A New Neuraminic Acid Derivative and Three Types of Glycopeptides Isolated from the Cuvierian Tubules of the Sea Cucumber *Holothuria forskali*

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The Cuvierian tubules of *Holothuria forskali* Della Chiaje, a sea cucumber found in the Adriatic Sea, were investigated with regard to their carbohydrate moieties. From a Pronase digest of these tubules three types of carbohydrate units were isolated and characterized. 1. A high-molecular-weight glycopeptide fraction was shown to contain sulphated polyfucose, galactosamine, a uronic acid and a previously unknown neuraminic acid derivative. The sulphate was shown by i.r. analysis to be present as an *O*-ester. The carbohydrate unit was linked *O*-glycosidically to threonine and serine residues in the polypeptide chain. The hitherto unknown neuraminic acid derivative (Hf-neuraminic acid) was resistant to enzymic cleavage by neuraminidase, even after mild alkaline hydrolysis for the removal of *O*-acyl residues. However, the glycosidic linkage of this compound to the other part of the carbohydrate moiety was readily cleaved by mild acid hydrolysis. Its chromatographic properties distinguished Hf-neuraminic acid from other known neuraminic acid derivatives (*N*-acetyl-, *NO*-diacetyl-, *NOO*-triacetyl- and *N*-glycollyl-neuraminic acid). Further, this acidic sugar was shown to possess neuraminic acid as its basic structure. Thus, an as yet unknown substituent lends the distinct properties to Hf-neuraminic acid. 2. The carbohydrate composition of a second glycopeptide fraction consisting of a derivative of neuraminic acid, galactose, mannose and glucosamine was similar to that of the well-known carbohydrate groups of the globular glycoproteins. 3. The third fraction contained two glycopeptides containing the disaccharide, glucosylgalactose, which was shown to be linked to the hydroxyl group of hydroxylysine residues of a collagen-like protein. Approximately half of these residues were glycosylated. In addition to these glycopeptides, a small amount of a third glycopeptide that carried only a galactosyl residue was detected. The amino acid sequence of the two major compounds were found to be Gly-Ala-Hyl*-Gly-Ser and Gly-Pro-Hyl*-Gly-Asp, where Hyl* represents a glycosylated amino acid residue.

The sea cucumber *Holothuria forskali*, like other members of the class of the Holothurioidea (phylum Echinodermata), can eject tubules from its Cuvierian system (Müller *et al.*, 1970) for its defence. The Cuvierian organ, its tubules, morphology and function have been described in detail by Müller *et al.* (1970). The overall goal of the present investigation is the detailed characterization of the Cuvierian tubules, particularly their striking capability to adhere to almost any object (Müller *et al.*, 1972). In the present paper, an extensive study of the carbohydrate moieties of these tubules, of which very little is known, is described.

Experimental

General methods

Collection of the sea cucumbers (Holothuria forskali Della Chiaje) and the Cuvierian tubules. Holothuria

forskali Della Chiaje were collected at the coast of Rovinj, Istria, Yugoslavia, and stored in sea water until used (Müller *et al.*, 1972). In this biotope, there are three species of *Holothuria* which were identified by the characteristic spicules of their carcasses. Only *Holothuria forskali* Della Chiaje were used for the present study. The Cuvierian tubules were obtained as described (Müller *et al.*, 1972) except that the ejected tubules were collected in a vessel filled with propan-2-ol. The tubules were completely dehydrated by several changes of this solvent and finally with diethyl ether and then dried.

Pronase digestion of the Cuvierian tubules. Dried Cuvierian tubules (5.2g) were cut into small pieces (0.5–1.0cm) and allowed to swell at room temperature in 500ml of water containing a few drops of toluene. The suspension was continuously shaken, and after 4h the water was decanted to remove the extracted salt and other soluble constituents. This process was repeated and the swollen tubules were then suspended

in 200 ml of water, homogenized and centrifuged at 5000 rev./min for 20 min. The resulting viscous supernatant solution and precipitate were freeze-dried, giving 0.6 and 3.5 g respectively.

The freeze-dried precipitate (3.3 g) was suspended in 400 ml of water containing 1 drop of toluene and digested with Pronase (Kaken Kagaku Co., Tokyo, Japan; 70000 p.u.k./g at pH 8.0 and 37°C). The pH was maintained constant by addition of saturated Ba(OH)₂ with the aid of a Radiometer pH-stat (model TTT1c). Initially 30 mg of the enzyme was added followed by further 60 mg given in three batches after intervals of 2 h. After 4 h the viscosity of the solution was considerably decreased, and after 20 h it was negligible, and a clear solution was obtained. After 30 h the pH of the digest was lowered to 6 by addition of 0.1 M-H₂SO₄. The BaSO₄ precipitate was removed by centrifuging, and washed with water. The supernatant solution and washings were combined and freeze-dried, yielding 3.3 g.

Isolation of the glycopeptides. The freeze-dried digest (3.1 g) described above was dissolved in 8 ml of 0.1 M-pyridine acetate buffer, pH 6.0, applied to a Sephadex G-50 column and eluted with the same buffer (Fig. 1). The effluent was monitored for protein content at 280 nm with a LKB Uvicord instrument, for free amino groups by the ninhydrin method (Yemm & Cocking, 1955) and for neutral sugars by the phenol-H₂SO₄ procedure (Hodge & Hofreiter, 1962). The two main glycopeptide fractions (1 and 3) which were obtained were subjected to further separation. Fraction 1 was subfractionated in 0.1 M-pyridine acetate buffer, pH 6.0, by passage through a Sephadex G-100 column (Fig. 2). Four subfractions (1A, 1B, 1C and 1D) were obtained. Fraction 3 (300 mg) was dissolved in 5 ml of 0.1 M-pyridine formate buffer, pH 3.2, applied to an AG-Dowex 50W (X2) column (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and yielded four glycopeptide fractions (3A, 3B, 3C and 3D, Fig. 4). The glycopeptides present in fraction 3D were further fractionated by chromatography on a Dowex 1 (X2) column (Fig. 5), and finally purified by gel filtration through a Sephadex G-25 column (1.0 cm × 113 cm) with water as eluent.

