Evidence for the Linkage of a Disaccharide to Hydroxylysine in Tropocollagen*

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SUMMARY

Soluble collagen from guinea pig skin has been subjected to sequential digestion with purified collagenase and trypsin. The small quantity of hexose present in the collagen can be detected in the digest largely as relatively basic glycopeptides. One of these glycopeptides has been obtained in pure form and accounts for at least 30% of the hexose of the original collagen. This glycopeptide has been shown to consist of a disaccharide of glucose and galactose linked O-glycosidically to the hydroxyl group of a hydroxylysine residue in a special peptide region of the collagen molecule. The amino acid composition and probable sequence in this region was shown to be

-Gly-Met-Hyl(Glc, Gal)-Gly-His-Arg-

A possible role for this structure in the formation of cross-links during collagen “maturation” is described.

After an examination of the levels of hexose and hexosamine during repeated replications of acid-soluble collagen, Kühn, Grassmann, and Hofmann (1) concluded that a small quantity of hexose, but no hexosamine, is covalently attached to the polypeptide chain. Both n-glucose and n-galactose (2-4) have subsequently been shown to be present. Following studies of isothyocyan (mol wt 320,000), Blumenfeld et al. (4) concluded that there are 7 residues of galactose and 5 of glucose attached glycosidically to the protein as monosaccharides. Evidence presented by Grassmann, Hörmann, and Hafter (5) suggests that the hexoses are linked to tropocollagen through an O-glycosidic bond. Since periodate under certain conditions was observed to solubilize a preparation of insoluble (mature) collagen, Hörmann (6) postulated that the glycosidically linked monosaccharides participate in the formation of intramolecular and intermolecular cross-links, which are important in stabilizing the network of mature collagen fibers.

In the present communication evidence is presented that at least 30 to 40%, and possibly all, of the hexose in tropocollagen derived from guinea pig skin occurs as a disaccharide, composed of glucose and galactose, which is linked O-glycosidically to the hydroxyl group of hydroxylysine. Information concerning the sequence of amino acids about this hydroxylysine residue is also presented. Preliminary reports of these studies have appeared (7, 8).

MATERIALS

Citrate-soluble collagen was prepared by the method of Gallop and Seifter (9) from the skins of guinea pigs weighing between 250 and 400 g. The preparation contained 0.45% hexose as measured by the orcinol procedure of Judd et al. (10) and expressed as galactose equivalents. The amino acid analysis of such a preparation is compared with that reported for rat skin acid-soluble collagen by Piez et al. (11) in Table I. After lyophilization, preparations of tropocollagen were stored in dessicators at 4°.

Collagenase was prepared from commercial preparations of crude Clostridium histolyticum collagenase by a modification of the gel filtration procedure of Keller and Mandl (12). A solution of 200 mg of crude collagenase in 20 ml of 0.05 M Tris HCl, pH 7.4, containing 0.15 M CaCl₂, was chromatographed on a Sephadex G-200 column (5 x 93 cm; bed volume, 2000 ml) equilibrated and eluted with the Tris-Ca++ buffer mentioned above. Collagenase activity was estimated in the fractions from chromatography by incubating 10 mg of tendon collagen (Worthington) with 2.5 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01 M CaCl₂ and a 0.2-ml aliquot of the fraction to be tested, for 8 hours at 37°. After filtration of the suspension, the ninhydrin-positive material released by the action of collagenase was estimated by the method of Moore and Stein (13). A small protein peak emerging from the column between 920 to 1200 ml was found to contain all the collagenase activity. The collagenase from six such columns was pooled, dialyzed extensively against water at 4°, and lyophilized.

The enzymatic activity of the residual white powder (162 mg) was assayed by the “20-min suspension” method of Gallop, Seifter, and Meilman (14) and was found to be 118 units per mg of protein. The enzyme would, however, not digest denatured ovalbumin and was thus judged to be free of any nonspecific protease.

Crude collagenase, trypsin, carboxypeptidase B, Galactostat, and Glucostat were purchased from Worthington and Pronase.
was purchased from Calbiochem. Oreinol was purchased from Matheson, Coleman, Bell, ninhydrin and dl-(+)-allo-\(\Delta\)-hydroxylysine HCl from Mann, carboxymethyl cellulose from Gallard-Schlesinger, and Sephadex gels from Pharmacia.

