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Studies on the Composition and Structure of Whale Cartilage Keratosulfate

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Introduction

Keratosulfate was first isolated from bovine cornea in 1953 by Meyer *et al.*[1] The water extract of bovine cornea was digested with proteolytic enzymes and deproteinized. Keratosulfate was separated from other mucopolysaccharides by ethanol fractionation, and its calcium salt was precipitated from 40 to 50% ethanol concentration. Later, keratosulfate was found from nucleus pulposus[2,3,4,5], costal cartilage[6], shark cartilage[7] and bovine nasal septum[8]. It became apparent that keratosulfate occurs more widely in connective tissues that first expected. It was also found that the contents of keratosulfate of human cartilage and nucleus pulposus increase with age and that keratosulfate is related to the aging[5,9,10].

In regard to the structure, methylation studies were performed on the native and desulfated keratosulfate from bovine cornea and its repeating unit was shown to be (1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate[11]. Since the yields of methylation products were rather low, further investigations were carried out on corneal keratosulfate and skeletal keratosulfate in recent years by using methylation and partial acid hydrolysis, and the proposed structure of repeating unit was confirmed once again[12,13].

From the point of sulfate content, oversulfated keratosulfates were isolated from various sources, especially in skeletal keratosulfates[5,7,14,15]. On the other hand, less-sulfated keratosulfates were found from cornea and nasal septum[5,16]. The methylation studies and isolation of galactose

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Abbreviation

Gal, galactose; GA, glucuronic acid; HexN, hexosamine; GN, glucosamine; GalN, galactosamine; GNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; SA, sialic acid; NANA, N-acetylneuraminic acid; Fuc, fucose; Man, mannose; Gal-S, galactose sulfate; GNAc-S, N-acetylglucosamine sulfate; KS, keratosulfate; TBA, thiobarbituric acid; Res, resorcinol.

6-sulfate from partial hydrolyzates of keratosulfates[12,13] showed that oversulfated keratosulfate contains both N-acetylglucosamine 6-sulfate and galactose 6-sulfate. It was further shown that keratosulfates contain varying amounts of galactosamine, sialic acid, methylpentose and amino acids, in addition to glucosamine and galactose which are constituents of keratosulfate[14,15]. Fucose was isolated as a methylpentose[17], and a small amount of mannose was isolated as a hexose other than galactose[13].

Recently, much interest has been concentrated on the linkage region between carbohydrate and peptide of mucopolysaccharides in close relation with biosynthesis of mucopolysaccharides. The linkage region of chondroitin 4-sulfate was first clarified in 1966 by Rodén and Smith[18] to be glucuronosyl-galactosyl-galactosyl-xylosyl-serine by its isolation and identification. Soon after, the linkage regions of chondroitin 6-sulfate, heparin[19], heparitin sulfate[20] and dermatan sulfate[21] were reported to be similar to that of chondroitin 4-sulfate. The linkage region of skeletal keratosulfate was considered that carbohydrate moiety is mainly linked to peptide glycosidically *via* the hydroxyl group of serine and threonine from the results of destruction of serine and threonine by alkali treatment[14,22]. In contrast, the amino acid content of bovine corneal keratosulfate was not decreased by alkali treatment. From these results and dinitrophenylation studies, it was proposed that the carbohydrate moiety of corneal keratosulfate would be linked to peptide *via* asparagine and glutamine, or aspartic acid and glutamic acid[14]. As to the linkage region of human skeletal keratosulfate, it was further reported that N-acetylgalactosamine appeared to link to the hydroxyamino acids, from the formation of direst Ehrlich's chromogen after alkali treatment[17].

In this study, the isolation of keratosulfate from whale nasal cartilage was carried out and purified keratosulfate fraction was obtained in good yield. The whale keratosulfate contained considerable amounts of galactosamine, sialic acid and amino acids, in addition to the constituents ; glucosamine, galactose and sulfate, and small amounts of mannose and fucose. The content of sialic acid was remarkably high and sialic acid was isolated and identified. The relation between sialic acid and the keratosulfate fraction was investigated by mild alkali and enzymatic treatments. Few reports are available on the problem. The distribution of sialic acid in the mucopolysaccharides of whale cartilage, and the sialic acid contents of some other cartilages are described. The linkage region between carbohydrate and peptide of this keratosulfate was also investigated. From the changes of carbohydrate and peptide moiety after mild alkali treatment in the presence and absence of sodium borohydride, it is considered that the majority

of linkages in this keratosulfate are α-glycosidic bonds of N-acetylhexosamine to both threonine and serine, and that N-acetylhexosamine may be N-acetylgalactosamine.

Materials and Methods

Materials

Nasal cartilage of adult whale (*Balaenoptera physuls*) was freed from extraneous tissues, cut into fine pieces, homogenized with acetone in Waring blendor and dried. Other specimens of cartilage from different sources were obtained from the following animals belonging to the mammals, elasmobranches and cephalopods; nasal cartilage of fetal whale (*Balaenoptera physuls*), nasal and femoro-articular cartilage of adult cattle, cranial cartilage of adult shark (*Prionece glauca*), adult ray (*Dasyatus akajei*) and adult squid (*Ommastrephes sloani pacificus*), which were stored at $-20°$ until used.

All chemicals for analysis were analytical grade reagents. Resorcinol was recrystallized from water. Neuraminidase from *Clostridium perfringens* was purchased from Sigma Chemical Co., pronase P from Kaken Chemical Co., Ltd.

Galactose 6-sulfate: Galactose 6-sulfate was prepared by the direct sulfation of D-galactose and purified by ion exchange chromatography essentially according to the procedure of Lloyd[23]. Materials were dried as follows: Galactose on phosphorous pentaoxide; pyridine on potassium hydroxide; chloroform on anhydrous calcium sulfate. Galactose (3.6 g) was dissolved in 280 ml of dry pyridine and 3 ml of dimethylsulfoxide in the presence of 400 mg of anhydrous calcium sulfate and after cooling to 5°, 1.4 ml of chlorosulfonic acid in dry chloroform (1.8 ml) was added dropwise. The mixture was stirred at 5° for 30 min and at 25° for 4 h. The upper pyridine layer was removed and oily lower layer was dissolved in water. The pH of the solution was adjusted to 8.0 with saturated aqueous barium hydroxide and precipitated barium sulfate was removed by centrifugation. Excess barium ion was precipitated as barium carbonate with dry ice. The clear supernatant was passed through a Dowex 50 column (H^+ form, 1.7×24 cm). The acidic eluate and washings were combined, adjusted to pH 7.0 with potassium hydroxide solution and concentrated below 40°. Then it was applied on a Dowex 1 column (OH^- form, 1×25 cm). The column was washed with water and eluted with 0.036*N*, 0.05*N* and 0.5*N* H_2SO_4. Each eluate was neutralized with barium hydroxide solution and converted to potassium salt as described above. Galactose sulfate (potassium salt) was obtained by freeze-drying.

N-acetylglucosamine 6-sulfate: N-acetylation of glucosamine-HCl was performed according to Roseman *et al.*[24]. Glucosamine-HCl (1.1 g)

dissolved in 25 ml of water was stirred for 90 min at 5° with 2.5 ml of methanol, 30 ml of Dowex 1 (bicarbonate form) and 0.65 ml of acetic anhydride. The mixture was filtered and the filtrate and washings were passed through an Amberlite IR-120 column (H^+ form, 1×20 cm). The effluent and washings were concentrated to dryness *in vacuo*. The residue was used for the succeeding direct sulfation which was performed by the same manner as the direct sulfation of galactose.

Galactose 6-sulfate and N-acetylglucosamine 6-sulfate were eluted from Dowex 50 column with 0.05*N* H_2SO_4. They gave only one spot by high voltage electrophoresis and paper chromatography with solvent C.

Analytical methods

Galactose was determined by the anthrone reaction[25] and the secondary L-cysteine-sulfuric acid reaction[26]. Hexosamines were separated on Dowex 50 (H^+) and determined by a modified Elson-Morgan reaction according to the procedure of Gardell[27] after hydrolysis in 3*N* HCl at 100° for 15 h. Uronic acid was determined by the modified carbazole reaction[28] using gluculonolactone as the standard. Sulfate was determined by the method of Dodgson and Price[29] and protein by the method of Lowry *et al.*[30] using serum albumine as the standard. Sialic acid was determined by the thiobarbituric acid method according to Warren[31] after hydrolysis in 0.1*N* H_2SO_4 at 80° for 1 h and also by the resorcinol method[32]. N-Acetylhexosamine was estimated by the Morgan-Elson reaction modified by Reissig *et al.*[33].

Electrophoresis

Electrophoresis was performed on a cellulose acetate strip with 0.1*M* acetic acid-pyridine buffer, pH 5.0, 160 V. for 1 h. Mucopolysaccharides were detected with 0.5% toluidine blue in 3% acetic acid.

High voltage electrophoresis was performed on Toyo No. 51A paper with acetic acid-pyridine buffer (acetic acid : pyridine : water, 4 : 100 : 900), pH 6.5, 3000 V. for 50 min. Sugars were detected with alkaline silver nitrate[34].

Paper chromatography

Paper chromatography was carried out on Toyo No. 51 paper by descending method, using the following solvent systems; solvent A, n-butanol-n-propanol-0.1*N* HCl (1 : 2 : 1)[35] and solvent B, n-butylacetate-acetic acid-water (3 : 2 : 1)[36] for sialic acid, solvent C, n-butanol-acetic acid-water (50 : 12 : 25) for sugars and sugar sulfates. Sialic acid was detected with the Ehrlich's reagent[35] and alkaline silver nitrate[34]. Sugar and sugar sulfate were detected with alkaline silver nitrate[34].