Analytical methods

Neutral sugars. These were determined in the various preparations as follows. The glycoproteins and glycopeptides were hydrolysed (1 M-H₂SO₄, 100°C, 4 h, in an evacuated and sealed tube), the resulting hydrolysates passed through a Dowex 1 (X2; acetate form) column and then a Dowex 50W (X2; H⁺ form) column, and the effluents combined and evaporated (Spiro, 1966). The residues obtained were analysed by g.l.c. (Isemura & Schmid, 1971). The glycopeptides were also analysed by g.l.c. after

methanolysis (i.e., without prior acid hydrolysis) and by paper chromatography.

Hexosamines. These were identified and determined by analysing acid hydrolysates (4 M-HCl, 100°C, 4 h) of the various preparations with a Technicon amino acid autoanalyser.

Fucose. This was determined by g.l.c. as mentioned above, and was further identified by chromatography on a Dowex 1 column (borate form) (Walborg *et al.*, 1965). As control for this and the determinations described above α_1 -acid glycoprotein (Schmid *et al.*, 1962, 1968) was used.

Hf-neuraminic acid. The hitherto unknown neuraminic acid, Hf-neuraminic acid, was measured colorimetrically by the Warren (1959) procedure with *N*-acetylneuraminic acid as standard. For its identification, this acidic sugar was purified by absorption on a Dowex 1 (X4; acetate form) column and elution with 1 M-pyridine acetate buffer, pH 4.5, and subsequently analysed by paper chromatography and t.l.c. in solvents 1, 2, 4 and 5 (see below) and high-voltage electrophoresis at pH 3.6 (see below). Further, the purified Hf-neuraminic acid was methanolysed with 0.5 M-HCl in methanol at 65°C for 16 h, trimethylsilylated and subjected to g.l.c. as indicated above.

Uronic acid. This was determined by the carbazole method, with glucuronolactone as standard (Dische, 1947). To eliminate the interference by neutral sugars and amino acids and to minimize the degradation of uronic acid, the uronic acid-containing fraction was prepared as follows. The glycopeptide fraction was heated in methanolic 1 M-HCl at 65°C for 16 h. After evaporation of the solvent, the residue was dissolved in 0.05 M-KOH. The solution was left for 1 h, and then passed through a column (1 cm × 8 cm) of Dowex 1 (X2; acetate form). The uronic acid was eluted with 1 M-acetic acid. In addition, the presence of uronic acid in the glycopeptide fraction was also demonstrated by high-voltage electrophoresis at pH 6.5 (see below for conditions) of acid hydrolysates (1 M-HCl, 100°C, 2 h) of this fraction.

Amino acid composition. The amino acid compositions of the Pronase digest and the glycopeptides isolated therefrom were determined with the aid of a Technicon or Hitachi Model KLA-3A autoanalyser after hydrolysis under N₂ with redistilled HCl at 108°C in evacuated and sealed tubes for 20 h. The amino acid analyses of the small peptides remaining after subtractive Edman degradation were determined by g.l.c. (Gehrke *et al.*, 1968) on a Tabsorb (Regis, Chicago, Ill., U.S.A.) column. The histidine and arginine contents of these peptides cannot be measured by the latter procedure, and were assessed by the Pauly (Mann & Leone, 1953) and Sakaguchi (Jepson & Smith, 1953) tests, respectively. The presence of thiol groups was determined by the nitroprusside test. Protein-bound tryptophan was measured colorimetrically by the procedure of Graham

et al. (1947). Half-cystine was determined as cysteic acid after oxidation with performic acid (Hirs, 1967). Because of the insolubility of the tubules, a Pronase digest of pooled tubules was used for the latter two analyses.

N-Terminal sequences of peptides. These were established by the subtractive Edman procedure in conjunction with amino acid analyses (Elzinga *et al.*, 1968) and by the direct Edman technique (Iwanaga *et al.*, 1969). The phenylthiohydantoin derivatives of the amino acids resulting from the latter method were identified by t.l.c. (Uniplates, 250 μ m thick, Silica Gel GF, purchased from Analtech, Newark, Del., U.S.A.) in solvents II and III of Brenner *et al.* (1962) and D and E of Edman & Sjöquist (1956).

C-Terminal amino acids. For the determination of these a glycopeptide (about 0.1 μ mol) was incubated with carboxypeptidase A (about 100 μ g) in 0.1 M-N-ethylmorpholine-acetate buffer, pH 8.0, at 37°C for 24 h or with carboxypeptidase C (about 100 μ g) in 0.5 M-pyridine acetate buffer, pH 5.3, for 24 h. The reaction mixtures were analysed by paper chromatography and high-voltage electrophoresis. As control, both the enzyme and glycopeptide were analysed simultaneously. Carboxypeptidase A was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and carboxypeptidase C was a gift from Dr. H. Zuber.

