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ON THE REACTION OF *N*-ACETYLCHONDROSINE, *N*-ACETYLCHONDROSINE 6-SULFATE, CHONDROITIN 6-SULFATE, AND HEPARIN WITH 1-(3-DIMETHYLAMINOPROPYL)-3-ETHYLCARBODIIMIDE

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ABSTRACT

N-Acetylchondrosine was activated at pH 4.75 with excess 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride to give an *O*-acylisourea that consists of equimolar amounts of *N*-acetylchondrosine and 1-(3-dimethylaminopropyl)-3-ethylurea, with concomitant uptake of 0.94 mol of hydrogen ion per mol of *N*-acetylchondrosine. The product was treated with sodium borohydride to give a carboxyl-reduced disaccharide, but it did not react with a nucleophile reagent, such as glycine ethyl ester, over the pH range of 4.75–11.0. The *O*-acylisourea was hydrolyzed mostly into *N*-acetylchondrosine and 1-(3-dimethylaminopropyl)-3-ethylurea with 0.1M sodium carbonate overnight at room temperature, but a small proportion was transformed into the *N*-acylurea. *N*-Acetylchondrosine 6-sulfate, chondroitin 6-sulfate, and heparin were also activated at pH 4.75 with excess 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride to give the corresponding *O*-acylisoureas containing one mol of 1-(3-dimethylaminopropyl)-3-ethylurea moiety per mol of uronic acid residue, respectively.

INTRODUCTION

The reaction of uronic acid residues in glycosaminoglycuronans with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (**1**), followed by reduction with sodium borohydride, is a conventional method for conversion of uronic acid into hexose units[1–3]. Based on the observed stoichiometry of hydrogen-ion uptake in the reaction of the uronic acid-carboxyl group with **1**, Taylor and Conrad[1] suggested that the reaction between the carboxyl group and **1** does not involve an intermediate, activated ester, but a lactone or intramolecular ester, which is formed by nucleophilic attack of a hydroxyl group at the activated carboxyl group, and this reacts with sodium borohydride to afford the carboxyl-reduced product.

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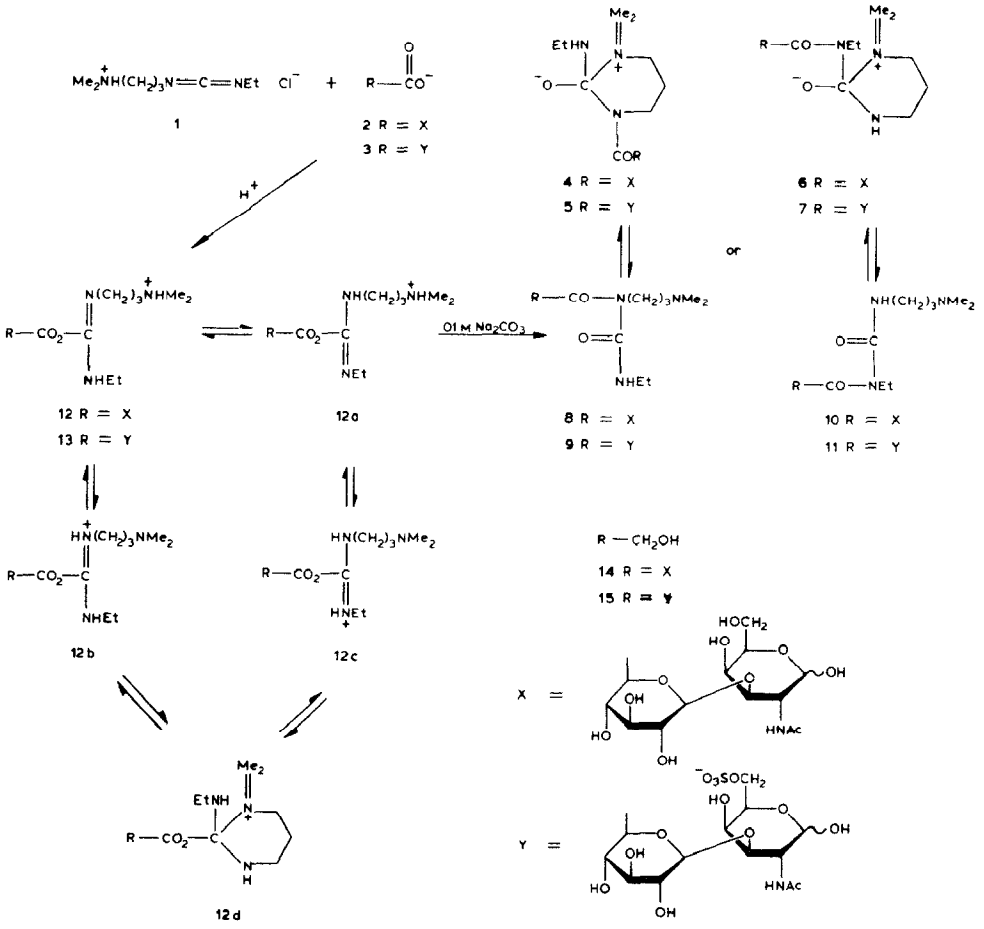
Recently, we observed that the reaction product between chondroitin 6-sulfate and **1** migrated electrophoretically to a position that was unexpected for the lactone or intramolecular ester suggested by Taylor and Conrad[1]. Since, we have investigated the reaction of *N*-acetylchondrosine (**2**) and its 6-sulfate (**3**), chondroitin 6-sulfate, and heparin with **1**, and the present report describes the isolation and characterization of the reaction products.



