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Mucopolysaccharides from the Connective Tissues of the Squid, *Ommastrephes sloani pacificus*

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In recent years, many papers on amino sugar-containing mucopolysaccharides have been published, reporting the isolation of hyaluronic acid, chondroitin, three types of chondroitin sulfate, heparin, heparitin sulfate and kerato sulfate. By the use of various techniques, the chemical structures of most of these substances have been elucidated. However, these studies have generally been limited to vertebrate tissues, and very little is known about the mucopolysaccharides of invertebrates[1-3].

The connective tissues of mammls contain mainly hyaluronic acid and chondroitin sulfates, and the mucopolysaccharides previously isolated from the skin are hyaluronic acid and chondroitin sulfate B[4]. In connection with the skin mucopolysaccharides of animals other than mammals, there is only one report that hyaluronic acid and over-sulfated chondroitin sulfate B were obtained from the skin of two elasmobranch fishes, the Blue shark and Sandbar shark[5].

Three isomeric chondroitin sulfates termed chondroitin sulfate A, B and C are known[6,7]; each of these contains acetylgalactosamine, uronic acid and sulfate in equimolar proportions. However, that the chondroitin sulfates can not be distinctly classified into the above three types has been made evident by the discovery of the presence of over-sulfated chondroitin sulfate containing an extra sulfate group. The first example of over-sulfated chondroitin sulfate was found in shark cartilage by Soda *et al.*[8] in 1940, and later a preparation with a sulfate-to-hexosamine ratio of 1.45 was obtained by further fractionation[9]. From its infrared spectrum[10], Mathews[11] suggested the shark chondroitin sulfate to be C type. Suzuki[12] showed that most of the ester sulfate is in the C6 position of the acetylgalactosamine residue and part of it is at the C2 or C3 position of the glucuronic acid residue, and he designated this substance as chondroitin sulfate D.

Another type of over-sulfated chondroitin sulfate has been found in chondroitin sulfate B preparations. Suzuki[12] obtained evidence sug-

gesting the presence of a disaccharide bearing two sulfate residues in the chondroitinase digests of chondroitin sulfate B from beef lung, and Seno and Meyer[5] obtained chondroitin sulfate B fractions with sulfate-to-hexosamine ratios of 1.42–1.62 from the skin of elasmobranch fishes.

Mathews *et al.*[3] showed in their studies on invertebrates that cartilage of the squid (*Loligo*) and of the horse-shoe crab (*Limlus*) contain an over-sulfated chondroitin sulfate which seems to be different from that of shark cartilage.

In the present work, the mucopolysaccharides were isolated from the connective tissues of a squid, *Ommastrephes sloani pacificus*, and their chemical structures were studied in detail. The main mucopolysaccharide component of the skin was non-sulfated chondroitin, and neither hyaluronic acid nor chondroitin sulfate B was detected[13]. From the cartilage, a novel chondroitin polysulfate containing 4, 6-disulfated acetylgalactosamine was isolated and designated as "chondroitin sulfate E"[14,15].

EXPERIMENTAL

Materials:

Adult squid, *Ommastrephes sloani pacificus*, kindly supplied by Taiyo Gyogyo Co. Ltd., Tokyo, were kept at −20°C until used.

Potassium hyaluronic acid was prepared from human umbilical cords, and sodium chondroitin sulfate A from whale cartilage, according to the procedure of Meyer *et al.*[4]. Sodium chondroitin sulfate C was obtained from shark cartilage by fractionation with cetylpyridinium chloride[16]. Bovine cornea chondroitin was kindly given by Prof. K. Meyer of Columbia University.

Pronase [EC class 3. 4. 4] was purchased from Kaken Kagaku Co. Ltd., Tokyo. The testicular hyaluronidase [EC 3. 2. 1. 35] was commertial preparation (Worthington Biochemical Co. Ltd.) assaying 300 USP per mg.. Chondroitinase-ABC, chondro-4-sulfatase and chondro-6-sulfatase were prepared from *Proteus vulgaris* and glucuronidase was obtained from *Flavobacterium heparinum*[17].

Unsaturated disaccharides, △Di-0S*, △Di-4S and △Di-6S, were prepared by chondroitinase digestion of chemically desulfated chondroitin, chondroitin sulfate A and chondroitin sulfate C, respectively[12,17]. Acetylgalactosamide 6-sulfate was synthesized by the method of Lloyd[18]. Acetylgalactosamine 4-sulfate was prepared by hydrolysis of UDP-acetylgalactosamine-4-sulfate with weak acid essentially as described

* Abbreviations used are: △Di-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; △Di-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; △Di-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose.

by Strominger[19], and acetylgalactosamine 4, 6-disulfate from UDP-acetylgalactosamine-4, 6-disulfate by the method of Harada *et al.*[20].

Analytical Methods:

Analyses were carried out by the method of micro-Kjeldahl for nitrogen, the Elson-Morgan reaction[21] for hexosamine, the carbazole[22] and orcinol[23] reactions for uronic acid, and the method of Dodgson and Price[24] for ester sulfate content. The reducing sugar was determined by the method of Park and Johnson[25] and *N*-acetylhexosamine by the Morgan-Elson reaction[26].

The hexosamine was identified by the method of Stoffyn and Jeanloz[27] and by ion exchange chromatography according to Gardell[21].

Fractionation of Mucopolysaccharides:

The mucopolysaccharides were fractionated by Dowex 1 (X2 ; 200–400 mesh, Cl^- form) column chromatography or with cetylpyridinium chloride essentially according to Schiller *et al.*[16].

Paper Chromatography:

Descending paper chromatography for enzymic digests of mucopolysaccharides was carried out on Toyo No. 51A or No. 50 paper (60 cm. long) in the following solvents : I, 1-butyric acid-0.5 *N* ammonia (5 : 3) ; and II, 1-butanol-ethanol-water (52 : 32 : 16). Compounds containing $\triangle$4, 6-glucuronic acid were detected by ultraviolet absorption photography[28] or by viewing under a Mineralight Model S-2537. Reducing sugars were detected by staining with aniline hydrogen phthalate reagent[29] and also with silver nitrate reagent[28].

Electrophoresis:

Electrophoresis of mucopolysaccharides was performed on cellulose acetate strip (15 cm. long) in 1.0 *M* acetic acid-pyridine, pH 3.5, at 100 volt for 1 hour[5]. Mucopolysaccharides were detected with 0.5% Alcian blue 8 GN in 3% acetic acid.

Paper electrophoresis after enzymic degestion of mucopolysaccharides was carried out on Toyo No. 51A paper (60 cm. long) in 0.05 *M* ammonium acetate-acetic acid buffer, pH 5.0, at a potential gradient of 30 volts per cm. for 45 minutes[30]. Unsaturated glucuronic acids and reducing sugars were detected by the same methods as used for paper chromatography.

Infrared Spectroscopy:

Spectra were obtained with KBr disk on either a Perkin-Elmer model 21 or a Hitachi model EPI-S2 spectrophotometer.

Identification of Repeating Unit:

The constituent disaccharide was isolated on partial acid hydrolysis of the mucopolysaccharides according to the procedure of Davidson and Meyer[31]. Chondrosin[32], prepared from chondroitin sulfate A or C, was used as a standard for identification of the disaccharide by chemical

analysis, paper chromatography, optical rotation and infrared spectrum.

Rate of Ester Sulfate Hydrolysis:

Sulfated mucopolysaccharides were hydrolyzed in 0.25 *N* hydrochloric acid at 100°C in sealed tubes[33] and released sulfates at the time intervals were determined by a modified turbidimetric method[34].

Enzymic Digestion:

Mucopolysaccharides were hydrolyzed with testicular hyaluronidase at 37°C in 0.1 *M* acetate buffer, pH 5.0, containing 0.15 *M* sodium chloride. Reaction mixtures contained 1.5 mg. of substrate and 150 μg. of enzyme in 10 ml. of the above buffer. The increase in reducing sugar with time was measured as glucose[25] and at the end of digestion the Morgan-Elson color density[26] was determined as N-acetylglucosamine[35].