Gas-liquid chromatography of neutral sugars

A sample (5 mg) was hydrolyzed in 1 ml of 1*N* HCl at 100° for

3 h, neutralized with 1*N* NaOH and desalted with Amberlite MB-3 (0.7×10 cm), the effluent was evaporated to dryness below 40° and placed in a vacuum desiccator over P_2O_5 overnight. Trimethylsilylation was carried out by the method of Yamakawa and Ueta[37]. Gas-liquid chromatographic analysis of TMS-sugars was performed on a Shimazu model GC-1C chromatograph with hydrogen flame detector using stainless steel column (4 mm×225 cm) packed with 3% SE-52 on Chromosorb W (80-100 mesh). The column temperature was maintained at 150° for 20 min., then programmed 2°/min. to a maximum of 180°. Nitrogen was used as a carrier gas and its flow rate was 55 ml/min. The flow rate of hydrogen was 34 ml/min. Fucose, xylose, mannose, galactose and glucose were used as the standards, which were dissolved in water, left overnight to equilibrate, evaporated and then trimethylsilylated.

Amino acid analysis

A sample was hydrolyzed in 6*N* HCl at 110° for 22 h in a vacuum-sealed tube. After being removed hydrochloric acid, the hydrolyzate was applied to an amino acid analyzer.

Infrared spectroscopy

Infrared spectrum of sample was obtained with KBr disk on a Hitachi model EPI-S2 spectrophotometer and a JASCO IR-G instrument.

Specific rotation

Specific rotation of sample was measured in aqueous solution on a Rudolph polarimeter.

Extraction of mucopolysaccharides from whale nasal cartilage

One hundred g of acetone dried cartilage was homogenized in ten times its weight of water, and heated for 1 h in a boiling water bath. The pH was adjusted to 7.8 with 1*N* NaOH and 150 mg of pronase was added. The mixture was covered with toluene layer and incubated at 40°. The pH was adjusted at intervals to 7.8 with NaOH. One hundred and fifty mg of pronase was added one day after and incubation was continued for 2 more days. The digestion mixture was filtered and calcium acetate was added to the filtrate to 2.5% and acetic acid to pH 4.5. The polysaccharides were precipitated with 2 volumes of ethanol, left overnight at 4° and centrifuged. The precipitate was washed with 80 and 99% ethanol successively, and dried in a vacuum desiccator over conc. H_2SO_4. It was redissolved in 330 ml of 5% sodium acetate containing 0.5*N* acetic acid and deproteinized by Sevag's method[1]: The solution was stirred with 120 ml of isoamyl-alcohol and chloroform (1 : 3) for 30 min and centrifuged. To the supernatant thus obtained 2 volumes of ethanol were added. The

precipitate was washed with ethanol and dried. Then it was dissolved in 500 ml of buffer and stirred with 16 g of Lloyd's reagent and kaolin (3 : 1) for 30 min. After centrifugation at 5000 r. p. m. for 20 min., the supernatant was treated with 2 volumes of ethanol. The precipitate was centrifuged and dried in a vacuum desiccator over conc. H_2SO_4. The mucopolysaccharide sodium salts were thus obtained.

Ethanol fractionation of mucopolysaccharides on cellulose column

Separation of keratosulfate fraction from mucopolysaccharide mixture was performed as follows modifying the Gardell's method[38]. Cellulose powder (Whatman) was suspended in water and poured into a suction flask. The flask was connected to a water pump and gently rotated during evacuation to remove the air present in the cellulose. The slurry was packed into a column caring about bubbles. The column was washed with water until the anthrone test of the effluent was negative and then equilibrated with 80% ethanol containing 0.3% barium acetate. According the amount of mucopolysaccharides, two columns of different size were used.

(1) Two g of mucopolysaccharides was dissolved in 5 ml of 0.3% aqueous solution of barium acetate and then added on the top of a column (3.6×20 cm) to precipitate. The column was washed with 400 ml of 80% ethanol containing 0.3% barium acetate. The elution of the material was performed with 800 ml of 35% ethanol containing 0.3% barium acetate and then 1000 ml of water. The flow rate was 60–120 ml/h. The carbazole and anthrone reactions were carried out on the eluates.

(2) For large scale preparations, 20 g of mucopolysaccharides was dissolved in 1000 ml of 2% barium acetate, to which ethanol was added to 20%. After removal of resulting precipitate, chondroitin sulfate A (Ba-salt), ethanol was added up to 50% to the supernatant. The precipitate was dissolved in 15 ml of 0.3% barium acetate and applied on a column (6.6×40 cm). The column was eluted with 80% ethanol, 35% ethanol, each containing 0.3% barium acetate, and with water as described above. The flow rate was 200–300 ml/h. Mucopolysaccharides of each fraction were precipitated with ethanol, and then dissolved in water and dialyzed against tap water and distilled water. The purified mucopolysaccharides were obtained from non-dialyzable fractions after freeze-drying.

Fractionation of keratosulfate on Dowex 1 column

The fractionation of the keratosulfate fraction on Dowex 1 was performed by the method of Schiller *et al.*[39]. Dowex 1 resin (200–400 mesh) was regenerated with 1*N* HCl and 1*N* NaOH at least 2 cycles. Finally the resin was converted to the chloride form with 1*N* HCl,

washed with water until chloride ion test was negative and packed into a column (2.3×44 cm). The keratosulfate fraction (0.2 g) was applied to the top of the column, washed with water and then eluted stepwise with increasing concentration of NaCl in the following order: 0.4, 1.0, 1.5, 2.0 and 3.0*M*. The flow rate was 60 ml/h, and each 15 ml was collected and screened by the anthrone reaction. After negative anthrone reaction was obtained, the concentration of sodium chloride solution as eluate was changed, thus, 300 ml of water, 300 ml of 0.4*M* NaCl, 500 ml of 1.0*M* NaCl, 600 ml of 1.5*M* NaCl, 350 ml of 2.0*M* NaCl and 300 ml of 3.0*M* NaCl were used as eluates. Last of all, the column was eluted with 5.0*M* NaCl.

Detection of galactose 6-sulfate in the keratosulfate fraction

The keratosulfate fraction (1.0 mg) was hydrolyzed in 1 ml of 0.5*N* HCl at 100° for 1 h. The products were examined by paper chromatography in solvent C and high voltage electrophoresis. The eluate from the paper was hydrolyzed in 1*N* HCl at 100° for 3 h. The component sugar in hydrolyzate was examined by paper chromatography.

Isolation and identification of sialic acid

The keratosulfate fraction was dissolved in 2 ml of 0.1*N* H_2SO_4 and heated at 80° for various periods. The optimal time was determined for 45 min from the maximum liberation of sialic acid. The released sialic acid was determined by the thiobarbituric acid reaction. In order to identify the nature of sialic acid, isolation of sialic acid from the keratosulfate fraction was performed. The keratosulfate fraction (20 mg) was hydrolyzed in 2 ml of 0.1*N* H_2SO_4 at 80° for 45 min. The hydrolyzate was neutralized to pH 5.4 with barium hydroxide solution and the resulting barium sulfate was removed by centrifugation. The sialic acid fraction was separated by the following two methods.

(1) The supernatant and washings were passed through a Dowex 50 column (H^+ form, 200–400 mesh, 1.3×24 cm) and the effluents which were positive for the resorcinol reaction were collected and then passed through a Dowex 1 column (formate form, 200–400 mesh. 1.3×24 cm) according to the Svennerholm's method[40]. After being washed with water, the column was eluted with 0.3*N* formic acid and each 5 ml was collected. The positive fractions for the resorcinol reaction were collected and concentrated *in vacuo* below 40° repeatedly to remove formic acid.

(2) The supernatant and washings were passed through a column of Sephadex G-10 (2.7×120 cm) and eluted with water. The flow rate was 20 ml/h and each 5 ml was collected and assayed by the resorcinol and anthrone reactions.

On the sialic acid fraction obtained, paper chromatography was performed using the solvents A and B. The detection and estimation

of glycolyl group of the sialic acid fraction was performed by the method of Klenk and Uhlenbruck[41]. Sample (500 μg as sialic acid) was dissolved in 0.5 ml of 1*N* H_2SO_4 and heated for 1 h in a boiling water bath, to which 2 ml of water was added. To 0.2 ml of this diluted hydrolyzate, 4 ml of Eegriwe's reagent (10 mg of recrystallized 2, 7-dihydroxynaphthalene in 100 ml of conc. H_2SO_4) was added. The mixture was heated for 20 min in a boiling water bath and measured at 546 mμ. Sodium glycolate was used as a standard.

Enzymatic treatment of the keratosulfate fraction

(1) Pronase digestion: The keratosulfate fraction (105 mg) was dissolved in 5 ml of water and digested with 1.2 mg of pronase P as described above. After 24 h, one third volume of 40% trichloroacetic acid was added to the mixture in an ice bath and stored overnight at 4°. After centrifugation, the supernatant was dialyzed against running water and then distilled water. From the non-dialyzable solution keratosulfate fraction was precipitated with 50–60% ethanol concentration in the presence of 5% sodium acetate.

(2) Neuraminidase-pronase digestion: The keratosulfate fraction (150 mg) was dissolved in 15 ml of 0.1*M* acetate buffer, pH 4.7, and digested with 1.1 mg of neuraminidase from *Clostridium perfringens*[42]. Immediately after mixing, and after 0.5, 2.5, 8, 24 and 48 h of digestion, 0.2 ml of the mixture was taken and released sialic acid was determined by the thiobarbituric acid reaction of Warren except preliminary acid hydrolysis. After 70 h, the pH of the mixture was adjusted to 7.8 and the digestion with 1.5 mg of pronase P was performed as described above. The keratosulfate fraction recovered by precipitation with 50–65% ethanol concentration was further purified using a column of Sephadex G-50. Two mg of the recovered keratosulfate fraction was applied on a column of Sephadex G-50 (0.6×97 cm) and eluted with water. The flow rate was 4.6 ml/h and each 2 ml was collected and assayed by the anthrone reaction. In preparative scale, Sephadex G-50 column (1.3×100 cm) was used for purification of 50 mg of the keratosulfate fraction.

