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STUDIES ON THE CHEMICAL STRUCTURE OF MUCOPOLYSACCHARIDES.

TOHRU KOMANO

1962

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CONTENTS

I. Introduction.

1. Biochemistry of the Mucopolysaccharides.

Of the various polysaccharides found in living organisms, the mucopolysaccharides are of significance from biochemical and physiological points of view.(1) These substances are known to exist widely in animal kingdom, and the modes of their existence are different, being due to their sources. Mostly, they are considered to exist in conjugated form with proteins(2-4) or lipids(4) in living organisms. These substances have been classified and defined by Meyer,(5) Stacey(6) and Masamune,(7) respectively, and in most cases the mucopolysaccharides contain 2-amino-2-deoxy-D-hexose (hexosamine) as a component.

In this dissertation, acid mucopolysaccharides consisting of 2-amino-2-deoxy-D-hexose and uronic acid, some of which contain sulfate ester group, are studied for their chemical structure and the elucidation of the structure is referred to chondroitin sulfate C obtained from shark cartilage.

Chondroitin sulfates are present in cartilage, tendon, aorta, skin and other connective tissues.(1,8-11) These are classified according to their physical and chemical properties into chondroitin sulfate A, B (β-heparin) and C.(12,13)

Hyaluronic acid is distributed widely without or with chondroitin sulfates in skin, connective tissues,(1) hyaline cartilage,(14) umbilical cord,(15) synovial fluid,(16) hemolytic streptococci,(3,17) and other tissues.

Heparin(1,18) is found in lung and liver, and has the blood anticoagulant activity, and, moreover, it has a relation with lipid metabolism in circulatory tissues.

Along with the development of enzyme chemistry, the enzymic degradation and biosynthesis of mucopolysaccharides are also studied by many investigators. Hyaluronic acid and chondroitin sulfate A and C are hydrolyzed by hyaluronidases from mammalian testis and leech to give mainly tetrasaccarides,(19,20) while by bacterial hyaluronidases (Pneumococci, Streptococci, Staphyrococci, etc.) it yields Δ4-5 unsaturated disaccharide (Fig. 1.2).(19-20) Chondroitin sulfatase(24-26) and heparinase(19,27) have also been reported.

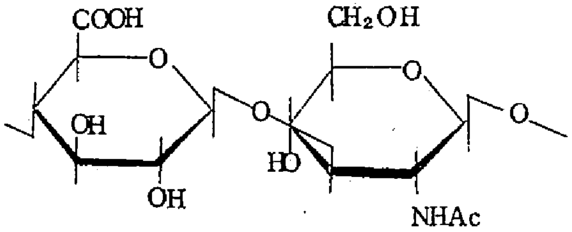
Studies on the metabolism of 2-amino-2-deoxy-D-glucose(28-30) and D-glucuronic acid(31-33) made it possible to investigate the biosyntheses of mucopolysaccharides. Biosynthesis of hyaluronic acid(28,34,35) was successfully worked out through uridine diphospho-2-acetamino-2-deoxy-D-glucose and uridine diphospho-D-glucuronic acid. The biosynthesis of chondroitin sulfate(36-39) and heparin(39,40) is also undertaken by many biochemists.

2. Chemical Structure of the Mucopolysaccharides.

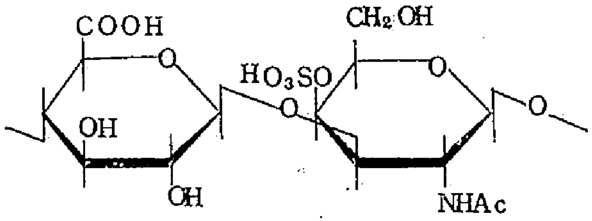
Although biosynthesis and degradation mechanism are known to some extent, chemical structure of the mucopolysaccharides has not been solved until recently.

Hyaluronic acid (Fig. I.1.I), which is composed of 2-acetamino-2-deoxy-D-glucopyranose and D-glucuronic acid, was the first in this field whose chemical structure was clarified. Weissmann and Meyer isolated a hyalobiuronic acid(41) from the acid hydrolysate of hyaluronic acid and characterized it as 3-O-(β-D-glucuronosyl)-2-acetamino-2-deoxy-D-glucose,(42) and afterwords, Hoffman and Hirano(43) determined 2-amino-2-deoxy-D-glucosidic linkage of hyaluronic acid as 4-O-(β-2-acetamino-2-deoxy-D-glucosyl)-D-glucuronic acid.

Chondroitin sulfate A and C (Fig. I.1.II, IV) are consisted of 2-acetamino-2-deoxy-D-galactose, D-glucuronic acid and sulfate. The marked difference of these substances was



I. I



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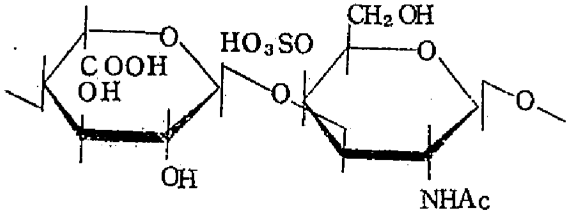

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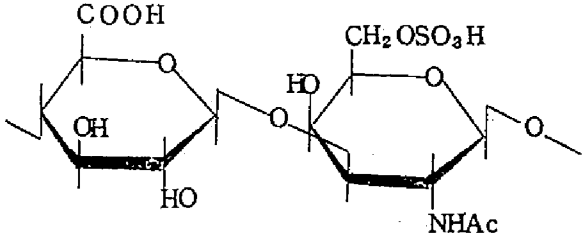
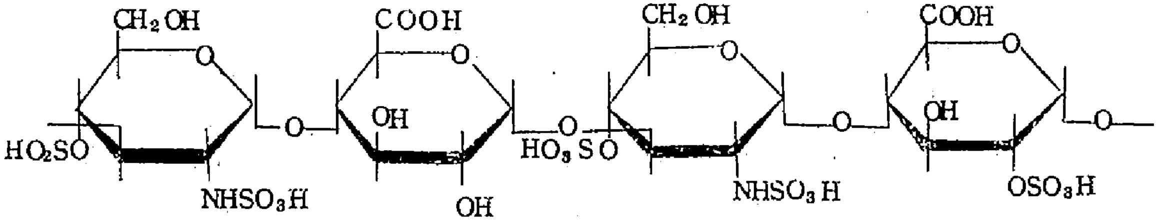


I. IV



I. V

Fig. I. 1

observed in $[\alpha]_D$ and in infrared spectrum. The former substance has $[\alpha]_D$ -30°, while the latter $[\alpha]_D$ -20°. Infrared spectrum of chondroitin sulfate C(44,45) shows equatorial sulfate absorption bands at 1000 $cm.^{-1}$, 820 $cm.^{-1}$ and 775 $cm.^{-1}$, which would indicate that the sulfate group is conjugated to equatorial hydroxyl group of carbon 6 of 2-acetamino-2-deoxy-D-galactose. While chondroitin sulfate A shows absorption bands at 928 $cm.^{-1}$, 852 $cm.^{-1}$ and 725 $cm.^{-1}$, which would indicate that the sulfate group does not attach to equatorial hydroxyl group, namely, it would attach to carbon 4 of 2-acetamino-2-deoxy-D-galactose. These facts were supported further by Strominger and Suzuki(46) by periodate oxidation studies.

The linkage of D-glucuronic acid to 2-acetamino-2-deoxy-D-galactose was investigated with chondroitin sulfate A. Before the report of Davidson and Meyer was published(50), there had been some arguments about the position which links each other.(47-49) Chondrosin isolated from chondroitin sulfate A by acid hydrolysis was revealed to be 3-O-(β-D-glucuronosyl)-2-acetamino-2-deoxy-D-galactose, and this structure was supported by Seno(51) with periodate oxidation of chondrosin. On the other hand, 2-acetamino-2-deoxy-D-galactosidic linkage could be speculated to be β-1:4 from the result obtained from the studies of bacterial hyaluronidase, which gave Δ4-5 unsaturated D-glucuronide disaccharide (Fig. I.2).(19-21)

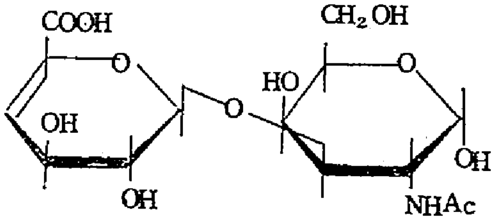


Fig. I.2 Δ4-5 Unsaturated D-glucuronide disaccharide

Chondroitin sulfate B or β-heparin (Fig. I.1.III) is consisted of 2-acetamino-2-deoxy-D-galactose and L-iduronic acid,(54) and carbon 4 of 2-acetamino-2-deoxy-D-galactose moiety is considered to be esterified with sulfate group. Total methylation of this substance with dimethyl sulfate and alkali was performed, and 2-amino-2-deoxy-4.6-di-O-methyl-D-galactose hydrochloride was isolated.(55) This result indicates that L-iduronic acid has the 1:3 glycosidic linkage.

The chemical structure of heparin has been proposed by Wolfrom and his co-workers(56) as shown in Fig. I.1.V. It is considered that 2-amino-2-deoxy-D-glucose and D-glucuronic acid are linked in α-type, and that the amino group of 2-

amino-2-deoxy-D-glucose is substituted by sulfate group. The only evidence known about the glycosidic linkage is that there exists 4-O-(α-D-glucuronosyl)-2-acetamino-2-deoxy-D-glucose.(57) However, futher investigations on chemical structure of this substance are desired.

3. The Object and Significance of the Present Studies.

Although the mucopolysaccharides are known to play a important role in living organisms, their chemical structures have not yet been solved completely, except hyaluronic acid.

In studying chemical structure of the mucopolysaccharides, isolation of unit disaccharide is an essential problem. Since the mucopolysaccharides are heteroglycans consisting of 2-amino-2-deoxy-D-hexose and uronic acid, the ease of acid hydrolysis of the glycosidic linkages is different.. The only obtained unit disaccharide was uronide disaccharide such as hyalobiuronic acid and chondrosin. Isolation of an alternative 2-amino-2-deoxy-D-hexoside disaccharide is also desired, but the hydrolytic condition has not been established.

Accordingly, it is necessary to know the difference of the ease in acid hydrolysis of methyl glycosides of 2-amino-

2-deoxy-D-glucose and of D-glucuronic acid.

At the first step in the present studies, syntheses of glycosides of 2-amino-2-deoxy-D-glucose and of D-glucuronic acid were carried out.(58-60) Then, acid hydrolysis with various concentrations of hydrochloric acid was performed with synthesized glycosides.(60) The results of acid hydrolysis (Table III.I) would indicate that the hydrolytic behavior depends upon the nature of 2-amino-2-deoxy-D-glucose. If the amino-group of 2-amino-2-deoxy-D-hexose is substituted with acetyl group, the obtained unit disaccharide would be an uronide disaccharide because of the resistance of methyl D-glucopyranoside uronamide to acid hydrolysis. When the amino group is not substituted or substituted with acid labile group, 2-amino-2-deoxy-D-hexoside disaccharide could be obtained as the unit disaccharide because of the resistance of methyl 2-amino-2-deoxy-D-glucopyranoside to acid hydrolysis.

Based on the working hypothesis deduced from the fundamental data, acid hydrolysis of chondroitin sulfate from shark cartilage (chondroitin sulfate C) was performed, and the uronide disaccharide, chondrosin, was isolated. This was characterized as chondrosin methyl ester hydrochloride.

On the other hand, hydrazinolysis of carboxyl-reduced chondroitin was carried out in order to remove acetyl group attached to amino group of 2-amino-2-deoxy-D-galactose. Acid hydrolysis of this hydrazinolyzed carboxyl-reduced chondroitin was performed, and 2-amino-2-deoxy-D-galactoside disaccharide, "carboxyl-reduced isochondrosin", was obtained.

Finally, methylation of carboxyl-reduced chondroitin was performed and 2.3.6-tri-O-methyl-D-glucopyranose was identified as a product in the hydrolysate of the methylated compound. This fact would indicate that 2-acetamino-2-deoxy-D-galactoside linkage of chondroitin sulfate is 1:4.(61)

From these results, it is concluded that the chemical structure of chondroitin sulfate from shark cartilage is 4-O-[β-D-glucuronosyl-3-O-(β-2-acetamino-2-deoxy-D-galactopyranosyl)] (Fig. I.1.IV).

It seems possible to elucidate the chemical structure of other mucopolysaccharides with the application of the procedures described above, and it is our hope that the biological and physiological significances of the mucopolysaccharides will be revealed in near future.

II. Syntheses of Methyl Glycosides of Component Sugars of the Mucopolysaccharides.

1. Historicals.

Methyl glycosidation has hitherto been performed by several ways, and the most commonly used method which was established by E. Fischer(62) is that in which methanolic hydrogenchloride is used. However, the yield of glycosides is rather poor and the mixture of α- and β-glycosides is generally obtained. β-Glycoside was synthesized specifically by Königs-Knorr reaction,(63) but an acylmigration was observed under hydrous conditions(64-66) with 1-α-bromo-2-acetamino-2-deoxy-3.4.6-tri-O-acetyl-D-hexopyranose, which undergoes no acylmigration under anhydrous conditions. β-Glycoside was also expected to be synthesized by trichloroacetylsugars.(67)

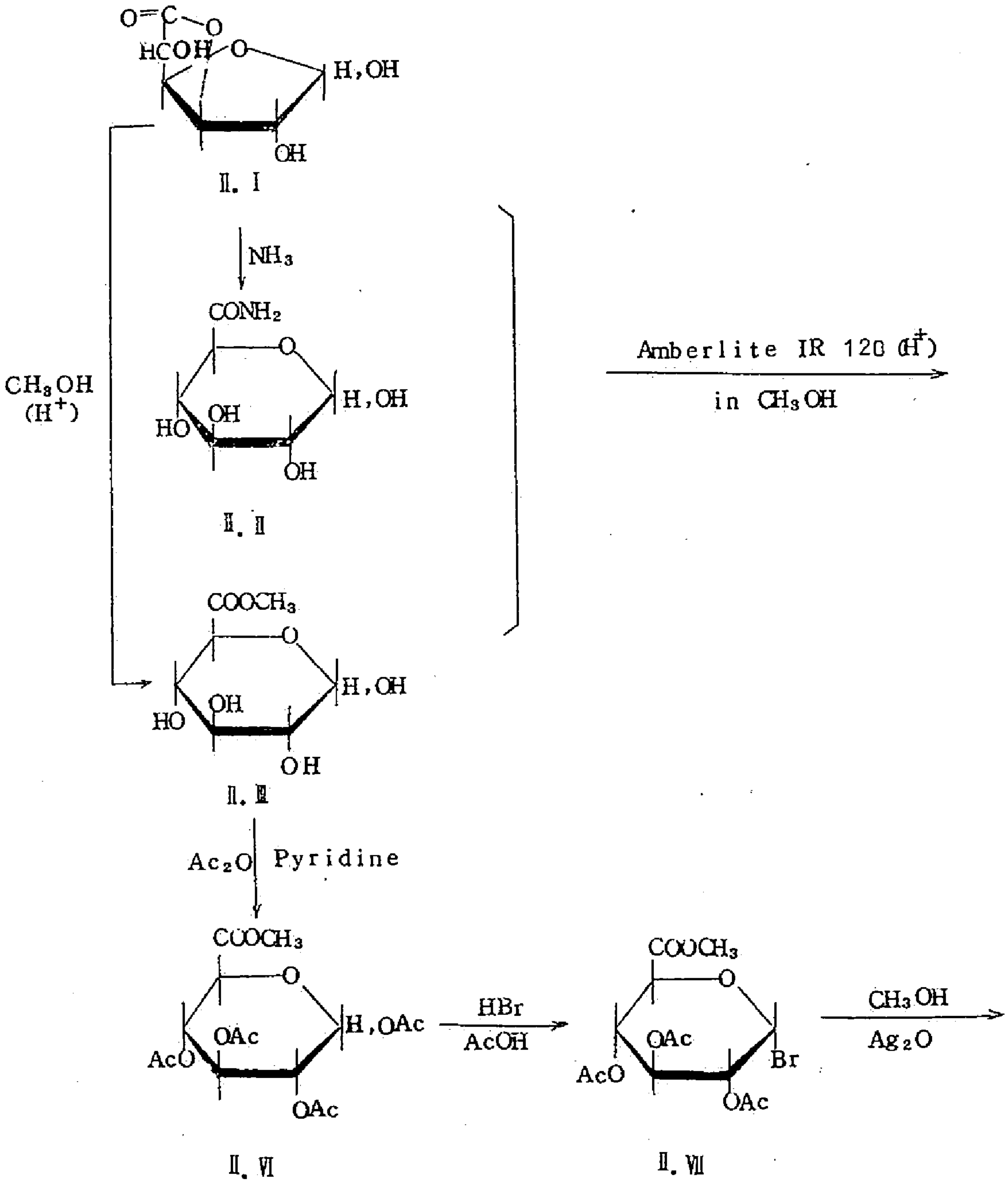
A novel and simpler method has been developed for synthesizing methyl glycosides of the component sugars of the mucopolysaccharides. Strong cation ion-exchange resin Amberlite IR 120 (H^+) or Dowex 50 (H^+) was used as a catalyst in methanol solution.(58,59,68-70) In this reaction, a mixture of α-

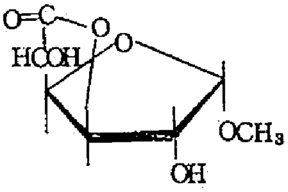
and β-glycosides of 2-acetamino-2-deoxy-D-glucose was formed and, besides, furanoside was also obtained in a small amount.(71) In the synthesis of glucuronide, however, α-D-glucopyranoside could not be obtained.(58)

Methyl α- and β-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranosides were synthesized by using 0.7% methanolic hydrogenchloride but the yields were very low.(72,73) Refluxing 2-benzyloxycarbonylamino-2-deoxy-D-glucose with Amberlite IR 120 (H^+) and methanol, the mixture of methyl 2-benzyloxycarbonylamino-2-deoxy-α- and β-D-glucopyranosides was obtained in good yield, and the separation of both the anomers was successful by the recrystallization of the acetates(58) from ethanol.