RESULTS AND DISCUSSION

Reaction of N-*acetylchondrosine* (**2**) *with* **1**. — *N*-Acetylchondrosine (**2**) was activated at pH 4.75 with an excess of **1** to give a cationic product that migrated to the cathode on paper electrophoretogram (Fig. 1). During the reaction, ~0.94 mol of hydrogen ions per mol of **2** was taken up. The reaction proceeded with a hydrogen-ion uptake corresponding to the degree of ionization of the uronic acid carboxyl-group of the disaccharide. The cationic reaction-product was separated

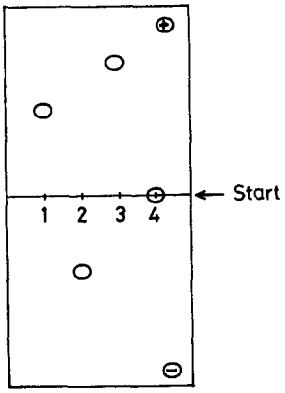


Fig. 1. Paper electrophoretogram of *N*-acetylchondrosine (**2**) and its derivatives: (1) *N*-acetylchondrosine, (2) the reaction product of **2** treated with **1**, (3) *N*-acetylchondrosine 6-sulfate (**3**), and (4) the reaction product of **3** treated with **1**.

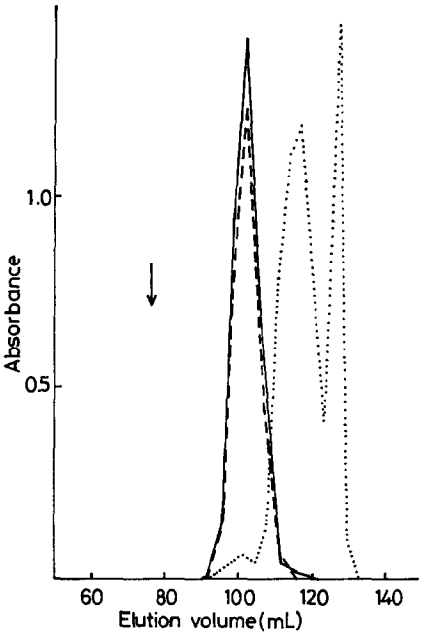


Fig. 2. Gel-filtration, on Sephadex G-15, of the reaction product of *N*-acetylchondrosine (**2**) treated with **1**: (———) carbazole reaction (absorbance at 530 nm), (– – – –) Morgan-Elson reaction (absorbance at 585 nm), and (.....) coloration of tertiary amine (absorbance at 530 nm). The arrow indicates the elution position of standard **2**.

from the hydrate of excess **1** [1-(3-dimethylaminopropyl)-3-ethylurea hydrochloride], by passing the reaction mixture through a column of Sephadex G-15 (Fig. 2). The analytical data of the compound formed showed that it contained one residue of **2** and one ureido residue (Table I). Therefore, a probable structure of the product is

TABLE I

N.M.R. DATA (δ) FOR *O*-ACYLISOUREAS OF *N*-ACETYLCHONDROSINE (**2**), *N*-ACETYLCHONDROSINE 6-SULFATE (**3**), CHONDROITIN 6-SULFATE, AND HEPARIN, AND OF THEIR ALKALINE MIGRATION PRODUCTS[a]

Compound	$-NHCH_2CH_3$	$-CH_2CH_2CH_2-$ *and* $-NHCOCH_3$	$-N^+(CH_3)_2H$	*Molar quantity of EDU[b]/disaccharide unit*
O-Acylisourea of **2** (**12**)	1.19 (3 H) (*J* 7.2)	2.03 (5 H)	2.88 (6 H)	1.0
Alkaline migration product (**8**) of **12**	1.09 (3 H) (*J* 7.2)	2.03 (5 H)	2.88 (6 H)	1.0
O-Acylisourea of **3** (**13**)	1.17 (3 H) (*J* 7.5)	2.00 (5 H)	2.87 (6 H)	1.0
O-Acylisourea of chondroitin 6-sulfate	1.22 (3 H) (*J* 7.2)	1.95 (5 H)	2.90 (6 H)	1.0
Alkaline migration product of *O*-acylisourea of chondroitin 6-sulfate	1.15 (0.3 H) (*J* 6.6)	1.95 (3.2 H)	2.81 (0.6 H)	0.10
O-Acylisourea of heparin	1.22 (3 H) (*J* 7.0)	2.05 (2.5 H[c])	2.92 (6 H)	1.0
Alkaline migration product of *O*-acylisourea of heparin	1.22 (0.8 H) (*J* 7.2)	2.05 (1.1 H)	2.92 (1.7 H)	0.28

[a] *J* values in Hz. [b] EDU, 1-(3-dimethylaminopropyl)-3-ethylurea. [c] Starting heparin contained 0.17 molar *N*-acetyl group per disaccharide unit.

