Studies on the Carbohydrate of Collagens

CHARACTERIZATION OF A GLUCURONIC ACID-MANNOSE DISACCHARIDE UNIT FROM NEREIS CUTICLE COLLAGEN

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Treatment of cuticle collagen from Nereis virens (clam worm) with alkaline borohydride at 37°C resulted in the release of 80% of its carbohydrate, indicating the presence of O-glycosidically linked units. The reduced saccharides formed were found to consist of two types: neutral mono-, di-, and trisaccharides of galactose similar to those observed in Lumbricus terrestris (earworm) cuticle collagen (Muir, L., and Lee, Y. C. (1970) J. Biol. Chem. 245, 502-509) and a novel acid disaccharide which was shown to be glucuronosylmannitol.

The acidic carbohydrate unit could also be obtained in high yield as glucuronosylmannose after partial acid hydrolysis of the collagen. Reduction of its methyl ester converted the reduced disaccharide to its glucosylmannitol derivative while studies employing periodate oxidation, methylation, and glycosidase digestion indicated that the structure of the disaccharide was 6-O-α-D-glucuronicyl-D-mannose.

Two glycopeptides containing the glucuronic acid-mannose disaccharide unit were isolated by gel filtration and Dowex 1 chromatography after collagenase and pronase digestions. Alkali and alkaline sulfite treatment of these glycopeptides demonstrated that the disaccharide was O-glycosidically linked to a threonine residue and the amino composition suggested that this attachment was within or adjacent to a characteristic collagen tripeptide sequence.

Comparative studies which we carried out on Nereis (a polychaeta) and Lumbricus (an oligochaeta) indicated that there are differences in the nature as well as the number of O-glycosidically linked carbohydrate units in the cuticle collagens of the two annelids. Lumbricus did not contain the acidic disaccharide but had more of the galactose units and a different distribution among the mono-, di-, and trisaccharides forms of this sugar.

It has been known for some time that most members of the collagens family contain covalently linked carbohydrate and can therefore be considered as glycoproteins (2). The presence of saccharide units in collagens from widely differing sources ranging from sponges to higher vertebrates suggests that the carbohydrate may have important biological roles and focuses attention on its nature and peptide attachment.

Although in most collagens carbohydrate occurs in the form of disaccharides (2-O-α-D-glucosyl-D-galactose) and monosaccharides (D-galactose) O-glycosidically linked to hydroxyl-

EXPERIMENTAL PROCEDURES

Preparation of Nereis Cuticle Collagen - The Nereis virens were dissected at a local beach and collected as described previously [2]. Homogenization of 10-g samples of cuticle was performed at 4°C using a Potter-Elvehem homogenizer. The samples were then washed with distilled water. The collagen was extracted by 0.5 M sodium chloride buffer, pH 7.0, extensively dialyzed against distilled water, and lyophilized.

Preparation of Reduced Oligosaccharides by Alkaline Borohydride Treatment - The collagen solution was adjusted to pH 7.0, incubated for 160,000 g centrifugation, and then incubated with 0.6 M NaOH for 1 h. The protein was precipitated by addition of 0.5 M sodium acetate at pH 5.0 to 0.5 M sodium chloride buffer and dialyzed against sodium acetate buffer, pH 5.0.

Preparation of Reduced Disaccharides by Alkaline Hydroxylamine Treatment - The collagen solutions were dialyzed against 0.075 M sodium chloride buffer, pH 7.0, for 16 h to extract the collagen the residue was dialyzed against distilled water in order to extract the disaccharide was 0.6 M sodium acetate, and then the residue was dialyzed again against distilled water.

Preparation of Glycopeptide - The collagen solution was adjusted to pH 7.0, incubated with 0.6 M NaOH for 1 h, and then incubated with 0.6 M NaOH for 1 h. The protein was precipitated by addition of 0.5 M sodium acetate at pH 5.0 and then dialyzed against sodium acetate buffer, pH 5.0.

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were blochemi for continued wth 0.01 wrh (formate form). After passage of the sample the column was washed with 10 ml of 0.01 M sodium acetate, pH 5.0 and then equilibrated with the same buffer. The enzyme was added Funionally to the column by the carbazole reaction (10) with brief (3 m) periodical pulsation.