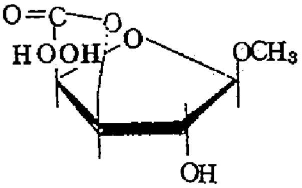
1.3.4.6-Tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose was expected to form 1-α-bromo-3.4.6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose by the usual glycosylbromide formation reaction with acetic acid-hydrogen bromide. However, in this reaction debenzyloxycarbonylation occurred with concomitant evolution of carbon dioxide and formation of benzylbromide. A similar reaction was reported by Weidmann and Zimmerman Jr.(74) with 1.3.4.6-tetra-O-benzoyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose, and May and Mosettig(75) with 1.3.4.6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose.

Fig. II. 1

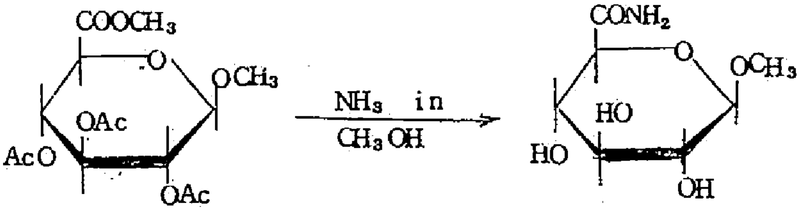

II. I
NH₃
II. II
CH₃OH (H⁺)
II. III
Amberlite IR 120 (H⁺)
in CH₃OH
Ac₂O
Pyridine
II. VI
HBr
AcOH
II. VII
CH₃OH
Ag₂O



II. IV



II. V



II. VIII

II. IX

2. Syntheses of Methyl D-glucuronides.

(a) Synthesis of Methyl α- and β-D-glucofururonoside-γ-lactones(68,69)

Procedure A —— D-glucurono-γ-lactone (II.I)(10 g.) was suspended in methanol (500 ml.) and to this was added Amberlite IR 120 (H^+)(10 g.). The mixture was refluxed for 2.5 hr., and during the reaction time the solution became slightly yellowish. The reaction mixture was then cooled to room temperature. Ion-exchange resin was separated by filtration and was washed thoroughly with methanol. The filtrate was concentrated to a sirup. It was dissolved in dioxane or ethanol and the solution was decolorized. To this was added a small amount of ether and the solution was kept in a refrigerator overnight. White prisms deposited. Upon recrystallization from dioxane or ethanol-ether white prisms were obtained. Yield, 5.5 g. (51 %), m.p. 138°, $[\alpha]_D^{23}$ -55° (c 1, water). Anal. Calcd. for $C_7H_{10}O_6$, C, 44.21; H. 5.30. Found, C, 44.37; H, 5.38. $[\alpha]_D$ showed that the above crystal was methyl β-D-glucofururonoside-γ-lactone (II.V).

The filtrate was concentrated under reduced pressure to a small volume and the solution was kept in a refrigerator.

A white crystalline substance deposited. Upon recrystallization from dioxane or ethanol-ether fine needles were obtained. Yield, 1.1 g. (10 %), m.p. 148°, $[\alpha]_D^{23}$ + 148.2° (c 1, water). Anal. Found, C, 44.24; H, 5.47. $[\alpha]_D$ showed that the above crystal was methyl α-D-glucofururonoside-γ-lactone (II.IV).

Procedure B(58) --- D-glucuronamide(76,77) (II.II)(5 g.) was suspended in methanol (400 ml.) and the solution was refluxed for 15 hr. in the presence of Amberlite IR 120 (H^+)(10 g.). D-Glucuronamide dissolved gradually in methanol. The reaction mixture was then treated in a similar procedure as that described in procedure A. Upon recrystallization from ethanol-ether white prisms were obtained. Yield, 2.6 g., m.p. 137-138°, $[\alpha]_D^{23}$ -54.5° (c 1, water). Anal., Found, C, 44.17; H, 5.39. The melting point on admixture with the authentic specimen of methyl β-D-glucofururonoside-γ-lactone (II.V) showed no depression.

The filtrate was treated in a similar procedure as that described in procedure A. A white crystalline substance deposited. Recrystallization was effected from ethanol-ether which afforded fine needles. Yield, 0.6 g. (12 %), m.p. 148°, $[\alpha]_D^{23}$ + 148° (c 1, water). Anal., Found, C, 44.04; H, 5.47. The melting point on admixture with the authentic specimen of methyl α-D-glucofururonoside-γ-lactone (II.IV) showed no depression.

Procedure C —— D-Glucuronic acid methyl ester (II.III)(78-80) (13 g.) was dissolved in methanol (500 ml.) and the solution was refluxed for 3 hr. in the presence of Amberlite IR 120 (H^+) (26 g.). The reaction mixture was treated in a similar procedure as that described in procedure A. White prisms were obtained. Yield, 6 g. (43 %), m.p. 137-138°, $[\alpha]_D^{23}$ -55° (c 1, water). Anal., Found, C, 44.25; H. 5.42. The melting point on admixture with the authentic specimen of methyl β-D-glucofururonoside-γ-lactone (II.V) showed no depression.

Upon treating the filtrate with the procedure A described above, fine needles separated. Yield, 1.5 g. (11 %), m.p. 147-148°, $[\alpha]_D^{23}$ + 148.1° (c 1, water). Anal., Found, C, 44.28; H, 5.33. The melting point on admixture with the authentic specimen of methyl α-D-glucofururonoside-γ-lactone (II.IV) showed no depression.

(b) Synthesis of Methyl β-D-glucopyranoside uronamide (II.IX).

(b.1) 1-α-bromo-2.3.4-tri-O-acetyl-D-glucuronic acid methyl ester (II.VII).

α,β-1.2.3.4-Tetra-*O*-acetyl-*D*-glucuronic acid methyl ester (II.VI)(78-80)(22.5 g.) was suspended in acetic acid saturated with dry hydrogen bromide (120 ml.), and the reaction mixture was shaken for 24 hr. under the exclusion of moisture. The solution changed to yellow, and to this was added chloroform (50 ml.) and the solution was washed with a saturated aqueous solution of sodium bicarbonate. Extraction with chloroform was repeated and the chloroform layer was separated and washed with water twice, and dried with anhydrous sodium sulfate. Chloroform was evaporated under reduced pressure and a sirup was obtained. Yield, 11.9 g. (50 %). This sirup was dried over phosphorous pentoxide and was used for the next reaction.

(b.2) Methyl 2.3.4-tri-*O*-acetyl-β-*D*-glucuronic acid methyl ester (II.VIII).(81)

The above obtained dried sirup (II.VII)(29 g.), silver oxide (9 g.) and methanol (40 ml.) were added to chloroform (50 ml.), and the mixture was shaken for 24 hr. Silver oxide was removed by filtration, and the chloroform solution was washed with water, dried with anhydrous sodium sulfate, and concentrated under reduced pressure to a small volume until fine needles separated. Yield, 12.6 g. (60 %). Recrystallization was effected from hot ethanol. M.p. 150-151°, $[\alpha]_D^{18}$ -28.7° (c 1, chloroform). Anal., Calcd. for $C_{14}H_{20}O_{10}$, C, 48.27; H, 5.79. Found, C, 48.16; H, 5.97.

(b.3) Methyl β-D-glucopyranoside uronamide (II.IX).

Methyl 2.3.4-tri-O-acetyl-β-D-glucuronic acid methyl ester (II.VIII)(5 g.) was suspended in methanol (125 ml.), and the solution was cooled with ice-water and then saturated with dry ammonia gas. The reaction mixture was left to stand at room temperature for 3 hr., and was concentrated under reduced pressure to a small volume. Upon cooling with ice, a crystalline substance separated. Recrystallization from methanol afforded fine needles. Yield, 2.1 g., m.p. 194-196°, $[\alpha]_D^{11}$ -53.9° (c 1, water). Anal., Calcd. for $C_7H_{13}O_6N$, C, 40.58; H, 6.32; N. 6.76. Found, C, 40.30; H, 6.60; N, 6.49.

3. Syntheses of Methyl 2-amino-2-deoxy-D-glucopyranosides.

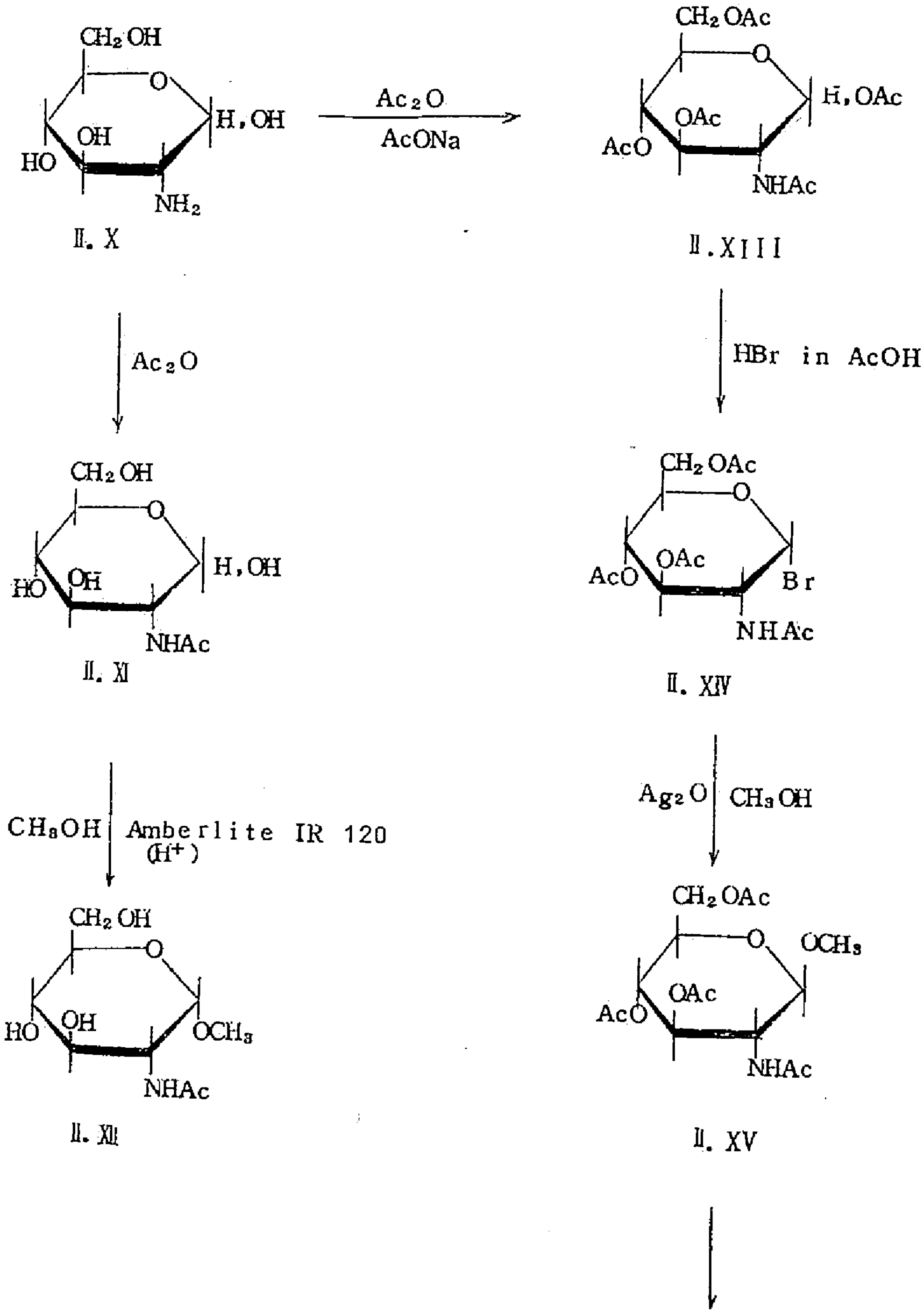
(a) Synthesis of Methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides (II.XII, XVI).

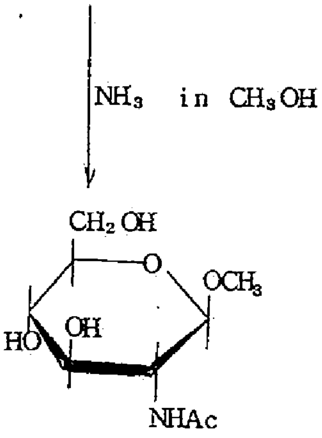
(a.1) Methyl 2-acetamino-2-deoxy-α-D-glucopyranoside (II.XII).

Methyl 2-acetamino-2-deoxy-α-D-glucopyranoside (II.XII) was prepared by the reported procedures.(70,82-85) The synthetic processes are shown in Fig. II.2. The product showed m.p. 178°, $[\alpha]_D^{20}$ +131.5° (c 1, water). These constants were the same as that reported previously.(70)

(a.2) Methyl 2-acetamino-2-deoxy-β-D-glucopyranoside (II.XVI).

Methyl 2-acetamino-2-deoxy-β-D-glucopyranoside (II.XVI) was prepared by the reported procedures.(65,70,86-88) The synthetic processes are shown in Fig. II.2. The product showed m.p. 199-200°, $[\alpha]_D^{9}$ -39.3° (c 1, water). These constants were the same as that reported previously.


CH2OH
O
H,OH
OH
HO
NH2
II. X
Ac2O
AcONa
CH2OAc
O
H,OAc
OAc
AcO
NHAc
II.XIII
Ac2O
HBr in AcOH
CH2OH
O
H,OH
HO
OH
NHAc
II. XI
CH2OAc
O
OAc
AcO
Br
NHAc
II. XIV
CH3OH
Amberlite IR 120 (H+)
Ag2O
CH3OH
CH2OH
O
HO
OH
OCH3
NHAc
II. XII
CH2OAc
O
OCH3
OAc
AcO
NHAc
II. XV



II. XVI

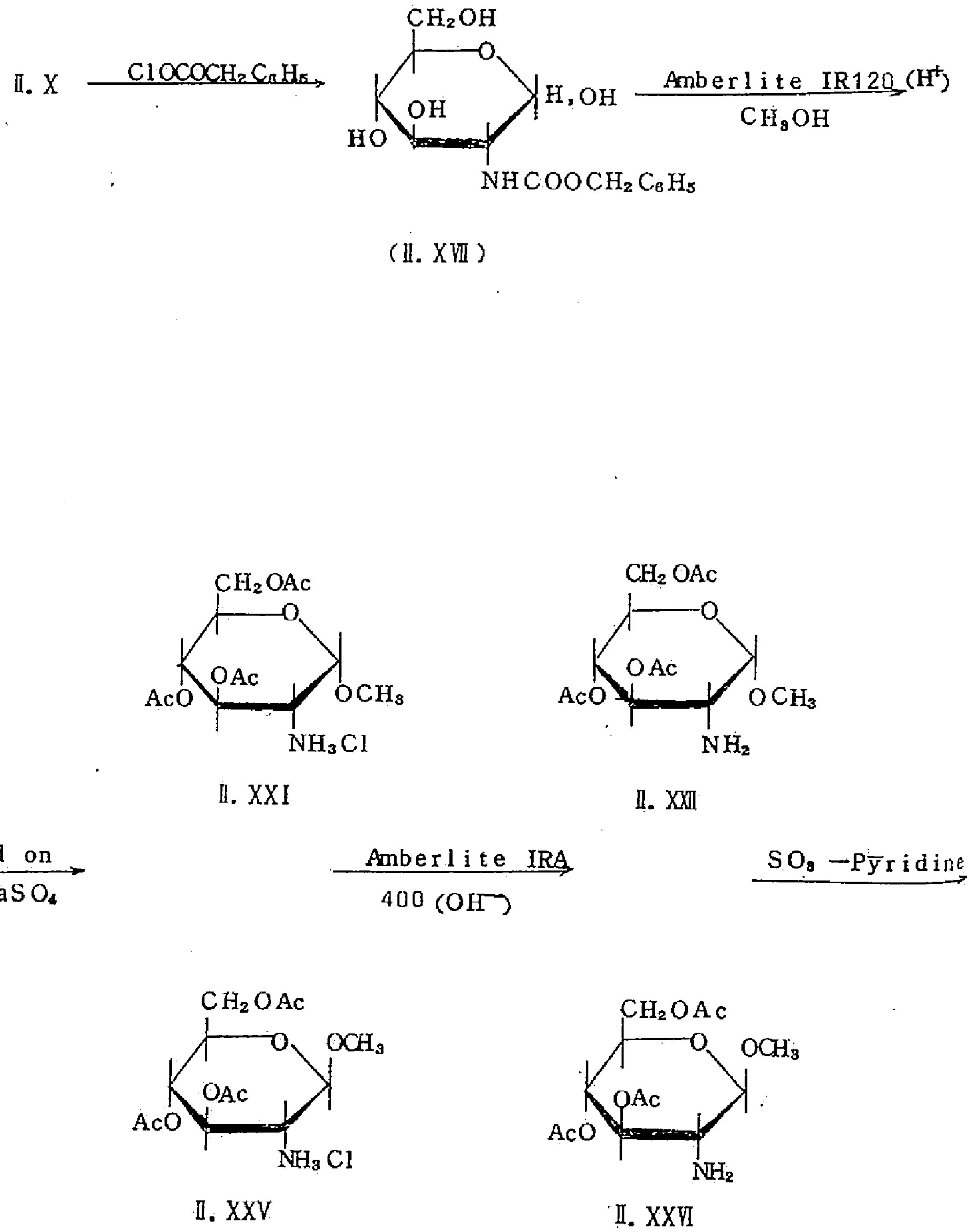
Fig. II. 2

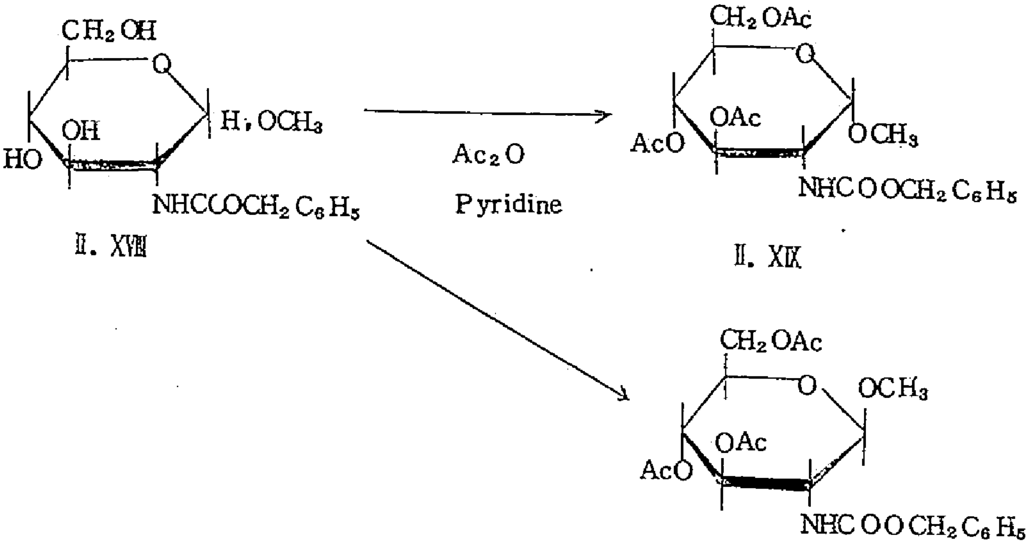
(b) Synthesis of Methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts (II.XXIV, XXVIII).(59)

(b.1) 2-Benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XVII).(72)

2-Amino-2-deoxy-D-glucopyranose hydrochloride (II.X) (107.6 g.) dissolved in water (500 ml.) was treated in the usual procedure with sodium hydroxide (50 g.) and benzylchloroformate (124 g.).(89) Two recrystallizations from methanol-water afforded white crystals (XVII). Yield, 164 g.