Alkali treatment of the keratosulfate fraction

(1) Alkali treatment at 4°: Seventy mg of the keratosulfate fraction was dissolved in 4 ml of 0.5*N* NaOH in a glass stoppered flask and kept in refrigerator. The solution was passed through a column of Amberlite IR-120 (H^+) to remove cations. Resulting acidic effluent was neutralized with barium hydroxide and concentrated. The keratosulfate was reisolated by the ethanol fractionation method on a cellulose column as described above.

(2) Alkali treatment at 25°: Seventy mg of the keratosulfate

fraction was dissolved in 3.5 ml of 0.5*N* NaOH in glass-stoppered flask, kept at 25° and treated as described above. The keratosulfate fraction was reisolated by passing through a column of Sephadex G-50 (2.2× 87.5 cm). The column was eluted with water at the flow rate, 20 ml/h. Fifty mg of 1.5*M* NaCl fraction of keratosulfate from Dowex 1 was also treated by the same way.

(3) Analysis of amino acid, hexosamine and chromogen after alkali treatment of keratosulfate without previous isolation:

Amino acid analysis: At the end of alkali treatment the solution was mixed with equal volume of 12*N* HCl in an ice bath, and the tube was sealed in vacuum in dry ice-acetone. Following these treatments, hydrolysis was carried out at 105°–110° for 22 h. Analysis was made by an amino acid analyzer.

Hexosamine analysis: At the end of the alkali treatment, the solution was mixed with an equal volume of 6.5*N* HCl and hydrolyzed at 100° for 15 h. The hydrolyzate was diluted with 9 volumes of water and hexosamine was analyzed on Dowex 50 according to the modified Elson-Morgan reaction by Gardell[27].

Chromogen analysis: At the end of alkali treatment, the solution was neutralized with 0.5*N* HCl to pH 5.8 and the chromogen formed was analyzed by a modified Reissig *et al.* test[33]. The modification consisted in not heating the sample after the addition of potassium tetraborate[17].

NH_2-terminal amino acid assay

Ten mg of the keratosulfate fraction was dissolved in 0.25 ml of 1% trimethylamine (aqueous solution). To the solution, 0.5 ml of 5% fluorodinitrobenzene (ethanol solution) was added and kept at room temperature in the dark for 24 h[43]. Excess fluorodinitrobenzene was extracted with 1 ml of ether three times. The solution was dried up *in vacuo* and the residue was hydrolyzed in 1 ml of 6*N* HCl at 105° for 16 h in a vacuum-sealed tube. After the hydrolyzate was diluted with 5 ml of water, extraction was performed with 4 ml of ether three times. The ether layer and the aqueous layer were evaporated and each residue was dissolved in acetone and water respectively. The analysis of the DNP-amino acids was carried out by two dimensional paper chromatography with a toluene solvent system and 1.5*M* phosphate buffer, pH 6.0[44]. The DNP-amino acids were detected under ultraviolet lamp (3850Å).

Results

Separation of keratosulfate on cellulose column

From 100 g of the acetone dried whale cartilage, 40.5 g of the

mixed mucopolysaccharides was obtained. The yield and analytical values of three fractions obtained by ethanol fractionation on a cellulose column are shown in Table 1.

Table 1. Analysis of mucopolysaccharides on cellulose column.

	Yield	Gal	UA	SA
80% EtOH fraction	1.3%	14.2%	3.1%	—%
35% EtOH fraction	7.1	25.7	<1.0	11.7
H_2O fraction	73.4	6.0	40.2	<0.6

Thirtyfive % ethanol fraction seemed to correspond to keratosulfate based on the analytical results, shown in Table 2. As uronic acid was less than 1%, contamination of chondroitin sulfate seemed to be negligible. The ratio of sulfate to hexosamine was 1.20, suggesting the presence of oversulfated keratosulfate. It was found that this keratosulfate contained galactosamine which was one sixth of total

Table 2. Analytical values of 35% EtOH fraction.

Gal	25.7%
UA	<1.0
HexN	22.7
GN	19.0
GalN	3.7
GalN/GN	0.19
SO_4	14.7
SO_4/HexN	1.2
SA (TBA)	11.7
(Res)	14.0
Lowry protein	11.0

Neutral sugars (Molar ratio)

Gal	100.0
Man	9.4
Fuc	3.1

$[\alpha]_D$ −15.1°
(C 1.5, H_2O)

hexosamine, 11.7% of sialic acid, 11% of protein and small amounts of neutral sugars, besides galactose and glucosamine which are constituent repeating units of keratosulfate. Neutral sugars other than galactose were identified as mannose and fucose by gas-liquid chromatography.

On electrophoresis the keratosulfate fraction gave one spot migrating slower than chondroitin sulfate. Infrared spectrum of the keratosulfate fraction is shown in Fig. 1. It showed the absorption bands at 1240 cm^{-1} corresponding to the S=O vibration and at 820 cm^{-1} corresponding to the equatorial C-O-S vibration, but no absorption bands at 850 and 928 cm^{-1} corresponding to the axial C-O-S vibration were observed.

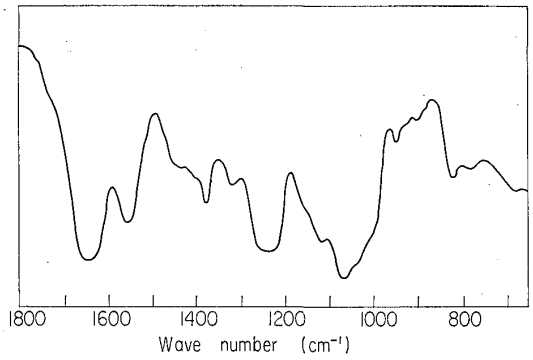

Fig. 1. Infrared spectrum of the keratosulfate fraction. The spectrum was obtained with KBr disk containing 1.5 mg of sample on a Hitachi model EPI-S2 spectrophotometer.

Fractionation of the keratosulfate fraction on Dowex 1 column

As the keratosulfate fraction seemed to contain oversulfated keratosulfate from the ratio of sulfate to hexosamine, it was fractionated on the Dowex 1 column. Its elution curve was shown in Fig. 2. The yield and the analytical values of each fraction are given in Table 3. All the fractions contained various amounts of sulfate and sialic acid. The contents of galactosamine and neutral sugars other than galactose were relatively lower, as the concentration of NaCl was increased.

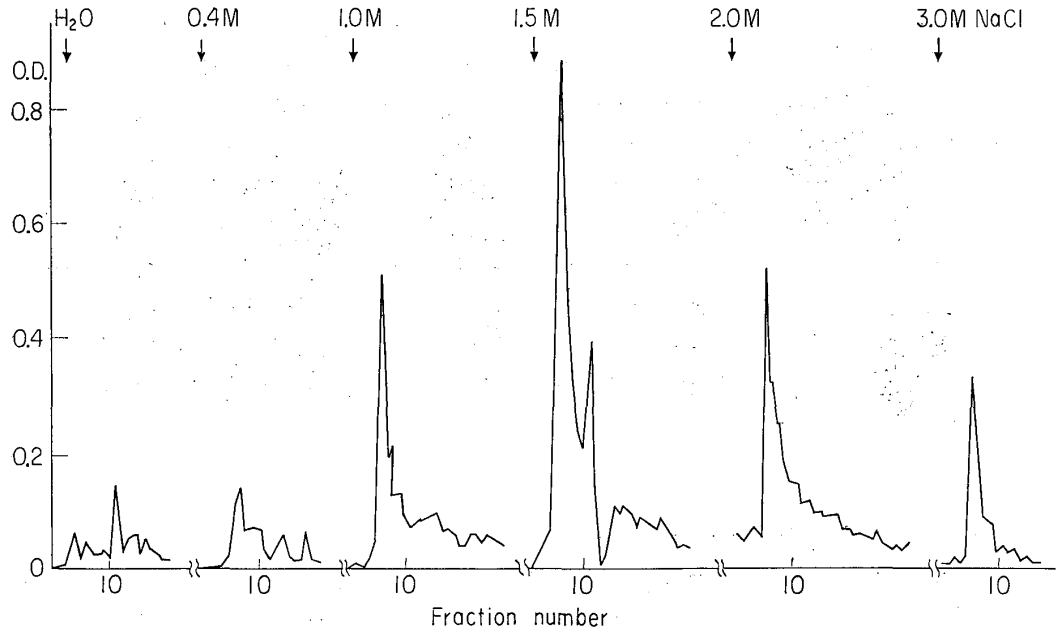

Fig. 2. Chromatography of the keratosulfate fraction on Dowex 1 column. Sample (200 mg) was applied on the Dowex 1 column (2.3×44 cm) and stepwise elution was used with NaCl solution of increasing concentration. The flow rate was 60 ml/h. Each 15 ml was collected and screened by the anthrone reaction.

Table 3. Analytical values of keratosulfate fractions obtained by Dowex 1 chromatography.

Fraction	1.0*M* NaCl	1.5*M* NaCl	2.0*M* NaCl	3.0*M* NaCl
Yield	15 %	28 %	16 %	6 %
Gal	24.9	23.7	27.3	25.1
HexN	20.9	22.8	22.3	20.9
GN	16.1	18.1	19.3	19.3
GalN	4.8	4.7	3.0	1.6
Gal/GN	0.30	0.24	0.15	0.08
SO_4	8.6	13.1	17.5	20.6
SO_4/HexN	0.75	1.05	1.44	1.80
SA (TBA)	17.0	12.0	13.0	9.0
(Res)	18.2	12.7	13.7	10.0
Lowry protein	10.9	7.7	7.7	7.1
Neutral sugars (Molar ratio)				
Gal	100.0	100.0	100.0	
Man	12.5	9.1	3.8	
Fuc	7.8	1.6	3.3	

Detection of galactose 6-sulfate in the keratosulfate fraction

The partial acid hydrolyzate of keratosulfate in 0.5*N* HCl gave spots corresponding to galactose 6-sulfate and N-acetylglucosamine 6-sulfate by high voltage electrophoresis and paper chromatography with solvent C as shown in Fig. 3. Since high voltage electrophoresis was not enough to isolate galactose 6-sulfate in preparative scale, eluates from the electrophoretogram were further subjected to paper chromatographic separation. The material obtained from the paper gave only one spot corresponding to galactose 6-sulfate. The hydrolyzate of the latter material gave only one spot corresponding to galactose on a paper chromatogram. The results indicated that the keratosulfate fraction contained galactose sulfate. The ester sulfate of whale keratosulfate was considered to be equatorial from the infrared spectrum. From these results and considering the previous report[13], the keratosulfate would contain galactose 6-sulfate other than N-acetylglucosamine 6-sulfate.

