Carbohydrate Research, 105 (1982) 69–85
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

PREPARATION AND PROPERTIES OF FLUORESCENT GLYCOSAMINOGLYCURONANS LABELED WITH 5-AMINOFLUORESCEIN

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(Received June 19th, 1981; accepted for publication in revised form, October 15th, 1981)

ABSTRACT

The uronic acid residues of all known glycosaminoglycuronans reacted with 5-aminofluorescein to yield fluorescent glycosaminoglycuronan derivatives, which showed fluorescence characteristics identical to those of fluorescein or 5-acetamidofluorescein. The fluorescent products could be purified by chromatography on Octyl-Sepharose; three preparations of labeled chondroitin 6-sulfate having different degrees of substitution, and a labeled heparin were obtained. Fluorescent hyaluronic acid containing labeled and unlabeled molecules was digested with testicular hyaluronidase to give fluorescent oligosaccharides. Fluorescent chondroitin 6-sulfate was treated with chondroitinase AC to give a nonfluorescent disaccharide and a minor proportion of fluorescent octasaccharide. Fluorescent heparin retained its anticoagulant activity, which was similar to that of the starting heparin; its half-life in circulating rabbit blood was 36 min (by fluorometry) and 45 min (by clotting-time assay).

INTRODUCTION

Various biologically significant proteins have been labeled with fluorescent substances in order to apply them as a fluorescent probe in immunological studies. In the field of carbohydrates, one of the first attempts to prepare fluorescent dextran and hyaluronic acid employed isothiocyanatofluorescein[1,2]. An attempt to label the free amino groups of heparin with 5-isothiocyanatofluorescein was reported from our laboratory[3]. Since then, we have been investigating the labeling of glycosaminoglycuronans in order to use the fluorescent products obtained for studying the function and metabolism of exogenously administered glycosaminoglycuronans.

5-Aminofluorescein (**2**) is slightly fluorescent [1/150 of the relative fluorescence intensity of fluorescein (**1**)], but its *N*-acetyl derivative, 5-acetamidofluorescein (**3**), has been found to be strongly fluorescent (Table I). In our procedure for the preparation of fluorescent glycosaminoglycuronans, the amino group of 5-aminofluorescein reacted with the carboxyl group of uronic acid residues in the polysaccharides in the

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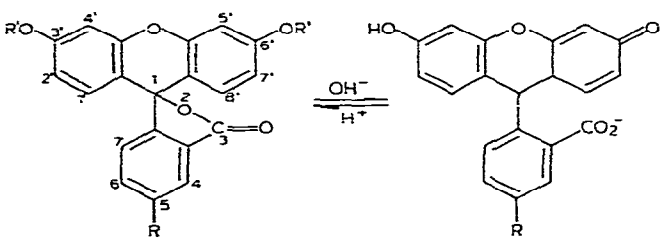
0008-6215/82/0000–0000/$ 02.75,

TABLE I

U.V. ABSORPTION AND FLUORESCENCE DATA FOR FLUORESCEIN AND ITS DERIVATIVES[a]

Compound	*Absorbance*		*Fluorescence*		
	λ_{max} *(nm)*	*Molar absorption coefficient* ($\times 10^{-4}$)	λ_{ex} *(nm)*	λ_{em} *(nm)*	RFI^b/A_{490}
1	490	8.79	491	515	2900
2	490	9.10	491	515	20
3	490	8.94	491	515	2900

[a]Measurement of absorbance and fluorescence intensity of each compound was made for solutions in 50mM borate buffer, pH 10.0. [b]Relative fluorescence intensity.



1 R = R' = H
2 R = NH_2, R' = H
3 R = NHAc, R' = H
4 R = NHAc, R' = Ac

presence of suitable condensing reagents, *e.g.*, 3-(3-dimethylaminopropyl)-1-ethyl carbodiimide. The present report describes the preparation of fluorescent derivatives of typical glycosaminoglycuronans, their analytical properties compared with those of the parent polysaccharides, and some basic experiments in biological systems.

EXPERIMENTAL

Materials. — Hog mucosal heparin (anticoagulant activity, 161 USP units/mg) and 5-aminofluorescein were purchased from Sigma Chemical Co., St. Louis, MO 63178. Hyaluronic acid (rooster comb, mol. wt. 2 000 000 by viscosity measurement), chondroitin 4-sulfate (mol. wt. 64 500 by the Nelson–Somogyi method), chondroitin 6-sulfate (mol. wt. 65 000 by the light-scattering method), dermatan sulfate (hog skin), and heparan sulfate (bovine kidney, 1.25M sodium chloride fraction from Dowex 1 column) were supplied by Seikagaku Kogyo Co. Ltd., Tokyo, Japan. 3-(3-Dimethyl-aminopropyl)-1-ethyl-carbodiimide was obtained from the Protein Research

Foundation, Osaka, Japan. Bovine testicular hyaluronidase (Type IV) and chondroitinase AC (*Arthrobacter aurescens*) were purchased from Sigma Chemical Co. and Seikagaku Kogyo Co. Ltd., respectively. The methyl glycoside of *N*-acetylchondrosine was obtained[4] by solvolysis of chondroitin 4- or 6-sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of methanol for 18 h at 95°. Standard *N*-acetylchondrosine and its oligomers were obtained by solvolytic depolymerization of chondroitin 6-sulfate (pyridinium salt) in 9:1 water–dimethyl sulfoxide for 14 h at 90°, followed by separation on AG 1-X4 ion-exchange resin[5].