Fig. Ⅱ. 3

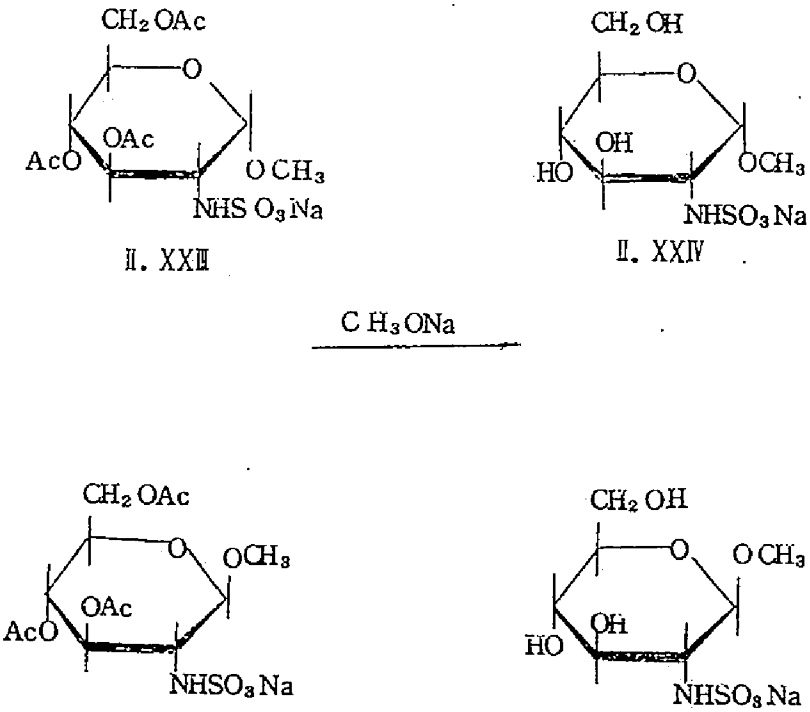

Ⅱ. X
ClOCOCH₂C₆H₅
CH₂OH
O
H, OH
OH
HO
NHCOOCH₂C₆H₅
Amberlite IR120 (H⁺)
CH₃OH
(Ⅱ. XⅧ)
CH₂OAc
OAc
AcO
OCH₃
NH₃Cl
Ⅱ. XXI
NH₂
Ⅱ. XXⅡ
Pd on BaSO₄
Amberlite IRA 400 (OH⁻)
SO₃ –Pyridine
Ⅱ. XXV
Ⅱ. XXⅥ



II. XVIII

II. XIX

II. XX



II. XXIII

II. XXIV

II. XXVII

II. XXVIII

(95 %), m.p. 214°. Anal., Calcd. for $C_{14}H_{19}O_7N$, C, 53.67; H, 6.11; N, 4.47. Found, C, 53.44; H, 6.30; N. 4.28.

(b.2) Methyl 2-benzyloxycarbonylamino-2-deoxy-α,β-D-glucopyranoside (II.VIII).

2-Benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XVIII) (10 g.) was dissolved in methanol (500 ml.) and the solution was refluxed for 3 to 3.5 hr. with Amberlite IR 120 (H^+)(20 g.) as the catalyst.(68-70) After removal of ion-exchange resin, the pale yellow methanolic solution was decolorized and was concentrated under reduced pressure to dryness. The white pasty product was dried in a vacuum desiccator to yield white powder. Recrystallization from ethanol afforded white crystals. Yield, 104. g. (92 %), m.p. 156-159°. $[\alpha]_D^{28}$ +18° (c 1, pyridine). Anal., Calcd. for $C_{15}H_{21}O_7N$, C, 55.04; H, 6.14; N, 4.28. Found, C, 55.13; H, 5.91; N, 4.26.

Although the product obtained from this reaction was in good agreement with analytical value, the melting point showed neither that of α-glycoside (m.p. 154-155°,(88) 158-159°(73)), nor of β-glycoside (m.p. 166-168°,(88) 168-170°(73)), and

$[\alpha]_D$ showed neither that of α-glycoside ($[\alpha]_D$ +80°,(88) + 88°(73), pyridine), nor of β-glycoside ($[\alpha]_D$ -38°,(88) -35°(73), pyridine). Consequently, this substance was considered to be a mixture of methyl 2-benzyloxycarbonylamino-2-deoxy-α- and β-D-glucopyranosides. The separation of α- and β-isomers was not successful at this step.

(b.3) Methyl 2-benzyloxycarbonylamino-2-deoxy-3.4.6-tri-O-acetyl-α- and β-D-glucopyranosides (II.XIX, XX).

A mixture of methyl 2-benzyloxycarbonylamino-2-deoxy-α- and β-D-glucopyranosides (II.XVIII) (10 g.) was acetylated with pyridine (50 ml.) and acetic anhydride (50 ml.) at room temperature for 20 hr. The reaction mixture was poured onto ice-water and was stirred vigorously. The solid matter was collected by filtration, and then was dissolved in a large amount of hot ethanol and was decolorized. After standing the solution in a refrigerator white crystalline matter separated. This was collected by filtration and was recrystallized from ethanol. Yield, 3.3 g. (50 %), m.p. 147-148°, $[\alpha]_D^{29}$ + 3.4° (c 1, pyridine). Anal., Calcd. for $C_{21}H_{27}O_{10}N$, C, 55.62; H, 6.00; N, 3.09. Found, C, 55.12;

H, 5.93; N, 3.33. $[\alpha]_D$ showed that the above crystal was the β-form (II.XX).

The filtrate was concentrated under reduced pressure to a small volume and was placed in a refrigerator, whereupon crystals separated. Repeated recrystallization from ethanol yielded 3.0 g. (45 %) of fine needles. M.p. 102-105°, $[\alpha]_D^{29}$ +104° (c 1, pyridine). Anal., Found, C, 55.39; H, 5.95; N, 3.30. The constants showed that the crystal was the α-isomer (II.XIX).(89)

(b.4) Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside hydrochloride (II.XXI).

Methyl 2-benzyloxycarbonylamino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside (II.XIX)(5 g.) was dissolved in methanol (100 ml.) and to this was added palladium-on-barium-sulfate catalyst (1 g.). The solution was stirred for few minutes, being passed with dry hydrogen gas, and to this was added a half an amount of 0.011 mole of dry methanolic hydrogenchloride. Another half portion of methanolic hydrogenchloride was added after 1.5 hr., and the reaction was completed within 3 hr. After removal of catalyst the solution was concentrated under reduced pressure to a small volume, and to this was added a large amount of ether. A white crystalline substance separated. Recrystallization was effected from ethanol-ether. Yield, 3.1 g. (80 %), m.p. 225-233°(decomp.). $[\alpha]_D^{23}$ +157.8° (c 1, water). Anal., Calcd. for $C_{13}H_{22}O_8NCl$, C, 43.89; H, 6.23; N, 3.94. Found, C, 43.79; H, 6.28; N, 3.85.

(b.5) Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside (II.XXII).

Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside hydrochloride (II.XXI)(5 g.) was dissolved in a small amount of 50 % aqueous methanol and the solution was passed through a column (20 x 2 cm. diam.) of Amberlite IRA 400 (OH^-) and the effluent was concentrated under reduced pressure. The product was a sirup, dried over phosphorous pentoxide, and was used in the next reaction.

(b.6) Methyl 2-deoxy-2-sulfamino-3.4.6-tri-O-acetyl-α-D-glucopyranoside sodium salt (II.XXIII).

Sulfation processes have hitherto been reported by several investigators, but selective sulfation is needed to prepare methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside (II.XXII). For this purpose mild sulfation condition is prefarable. Therefor, the use of sulfur trioxide-pyridine complex(73,89-93) seems suitable instead of using chlorosulfonic acid(94-99) and sulfamine(100), which are suitable for the reaction under rather severe conditions.

Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside (II.XXII) was dissolved in anhydrous pyridine (60 ml.) and to this was added the sulfation reagent(89,90) which was freshly prepared by slow addition of sulfur trioxide 63 ml.) to anhydrous pyridine (50 ml.) previously cooled to -15°. The reaction mixture was shaken for 20 hr. at room temperature with the exclusion of moisture. A pale-yellow sirupy substance was obtained. This was poured into water (700 ml.) containing sodium bicarbonate (4 g.), and the resultant solution was concentrated under reduced pressure. It was extracted three times with ethylacetate-methanol, and the extract was concentrated under reduced pressure to a sirup.

(b.7) Methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside sodium salt (II.XXIV).

The above obtained sirup (II.XXIII) was dissolved in methanol and to this was added 0.05 mole of sodium methoxide to effect deacetylation. The reaction mixture was kept at room temperature for 20 hr., and the solvent was removed under reduced pressure to give a sirup. To this was added a large amount of ethanol, and the solution was stored overnight in a refrigerator. The amorphous precipitate was collected by centrifugation. Repeated recrystallizations from methanol-ethanol yielded 2.3 g. (4.6 %, based on II.XXI) of white powder, which was very hygroscopic. The product was negative for the ninhydrin test and the Elson-Morgan reaction, but was positive for both the tests after hydrolysis with 2.5 N hydrochloric acid at 100°. M.p. 175-178° (decomp.), $[\alpha]_D^{25}$ +105.9° (c 1, water). Anal., Calcd. for $C_7H_{14}O_8NSNa \cdot H_2O$, C, 26.8; H, 5.1; N, 4.5; Na, 7.3; S, 10.2. Found, C, 27.3; H, 5.3; N, 4.4; Na, 7.7; S, 10.1. This compound seemes to be a monohydrate.

(b.8) Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside hydrochloride (II.XXV).

Methyl 2-benzyloxycarbonylamino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside (II.XX)(5 g.) was dissolved in methanol (100 ml.) and to this was added palladium-on-barium sulfate catalyst (1 g.). The reaction mixture was treated in a similar procedure as that described in (b.4). Yield, 3.0-3.2 g. (78-83 %), m.p. 216-227° (decomp.). $[\alpha]_D^{25}$ + 10° (c 1, water). Anal., Calcd. for $C_{13}H_{22}O_8NCl$, C, 43.89; H, 6.23; N, 3.94. Found, C, 43.99; H, 6.45; N, 4.11.

(b.9) Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside (II.XXVI).

Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside hydrochloride (II.XXV)(5 g.) was dissolved in a small amount of 50 % aqueous methanol and the solution was treated in a similar procedure as that described in (b.5). The product was a sirup.

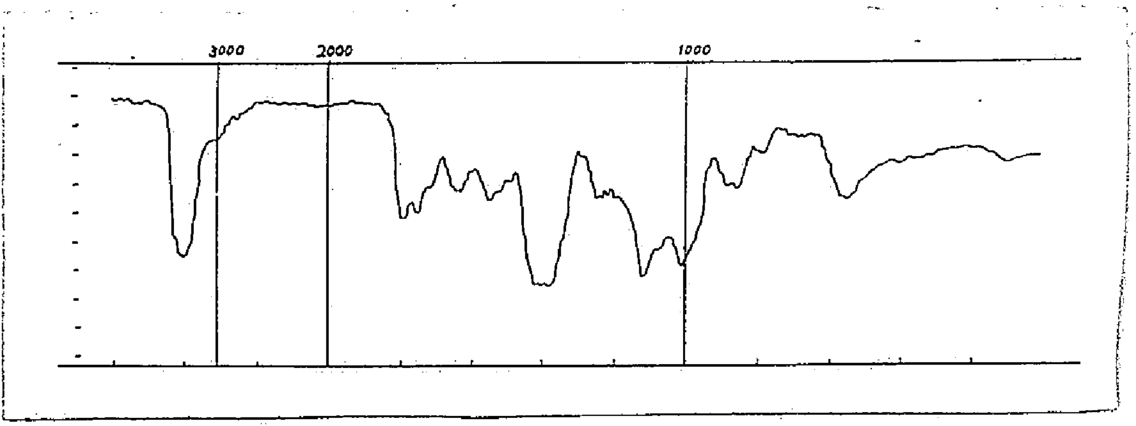
(b.10) Methyl 2-deoxy-2-sulfamino-3.4.6-tri-O-acetyl-β-D-glucopyranoside sodium salt (II.XXVII).

Methyl 2-amino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside (II.XXVI) obtained above was dissolved in anhydrous pyridine (60 ml.) and to this was added the sulfation reagent. The reaction mixture was treated in a similar procedure as that described in (b.6). The product was also a sirup.

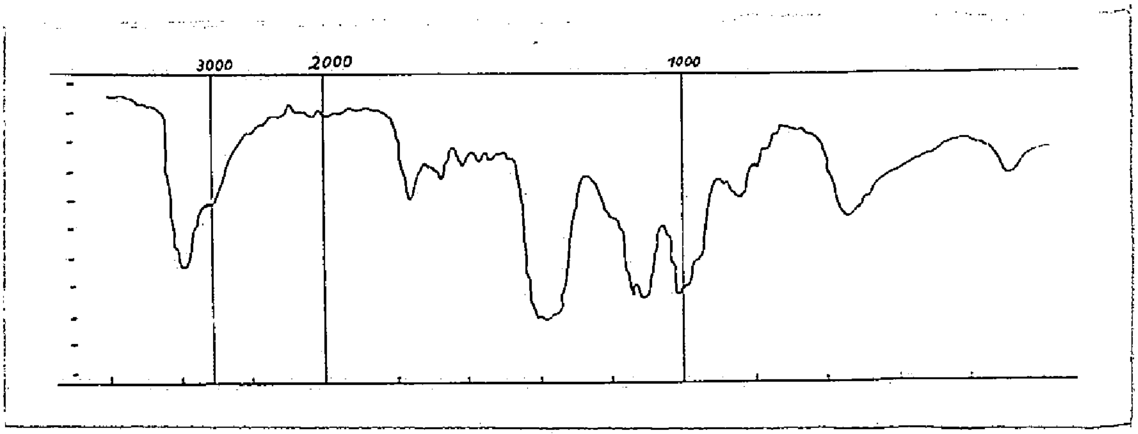
(b.11) Methyl 2-deoxy-2-sulfamino-β-D-glucopyranoside sodium salt (II.XXVIII).

Methyl 2-deoxy-2-sulfamino-3.4.6-tri-O-acetyl-β-D-glucopyranoside sodium salt (II.XXVII) was dissolved in methanol and to this was added 0.05 mole of sodium methoxide to effect deacetylation. The reaction mixture was treated in a similar procedure as that described in (b.7). Yield, 2 g. (4 %, based on II.XXV). M.p. 193-197° (decomp.), $[\alpha]_D^{25}$ +5.0° (c 1, water). Anal., Calcd. for $C_7H_{14}O_8NSNa \cdot H_2O$, C, 26.8; H, 5.1; N, 4.5; Na, 7.3; S, 10.2. Found, C, 27.3; H, 5.3; N, 4.3; Na 7.4 ; S, 10.3. This compound seems to be a monohydrate.

Methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside sodium salt (monohydrate)(II.XXIV) (KBr)

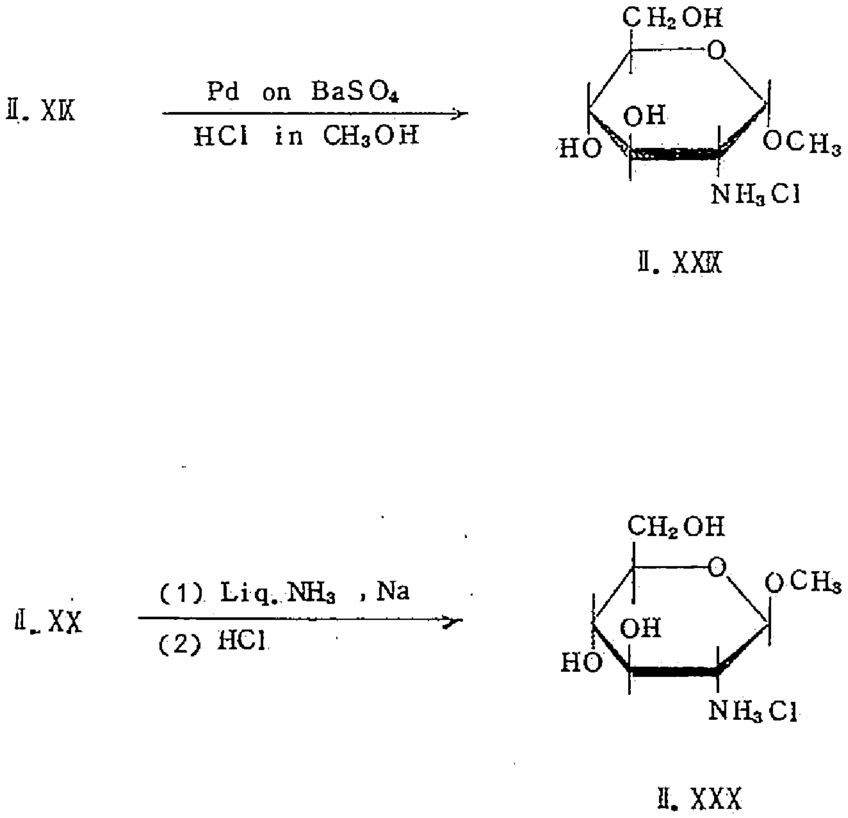


Methyl 2-deoxy-2-sulfamino-β-D-glucopyranoside sodium Salt (monohydrate)(II.XXVIII) (KBr)



(c) Synthesis of Methyl 2-amino-2-deoxy-α- and β-D-glucopyranoside hydrochloride (II.XXIX, XXX).

Fig. II. 4



(c.1) Methyl 2-amino-2-deoxy-α-D-glucopyranoside hydrochloride (II.XXIX).

Methyl 2-benzyloxycarbonylamino-2-deoxy-3.4.6-tri-O-acetyl-α-D-glucopyranoside (2 g.) was dissolved in methanol (50 ml.) and to this was added palladium-on-barium sulfate catalyst (0.5 g.). The solution was stirred for few minutes being passed in with dry hydrogen gas, and to this was added an excess amount of methanolic hydrogen chloride (0.01 mole). Reduction was performed under 5°C and was completed within 3 hr. Catalyst was removed by filtration and the solution was evaporated under reduced pressure. A white sirup was obtained, which was very hygroscopic. This was again dissolved in anhydrous methanol and the solution was evaporated to dryness. This process was repeated in order to free from hydrogenchloride remained. A sirup was dried over phosphorous pentoxide and was stored in a vacuum desiccator.

(c.2) Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride (II.XXX).

Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride (II.XXX) was prepared by the reported procedure(73) from methyl 2-benzyloxycarbonylamino-2-deoxy-3.4.6-tri-O-acetyl-β-D-glucopyranoside (II.XX) with liquid ammonia and metallic sodium. The product showed m.p. 189-190°, $[\alpha]_D^{24}$ -17.4°. These constants were the same as those reported previously.(73)

4. Debenzyloxycarbonylation of 1.3.4.6-Tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-hexopyranose in the Conversion of α,β-Acetoxy to Glycosyl Bromide.

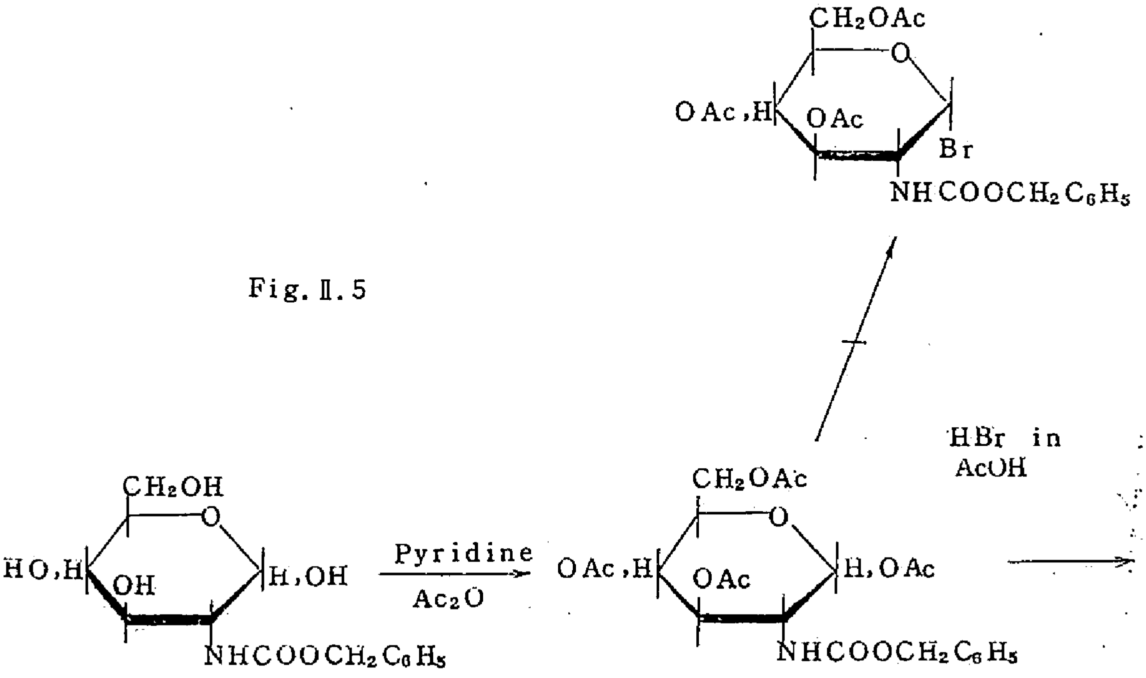
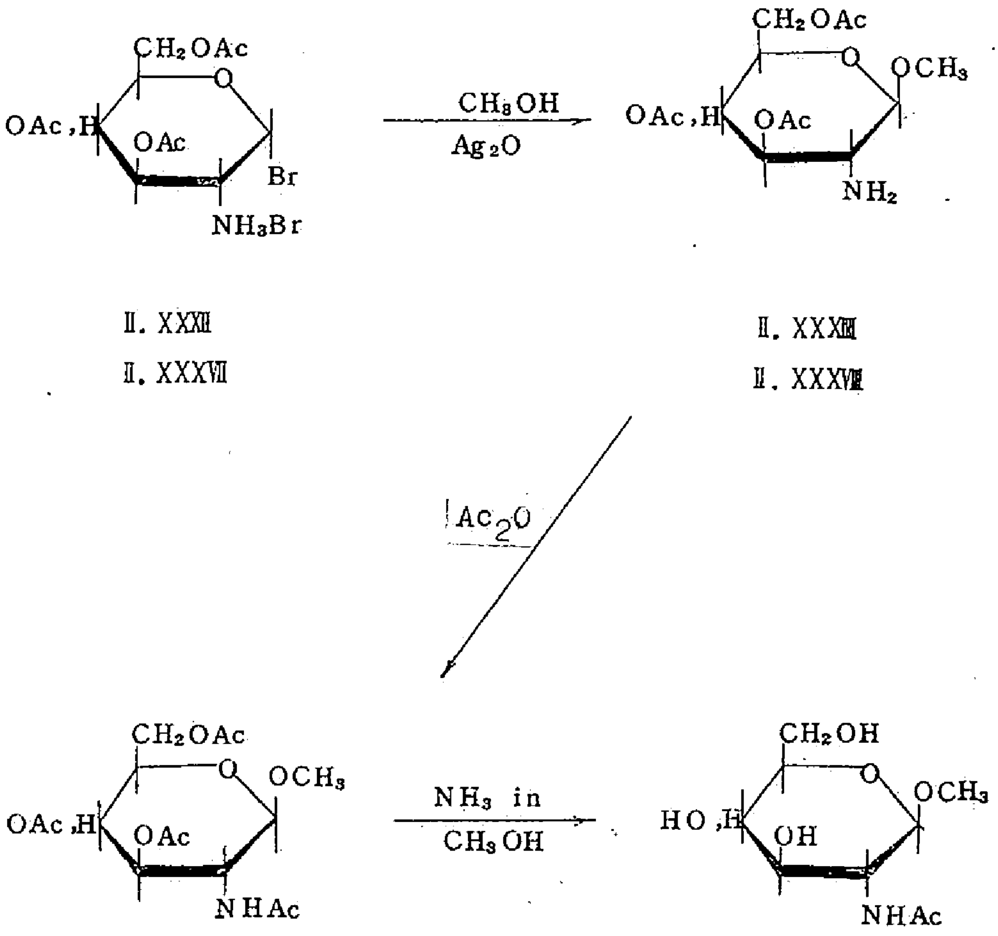


Fig. II.5

II. XVIII
II. XXXV

II. XXXI
II. XXXVI



II. XXXII
II. XXXVII

II. XXXIII
II. XXXVIII

II. XXXIV
II. XXXIX

II. XXXX

The Königs-Knorr reaction is widely used in synthesizing β-glycosides of sugars. A similar reaction could be expected with α,β-1.3.4.6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XXXI) to yield 1-α-bromo-3.4.6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XXXXI), and the glycosidation reaction gives methyl 3.4.6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (II.XX). However, in the glycosylbromide formation reaction with acetic acid and hydrogen bromide, a vigorous evolution of carbon dioxide was observed. To the reaction mixture was added a large amount of ether and fine needles were obtained. The analysis of this compound revealed that it was 1-α-bromo-3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-glucopyranose hydrobromide (II.XXXII).

A similar reaction was expected to α,β-1,3,4,6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-galactopyranose (II.XXXVI), and 1-α-bromo-3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-galactopyranose hydrobromide (II.XXXVII) was obtained with concurrent evolution of carbon dioxide and formation of benzylbromide.

In order to confirm this reaction, the resulting compound (II.XXXII, XXXVII) was treated in a chloroform solution with methanol and silver oxide. The obtained methyl glycoside (II.

XXXIII, XXXVIII) was acetylated to yield 2-acetamino derivative (II.XXXIV, XXXIX). II.XXXIV was identical with an authentic specimen and II.XXXIX showed the same constants as those reported in the literature.(101) Deacetylation of II.XXXIX gave methyl 2-acetamino-2-deoxy-β-D-galactopyranoside (II.XXXX).(101)

(a.1) α,β-1.3.4.6-Tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XXXI).

2-Benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XVII) (10 g.) was dissolved in pyridine (50 ml.) and to this was added dropwise with cooling acetic anhydride (50 ml.). The reaction mixture was kept at room temperature for 20 hr. and then was added to 1 l. of ice water. The precipitated sirupy substance was washed thoroughly with water and extracted with chloroform, which was washed with a saturated aqueous sodium bicarbonate solution, and then with 1 N hydrochloric acid. The dried chloroform solution upon evaporation gave a sirup which was dried over phosphorous pentoxide. Yield, 12.2 g. (80 %). This substance was considered to be the mixture of α- and β-form, and could be obtained in a low yield in crystalline form by repeated recrystallizations from ethanol, m.p. 148°.

(a.2) 1-α-Bromo-3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-glucopyranose hydrobromide (II.XXXII).

α,β-1.3.4.6-Tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (II.XXXI)(5 g.) was dissolved in 100 ml. acetic acid saturated with dry hydrogen bromide. The reaction mixture was kept at room temperature for 24 hr. with exclusion of moisture and was often shaken vigorously. When the reaction was over, the reaction mixture became yellowish red, and fine needles separated. After standing for 20 hr., a large amount of ether was added and the mixture was cooled with ice. The crystals were collected on a glass filter, washed with ether until the washings became negative for bromine ion test, and were dissolved in hot ethanol. Fine needles were obtained. Yield, 3.6 g. (66 %), m.p. 150° (decomp.). $[\alpha]_D^{20}$ +151° (c 1, ethyl acetate). Anal., Calcd. for $C_{12}H_{19}O_7NBr_2$, C, 32.09; H, 4.26; N, 3.12. Found, C, 31.75; H, 4.69; N, 3.11. Reported m.p. 149-150° (decomp.), $[\alpha]_D$ + 152.8° (c 1.096, ethyl acetate).(102)

(a.3) Methyl 3.4.6-tri-*O*-acetyl-2-amino-2-deoxy-β-*D*-glucopyranoside (II.XXXIII).

1-α-bromo-3.4.6-tri-*O*-acetyl-2-amino-2-deoxy-*D*-glucopyranose hydrobromide (II.XXXII)(1.5 g.) was dissolved in chloroform, and to this were added methanol (1 ml.), silver oxide (2 g.), and a small amount of anhydrous sodium sulfate. After stirring for 6 hr., the reaction mixture was filtered and the filtrate was decolorized. Silver oxide remained in the solution was removed with hydrogen sulfide. The resulting methyl 3.4.6-tri-*O*-acetyl-2-amino-2-deoxy-β-*D*-glucopyranoside (II.XXXIII) was a sirupy substance.

(a.4) Methyl 3.4.6-tri-*O*-acetyl-2-acetamino-2-deoxy-β-*D*-glucopyranoside (II.XXXIV).

Methyl 3.4.6-tri-*O*-acetyl-2-amino-2-deoxy-β-*D*-glucopyranoside (II.XXXIII) was dissolved in chloroform and to this was added acetic anhydride (1.5 moles) and the solution was refluxed for 30 min. The solution was washed twice with a saturated aqueous sodium bicarbonate solution, dried with anhydrous sodium sufate, and concentrated under reduced pressure to dryness. The residue was dissolved in a small amount of methanol and the solution was decolorized, and to this was added a large amount of ether which precipitated a crystalline substance. Recrystallization was effected from methanol-ether. Yield, 0.72 g. (60 % based on II.XXXII). M.p. 160°, $[\alpha]_D^{20}$ -24° (c 1, chloroform). Anal., Calcd. for $C_{15}H_{23}O_9N$, C, 49.86; H, 6.42; N, 3.88. Found, C, 50.15; H, 6.42; N, 4.06. The melting point on admixture with the compound prepared from 1-α-bromo-3.4.6-tri-O-acetyl-2-acetamino-2-deoxy-D-glucopyranose showed no depression.

(b.1) 2-Benzyloxycarbonylamino-2-deoxy-D-galactopyranose (II.XXXV).

2-Amino-2-deoxy-D-galactopyranose hydrochloride (5 g.) was dissolved in water (25 ml.) and was treated in the usual procedure with sodium hydroxide (2.5 g.) and benzylchloroformate (6.2 g.). Recrystallization from methanol-water gave fine needles. Yield, 5.9 g. (80 %), m.p. 183°. This compound was more soluble in water than 2-benzyloxycarbonylamino-2-deoxy-D-glucose (II.XVII). Anal., Calcd. for $C_{14}H_{19}O_7N$, C, 53.67; H, 6.11; N, 4.47. Found C, 53.46;

H, 6.16; N, 4.75.

(b.2) α,β-1.3.4.6-Tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-galactopyranose (II.XXXVI).

Acetylation of 2-benzyloxycarbonylamino-2-deoxy-D-galactopyranose (II.XXXV)(2 g.) was carried out with acetic anhydride and pyridine. Repeated recrystallizations from ethanol gave crystals. Yield, 1.6 g. (51 %), m.p. 133°. Anal., Calcd. for $C_{22}H_{27}O_{11}N$, C, 54.86; H, 5.65; N, 2.91 Found, C, 54.72; H, 5.61; N, 2.93.

(b.3) 1-α-Bromo-3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-galactopyranose hydrobromide (II.XXXVII).

1-α-Bromo-3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-galactopyranose hydrobromide (II.XXXVII) was prepared from α,β-1.3.4.6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-galactopyranose (II.XXXVI) by a similar procedure described in (a.2). This compound was unstable in moist air and turned fairly fast to a reddish-colored sirup. The crude substance which melted at 161° (decomp.) was dried in vacuo over phosphorous pentoxide.

(b.4) Methyl 3.4.6-tri-O-acetyl-2-amino-2-deoxy-β-D-galactopyranoside (II.XXXVIII).

This compound was prepared from the above dried substance (II.XXXVII) by a similar procedure as described in (a.3). The product was hygroscopic, dried over phosphorous pentoxide, and used as such in the next reaction.

(b.5) Methyl 3.4.6-tri-O-acetyl-2-acetamino-2-deoxy-β-D-galactopyranoside (II.XXXIX).

This compound was prepared from methyl 3.4.6-tri-O-acetyl-2-amino-2-deoxy-D-galactopyranoside (II.XXXVIII) by a similar procedure as that described in (a.4). Yield, 85 mg. (19.7% based on II.XXXVI), m.p. 215°. Anal., Calcd. for $C_{15}H_{23}O_9N$, N, 3.88. Found, N, 3.92. The reported m.p. 216-217°.(101)

(b.6) Methyl 2-acetamino-2-deoxy-β-D-galactopyranoside (II.XXXX).

Deacetylation of methyl 3.4.6-tri-O-acetyl-2-acetamino-2-deoxy-β-D-galactopyranoside (II.XXXIX)(50 mg.) was performed with 0.5 N sodium methoxide at room temperature for 20 hr. The reaction mixture was concentrated under reduced pressure and to this was added a large amount of ether. Upon standing in a refrigerator crystals separated. Yield, 22 mg. (70 %), m.p. 191°, $[\alpha]_D^{31}$ -13.5° (c 0.7, methanol). Anal., Calcd. for $C_9H_{17}O_6N$, N, 5.96. Found, N, 6.04.
The reported m.p. 191-192°, and $[\alpha]_D^{23}$ -12°(c 1.05, methanol).(101)

5. Summary

Methyl α- and β-D-glucofururonoside-γ-lactones, methyl β-D-glucopyranoside uronamide, methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides, methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts, and methyl 2-amino-2-deoxy-α-and β-D-glucopyranoside hydrochlorides were synthesized.

In methyl glycosidation of these substances, strong cation exchange resin Amberlite IR 120 (H^+) was effectively used as an acid catalyst.

Debenzyloxycarbonylation of 1.3.4.6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-hexose (D-glucose and D-galactose) was observed during glycosylbromide formation reaction with acetic acid-hydrogen bromide.