Sialic acid in the keratosulfate fraction

The existence of sialic acid in the keratosulfate fraction was suggested by the color reactions with thiobarbituric acid and resorcinol (Tables 1 and 2) and the spectra were identical to those of N-acetylneuraminic acid (Fig. 4). In order to characterize the sialic acid, the isolation of sialic acid was performed. The resorcinol positive fraction was eluted from the tube number 14 to 19 from Dowex 1 column

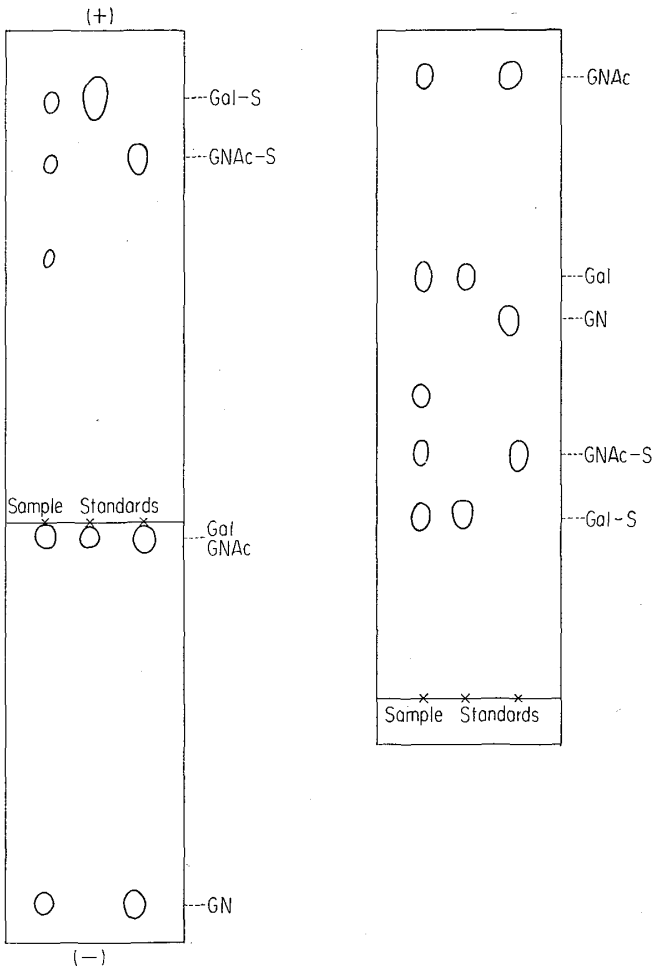

Fig. 3. Electrophoretogram (*left*) and paper chromatogram (*right*) of the hydrolyzate of the keratosulfate fraction. Electrophoresis was performed in pyridine-acetic acid-water (100 : 4 : 900), pH 6.5 at 3000 V for 50 min on Toyo No. 51A paper. Paper chromatography was performed with solvent C for 24 h on Toyo No. 51 paper by descending method. Sugar and sugar sulfate were detected with alkaline silver nitrate.

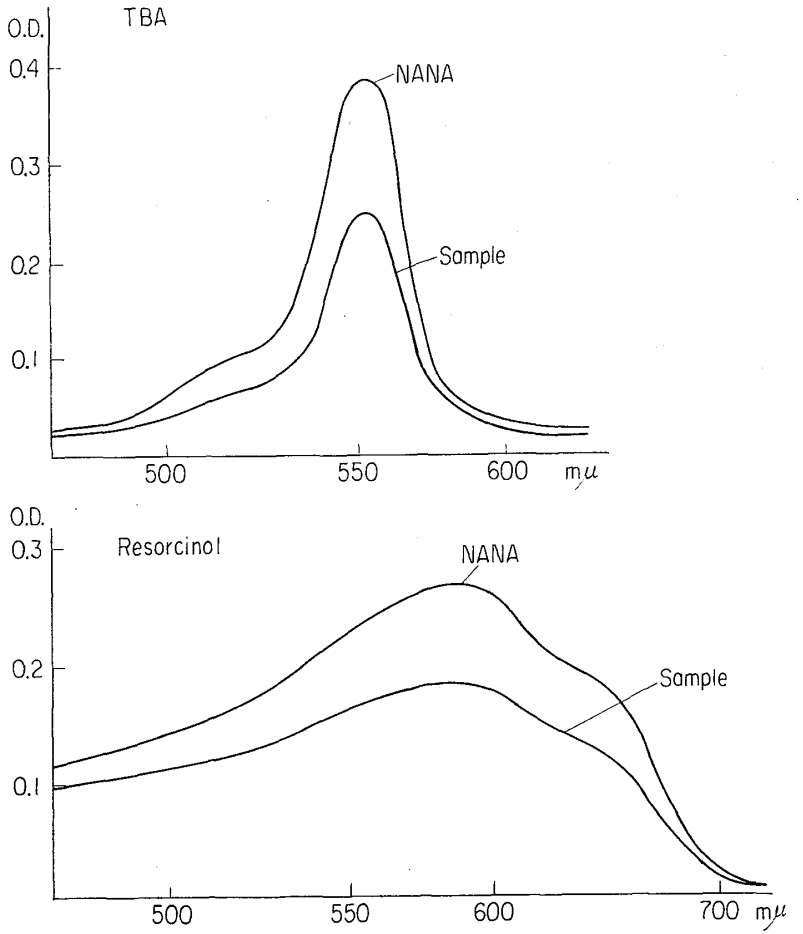

Fig. 4. Absorption spectra of sialic acid of the keratosulfate fraction by the thiobarbituric acid reaction and the resorcinol reaction. Standard solution contained 80 μg of N-acetylneuraminic acid and sample, 45.3 μg of the keratosulfate in the thiobarbituric acid reaction, and in the resorcinol reaction, 53.3 μg and 259 μg, respectively.

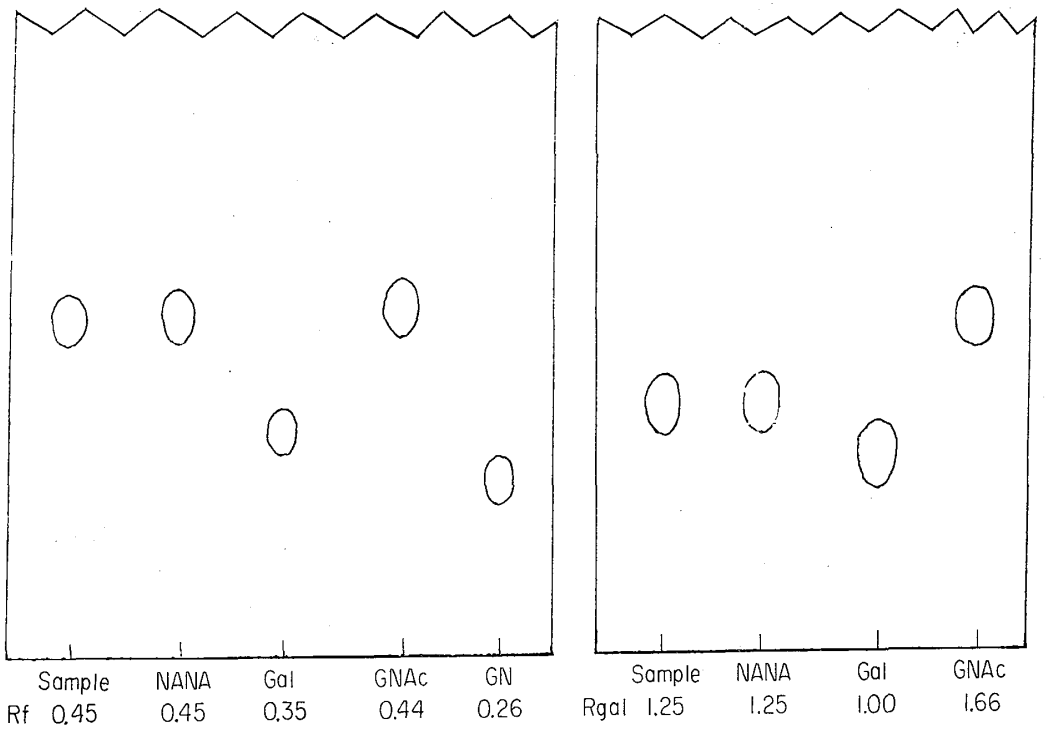

Fig. 5. Paper chromatogram of sialic acid fraction. Paper chromatography was performed with solvent A (*left*) and B (*right*) for 24 h on Toyo No. 51 paper by descending method. Sialic acid was detected with the Ehrlich's reagent and alkaline silver nitrate.