Analytical methods. — The uronic acid content was determined by the carbazole method[6] with D-glucurono-6,3-lactone as standard. Hexosamine determination of the glycosaminoglycuronans and oligosaccharides carrying 5-aminofluorescein groups was performed after removal of the bound fluorescein groups. After hydrolysis with 3M hydrochloric acid for 16 h at 100° and complete removal of hydrochloric acid, a solution of the hydrolyzed product in water (2 mL) was loaded onto a column (0.4 × 18 cm) of Dowex 50W-X8 (H^+, 200–400 mesh) cation-exchange resin, which was eluted successively with water (30 mL) and 0.3M hydrochloric acid (90 mL). The fraction eluted with 0.3M hydrochloric acid was subjected to hexosamine determination by a modification of the Elson–Morgan procedure[7]. The hexosamine content of the reduced fluorescent oligosaccharides was determined by the aforementioned procedure, after reduction with sodium borohydride[8]. The total sulfate content was determined by the turbidimetric method[9], and the *N*-sulfate content by the previously reported method[10]. The degree of substitution of fluorescent glycosaminoglycuronans was expressed as the molar ratio of bound 5-aminofluorescein groups to disaccharide units. The number of bound 5-aminofluorescein groups was calculated from the molar absorption coefficient of 5-acetamidofluorescein (89 400 in 50mM borate buffer, pH 10, at 490 nm), and the number of disaccharide units by hexosamine determination. The fluorescence intensity at 515 nm was measured for a solution in 50mM borate buffer (pH 10.0), under excitation at 491 nm, with a Hitachi 204 spectrofluorometer.

Cellulose acetate membrane electrophoresis was carried out on Separax strips (Fuji Photo Film Co., Tokyo, Japan) for a solution in 50mM lithium chloride–10mM hydrochloric acid (pH 2.0) or 0.3M pyridine–0.44M acetic acid (pH 5.2) with a current of 1 mA/cm for 20 min. The fluorescent bands on a strip were located under u.v.-irradiation at 365 nm, after exposure to ammonia vapor, and then the strip was stained with a 0.5% solution of Alcian Blue.

Analytical gel-chromatography was performed on the gel selected. Each sample (2 mg), dissolved in 0.15M sodium chloride (1 mL), was applied to a column (1.5 × 80 cm) of the gel prepared in 0.15M sodium chloride and eluted with the same solution at a flow rate of 20 mL/h. Fractions (2 mL per tube) were analyzed for uronic acid and for relative fluorescence-intensity.

T.l.c. was performed on precoated Silica gel 60 plates (Merck, Darmstadt) developed with 3:1 (v/v) chloroform–ethanol (solvent *A*) or 50:10:1 (v/v) ethyl acetate–methanol–butyl acetate (solvent *B*); and on precoated cellulose plates

(Merck) developed with 5:3 (v/v) butyric acid–0.5M ammonium hydroxide (solvent *C*) or 15:10:3:12 (v/v) 1-butanol–pyridine–acetic acid–water (solvent *D*). For detection of faintly fluorescent 5-aminofluorescein, the plate was sprayed with acetic anhydride and heated for 10 min at 80°, and then observed under u.v. irradiation. Oligosaccharides were detected with the *p*-aminohippuric acid–phthalic acid[11] or alkaline silver nitrate reagent[12] with *N*-acetylchondrosine and its oligomers as standards.

The anticoagulant activity was assayed by the U.S. Pharmacopoeia whole-blood assay method.

5-Acetamidofluorescein (**3**). — 5-Aminofluorescein (**2**) (500 mg) dissolved in 0.1M sodium hydroxide (50 mL) was treated with acetic anhydride (2 mL) with stirring at room temperature for 30 min. The pH of the reaction mixture was kept at 6.5–7.0 by adding 0.5M sodium hydroxide as required during the reaction. The precipitate formed by acidification (pH 4.5) with acetic acid was separated and recrystallized three times from 2:1 (v/v) acetone–water, to afford orange-yellow crystals (262 mg), m.p. 237–240° (dec.), R_F 0.40 (solvent *A*), 0.33 (solvent *B*).

Anal. Calc. for $C_{22}H_{15}NO_6$: C, 67.87; H, 3.88; N, 3.60. Found: C, 67.92; H, 3.90; N, 3.52.

5-Acetamidofluorescein-3′,6′-diyl diacetate (**4**). — To a solution of 5-aminofluorescein (**2**) (500 mg) in pyridine (10 mL), was added acetic anhydride (4 mL) with stirring, and the reaction mixture was boiled under reflux for 1 h at 120°. It was then poured into cold water (3 vol.). The precipitate (412 mg) was recrystallized twice from 1:1 (v/v) acetone–water to afford slightly brown crystals (180 mg), m.p. >300°, R_F 0.64 (solvent *B*).

Anal. Calc. for $C_{26}H_{19}NO_8$: C, 65.96; H, 4.05; N, 2.96. Found: C, 66.02; H, 4.15; N, 2.88.

This compound is soluble in acetone, but insoluble in water. It dissolved in 0.1M sodium hydroxide within 2 min at 30°, and decomposed into 5-acetamidofluorescein.

5-Aminofluorescein-labeled methyl glycoside of N*-acetylchondrosine*. — To a solution of the methyl glycoside of *N*-acetylchondrosine (100 mg) in 20 mL of 3:1 (v/v) M hydrochloric acid–pyridine, 5-aminofluorescein (95 mg) dissolved in 1:1 (v/v) M hydrochloric acid–pyridine (4 mL) was added. The pH of the solution was adjusted to 4.75 with 12M hydrochloric acid. 3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide (0.27 mL) was added to the reaction mixture with stirring at room temperature, and the pH was maintained at 4.75 by the addition of 6M hydrochloric acid. After 1 h, the reaction mixture was concentrated under diminished pressure and extracted with ethyl acetate until the organic layer showed no color. The aqueous layer was chromatographed on a column (1.5 × 30 cm) of Amberlite XAD-4 (polymerized styrene and divinylbenzene, a packing material for adsorption chromatography) with a stepwise elution with water (60 mL), 1:4 (v/v) methanol–water (60 mL), and 1:2 (v/v) methanol–water (60 mL). The fractions eluted with 1:2 (v/v) methanol–water were combined and evaporated under diminished pressure to

TABLE II

ANALYTICAL DATA FOR 5-AMINOFLUORESCEIN-LABELED OLIGOSACCHARIDES ISOLATED FROM ENZYMIC DIGESTS OF FLUORESCENT HYALURONIC ACID AND FLUORESCENT CHONDROITIN 6-SULFATE BY GEL CHROMATOGRAPHY ON SEPHADEX G-25