III. Acid Hydrolysis of Methyl Glycosides and Mucopolysaccharides with special reference to Chondroitin Sulfate (from Shark Cartilage).

1. Scope of Acid Hydrolysis of the Mucopolysaccharides.

(a) Acid Hydrolysis of Glycosides.

Acid hydrolysis of glycosides has been performed by many investigators in the purpose to determine the structures of polysaccharides and to examine and measure the stability of the glycosidic linkages.(62) Investigations by acid hydrolysis were carried out with the monosaccharides such as methyl-, ethyl-, benzyl- and phenyl-glucosides, and it has been recognized that the glycosides which have the aliphatic aglycon groups are more resistant to acid hydrolysis than those which have the aromatic aglycon groups.(103)

Besides the problems of substituents on carbon 1, the conformation of sugar moiety has an important effect on acid hydrolysis. Reeves(104) stated that the pyranose ring has eight possible strainless ring forms and C1 conformation is the most stable in case of D-glucose. In C1 conformation of D-glucose, hydroxyl groups of carbon 1,2,3 and 4, and hydroxymethyl group of carbon 5 are all equatorial in α-form, while in β-form hydroxyl group of carbon 1 is axial. Therefore, it would be easily understood that β-glycosides which contain aliphatic aglycon groups are generally hydrolyzed faster than α-glycosides.

If acid hydrolysis proceeds through the intermediate carbonium cation form(105) (Fig. III.1.III.III), the cleavage of the cyclized oxgen bond would initiate the reaction. Therefore, the nature of the substituents on carbon 5 also exerts a marked effect on the stability of the sugar molecule. In contrast with the aliphatic aglycon group, the aromatic as well as the sugar aglycon groups give considerable different behaviors in the ease of hydrolysis. It is noted that the instability of the ring conformation is caused by axial hydroxyl groups, especially hydroxyl group of carbon 2. Although in cellobiose molecule both the glucose units link in β-form, it is more resistant to acid hydrolysis than maltose, in which both the glucose units link in α-form.(1,38) This would be due to the difference between maltose and cellobiose with respect to the conformation of the non-reducing glucose moiety.(106) In maltose, the non-reducing glucose

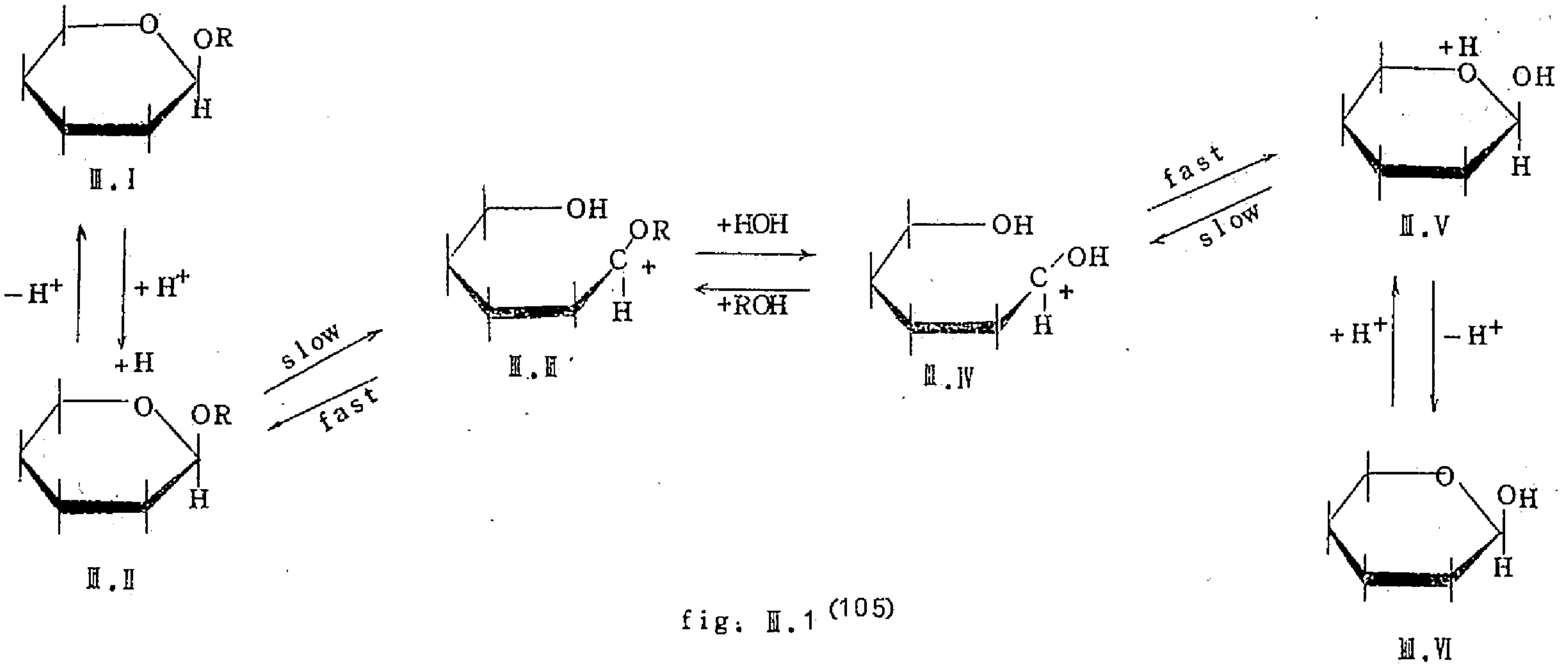

OR
H
III.I
−H$^+$
+H$^+$
+H
OR
H
III.II
slow
fast
OH
OR
C
+
H
III.III
+HOH
+ROH
OH
OH
C
+
H
III.IV
fast
slow
+H
O
OH
H
III.V
+H$^+$
−H$^+$
O
OH
H
III.VI

fig: III.1 (105)

unit might be a boat form (B1 or B3), and then the hydroxyl group of carbon 2 is axial and the reducing glucose unit is of C1 conformation. On the other hand, in cellobiose both the glucose units are of C1 conformation.

Furthermore, the ring structure has an important role for the stability and the ease of hydrolysis. Aldofuranosides are hydrolyzed faster than aldopyranosides. These facts suggest that the furanose ring is essentially planner and the ring has a strain which is a cause of the instability of the molecule.(62,105)

(b) Acid Hydrolysis of the Mucopolysaccharides in Relation to the Isolation of Unit Disaccharides.

The most important step in any structural investigations of polysaccharides is the isolation and identification of the component sugars. Complete hydrolysis of the mucopolysaccharides has been carried out by several ways in acidic conditions. Since the mucopolysaccharides are heteroglycans consisting of 2-amino-2-deoxy-D-hexose and uronic acid, or other neutral sugars, hydrolytic process is rather complicated. Acids usually used are hydrochloric acid,(107-109) sulfuric acid(110) and oxalic acid,(111) and in our experiments hydrochloric acid was used in all series of acid hydrolysis.

In structural studies of polysaccharides, the isolation of the unit disaccharides is also essential. Especially, in the mucopolysaccharides the alternative unit disaccharide is required to determin both the glycosidic linkages which are uronidic and 2-amino-2-deoxy-hexosidic linkages. Acid hydrolysis of the mucopolysaccharides has hitherto given a sole kind of unit disaccharide which is characterized as β-1:3 uronides.(42,50,55) Isolation of the 2-amino-2-deoxy-hexoside disaccharide has not yet been reported. It is reasonable to consider that the failure of obtaining 2-amino-2-deoxy-hexoside disaccharide is due to the ease for acid hydrolysis of the 2-amino-2-deoxy-hexosidic linkage as compared with the uronidic linkage in mucopolysaccharides.

Glucuronic acid moiety in mucopolysaccharides is readily decarboxylated under the acid hydrolytic conditions(112,113) and therefore is reduced beforehand to hexose to avoid decarboxlation.(114-116) The carboxyl-reduced mucopolysaccharide would be of interest for the study of acid hydrolysis which might give an alternative unit disaccharide. It may be possible to obtain 2-amino-2-deoxy-hexoside disaccharide if there is a difference in the ease of mucopolysaccharide hydrolysis which favors scission of the uronidic linkage. No report has been published concerning the difference in acid hydrolysis of both the glycosidic linkages. It is probable that such an investigation would give a fundamental information on the nature of glycosidic linkages in mucopolysaccharides.

2. Acid Hydrolysis of Methyl D-glucuronides and Methyl 2-amino-2-deoxy-D-glucopyranosides.

Acid hydrolysis of methyl glycosides which have relation to the component sugars of mucopolysaccharides has been reported by several investigators.

Haworth and Hirst(117) reported the acid hydrolysis of methyl α- and β-D-glucopyranosides, Neuberger and Rivers(118) that of methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride, and Foster, Horton and Stacey(73) mentioned about the influence of the substituents on amino group on the acid hydrolysis of methyl 2-amino-2-deoxy-α- and β-D-glucopyranosides. However, hydrolysis of these substances has been done under the different conditions. Therefore, it is necessary to study about the ease of hydrolysis of the standard glycosides under the same conditions.

In the experiments herein described the acid hydrolysis was performed with the synthesized substances described in Chapter II. These are methyl α- and β-D-glucofururonoside-γ-lactones, methyl β-D-glucopyranoside uronamide, methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides, methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside hydrochlorides and methyl α- and β-D-glucopyranosides.

Glycoside (100 mg.) was dissolved in hydrochloric acid (25 ml.) of 2.49 N (9.11 %), 0.996 N (3.64 %), and 0.463 N (1.60 %), respectively. The solution was transferred to a pyrex flask with a condenser and hydrolysis was performed on a boiling water bath. One ml. of the solution was taken out at intervals and neutralized with 1 N sodium hydroxide.

The reducing sugar was determined by the Somogyi's method(119) and 2-amino-2-deoxy-D-glucose was determined by the Elson-Morgan reaction.(120,121) The sulfate ion was determined by the Lloyd's method.(122)

The rate of hydrolysis was expressed as half hydrolysis time as shown in Table III.I.

Methyl β-D-glycopyranoside was hydrolyzed in all the three hydrochloric acid concentrations faster that methyl α-D-glucopyranoside.(117) A similar relationship was observed between methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides,

Table III.I Hydrolysis of Methyl Glycosides with 2.493 N, 0.996 N and 0.463 N Hydrochloric Acid

	Half hydrolysis time[a] (min.)		
	Conc. of HCl (N)		
	2.493	0.996	0.463
Methyl α-D-glucopyranoside	48	127	282
Methyl β-D-glucopyranoside	24	60	135
Methyl 2-acetamino-2-deoxy-α-D-glucopyranoside	18	39.5	68
Methyl 2-acetamino-2-deoxy-β-D-glucopyranoside	4	9.5	14.5
Methyl 2-amino-2-deoxy-α-D-glucopyranoside hydrochloride	540 [4.5 %[b]]	-	-
Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride	540 [5.5 %]	-	-
Methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside sodium salt	540 [7.0 %]	-	-
Methyl 2-deoxy-2-sulfamino-β-D-glucopyranoside sodium salt	540 [13.0 %]	-	-
Methyl α-D-glucofururonoside-γ-lactone[c]	1.1	2.1	2.7
Methyl β-D-glucofururonoside-γ-lactone[c]	2.3	5.9	8.8
Methyl β-D-glucopyranoside uronamide	25	95	225

a) Half hydrolysis time represents the time required for 50 % hydrolysis of the compound.

b) The percentage of reducing sugars produced on 540 min.-hydrolysis with 2.493 N hydrochloric acid.

c) Unhydrolyzed methyl α- and β-D-glucofururonoside-γ-lactones exhibit reducing power 2.2 % and 17.0 %, respectively, of the hydrolyzed sugar, as estimated (cf. Ref. 69). The hydrolysis rate described here was corrected in respect to these values.

as well as methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts and methyl 2-amino-2-deoxy-α- and β-D-glucopyranoside hydrochlorides. On the contrary, with methyl α- and β-D-glucofururonoside-γ-lactones, the α-glycoside was hydrolyzed faster than the β-glycoside. It is suggested that this is due to the furanoside structures of the compounds (c.f., III.(a)).

Of a series of methyl 2-amino-2-deoxy-D-glucopyranosides, methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides were easily hydrolyzed with a rate comparable to that of methyl α- and β-D-glucopyranosides, while methyl 2-amino-2-deoxy-α- and β-D-glucopyranoside hydrochlorides as well as methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts were slowly hydrolyzed. The rate with which methyl 2-amino-2-deoxy-D-glucopyranosides are hydrolyzed appears to depend upon the nature of the substituents attached to the amino group. As Foster, Horton and Stacey(73) and Stacey(123) metnioned, the glycosidic linkage in methyl 2-acetamino-2-deoxy-D-glucopyranoside would be cleaved faster than would be *N*-acetyl group. On the other hand, the *N*-sulfate group of methyl 2-deoxy-2-sulfamino-D-glucopyranoside sodium salt was very readily hydrolyzed, as shown in Table III.II, and the hydrolysis resulted in production of methyl 2-amino-2-deoxy-

D-glucopyranoside hydrochloride.

Table III.II. Hydrolysis of Sulfate Esters of Methyl 2-deoxy-2-sulfamino-D-glucopyranoside Sodium Salts by 2.493 N Hydrochloric Acid.[a)]

Time (min.)	Methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside sodium salt	Methyl 2-deoxy-2-sulfamino-β-D-glucopyranoside sodium salt
0	0 (%)[b)]	0 (%)[b)]
5	70.3	72.7
10	100	100
15	100	100

a) Hydrolysis was performed in a boiling water bath at 100°C.

b) These values show the percentage of sulfate anion released.

Consequently, as Moggridge and Neuberger(124) reported, the latter compound is hardly hydrolyzed on account of the formation of NH_3^+ group on carbon 2 which would interfere the approach of H^+ ion towards glycosidic bond.

In comparison of the rate of hydrolysis of methyl α- and β-D-glucofururonoside-γ-lactones with that of methyl β-D-glucopyranoside uronamide, the former two compounds are much more rapidly hydrolyzed than the latter compound. It is mentioned that there has been observed a marked difference in hydrolysis rate between D-glucopyranoside and D-glucofuranoside, and that the latter compound is more rapidly hydrolyzed than the former compound (c.f., III.(a)). A similar result was observed with the case of methyl D-glucuronides.

There have been some arguments about unit disaccharides isolated from the acid hydrolysis products of mucopolysaccharides.(42,49,50,125) The results obtained with the hydrolysis of methyl β-D-glucopyranoside uronamide and of methyl 2-acetamino-2-deoxy-β-D-glucopyranoside show that at a relatively low concentration of hydrochloric acid, the former compound is more slowly hydrolyzed than the latter compound. At a relatively high concentration of hydrochloric acid (2.493 N) the difference of the hydrolysis rate is decreased. The implication of this fact suggests that the uronidic linkage might be more resistant to acid hydrolysis at a low concentration of acid than the 2-amino-2-deoxy-D-hexosidic linkage, and at a high concentration of acid (e.g., 2.493 N) both the glycosidic linkages would be cleaved at a comparable rate.

Therefore, it would be reasonable to expect that, on hydrolysis with low concentration of acid, the mucopolysaccharides will produce urnoide disaccharide, and, with high concentrations of acid and under appropriate conditions, both kinds of the unit disaccharide (uronide and 2-amino-2-deoxy-hexoside) might be produced. This assumption would not be in contradiction with the facts reported by Meyer and his co-worders(42,50) and by Masamune and his co-workers.(125)

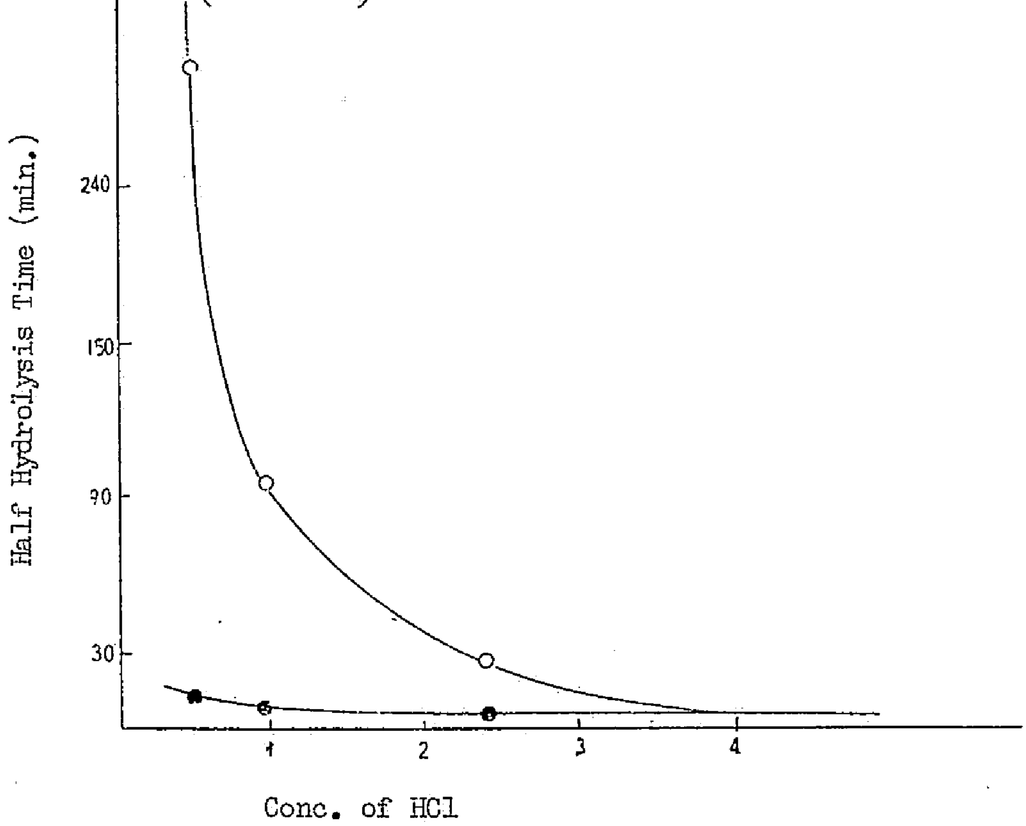
3. Acid Hydrolysis of the Mucopolysaccharides with special reference to Chondroitin Sulfate (from Shark Cartilage).