The digestion of mucopolysaccharides by chondroitinase-ABC[17] was carried out at 37°C in the following conditions. Incubation mixtures of final 50μl. contained substrate polysaccharide, 0.1μmole (as uronic acid); Tris-HCl (pH 8.0), 2.5μmoles; sodium acetate, 3μmoles; bovine serum albumin, 5μg; and 0.08 unit of purified chondroitinase-ABC. Incubation was stopped adding 0.45 ml. of 0.05 *M* KCl-HCl buffer (pH 1.8). It was then centrifuged at 10,800 g for 10 minutes and the increase in absorption at 232 mμ of the supernatant with time was measured. The products of enzymic digestion were subjected to paper chromatography and paper electrophoresis[12]. To isolate the degradation products, incubation in large scale was carried out essentially under the same conditions.

Unsaturated disaccharides were desulfated by chondro-4-sulfatase and chondro-6-sulfatase which were prepared from *P. vulgaris*[17]. Incubation mixtures contained the following components, in final volumes of 60μl.: unsaturated disaccharide, 0.2μmole; Tris-HCl (pH 8.0), 3μmoles; sodium acetate, 3μmoles; bovine serum albumin, 5μg.; and enzyme. Incubation was carried out at 37°C and the digests thus obtained were subjected to paper electrophoresis.

Unsaturated disaccharides were degraded by glucuronidase from *F. heparinum*[17]. Incubation mixtures contained the following components, in final volumes of 0.2 ml.: unsaturated disaccharide, 0.3μmole; acetate buffer (pH 5.2), 10μmoles; sodium fluoride, 10μmoles; and enzyme, 0.023 unit. The samples were incubated at 37°C and diluted with 0.05 *M* KCl-HCl buffer (pH 1.8) to measure absorption at 232 mμ. The digested samples were also subjected to paper chromatography and paper electrophoresis.

RESULTS

Isolation of Mucopolysaccharides from Squid Skin:

The skin of squid was defatted with acetone and dried in a desiccator. The dried skin cut up with scissors (50 g., from 135 animals) was suspended in 500 ml. of water and heated in a boiling water bath for 1 hour. The red-brown suspension was adjusted to pH 7.8 with sodium hydroxide, and incubated with 500 mg. of pronase (1% of dry skin) at 40°C for 24 hours in the presence of final 5% of ethanol to prevent bacterial growth. The incubation was repeated with half the original amount of pronase. After centrifugation, calcium acetate was added to 2.5% and acetic acid to 0.25 *N* followed by 2 volumes of ethanol to the clear supernatant. The mixture was kept in a refrigerator overnight and centrifuged ; the precipitate was dissolved in water and the insoluble material was centrifuged off. Additional protein was removed by treatment with 10% trichloroacetic acid and after dialysis the clear solution was stirred with 3 g. of Lloyd's reagent and 1 g. of kaolin for 30 minutes. The mixture was then centrifuged and the precipitate was washed with water. The washings were combined with the supernatant and sodium acetate was adedd to 1%. A mucopolysaccharide fraction was precipitated from the combined solution by adding 3 volumes of ethanol and keeping the mixture in a refrigerator overnight. The precipitate was collected by centrifugation, washed with ethanol, and dried in a desiccator. The yield was 1.2 g.

In order to fractionate with cetylpyridinium chloride, the mucopolysaccharide fraction, 350 mg. (106 mg. as uronic acid), was dissolved in 200 ml. of 0.03 *M* sodium chloride, and 10 ml. of 10% cetylpyridinium chloride was added. After the mixture was incubated at 37°C for 1 hour, the cetylpyridinium-complex was precipitated together with 7 g. of Celite No. 535 and the mucopolysaccharides were extracted stepwise with each 200 ml. of 0.3 *M*, 1.2 *M* and 2.1 *M* sodium chloride containing 0.1% cetylpyridinium chloride. The results are shown in Table 1. The fraction soluble in 0.3 *M* sodium chloride contained 77% of the total uronic acid and small amounts of uronic acid were also present in the fractions soluble in 1.2 *M* and 2.1 *M* sodium chloride. The main fraction was collected and stirred with Lloyd's reagent to remove excess of cetylpyridinium chloride. After centrifugation, the clear supernatant was dialyzed against distilled water until chloride ions were not detected and concentrated *in vacuo*. The yield of the squid skin mucopolysaccharide which was precipitated with 3 volumes of ethanol in the presence of 1% sodium acetate was 220 mg.

Isolation of Mucopolysaccharides from Squid Cartilage:

The cranial cartilage of squid was freed from other tissues. The

Table 1. Cetylpyridinium Chloride Fractionation of Squid Skin Mucopolysaccharides.

Flask No.	Solvent in 0.1% CPC*	Volume	Total uronic acid	Recovery
		ml.	mg.	%
1	Supernatant	170	7.4	7.0
2	0.03 *M* NaCl	180	1.5	1.4
			8.9	8.4
3	0.3 *M* NaCl	176	64.2	60.6
4	„	200	15.2	14.3
5	„	196	2.2	2.1
6	„	186	0	0
			81.6	77.0
7	1.2 *M* NaCl	181	5.2	4.9
8	„	181	1.5	1.4
9	„	181	0	0
			6.7	6.3
10	2.1 *M* NaCl	185	7.5	7.1
11	„	186	0	0
			7.5	7.1

* Abbreviation of cetylpyridinium chloride.

wet cartilage (100 g., from 80 animals) was homogenized with 150 ml. of water in a Waring blendor. The suspension was heated in a boiling water bath for 1 hour and, after cooling, extracted with 2% solution of sodium hydroxide stirring at 4–5°C for 24 hours. The extract which was almost liquefied was centrifuged and digested with pronase in the same conditions as the case of skin. The amounts of pronase used were 40 mg. (5 mg. for each g. of protein) in the first incubation and 20 mg. in the second one. At the end of the digestion, insoluble material was removed and trichloroacetic acid was added to a concentration of 10%. After centrifugation, the supernatant was dialyzed against running water for 3 days and distilled water for 1 day. The clear solution was concentrated *in vacuo* to approximately 100 ml. and sodium acetate was added to 1%. A mucopolysaccharide fraction was obtained by precipitating it with 3 volumes of ethanol in a refrigerator, washed and dried as before. The yield was 1.5 g.

To fractionate the mucopolysaccharides, 100 mg. (26.5 mg. as uronic acid) was applied on a column, 2.0 cm. in diameter and 50.0 cm. in length, of Dowex 1 in chloride form. The column was washed with water and eluted stepwise with increasing concentrations of sodium chloride: 0.5 *M*, 1.5 *M*, 2.0 *M*, 3.0 *M* and 4.0 *M*. Fractions of 5.0 ml. were collected and the uronic acid contents were determined by the carbazole reaction. Fig. 1 demonstrates the elution pattern. Large amounts of mucopolysaccharide (17.8 mg. as uronic acid) were eluted with 3.0 *M* sodium