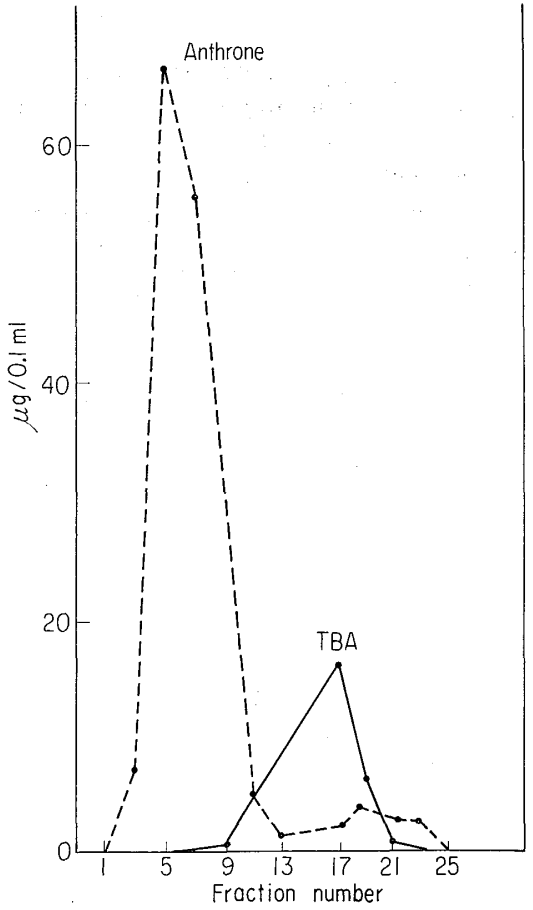

Fig. 6. Gel filtration of the hydrolyzate of the keratosulfate fraction on Sephadex G-10 column. Sample (50 mg) was hydrolyzed in 5 ml of 0.1 N H_2SO_4 at 80° for 1 h, applied on the Sephadex G-10 column (2.7×120 cm) and eluted with water. The flow rate was 20 ml/h. Each 5 ml was collected and screened by the anthrone (- - -) and thiobarbituric acid (——) reaction.

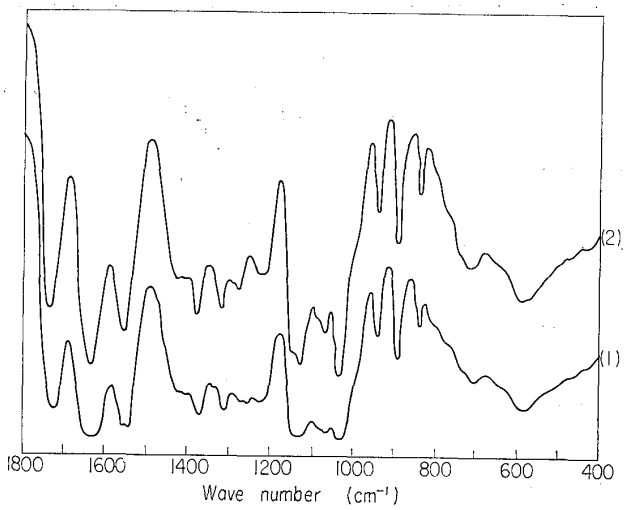

Fig. 7. Infrared spectra of sialic acid fraction (1) and authentic N-acetylneuraminic acid (2). The spectrum was obtained with KBr disk containing 1 mg of sample on a JASCO IR-G instrument. N-acetylneuraminic acid was used after equibrated with water.

(formate form, 1.3×24 cm). Paper chromatograms with solvents A and B are shown in Fig. 5. The R_f values of the sialic acid fraction coincided with those of authentic N-acetylneuraminic acid. No spots corresponding with N-glycolylneuraminic acid nor N, O-diacetylneuraminic acid were detected in both solvents. Furthermore, glycolyl group could not be detected in its spectrum obtained by the method of Klenk and Uhlenbruck[41]. Infrared spectrum (Fig. 6) of the sialic acid fraction which was separated by gel filtration (Fig. 7) was identical with that of N-acetylneuraminic acid equilibrated with water. From these results the kind of sialic acid of the keratosulfate fraction was identified as N-acetylneuraminic acid and not N-glycolylneuraminic acid.

Distribution of sialic acid in cartilage from different sources

Sialic acid in the mucopolysaccharides of whale nasal cartilage: The sialic acid content of whale nasal cartilage determined by the thiobarbituric acid reaction was approximately 0.7%. As shown in Table 1, the content of sialic acid in the chondroitin sulfate fraction was less than 0.6% and that of the keratosulfate fraction was 11.7%. Considering the yield of sialic acid, the latter value accounted for nearly half of the sialic acid originally present in the whale cartilage.

Sialic acid content of various cartilage: The sialic acid was determined on the cartilages from various sources (Table 4). The dry cartilage (30 mg) was hydrolyzed in 5 ml of 0.1*N* H_2SO_4 at 80° for 1 h, purified with a Dowex 1 column and sialic acid was estimated by the resorcinol reaction. The cartilages of whale and cattle (*mammals*) contained more sialic acid than the cartilages of shark and ray (*elasmo-*

Table 4. The contents of sialic acid and hexosamine of cartilages.

	Sialic acid		GN*	GalN*
	(TBA)	(Res)		
Nasal cartilage of adult whale	0.69%	0.96%	2.5%	14.4%
Nasal cartilage of fetal whale	0.68	0.83	1.6	17.4
Nasal cartilage of adult cattle	0.54	0.61	1.8	11.5
Femoro-articular cartilage of adult cattle	0.41	0.32	2.1	2.7
Cranial cartilage of adult shark	0.11	0.08	1.2	14.6
Cranial cartilage of adult ray	0.18	0.15	0.8	9.0
Cranial cartilage of adult squid	0.02	0.03	0.3	5.2

* These values are quoted from "Biochemistry and Medicine of Mucopolysaccharides" p. 29 (1962) by K. Anno and N. Seno.

blanches) and the cartilage of squid (*cephalopod*) had the least sialic acid. It seemed that the sialic acid contents are parallel with the glucosamine contents of the cartilages as shown in Table 4.

Association of sialic acid with whale keratosulfate fraction

In order to examine whether the sialic acid links to carbohydrate moiety in the keratosulfate fraction or to the peptide portion, following experiments were performed.

(1) The effects of further pronase digestion on the keratosulfate fraction was examined. If the sialic acid is present in carbohydrate moiety, its content will not decrease in the reisolated keratosulfate fraction after pronase digestion, but if in peptide moiety, it will decrease in the reisolated keratosulfate fraction after this treatment. The analytical data before and after pronase digestion shown in Table 5 indicated that the protein content decreased from 11% to 9%, but no decrease of sialic acid was observed. The results suggested that the sialic acid did not link to the peptide portion which was digested with pronase.

(2) The digestion of the keratosulfate fraction with neuraminidase followed with pronase was performed. The released sialic acid was determined at intervals by the thiobarbituric acid method except preliminary acid hydrolysis. As shown in Fig. 8, 91% of total sialic acid of the keratosulfate fraction was released after 24 h and 92% after

Table 5. Analytical values after enzymatic treatments of the keratosulfate fraction.

	Before	Pronase	Neuraminidase-Pronase
SA (TBA)	11.7%	12.1%	0.7%
(Res)	14.0	12.9	——
Lowry protein	11.4	9.1	9.7
GN	19.0	19.4	23.9
GalN	3.7	3.3	3.5
GalN/GN	0.19	0.17	0.15
Gal	25.7	29.0	32.1

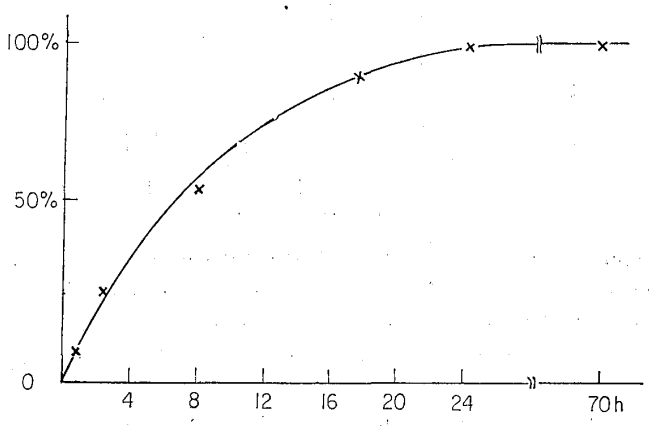

Fig. 8. Release of sialic acid by neuranimidase. Keratosulfate was digested with neuraminidase from *Cl. perfringence* as described in the text. The released sialic acid was determined by the thiobarbituric acid reaction without preliminary hydrolysis.

70 h. After neuraminidase-pronase digestion the keratosulfate fraction was precipitated with 50–65% ethanol concentration and then the precipitate was further purified by passing through a Sephadex G-50 column. The elution curves were shown in Fig. 9 before and after the treatment. As shown in Table 5, the reisolated keratosulfate fraction was almost free from sialic acid. Even after neuraminidase digestion, however, pronase had little effect on the keratosulfate fraction. From above results that it was readily released with neuraminidase, the sialic acid seemed to link to non-reducing end of keratosulfate with α-ketosidic linkage.

(3) The keratosulfate fraction was treated with 0.5*N* NaOH at 4° for 19 h and at 25° for 48 h. The sialic acid content together with amino acid content of reisolated keratosulfate after this treatment is shown in Table 6. The amino acid contents decreased from 11.4% to 10.0% at 4° and to 6.0% at 25°, though the sialic acid did not decrease in both treatments. Therefore, the peptide moiety which was elimi-

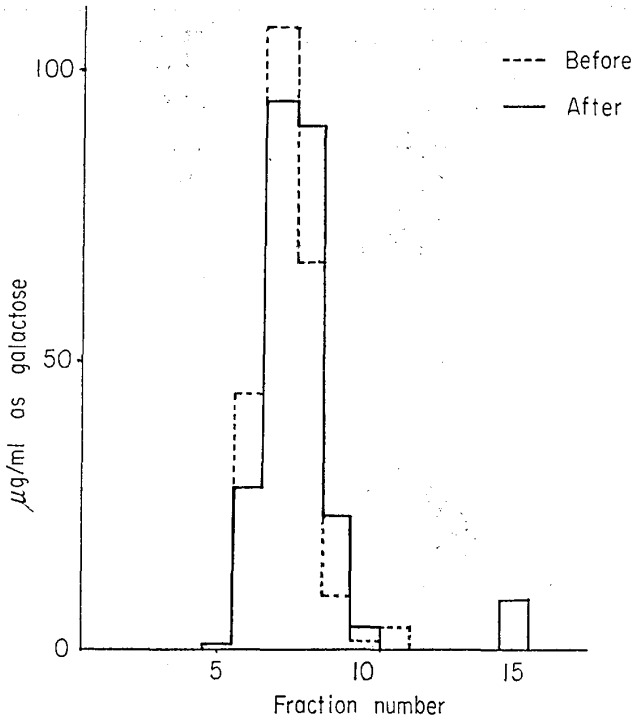

Fig. 9. Gel filtration of keratosulfate before and after neuraminidase-pronase digestion on Sephadex G-50 column. Sample (2 mg) was applied on the Sephadex G-50 column (0.6×97 cm) and eluted with water at the flow rate, 4.7 ml/h. Each 2 ml was collected and screened by the anthrone reaction. *Dashed line*, untreated keratosulfate; *solid line*, keratosulfate precipitated with ethanol after neuraminidase-pronase digestion.