(a) Complete Hydrolysis of Chondroitin Sulfate.

From the fundamental data obtained from acid hydrolysis of methyl glycosides, it is concluded that the acid concentration for the complete hydrolysis of the mucopolysaccharides is required to be in the strength of 4 N or higher concentrations of hydrochloric acid, as shown in Fig. III.2.

According to this assumption, complete hydrolysis of chondroitin sulfate (IV.(a)) and desulfated chondroitin

Fig. III.2 Comparison of the Ease of Acid Hydrolysis of Methyl 2-acetamino-2-deoxy-β-D-glucopyranoside (—●—●—) and Methyl β-D-glucopyranoside uronamide (—○—○—)



methyl ester (IV.(b)) was performed by 4 N and 6 N hydrochloric acid, respectively. In each case 100 mg. of sample was dissolved in 2 ml. of corresponding concentrations of hydrochloric acid and was hydrolyzed on a boiling water bath at 100°. The results are shown in Table III.III.

The yield of 2-amino-2-deoxy-D-galactose was 60–80% theoretical and D-glucuronic acid was decomposed under these conditions to yield only a small amount. Boas stated that 2-amino-2-deoxy-D-glucose was decomposed about 20–25% under refluxing with 6 N hydrochloric acid at 100°.(109) Accordingly, above obtained data would be reliable, and it would suggest that complete hydrolysis of the mucopolysaccharide should be performed with 4 N or higher concentrations of hydrochloric acid.

Table III.III. Complete Hydrolysis of Chondroitin Sulfate and Desulfated Chondroitin Methyl Ester.

	Yield (%)		
	conc. of HCl	2-amino-2-deoxy-D-galactose[a)]	D-glucuronic acid[b)]
Chondroitin Sulfate	4 N	69.3	6.1
	6 N	55.7	6.2
Desulfated Chondroitin Methyl Ester	4 N	83.0	7.3
	6 N	67.5	5.0

a). 2-Amino-2-deoxy-D-galactose was determined by Eslon-Morgan reaction.(119,128)

b). D-Glucuronic acid was determined by Dische's method.(126)

(b) Isolation of the Unit Disaccharide from Chondroitin Sulfate.

As it has been predicted that the unit disaccharide of chondroitin sulfate exists in two forms, i.e.; the D-glucuronide disaccharide and the 2-amino-2-deoxy-D-galactoside disaccharide. By acid hydrolysis of chondroitin sulfate A, a unit disacchride was isolated by Leven 1914,(108) and, afterwords, by K.H.Meyer, Felling and Fischer,(127) and by Wolfrom, Madison and Cron,(49) respectively. The glycosidic linkage of the isolated unit disaccharide was considered to be 4-O-(β-2-acetamino-2-deoxy-D-galactopyranosyl)-D-glucuronic acid. However, this was reinvestigated by Meyer and his co-workers(42, 50) by the isolation of hyarobiuronic acid from hyaluronic acid, and chondrosin from chondroitin sulfate A. The former compound was determined as 3-O-(β-D-glucuronosyl)-2-acetamino-2-deoxy-D-glucose and the latter compound as 3-O-(β-D-glucuronosyl)-2-acetamino-2-deoxy-D-galactose. The results were supported by another investigators(51,125) and by our fundamental data of the acid hydrolysis of methyl glycosides.(58)

Although the above described evidences were proved with hyaluronic acid as well as chondroitin sulfate A, chondroitin sulfate C, which is different in infrared absorption spectrum and specific rotation from chondroitin sulfate A, remained unsolved in regards to the chemical structure. Chondroitin sulfate from shark cartilage was assumed to be chondroitin sulfate C in its physical and chemical properties. Therefore, isolation of chondrosin from chondroitin sulfate C by acid hydrolysis was performed and the D-glucuronidic linkage of this substance was investigated.

(b.1) Preparation of Chondrosin (III.VII).

Purified chondroitin sulfate (5 g.) was hydrolyzed by 100 ml. of 2 N hydrochloric acid for 2 hr. at 100°. Under these conditions a small amount of 2-amino-2-deoxy-D-galactose and oligosaccharide was detected. The solution was neutralized with a saturated aqueous solution of sodium bicarbonate to pH 5.6, and was evaporated under reduced pressure to dryness. The obtained substance was dried over phosphorous pentoxide and was used for the next reaction.

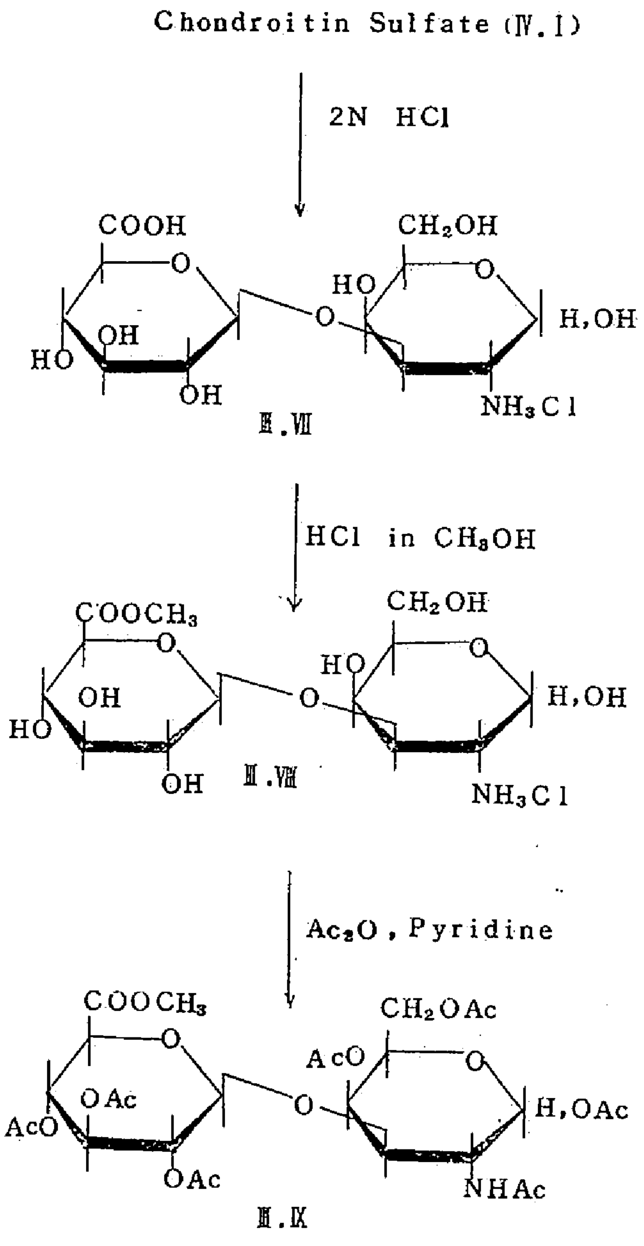

Chondroitin Sulfate (Ⅳ.Ⅰ)
2N HCl
COOH
HO
OH
OH
CH₂OH
HO
O
H,OH
NH₃Cl
Ⅲ.Ⅶ
HCl in CH₃OH
COOCH₃
OH
HO
OH
CH₂OH
HO
O
H,OH
NH₃Cl
Ⅲ.Ⅷ
Ac₂O, Pyridine
COOCH₃
OAc
AcO
OAc
CH₂OAc
AcO
O
H,OAc
NHAc
Ⅲ.Ⅸ

Fig. Ⅲ.3

(b.2) Chondrosin Methyl Ester Hydrochloride (III.VIII).

The above dried substance (1 g.) was suspended in 100 ml. of 0.02 N methanolic hydrogen chloride. The reaction mixture was shaken for 20 hr. The undissolved substance was separated by filtration and the pale yellow solution was evaporated under reduced pressure to obtain white powder. This was again dissolved in hot ethanol, decolorized and ethanol was evaporated. Repeated recrystallizations gave 2.5 g. of chondrosin methyl ester hydrochloride (III.VIII). Yield, 0.83 g. (80 %), m.p. 159-160°. $[\alpha]_D^{20}$ + 40° (c 1, water), after 18 hr., +35°. Anal., Calcd. for $C_{13}H_{24}O_{11}NCl$, C, 38.48; H, 5.96; N, 3.45. Found, C, 38.62; H, 6.07; N, 3.34. The reported constants for this compound are m.p. 159-161°, $[\alpha]_D^{23}$ +42° (c 2, water)(50), m.p. 155-156°, $[\alpha]_D^{25}$ +39° (c 4, methanol).(49)

It is apparent that the constants of chondrosin methyl ester hydrochloride prepared from chondrotin sulfate from shark cartilage (chondroitin sulfate C) are in good agreement with the reported constants. Consequently, the substance is considered to be identical with chondrosin methyl ester hydrochloride obtained from chondroitin sulfate A. This indicates that the chemical structure of chondrosin is 3-O-(β-D-glucuronosyl)-2-acetamino-2-deoxy-D-galactose.

(b.3) Heptaacetyl Chondrosin Methyl Ester (III.IX).

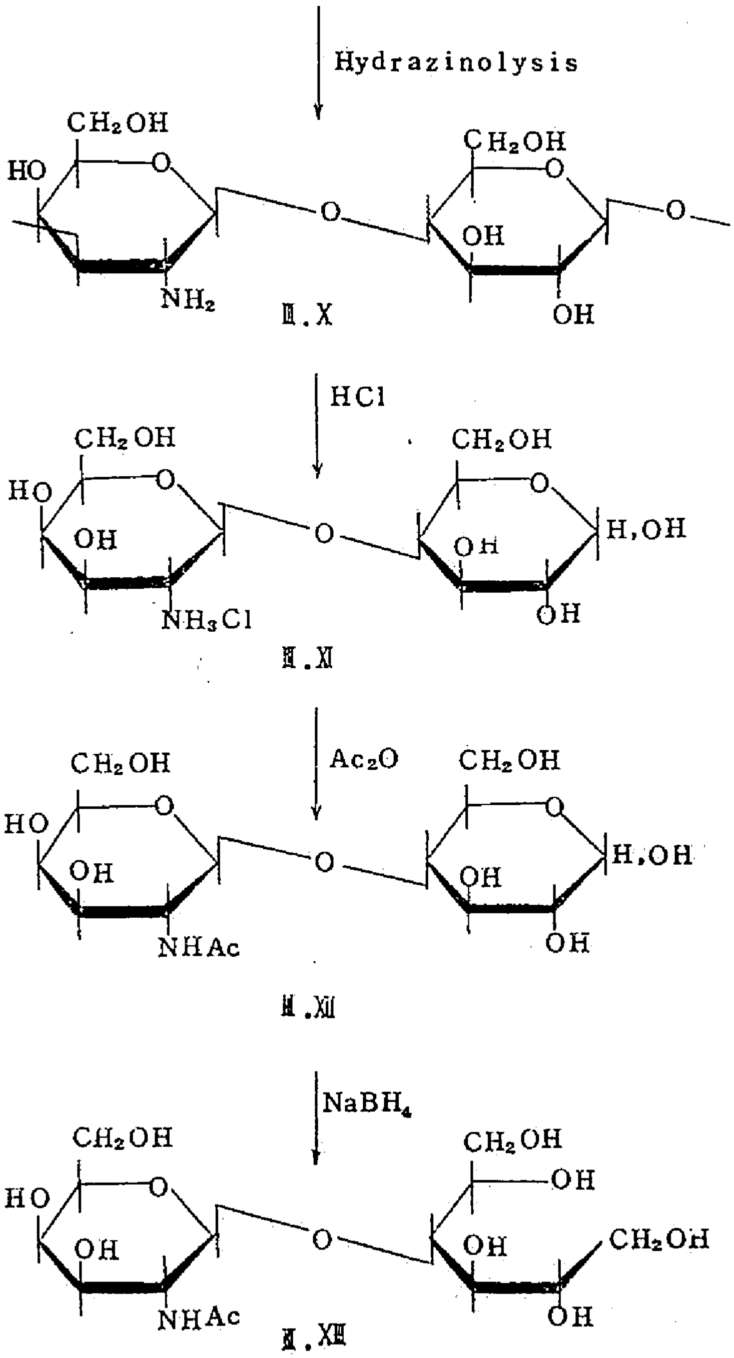
Chondrosin methyl ester hydrochloride (1 g.) was acetylated by pyridine (5 ml.) and acetic anhydride (5 ml.) by usual procedures. A fine crystalline substance was obtained by recrystallization from ethanol. Yield, 1.1 g. (70 %), m.p. 190-192°. Anal., Calcd. for $C_{27}H_{37}O_{18}N$, C, 48.87; H, 5.62; N, 2.11. Found, C, 48.70; H, 5.92; N, 2.20. $[\alpha]_D^{20}$ +24° (c 0.5, methanol).

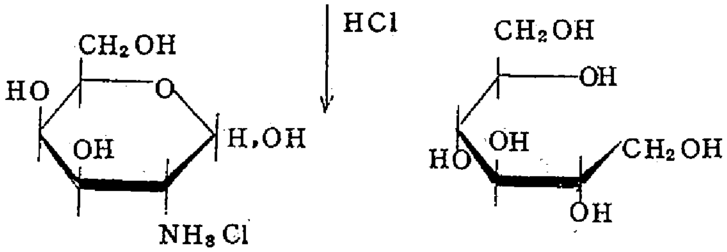
4. Acid Hydrolysis of Hydrazinolyzed Carboxyl-reduced Chondroitin.

Anhydrous hydrazine had a week reducing power and the use of this character was first introduced into protein chemistry by Akabori and his co-workers.(128) Afterwords, Matushima and Fujii(129) applied the hydrazinolysis to chondroitin sulfate C and found to cause a low but successful deacetylation. Wolfrom and Juliano(115) investigated further

Fig. Ⅲ.4

Carboxyl—reduced Chondroitin (Ⅳ.Ⅲ)





XIII, XIV

other mucopolysaccharides and the isolation of 2-amino-2-deoxy-D-hexodide disaccharide has reported by Yoshizawa,(130) and Yoshizawa and Sato.(131) They obtained the new disaccharide by hydrazinolysis and successive acid hydrolysis of blood-group A substance of hog gastric mucosa. The yield was very poor and the hydrolysate contained several kinds of substances, and, futhermore, unknown byproducts were also presented. Nevertheless, as has been suggested from the result of acid hydrolysis of methyl 2-amino-2-deoxy-D-glucose hydrochloride, 2-amino-2-deoxy-D-hexoside disaccharide is expected to be obtained by acid hydrolysis of hydrazinolyzed mucopolysaccharides.

The hydrazinolyzed carboxyl-reduced chondroitin was hydrolyzed with 3 N hydrochloric acid at 100° for 75 min. and hydrolysate was examined by paper chromatography. There were detected mainly three spots, which were D-glucose, 2-amino-2-deoxy-D-galactose and an unknown substance. This substance reduced the Fehling's solution and gave positive test for the Molish reaction. This was considered to be 2-amino-2-deoxy-D-galactoside disaccharide and detailed characterizations were carried out as described below.

(a) N-Deacetylated Carboxyl-reduced Chondroitin (Hydrazinolized Carboxyl-reduced Chondroitin)(III.X).

Carboxyl-reduced chondroitin (IV.III)(10 g.) was heated with anhydrous hydrazine (50 ml.)(132) in an autoclave for 15 hr. at 100-110°. The reaction mixture was cooled to room temperature and was concentrated under reduced pressure to dryness to remove the excess of hydrazine. The obtained brown sirup was dissolved in water (50 ml.) and was dialyzed against running water for 24 hr. and then against distilled water for 24 hr. A small amount of precipitate was separated by filtration. Slightly brown colored solution was concentrated under reduced pressure to a small volume, and to this was added a large amount of ethanol-ether (40:60 V/V). Light-yellow precipitate was collected by centrifugation and was dried over phosphorous pentoxide. Yield, 4.8 g. (53 %), $[\alpha]_D^{12}$ +7.8° (c 0.5, 0.3 N HCl). Anal., Calcd. for $C_{12}H_{21}O_9N$, N, 4.33. Found, N, 4.55.

(b) Acid Hydrolysis of N-Deacetylated Carboxyl-reduced Chondroitin.

N-Deacetylated carboxyl-reduced chondroitin (III.X)(10 g.) was dissolved in 1.5 N hydrochloric acid (100 ml.) and was hydrolyzed for 100 min. on a boiling water bath. Paper chromatographic examination of the hydrolysate revealed the presence of D-glucose (Rf, 0.17), 2-amino-2-deoxy-D-galactose (Rf, 0.12) and two unknown spots (Rf, 0.07, 0.03)(solvent; n-butanol:acetic acid:water = 40:10:50), and two of them were positive for ninhydrin and o-aminodiphenyl reagents.

(c) Isolation of N-Acetyl-Carboxyl-reduced Isochondrosin [tentatively] (4-O-(β-2-Acetamino-2-deoxy-D-galactopyranosyl)-D-glucopyranose)(III.XII).

The hydrolysate obtained above was neutralized with N sodium hydroxide to pH 5.8 and the solution was passed through a Dowex 50 (H^+) column (3 x 20 cm.). The column was washed with water thoroughly and the eluate was concentrated under reduced pressure to dryness. White precipitate was characterized as D-glucose by means of paper chromatography. Yield, 2 g. (18 %). Total acetylation of this substance gave 1.2.3.5.6-penta-O-acetyl-D-glucose, m.p. 136°, and the melting point on admixture with the authentic specimen showed no depression.