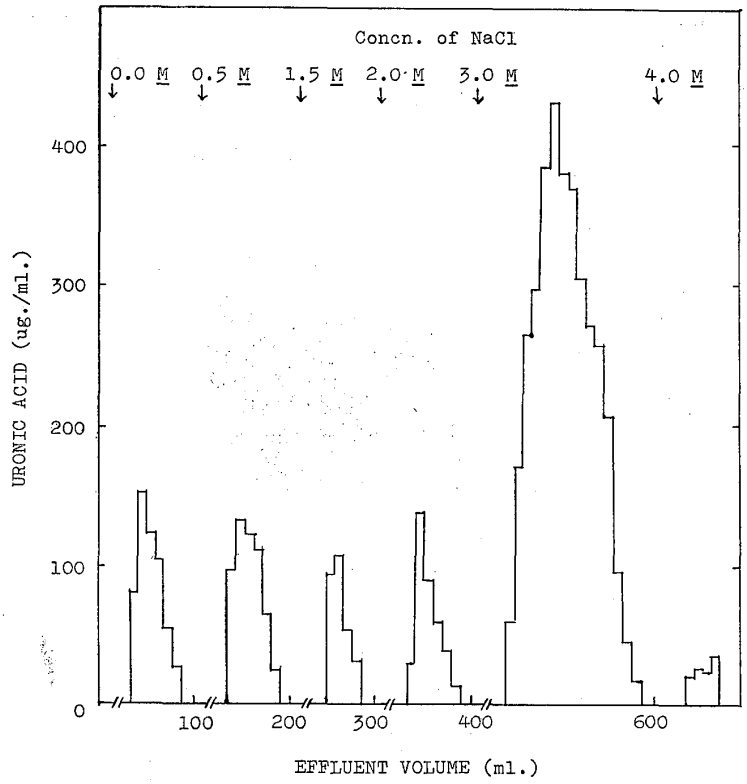

Fig. 1. Dowex 1 column chromatography of 100 mg. of squid cartilage mucopolysaccharide. Column size was 2×50 cm. and stepwise elution was used with NaCl solution of increasing concentration. Each fraction contained 5.0 ml. and flow rate was 0.3 ml. per minute.

chloride and relatively small amounts were eluted in the other fractions. The main fraction was concentrated *in vacuo* and dialyzed against distilled water until chloride ions were not detected. The clear solution was lyophilized. The yield of the squid cartilage mucopolysaccharide was 63 mg.

Properties of the Mucopolysaccharide of Squid Skin and of Squid Cartilage:

The chemical analysis of squid skin mucopolysaccharide is shown in Table 2. The molar ratio of uronic acid to hexosamine was 0.98 and the sulfate content was very low. From the molar ratio of nitrogen

Table 2. Analysis of Squid Skin Mucopolysaccharide.

	%	Molar ratio
Hexosamine	29.8	1.00
Uronic acid (carbazole)	31.4	0.98
Uronic acid (orcinol)	19.2	—
Nitrogen	2·5	1.06
Sulfate	<0.7	<0.04
$[\alpha]_D$ in water (c, 1)	−23°	

Table 3. Analysis of Squid Cartilage Mucopolysaccharide.

	Mixed mucopolysaccharide		Main mucopolysaccharide	
	%	Molar ratio	%	Molar ratio
Hexosamine	24.6	1.00	28.5	1.00
Uronic acid (carbazole)	26.5	0.99	29.8	0.97
Uronic acid (orcinol)	16.5	—	—	—
Nitrogen	2.0	1.04	—	—
Sulfate	20.0	1.50	23.7	1.55
$[\alpha]_D$ in water (c, 1)	−38°		−36°	

to hexosamine, it was indicated that the skin mucopolysaccharide contains practically no protein. In Table 3, the chemical analysis of the main mucopolysaccharide component of squid cartilage, as well as the mixed mucopolysaccharides, is presented. The molar ratios of uronic acid and sulfate to hexosamine of the sample were 0.97 and 1.55, respectively, suggesting that the cartilage mucopolysaccharide is over-sulfated. The nitrogen content indicated that the cartilage mucopolysaccharide contains no protein.

The optical rotations of the skin and cartilage mucopolysaccharides were −23° and −36° (c 1, water), respectively.

In order to determine the type of amino sugar, the skin and cartilage mucopolysaccharides were hydrolyzed with 3 N hydrochloric acid at 100°C for 16 hours and subjected to ninhydrin degradation. The hydrolyzates were completely freed of acid by alternate addition of water and evaporation *in vacuo*, and heated in sealed tubes with 8% ninhydrin–4% pyridine solution at 100°C for 1 hour. In both reaction mixtures, only one spot was obtained by paper chromatography using acetone–1–butanol–water (7 : 5 : 2) and the Rf values were in accord with that of authentic lyxose. The acid hydrolyzates were also applied on Dowex 50 (X8 ; 200–

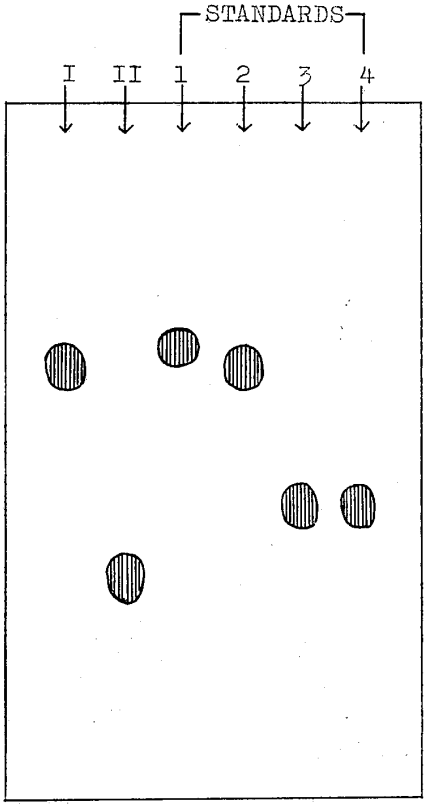

Fig. 2. Schematic representation of electrophoretogram on cellulose acetate strips of squid skin mucopolysaccharide (I) and squid cartilage mucopolysaccharide (II). Electrophoresis was performed in 1.0 M acetic acid-pyridine (pH 3.5) at 100 volt for 1 hour. Mucopolysaccharides were detected with 0.5% Alcian blue 8 GN in 3% acetic acid. The standards used are ; 1, hyaluronic acid ; 2, bovine cornea chondroitin ; 3, chondroitin sulfate A ; 4, chondroitin sulfate C.

400 mesh, H^+ form) column and eluted with 0.3 *N* hydrochloric acid. A single peak corresponding to galactosamine was obtained from both mucopolysaccharide samples. From these data the hexosamine of the skin and cartilage mucopolysaccharides was identified as galactosamine ; no other amino sugar was detected.

The mucopolysaccaride samples from squid skin and cartilage gave in each case a single spot by electrophoresis on a cellulose acetate strip as shown in Fig. 2, suggesting that they are homogenious in regard to acidity. The spot of the skin mucopolysaccharide moved as those of hyaluronic acid and bovine cornea chondroitin. The mobility of the cartilage mucopolysaccharide, on the other hand, was larger than that of chondroitin sulfate A or C, as would be expected from the difference in sulfate contents of these mucopolysaccharides.

The cartilage mucopolysaccharide was also indicated to be homogenious by cetylpyridinium chloride fractionation. When the cartilage mucopolysaccharide (6.4 mg. as uronic acid) was fractionated with cetylpyridinium chloride, 94% of the total uronic acid was extracted with 2.1 *M* sodium chloride and negligible amounts with 0.3 *M*, 1.2 *M* and 3.0 *M* sodium chloride.

The infrared spectrum of the skin mucopolysaccharide, as shown in Fig. 3, showed absorptions neither at 1240 $cm.^{-1}$ for S = O stretching vibration nor at 928 $cm.^{-1}$, 850 $cm.^{-1}$ and 820 $cm.^{-1}$ for ester sulfate groups. The spectrum was identical with that of bovine cornea chondroitin except for a slight difference at 1240 $cm.^{-1}$, the peak of the squid sample being much smaller than the corresponding peak of the