**METHODS**

**Preparation of Glycopeptides**—Collagen was digested by suspending 1.5 g of the protein in 25 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01 M CaCl\(_2\) and then incubating it with collagenase (1% by weight) at 37°C for 24 hours in an atmosphere saturated with toluene. Then the pH of the digest was adjusted to 8.0, crystalline trypsin (1% or 2%) was added, and digestion at 37°C was continued for an additional 24 hours. The pH of the digest was then lowered to 5.0, and a small amount of precipitate was removed by centrifugation.

The digest was subjected to gel filtration on a Sephadex G-25 column (1.8 x 75 cm; bed volume, 220 ml), equilibrated, and eluted with 0.1 M pyridine acetate buffer, pH 5.0. Ninhydrin-positive material was estimated by the method of Moore and Stein (13). A typical experiment is illustrated in Fig. 1. The major portion of the ninhydrin-positive material was separated from the hexose-containing material in this way. The hexose peak, indicated by the *arrow*, accounted consistently for 70 to 80% of the hexose in collagen. This glycopeptide-containing fraction was lyophilized, dissolved in citrate buffer, and further resolved by chromatography on carboxymethyl cellulose. A typical experiment is illustrated in Fig. 2. Three major glycopeptide peaks which accounted for more than 90% of the applied hexose were repeatedly observed. The last hexose peak to emerge from the column (Peak 3) consistently accounted for 40 to 50% of the applied hexose. The majority of the ninhydrin-positive material emerged before the glycopeptide peaks.

Table I illustrates the yields of hexose obtained at different stages of a typical experiment. The three glycopeptide peaks observed in the carboxymethyl cellulose chromatography contained a majority of the hexose present in the original collagen, the glycopeptide of Peak 3, itself, representing at least one-third of the hexose of collagen.

**Identification and Quantitation of Hexoses and Hexosamines**—For identification of the hexoses of a glycopeptide, a sample containing 0.4 \(\mu\)mole of hexose was hydrolyzed in 2 \(\times\) HCl for 2 hours in a sealed evacuated ampule at 110°C. After removal of the excess HCl by evaporation under reduced pressure on a rotary evaporator, the hydrolysate was dissolved in a minimum of water out in l-butanol-pyridine-water (6:4:3) and in ethyl acetate-water (12:8:4). Chromatograms were sprayed with aniline-phthalate for detection of hexoses.

Table II illustrates the yields of hexose obtained at different stages of a typical experiment. The three glycopeptide peaks observed in the carboxymethyl cellulose chromatography contained a majority of the hexose present in the original collagen, the glycopeptide of Peak 3, itself, representing at least one-third of the hexose of collagen.

### Table I

**Amino acid analyses of guinea pig skin and rat skin acid-soluble collagens**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Guinea pig</th>
<th>Rat*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>331</td>
<td>334</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>75</td>
<td>71</td>
</tr>
<tr>
<td>Proline</td>
<td>125</td>
<td>121</td>
</tr>
<tr>
<td>Proline</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>Serine</td>
<td>123</td>
<td>121</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Threonine</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>321</td>
<td>334</td>
</tr>
<tr>
<td>Alanine</td>
<td>112</td>
<td>106</td>
</tr>
<tr>
<td>Valine</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.6</td>
<td>7.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Lysine</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>5.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>Arginine</td>
<td>51</td>
<td>51</td>
</tr>
</tbody>
</table>

* From Piez et al. (11).
TABLE II

Yields of hexose in various fractions during typical preparation of glycopeptides from collagen

<table>
<thead>
<tr>
<th>Step</th>
<th>Hexose recovered</th>
<th>mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td></td>
<td>15.2</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-25 chromatography, major hexose fraction</td>
<td>10.6</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>CM-cellulose peaks</td>
<td></td>
<td>2.1</td>
<td>14</td>
</tr>
<tr>
<td>Peak 1</td>
<td>2.0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Peak 2</td>
<td>5.0</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Hexose recovered</td>
<td></td>
<td>2.16 f 0.18</td>
<td>2.16 f 0.26</td>
</tr>
</tbody>
</table>

* Mean ± S.D. No other amino acids were present in amounts exceeding 0.05 mole per mole of hydroxylysine.

TABLE III

Amino acid and hexose composition of glycopeptides (Peak 3) derived from collagen

<table>
<thead>
<tr>
<th>Component</th>
<th>% collagenase +1% trypsin</th>
<th>% collagenase + 2% trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole hydroxylysinea</td>
<td></td>
</tr>
<tr>
<td>Hydroyxlysine</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.29 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.16 ± 0.07</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.87 ± 0.08</td>
<td>0.67 ± 0.20</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.87 ± 0.18</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.45 ± 0.06</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.39 ± 0.06</td>
<td>2.45 ± 0.18</td>
</tr>
<tr>
<td>Methioninebe</td>
<td>1.02 ± 0.04</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>Hexosec</td>
<td>2.16 ± 0.18</td>
<td>2.16 ± 0.26</td>
</tr>
</tbody>
</table>

* Mean ± S.D. No other amino acids were present in amounts exceeding 0.05 mole per mole of hydroxylysine.