5-Aminofluorescein-labeled oligosaccharide	R_F *value*[a]	*Hexosamine (mmol/g)*			*Hexosamine/fluorescein group (mol/mol)*
		Before reduction (A)	*After reduction (B)*	*B/A*	
5-Aminofluorescein-labeled methyl glycoside of *N*-acetylchondrosine	0.83	1.12(1.29)[b]	0(0)	0	1.05(1.0)
Peaks of the chromatogram of hyaluronidase-digests of fluorescent hyaluronic acid					
Peak 4 (octasaccharide)	0.54	2.12	1.67	0.77(0.75)	3.90(4.0)
Peak 5 (hexasaccharide)	0.64	1.89	1.30	0.69(0.67)	3.04(3.0)
Peaks of the chromatogram of chondroitinase-digests of fluorescent chondroitin 6-sulfate					
Peak 2 (octasaccharide)	0.53	1.54	1.12	0.73(0.75)	3.74(4.0)
Peak 3 (tetrasaccharide)	0.71	1.40	0.69	0.49(0.50)	2.08(2.0)

[a]Obtained by t.l.c. on cellulose plate with solvent *D*. [b]Theoretical values are given in parentheses.

dryness. The residue was dissolved in 0.1M sodium hydroxide (10 mL) and incubated for 20 h at 30°. The mixture, made neutral with M hydrochloric acid, was applied to a column (2.5 × 85 cm) of Toyopearl HW-40 (polymerized hydrophilic vinyl monomer, a packing material for gel filtration, Toyo Soda Kogyo Co., Ltd., Tokyo, Japan), prepared in 1:3 (v/v) ethanol–water and eluted with the same solvent. The fractions showing a high intensity of fluorescence (K_{av} 0.81) were collected and rechromatographed under the same conditions. The purified material was lyophilized to give a yellow powder (28 mg). The analytical data for this material are listed in Table II.

Comparison of alkaline stability of 5-aminofluorescein and its acetylated derivatives. — To each solution of **2**, **3** and **4** (2 mg each) in acetone (1 mL, was added 0.2M sodium hydroxide (1 mL). Each of the reaction mixtures was incubated at 30° and analyzed by t.l.c. at intervals of 0 min (solvent *A*), 2 min (solvent *A*), and 24 h (solvents *A* and *B*) after incubation had been started.

Preparation of fluorescent glycosaminoglycuronans. — The following procedure is given as an example for labeling chondroitin 6-sulfate (changes for labeling other glycosaminoglycuronans are given in Table III). To a solution of chondroitin 6-sulfate (100 mg, sodium salt) in 3:1 (v/v) M hydrochloric acid–pyridine (20 mL), was added 5-aminofluorescein (**2**) (114 mg, 1.6 mol. equiv. per disaccharide unit) in 1:1 (v/v) M hydrochloric acid–pyridine (4 mL), and the pH was adjusted to 4.75

TABLE III

LABELING CONDITION OF GLYCOSAMINOGLYCURONANS WITH 5-AMINOFLUORESCEIN AND PROPERTIES OF FLUORESCENT PRODUCTS

Compound	*S (%)*	*Labeling conditions*			*Fluorescent product*				
		Amount of cpd. (mg)	*Reagents (mol/mol HexN)* 2	*DEC*[a]	*Yield (mg)*	*S (%)*	*D.S.*[b]	*RFI/A*$_{490}$	*RFI/μg*[b]
Hyaluronic acid		200	1.6	3.0	140(70%)		0.0086	2200	3.8
Chondroitin 4-sulfate	6.15	500	1.6	3.0	402(80%)	6.05	0.021	2600	10.6
Chondroitin 6-sulfate	6.11	500	1.6	3.0	389(78%)	6.09	0.015	2300	7.5
Chondroitin 6-sulfate	6.11	100	1.6	9.8	83(83%)	5.95	0.039	2300	10.8
Dermatan sulfate	6.31	300	1.6	9.8	240(82%)	6.02	0.017	1300	4.9
Heparan sulfate	6.08	50	1.6	9.8	39(78%)	5.77	0.050	2000	14.9
Heparin	12.33	500	1.6	3.0	350(70%)	12.21	0.016	2400	5.7
Heparin	12.33	500	1.6	9.8	364(73%)	12.10	0.039	2500	9.9
Heparin	12.33	500	3.9	9.8	355(71%)	12.12	0.040	2500	9.6

[a]3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide. [b]Abbreviations: D.S., degree of substitution; and RFI, Relative fluorescence intensity.

with 12M hydrochloric acid. 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide (0.37 mL, 9.8 mol. equiv. per disaccharide unit) was added to the mixture with stirring at room temperature, and the pH was maintained at 4.75 by the addition of 6M hydrochloric acid. After 1 h, the mixture was transferred to a dialysis bag (Visking tube, 30/20) and was dialyzed against running distilled water (not tap water!) for 24 h. The dialyzate was mixed with cold ethanol (3 vol.) containing 1.25% of sodium acetate. The precipitate was centrifuged off, washed with ethanol, and dissolved in 0.1M sodium hydroxide (20 mL). The solution was kept for 24 h at 30°, and then made neutral with 0.1M hydrochloric acid. Cold ethanol containing 1.25% of sodium acetate (3 vol.) was added, the precipitate was centrifuged off, washed three times with ethanol, and then dissolved in water (100 mL). The solution was dialyzed against distilled water for 24 h. After adjustment of the pH to 6.5 with 0.1M sodium hydroxide, the dialyzate was lyophilized to give the sodium salt of fluorescent chondroitin 6-sulfate (83 mg) as a yellow powder. No contamination with free **2** was detected by t.l.c. (solvents *A* and *B*). Labeling conditions and analytical data for the fluorescent preparations are listed in Table III.