Table 6. The contents of sialic acid and amino acids before and after alkali treatment (0.5*N* NaOH)

	Before	4°, 19h	25°, 48h
SA (TBA)	11.7%	13.4%	11.7%
(Res)	14.0	14.6	14.1
Amino acids	11.4	10.0	6.0

nated with alkali may not contain sialic acid, and it was considered from the results that the sialic acid might link to non-reducing end of carbohydrate chain of the keratosulfate fraction.

Effect of alkali treatment —Carbohydrate-peptide linkage—

The keratosulfate fraction obtained from whale nasal cartilage contained not only sialic acid but also galactosamine which occupied one sixth of total hexosamine and 11.4% of amino acids, as described above. Then alkali treatment was performed to investigate the relation between these components and the repeating constituents, especially the linkage region between the carbohydrate and peptide. When the keratosulfate fraction was dissolved in 0.5*N* NaOH and left at 25° for

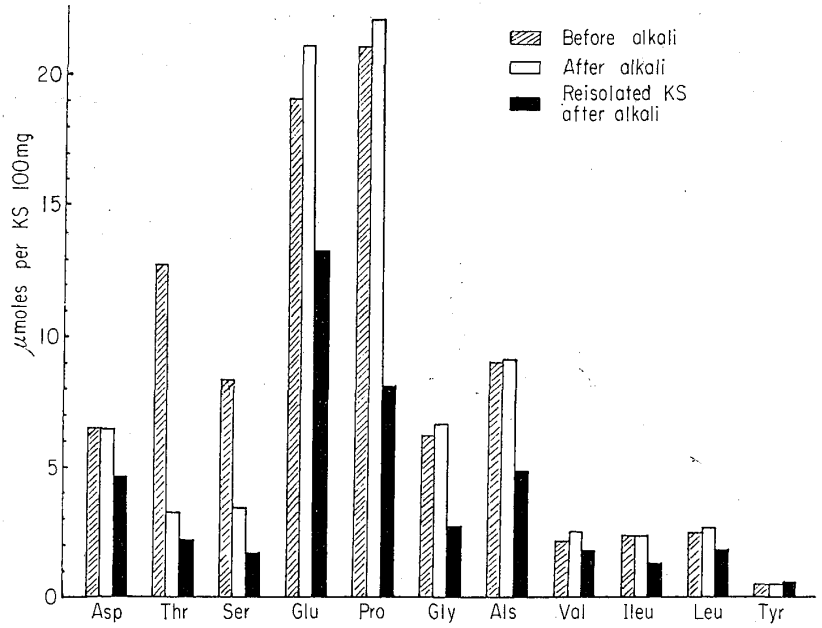

Fig. 10. Changes of amino acid contents of keratosulfate by alkali treatment. *Striped bar,* untreated keratosulfate; *open bar,* keratosulfate treated with 0.5*N* NaOH at 25° for 48 h and analyzed directly for amino acids; *black bar,* keratosulfate reisolated after alkali treatment. Details are described in the text.

48 h, 75% of threonine and 54% of serine decreased but the other amino acids did not decrease (Fig. 10). The treatment at 4° for 19 h resulted in destruction of 20% of threonine and 23% of serine. The degree of destruction at 4° was less than that of 25°. These data could be explained by the β-carbonyl elimination of the carbohydrate moiety from the hydroxyl groups of threonine and serine in the keratosulfate fraction to yield α-aminoacrylic acid from serine and α-aminocrotonic acid from threonine. This hypothesis was confirmed by the formation of alanine and α-aminobutyric acid after 0.5*N* NaOH and 0.3*M* $NaBH_4$ treatment as shown in Table 7. The keratosulfate fraction treated at 25° was reisolated with Sephadex G-50 column. The elution curves given in Fig. 11 showed that the molecular weight of the keratosulfate fraction became a little smaller after alkali treatment.

Table 7. The changes of amino acids after alkali treatment in the presence and absence of sodium borohydride at 25° for 48h.

	Before	NaOH	NaOH-$NaBH_4$
Threonine	12.7	3.2	3.4
Serine	7.3	3.4	3.7
Alanine	8.9	8.9	10.0
α-Aminobutylic acid	—	—	2.7

Figures are expressed as μmoles/100 mg KS.

Then the analyses of the reisolated keratosulfate fraction after alkali were performed. The amino acid contents are shown in Fig. 10. All of the amino acids decreased and the total of them became 6.0% from

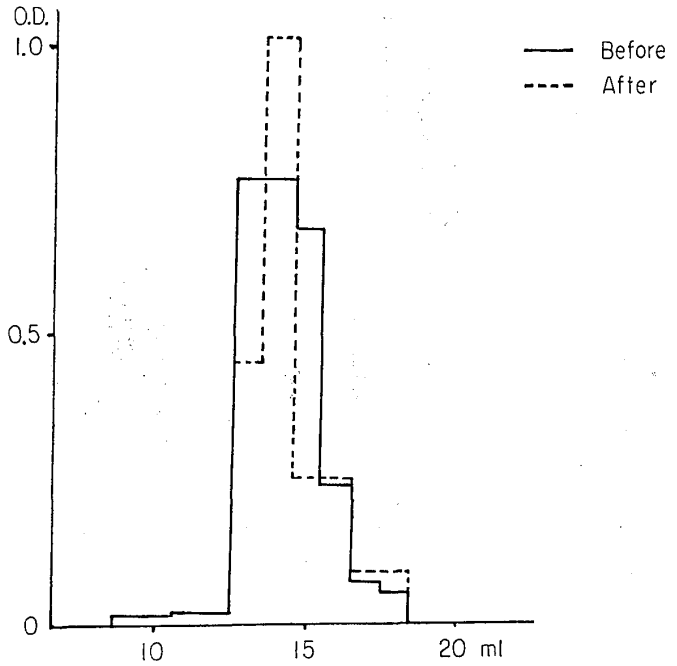

Fig. 11. Gel filtration of keratosulfate before and after alkali treatment on Sephadex G-50 column. Sample (2 mg) was applied on the Sephadex G-50 column (1.3×100 cm) and eluted with water at the flow rate, 5 ml/h. The eluate was screened by the anthrone reaction. *Solid bar*, untreated keratosulfate; *dashed bar*, keratosulfate treated with 0.5*N* NaOH at 25° for 48 h.

Table 8. Analytical values of KS before and after alkali treatment.

	Before treatment	Reisolated KS after treatment	
		4°, 19h	25°, 48h
Yield		70 %	50 %
Gal	25.7%	26.9%	25.3%
HexN	22.7	22.0	21.7
GN	19.0	18.7	20.5
GalN	3.7	3.3	1.2
GalN/GN	0.19	0.17	0.06
SO_4	14.7	12.9	13.7
SA (TBA)	11.7	13.4	11.7
(Res)	14.0	14.6	14.1
Amino acids	11.4	10.0	6.0
Neutral sugars (Molar ratio)			
Gal	100.0		100.0
Man	9.4		8.6
Fuc	3.1		5.3

11.4%. Seventy % of galactosamine was also lost by the treatment at 25°. Sialic acid and neutral sugars did not decrease (Table 8). These facts were further confirmed on the 1.5*M* NaCl fraction from Dowex 1; the decrease of threonine, serine and galactosamine were observed after alkali treatment (Fig. 12 and Table 9).

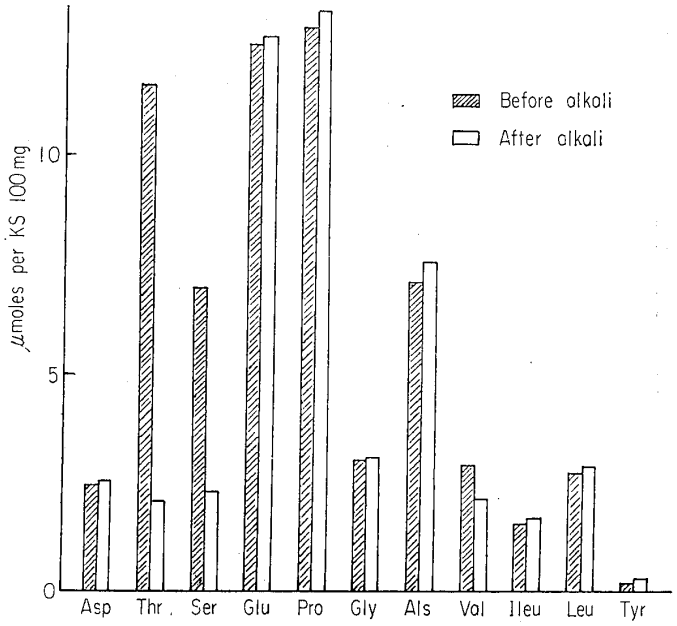

Fig. 12. Changes of amino acid contents of 1.5*M* NaCl fraction by alkali treatment. *Striped bar,* untreated keratosulfate; *open bar,* 1.5*M* NaCl fraction treated with 0.5*N* NaOH at 25° for 48 h.