T.l.c. and paper chromatography. Whatman no. 1 and 3 MM paper and the following solvent systems were used: (1) butan-1-ol-acetic acid-water (3:1:1, by vol.), (2) butan-1-ol-pyridine-acetic acid-water (15:10:3:12, by vol.), (3) butan-1-ol-ethanol-water (10:1:2, by vol.), (4) butan-1-ol-propan-1-ol-0.1 M-HCl (1:2:1, by vol.), (5) phenol-water (7:3, v/v), (6) butan-1-ol-pyridine-water (5:3:2, by vol.). Solvents (2) and (6) were used for paper chromatography of the neutral sugars, solvents (1), (2) and (4) for Hf-neuraminic acid, solvents (1) and (3) for amino acids and glycopeptides, and solvent (5) for Hf-neuraminic acid on t.l.c. For the detection on the paper chromatograms of the various constituents, the following reagents were utilized: (1) cadmium-ninhydrin for amino acids and peptides (Dreyer & Bynum, 1967), (2) silver nitrate-NaOH (Trevelyan *et al.*, 1950), diphenylamine-aniline-H₃PO₄ (Bailey & Bourne, 1960), and aniline-hydrogen phthalate (Partridge, 1949) for reducing sugars and neuraminic acids and (3) periodate-thiobarbiturate for neuraminic acids (Anderson, 1966).

High-voltage electrophoresis. This was carried out at 50 V/cm for 1.5 h in a horizontal Savant apparatus with Whatman 3 MM paper and the following buffers: (1) pH 1.8, 1 M-formic acid; (2) pH 3.6, pyridine-acetic acid-water (1:10:89, by vol.); (3) 0.1 M-pyridine adjusted to pH 5.3 with addition of acetic acid; (4) pH 6.5, pyridine-acetic acid-water (25:1:225, by vol.).

Low-voltage electrophoresis. This was carried out on the high-molecular-weight glycopeptide fraction at 15 V/cm for 1 h in a horizontal apparatus on cellulose acetate sheets (6 cm $\times$ 21 cm) in either the pH 1.8 or the pH 3.6 buffer described above. Sodium chondroitin sulphate A was utilized as reference substance. The glycopeptides were detected by the meta-chromatic stain by using 0.06% Toluidine Blue in 0.5% acetic acid (Reinitz, 1953).

Sulphate. This was determined as follows. (a) The glycopeptide was dissolved in water and passed through a column of Dowex 50 (X2; H⁺ form) to remove cations. The effluent and water washings were combined and concentrated under reduced pressure. The resulting residue was hydrolysed with 2 M-HCl (1 ml/mg of compound) at 110°C for 5 h, and the hydrolysate was quantitatively analysed by the benzidine-sulphate method (Dodgson & Spencer, 1953). (b) The glycopeptide was treated with 0.06 M-HCl in methanol (1 ml/mg of compound) at 25°C for 40 h to remove possible O-sulphate (Kantor & Schubert, 1957). The precipitate obtained after addition of ether was washed several times with diethyl ether-methanol (2:1, v/v) and dried. The i.r. spectrum of this precipitate obtained with a KBr disc on a Hitachi model EPI-S₂ spectrophotometer was compared with that of non-treated glycopeptide.

Procedures used for studying the carbohydrate-peptide linkages

β -Elimination. This reaction was done under the following three sets of conditions: (a) 0.5 M-NaOH, 4°C for 40 h; (b) 0.5 M-NaOH, 25°C for 40 h, NaBH₄ added (10 mg/mg of compound); and (c) 0.5 M-NaOH, 4°C for 40 h, NaBH₄ added (10 mg/mg of compound) (low-molecular-weight glycopeptides). After neutralization with 1 M-acetic acid, the samples were desalted by passage through a Sephadex G-25 column equilibrated with 0.1 M-pyridine acetate buffer, pH 6. Appropriate portions of the glycopeptide fractions were freeze-dried and analysed for amino acids and hexosamine. Boric acid was removed as the methyl ester by repeated evaporation with methanol. The neutral sugars were analysed by g.l.c. (Isemura & Schmid, 1971).

Determination of degree of glycosylation of hydroxylysine residues

For this procedure alkaline hydrolysis of the Cuvierian tubules was done with 2 M-NaOH at 100°C for 20 h. Appropriate correction for the degradation of hydroxylysine was applied, leucine being the standard for the amino acid analysis (Spiro, 1969).

Procedures used for studying certain glycosyl linkages

Enzymic removal of neuraminyl residues. This was attempted by incubation with *Clostridium perfringens*

neuraminidase (Cassidy *et al.*, 1965). To a glycopeptide (1.5 μ mol) dissolved in 9.5 ml of 0.1 M-sodium acetate buffer, pH 4.5, neuraminidase (0.1 ml; specific activity 15 as defined by Cassidy *et al.*, 1965) was added and incubated at 40°C. At various periods of

incubation, portions (0.2 ml) of the digest were withdrawn and the liberated neuraminic acid was measured by the periodate–thiobarbiturate reaction (Warren, 1959). α_1 -Acid glycoprotein (Schmid *et al.*, 1962) was used as a control.

Table 1. *Amino acid and carbohydrate composition of the Cuvierian tubules of Holothuria forskali and the four fractions obtained by gel filtration of a Pronase digest of pooled Cuvierian tubules (Fig. 1)*

For experimental details see text. For this study the Cuvierian tubules were extracted with water before analysis. The amino acid composition is given in number of residues/1000 residues. These data and those of the carbohydrate composition are corrected for moisture content of the preparations, except when indicated otherwise. The recovery of fucose, mannose, galactose, glucose, galactosamine, glucosamine and *N*-acetylneuraminic acid of fractions 1 to 4 totalled 102, 96, 96, 100, 99, 103 and 94%, respectively, of the content of these monosaccharides in the whole tubules. The content of uronic acid in the whole tubules could not (N.D.) be determined because of the insolubility of the tubules under conditions used for methanolysis. The total content of amino acids and carbohydrates were not corrected for water taken up during hydrolysis.