RESULTS

Composition of Peak 3 Glycopeptide—The mean values for the amino acid and hexose analyses of preparations of the glycopeptide of Peak 3 from digests containing both 1% and 2% trypsin are compared in Table III. The original digestion mixture contained only 1% trypsin, and the resulting Peak 3 glycopeptide contained almost 2 moles of arginine, 1 mole each of histidine, hydroxylysine, and methionine, 3 moles of glycine, 2 moles of hexose, and small but persistent amounts of lysine and glutamic acid. When the amount of trypsin in the digest was increased to 2%, the arginine value approached 1, the glycine value was approximately 2, and the amounts of glutamic acid and lysine were significantly reduced. The excess amounts of arginine and glycine and the small amounts of glutamic acid and lysine found in the glycopeptide prepared from a digest containing 1% trypsin presumably reflect incomplete hydrolysis by this enzyme at this concentration. All further studies reported here were conducted on preparations of the glycopeptide of Peak 3 obtained from digests containing 2% trypsin. These data indicate that the composition of the glycopeptide in the immediate vicinity of the hexose is: hydroxylysine, 1; arginine, 1; histidine, 1; methionine, 1; glycine, 2; and hexose, 2.

The hexoses of the glycopeptide were identified as glucose and galactose by paper chromatography in the two solvents listed in “Methods.” The glycopeptide contained no glucosamine or galactosamine.

When the glycopeptide of Peak 3 was subjected to high voltage paper electrophoresis at pH 3.6 or 6.5, a single ninhydrin-positive compound was detected. The mobility of this compound at pH 3.6 was slightly less than that of basic amino acid controls (see Peak 3, Fig. 4). This observation is consistent with the presence of several basic amino acids in the glycopeptide.

Carbohydrate-Peptide Bond of Peak 3 Glycopeptide—Samples of collagen and of the Peak 3 glycopeptide were treated with periodate in order to determine if the side chains of the hydroxylysine residues in these materials were unsubstituted. Oxidation by periodate would indicate that both the hydroxyl group and amino group of the hydroxylysine side chain were free. The treatment of gelatin derived from guinea pig skin collagen with periodic acid resulted in the destruction of all but a small amount (about 9%) of the hydroxylysine in this protein (Table IV); on the other hand about 90% of the hydroxylysine of Peak 3 is stable to such
treatment. The hydroxylysine in the Peak 3 glycopeptide (calculated from the hexose and hydroxylysine contents of collagen and from the actual yield of hexose in the Peak 3 glycopeptide) represents about 9% of the total of this amino acid in collagen, which is in agreement with the amount stable toward periodate in intact collagen.

Hydrolysis of the glycopeptide with 2 N HCl at 110° for 30 min released about 2 moles of reducing sugar per mole of hydroxylysine as measured by the Park-Johnson method (17). These results suggest that the carbohydrate of this glycopeptide is linked glycosidically to the hydroxylysine residue.

Further evidence for a glycosidic linkage of hexose to hydroxylysine was afforded by studies of alkaline hydrolysates. The glycopeptide, 1.8 µmoles, was heated in 2 N NaOH (total volume, 1.5 ml) at 90° for 10 hours in a covered, Vycor test tube. A hexose-containing compound was then isolated by Sephadex chromatography (for a similar experiment see Fig. 3) of the alkaline hydrolysate. Subsequent acid hydrolysis and amino acid analysis of this derivative (Table V) revealed no significant quantity of any amino acid except hydroxylysine, indicating that the hexoses are attached to hydroxylysine in the original glycopeptide. The hydroxylysine to glucose to galactose ratio of 1:1:1 suggests that the carbohydrate moiety of the glycopeptide is a disaccharide composed of glucose and galactose.