Comparison of the stabilities, in alkaline medium, of chondroitin 6-sulfate labeled with fluorescein (**1**) *or 5-aminofluorescein* (**2**). — Chondroitin 6-sulfates labeled either with **1** or **2** according to the labeling procedure described in the preceding paragraph, but without the alkaline treatment (0.1M sodium hydroxide, 24 h, 30°), were used in this experiment. The details of the incubation procedure are given in the legend to Fig. 1. After incubation, the pH of the reaction mixture was adjusted to 7.0 with 0.5M acetate buffer (pH 6.0), and cold ethanol (3 vol.) containing 1.25% sodium acetate was added. The precipitate formed was centrifuged off, and washed

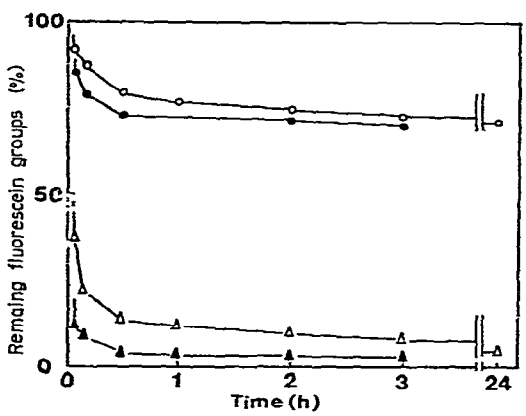


Fig. 1. Comparison of the stability, under alkaline conditions, of chondroitin 6-sulfates labeled with fluorescein (**1**) and 5-aminofluorescein (**2**). The samples (2 mg each) were treated with 0.1M sodium hydroxide (1 mL) or M hydroxylamine (adjusted to pH 12.0 with 2M NaOH) (1 mL) at 30° for the periods indicated. The proportion (%) of fluorescein groups remaining in each reaction product was determined by the procedure described in the Experimental section: Chondroitin 6-sulfate labeled with **1** treated with: (△) 0.1M sodium hydroxide, and (▲) M hydroxylamine (pH 12.0). Chondroitin 6-sulfate labeled with **2** treated with: (○) 0.1M sodium hydroxide, and (●) M hydroxylamine (pH 12.0).

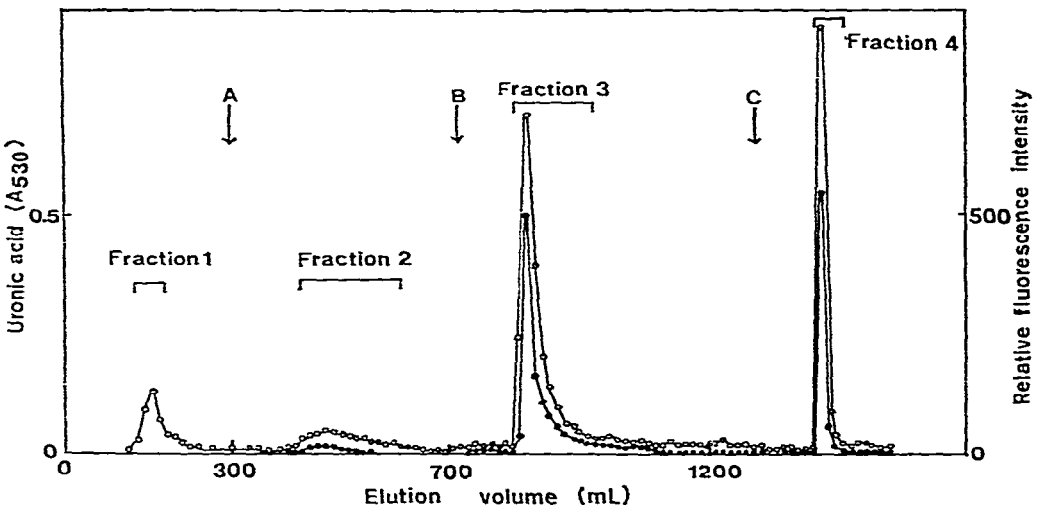


Fig. 2. Separation of fluorescent chondroitin 6-sulfate into 5-aminofluorescein-labeled and unlabeled fractions on Octyl-Sepharose CL-4B. Fluorescent chondroitin 6-sulfate (degree of substitution 0.039) (78 mg) was chromatographed in a column (1.5 × 80 cm) of Octyl-Sepharose CL-4B prepared in 2.0M ammonium sulfate in 50mM phosphate buffer (pH 6.2), and eluted stepwise with 2.0M (2 bed vol.), 1.8M (A, 2.8 bed vol.), M (B, 3.5 bed vol.), and no ammonium sulfate in 50mM phosphate buffer (C, 3.0 bed vol.) at a flow rate of 50 mL/h. Fractions (15 mL per tube) were analyzed for uronic acid (○) and relative fluorescence intensity at 515 nm (●). Each of the pooled fractions indicated by brackets was processed as described in the Experimental section.

four times with ethanol and once with ether. The ratio of absorbance at 490 nm for a solution in 50mM borate buffer (pH 10.0) to absorbance of the product of the carbazole reaction of the precipitate was determined, and is expressed as percentage with reference to the ratio for the starting sample (Fig. 1).

Separation of 5-aminofluorescein-labeled from unlabeled fractions of fluorescent glycosaminoglycuronans. — The fluorescent chondroitin 6-sulfate having a degree of substitution 0.039 (78 mg) was chromatographed on a column of Octyl-Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as described in the legend to Fig. 2. Fractions corresponding to each peak (Fig. 2) were combined, diluted ten times with an aqueous solution of cetylpyridinium chloride to reach a 0.3% concentration of cetylpyridinium chloride, and kept overnight at 37°. After centrifugation, each precipitate was collected, washed with 0.1% cetylpyridinium chloride solution, and redissolved in 2.1M sodium chloride (10–20 mL). After addition with stirring of cold ethanol (3 vol.), the mixture was kept for 16 h at 4°. The precipitate was centrifuged off, washed with 3:1 (v/v) ethanol–water, and dissolved in water (10 mL). The solution was desalted by passage through a column (2.6 × 92 cm) of Sephadex G-25, and lyophilized. Analytical data for Fractions 1–4 (see Fig. 2) are reported in Table IV.