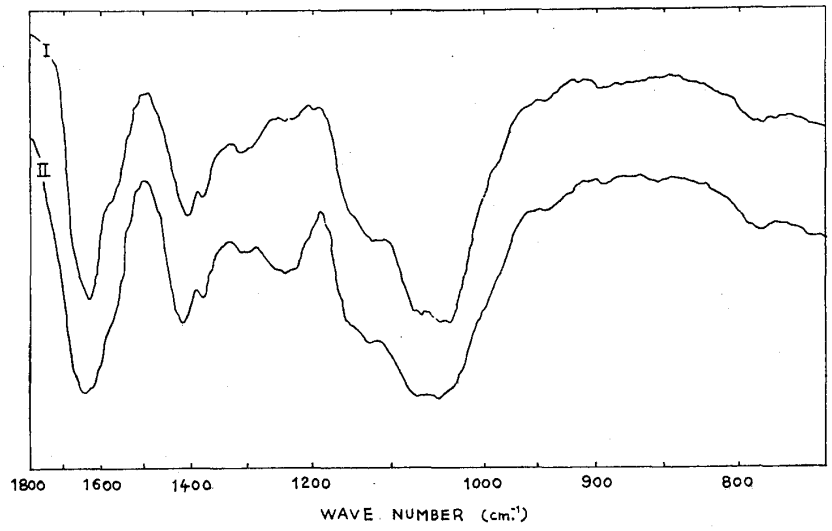

Fig. 3. Infrared spectra of squid skin mucopolysaccharide (I) and bovine cornea chondroitin (II). The spectra were obtained with KBr disk containing each 0.8 mg. of mucopolysaccharide on a Perkin-Elmer model 21 spectrophotometer.

cornea sample. The difference may be explained from that the cornea chondroitin contained some quantity of sulfate group[31] whereas the sulfate content of squid skin mucopolysaccharide is negligible. The infrared spectrum of cartilage mucopolysaccharide, as well as that of chondroitin sulfate A and C, is shown in Fig. 4. The spectrum of cartilage mucopolysaccharide had absorption bands for ester sulfate group not only at 928 cm.$^{-1}$ and 850 cm.$^{-1}$, as those found in chondroitin sulfate A, but also 820 cm.$^{-1}$ as in chondroitin sulfate C. This indicates that the squid cartilage mucopolysaccharide has both axial and equatorial sulfate groups.

In order to isolate the constituent disaccharide, each of 150 mg. of the skin and cartilage mucopolysaccharides hydrolyzed with 5 ml. of 1 *N* sulfuric acid at 100°C for 4 hours. After neutralyzation with

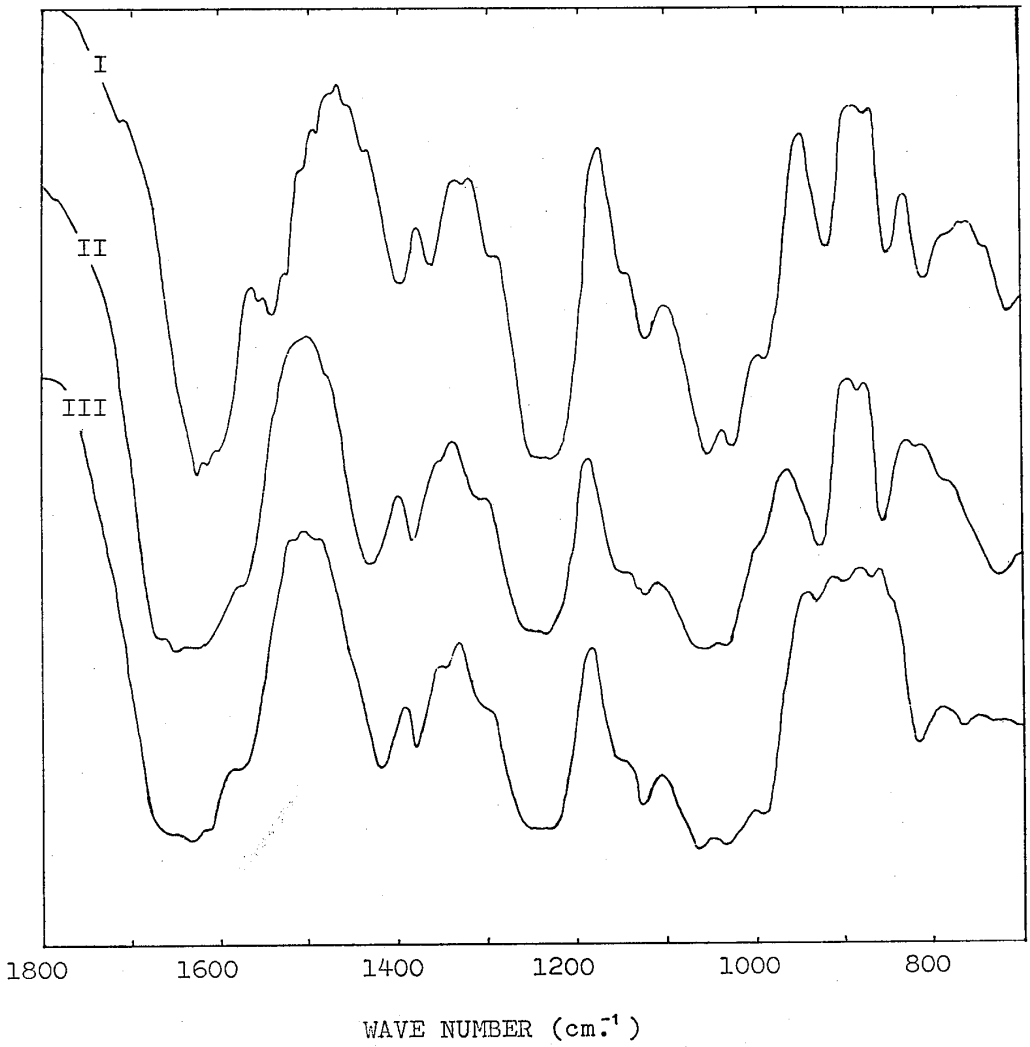

Fig. 4. Infrared spectra of squid cartilage mucopolysaccharide (I), chondroitin sulfate A (II) and C (III). The spectra were obtained with KBr disk containing each 2 mg. of mucopolysaccharide on a Hitachi model EPI-S2 spectrophotometer.

saturated barium hydroxide solution to pH 4.8, the supernatant was placed on a column (1.2 cm. × 17.5 cm.) of Dowex 50 (200–250 mesh, H^+ form). The column was washed with water until the Molisch test for degraded sugars became negative and then eluted with 0.01 *N* acetic acid. The fractions which were positive with the ninhydrin reagent were collected and concentrated to dryness *in vacuo*. The concentration was repeated after adding a small amount of water to remove acetic acid and the resulted neutral solution was lyophylized. A disaccharide, which has been identified as chondrosin by chemical analysis, paper chromatography, optical rotation and infrared spectrum, was obtained from the skin and cartilage mucopolysaccharides in the yields of 69% and 45%, respectively. Thus, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose appears to be the repeating units of both skin and cartilage mucopolysaccharides, as is the case of chondroitin sulfate A or C.

The mucopolysaccharides from squid skin and cartilage were incubated in buffer with the purified preparation of chondroitinase-ABC and the increase at 232 mμ with time was measured. The results are

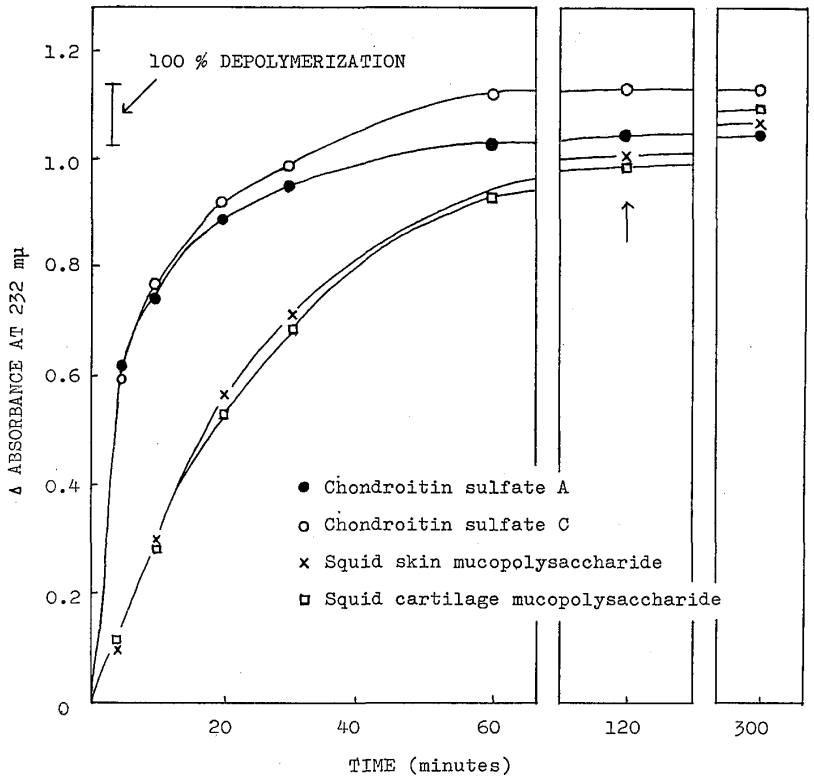

Fig. 5. Degradation of mucopolysaccharides by chondroitinase-ABC. Incubation mixtures and conditions are described in the text. Absorption at 232 mμ was measured against the corresponding blank mixture which contained heat inactivated enzyme. At the time indicated by the arrow, 0.075 unit of enzyme was added to the incubation mixture.