Separation of Glycopeptides - The Nereis cuticle collagen was digested with collagenase (10 mg/ml type IV) (Hormon, Stockholm) on the conditions previously described (9). The resulting material was separated by centrifugation and was then followed on a column of Sephadex G-25. The major glucuronic acid fraction (fraction 1) was digested with pronase (100 mg/ml) at 37°C. A total of 20 ml of water was used initially and two further additions were made at 2 h. The fractions were collected at 100 ml and neutralized by 2% pyridine acetate buffer at pH 5.0. The combined effluent and water washes were lyophilized and then applied to a column of Sephadex G-25 in buffers saturated with toluene to prevent bacterial growth.

Composition of Collagen - The earthworm collagen yielded primarily the reduced di- and trisaccharides of galactose, in agreement with work of Muir and Lee (7), where Nereis (clam worm) yielded in addition to these galactosaccharides a slower moving component which after isolation by preparative paper chromatography was identified as glucuronic acid by anion exchange chromatography after acid hydrolysis. No sulfate esters (less than 0.01%) were found in either of the two cuticles.

Characterization of Oligosaccharides Released by Alkaline Borohydride Treatment - Alkaline borohydride liberated several reduced saccharide components from both Nereis and Lumbricus cuticle collagen which were resolved by paper chromatography (Fig. 1). The earthworm collagen yielded primarily the reduced di- and trisaccharides of galactose, in agreement with work of Muir and Lee (7), where Nereis (clam worm) yielded in addition to these galactosaccharides a slower moving component which after isolation by preparative paper chromatography was identified as glucuronic acid by anion exchange chromatography after acid hydrolysis. No sulfate esters (less than 0.01%) were found in either of the two cuticles.

Table 1

| Component     | Nereis | Lumbricus |
|---------------|--------|-----------|
| Galactose      | 12.4%  | 11.9%     |
| Glucuronic Acid| 0.8%   | 12.1%     |
| Mannose        | 4.3%   | 4.4%      |
| Glucosamine    | 1.3%   | 1.4%      |
| Glucose        | 1.2%   | 1.3%      |
| Glucomannan    | 0.9%   | 0.8%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
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| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
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| Mannitol       | 0.3%   | 0.4%      |
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| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
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| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
| Glucose        | 0.3%   | 0.4%      |
| Glucomannan    | 0.3%   | 0.4%      |
| Mannitol       | 0.3%   | 0.4%      |
| Glucosamine    | 0.3%   | 0.4%      |
| Mannose        | 0.3%   | 0.4%      |
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| Glucomannan    | 0.3%   | 0.4%      |
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Nereis by alkaline borohydride treatment of the migrating oligosaccharides produced by alkaline borohydride tose and galactitol in molar rates of

Solvent System was consistent with their identity as galactobiitol and galactotriitol, respectively. Calactitol which was also present in both collagen samples ran off the paper during this period of Chromatography. The glucuronosylmannitol which was also present in both collagen samples ran off the paper during this period of chromatography.

amounts of glucuronic acid and a neutral nonreducing component which migrated to the position of mannitol on paper chromatography (Fig. 2). In contrast, the two more rapidly migrating oligosaccharides produced by alkaline borohydride treatment of the Nereis collagen yielded on hydrolysis galactose and galactitol in molar ratios of 1:1 and 2:1 (Fig. 2) which was consistent with their identity as galactobitol and galactotriitol, respectively.

When the products of alkaline borohydride treatment were separated by borate complex anion exchange chromatography the Nereis collagen demonstrated a peak appearing after the di- and trisaccharides of galactose which was not present in the elution curve from Lumbricus (Fig. 3). The glucuronosylmannitol obtained by preparative paper chromatography emerged from the column in the same position as this additional component.

Quantitation by borate complex anion exchange chromatography indicated that a large portion of the carbohydrate present in both the Nereis and Lumbricus collagens was present in oligosaccharide units released by the mild alkaline borohydride treatment (Table II). In addition to the galactose di- and trisaccharides liberation of some monosaccharide in the form galactitol was observed. Since this reduced monosaccharide could not be measured by the orcinol reagent employed in the borate complex anion exchange chromatography it was analyzed by the periodic acid-chromotropic acid procedure after elution from paper chromatographs. Essentially all of the glucuronic acid and most of the mannose (86%) present in Nereis collagen was recovered in the acidic disaccharide while the various released galactose-containing units accounted for 92% of the galactose present in the intact protein (Table II). A similar high recovery was observed for the Lumbricus collagen which yielded 96% of its galactose in the form of reduced mono-, di-, and trisaccharides after the alkaline borohydride treatment.