either the *O*-acylisourea **12**, which has been known as an unstable intermediate, or the *N*-acylurea **8** or **10**, which is produced by migration of the acyl group to either nitrogen atom of the 1-(3-dimethylaminopropyl)-3-ethylureido residue. Upon gel-filtration with Sephadex G-15, the reaction product was eluted later than **2**, in spite of its larger mol. wt. Such behavior may be due to some interaction between the ureido residue and the Sephadex gel-matrix in the medium used. The reaction product treated with hydroxylamine yielded a hydroxamate that gave a positive (characteristic red color) ferric ion test, as expected for esters or lactones. Sodium borohydride reduction of the adduct afforded the carboxyl-reduced disaccharide **14**, which gave a single peak, positive to the anthrone reaction, upon gel-filtration on Sephadex G-15. By treatment with 0.1 M sodium carbonate overnight at room temperature, the reaction product gave **2** and 1-(3-dimethylaminopropyl)-3-ethylurea, in addition to a cationic substance, presumably a product of rearrangement. On paper electrophoresis, the cationic substance migrated to the same position as that of the original product of reaction, **1** and **2**, but gave a coloration obviously different with the *p*-aminohippuric acid reagent. The cationic substance was separated from **2** and 1-(3-dimethylaminopropyl)-3-ethylurea by successive treatment with Sephadex G-15, Dowex 1-X8 (Cl^-), and again Sephadex G-15, as described in the Experimental section (Figs. 3a, b,

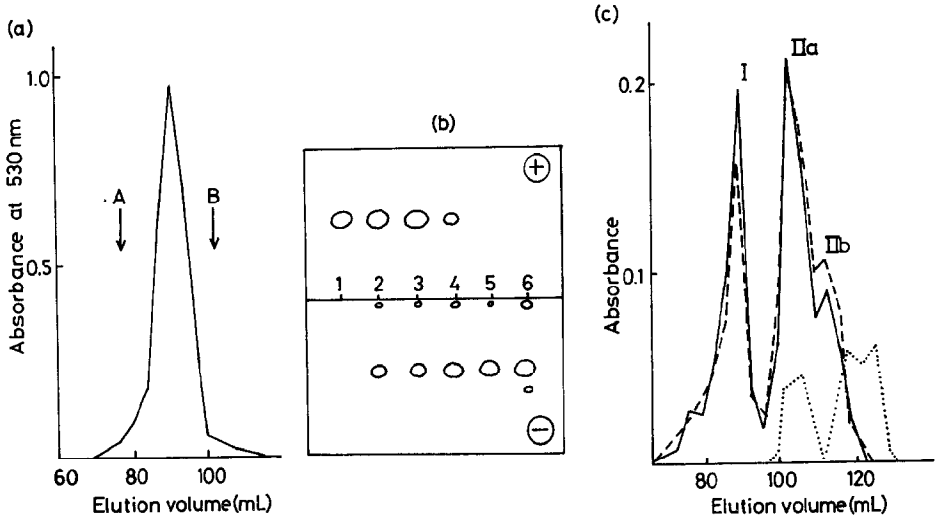


Fig. 3. Chromatographic properties of products obtained by alkaline treatment of *O*-acylisourea of *N*-acetylchondrosine (**2**): (a) the first gel-filtration on Sephadex G-15, (b) paper electrophoretogram, and (c) the second gel-filtration on Sephadex G-15 (see text). Fig. 3a: the arrows A and B indicate the elution positions of **2** and the reaction product **12**, respectively. Fig. 3b: (1) standard **2**, (2) after treatment with 0.1M sodium carbonate overnight, (3) fractions (75–100 mL) obtained from the first gel-filtration on Sephadex G-15, (4) eluates obtained from the Dowex 1-X8 (Cl^-) column, (5) fractions (99–113 mL) obtained from the second gel-filtration on Sephadex G-15, and (6) after a further treatment of the migration product **8** or **10** with 0.1M sodium carbonate overnight. Fig. 3c: (———) carbazole reaction (absorbance at 530 nm), (– – – –) Morgan-Elson reaction (absorbance at 585 nm), and (.....) coloration of tertiary amine (absorbance at 530 nm).

and c). The material of Peak I in Fig. 3c was identified as **2** by paper electrophoresis. The materials of Peaks IIa and IIb behaved like two isomeric forms of a single compound, presumably, the cyclic (**4** or **6**) and the open chain form (**8** or **10**), because their electrophoretic behavior and coloration with the *p*-aminohippuric acid reagent were identical. In addition, when a solution of the materials of combined Peaks IIa and IIb in 0.1M sodium carbonate was chromatographed on Sephadex G-15, a single peak was obtained at a position coinciding with that of Peak IIb. On the other hand, a solution of the materials in 0.1M hydrochloric acid gave, on gel-filtration, a single peak at a position coinciding with that of Peak IIa. The n.m.r. spectrum of the material of combined Peaks IIa and IIb indicated that they derived from an equimolar amount of **2** and 1-(3-dimethylaminopropyl)-3-ethylurea (Table I).