shown in Fig. 5, which includes comparison curves showing the degradation of chondroitin sulfate A and C. Both mucopolysaccharides were degraded much more slowly than chondroitin sulfate A or C, but after prolonged incubation the level of unsaturated disaccharide rose to nearly 100% of the total uronic acid. This indicates that every repeating unit in mucopolysaccharides quantitatively converted to unsaturated disaccharides.

The time course of testicular hyaluronidase digestion of several mucopolysaccharides including the squid skin and cartilage mucopolysaccharides are presented in Fig. 6. The skin mucopolysaccharide was hydrolyzed at a rate comparable to that of hyaluronic acid, whereas the digestion rate of the cartilage mucopolysaccharide was very slow. After 48 hours the reducing value of the latter was about one-fourth of that of hyaluronic acid and half of that of chondroitin sulfate A or C, and only after a 172-hour incubation with further addition of

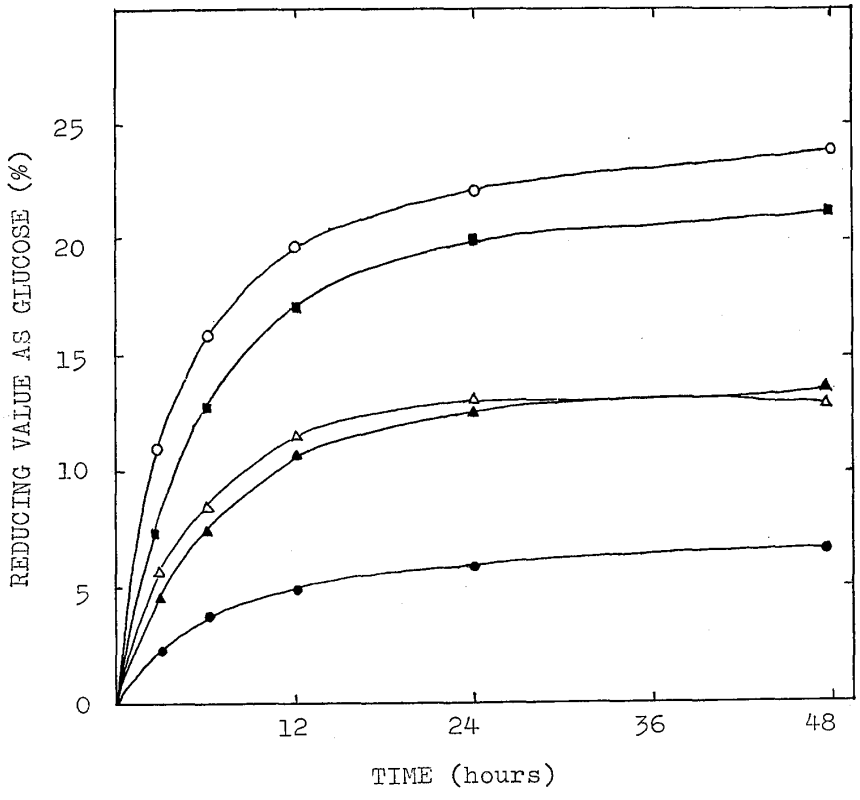

Fig. 6. Hyaluronidase digestion of mucopolysaccharides. Incubation mixtures and conditions are described in the text. Reducing sugar as glucose was measured at the indicated time.

○: Hyaluronic acid
▲: Chondroitin sulfate A
△: Chondroitin sulfate C
■: Squid skin mucopolysaccharide
●: Squid cartilage mucopolysaccharide

enzyme, it reached to nearly the same extent as chondroitin sulfate A or C. The apparent difference in the rate of digestion between the two groups of mucopolysaccharide is likely coming from the difference in sulfate contents.

Positions of Sulfate Group in the Squid Cartilage Mucopolysaccharide:

The sulfate positions in the squid cartilage mucopolysaccharide were suggested by estimating the rate of acid hydrolysis of ester sulfate. The cartilage mucopolysaccharide was dissolved in 0.2 ml. of 0.25 *N* hydrochloric acid (each containing 1.7 mg. of the sample) and hydrolyzed in a sealed tube at 100°C. At the time intervals, the tube was cooled, and to remove the undegraded mucopolysaccharide 0.3 ml. of 1% cetylpyridinium chloride was added. After centrifuging off cetylpyridinium complex, 3.8 ml. of 0.2 *N* hydrochloric acid was added to 0.2 ml. of the supernatant and the released inorganic sulfate was determined by adding the barium chloride-gelatin reagent, according to Dodgson and Price[24]. The results are shown in Fig. 7. The two distinct half lives were obtained, which were calculated, according to Rees[33], to be 0.9 hour and 2.3 hours, respectively. This result indicates that the cartilage mucopolysaccharide contains two types of sulfate group. These values correspond to those of sulfate esters of secondary axial and primary hydroxyl groups, respectively[33]; probably, the sulfate groups in the cartilage mucopolysaccharide are not present in the glucuronic acid residue but at both C4 and C6 positions of the acetylgalactosamine residue.

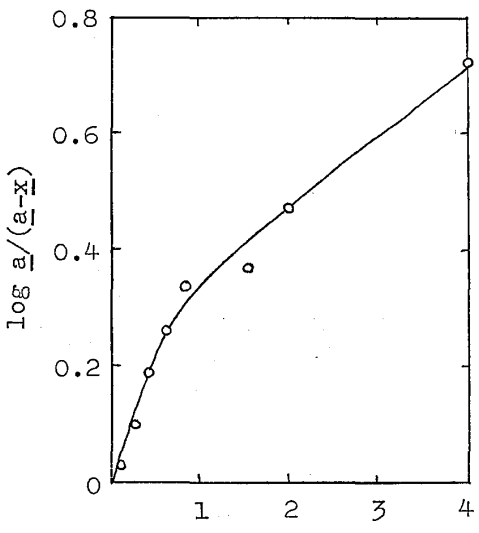

Fig. 7. The release of free sulfate on acid hydrolysis of squid cartilage mucopolysaccharide. The mucopolysaccharide was hydrolyzed with 0.25 *N* hydrochloric acid (1.7 mg. in 0.2 ml.) in a sealed tube at 100°C. At the indicated time, the free sulfate was determined by the modified turbidimetric method.

The ratio of the *N*-acetylglucosamine value to the glucose value given by the products of hyaluronidase digestion was 0.25 in the cartilage mucopolysaccharide as shown in Table 4. This value (0.25) was higher than that of chondroitin sulfate A (0.07) in which the C4 position of the acetylgalactosamine is substituted, and lower than that of chondroitin sulfate C (1.07) in which the C4 position is free. From these results it is assumed that about 80% of the acetylgalactosamine

Table 4. Morgan-Elson Reaction and Reducing Power of Hyaluronidase Digests of Chondroitin Sulfates.