Isolation of Glucuronosylmannose—Since it was observed that the glucuronosylmannitol isolated from the released products of alkaline borohydride-treated Nereis collagen was only partially cleaved into glucuronic acid and mannitol during hydrolysis in 2 N H2SO4 for 4 h at 100°C, these conditions were employed to prepare this acidic disaccharide in its unreduced form directly from the cuticle collagen. After such partial acid hydrolysis paper chromatography of the acidic saccharide fraction, eluted from Dowex 1 with formic acid, revealed free glucuronic and a slower component (Rfoc = 0.56) (Fig. 4) which upon elution and reduction with sodium borohydride was found to migrate to the same position (Rfoc = 0.46) as the glucuronosylmannitol obtained by alkaline borohydride treatment of the Nereis collagen. Approximately 40% of the glucuronic acid present in the cuticle collagen was recovered in the disaccharide isolated after this procedure of incomplete acid hydrolysis.

Characterization of Glucuronosylmannose—The acidic disaccharide obtained by partial acidic hydrolysis of Nereis collagen as well as the one recovered after alkaline borohydride treatment of the Nereis cuticle collagen was recovered in the acidic disaccharide isolated after this procedure of incomplete acid hydrolysis.

Fig. 1. Paper chromatography of oligosaccharides released by alkaline borohydride treatment of Lumbricus (EW) and Nereis (CW) cuticle collagens. Chromatography was performed in Solvent System A for 48 h and the paper was stained with the silver reagent. St, standard sugars; Lac, lactose; Gal-Gal-H2, galactobitol; (Gal)2-Gal-H2, galactotriitol; GlcUA-Man-H2, glucuronosylmannitol. Galactitol which was also present in both collagen samples ran off the paper during this period of chromatography.

Fig. 2. Paper chromatography of neutral sugars from acid hydrolysates of oligosaccharides isolated from alkali borohydride-treated Nereis cuticle collagen. The oligosaccharides hydrolyzed are identified in Fig. 1 and were as follows: 1, galactobitol; 2, galactotriitol; 3, glucuronosylmannitol. Chromatography was carried out in Solvent System B for 5 days and the sugars were detected with the silver reagent. St, standard sugars; Man, mannose; Man-H2, manitol; Gal-H2, galactitol; Gal, galactose; GlcUA, glucuronic acid.
Further information about the \textit{Nereis} collagen disaccharide was obtained by methylation which yielded after acid hydrolysis a single neutral component migrating on thin layer chromatography to the position of 2,3,4,tri-O-methylmannose ($R\text{_{Terr}-Me-Man} = 0.78$) and clearly separated from the 3,4,6- and 2,3,6-tri-O-methyl derivatives in Solvent System C (17). The identification of this tri-O-methylmannose derivative is consistent with the results of the periodate oxidation studies on the occurrence of a $1 \rightarrow 6$ glucuronosylmannose linkage.

When the disaccharide (0.12 \( \mu \text{mol} \)) was reacted with triphenyltetrazolium, it was found to reduce this compound to its red formazan derivative (color yield 1.7 times that of a mannose standard) indicating that indeed C-2 of the mannose residue is unsubstituted (25). As expected, a number of $1 \rightarrow 4$- and $1 \rightarrow 6$-linked disaccharides tested also reduced the triphenyltetrazolium while standard saccharides with substitution at C-2 which were tested (kojibiose, 2-deoxyglucose, and 2,3,4-tri-O-methylmannose) yielded no color in this reaction.