	Whole tubules (pooled)	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Hydroxyproline	45	0	23	56	29
Aspartic acid	85	109	128	104	65
Threonine	60	67	50	38	58
Serine	53	49	49	45	42
Glutamic acid	99	90	71	111	88
Proline	77	68	53	126	84
Glycine	247	142	109	335	281
Alanine	97	72	45	77	109
Cystine (half)	35	98	68	16	3
Valine	15	42	29	9	36
Methionine	9	10	5	2	8
Isoleucine	22	40	26	6	24
Leucine	33	42	23	6	33
Tyrosine	14	40	25	2	14
Phenylalanine	15	18	14	2	18
Hydroxylysine	6	0	8	16	4
Lysine	21	51	121	13	22
Histidine	12	15	42	7	10
Arginine	54	43	106	28	70
Tryptophan	2*	4	5	1	2
Fucose	1.00	12	1.0	0.12	0.05
Mannose	0.10	0.40	0.6	0.14	0.05
Galactose	0.65	0.61	1.4	2.9	0.06
Glucose	0.68	0.90	0.9	3.1†	0.06
Galactosamine	0.72	4.7	1.3	0.0	0.49
Glucosamine	0.14	0.36	11.6	0.0	0.00
Hf-Neuraminic acid	0.14	1.5	1.1	0.0	0.00
Uronic acid	N.D.	1.5	0.0	0.0	0.00
Amino acids, total (%)	106	50.5	83.0	102.9	114.2
Carbohydrates, total (%)	3.4	22.0	17.9	6.3	0.7
Total (%)	‡	102.5§	100.9	109.2	114.9
Amount of each fraction, weight %	(100)	8	1	19	72

* An appropriate correction for the content of tryptophan of Pronase was applied.

† Corrected for the glucose content of the Pronase preparation.

‡ Sulphate and ash of the tubules were not determined.

§ This value includes 17% sulphate and 12% ash.

Alkaline hydrolysis of O-acyl groups of Hf-neuraminyl residues of glycopeptides. This was carried out as follows. The glycopeptide was heated in 0.05M- Na_2CO_3 at 100°C for 1 h (Schauer & Faillard, 1968). After cooling, acetic acid was added to the solution to adjust the pH to 4.5, followed by digestion with neuraminidase as described above.

Periodate oxidation. This was done with a large excess of NaIO_4 (molar ratio 1:50) in 0.1M-sodium acetate buffer, pH4.5, at 6°C in the dark (Isemura & Schmid, 1971). After the desired period of oxidation, a twofold molar excess of ethylene glycol over NaIO_4 was added to the reaction mixture followed by NaBH_4 (10mg/mg of compound). After 20h at room temperature, the solution was neutralized with acetic acid and passed through a column (1.2cm $\times$ 5cm) of Dowex 1 (X2; formate form). The effluent and washings were combined and evaporated repeatedly with methanol, and the final residue was hydrolysed in a sealed tube with 1M- H_2SO_4 for carbohydrate analysis by g.l.c.

Results

Chemical composition of the Cuvierian tubules

The amino acid composition of the Cuvierian tubules (Table 1) is very similar to that reported by Watson & Silvester (1959), except for the content of

tryptophan, which had previously not been determined, but differs significantly from that described by Gross (1963), who studied a different species of the *Holothuria forskali*. The relatively high content of glycine, hydroxylysine, proline and hydroxyproline is suggestive of the presence of collagen (Tristram & Smith, 1963). No free thiol groups could be detected.

The carbohydrate composition of the Cuvierian tubules (Table 1) differed from that reported by Watson & Silvester (1959) with regard to the content of the neutral hexoses and fucose. This difference may be explained by the extraction of certain constituents from the tubules before our analysis. Fucose was the only deoxyhexose identified, and no xylose could be detected.

A Pronase digest of pooled Cuvierian tubules yielded on gel filtration two major (nos. 1 and 3) carbohydrate-containing fractions (Fig. 1) whose recoveries were 8 and 18%, respectively, of the total weight. The carbohydrate and amino acid compositions (Table 1) of the four fractions obtained showed the following characteristics. Fraction 1 (Table 1) contained essentially all the fucose, galactosamine and uronic acid and possessed a large percentage of Hf-neuraminic acid. It was devoid of hydroxylysine and hydroxyproline, a result indicating that the collagen of the Cuvierian tubules was completely digested by Pronase paralleled by the mentioned

Fig. 1. Gel filtration through Sephadex G-50 of a Pronase digest of Cuvierian tubules

The column dimensions were 3cm $\times$ 100cm. The elution was performed with 0.1M-pyridine acetate buffer, pH6.0, and 10ml fractions were collected. The flow rate was 6ml/h. Samples of 0.2ml were analysed for neutral sugars (E_{490} , ●) and of 0.6ml for free amino groups (E_{570} , ○). The effluent was also monitored at 280nm for protein (---). The void volume of the column is indicated by the arrow.