The fluorescent heparin having a degree of substitution 0.039 (100 mg) was fractionated on the Octyl-Sepharose column by use of nearly the same procedure as

TABLE IV

SEPARATION OF FLUORESCENT GLYCOSAMINOGLYCURONANS ON OCTYL-SEPHAROSE CL-4B[a]

Fluorescent glycosaminoglycuronan	*Yield (mg)*	*S (%)*	*N-bound S (%)*	*D.S.*	*RFI/A_{400}*	*RFI/μg*	*Anticoagulant activity (USP units/mg)*
Starting chondroitin 6-sulfate		6.11					
Fluorescent chondroitin 6-sulfate	78.0	5.95		0.039	2300	10.9	
Fluorescent chondroitin 6-sulfate chromatographed on Octyl-Sepharose CL-4B							
Fraction 1	5.4	6.19		0	0	0	
Fraction 2	6.4	5.98		0.012	2100	3.8	
Fraction 3	30.0	5.74		0.028	2300	9.7	
Fraction 4	16.6	5.20		0.047	1700	12.0	
Starting heparin		12.33	4.35				161.1
Fluorescent heparin	100	12.10	4.15	0.039	2500	9.9	131.1
Fluorescent heparin chromatographed on Octyl-Sepharose CL-4B							
Fraction 1	31.7	12.17	4.28	0	0	0	102.5
Fraction 2	36.9	11.98	4.03	0.057	2700	18.0	161.1
Fraction 3	4.3	11.22	3.81	0.074	1600	13.1	81.4

[a]For abbreviations, see footnote to Table III.

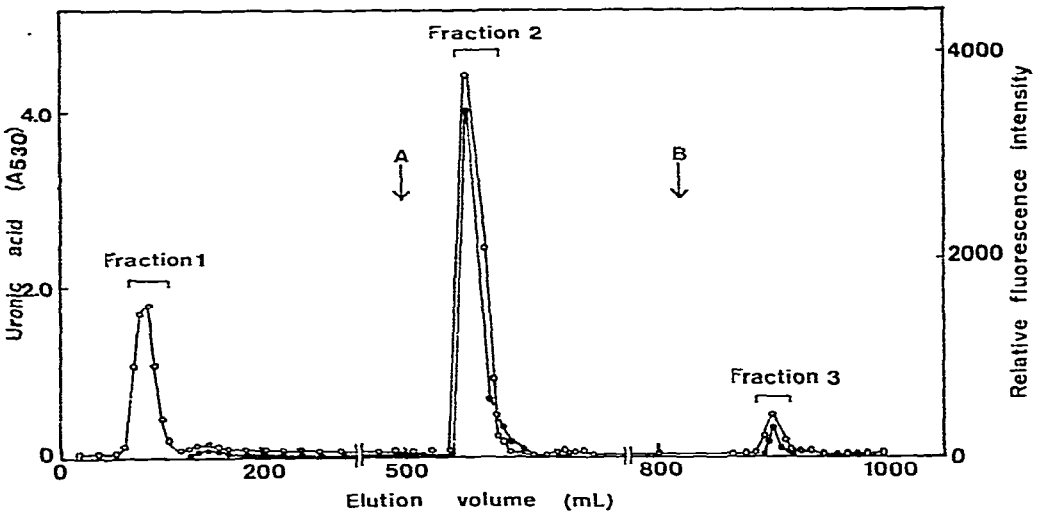


Fig. 3. Separation of fluorescent heparin into 5-aminofluorescein-labeled and unlabeled fractions on Octyl-Sepharose CL-4B. Fluorescent heparin (degree of substitution 0.039) (100 mg) was chromatographed according to the same procedure as described in the legend to Fig. 2, except for the stepwise elution system of 3.0M (3.5 bed vol.), M (A, 2.5 bed vol.), and no ammonium sulfate in 50mM phosphate buffer (B, 1.4 bed vol.).

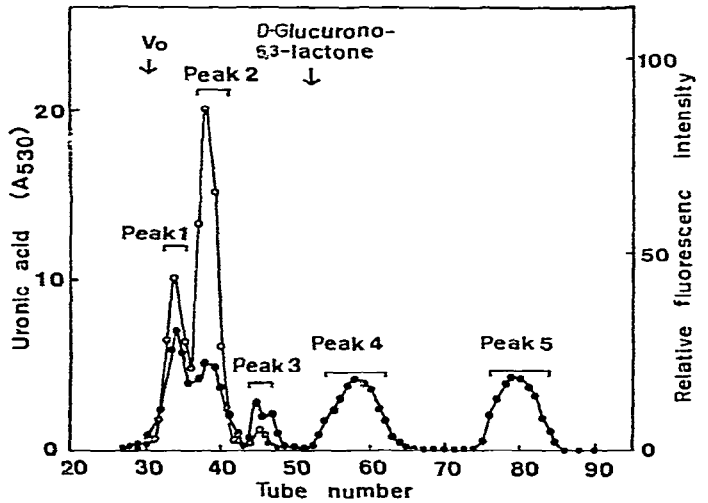


Fig. 4. Gel chromatography, on Sephadex G-25, of the digestion products of fluorescent hyaluronic acid with testicular hyaluronidase. The digestion mixture, which had been centrifuged, was loaded onto a column (2.5 × 80 cm) of Sephadex G-25 prepared in 0.15M ammonium carbonate, and eluted with the same solution at a flow rate of 30 mL/h. The eluent was collected (4.5-mL fractions) and was analyzed for uronic acid (○) and relative fluorescence intensity at 515 nm (●). The fractions corresponding to each peak indicated by brackets were combined and lyophilized.

