylated trisaccharide, the acetamido group was reduced to an ethylamine which served to protect the glycosidic linkage from hydrolytic cleavage and resulted in low yields of methylated monosaccharide products. The report[7] that lithium borohydride reduced methyl ester groups but did not affect acetamido groups was verified on methylated I in the present study and high yields of methylated monosaccharides were obtained from reduced,

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