Incubation of the glucuronosylmannose with purified liver \( \beta \)-glucuronidase did not result in any cleavage of the disaccharide while digestion with crude enzymes either from limpet or \textit{Helix pomatia} resulted in the release of glucuronic acid and mannose so that after 48 h approximately 75\% hydrolysis had been achieved. Since the liver enzyme was found to contain only \( \beta \)-glucuronidase activity while the crude mollusk

**TABLE II**

\textit{Distribution of carbohydrate of \textit{Nereis} and \textit{Lumbricus} cuticle collagens among O-glycosidically linked units}

|          | Gal | (Gal)$_2$ | (Gal)$_3$ | GlcUA-Man | Total |
|----------|-----|-----------|-----------|------------|-------|
| \textit{Nereis} | 0.7 | 1.6       | 1.5       | 1.9        | 5.7   |
| \textit{Lumbricus} | 1.1 | 19.0      | 5.2       | -$^b$      | 25.3  |

$^a$ (Gal)$_2$ and (Gal)$_3$ refer to the di- and trisaccharide units, respectively.

$^b$ Component was below the range of detection.

dride treatment of this protein were both cleaved incompletely into glucuronic acid and mannose or glucuronic acid and mannitol by subsequent acid hydrolysis. To explore the basis of this relative acid resistance, the disaccharide was converted to its methyl ester and then reduced with sodium borohydride to form a neutral component (85\% yield) which had a substantially more rapid migration on paper chromatography than the original compound (Fig. 5) and which was hydrolyzed completely under our standard conditions to equimolar amounts of glucose and mannitol. This increase in susceptibility to acid hydrolysis brought about by conversion of the glucuronic acid to glucose is consistent with the known stability of the uronic linkage (20).

Periodate oxidation of the glucuronosylmannose disaccharide resulted in complete destruction of the mannose residue; glycerol but no erythritol was observed upon paper chromatography of the neutral sugar fraction obtained after acid hydrolysis of the reduced oxidation products. Furthermore, when the reduced disaccharide (glucuronosylmannitol) was submitted to periodate oxidation, only 1 mol of formaldehyde was formed (1.15 \( \mu \text{mol}/\mu \text{mol disaccharide} \)) under conditions which released 1.96 \( \mu \text{mol} \) and 1.17 \( \mu \text{mol} \) of formaldehyde/mol of disaccharide from lactitol and melibiose, respectively. These observations would indicate that glucuronic acid is attached to C-6 of the mannose residue.

**FIG. 4.** Paper chromatography of acidic sugar fraction (Dowex 1, formic acid eluate) obtained after partial hydrolysis (2 \( N \) \( \text{H}_2\text{SO}_4, \) 4 h, 100°C) of \textit{Nereis} cuticle collagen (Hyd). Chromatography was performed in Solvent System A for 24 h and the paper was stained with the silver reagent. St, standard sugars.
enzymes were active toward both synthetic α- and β-glucuronides, as previously reported (28), splitting of the disaccharide by the latter enzymes can be attributed to the existence of an α-glucuronidic linkage in this compound.

Confirmation of the presence of an α-glycosidic bond in the

![Image: GlcUA-Man-H2, glucuronosylmannitol; Glc-Man-H2, glucosylmannitol; St, standard sugars.](image)

**Fig. 5.** Comparison of paper chromatographic migration of reduced acidic disaccharide from *Nereis* cuticle collagen before (1) and after (2) its conversion to a neutral component by esterification and subsequent reduction. Chromatography was performed in Solvent System A for 26 h and the paper was stained with the silver reagent. Glc-Man-H2, glucosylmannitol; GlcUA-Man-H2, glucuronosylmannitol; St, standard sugars.

![Image: Gel filtration on Sephadex G-25 of collagenase digest of *Nereis* cuticle collagen. The sample representing 1 g of the collagen was placed on a column (2.1 x 125 cm) which was equilibrated and eluted with 0.1 M pyridine acetate buffer, pH 5.0, at a flow rate of 15 ml/h. Each fraction was analyzed for hexose by the anthrone reaction and for hexuronic acid and peptide by the carbazole and ninhydrin reagents, respectively. Numbered areas designate tubes which were pooled; Fraction 2 was studied further after pronase digestion. The recoveries from the column were: hexose, 87%; hexuronic acids, 91%.

**Fig. 6.** Gel filtration on Sephadex G-25 of collagenase digest of *Nereis* cuticle collagen. The conditions of the electrophoresis are specified under "Experimental Procedures." The paper was stained with the ninhydrin reagent.