Table 9. Analytical values of 1.5*M* NaCl fraction before and after alkali treatment (0.5*N* NaOH 25°, 48h).

	before treatment	after treatment
Yield		72%
Gal	20.7%	24.2%
HexN	22.8	20.9
GN	18.1	18.9
GalN	4.7	2.0
GalN/GN	0.24	0.10
SO_4	13.1	15.6
SA (TBA)	12.0	13.3
(Res)	12.7	13.8
Amino acids	8.8	6.7
Neutral sugars (Molar ratio)		
Gal	100.0	100.0
Man	9.1	9.9
Fuc	1.6	2.3

The keratosulfate fraction reisolated after alkali treatment gave color by the modified Morgan-Elson reaction. The spectrum was identical with that of N-acetylglucosamine and N-acetylgalactosamine on the normal Morgan-Elson reaction as shown in Fig. 13. On the other hand, the keratosulfate fraction before alkali treatment did not give color with this modified reaction (Table 10). Considering the

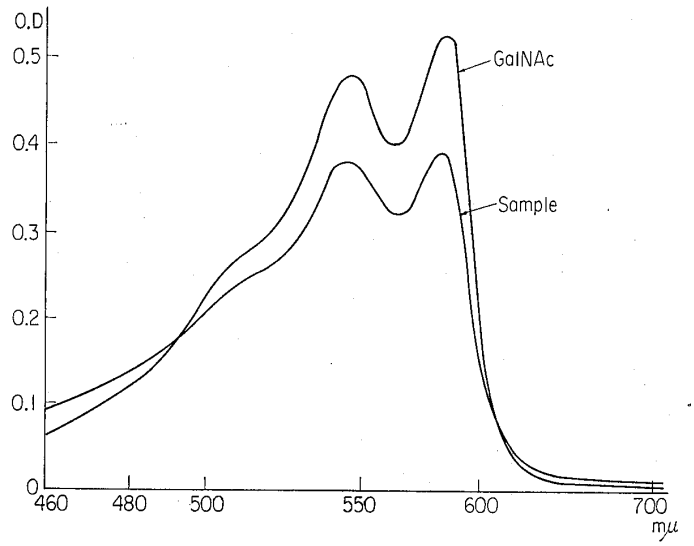

Fig. 13. Absorption spectra of the keratosulfate fraction after alkali treatment by the modified Morgan-Elson reaction and of N-acetylgalactosamine by the Morgan-Elson reaction. The modification consisted in not heating the sample after the addition of potassium tetraborate. Standard solution contained 85.4 μg of N-acetylgalactosamine and the sample, 2230 μg.

Table 10. The modified Morgan-Elson and the Morgan-Elson reaction of the keratosulfate fraction after alkali treatment (0.5*N* NaOH, 48h).

	modified Morgan-Elson			Morgan-Elson	
	Before	4°	25°	Before	25°
as GalNAc	0.2%	0.7	2.0	0.2	2.0

chromogen formation and the loss of galactosamine in the keratosulfate fraction after alkali treatment, it was presumed that N-acetylgalactosamine linked to threonine and serine, and that N-acetylgalactosamine was converted to Kuhn's chromogen by β-elimination. The time course of the formation of chromogen and of the loss of threonine and serine are shown in Fig. 14. The chromogen gave maximum amount after 24 h and decreased thereafter. The destruction of threonine and serine reached nearly maximum after 48 h. As shown in Table 11 the amounts of chromogen formed were parallel with the amounts of hydroxyamino acids destroyed after 24 h and 48 h.

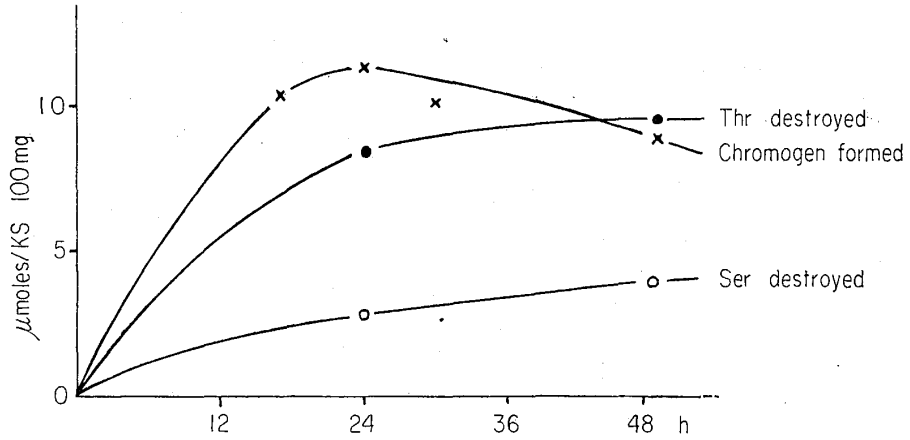

Fig. 14. Destruction of hydroxyamino acids and formation of direct Ehrlich's chromogen by alkali treatment of keratosulfate.

Table 11. Changes of galactosamine and hydroxyamino acids with alkali.

	Chromogen formed	GalN destroyed	Hydroxy-amino acids destroyed	Thr destroyed	Ser destroyed
24h	11.3	14.1	11.2	8.4	2.8
48h	9.6	15.1	13.4	9.5	3.9

Figures are expressed as μmoles/100 mg KS.

Table 12. Changes of hexosamine contents with alkali (0.5*N* NaOH, 25°, 24h).

Fr.		Before alkali	After alkali			
				loss of original	amount lost	chromogen formed
		%	%	%	μmoles/KS 100 mg	
1.0*M* NaCl	GN	16.1	17.9	+11		
	GalN	4.8	1.5	−69	18.5	14.6
	GalN/GN	0.30	0.08			
1.5*M* NaCl	GN	18.1	17.7	− 2		
	GalN	4.7	2.2	−53	13.9	11.3
	GalN/GN	0.24	0.12			
2.0*M* NaCl	GN	19.3	17.6	− 9		
	GalN	3.0	1.1	−63	11.5	10.9
	GalN/GN	0.15	0.06			
3.0*M* NaCl	GN	19.3	18.1	− 6		
	GalN	1.6	0.5	−69	6.2	8.4
	GalN/GN	0.08	0.03			

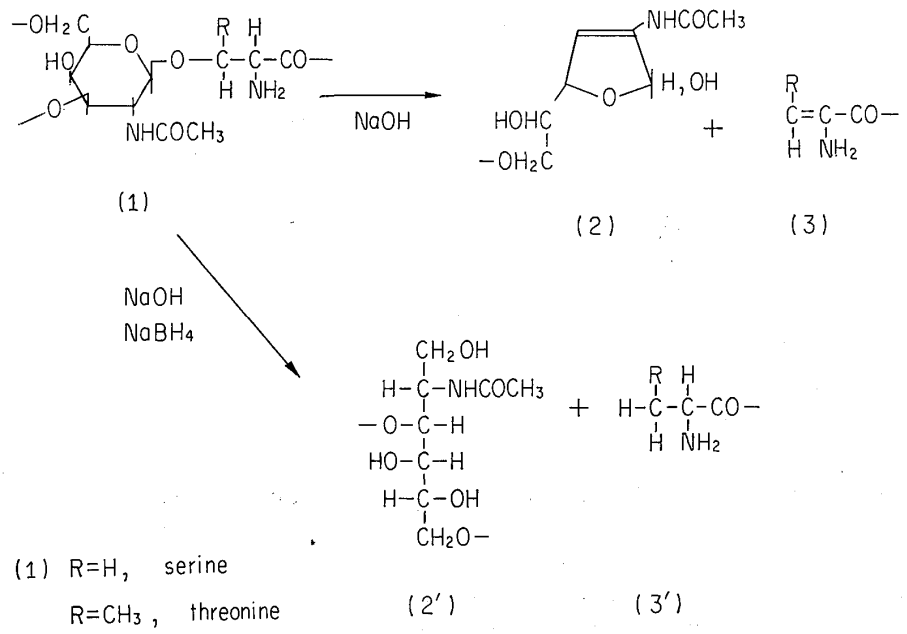

Fig. 15. Schema of alkaline elimination of the linkage region of keratosulfate.

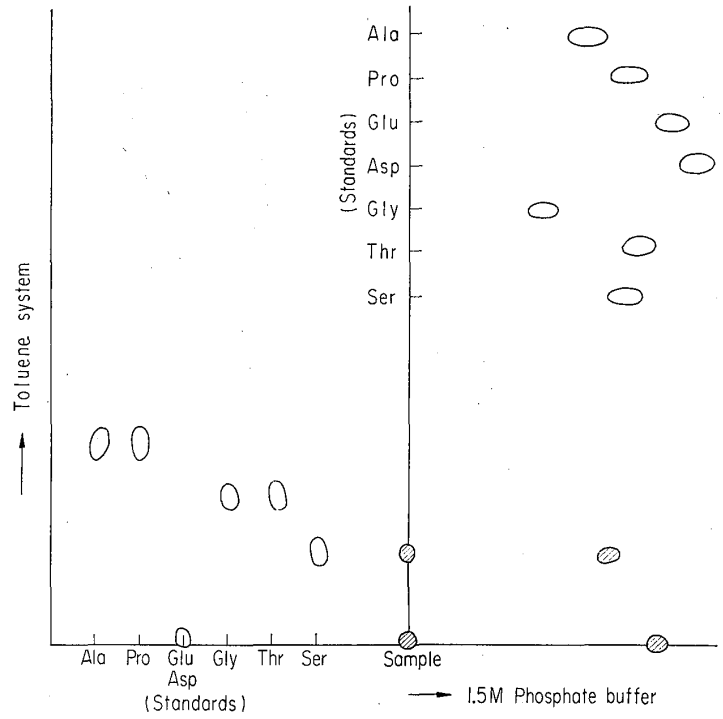

Fig. 16. Paper chromatogram of DNP-amino acids of the keratosulfate fraction. Two dimensional paper chromatography was performed with a toluene solvent system and 1.5*M* phosphate buffer, pH 6.0. DNP-amino acids were detected under ultraviolet lamp.