Fig. 2. Gel filtration of fraction 1 through Sephadex G-100

The column dimensions were 1 cm $\times$ 60 cm, and 70 mg of the fraction were applied. The effluent, which was monitored for E_{280} (---), was collected as 2 ml fractions at a flow rate of 1.5 ml/h. Samples (0.2 ml) of the effluent were analysed for neutral hexoses (E_{490} , ●) and free amino groups (E_{570} , ○).

disappearance of the viscosity of the extract. Fraction 1 was further distinguished by the presence of 17% sulphate and a sulphur content of 7.0%. The minor fraction 2 (Fig. 1) contained essentially all the glucosamine and a large relative percentage of the lysine, histidine and arginine (Table 1). Fraction 3 contained most of the galactose and glucose of the tubules and had a high content of hydroxylysine, hydroxyproline, glutamic acid, proline and glycine. Fraction 4, whose carbohydrate content was very low, consisted of peptides and free amino acids, the latter being identifiable directly by g.l.c.

Isolation from fraction 1 of high-molecular-weight glycopeptides and their partial characterization

Fractionation of fraction 1 by gel filtration at pH 6.0 (Fig. 2) yielded four subfractions (1A, 1B, 1C and 1D) whose recoveries were 64, 4, 10 and 25%, respectively. The amount of fraction 1B was too small to be studied further, and fraction 1D, which was composed of low-molecular-weight carbohydrate-free peptides, was also not further investigated.

Fraction 1A, which consisted primarily of high-molecular-weight (3S) constituents, contained essentially all the fucose, glucose, galactosamine (recovery 96%), uronic acid and sulphate of fraction 1, but was devoid of mannose (Table 2). The high content of fucose and of sulphate (8.5% sulphur) was striking.

The molar proportions of fucose to sulphate to galactosamine were approx. 3:6:1. These results together with the elution characteristics on gel filtration suggested the presence of polyfucose-sulphate-protein complexes in the tubules (see below for subfractions of fraction 1A).

Fraction 1C accounted essentially for the total content of mannose, galactose and glucosamine (Table 2). The recovery of Hf-neuraminic acid of fractions 1A and 1C accounted for 83% of that of fraction 1 or 71% of that of the whole tubules.

Further investigations on the carbohydrate moiety of fraction 1A

Fraction 1A showed metachromasia with Toluidine Blue similar to that of acid mucopolysaccharides. On low-voltage electrophoresis at pH 3.6 (Fig. 3), this fraction appeared monodisperse, a single band migrating towards the anode slightly faster than (sodium) chondroitin sulphate A. At pH 1.8, however, two components were observed, the major one of which exhibited an electrophoretic mobility like that of chondroitin sulphate A whereas the minor component moved slightly faster.

For the i.r. analysis the reduced and carboxymethylated forms (RCM-1A) of fraction 1A were used to minimize the influence of the peptide moiety of the glycopeptide. [After reduction and carboxy-

methylation of fraction 1A, the glycopeptide (RCM-1A) was separated from the peptide(s) by gel filtration through a Sephadex G-25 column.] The spectrum of this compound showed absorption bands at 820, 840 and 1230–1250 cm^{-1} characteristic of axial and equatorial sulphate esters (Kawai *et al.*, 1966). After desulphation of this fraction in methanolic hydrogen chloride (Kantor & Schubert, 1957), these bands largely disappeared, confirming the

presence of sulphate as an *O*-ester in fraction RCM-1A, and hence clearly indicating that the polysaccharide moiety of fraction 1A contained sulphate ester groups.

To elucidate the type of linkage between the carbohydrate and polypeptide moieties of the glycopeptides of fraction 1A, fraction RCM-1A was subjected to β -elimination. A comparison of the content of five amino acids of this fraction with those of the same fraction obtained after β -elimination carried out at 4°C in the absence of NaBH_4 under the conditions described above indicated that about 20% of the serine was destroyed, whereas amounts of all other amino acids remained essentially unchanged. In the presence of NaBH_4 and at 25°C, the destruction by the β -elimination reaction of serine and threonine was 26 and 56%, respectively. The content of alanine increased by about 30%, representing the expected amount of hydrogenated 2-aminoacrylic acid (Table 3). A small amount of α -aminobutyric acid was observed, accounting for the hydrogenated 2-aminocrotonic acid (Tanaka *et al.*, 1964). These results suggested that threonine and serine are involved in *O*-glycosidic, alkali-sensitive linkages between carbohydrate and peptide moieties of the glycopeptides of fraction 1A.

Partial characterization of the Hf-neuraminic acid

The Hf-neuraminic acid residue of fraction 1 was not cleaved by neuraminidase even after mild alkaline hydrolysis for the removal of any *O*-acyl residues of the neuraminic acid (Schauer & Faillard, 1968). In a control experiment the sialic acid of α_1 -acid glycoprotein was completely removed. The Hf-neuraminic acid isolated from a mild acid hydrolysate of a Pronase digest of the Cuvierian tubules afforded, with the Warren reagents, a chromogen that had an absorption maximum at the same position as that of *N*-acetylneuraminic acid. On paper chromatography in solvent 4 this acidic sugar

Table 2. *Amino acid and carbohydrate composition of fractions 1A and 1C, the two glycopeptide fractions from fraction 1 (Fig. 3) derived from a Pronase digest of cuvierian tubules of Holothuria forskali*

The amino acid composition is given in number of residues/1000 residues of the fraction investigated. The content of the monosaccharides is expressed in percent of the analysed preparation. N.D. indicates that the tryptophan contents of the two fractions were not determined.