Origin of oligosaccharide	*N*-acetyl-glucosamine (A)	Glucose (G)	(A)/(G)
	μM/ml.	μM/ml.	
Chondroitin sulfate A	0.01	0.15	0.07
Chondroitin sulfate C	0.16	0.15	1.07
Squid cartilage mucopolysaccharide	0.03	0.12	0.25

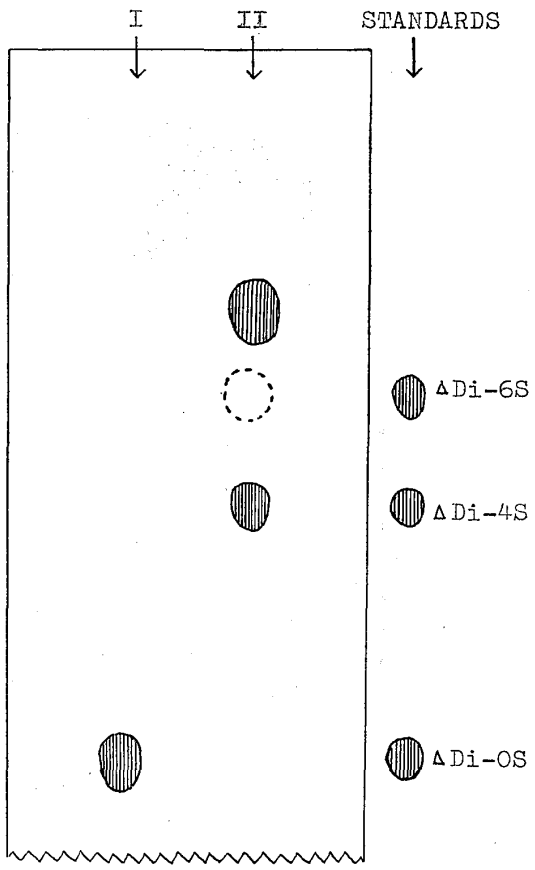

Fig. 8. Tracing of a paper chromatogram is Solvent I of the chondroitinase digests. I: Digest of squid skin mucopolysaccharide; II: Digest of squid cartilage mucopolysaccharide. Ultraviolet absorption print is schematically represented.

is sulfated at the C4 position.

From the above results of sulfate positions and the value of sulfate content, it is considered that the squid cartilage mucopolysaccharide is a novel type of chondroitin polysulfate which contains a 4, 6-disulfated acetylgalactosamine residue. This polysaccharide is henceforth designated as "chondroitin sulfate E".

Isolation and Identification of Unsaturated Disaccharides Produced by Chondroitinase Digestion:

In order to confirm the structure of chondroitin sulfate E, degradation products by chondroitinase-ABC digestion were isolated and identified.

After the chondroitin sulfate E was exhaustively digested with chondroitinase-ABC, aliquots of the reaction mixture containing 0.5μ mole of disaccharides were chromatographed in Solvent I. As shown in Fig. 8, the digest of chondroitin sulfate E gave three spots on the chromatogram. The fastest moving spot corresponded to that of △Di-4S and the faint middle spot to that of △Di-6S (these components will be referred to as △Di-$4S_E$ and △Di-$6S_E$, respectively). The slowest component had a strongest absorption under an ultraviolet lamp. This will be referred to as △Di-diS_E.

On the other hand, the squid skin mucopolysaccharide was digested with chondroitinase-ABC and subjected to paper chromatography as described above. The chromatogram of digests showed only one spot which was in accord with that of non-sulfated disaccharide, △Di-0S (Fig. 8). This indicates that the skin mucopolysaccharide contains only non-sulfated repeating unit.

The characterization of unsaturated disaccharides produced from chondroitin sulfate E was carried out on the paper chromatogram. When the chromatogram was sprayed with aniline hydrogen phthalate reagent, △Di-$4S_E$ and △Di-diS_E gave purple color and △Di-$6S_E$, in contrast, gave brown color. It has already been reported[36] that acetylgalactosamine 4-sulfate and acetylgalactosamine 4, 6-disulfate yielded a purple color, whereas acetylgalactosamine 6-sulfate yielded a brown color under the same conditions. Therefore, △Di-$4S_E$, △Di-$6S_E$ and △Di-diS_E appear to contain, as a reducing sugar moiety, acetylgalactosamine 4-sulfate, acetylgalactosamine 6-sulfate and acetylgalactosamine 4, 6-disulfate, respectively.

After development of the chromatogram of chondroitin sulfate E digests, each of the ultraviolet absorbing regions was cut out into strips and eluted by immersing in 0.01 *N* hydrochloric acid at 50°C for 10 minutes[37] to determine the content of ultraviolet absorbing material. The yields for 0.5μmole (as glucuronic acid) of chondroitin sulfate E calculated from glucuronic acid determination[22], were 0.15μmole of △Di-$4S_E$, 0.04μmole of △Di-$6S_E$ and 0.30μmole of △Di-diS_E.

To isolate the degradation products for further characterization, 150 mg. of chondroitin sulfate E was incubated in 15 ml. of 0.05 *M* Tris-HCl (pH 8.0), containing 1.5 mg. of bovine serum albumin, 0.75 m mole of sodium acetate, and 18 units of chondroitinase-ABC at 37°C for 2 hours. The mixture was heated in a boiling bath for 2 minutes and the precipitate was removed by centrifugation. The supernatant was concentrated *in vacuo* and applied to chromatography as bands (50 cm.) on 6 sheets of Toyo No. 50 paper in Solvent I for 48 hours. The products located by viewing under ultraviolet light, △Di-$4S_E$, △Di-$6S_E$ and △Di-diS_E, were eluted from the chromatogram with water. Chromatography in Solvent I, followed by eluting with water, was repeated with each product to remove small amounts of contaminants. The samples were then desalted separately by paper chromatography in Solvent II and further purified by paper electrophoresis in 0.05 *M* ammonium acetate-acetic acid buffer (pH 5.0) (cf. Fig. 9). After thoroughly desalted again by paper chromatography in Solvent II as described above, the samples were eluted from the paper with water and lyophilized.

The results of chemical analysis of △Di-diS_E indicated that molar

ratios of galactosamine and sulfate to glucuronic acid were 0.87 and 1.94, respectively, indicating that $\triangle$Di-diS$_E$ is a disulfated disaccharide. That $\triangle$Di-4S$_E$ and $\triangle$Di-6S$_E$ are monosulfated was also indicated by the analyses.

All the compounds, $\triangle$Di-4S$_E$, $\triangle$Di-6S$_E$ and $\triangle$Di-diS$_E$, showed an ultraviolet absorption spectrum with a maximum at 232 mμ which is characteristic of a disaccharide unsaturated α, β to the carboxyl group of the uronic acid moiety[12.38.39].

In the Morgan-Elson reaction, $\triangle$Di-4S$_E$ and $\triangle$Di-diS$_E$ had extremely low absorption coefficients at 585 mμ, whereas $\triangle$Di-6S$_E$ showed a strong absorption with a maximum at 585 mμ (m$\varepsilon_{585m\mu}$; about 20). The low value of $\triangle$Di-diS$_E$ suggests that the C4 position of the acetyl-