Alkali treatment was further performed on the four fractions obtained by Dowex 1 chromatography. The changes of the amounts of hexosamines before and after alkali treatment are listed on Table 12. All the fractions showed the considerable loss of galactosamine but little change of glucosamine. All the fractions also gave color by the modified Morgan-Elson reaction as shown in Table 12. The results indicate that all the fractions contain the linkage between hydroxy-amino acids and N-acetylgalactosamine (See Fig. 15).

NH_2-terminal amino acids

The paper chromatogram of DNP-amino acids is shown in Fig. 16. The DNP-amino acids obtained from the DNP-keratosulfate fraction were identified as DNP-glutamic acid and DNP-serine.

Discussions

Mucopolysaccharides separated from whale nasal cartilage was fractionated with ethanol on cellulose column and purified keratosulfate was obtained in good yield. The glucuronic acid content was less than 1%, so the contamination of chondroitin sulfate seemed to be negligible. The keratosulfate gave only one spot having slower mobility than that of chondroitin sulfate by electrophoresis and no spot corresponding to chondroitin sulfate was detectable.

It was reported that skeletal keratosulfates varied in the contents of sulfate, sialic acid, methylpentose and amino acids and in the ratio of galactosamine to total hexosamine[13,14]. The keratosulfate prepared from whale nasal cartilage was also oversulfated, having the ratio of sulfate to hexosamine 1.2. Its galactosamine content was one sixth of the total hexosamine. It contained about 10% of sialic acid, 10% of amino acids and small amounts of neutral sugars other than galactose. These neutral sugars were identified by gas-liquid chromatography to be mannose and fucose.

Since the presence of oversulfated keratosulfate could be considered from the ratio of sulfate to hexosamine, fractionation on Dowex 1 was performed. The keratosulfate was fractionated into at least four fractions whose ratio of sulfate to hexosamine varied from 0.75 to 1.8. Galactosamine, sialic acid and neutral sugars other than galactose showed a tendency to be plenty in the less-sulfated fraction. The protein contents of four fractions were almost similar. But the contamination of glycoproteins was not taken into consideration, because the decrease of protein accompanied no decrease of these components after alkali or enzymatic treatment.

The infrared spectrum of the keratosulfate showed only the bands corresponding to the equatorial ester sulfate groups. From the partial

acid hydrolyzate of the keratosulfate, the spot corresponding to galactose 6-sulfate in addition to N-acetylglucosamine 6-sulfate was detected by electrophoresis and by paper chromatography. The eluate corresponding to the spot of galactose 6-sulfate was hydrolyzed to give only galactose. Therefore, the oversulfated keratosulfate from whale cartilage seemed to contain both N-acetylglucosamine 6-sulfate and galactose 6-sulfate.

The content of sialic acid in whale nasal cartilage was approximately 0.7%. After fractionation on the cellulose column, the chondroitin sulfate fraction contained only a little sialic acid less than 0.6%. In contrast, the keratosulfate fraction contained as much as 11.7%. It seemed interesting that nearly half of the sialic acid in whale cartilage was contained in the keratosulfate fraction. As the sialic acid content of the keratosulfate was remarkably high in contrast with that of the corneal keratosulfate (less than 1%), its separation was carried out by the method of Svennerholm and by gel filtration on Sephadex G-10. The isolated sialic acid was identified as N-acetylneuraminic acid by absorption spectra and paper chromatography, and no glycolyl group was detectable. Sialic acids are found widely distributed throughout the animal kingdom, especially as components of glycoproteins present in biological fluids. It has been known that N-acetylneuraminic acid and N-glycolylneuraminic acid exist together in many cases[45,46]. Nakagawa *et al.*[47] isolated N-acetylneuraminic acid directly from whale nasal cartilage and crystallized. They also reported that no N-glycolylneuraminic acid was detectable. Our results consisted with them.

The sialic acid content was not decreased by pronase digestion nor by alkali treatment though the protein content was decreased, especially by alkali treatment to half of the original. Therefore the sialic acids were assumed to be attached not to the released protein moiety but to the remaining carbohydrate moiety. In addition, the fact that almost all of sialic acids were easily released by neuraminidase showed that they were linked to non-reducing end of carbohydrate chains and/or side chains[48]. It was further confirmed by the elution curves on gel filtration before and after neuraminidase-pronase digestion in Fig. 9, which indicated no occurrence of considerable degradation but of cleavage to a little lower molecular weight compound, and its molecular weight was roughly estimated as about 10,000. Anderson[49] reported the relationship between sialic acid and the chondromucoprotein in human cartilage by the methods of acid and alkali treatments and neuraminidase digestion. By alkali treatment (0.5*N* KOH, at 25°) bound sialic acid was liberated in a diffusible form. The sialic acid was susceptible to neuraminidase action and only after digestion, free sialic acid could be detectable by the thiobarbituric acid reaction. He

proposed a hypothesis that the protein component of the chondromucoprotein molecule was a sialoprotein. In the case of the keratosulfate of whale, however, the sialic acid still remained in the keratosulfate fraction after alkali treatment, therefore it was assumed that sialic acid might be attached to the carbohydrate moiety of the keratosulfate.

The study of the linkage region between carbohydrate and peptide of the keratosulfate fraction was performed by alkali treatment. The treatment of keratosulfate with 0.5*N* NaOH brought about the decrease of threonine and serine and the treatment with 0.5*N* NaOH and 0.3*M* $NaBH_4$ the decrease of threonine and serine accompanied by the formation of α-aminobutyric acid and alanine, which suggested that threonine and serine were included in the linkage region. After the treatment with 0.5*N* NaOH, the keratosulfate gave color directly with *p*-dimethylaminobenzaldehyde reagent in the Morgan-Elson reaction without heating, which indicated that Kuhn's chromogen was formed at the reducing end. It suggested that N-acetylhexosamine was involved in the linkage region of carbohydrate moiety, that is, N-acetylhexosamine was linked to the hydroxyl groups of threonine and serine *via* the O-glycosidic bonds. By β-elimination with alkali, transformation of N-acetylhexosamine to Kuhn's chromogen and that of threonine and of serine to α-aminocrotonic acid and α-aminoacrylic acid respectively occurred. In the presence of $NaBH_4$, α-aminocrotonic acid was reduced to α-aminobutylic acid and α-aminoacrylic acid to alanine. The scheme is shown in Fig. 15. When alkali treated keratosulfate was heated with borate buffer and then added *p*-dimethylaminobenzaldehyde reagent, the color yield was the same as that obtained without heating. It suggested that N-acetylhexosamine at the reducing end of carbohydrate moiety by alkali treatment was all changed to the chromogen and did not remain as N-acetylhexosamine. The chromogen formed with alkali was recovered in polymer keratosulfate fraction by Sephadex G-25, therefore it seemed still to attach itself to the keratosulfate by the alkali-stable linkage. As it was reported that the substituent at C-3 was facile in alkali[50], N-acetylhexosamine might be linked at C-6. After alkali treatment, the decrease of galactosamine was remarkable, whereas the decrease of glucosamine seemed to be insignificant, which suggested that N-acetylhexosamine in the linkage may be N-acetylgalactosamine and not N-acetylglucosamine. In order to confirm this suggestion, further studies are necessary, for example, the identification of N-acetylgalactosaminitol produced by alkaline reductive treatment. For this purpose the separation and identification of N-acetylgalactosaminitol, N-acetylglucosaminitol, N-acetylgalactosamine and N-acetylglucosamine using gas-liquid chromatography have been examined but have not succeeded yet. In the Morgan-Elson

reaction the color yield of N-acetylgalactosamine differs from that of N-acetylglucosamine and is affected by the position of substituent, therefore the estimation of chromogen is difficult. In the present study, the color yield was estimated on the basis of that of N-acetylgalactosamine, which seemed proper because the decreased amount of galactosamine was nearly equal to that of threonine and serine. The cleavage of linkage region in chondroitin 4-sulfate sufficiently occurred with 0.5*N* NaOH at 4°, while most of the cleavage in whale keratosulfate occurred at 25°. It seemed to be the influence of substituents on the amino and carboxyl groups of the hydroxyamino acid residues[52] in the linkage region, that is, both the amino and carboxyl groups of serine in chondroitin 4-sulfate were substituted[51]. On the other hand, amino-terminal of serine in whale keratosulfate was free by the fact that DNP-serine was obtained from DNP-whale keratosulfate. DNP-glutamic acid was identified besides DNP-serine. It might mean that whale keratosulfate would further contain the same linkage region as corneal keratosulfate which would include glutamine or glutamic acid[14].

Summary

From whale nasal cartilage, keratosulfate barium salt was obtained in good yield by ethanol fractionation on cellulose column. The keratosulfate was further fractionated by anion exchange (Dowex 1) column chromatography. All the fractions obtained from the Dowex 1 column contained the various amounts of galactosamine, sialic acid, mannose, fucose and protein, in addition to the constituents; glucosamine, galactose and sulfate. The ratios of sulfate to hexosamine in each fraction differed from 0.75 to 1.8. As sugar sulfates, galactose 6-sulfate other than N-acetylglucosamine 6-sulfate was detected in partial acid hydrolyzate by electrophoresis and by paper chromatography.

The high content (11.7%) of sialic acid was remarkable in contrast with corneal keratosulfate in which the content of sialic acid was less than 1%. The sialic acid was isolated from whale cartilage keratosulfate and identified to be N-acetylneuraminic acid by paper chromatography, color reactions and infrared spectrum. The results of pronase and alkali treatments suggest that the sialic acid may be present in the carbohydrate moiety and not in the alkali labile peptide moiety of the keratosulfate. The sialic acid was easily released by neuraminidase digestion, which indicates that the sialic acid attaches to the non-reducing end of carbohydrate. In connection with sialic acid in cartilage, the sialic acid contents of cartilages from various sources were determined.

The linkage between carbohydrate and peptide in the keratosulfate fraction was investigated by the action of alkali in the presence and

absence of sodium borohydride. N-acetylhexosamine seemed to link to threonine and serine, and N-acetylhexosamine was assumed to be N-acetylgalactosamine.

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