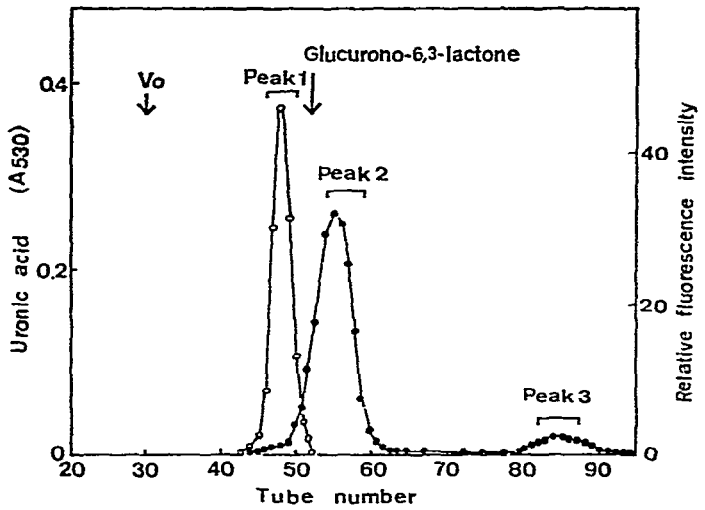


Fig. 5. Gel chromatography, on Sephadex G-25, of the digestion products of 5-aminofluorescein-labeled chondroitin 6-sulfate with chondroitinase AC. The digestion mixture was chromatographed according to the same procedure as described in the legend to Fig. 4: (○) uronic acid; (●) relative fluorescence intensity at 515 nm.

that just described, except for the molar concentrations of ammonium sulfate used for elution (Fig. 3).

Digestion of fluorescent hyaluronic acid with testicular hyaluronidase. — Fluorescent hyaluronic acid having a degree of substitution 0.0086 (50 mg) was dissolved in 20mM sodium chloride–60mM acetate buffer (10 mL) (pH 5.0), and incubated with 10 000 units of bovine testicular hyaluronidase for 24 h at 37°. After neutralization with 0.2M sodium hydroxide and heating in boiling water for 15 min, the mixture was centrifuged, and the supernatant solution was chromatographed on a column of Sephadex G-25 as described in the legend to Fig. 4. The fractions corresponding to each peak in the Figure were combined and lyophilized. The fractions corresponding to Peaks 4 and 5 were analyzed for hexosamine before and after reduction, and for absorbance at 490 nm. These fractions were also analyzed by t.l.c. (solvents *C* and *D*). Analytical data of the fractions are listed in Table II.

Digestion of 5-aminofluorescein-labeled chondroitin 6-sulfate with chondroitinase AC. — The labeled fraction of chondroitin 6-sulfate having a degree of substitution 0.028 (Fraction 3 in Table IV) (30 mg) was dissolved in enriched Tris buffer[13] (pH 8.0, 14 mL) and incubated with chondroitinase AC (20 units) for 24 h at 37°. After being heated for 2 min in boiling water, the mixture was centrifuged, and the supernatant solution was chromatographed on Sephadex G-25 as described in the legend to Fig. 5. The fractions corresponding to each peak in the Figure were combined and lyophilized. Analytical data for these fractions are listed in Table II.

Examination of the stability of the amide linkage between 5-aminofluorescein and glycosaminoglycuronans in biological systems. — The liver and kidney obtained

from a male rabbit were each homogenized with 0.1M sodium chloride–50mM phosphate buffer (4 vol.) (pH 7.0), and the homogenate was centrifuged at 4 500*g* for 20 min. The labeled fractions of heparin (Fraction 2 in Table IV) and of chondroitin 6-sulfate (Fraction 3 in Table IV) were each dissolved in 0.1M sodium chloride–50mM phosphate buffer (pH 7.0) at a concentration of 2 mg/mL. To 0.5 mL of each solution was added rabbit plasma, liver homogenate, or kidney homogenate (0.5 mL each). Toluene (50 μL) was added to the mixture, which was then incubated for 24 h at 30°, dipped in ice–water, and then mixed with cold ethanol (3 vol.). After being kept for 1 h at 0°, the precipitate formed was centrifuged off, and the supernatant solution was evaporated to dryness. The ethanol-soluble fraction of each residue was analyzed for free 5-aminofluorescein by t.l.c. on silica gel plates in solvents *A* and *B*.

Analysis of time-dependency of concentration, and of anticoagulant activity of 5-aminofluorescein-labeled heparin in blood circulation. — Male rabbits (3–3.5 kg) were anesthetized with urethan and were cannulated into the carotid. The labeled fraction of heparin (Fraction 2 in Table IV) (3 mg/kg) was injected intravenously *via* the auricular vein. Preparation of plasma samples, and measurements of fluorescence intensity and of clotting time of the plasma samples were carried out as described previously[3].

RESULTS AND DISCUSSION

Preparation of fluorescent glycosaminoglycuronans. — In order to form the amide linkage between 5-aminofluorescein (**2**) and the carboxyl group of uronic acid residues in glycosaminoglycuronans by treatment with 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide, the pH was maintained constant with a reaction medium containing pyridine and hydrochloric acid which also enhanced the solubility of 5-aminofluorescein in the medium. An ester may be formed between the carboxyl (of 5-aminofluorescein and glycosaminoglycuronans), hydroxyl (of polysaccharides), and phenolic groups (of 5-aminofluorescein) in addition to the amide linkage, but the marked difference in stability between ester and amide linkages in alkaline media was confirmed by the hydrolysis of 5-acetamidofluorescein-3′,6′-diyl diacetate (**4**) into 5-acetamidofluorescein (**3**) with 0.1M sodium hydroxide within 2 min at 30°. 5-Acetamidofluorescein formed was stable for 24 h under the same conditions.

Fluorescein (**1**) itself binds to glycosaminoglycuronans, presumably through an ester linkage under the conditions employed for 5-aminofluorescein. The results (Fig. 1) show distinct differences in the stability to alkali between the fluorescein groups in chondroitin 6-sulfate labeled with **1** and those in chondroitin 6-sulfate labeled with **2**. The ester bonds in the former compound were rapidly cleaved to release fluorescein in alkaline hydroxylamine solution and in 0.1M sodium hydroxide at 30°. In contrast, 71% of the 5-aminofluorescein bound to chondroitin 6-sulfate remained bound in both media for 24 h. Such stability corresponds closely to that of 5-acetamidofluorescein, suggesting that the alkali-stable fluorescein group in the

latter compound is bound to chondroitin 6-sulfate through an amide linkage. These observations served as a basis for the preparation of fluorescent glycosaminoglycuronans.