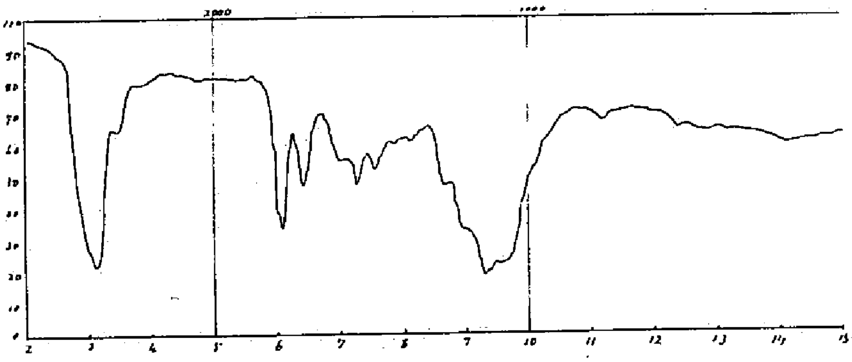
The column was then eluted with 0.5 N hydrogen chloride until the eluate became negative for ninhydrin test. The eluate was concentrated under reduced pressure to dryness. The crude substance weighing 4.5 g. (41 %) was again dissolved in water, decolorized and the solution was evaporated under reduced pressure. By the paper chromatographic examination of the product, three ninhydrin positive substances were detected, being contaminated with a small amount of D-glucose. The mixture was acetylated by usual procedures with acetic anhydride (16 ml.) and pyridine (16 ml.). The reaction mixture was poured onto ice-water and a small amount of solid matter was removed by filtration. The filtrate was extracted three times with chloroform (100 ml. for each time), and the chloroform solution was washed with a saturated aqueous solution of sodium bicarbonate, N hydrochloric acid and with water, and then was concentrated under reduced pressure to a small volume. A large amount of ether was added and white precipitate separated (3.5 g.). This was dried in a vacuum desiccator.

The above obtained substance was deacetylated with methanol saturated with ammonia and the solution was evaporated. White powder was obtained (2.5 g.). Paper chromatographic examination of this substance by using the solvent of n-butanol: ethanol:water (45:10:45) detected the spots of 2-acetamino-2-deoxy-D-galactose (Rf, 0.22), D-glucose (Rf, 0.18) and the substances whose Rf values were 0.10, and 0.06, respectively. The substance having Rf 0.10 was assumed to be 2-acetamino-2-deoxy-D-galactoside disaccharide and the substance having Rf 0.06 was considered to be an oligosaccharide.

The mixture of these four substances was dissolved in a solvent (n-butanol:ethanol:water = 45:10:45) and was separated by gradient elution of cellulose powder column (5 x 40 cm.)

using the same solvent. The eluate was fractionated in 50 ml. portions and was examined paper chromatographically. The separation was effected by this procedure and the fractions from 35 to 55 (total volume of 1 l.) were collected and concentrated to dryness. The yield of the crude substance was 750 mg. This was dissolved in hot ethanol, decolorized and precipitated by the addition of ethylacetate. Repeated recrystallizations from hot ethanol-ethylacetate afforded white powder, which gave a single spot on paper chromatogram, reduced the Fehling's solution and was positive for the Molisch test, m.p. 139-141°, $[\alpha]_D^{18}$ +73.1° (c 0.32, water)(15 min.) $\longrightarrow$ +43.8° (20 hr.). Anal., Calcd. for $C_{14}H_{25}O_{11}N \cdot H_2O$, C, 41.89; H, 6.78; N, 3.49. Found, C, 42.16; H, 6.87; N, 3.24. This compound seems to be a monohydrate.

N-Acetyl-Carboxyl-reduced Isochondrosin (III.XII)(KBr) pellet)



(d) Determination of the Reducing End of N-Acetyl-Carboxyl-reduced Isochondrosin.

The above obtained disaccharide (20 mg.) was dissolved in water (3 ml.) and to this was added sodium borohydride (20 mg.). The reaction mixture was stirred for 1 hr. with cooling and kept for 2 hr. at room temperature. After neutralizing with acetic acid, the solution was passed through a Dowex 50 (H^+) column and the eluate was concentrated under reduced pressure with occasional addition of methanol.

The obtained sirup was dissolved in 4 N hydrochloric acid (5 ml.) and was hydrolyzed for 3 hr. on a boiling water bath.

The solvent was removed under reduced pressure and the residue was examined paper chromatographically. The results are shown in Table III.IV.

Table III.IV. Rf Values of the Hydrolysate of the Reduced N-Acetyl-Carboxyl-reduced Isochondrosin (4-O-(β-2-acetamino-2-deoxy-D-galactopyranosyl)-D-glucitol)(III.XIII)

	Solvents	
	n-butanol:acetic acid: water (40:10:50)	n-butanol:ethanol: water (45:10:45)
	Rf a)	Rf b)
D-Glucose	0.24	0.14
D-Glucitol	0.24	0.14
Hydrolysate	0.24 0.17	0.14 0.09
2-Amino-2-deoxy-D-galactitol hydrochloride b)	0.13	0.05
2-Amino-2-deoxy-D-galactose hydrochloride	0.17	0.09

a) The spraying reagents used were 0.1 % AgNO$_3$ in 50 % aqueous alcohol and 1 % KMnO$_4$ in 2 % aqueous Na$_2$CO$_3$.

b) 2-Amino-2-deoxy-D-galactitol hydrochloride was prepared by the reported procedure.(133)

D-Glucose and D-glucitol showed the same Rf value in the case of both the solvents, and there was detected 2-amino-2-deoxy-D-galactose hydrochloride. This fact indicates that 2-amino-2-deoxy-D-galactose moiety of carboxyl-reduced isochondrosin is not the reducing end, and consequently, the reducing end is D-glucose.

Accordingly, it is concluded that the above obtained new disaccharide is 2-amino-2-deoxy-D-galactoside disaccharide as has been expected from the fundamental studies on acid hydrolysis of methyl glycosides.

5. Discussion.

Fundamental studies on acid hydrolysis of methyl D-glucuronides and methyl 2-amino-2-deoxy-D-glucosides were carried out under the same conditions. It has been suggested that methyl D-glucopyranoside uronamide is more resistant to acid hydrolysis than methyl 2-acetamino-2-deoxy-D-glucopyranoside, provided the acid concentration is low. It has been reported that mild acid hydrolysis of chondroitin sulfate given chondrosin, D-glucuronide disaccharide.

Acid hydrolysis of chondroitin sulfate showed that under drastic conditions, D-glucuronic acid was readily decarboxylated. Therefore, D-glucuronic acid moiety was reduced beforehand to D-glucose to avoid decarboxylation.

Methyl 2-amino-2-deoxy-D-glucopyranoside hydrochloride, as well as methyl 2-deoxy-2-sulfamino-D-glucopyranoside, resisted strongly to acid hydrolysis. These results lead us to perform acid hydrolysis of deacetylated carboxyl-reduced chondroitin. Deacetylation with anhydrous hydrazine of the mucopolysaccharides was effectively carried out, but the yield was very low. During hydrazinolysis, destruction of molecule was also observed.(130,131) Nevertheless, a new 2-amino-2-deoxy-D-galactoside disaccharide was isolated from acid hydrolysate of hydrazinolyzed product of carboxyl-reduced chondroitin.

On acid hydrolysis of carboxyl-reduced heparin,(57) another product than hexoside disaccharide was also found. Futher characterization of this substance was not reported. It seems probable that this substance would be 2-amino-2-deoxy-D-hexoside disaccharide since sulfate ester group in heparin is very labile towards acid hydrolysis.

6. Summary.

Acid hydrolysis of methyl 2-acetamino-2-deoxy-α- and β-D-glycopyranosides, methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts, methyl 2-amino-2-deoxy-α- and β-D-glucopyranoside hydrochlorides, methyl α- and β-D-glucofururonoside-γ-lactones, methyl β-D-glucopyranoside uronamide and methyl α- and β-D-glucopyranosides was performed with 2.493 N 0.996 N and 0.463 N hydrochloric acid, respectively.

Taking into consideration the results obtained from acid hydrolysis of methyl glycosides (Table III.I), acid hydrolysis of chondroitin sulfate from shark cartilage and the deacetylated carboxyl-reduced chondroitin was carried out. Acid hydrolysis of chondroitin sulfate gave chondrosin which was characterized as chondrosin methyl ester hydrochloride (III.VII) and acid hydrolysis of the deacetylated carboxyl-reduced chondroitin gave 2-amino-2-deoxy-D-galactoside disaccharide (III.XIII), which is a novel unit disaccharide of chondroitin sulfate.

IV. Determination of Chemical Linkages of the Mucopolysaccharides with special reference to Chondroitin Sulfate (from Shark Cartilage) by Methylation Method.

1. Methylation of the Mucopolysaccharides.

Since Haworth and Leitch had published methylation of maltose with dimethylsulfate and alkali,(134) much of the contribution has been done to the field of carbohydrate chemistry, especially to the determination of glycosidic linkage of polysaccharides.(135-137) In this procedure free hydroxyl groups of polysaccharide are methylated by repeated methylation with dimethylsulfate and alkali. By acid hydrolysis of the methylated product, partially methylated monosaccharide is expected to be produced. This accounts for the position of hydroxyl group which is responsible for glycosidic linkage. Therefore, when the obtained partially methylated sugar is compared with the reference compound, the glycosidic linkage of original polysaccharide is to be determined.

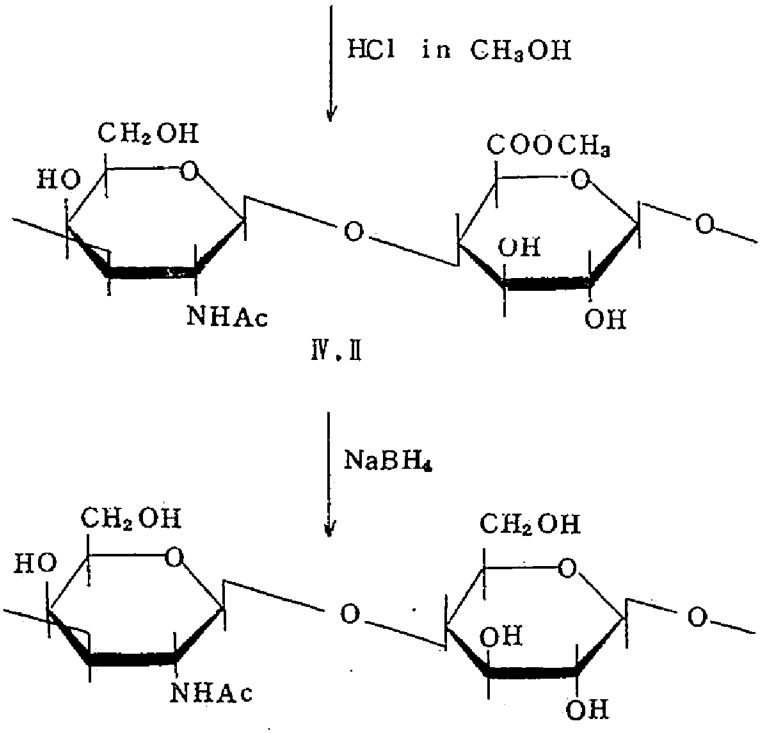
Methylation of starch by this method gave very low yield of methylated product. This was considered to be due to the alkali elimination of glycosidic linkage which caused the destruction of molecule. In order to avoid the destruction of molecule, Freudenberg and Boppel,(138) and Hodge, Karjala and Hilbert(139) reported use of liquid ammonia, methyliodide and matallic sodium which enabled successful methylation. Most commonly used method is that in which silver oxide and methyliodide are used.(140-143) However, this is effective only for disaccharide or monosaccharide.

Methylation of the mucopolysaccharides has been tried by several investigators.(144-147) However, except β-heparin (chondroitin sulfate B),(147) most of the investigations were not successful. These fact would indicate that the glycosidic linkage is rather labile in alkaline conditions. Moreover, uronic acid moiety might, to some extent, be degradated in the methylation conditions. Therefore, mild methylation is desired.

In this investigation, the D-glucuronic acid moeity of chondroitin sulfate was reduced to D-glucose.(50,54,114) This carboxyl-reduced chondroitin was methylated with liquid ammonia, methyliodide and metallic sodium, and then with dimethylsulfate and alkali, and finally with silver oxide and methyliodide. The yield of methylated carboxyl-reduced chondroitin was very low. However, acid hydrolysis of this substance gave 2.3.6-tri-O-methyl-D-glucose, which was detected by paper chromatographic examination. This gives the unquestionable evidence that the 2-amino-2-deoxy-D-galactosidic linkage is 1:4.

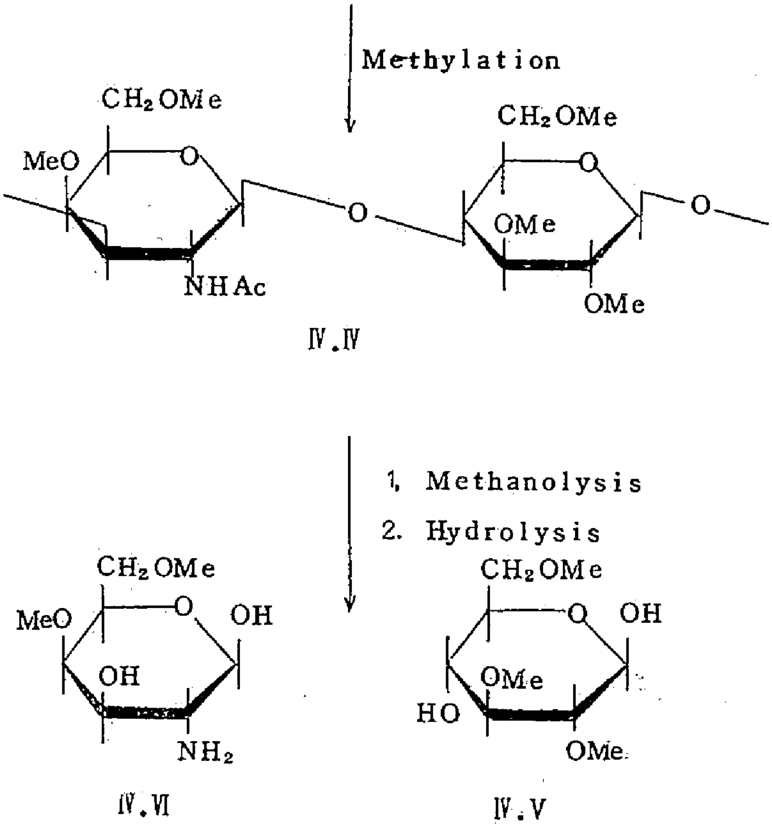
2. Chemical Structure of Chondroitin Sulfate (from Shark Cartilage) as proved by Methylation Method.

Chondroitin Sulfate (IV.I)



IV.II

IV.III



IV.IV

IV.VI

IV.V

(a) Purification of Chondroitin Sulfate (IV.I).

In Alkali extracted chondroitin sulfate were contaminated ashes and proteins. Sevag's method was applied to deproteinize crude chondroitin sulfate.(148)

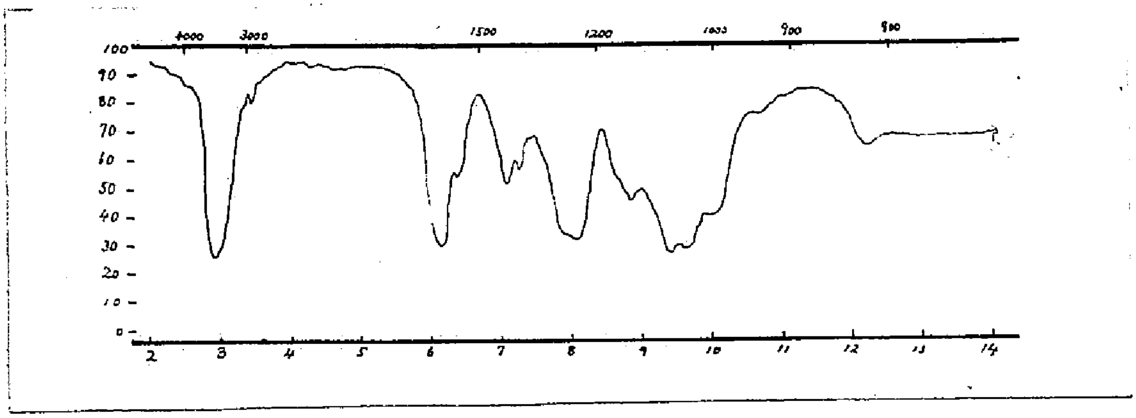
Crude chondroitin sulfate (30 g.) was dissolved in water

(500 ml.) and to this were added chloroform (180 ml.) and n-amylalcohol (60 ml.). The mixture was shaken for 20 hr. and the water layer was separated by centrifugation from the chloroform and denaturated protein layers. Ethanol (2 l.) was added to the separated water layer and to this was added with shaking a few drops of a saturated aqueous solution of sodium chloride. White precipitate was obtained and was positive for the biuret reaction. This was dissolved again in water and this process was repeated two times. White precipitate was collected by centrifugation and was negative for the biuret reaction. This was washed with ethanol and ether, and was dried over phosphorous pentoxide. Yield, 20 g. (67 %), $[\alpha]_D^{23}$ -12.4° (c 1, water). Anal., Calcd. for $C_{14}H_{19}O_{14}NNaS$, N, 2.78; S, 6.37. Found, N, 2.71; S, 6.12.

Infrared absorption bands of sulfate at 1230-1265 $cm.^{-1}$, 1000 $cm.^{-1}$, 820 $cm.^{-1}$ and 780 $cm.^{-1}$ were observed.

The data obtained from $[\alpha]_D$ and infrared spectrum showed that chondroitin sulfate extracted from shark cartilage was very much similar or same to chondroitin sulfate C.