![Image: Chromatography on Dowex 1-X2 of glycopeptides from *Nereis* cuticle collagen. The glycopeptides were derived from Fraction 2 on Sephadex G-25 (Fig. 6) after digestion with pronase and gel filtration on Sephadex G-15 (see "Experimental Procedures"). The sample containing 15 mg of hexose and 3 mg of hexuronic acid was placed on a column (1.1 x 102 cm) equilibrated with 0.002 M pyridine acetate buffer, pH 5.0. After elution with this buffer a linear concentration gradient at this pH was started (arrow) as described under "Experimental Procedures"; a pyridine acetate concentration of 0.24 M was reached at tube 95. A flow rate of 20 ml/h was maintained and aliquots of each fraction were analyzed for hexose by the anthrone reaction and hexuronic acids by the carbazole reagent. Numbered areas designate tubes which were pooled and these are subsequently referred as D-1, D-2, and D-3, respectively. The recoveries from the column were: hexose, 91%; hexuronic acids, 94%.

**Fig. 7.** Chromatography on Dowex 1-X2 of glycopeptides from *Nereis* cuticle collagen. The glycopeptides were derived from Fraction 2 on Sephadex G-25 (Fig. 6) after digestion with pronase and gel filtration on Sephadex G-15 (see "Experimental Procedures"). The sample containing 15 mg of hexose and 3 mg of hexuronic acid was placed on a column (1.1 x 102 cm) equilibrated with 0.002 M pyridine acetate buffer, pH 5.0. After elution with this buffer a linear concentration gradient at this pH was started (arrow) as described under "Experimental Procedures"; a pyridine acetate concentration of 0.24 M was reached at tube 95. A flow rate of 20 ml/h was maintained and aliquots of each fraction were analyzed for hexose by the anthrone reaction and hexuronic acids by the carbazole reagent. Numbered areas designate tubes which were pooled and these are subsequently referred as D-1, D-2, and D-3, respectively. The recoveries from the column were: hexose, 91%; hexuronic acids, 94%.

**Fig. 8.** Paper electrophoresis of *Nereis* collagen glycopeptides obtained by Dowex 1-X2 chromatography. The designations D-1, D-2, D-3 refer to the major glycopeptide peaks (Fig. 7) and Se designates the Sephadex fraction from which they were resolved. The conditions of the electrophoresis are specified under "Experimental Procedures." The paper was stained with the ninhydrin reagent.
Nereis disaccharide was obtained from the observation that its glucosylmannitol derivative was slowly hydrolyzed by α-glucosidase (35% in 48 hr) but was completely resistant to cleavage by β-glucosidase.

Isolation of Glycopeptides—In order to characterize further the glucuronylmannose disaccharide and determine the nature of its attachment to the polypeptide chain, Nereis cuticle collagen was digested with collagenase and glycopeptides containing this carbohydrate were investigated. Approximately 95% of the glucuronic acid present in the collagen were solubilized by incubation with this enzyme and filtration of the digest on Sephadex G-25 (Fig. 6) yielded a broad glycopeptide peak (Fraction 2) which was included in the gel and represented 62% of the uronic acid recovered from the column. After a digestion with pronase and removal of released amino acids and small peptides by filtration on Sephadex G-15 these glycopeptides were further purified on Dowex 1-X2 at pH 5.0 (Fig. 7). The glucuronic acid-containing glycopeptides were adsorbed on the resin and eluted primarily as two partially resolved peaks (Peaks D-2 and D-3) at a buffer concentration of 0.19 M and 0.21 M respectively, while the glycopeptides containing only neutral sugars emerged with the dilute buffer wash (Peak D-1).

On electrophoresis the two glucuronic acid-containing glycopeptides (D-2 and D-3) moved toward the anode as single components with mobilities increasing in order of their elution from the Dowex 1 column while the glycopeptides which were not adsorbed on the resin (D-1) remained close to the origin (Fig. 8).

Characterization of the Glucuronic Acid-containing Glycopeptides—As revealed by paper chromatography, alkaline borohydride treatment of the glycopeptides resulted in the release of only glucuronosylmannitol from the acidic glycopeptides (D-2 and D-3) while galactobiol and galactoxtitol were the oligosaccharide products obtained from the neutral glycopeptides (D-1) (Fig. 9).