The material of combined Peaks IIa and IIb did not form a hydroxamate and was not reduced with sodium borohydride. Upon further treatment with 0.1M sodium carbonate overnight at room temperature, they no longer released **2** (Fig. 3b). The amide linkage of the compound obtained by condensation of **2** with glycine ethyl ester by the method of Danishefsky and Siskovic[4] also was not reduced with sodium borohydride, nor was it cleaved by 0.1M sodium carbonate overnight at room temperature. This suggests that the condensation product of **1** and **2** is an ester of the uronic

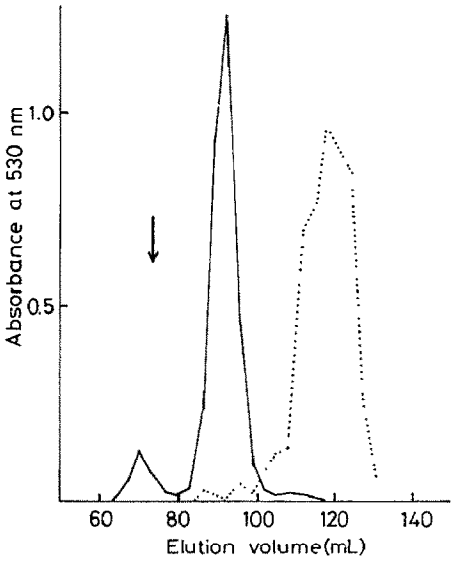


Fig. 4. Gel-filtration, on Sephadex G-15, of the reaction product of *N*-acetylchondrosine 6-sulfate (**3**) treated with **1**: (———) carbazole reaction and (.....) coloration of tertiary amine. The arrow indicates the elution position of **3**.

acid group of **2**, that is, the *O*-acylisourea **12**, and the product of alkaline migration is an amide of the uronic acid group, the *N*-acylurea **8** or **10**.

As mentioned earlier, successive treatment with Sephadex G-15, Dowex 1-X8 (Cl^-), and Sephadex G-15 columns was necessary for the separation of the product of alkaline migration. It was eluted with **2**, at a position intermediate between that of **2** and **12** (Fig. 3a), indicating the probable formation of an intermolecular salt with another molecule of **2** during gel-filtration at pH 5. This intermolecular salt is probably dissolved into **2** and the product of migration during passage through the column of Dowex 1-X8 (Cl^-), and then each compound is separated by subsequent treatment with a column of Sephadex G-15 (Fig. 3b).

No condensation took place between the isolated *O*-acylisourea **12** and glycine ethyl ester hydrochloride in acetate buffer (pH 4.75); phosphate buffers at pH 5, 6, 7, 8, and 9; 0.1 M sodium hydrogencarbonate; or 0.1 M sodium carbonate. Furthermore, no amide compound was formed by the addition of glycine ethyl ester to the reaction mixture previously prepared by the action of **1** on **2** (experiments not described). On the other hand, about one half of **2** was converted into the amide, and the other half into **12**, when **1** was added to a solution of **2** and glycine ethyl ester in water (pH 4.75), according to the procedure of Danishefsky and Siskovic[4]. This suggests that **12** is not reactive toward nucleophilic compounds, such as glycine ethyl ester, but that an active intermediate formed by **2** and **1** may, in part, react with the nucleophilic compound present in the reaction mixture to yield an amide, leading to a rapid transformation of the active intermediate into the stable *O*-acylisourea. Although the *O*-acylisourea **12** can be represented as several tautomeric forms (**12a**, **12b**,

12c, and **12d**), the real structure of the active intermediate that reacts with nucleophilic compounds, or that of the stable *O*-acylisourea, still remains unclear. The formation of a six-membered ring **12d**, may contribute, however, to the stabilization of **12**.

Reaction of N-*acetylchondrosine 6-sulfate* (**3**) *with* **1**. — Compound **3**, in water, was activated at pH 4.75 with an excess of **1** with an uptake of 0.91 mol of hydrogen ion per mol of **3**, to give a neutral product on paper electrophoresis (Fig. 1). The electrophoretic, analytical, and n.m.r. data of the product, which was isolated by gel-chromatography on a Sephadex G-15 column, showed that it derived from equimolar amounts of **3** and 1-(3-dimethylaminopropyl)-3-ethylurea which suggested the structure of *O*-acylisourea **13**. The compound reacted with sodium borohydride to give the carboxyl-reduced disaccharide **15**. Treatment with 0.1M sodium carbonate for 2 h at room temperature hydrolyzed most of **13** to give **3**, but a part was transformed into a product of migration that was isolated by column chromatography on Dowex 1-X8 (Cl^-), followed by preparative paper electrophoresis. The product migrated to the same position as that of **13** on paper electrophoretogram and was not reduced with sodium borohydride. These results show that **13**, as **12**, is, in part, converted into an *N*-acylurea (**9**, **11**, **5**, or **7**).

Glucose 6-sulfate was not activated with **1** (data not shown). The lithium salt of **2** was activated also with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *p*-toluenesulfonate to give a compound with an uptake of 0.90 mol of hydrogen ion per mol of **2**, which confirms the formation of the *O*-acylisoureas of **2**. On paper electrophoresis at pH 5.5, the *O*-acylisourea **12** migrates as a cation owing to the presence of the tertiary amino group in the urea residue, whereas the *O*-acylisourea **13** hardly migrates because of the formation of a neutral inner salt between the ureido residue and the sulfate group. These *O*-acylisoureas (**12** and **13**) are relatively stable in water or in acetate buffer (pH 4.75), and slowly hydrolyzed with 0.1M hydrochloric acid at room temperature to give **2** and **3**, respectively.