Chondroitin Sulfuric Acid Sodium Salt (IV.I)(KBr pellet)

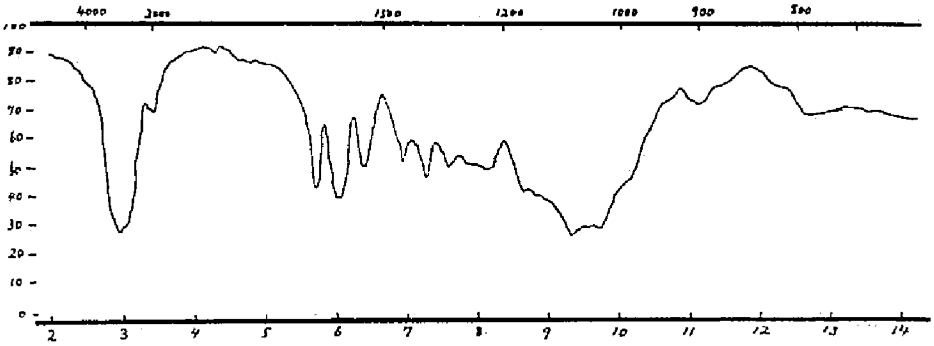


(b) Desulfation and Esterification of Chondroitin Sulfate (IV.II).

Powdered chondroitin sulfate (IV.I)(5 g.) was suspended in methanol (500 ml.) and to which was added methanolic hydrogenchloride(159) (final concentration was 0.06 mole) and the reaction mixture was shaken for 20 hr. Desulfated chondroitin methyl ester was collected by centrifugation. Desulation was repeated three times. The substance showed negative test sodium nitroprusside, but sulfate absroption band at 1265 cm.$^{-1}$ was shown very slightly by infrared analysis. Yield, 2.7 g. (84 %). $[\alpha]_D^{17}$ +25° (c 0.2, water).

Anal., Calcd. for $C_{15}H_{23}O_{11}N$, N, 3.65. Found, N, 3.96.

Desulfated Chondroitin Methyl Ester (IV.II)(KBr pellet)

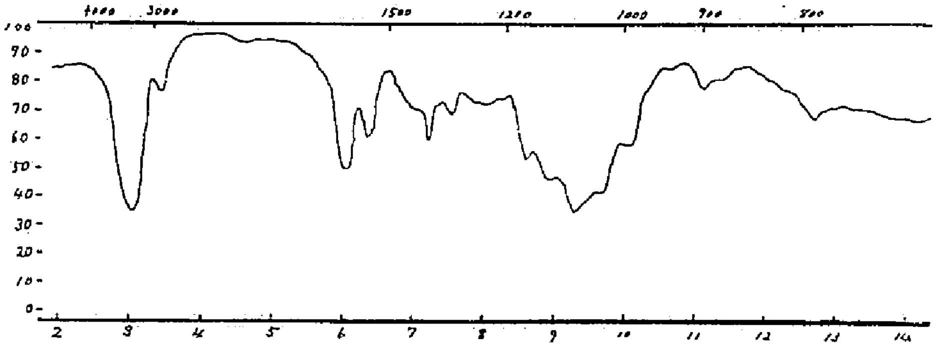


Sulfate absorption bands at 1230-1265 cm.$^{-1}$ became very weak and those at 1000 cm.$^{-1}$ and 820 cm.$^{-1}$ disappeared. Characteristic absorption bands of ester at 1740-1750 cm.$^{-1}$ and carbonyl group at 1445 cm.$^{-1}$ became apparent.

(c) Reduction of Desulfated Chondroitin Methyl Ester (IV.III).

Desulfated chondroitin methyl ester (IV.II)(10.8 g.) was dissolved in 0.4 M borin acid (180 ml.) and the solution was cooled with ice. To this was added dropwise 300 ml. (containing 3.9 g.) of aqueous solution of sodium borohydride with mechanical stirring during one hr. After the addition was over, stirring was continued for more 2 hr. at room temperature. The reaction mixture was then acidified with acetic acid to pH 5 and was kept in a refrigerator overnight. This was dialized for 24 hr. against running water and for five days against distiled water. The solution was concentrated to a small volume under reduced pressure and a large amount of ethanol was added to obtain white precipitate. This was collected by centrifugation, washed with ether and dried over phosphorous pentoxide. Yield, 12.3 g. (89 %), Anal., Calcd. for $C_{14}H_{23}O_{10}N$, N, 3.38. Found, N, 4.40.

Carboxyl-reduced Chondroitin (IV.III)(KBr pellet)



Absorption bands of ester at 1740-1750 cm.$^{-1}$ and carbonyl absorption band at 1445 cm.$^{-1}$ disappeared.

In order to reduce D-glucuronic acid moiety completely, esterification was again performed with carboxyl-reduced chondroitin as described in (b) of this chapter. Absorption bands of sulfate disappeared and absorption bands of ester and carboxyl group at 1740-1750 cm.$^{-1}$ and at 1445 cm.$^{-1}$ became very weak.

This was again reduced by sodium borohydride. Absorption bands of sulfate were not observed. Absorption bands of ester and carboxyl group disappeared. This substance hardly dissolved in water. Anal., Found, N, 3.79. $[\alpha]_D^{10}$ + 9.3° (c 0.2, water).

(d) Methylation of Carboxyl-reduced Chondroitin (IV.IV).

In the first step, carboxyl-reduced chondroitin (IV.III) (5 g.) was suspended in liquid ammonia (300 ml.) at around -60° and was soaked for 6 hr. with occasional stirring. To this were added carefully small pieces of metallic sodium (2.5 g.) within 30 min. and the mixture was stirred vigorously until blue color of sodamide disappeared. To this was added dropwise methyliodide (6.8 ml.) with shaking and the reaction mixture was left to stand for 1.5 hr. with occasional stirring. During the reaction time white gelatinous suspension was observed to occur. In the second step, metallic sodium (1.5 g.) and methyliodide (4.3 ml.) were further added and the similar process was repeated as described in the first step.

In the third and fouth steps, 1.3 g. of metallic sodium and 4.3 ml. of methyliodide were used. In the fifth and sixth step, 1.2 g. of metallic sodium and 4.3 ml. of methyliodide were used. After the reaction was over, liquid ammonia was evaporated at room temperature and the obtained solid matter was dried over conc. sulfuric acid. Dried substance was extracted repeatedly with chloroform, and no extractable substance was obtained. This was then dissolved in water (150 ml.) and the solution was dialyzed against running water for two days and against distilled water for one day. The solution was concentrated to a small volume under reduced pressure and to this was added ethanol-ether to obtain a white precipitate, which was collected and dried in a vacuum disiccator. Yield, 4 g. (67 %). Anal., Calcd. for $C_{14}H_{18}O_5N(OCH_3)_5$, OCH_3, 35.63. Found, OCH_3, 26.37. (74 % of theoretical amount).

In order to complete methylation, the above obtained substance was dissolved in water (50 ml.) and 30 %

aqueous solution of sodium hydroxide (100 ml.) was added with mechanical stirring. To the reaction mixture was added dropwise dimethylsulfate (30 ml.). It required 2 hr. A similar procedure was repeated four times under the same conditions. Throughout the reaction, temperature was kept under 30°. A pale yellow solution was neutralized with conc. hydrochloric acid and was dialyzed for two days against running water and for one day against distilled water. A small amount of precipitate was removed by filtration and the soltion was concentrated under reduced pressure to a small volume and to this was added ethanol-ether to obtain a white precipitate. This was collected by filtration, washed with ethanol-ether and was dried over phosphorous pentoxide. Yield, 0.2 g. 3.5 %). Anal., Found OCH_3, 29.3 (85 % of theoretical amount).

This was dissolved in a small amount of methanol and to this were added methyliodide (10 ml.) and silver oxide (5 g.) and the mixture was refluxed for 3 hr. To the reaction mixture were added again methyliodide (10 ml.) and silver oxide (5 g.) and the mixture was refluxed for 3 hr. To the reaction mixture were added the same reagents (10 ml. of methyliodide and 2 g. of silver oxide) and refluxing was continued for 3 hr. The last process was repeated again.

After cooling the reaction mixture to room temperature, the solid matter was removed by filtration and the filtrate was evaporated to dryness. The obtained substance was dissolved in a small amount of water and the solution was filtered. To the filtrate was added a large amount of ethanol-ether to obtain a white precipitate. This was collected by filtration and was dried in a vacuum desiccator. Yield, 80 mg. (1.4 %). Anal., Calcd. for $C_{14}H_{18}O_5N(OCH_3)_5$, N, 3.22. Found, N, 3.20, OCH_3, 30.64 (86 % of theoretical amount). Infrared absorption spectra of methylated carboxyl-reduced chondroitin showed that there still remained hydroxyl absorption band at 3400 $cm.^{-1}$

(e) Methanolysis and Acid Hydrolysis of Methylated Carboxyl-reduced Chondroitin.

Methylated carboxyl-reduced chondroitin (30 mg.) was dissolved in 2 % of methanolic hydrogenchloride (15 ml.) and the solution was refluxed for 8 hr. on a boiling water bath. After reaction was over, the solvent was evaporated under reduced pressure. Pale yellow sirup was obtained. This was dissolved in methanol, and the solution was decolorized and was evaporated.

Hydrolysis of this sirup was performed with 2 N hydrochloric acid (10 ml.) for 10 hr. on a boiling water bath. The solution was neutralized with silver oxide and the precipitated silver chloride was removed by filtration. The filtrate was decolorized and was extracted repeatedly with chloroform and the chloroform solution was dried with sodium sulfate and was concentrated under reduced pressure to dryness. A colorless sirup was obtained. This was dissolved in a small amount of water and the solution was passed through a Dowex 50 (H^+) (1 x 20 cm.) column, which was then washed with water. Neutral methylated sugar fractions were collected and the solution was concentrated to a small volume and was examined by paper chromatographic technique (Table IV.I).

The column was then eluted with 0.3 N hydrochloric acid until the eluate become negative for the ninhydrin test. The eluate was concentrated under reduced pressure and the obtained sirup was examined by paper chromatography (Table IV.I).

As shown in Table IV.I there existed several spots in hydrolysate. This would be due to the fact that methylation of carboxyl-reduced chondroitin was not complete. However, there was a substance showing R_G 0.75 with solvent A and 0.79 with solvent B, which were the same R_G values as those of 2.3.6-tri-O-methyl-D-glucose, but different from R_G values as those of 2.4.6-tri-O-methyl-D-glucose with both the solvents. This fact would indicate that one of the hydroxyl groups of the 2-acetamino-2-deoxy-D-galactose moiety links to 4-position of the D-glucose moiety in carboxyl-reduced chondroitin. Therefore, it is concluded that in chondroitin sulfate the 2-acetamino-2-deoxy-D-galactose moiety links to 4-position of the D-glucuronic acid moiety.

3. Discussion.

Methylation of β-heparin has been worked out by Jeanloz,(147) who failed to detect methylated L-iduronic acid in the molecule. It is considered that in alkaline medium the destruction of sugar molecule is observed, and moreover, in acid hydrolysis of uronic acid, decarboxylation is prone to occur. . These facts would indicate that the uronic acid moiety in mucopolysaccharides is rather labile, and, consequently, should be reduced to more stable form such as hexose. Reduction of the uronic acid moiety to hexose was carried out in good yield by using chondroitin methyl ester (IV.II) and sodium borohydride to yield carboxyl-reduced chondroitin (IV.III).

Table IV.I. R_G Values of the Hydrolysate of Methylated Carboxyl-reduced Chondroitin.

	Solvent A$^{b)}$		Solvent B$^{b)}$	
	R_G a)		R_G a)	
	o-amino-diphenyl	ninhydrin	o-amino-diphenyl	ninhydrin
2.4.6-Tri-O-methyl-D-glucose$^{c)}$	0.78		0.75	
2.3.6-Tri-O-methyl-D-glucose$^{d)}$	0.75		0.79	
	0.75		0.79	0.71
Hydrolysate	0.51	0.43	0.52	0.46
	0.29	0.29	0.35	0.26

a) R_G value was expressed as R_f value of methylated sugar/R_f value of 2.3.4.6-tetra-O-acetyl-D-glucose.

b) Solvent A; n-butanol:ethanol:water (50:10:40).
Solvent B; n-butanol:ethanol:ammonia:water (40:10:1:49).

c) 2.3.6-Tri-O-methyl-D-glucose and 2.3.4.6-tetra-O-methyl-D-glucose were prepared from methylation of cellobiose by the procedure of Haworth and Leitch.(134)

d) 2.4.6-Tri-O-methyl-D-glucose was prepared by the procedure of Freudenberg and Plankenhorn(150) and McCloskey.(151)

Methylation of carboxyl-reduced mucopolysaccharides has not been reported. Methylation of chitin(152) (consisting of 2-acetamino-2-deoxy-D-glucose) and of keratosulfate(153) (consisting of 2-amino-2-deoxy-D-glucose and D-galactose) gave methylated products in very poor yields. Although the alkali degradation of carboxyl-reduced chondroitin had occurred during the methylation reaction and the methoxyl content of the methylated product was 86 %. There was detected 2.3.6-tri-O-methyl-D-glucose from the hydrolysate. Therefore, it is linked through carbon 4 to carbon 1 of 2-amino-2-deoxy-D-galactose. From the results of investigations with hyaluronidase,(19,20) 2-amino-2-deoxy-D-galactosidic linkage of chondroitin sulfate C was proved to be β-type, and, consequently, 2-amino-2-deoxy-D-galactosidic linkage is evidenced to be β-1:4.

Methylation of carboxyl-reduced chondroitin was performed with great difficulity, and this fact would suggest that carboxyl-reduced mucopolysaccharide would be methylated by the herein described procedures which enable to elucidate the 2-amino-2-deoxy-hexosidic linkage in the molecule.

4. Summary.

Desulfation and methyl esterification of chondroitin sulfate from shark cartilage were performed and repeated reduction with sodium borohydride was effectively carried out with desulfated chondroitin methyl ester to yield carboxyl-reduced chondroitin. With this substance was performed methylation with liquid ammonia, matallic sodium and methyliodide, and with dimethylsulfate and alkali, and then with methyliodide and silver oxide. With the methylated product were performed methanolysis and hydrolysis with hydrochloric acid. The paper chromatographic examinations of this hydrolysate detected 2.3.6-tri-O-methyl-D-glucose.

From this result, it is concluded that 2-acetamino-2-deoxy-D-galactosidic linkage of chondroitin sulfate is β-1:4.

V. Conclusion.

In order to study the chemical structures of acid mucopolysaccharides, the isolation of alternative unit disaccharides is an essential problem. However, systematic investigation about acid hydrolysis of glycosides of component sugars of the mucopolysaccharides has not been reported. Therefore, present studies have been started to synthesize the fundamental compounds of methyl D-glucuronides and methyl 2-amino-2-deoxy-D-glucopyranosides, and to hydrolyze of these compounds.

1. Methyl α- and β-D-glucofururonoside-γ-lactones, methyl β-D-glucopyranoside uronamide, methyl 2-acetamino-2-deoxy-α- and β-D-glucopyranosides, methyl 2-deoxy-2-sulfamino-α- and β-D-glucopyranoside sodium salts, methyl 2-amino-2-deoxy-α- and β-D-glucopyranoside hydrochlorides, and methyl α- and β-D-glucopyranosides were synthesized.

In methylglycosidation reactions, strong cation exchange resin Amberlite IR 120 (H^+) was used effectively as a catalyst.

Separation of the mixture of methyl 2-benzyloxycarbonylamino-2-deoxy-α,β-D-glucopyranosides was performed successfully as triacetate, which enabled us to synthesize methyl 2-deoxy-

2-sulfamino-α- and β-D-glucopyranoside sodium salts.

2. Acid hydrolysis of synthesized methyl glycosides described above was performed with 2.493 N, 0.996 N and 0.463 N of hydrochloric acid, respectively. Hydrolysis rates were compared in terms of half hydrolysis time (Table III.I). When methyl D-glucuronide had the pyranose ring, it resisted to acid hydrolysis in comparison with methyl 2-acetamino-2-deoxy-D-glucopyranoside. However, when amino-group of methyl 2-amino-2-deoxy-D-glucopyranoside was free or substituted with acid labile group such as sulfate, it resisted strongly to acid hydrolysis. The possible cleavage of mucopolysaccharides by acid hydrolysis is that, if amino group of 2-amino-2-deoxy-D-hexose moiety is substituted with acetyl group, the obtained unit desaccharide would be uronide disaccharide, and that, if amino group is free or substituted with acid labile group, the obtained unit disaccharide would be 2-amino-2-deoxy-D-hexoside disaccharide.

According to this prediction, acid hydrolysis was performed with chondroitin sulfate from shark cartilage (chondroitin sulfate C) as well as deacetylated carboxyl-reduced chondroitin (hydrazinolized carboxyl-reduced chondroitin). From the former substance there was obtained uronide disaccharide, chondrosin, while from the latter there was obtained 2-amino-2-deoxy-D-galactoside disaccharide, carboxyl-reduced isochondrosin. Together with the results obtained from methylation study, this new disaccharide is proved to be 4-O-(β-2-acetamino-2-deoxy-D-galactopyranosyl)-D-glucopyranose.

3. Methylation of carboxyl-reduced chondroitin was performed with liquid ammonia, metallic sodium and methyliodide, and with dimethylsulfate and alkali, and then with methyliodide and silver oxide. From the reaction product of successive methanolysis and hydrolysis, 2.3.6-tri-O-methyl-D-glucose was detected. This fact would indicate that carbon 1 of 2-amino-2-deoxy-D-galactose links to carbon 4 of D-glucose; namely, in chondroitin sulfate, together with the fact that testicular hyaluronidase attacks β-2-amino-2-deoxy-D-hexosidic linkage, 2-amino-2-deoxy-D-galactosidic linkage is β-1:4.

Thus, chemical structure of chondroitin sulfate from **shark cartilage is concluded to be [-3-O-(β-D-glucuronosyl)-4-O-(2-acetamino-2-deoxy-β-D-galactopyranosyl)-]$_n$.**

It is our hope that some of the procedures herein described is applicable for the investigations of other mucopolysaccharides, and that the results would be useful for the progress and development of the chemistry and biochemistry of the mucopolysaccharides.

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