Compositional analyses of the acidic glycopeptides demonstrated that glucuronic acid and mannose in equimolar amounts were the only sugar components (Table III). Only a limited group of amino acids were observed and the presence of hydroxyproline as well as about one third of the residues as glycine indicated that the glycopeptides were indeed derived from a collagen polypeptide chain (Table III). NH₂-terminal analyses indicated that the glycopeptides contained a single disaccharide unit (Table III).

Alkaline treatment of the glycopeptides under conditions which promote β elimination resulted in a selective loss of threonine (Table III) suggesting that the disaccharide is O-glycosidically linked to this amino acid. The linkage amino acid was further identified by paper chromatography of its sulfonate derivative after alkaline sulfite treatment of the glycopeptides; glycopeptides D-2 and D-3 yielded an amino acid which migrated to the position of α-amino-β-sulfonobutyric acid, but no cysteic acid was observed. In contrast the neutral galactose-containing glycopeptides (D-1) were found to contain both cysteic acid and α-amino-β-sulfonobutyric acid after alkaline sulfite treatment.

**DISCUSSION**

The results of the present investigation indicate that the Nereis cuticle collagen contains a novel acidic carbohydrate unit in which the disaccharide, 6-O-α-D-glucuronosyl-D-mannose is O-glycosidically linked to threonine on the polypeptide chain (Fig. 10). This disaccharide could be obtained in high yield either by partial acid hydrolysis or alkaline borohydride treatment of the cuticle collagen. The latter procedure was shown to result in the release of the reduced disaccharide (6-O-α-D-glucuronosylmannitol) by cleavage through β elimination of its linkage to threonine. The unreduced form of the disaccharide was obtained by the acid hydrolysis due to the stability of the glucuronic bond (20, 29, 30) and indeed it could be shown that after reduction of its carboxyl group the resultant neutral disaccharide (glucosylmannitol) was much more susceptible to hydrolytic cleavage.

Information obtained by periodate oxidation, methylation, and triphenyltetrazolium chloride reduction studies clearly demonstrated that the glucuronic acid was linked to C-6 of the mannose while the α-anomeric configuration of this linkage

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**Table III**

| Component       | Glycopeptide |
|-----------------|--------------|
|                 | D-2 | D-3 |
| Glucuronic acid | 1.0 | 1.0 |
| Mannose         | 1.1 | 1.1 |
| Hydroxyproline  | 2.0 | 1.3 |
| Threonine       | 1.8 | 1.4 |
| Serine          | 0.8 | 0.8 |
| Glutamic acid   | 2.3 | 2.3 |
| Proline         | 1.2 | 1.0 |
| Glycine         | 5.4 | 5.0' |
| Alanine         | 1.4 | 1.4 |

*Glycopeptide peaks are shown in Fig. 7.*

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References

1. Mild alkaline treatment (0.1 N NaOH, 37°C, 48 h) of the glycopeptides resulted in the following amino acid destruction: D-2: threonine, 0.7 mol; serine, 0.0 mol; D-3: threonine, 0.8 mol; serine, 0.1 mol.

2. Subtractive Edman degradation indicated 1.1 mol of glycine in NH₂-terminal position.

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**Fig. 9. Identification by paper chromatography of oligosaccharides released by alkaline borohydride treatment of Nereis cuticle collagen glycopeptides from Dowex 1-X2 column.** D-1 and D-3 refer to the Dowex 1 glycopeptide peaks (Fig. 7) and Se represents the Sephadex fraction from which they were resolved. Chromatography was carried out in Solvent System A for 42 h and the paper was stained with the silver reagent.
was established from the action of various glycosidases both on the native disaccharide and on its glucosylmannitol derivative.

It was apparent from the analysis of the products released by alkaline borohydride treatment that all of the glucuronic acid and most of the mannose present in the Nereis cuticle collagen occurred in the form of the glucurono-0-mannosyl-threonine-saccharide; moreover over 90% of the galactose in the collagen could be accounted for by the mono-, di-, and trisaccharide units of this sugar. This would indicate that the bulk (80%) of the carbohydrate of the Nereis collagen occurs in the form of these O-glycosidically linked saccharide units.