Reaction of chondroitin 6-sulfate and heparin with **1**. — Chondroitin 6-sulfate was activated with an excess of **1** to give a compound having a mobility of 0.40 on Separax electrophoresis (Table II). During the reaction, an uptake of 0.79 mol of hydrogen ion per mol of uronic acid residue was observed. The data of sulfur content and n.m.r. indicate that the compound consisted of equimolar proportions of disaccharide units of the polysaccharide and 1-(3-dimethylaminopropyl)-3-ethylureido residues, which suggests an *O*-acylisourea. This compound reacted with sodium borohydride to give carboxyl-reduced chondroitin 6-sulfate, which migrated to the same position, on Separax electrophoretogram, as that of the compound obtained by successive treatment of chondroitin 6-sulfate with **1** and with sodium borohydride, and the contents of neutral sugars of these compounds were nearly identical (Table II). Upon treatment with 0.1M sodium carbonate for 64 h at room temperature, a large part of the ureido residue of the *O*-acylisourea was hydrolyzed to give a product having R_{C6S} 0.89 (Table II). Prolonged reaction did not change the migration distance of the product on Separax electrophoresis. The n.m.r. spectrum of the product

TABLE II

ANALYTICAL DATA OF CHONDROITIN 6-SULFATE, HEPARIN, AND THE DERIVATIVES OBTAINED BY TREATMENT WITH 1-(3-DIMETHYLAMINOPROPYL)-3-ETHYLCARBODIIMIDE HYDROCHLORIDE (**1**)

Compound	*Relative mobility*[a]	*S (%)*	*Neutral sugar (%)*
Chondroitin 6-sulfate	1.0 (C)	6.44	
O-Acylisourea of chondroitin 6-sulfate	0.40 (C)	5.04 (5.14[b])	
Treated with 0.1M Na_2CO_3	0.89 (C)	6.10 (5.86[b])	
Reduced with $NaBH_4$	0.75 (C)		29.9
Product of chondroitin 6-sulfate successively treated with **1** and $NaBH_4$	0.75 (C)		29.4
Heparin	1 (H)	12.46	
O-Acylisourea of heparin	0.72 (H)	10.11 (10.21[b])	
Treated with 0.1M Na_2CO_3	0.94 (H)	11.01 (11.24[b])	
Reduced with $NaBH_4$	0.99 (H)		
Product of heparin successively treated with **1** and $NaBH_4$	0.99 (H)		

[a]Electrophoretic mobility on cellulose acetate membrane relative to that of chondroitin 6-sulfate (C) or heparin (H). [b]In parentheses, calculated value for each of the polysaccharides carrying a 1-(3-dimethylaminopropyl)-3-ethylureido residue in the observed amount (see Table I).

isolated from the dialyzed reaction mixture revealed that it contained ~0.1 1-(3-dimethylaminopropyl)-3-ethylureido residue per uronic acid residue (Table I), indicating that 90% of the ureido residue in the *O*-acylisourea of chondroitin 6-sulfate was hydrolyzed, but a residual 10% of the ureido residue rearranged to give an *N*-acylurea, similar to the formation of *N*-acylureas from *N*-acetylchondrosine (**2**) or its 6-sulfate (**3**).

Heparin was also activated with an excess of **1** with an uptake of 0.78 mol of hydrogen ion per mol of uronic acid residue to give a substance showing R_{Hep} 0.72 on Separax electrophoresis (Table II). The product was found to be an *O*-acylisourea that consists of 1-(3-dimethylaminopropyl)-3-ethylureido residue per heparin disaccharide unit, on the basis of n.m.r. spectrum, sulfur content, and chemical properties. The *O*-acylisourea of heparin was treated with sodium borohydride to yield a product having R_{Hep} 0.99 that could not be separated on Separax electrophoresis from the carboxyl-reduced heparin obtained by successive treatment of heparin with **1** and with sodium borohydride (Table II). Under identical basic conditions, ~70% of the ureido residue of the *O*-acylisourea of heparin was hydrolyzed to give a product having R_{Hep} 0.94, which contained 0.28 ureido residue per mol of uronic acid residue (Table I). In addition, the stability toward alkali indicates that a part of the carboxyl groups in the *O*-acylisourea migrates to form a stable *N*-acylurea. As mentioned

earlier, both hydrolysis and rearrangement of the *O*-acylisoureas of the polysaccharides in alkaline medium are more difficult to perform than those of the *O*-acylisoureas of the disaccharides.

Taylor and Conrad[1] suggested that the reaction of polysaccharides containing uronic acid residues with **1** led to the formation of lactones or intramolecular esters. As mentioned earlier, we have obtained experimental evidence that conflicts with their conclusion. The present results indicate that the reaction of chondroitin 6-sulfate or heparin with **1** leads to the formation of the relatively stable *O*-acylisourea with an uptake of hydrogen ion corresponding to the proportion of carboxylate anion present. The *O*-acylisourea isolated is then reduced with sodium borohydride to give the carboxyl-reduced polysaccharide, and a smaller proportion of the acyl group of the *O*-acylisourea migrates to either nitrogen atom of the 1-(3-dimethylaminopropyl)-3-ethylureido residue of the compound to give the stable *N*-acylurea under mild alkaline conditions.