Fraction ...	1A	1C
Hydroxyproline	0	0
Aspartic acid	13	73
Threonine	11	48
Serine	9	40
Glutamic acid	13	48
Proline	13	36
Glycine	23	73
Alanine	11	53
1/2-Cystine	17	51
Valine	6	17
Methionine	2	4
Isoleucine	6	27
Leucine	5	21
Tyrosine	6	21
Phenylalanine	3	9
Hydroxylysine	0	0
Lysine	9	25
Histidine	4	10
Arginine	10	21
Tryptophan	N.D.	N.D.
Total amino acid residues	161	577
Fucose	20	2.8
Mannose	0.0	2.5
Galactose	0.6	4.2
Glucose	1.2	0.0
Galactosamine	7.5	Trace
Glucosamine	Trace	3.4
Hf-Neuraminic acid	1.2	5.3
Uronic acid	2.5	0.8
Total carbohydrate (%)	33	19
Total amino acids (%)	20	70
Sulphate (%)	25	5.3
Ash (%)	20	5
Total (%)	98	99

Table 3. *Effect of β -elimination on the amino acid composition of fraction RCM-1A*

The alkaline hydrolysis was done at 25°C in presence of NaBH_4 .

	Amino acid composition of glycopeptide (mmol/g)	
	Before β -elimination	After β -elimination
Serine	35	26
Threonine	46	25
Alanine	23	29
Aspartic acid	24	23
Glutamic acid	29	29

Fig. 3. Electrophoretogram of fractions 1A and RCM-1A on cellulose acetate

Spots were detected by the metachromatic stain with Toluidine Blue. (a) pH 3.6; (b) pH 1.8. Samples 1 and 3, sodium chondroitin sulphate A; samples 2 and 4, fraction 1A; sample 5, fraction RCM-1A.

Fig. 4. Chromatography on Dowex 50 of fraction 3 (Fig. 1) containing the low-molecular-weight glycopeptides of the Cuvierian tubules of *Holothuria forskali*

Fraction 3 (200mg) was applied to a column (1.8cm $\times$ 70cm) of Dowex 50W (X2) previously equilibrated with 0.1M-pyridine formate buffer, pH 3.2. After being washed with 160ml of the same buffer, a pH-ionic strength gradient using 900ml of this buffer and 1800ml of 2M-pyridine acetate buffer, pH 5.0, was applied at the position indicated by the arrow. Fractions (8.0ml) were collected at a flow rate of 20 ml/h. Samples (0.2 ml) were analysed for neutral sugars (E_{490} , $\bullet$) and for free amino groups (E_{370} , $\circ$). The fraction designated P contained Pronase as judged by the absence of galactose. The four glycopeptide fractions are marked 3A, 3B, 3C and 3D.

Fig. 5. Chromatography on Dowex 1 of fraction 3D (Fig. 4)

The Dowex 1 (X2) column (1.8 cm $\times$ 52 cm) previously equilibrated with 1% *N*-ethylmorpholine acetate buffer, pH 8.0, was washed with the same buffer (140 ml) to recover the non-retained fraction. A linear pH-ionic strength gradient (100 ml of this buffer and 100 ml of 0.2 M-acetic acid) was applied at the position indicated by the arrow. Fractions (4.6 ml) were collected at a flow rate of 10 ml/h. Samples (0.2 ml) were analysed for neutral hexoses (●).

Table 4. Chemical compositions of glycopeptides I and II

Compositions are expressed as molar ratios relative to hydroxylysine (1.00). Amino acids present at less than 0.1 mol/mol of hydroxylysine were not included. Amino acids and carbohydrate were determined by g.l.c., the sugars after methanolysis. The absence of histidine and arginine was established by specific colour tests. Values in parentheses were obtained without hydrolysis before g.l.c.

	Glycopeptide I	Glycopeptide II
Amino acid		
Hydroxylysine	1.00	1.00
Glycine	1.84	1.82
Alanine	0.97	—
Serine	0.86	—
Proline	—	0.92
Aspartic acid	—	0.99
Carbohydrate		
Glucose	0.93 (0.92)	0.96 (0.90)
Galactose	0.85 (0.20)	0.86 (0.22)

(R_F 0.31), after its purification by chromatography, appeared distinct from *N*-acetylneuraminic acid (R_F 0.49), *NO*-diacetylneuraminic acid (R_F 0.61), *NOO*-triacetylneuraminic acid (R_F 0.69), and *N*-glycolylneuraminic acid (R_F 0.37). Comparable differences were also observed on t.l.c. and on high-voltage electrophoresis. Further, t.l.c. in solvent 5 demonstrated that this unknown neuraminic

acid derivative was also different from the neuraminic acid (*N*-acetoglycolyl-4-methyl-4,9-dideoxyneuraminic acid) isolated from the jelly coat of a sea urchin (Hotta & Kurokawa, 1970) and from that (*N*-glycolyl-*O*-acetylneuraminic acid) described by Hakomori & Saito (1969).

After methanolysis effecting cleavage of the *N*-acyl residue, any *O*-acyl residues and subsequent trimethylsilylation and g.l.c., the Hf-neuraminic acid gave a compound that was characterized by retention times on an SE-30 and on an OV-17 column that were essentially identical with those of the neuraminic acid of *N*-acetylneuraminic acid prepared by the same procedure (Isemura & Schmid, 1971). These findings suggest that the basic structure of Hf-neuraminic acid is most likely neuraminic acid and that this acidic sugar carries an unknown substituent(s) which seems to prevent its cleavage by neuramidase. The possible positions of the substituents of neuraminic acid leading to this inhibition have been discussed by Schauer & Faillard (1968) and Brossmer & Holmquist (1971).