Compounds having comparable degrees of substitution were obtained under the same labeling conditions from all glycosaminoglycuronans, except hyaluronic acid which seemed less reactive toward 5-aminofluorescein (Table III). An increase in the ratio of 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide to the polysaccharides resulted in an increase of the degree of substitution for chondroitin 6-sulfate and heparin. Reversing the order of addition of the reagents, 5-aminofluorescein and 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide, did not influence the degree of substitution in all cases (data not shown). The total sulfate content in the fluorescent polysaccharides and their parent materials indicate that no appreciable desulfation had taken place during the labeling procedure.

Properties of fluorescent glycosaminoglycuronans. — Each of the fluorescent glycosaminoglycuronans exhibited fluorescence of λ_{em} 513–515 nm under excitation at 491 nm, and the relative fluorescence intensities of these preparations showed their maximum value at pH 9–11. These properties are in accord with those of fluorescein and 5-acetamidofluorescein. The fluorescence emission properties of proteins labeled with fluorochromes are known to depend on the molecular environment of the fluorescent tag[14]. The ratio of relative fluorescence intensity at 515 nm to the absorbance at 490 nm (RFI/A_{490}) of each preparation is considered to reflect the fluorescence intensity of the fluorescein residue, namely, the relative fluorescence quantum yield. The value of relative fluorescence intensity per A_{490} of 5-acetamidofluorescein is 2 900, which is comparable to that of fluorescein (Table I). The data (Table III) reveal that the value, relative to the fluorescence intensity per A_{490}, of each preparation corresponds to 70–90% of that of 5-acetamidofluorescein, except for dermatan sulfate (44.8%). The relative fluorescence intensity per weight ($RFI/\mu g$) shown in Table III is dependent on both degree of substitution and relative fluorescence intensity per A_{490}.

The solubilities of the fluorescent derivatives in aqueous solution are comparable to those of the starting materials, except for fluorescent hyaluronic acid, which is more soluble than the starting hyaluronic acid, possibly owing to partial depolymerization, as described later. The amide linkage in the fluorescent polysaccharides could be hydrolyzed quantitatively with M hydrochloric acid for 12 h at 100°, and one of the hydrolysis products was identified as 5-aminofluorescein by t.l.c. Under these conditions, the ester sulfate groups of the fluorescent polysaccharides were also completely hydrolyzed.

The electrophoretograms of the fluorescent derivatives at pH 5.2 and 2.0 showed, in each case, a single, fluorescent band overlapping with that made visible by Alcian Blue staining. Each front of these bands was slightly retarded compared with that of the respective starting-material, except fluorescent hyaluronic acid, which had a mobility comparable to that of starting hyaluronic acid at both pH values

(data not shown), possibly owing to a partial depolymerization of the polysaccharide chain as described in the next paragraph.

Gel chromatography of the fluorescent derivatives listed in Table III was performed in order to compare their elution patterns with those of the starting materials (data not shown). Hyaluronic acid was eluted from Sepharose 2B at the void volume, whereas fluorescent hyaluronic acid was slightly retarded. On the other hand, both samples were eluted from Sepharose 6B near the void volume. Therefore, a limited degradation of the polysaccharide chain had probably occurred during the preparation. The elution profiles, in gel chromatography, of other fluorescent glycosaminoglycuronans coincided with those of the respective starting materials, indicating that no depolymerization had occurred during the preparation. The elution diagrams obtained by monitoring the carbazole reaction and fluorescence intensity of each fluorescent preparation almost overlapped.

Separation of fluorescent glycosaminoglycuronans composed of 5-aminofluorescein-labeled molecules, and their properties. — Hydrophobic interaction chromatography on Octyl-Sepharose, which has been successfully used for fractionating fluorescent heparin labeled with isothiocyanatofluorescein[15], was applied to fluorescent chondroitin 6-sulfate and heparin prepared by the present method. In all cases, 5-aminofluorescein-labeled and unlabeled fractions could be clearly separated (Figs. 2 and 3). The unlabeled fraction of chondroitin 6-sulfate (eluted with 2.0M ammonium sulfate) showed an affinity to Octyl-Sepharose gels higher than that of the unlabeled fraction of heparin (eluted with 3.0M ammonium sulfate). The high affinity of the former fraction to the gel may be due to its larger molecular size and higher *N*-acetyl group content[16,17]. The labeled fractions of both preparations retained on the gels were eluted stepwise with decreasing concentrations of ammonium sulfate. Rechromatography of each fraction of the labeled fractions indicated the absence of any unlabeled fraction. Analytical and biological data for the fractions obtained are reported in Table IV.

The molar proportion of bound 5-aminofluorescein groups in labeled fractions of chondroitin 6-sulfate (Fractions 2–4 in Table IV) was estimated to be ~1.5, ~3.6, and ~5.9 per molecule of polysaccharide from the degree of substitution and mol. wt. of the starting chondroitin 6-sulfate, 65 000. The amount (51.2%) of Fraction 3 is about one half of the total amount of products, and this yield is similar to that of Fraction 2 of heparin (50.6%) as shown in Table IV. The values of relative fluorescence intensity per A_{490} of Fractions 2–4 of labeled chondroitin 6-sulfate suggest the existence of an optimum degree of substitution corresponding to the highest, relative-fluorescence quantum yield. The electrophoretic mobilities of the labeled fractions relative to that of starting chondroitin 6-sulfate at pH 2.0 varied, depending on the degree of substitution and sulfate content (data not shown).