EXPERIMENTAL

Materials. — Chondroitin 6-sulfate and chondro-6-sulfatase (E.C. 3.1.6.10) were obtained from Seikagaku Kogyo Co. (Tokyo), hog-mucosal heparin and glucose 6-sulfate from Sigma Chemical Co. (St. Louis, MO 63178), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (**1**) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *p*-toluenesulfonate from Nakarai Chemical Co. (Kyoto). Glycine ethyl ester hydrochloride was obtained from Nutritional Biochemicals Co. (Cleveland, OH 44128). *N*-Acetylchondrosine (lithium salt) was prepared from chondroitin 6-sulfate by the method of Inoue and Nagasawa[5].

N-Acetylchondrosine 6-sulfate (lithium salt) was prepared by the direct sulfation[6] of *N*-acetylchondrosine with pyridine–sulfur trioxide, followed by fractionation in a column of AG 1-X4 anion-exchange resin with a linear gradient (0–2M lithium chloride), and was isolated in 14% yield.

Anal. Calc. for $C_{14}H_{22}LiNO_{15}S \cdot H_2O$: C, 33.54; H, 4.83; N, 2.79; S, 6.40. Found: C, 33.25; H, 4.92; N, 2.77; S, 6.22.

The compound was digested with chondro-6-sulfatase to give a single product, *N*-acetylchondrosine, detected on a paper chromatogram[7]. All analytical samples, except chondroitin 6-sulfate and heparin were dried in the presence of phosphorus pentaoxide, in a vacuum desiccator, overnight, at room temperature. Both of the polysaccharides were dried in the presence of phosphorus pentaoxide, under reduced pressure, for 2 h at 100°.

Methods. — Uronic acid and sulfur content were determined by the methods reported previously[8], and hexose content by a modification of the method of Trevelyan and Harrison[9]. Tertiary amine was measured by color formation with 2% citric acid in acetic anhydride and a 5-min boiling time[10]. N.m.r. spectra were recorded at 22° for the disaccharides and at 100° for the polysaccharides with a Varian EM-90 n.m.r. spectrometer for solutions in deuterium oxide containing sodium 4,4-dimethyl-4-

silapentane-1-sulfonate as the internal standard. Cellulose acetate membrane electrophoresis was performed on Separax strips (Fuji Photo-Film Co., Tokyo) in 0.1M phosphate buffer (pH 8.0) with a current of 1 mA/cm for 25 min. The strips were stained with 0.5% solution of Alcian Blue. Analytical and preparative paper electrophoreses were performed on Toyo Roshi No. 51 paper (12 × 25 cm) in 5:1:5:250 (v/v) pyridine–acetic acid–1-butanol–water (pH 5.5), at a potential of 70 V/cm for 50 min at 4°. The papers were stained with the *p*-aminohippuric acid–phthalic acid reagent[11]. Gel chromatography on Sephadex G-15 was carried out at 4° with 1:10 (v/v) ethanol–water as the eluent.

Reaction of N-*acetylchondrosine* (**2**) *with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride* (**1**). — Compound **2** (lithium salt, 55 mg) was dissolved in water (2 mL), and the pH of the solution was adjusted to 4.75 by the addition of 0.1M sodium hydroxide. To this solution, **1** (102 mg)* was added in several portions at room temperature. The pH was maintained at 4.75 by titration with 0.1M hydrochloric acid. The uptake of acid (~0.89 mol of hydrogen ion per mol of **2**) stopped 1 h after the first addition of **1**. The mixture was lyophilized to a small volume, and the residue applied to a column (1.5 × 95 cm) of Sephadex G-15 at 4°, which was eluted with 1:10 (v/v) ethanol–water (Fig. 2). Each fraction was analyzed for uronic acid and tertiary amine content. The fractions of 92–105 mL were combined and lyophilized to give **12** as a white powder (74 mg, 91.4%).

Anal. Calc. for $C_{22}H_{41}ClN_4O_{12} \cdot H_2O$: C, 43.53; H, 7.14; N, 9.23. Found: C, 43.32; H, 6.95; N, 8.93.

Reaction of N-*acetylchondrosine 6-sulfate* (**3**) *with* **1**. — Compound **3** (lithium salt, 23.4 mg) was dissolved in water (1 mL), and the pH of the solution was adjusted to 4.75 by the addition of 0.1M sodium hydroxide. To this solution was added **1** (40 mg) in several portions at room temperature. The pH was maintained at 4.75 by titration with 0.1M hydrochloric acid. After 0.91 mol of hydrogen ions per mol of **3** had been taken up, the reaction mixture was treated as just described to give **13** (24.0 mg, 78.7%).

Anal. Calc. for $C_{22}H_{41}N_4O_{15}S \cdot 1.5\ H_2O$: C, 40.00; H, 6.71; N, 8.48; S, 4.85. Found: C, 39.94; H, 6.37; N, 8.34; S, 4.68.