Studies on the low-molecular-weight glycopeptides

For the isolation of these glycopeptides, fraction 3 was chromatographed on a Dowex 50 column (Fig. 4) yielding, in addition to many carbohydrate-free peptides, four heterogeneous glycopeptide fractions (3A, 3B, 3C and 3D), whose recoveries in terms of neutral hexoses of fraction 3 were 5, 10, 20 and 30%, respectively. The remaining carbohydrate was found in the fractions eluted later. Chromatography of fraction 3D on Dowex 1 yielded three glycopeptide fractions (Fig. 5), two of which appeared homogeneous as judged by high-voltage electrophoreses at pH 6.5, 3.6 and 1.8. After final purification by gel filtration through a Sephadex G-25 column, these glycopeptides designated I and II accounted for 30 and 36%, respectively, in terms of glucose of fraction 3D and for 3 and 4%, respectively, of the neutral hexoses of the starting material. The amino acid and carbohydrate composition of these two compounds are listed in Table 4. These glycopeptides were free of fucose, Hf-neuraminic acid and hexosamines.

Characterization of glycopeptides I and II

The amino acid sequence of glycopeptide I was established by the subtractive Edman procedure (Table 5) and is described as item 1 of Table 7. The presence of the C-terminal serine was confirmed by digestion with carboxypeptidase C (Zuber, 1968) releasing 0.2 mol of serine/mol of peptide. No glycine was cleaved. Carboxypeptidase A, even at an enzyme/substrate ratio 10-fold higher than normal, did not release any amino acids. The amino acid sequence of glycopeptide II was established (Table 6) and is given as item 2 of Table 7.

Table 5. *Amino acid sequence of glycopeptide I*

Results are expressed in mol of amino acid/mol of glycopeptide.

	Subtractive Edman degradation				Direct Edman (Phenylthiohydantoin- amino acid recovered)
	Gly	Ala	Hyl	Ser	
Composition of glycopeptide	2.13	1.13	1.16	1.00	
Step 1	1.05	1.14	0.23*	1.00	Gly
Step 2	1.06	0.28	0.0	1.00	Ala
Step 3	1.05	0.0	0.0	1.00	†
Step 4	0.60	0.0	0.0	1.00	Gly

* This value is low because of the partial reaction of phenylisothiocyanate with the ϵ -amino group. The resulting ϵ -substituent is not cleaved under the conditions used for the hydrolysis of peptides for amino acid analysis.

† No extractable phenylthiohydantoin-amino acid was obtained at this step. Hence, hydroxylysine was assumed to be the third residue.

Table 6. *Amino acid sequence of glycopeptide II*

Results are expressed in mol of amino acid/mol of glycopeptide.

	Subtractive Edman degradation				Direct Edman (Phenylthiohydantoin- amino acid recovered)
	Gly	Pro	Hyl	Asp	
Composition of glycopeptide	1.83	0.93	1.01	1.00	
Step 1	1.16	0.93	0.69*	1.00	Gly
Step 2	1.02	0.29	0.21	1.00	Pro
Step 3	1.10	0.0	0.0	1.00	†
Step 4	0.54	0.0	0.0	1.00	Gly

* † For explanation see footnotes of Table 5.

The carbohydrate moiety of both glycopeptides was shown by g.l.c. both on OV-17 and SE-30 columns to contain equimolar amounts of glucose and galactose, suggesting that the disaccharide, Glc-Gal, is linked to hydroxylysine as previously found in the study of glycopeptides derived from other collagens (Spiro, 1970). The high stability of the Gal-Hyl linkage towards mild acid hydrolysis, which has been reported for other collagens by Butler & Cunningham (1966) and Spiro (1967*b*), was demonstrated for Glc-Gal-Hyl (Fig. 6). A similar stability was observed when glycopeptides I and II were subjected to methanolysis (0.5M-HCl in methanol, 65°C, 16h). Essentially all glucose was released, whereas only 0.2mol of galactose was liberated (Table 4). Direct evidence for the presence of this Gal-Hyl bond in both glycopeptides was obtained by a study of their alkaline-hydrolysis products. Under the conditions of the β -elimination in the presence of NaBH₄ these two

glycopeptides did not yield any galactitol or glucitol. Thus, the results confirmed not only that this carbohydrate-peptide linkage is stable towards alkali but also that the glucose and galactose of these two glycopeptides are indeed linked as a disaccharide to the hydroxyl group of hydroxylysine. On periodate oxidation of Glc-Gal-Hyl, both sugars were destroyed to an extent of approximately 90%, whereby a (1→3) glycosidic bond could be excluded. Since no threitol was observed on g.l.c. of the oxidation products, a (1→4) linkage could also be excluded. Hence, glucose and galactose were linked either through a (1→2) or (1→6) linkage, agreeing with the reports by Spiro (1967*a*) and Katzman *et al.* (1972). In a further experiment on the isolation of Glc-Gal-Hyl from the Pronase digest, it was noted that some of the fractions eluted last with dilute NH₃ from the Dowex 50 column contained, in addition to Glc-Gal-Hyl, a compound which migrated on high-voltage electrophoresis like

Fig. 6. High-voltage electrophoretogram of Gly-Gal-Hyl and the products derived from it by mild acid hydrolysis

Conditions of electrophoresis were pH 6.5, 50 V/cm, 1 h. Positions 1 and 6, from left to right: glycine, arginine and lysine. Positions 2 and 5: hydroxylysine. Position 3: partial acid hydrolysate of Glc-Gal-Hyl (0.05M-H₂SO₄, 100°C, 14 h; Spiro, 1967b). Position 4: Glc-Gal-Hyl. The line of application is indicated by the arrow. The electrophoretogram was stained with ninhydrin. Under the conditions selected lysine migrated 12 cm.