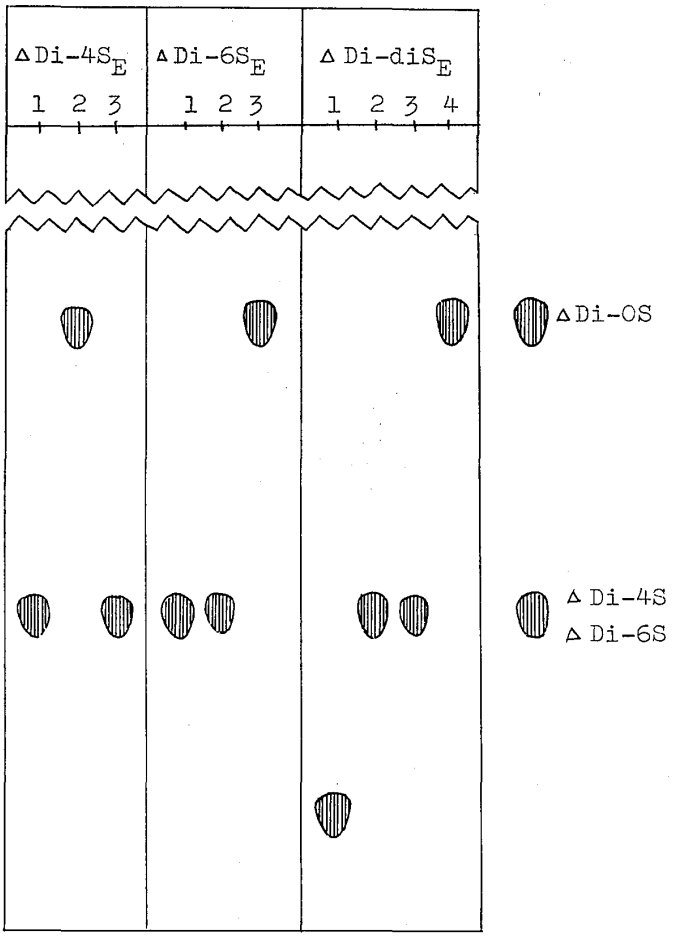

Fig. 9. Tracing of paper electrophoretogram of chondro-4-sulfatase and chondro-6-sulfatase digests of unsaturated disaccharides. Incubation mixtures and conditions are described in the text. 1: No enzyme added; 2: Chondro-4-sulfatase; 3: Chondro-6-sulfatase; 4: Chondro-4-sulfatase plus chondro-6-sulfatase. Ultraviolet absorption prints are schematically represented.

galactosamine residue is substituted.

The electrophoretogram of the products by chondro-4-sulfatase and chondro-6-sulfatase digestions of $\triangle$Di-4S_E, $\triangle$Di-6S_E and $\triangle$Di-diS_E are shown in Fig. 9. On exhaustive digestion with chondro-4-sulfatase, $\triangle$Di-diS_E as well as $\triangle$Di-4S_E were each converted to compounds with the lower electrophoretic mobilities, which were identified as $\triangle$Di-6S and $\triangle$Di-0S, respectively, by electrophoretic mobilities, chemical analyses and digestion with *F. heparinum* glucuronidase (see below). $\triangle$Di-6S_E was, however, remained unaffected. With chondro-6-sulfatase, on the other hand, $\triangle$Di-diS_E as well as $\triangle$Di-6S_E were converted to compounds with the lower electrophoretic mobilities, which were identified as $\triangle$Di-4S and $\triangle$Di-0S, respectively, while $\triangle$Di-4S_E was not affected. All these data indicate that the sulfate residues of $\triangle$Di-diS_E are located at the C4 and C6 positions of the acetylgalactosamine moiety and removed by chondro-4-sulfatase producing $\triangle$Di-6S and chondro-6-sulfatase producing $\triangle$Di-4S, respectively.

Further information on the structure of $\triangle$Di-diS_E was given by the digestion of *F. heparinum* glucuronidase and by the mild acid hydrolysis.

When 0.3μmole of $\triangle$Di-diS_E was incubated with 0.03 unit of *F. heparinum* glucuronidase at pH 5.2 for 80 minutes, a new spot was detected on paper chromatogram and paper electrophoretogram (Fig. 10). This product was assumed to be a mixture of acetylgalactosamine 6-sulfate and α-keto acid on the basis of the electrophoretic mobility and the purple color reaction with the o-phenylendiamine reagent[36,40]. Since this glucuronidase is known to degrade an unsaturated disaccharide, $\triangle$Di-0S, to acetylgalactosamine and α-keto acid[17,40], acetylgalactosamine 4, 6-disulfate and α-keto acid must be produced from $\triangle$Di-diS_E. In this experiment, however, acetylgalactosamine 6-sulfate instead of acetylgalactosamine 4, 6-disulfate was detected in the $\triangle$Di-diS_E digests. It is probable that the sulfate at the C4 position of $\triangle$Di-diS_E is removed by a chondro-4-sulfatase-like contaminant[17] and only after the desulfation the specific glucuronidase affects it. This assumption is supported by the fact that $\triangle$Di-4S as compared with $\triangle$Di-0S or $\triangle$Di-6S was hydrolyzed very slowly with the glucuronidase[17].

A sample of $\triangle$Di-diS_E (0.15μmole) was hydrolyzed with 0.04 *N* hydrochloric acid (0.2 ml.) in a boiling water bath for 1 hour. The hydrolyzate was then freed of acid by alternate addition of water and evaporation *in vacuo* (3 cycles) and chromatographed on paper in Solvent I. As shown in Fig. 10, two new spots were observed on the chromatogram by staining with the aniline hydrogen phthalate reagent as well as with the silver nitrate reagent. The faster moving spot coincided in mobility with acetylgalactosamine 6-sulfate and the slower

spot with acetylgalactosamine 4, 6-disulfate. The same results were also obtained from paper electrophoresis (Fig. 10). These observations indicate that on acid hydrolysis the glucuronic acid moiety of $\triangle$Di-diS_E is degraded to produce acetylgalactosamine 4, 6-disulfate and acetylgalactosamine 6-sulfate. The production of acetylgalactosamine 6-sulfate without acetylgalactosamine 4-sulfate showed that the sulfate of the C4 position of acetylgalactosamine is likely more acid-labile than that of the C6 position.

From all the data described above $\triangle$Di-4S_E, $\triangle$Di-6S_E and $\triangle$Di-diS_E were identified as 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4, 6-disulfo-D-galactose, respectively.

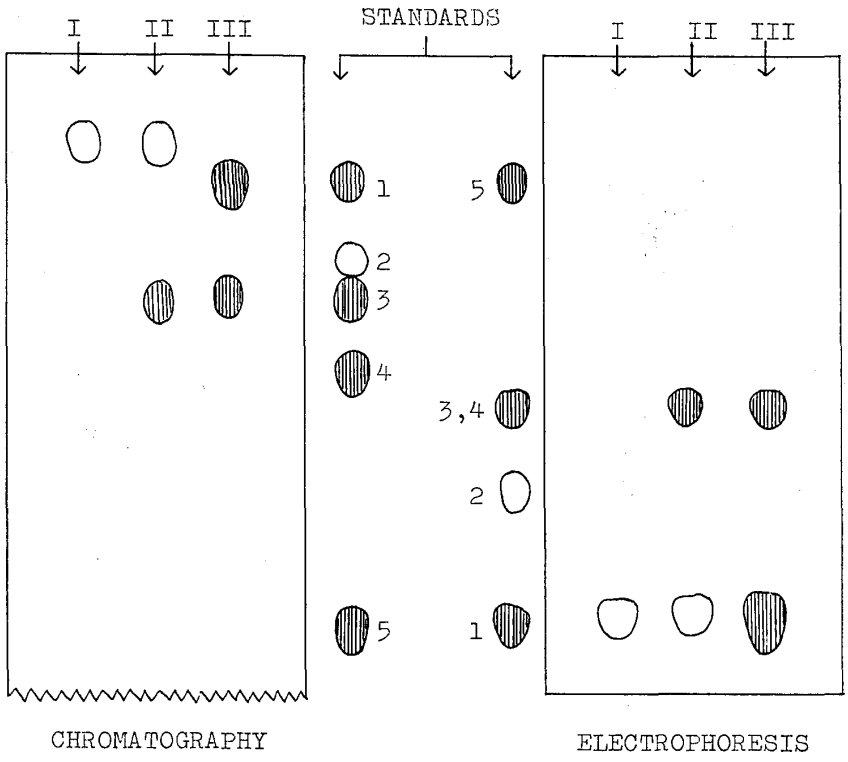

Fig. 10. Tracing of paper chromatogram (*left*) and paper electrophoretogram (*right*) of *F. heparinum* glucuronidase digest and acid hydrolyzate of the unsaturated disaccharide, $\triangle$Di-diS_E. Conditions are described in the text. I: Unaffected; II: Glucuronidase digestion; III: Acid hydrolysis. All the spots were detected by staining with the aniline hydrogen phthalate, and the spots corresponding to unsaturated disaccharides which could be detected also by viewing under an ultraviolet lamp are indicated by open symbols. The standards used are: 1, acetylgalactosamine 4, 6-disulfate; 2, $\triangle$Di-4S; 3, acetylgalactosamine 6-sulfate; 4, acetylgalactosamine 4-sulfate; 5, acetylgalactosamine.