The absence of hydroxylsine in annelid cuticle collagen a priori excludes the presence of carbohydrate units linked to this amino acid such as occurs in most other vertebrate and invertebrates collagens (2). The work of Muir and Lee has demonstrated that the carbohydrate of Lumaribus cuticle collagen is present primarily in the form of α(1→2)-linked di- and trisaccharides of galactose which are attached to threonine and serine residues on the peptide chain (7, 8). Our studies confirm these findings and demonstrate that such O-glycosidically linked galactose units also occur in the cuticle collagen of the clam worm, Nereis, a member of the class Polychaeta of the annelid phylum. Moreover found that both the Nereis and Lumaribus collagens contain a small number of monosaccharide units of galactose. As exemplified by these two collagens, the distribution of the O-glycosidically linked galactose-containing units differs between polychaete and oligochaete worms. Lumaribus cuticle collagen has a 7 times greater average density of these units than that of Nereis (25.3 compared to 3.8 units/1000 total amino acid residues) and moreover had a ratio of galactose mono-, di-, and trisaccharide of 1:17:5 in contrast to that of Nereis in which this ratio was approximately 1:2:2. However, the present study has shown that the major difference between the two cuticle collagens is to be found in the occurrence of the O-glycosidically linked glucurono-0-mannosyl-threonine-saccharide in the Nereis protein. From the amino acid composition of the glycopeptides containing this acidic carbohydrate unit it would appear likely that the threonine residue to which the glucurono-0-mannosyl-threonine unit is attached is located adjacent to or within a glycosylating galactose unit; in the two glycopeptides isolated approximately one-third of the residues were glycine while one-fifth were constituted by hydroxyproline plus proline. Since Nereis cuticle collagen has been reported to have a molecular weight of 1,400,000 (6) it can be calculated from our data that there are approximately 28 glucurono-0-mannosyl-threonine and 56 galactose-containing carbohydrate units in each protein molecule.

The annelid cuticle collagens appear to be the only members of the collagen family of proteins which contain carbohydrate units linked to threonine and serine residues. These saccharide units have in common with the more familiar hydroxyprolyl glycosides of collagens (2) an O-glycosidic carbohydrate-peptide bond and short sugar chains. This may have structural implications as such small saccharide units would be more likely accommodated into the three dimensional packing of collagen molecules than longer structures such as those which are characteristic of asparagine-linked carbohydrate (31). Although asparagine-linked oligosaccharides have been reported to occur in proteins of the collagen-type (32–35) they tend to be attached to more polar segments of the polypeptide chain outside of the helical domain. It may be noted that despite the unusual high content of hydroxyproline in the annelid cuticle collagen this amino acid does not appear to be involved in the attachment of carbohydrate to the polypeptide chain.

The occurrence of a carbohydrate-peptide bond involving mannose and a hydroxyamino acid as in the Nereis collagen has otherwise been only observed in the mannan-proteins of yeast cell walls (36). However, a recent report describing the isolation of mannotol-containing oligosaccharides from a rat brain proteoglycan fraction suggests the possibility that mannose-threonine (serine) linkages may be more widely distributed (37).

The finding of a glucuronic acid-containing disaccharide in the Nereis cuticle collagen helps to expand our concept of carbohydrate units of glycoproteins. Uronic acids have generally been considered to be components of the relatively large repeating units characteristic of proteoglycans (38) although a sulfated carbohydrate moiety (M, 5,800) in which glucuronic acid occurs has been found to be an integral part of human thyroglobulin (39). The glucurono-0-mannosyl-threonine unit of Nereis collagen being small in size and acidic in character seems to represent a structural pattern which in some ways resembles the acidic disaccharides of ovine submaxillary glycoprotein in which sialyl-N-acetylgalactosamine (α-N-acetylmuramyl-(2→6)-N-acetylgalactosamine) are O-glycosidically attached to threonine and serine residues on the polypeptide chain (40). Since sialic acids are not present in annelids (41) the uronic acid may serve an analogous function in forming a negatively charged saccharide unit.

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**Fig. 10. Proposed structure and peptide attachment of the acidic disaccharide unit of Nereis cuticle collagen: 8-O-α-glucuronosyl-0-β-mannosyl-threonine.**
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