Gal-Hyl. Relative amounts of Glc-Gal-Hyl and Gal-Hyl in fraction 3 were 95 and 5% as estimated by the hydroxylysine content of the corresponding spot on high-voltage electrophoresis.

Degree of glycosylation of the hydroxylysine residues of the Cuvierian tubules

The number of carbohydrate-carrying hydroxylysine residues per 1000 amino acid residues of Cuvierian tubules was assessed from the total hydroxylysine content (six residues; Table 1) minus that of the free hydroxylysine found after alkaline hydrolysis yielding the number of hydroxylysine residues that are glycosylated. The results indicated that half (48%) of the hydroxylysine residues carried a carbohydrate unit. This value is in good agreement with that (53%) calculated from the hydroxylysine content of the whole tubules and the galactose content (3.2 residues) of fraction 3 assuming that all galactose residues of this fraction are involved in this type of linkage (the galactose of fraction 1 is thought to be located in the core of the heteropolysaccharide units of the glycopeptides of this fraction).

Discussion

The study described on the Cuvierian tubules revealed the presence of at least three types of carbohydrate units attached through three different types of linkages to the proteins. The higher-molecular-weight glycopeptide fraction (fraction 1) had a carbo-

hydrate moiety that was mainly composed of fucose, galactosamine and sulphate in a molar proportion 3:1:6. This highly sulphated polyfucose associated with a peptide had an electrophoretic mobility and metachromasia similar to that of chondroitin sulphate A. Polyfucose (or fucoidan) sulphates have been isolated from the brown seaweeds (Percival & Ross, 1950; O'Neill, 1954; Schweiger, 1962), sea animals such as the sea urchin (Vasseur, 1948) and other species of sea cucumbers (Maki & Hiyama, 1956; Motohiro, 1960). This heteropolysaccharide may perhaps represent a counterpart to the well-known mucopolysaccharides of higher animals (Percival & Ross, 1950; Lash & Whitehouse, 1960; Inoue, 1965; Suzuki *et al.*, 1968; Katzman & Jeanloz, 1969; Obrink & Wasteson, 1971; Brandt & Muir, 1971; Mathews, 1971). These acidic polysaccharide units are linked to the polypeptide chain primarily by *O*-glycosidic bonds involving the hydroxyl groups of serine and threonine. Many acid mucopolysaccharides of higher animals have similar carbohydrate-protein linkages.

The second type of carbohydrate unit (fraction 1C), composed of Hf-neuraminic acid, galactose, glucosamine and mannose, resembles in its chemical composition those of the globular glycoproteins, such as the plasma glycoproteins, which possess the predominant terminal oligosaccharide sequence: NeuNAc-Gal-GlcNAc-Man (Schmid, 1972).

The smallest carbohydrate unit was the disaccharide, glycosylgalactose, which was linked to hydroxylysine. This disaccharide has been reported to be

Table 7. Comparison of the amino acid sequences of glycopeptides derived from invertebrate and vertebrate collagens

Amino acid sequence	Reference
Invertebrate	
-Gly-Ala-Hyl*Gly-Ser-	Present study
-Gly-Pro-Hyl*Gly-Asp-	
-Gly-Ala-Hyl*Gly-Asp-Arg-	Isemura <i>et al.</i> (1972)
-Gly-Glu-Hyl*Gly-Ala-Arg-	
-Gly-Gln-Hyl*Gly-Ala-Arg-	
-Gly-Pro-Hyl*Gly-Ala-Arg-	
Vertebrate	
-Gly-Met-Hyl*Gly-His-Arg-	Cunningham & Ford (1968)
-Gly-Phe-Hyl*Gly-Ile-Arg-	Butler & Cunningham (1966)
-Gly-Ile-Hyl*Gly-Ile-Arg-	
Hyl*Gly-His-Arg-	Common sequence
[-Gly- X -Hyl*Gly- Y -Arg-]	

* To this residue the disaccharide, Glc-Gal, is attached.

present in many collagens (Spiro, 1970; Butler & Cunningham, 1966) and its structure has been studied extensively (Spiro, 1970; Katzman *et al.*, 1972). A second compound, galactosylhydroxylysine, was present in small amounts in contrast to the finding of the high relative percentage of this compound in certain human tissues (Pinnell *et al.*, 1971). None of these three types of carbohydrate units possess detectable amounts of xylose.

The amino acid sequences of the two glycopeptides containing the hydroxylysine-disaccharide structure have been elucidated for the first time in invertebrate collagen. A comparison (Table 7) of these structures with those derived from vertebrate collagens (Morgan *et al.*, 1970) revealed the following noteworthy points. The amino acid sequence of the peptides that possess the hydroxylysine residue carrying the disaccharide all have certain features in common suggesting that this hexapeptide sequence acts as a signal for the glycosylation of the hydroxylysine residues. This sequence further suggests a high degree of specificity for the enzyme that catalyses the hydroxylation of these specific lysine residues. Although relatively less abundant amino acids (isoleucine, methionine and phenylalanine) have been found in the linkage region in certain vertebrate collagens (Morgan *et al.*, 1970), in the present study some of the abundant amino acids (alanine, serine, aspartic acid and proline) were found in these regions of the invertebrate collagen. The present results from

invertebrate collagen are compatible with those derived from bovine renal glomerular basement membrane (Spiro, 1967b) and from lens capsules (Spiro & Fukushi, 1969) in which aspartic acid and glutamic acid were found in the glycopeptides containing hydroxylysine-disaccharide. Preliminary experiments on cuttlefish skin collagen (Isemura *et al.*, 1972) also support this contention.

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