DISCUSSION

The present results indicate that the main mucopolysaccharide isolated from squid skin is non-sulfated chondroitin. Chondroitin was first isolated from bovine cornea by Davidson and Meyer[31] in 1954, but has not yet been isolated from other animal tissues. The sulfate content of bovine cornea chondroitin was reported to be 2%, whereas that of squid skin chondroitin is negligible. This difference may be explained as being due to the difference in the methods of fractionation, but it is uncertain at present whether or not chondroitin is in general sulfate-free.

In this study it is also indicated that the main mucopolysaccharide of squid cartilage which was isolated by eluting from the Dowex 1 column with 3.0 *M* sodium chloride is a chondroitin polysulfate which contains galactosamine, glucuronic acid and sulfate in molar ratios of 1.00 : 0.97 : 1.55. This substance was almost exclusively extracted from the cetylpyridinium-complex with 2.1 *M* sodium chloride and its electrophoresis gave only one spot moving faster than that of chondroitin sulfate A or C. Therefore, the mucopolysaccharide seems to be homogenious.

The chondroitin sulfate of squid cartilage showed absorption bands for both axial and equatorial sulfate groups in the infrared spectrum, and the data of the Morgan-Elson color reaction of hyaluronidase digests and the half lives of ester sulfate on acid hydrolysis suggest that the sulfate groups are probably located not in the glucuronic acid moiety, but at both C4 and C6 positions of the acetylgalactosamine moiety. Consequently, the chondroitin polysulfate of squid cartilage is concluded to be a new type of chondroitin polysulfate containing a 4, 6-disulfated acetylgalactosamine residue. Hence, the name "chondroitin sulfate E" has been suggested to this polysaccharide.

The above observations were further confirmed by the isolation of a novel disulfated disaccharide, $\triangle$Di-diS$_E$, with its two sulfate groups at the C4 and C6 positions of the acetylgalactosamine residue. This substance was obtained as a major product of chondroitinase-ABC digestion of chondroitin sulfate E. On the assumption that the disulfated disaccharide, $\triangle$Di-diS$_E$, and the monosulfated disaccharides, $\triangle$Di-4S$_E$ and $\triangle$Di-6S$_E$, are derived from a hybrid polysaccharide chain, it may be calculated from the analysis of the products of chondroitinase digestion that about 60% of the total acetylgalactosamine residue of chondroitin sulfate E are disulfated at the C4 and C6 positions, the remainder of the sulfate groups being located at either the C4 position (30%) or C6 position (8%). The molar ratio of sulfate to acetylgalactosamine in chondroitin sulfate E calculated from the proportion of the three kinds of sulfated disaccharide described above fairly well agreed with the value given by chemical analysis.

The results obtained from the Morgan-Elson color reaction of hyaluronidase digests indicate that about 80% of the acetylgalactosamine is sulfated at the C4 position. This value differs slightly from the 90% which is obtained from the quantity of the C4-sulfated disaccharides produced by chondroitinase digestion. The slightly lower value may be due to the fact that the testicular hyaluronidase digests consist mainly of tetra- and hexa-saccharides whose Morgan-Elson color density differs from that of disaccharides.

The disulfated disaccharides, $\triangle$Di-diS$_D$ and $\triangle$Di-diS$_B$, have been obtained by chondroitinase digestion from shark chondroitin sulfate D and chondroitin sulfate B prepared from beef lung, respectively. In these disaccharides, one sulfate group is located at the C6 position of the acetylgalactosamine residue in the former and at the C4 position in the latter; the second sulfate group being located at either the C2 or C3 position of the glucuronic acid residue in both. The disulfated disaccharide, $\triangle$Di-diS$_E$, obtained from chondroitin sulfate E of squid cartilage in the present study does not bear any sulfate group at the glucuronic acid moiety, and is apparently a new type of disaccharide.

From the viewpoint of comparative biochemistry and the structural variations of chondroitin sulfate, it is interesting that the first example of chondroitin in skin and an unusual type of chondroitin polysulfate in cartilage are found to occur as the main mucopolysaccharide components of the in ertebrate squid. The over-sulfated mucopolysaccharides which were obtained from the cartilage of the squid (*Loligo*) and the horse-shoe crab (*Limulus*), by Mathews *et al.*[3], resemble the chondroitin polysulfate of the squid (*Ommastrephes sloani pacificus*) cartilage in some properties. It is, however, still uncertain whether a high sulfate content reflects the molecular evolution of the mucopolysaccharides and such an over-sulfated mucopolysaccharide as chondroitin sulfate E should be thought of as characteristic of invertebrate tissues. In order to resolve this question it seems to be necessary to study in more detail the structure and distribution of mucopolysaccharides in invertebrate tissues. In relation to this problem, Mathews[41] has recently described that the various types of over-sulfated mucopolysaccharide occur in the connective tissues of several lower animals (invertebrates, cyclostomes, elasmobranchs and teleosts), in which all the backbone of the polysaccharide is closely similar to the chondroitin sulfate of mammalian tissues, but only the sulfate contents are different.

Recently, a polysaccharide was isolated from the squid, *Ommastrephes sloani pacificus*, liver which is the largest organ of this organism[42]. The liver polysaccharide was mainly consisted of nine sugars; glucosamine, galactosamine, galactose, mannose, glucose, arabinose, xylose, fucose and glucuronic acid, it being an unusually complex polysac-

charide. A polysaccharide containing pentose as a constituent sugar has not yet been found in animal origin. Consequently, the squid liver also appears to contain a novel type of polysaccharide.

The occurrence of non-sulfated chondroitin and over-sulfated chondroitin sulfate in squid connective tissues is also of interest in connection with the biosynthesis of chondroitin sulfates. If there is no other difference than the degree of sulfation in primary structures between chondroitin-protein complexes and chondroitin sulfate-protein complexes, it appears possible that the tissue distribution of chondroitin sulfates with varying degrees of sulfation is related to the cellular activity of sulfation. Thus, a lack or repression of sulfation enzymes in the skin cells may result in accumulation of non-sulfated or less sulfated polysaccharides. The occurrence of the characteristic chondroitin polysulfate in the cartilage, on the other hand, suggests that several specific sulfation enzymes are involved in the synthetic process being carried out by this particular tissue.

SUMMARY

The mucopolysaccharides of the connective tissues, skin and cartilge of the squid (*Ommastrephes sloani pacificus*) were isolated and characterized.

The main mucopolysaccharide component isolated from squid skin is indicated to be a non-sulfated chondroitin from the results of chemical analysis, $[\alpha]_D$, electrophoresis, infrared spectrum, identification of repeating unit, measurements of hydrolysis rate of hyaluronidase digestion and formation of non-sulfated disaccharide by chondroitinase digestion.

The mucopolysaccharide isolated from cartilage was found to be homogenious on electrophoresis and cetylpyridinium chloride fractionation. This substance was shown to be a new type of chondroitin polysulfate by the following evidence: 1. The ratios of uronic acid and sulfate to hexosamine were 0.97 and 1.55, respectively. 2. The infrared spectrum showed absorption bands for both axial and equatorial sulfate group. 3. The Morgan-Elson color density of hyaluronidase digests and the half lives of ester sulfate on acid hydrolysis indicated that the sulfate groups are associated not with the glucuronic acid moiety, but with the acetylgalactosamine residue at the C4 and C6 positions. 4. By chondroitinase digestion, a noval unsaturated disaccharide bearing two sulfate groups was derived as a major product, which was identified to be 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose. The name "chondroitin sulfate E" was suggested for this chondroitin polysulfate containing a 4, 6-disulfated acetylgalactosamine residue.

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