Reaction of chondroitin 6-sulfate with **1**. — Chondroitin 6-sulfate (sodium salt; S, 6.44; 143 mg) was dissolved in water (10 mL). The pH of the solution was adjusted to 4.75 by addition of 0.1M hydrochloric acid. To this solution was added **1** (230 mg) in several portions at room temperature. The pH was maintained at 4.75 by titration with 0.1M hydrochloric acid. After 0.79 mol of hydrogen ions per mol of uronic acid residue had been taken up, the solution was dialyzed against running tap-water overnight, and then against two changes of distilled water (2.5 L each). Lyophilization of the dialyzate gave a white powder (145 mg, 79.2%).

Anal. Calc. for an *O*-acylisourea containing one 1-(3-dimethylaminopropyl)-3-

*The molar proportion of water-soluble carbodiimide was 4 times that of uronic acid residue, unless otherwise noted.

ethylureido residue per uronic acid residue: S, 5.14; N, 8.47. Found: S, 5.04; N, 8.24.

Reaction of heparin with **1**. — Heparin (sodium salt; S, 12.46; 122 mg) was dissolved in water (10 mL). The pH of the solution was adjusted to 4.75 by addition of 0.1M hydrochloric acid. To this solution was added **1** (230 mg, 6 times molar quantity of uronic acid residue) in several portions at room temperature. The pH was maintained at 4.75 by titration with 0.1M hydrochloric acid. After 0.78 mol of hydrogen ions per mol of uronic acid residue had been taken up, the solution was treated as described for the reaction of chondroitin 6-sulfate. The *O*-acylisourea of heparin was obtained as a white powder (110 mg, 73.6%).

Anal. Calc. for an *O*-acylisourea containing one 1-(3-dimethylaminopropyl)-3-ethylureido residue per uronic acid residue: S, 10.21; N, 7.61. Found: S, 10.11; N, 7.56.

Reduction of O*-acylisoureas* (**12** *and* **13**) *and their products of alkaline migration* (**8** *and* **9**) *with sodium borohydride*. — Reduction of these disaccharide derivatives was performed at pH 7–8 by the alternate addition of 2M sodium borohydride (0.2 mL of 2M sodium borohydride per mg of the disaccharide) and M hydrochloric acid. After the addition of the reducing agent, the pH of the reaction mixture was adjusted to 7.0, and the solution was kept for 1 h. Excess sodium borohydride was decomposed by acidification with 1.5M sulfuric acid. The solution, made neutral with M sodium hydroxide, was applied to a column (1.5 × 95 cm) of Sephadex G-15, which was eluted with 1:10 (v/v) ethanol–water. Fractions (3.0 mL each) were analyzed by both the carbazole and anthrone methods.

Reduction of O*-acylisoureas of chondroitin 6-sulfate and of heparin with sodium borohydride*. — Sodium borohydride (75 mg) was added to a solution of the *O*-acylisourea of chondroitin 6-sulfate (32 mg) in water (4 mL). The solution was heated for 2 h at 50°, made acidic with 1.5M sulfuric acid and then neutral with M sodium hydroxide, dialyzed against running tap-water overnight, and then against two changes of distilled water (2.5 L each). Lyophilization of the dialyzate gave, as a white powder, carboxyl-reduced chondroitin 6-sulfate (sodium salt, 25 mg). The *O*-acylisourea of heparin (5 mg) was reduced with sodium borohydride (25 mg) by a method similar to that just described. The carboxyl-reduced heparin (sodium salt, 4.6 mg) was obtained as a white powder.

Reaction of chondroitin 6-sulfate and of heparin with **1**, *followed by reduction with sodium borohydride*. — A solution of chondroitin 6-sulfate (sodium salt, 50 mg) in water (8 mL) was treated with **1** (104 mg) as described in the preceding paragraph. After the hydrogen-ion uptake had ceased, solid sodium borohydride (151 mg) was added to the solution at room temperature. The mixture was heated for 2 h at 50°, made acidic with 1.5M sulfuric acid, neutral with M sodium hydroxide, dialyzed against running tap-water overnight, and then against two changes of distilled water (2.5 L each). Lyophilization of the dialyzate gave, as a white powder, carboxyl-reduced chondroitin 6-sulfate (sodium salt, 44 mg).

A solution of heparin (20 mg) in water (3 mL) was treated with **1** (60 mg), followed by reduction with sodium borohydride (240 mg) under the same conditions

as just described. Carboxyl-reduced heparin (sodium salt, 14 mg) was obtained as a white powder.

Alkaline treatment of O-*acylisoureas* **12** *and* **13**. — A solution of the *O*-acylisourea **12** (74 mg) in 0.1M sodium carbonate (1.0 mL) was kept overnight at room temperature. After the pH of the reaction mixture had been adjusted to 5.0 by the addition of Dowex 50W-X2 (H^+) cation-exchange resin, the resin was filtered off. The filtrate was lyophilized, and the residue was dissolved in 1 : 10 (v/v) ethanol–water (0.5 mL) and applied onto a column (1.5 × 95 cm) of Sephadex G-15 (Fig. 3a). The column was eluted with 1 : 10 (v/v) ethanol–water. The fractions of 75–100 mL, positive to the carbazole reaction, were pooled and lyophilized, and a solution in water of the residue was passed through a column of Dowex 1-X2 (Cl^-) anion-exchange resin. The eluate and water washings were lyophilized, and the residue was applied onto another column (1.5 × 95 cm) of Sephadex G-15 (Fig. 3c), which was eluted with 1 : 10 (v/v) ethanol–water. The pooled fractions corresponding to Peaks IIa and IIb were combined and lyophilized to give a white powder (14.5 mg).