Fluorescent heparin could be separated into an unlabeled (Fraction 1, 43.5%, based on uronic acid determination), and two labeled fractions (Fraction 2, 50.6% and Fraction 3, 5.9%). The mol. wt. of starting heparin was estimated to be ~14000 by comparing its gel filtration diagram with that of Cohelfred heparin (Cohelfred

Laboratories, Chicago, IL 60637, mol. wt. 14 000, by the light-scattering method). Fraction 2 corresponds to 90% of the total labeled fractions. It has ~1.3 bound 5-aminofluorescein groups per heparin molecule, assuming that the mol. wt. of heparin in this fraction is identical with that of the starting heparin. The electrophoretic mobility of this fraction was nearly the same as that of starting heparin at pH 2.0, and its anticoagulant activity retained the potency of the starting heparin (Table IV). *N*-Fluoresceinylthiocarbamoylheparin, prepared by labeling partially *N*-desulfated heparin with 5-isothiocyanatofluorescein, followed by separation on Octyl-Sepharose gel, retained almost the same activity as that of the parent heparin. The *N*-fluoresceinylthiocarbamoylheparin fraction, the yield of which corresponded to 80% of the total labeled fractions, was also found to have one *N*-fluoresceinylthiocarbamoyl group per heparin molecule[15]. The data (Table IV) for the fluorescent heparin preparations obtained by the present methods, together with those for the *N*-fluoresceinylthiocarbamoylheparin, indicate that such a minor proportion of fluorescein group seems to show little effect on the anticoagulant activity of heparin.

Analytical gel-chromatography of the fractions of fluorescent chondroitin 6-sulfate separated on Octyl-Sepharose CL-4B was performed on Sepharose 6B (data not shown). Comparison of elution profiles suggests that the molecular size of the unlabeled fraction of chondroitin 6-sulfate (Fraction 1) is rather smaller than those of the labeled fractions (Fractions 3 and 4), and the size of the highly labeled fraction (Fraction 4) seems to be slightly larger than that of the moderately labeled one (Fraction 3). A similar difference in molecular size was observed between the labeled and unlabeled fractions of heparin by comparing their gel chromatographic patterns on Sephadex G-100 (data not shown). These results indicate that the glycosaminoglycuronan of large molecular size containing more uronic acid residues is more likely to bind a 5-aminofluorescein group than the corresponding one smaller in molecular size. These results also explain the greater anticoagulant activity of the labeled fractions of heparin, as compared to that of the unlabeled fractions, as it is known that the anticoagulant activity of heparin fractions of different molecular size increases with an increasing degree of polymerization[18].

Enzymic degradation of some fluorescent glycosaminoglycuronans. — (*a*) *Hyaluronidase digestion of fluorescent hyaluronic acid.* The digestion products of fluorescent hyaluronic acid with bovine testicular hyaluronidase for 24 h were analyzed by gel chromatography on Sephadex G-25. The fractions corresponding to Peaks 1–3, monitored by both carbazole reaction and fluorescence measurement, were combined and lyophilized (Fig. 4). T.l.c. (solvents *C* and *D*) of each peak showed two spots; one was nonfluorescent and could be detected by the alkaline silver nitrate reagent or *p*-aminohippuric acid–phthalic acid reagent, and the other was fluorescent. By means of reference oligosaccharides, each nonfluorescent spot of Peaks 1–3 was identified as hexa-, tetra-, and di-saccharide, respectively. The materials isolated from Peaks 4 and 5, each of which showed a single fluorescent spot by t.l.c., were analyzed for the molecular ratio of bound 5-aminofluorescein group to hexosamine, and for the hexosamine content before and after reduction with sodium borohydride.

The results in Table II indicate that the materials corresponding to Peaks 4 and 5 are octa- and hexa-saccharides carrying one 5-aminofluorescein group, respectively. Marked retardation in the elution of these fluorescent oligosaccharides is probably due to an interaction between the 5-aminofluorescein group and Sephadex-gel matrix, as indicated by the K_{av} values of fluorescein and of 5-aminofluorescein-labeled methyl glycoside of *N*-acetylchondrosine determined on Sephadex G-25 gels (3.7 and 2.7, respectively).

(*b*) *Chondroitinase AC digestion of 5-aminofluorescein-labeled chondroitin 6-sulfate.* By gel chromatography, on Sephadex G-25, of the digestion product with chondroitinase AC, it was found that the moderately labeled fraction of chondroitin 6-sulfate (Fraction 3 in Table IV) was degraded almost completely into an unlabeled disaccharide (Peak 1 in Fig. 5). By analysis of the molar ratio of bound 5-aminofluorescein group to hexosamine, and of the hexosamine content before and after reduction with sodium borohydride (Table II), two fluorescent peaks (Peaks 2 and 3) were identified as octa- and tetra-saccharide, each carrying one 5-aminofluorescein group.

These results suggest that the enzymic degradation of labeled hyaluronic acid and chondroitin 6-sulfate is inhibited after the formation of hexa- and tetra-saccharides, respectively, as the smallest products carrying a fluorescent tag, presumably owing to restriction of the enzyme activity by the bulky 5-aminofluorescein group. Isolation and identification of the labeled oligosaccharides from the enzymic digests of labeled hyaluronic acid and chondroitin 6-sulfate also indicated that the fluorescein groups were introduced along the whole polysaccharide chain, not specifically in sugar residues near the linkage region.

Detection of 5-aminofluorescein-labeled heparin in circulating blood. — The biochemical stability of the fluorescein amide linkage in labeled chondroitin 6-sulfate and heparin was unequivocally established by the observation that 5-aminofluorescein was not liberated during incubation of these fluorescent materials with rabbit plasma or tissue homogenates.

Labeled heparin was intravenously injected into a male rabbit, and the elimination of fluorescent heparin from the blood was monitored by two methods; the half-life determined fluorometrically was 36 min, and by clotting-time measurement 45 min. Although the discrepancy between these two half-life values may be partly due to the heterogeneity in molecular species of labeled heparin, it may be mainly ascribed to the time-lag arising from rapid disappearance of the fluorescent heparin in blood vessel and to the duration of the anticoagulant effect induced by heparin, as discussed in the previous report on *N*-fluoresceinylthiocarbamoylheparin[3].

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