A solution of the *O*-acylisourea **13** (7.2 mg) in 0.1M sodium carbonate (0.5 mL) was kept for 2 h at room temperature. The reaction mixture was passed through a column (1 × 10 cm) of Dowex 1-X2 (Cl^-) anion-exchange resin. The eluate and water washings were lyophilized, and the residue was dissolved in a minimum volume of water and subjected to preparative paper electrophoresis. The material that migrated to the neutral zone of the filter paper was extracted with water, and the extract was lyophilized (yield, 0.80 mg).

Alkaline treatment of O-*acylisoureas of chondroitin 6-sulfate and of heparin*. — A solution of the *O*-acylisourea of chondroitin 6-sulfate (50 mg) in 0.1M sodium carbonate (2 mL) was kept for 64 h at room temperature. The solution was dialyzed against running tap-water overnight, and then against two changes of distilled water (2.5 L each). Lyophilization of the dialyzate gave a white powder (33 mg).

A solution of the *O*-acylisourea of heparin (50 mg) in 0.1M sodium carbonate (2 mL) was treated as just described, to give a white powder (40 mg).

Reaction of O-*acylisourea* (**12**) *with glycine ethyl ester*. — Samples of *O*-acylisourea **12** (3 mg) and glycine ethyl ester hydrochloride (0.7 mg) were dissolved in each of the following buffers (50 μL): 0.1M acetate (pH 4.75); 0.15M phosphate at pH 5, 6, 7, 8, and 9; 0.1M sodium hydrogencarbonate; and 0.1M sodium carbonate. Each solution was kept at room temperature, and aliquots were withdrawn after 1, 2, and 16 h for paper electrophoresis.

Reaction of N-*acetylchondrosine* (**2**) *with glycine ethyl ester hydrochloride in the presence of* **1**. — *N*-Acetylchondrosine (**2**, lithium salt, 20 mg) and glycine ethyl ester hydrochloride (35 mg, 5 times molar quantity of uronic acid residue) were dissolved in water (0.5 mL) and the pH was adjusted to 4.75 by addition of 0.1M sodium hydroxide. To the solution was added **1** (50 mg, 5 times molar quantity of uronic acid residue) in several portions. During the reaction, the pH was maintained at 4.75 by addition of 0.1M hydrochloric acid. After the hydrogen-ion uptake had ceased (0.4 mol of hydrogen ion per mol of **2**), the reaction mixture was applied to a

column (1.5 × 95 cm) of Sephadex G-15 and eluted with 1:10 (v/v) ethanol–water. The fractions corresponding to the main peak, which consisted of [(*N*-acetylchondrosine)glycine ethyl ester]amide and **12**, were collected and lyophilized. The residue was fractionated by preparative paper electrophoresis. The material that migrated to the neutral zone of the filter paper was extracted with water and the extract was lyophilized to give [(*N*-acetylchondrosine)glycine ethyl ester]amide (11 mg, 46%).

Treatment of [(N-acetylchondrosine)glycine ethyl ester]amide with sodium borohydride. — To a solution of glycine ethyl ester amide of *N*-acetylchondrosine (3 mg) in water (0.5 mL) was added 2M sodium borohydride (1.5 mL). During the reaction, the pH was maintained at 7–8 by the intermittent addition of M hydrochloric acid. After 1 h, the mixture was lyophilized to a small volume, applied onto a column (1.5 × 95 cm) of Sephadex G-15, and eluted with 1:10 (v/v) ethanol–water. Fractions were analyzed by the carbazole and anthrone methods.

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REFERENCES

1 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
2 R. L. TAYLOR, J. E. SHIVELY, AND H. E. CONRAD, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
3 R. L. TAYLOR, J. E. SHIVELY, H. E. CONRAD, AND J. A. CIFONELLI, *Biochemistry*, 12 (1973) 3633–3637.
4 I. DANISHEFSKY AND E. SISKOVIC, *Carbohydr. Res.*, 16 (1971) 199–205.
5 Y. INOUE AND K. NAGASAWA, *Carbohydr. Res.*, 97 (1981) 263–278.
6 R. L. WHISTLER, W. W. SPENCER, AND J. N. BEMILLER, *Methods Carbohydr. Chem.*, 2 (1963) 298–303.
7 H. SAITO, T. YAMAGATA, AND S. SUZUKI, *J. Biol. Chem.*, 243 (1968) 1536–1542.
8 Y. INOUE AND K. NAGASAWA, *Carbohydr. Res.*, 46 (1976) 87–95.
9 W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 50 (1952) 298–302.
10 S. OHKUMA, *Yakugaku Zasshi*, 75 (1955) 1124–1128.
11 L. SATTLER AND F. W. ZERBAN, *Anal. Chem.*, 24 (1952) 1862.