Since the bond linking the disaccharide is resistant to alkaline hydrolysis, it is almost certainly glycosidic in nature. When subjected to high voltage electrophoresis at pH 3.6 (see Fraction A, Fig. 4) the compound containing hydroxylysine, glucose, and galactose exhibited a mobility greater than that of glycine but slightly less than that of the basic amino acids. This mobility suggests that the ε-amino group on the hydroxylysine residue is unsubstituted; therefore, an N-glycosidic linkage of the disaccharide to hydroxylysine seems unlikely. Finally, reaction of purified Fraction A with fluorodinitrobenzene yielded a water-soluble dinitrophenyl derivative. Acid hydrolysis of this compound yielded an ethyl acetate-soluble derivative which was identical with α,ε-bis(dinitrophenyl)hydroxylysine in two chromatographic solvents.

These data show that the hexoses of Peak 3 occur as a disaccharide of glucose and galactose attached O-glycosidically to the hydroxyl group of hydroxylysine.

**Table IV**

<table>
<thead>
<tr>
<th>Amine acid</th>
<th>Collagen</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.360</td>
<td>0.030</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/ml</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.165</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.017</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.017</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.178</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.182</td>
</tr>
</tbody>
</table>

* No other amino acids were present in quantities exceeding 0.02 mole per mole of hydroxylysine.
* Measured with Galacostat, see "Methods."
* Measured with Glucostat, see "Methods."
TABLE VI
Amino acid compositions of Peak 3 glycopeptide before and after treatment with carboxypeptidase B and with Pronase

<table>
<thead>
<tr>
<th>Component</th>
<th>Untreated</th>
<th>Carboxypeptidase B</th>
<th>Pronase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole hydroxylysine</td>
<td>moles/mole hydroxylysine</td>
<td>moles/mole hydroxylysine</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.10 ± 0.02</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.11 ± 0.10</td>
<td>0.95</td>
<td>1.02</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.11 ± 0.07</td>
<td>Trace</td>
<td>0.95</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.14 ± 0.03</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.45 ± 0.18</td>
<td>2.22</td>
<td>1.41</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.81 ± 0.12</td>
<td>0.79</td>
<td>0.11</td>
</tr>
<tr>
<td>Hexose*</td>
<td>2.16</td>
<td>2.26</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* No other amino acids were present in amounts exceeding 0.10 mole per mole hydroxylysine.
* Average of Peak 3 glycopeptide (see Table III).
* Hexose was measured by the orcinol procedure of Judd et al. (10) and calculated as galactose equivalents.
* Hexose present but quantitative analysis not available.

TABLE VII
Composition of fractions isolated from partial alkaline hydrolysate of Peak 3 glycopeptide

<table>
<thead>
<tr>
<th>Component</th>
<th>Fraction A</th>
<th>Fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole hydroxylysine</td>
<td>moles/mole ornithine</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>1.0</td>
<td>Absent</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.39</td>
<td>Absent</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>Ornithine</td>
<td>Absent</td>
<td>1.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>Absent</td>
<td>0.95</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.11</td>
<td>Absent</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.09</td>
<td>Absent</td>
</tr>
</tbody>
</table>

* No other amino acids were present in ratios exceeding 0.01.
* Measured with Galactostat, see "Methods.
* Measured with Glucostat, see "Methods.

The glycopeptide of Peak 3 was established by digestion of 0.5 µmole of the glycopeptide with 2 mg of carboxypeptidase B (Lot 6079, 100 units per mg of protein) in 0.025 M Tris-HCl, 0.1 M NaCl buffer, pH 7.62 (final volume 1.3 ml) at 24° for 24 hours. Amino acids and the enzyme were separated from the altered glycopeptide by chromatography on Sephadex G-25. The amino acid analysis of such a preparation, given in Table VI, shows that only arginine was released from the glycopeptide by the action of carboxypeptidase B, and thus demonstrates that the arginine residue is located at the carboxyl-terminal end of the peptide. This observation is consistent with the known specificity of trypsin, used in the preparation of the digest from which the glycopeptide was obtained.

The Peak 3 glycopeptide was degraded with Pronase in the following manner: 1.5 µmoles of peptide were incubated with 1 mg of Pronase in 0.05 M Tris-HCl buffer, pH 8.5, containing 0.01 M CaCl₂ (final volume 2 ml) for 8 hours at 37°. Again the digestion mixture was subjected to gel filtration to separate the partly digested glycopeptide from free amino acids and enzyme. The amino acid analysis of the Pronase-digested glycopeptide, also given in Table VI, shows that approximately 1 mole of glycine and 1 mole of methionine were removed from the glycopeptide by the action of Pronase. Since the arginine residue in the peptide was unaffected by Pronase treatment, the glycine and methionine residues must be located on the amino-terminal end of the glycopeptide of Peak 3.

Partial alkaline hydrolysis of the glycopeptide was accomplished by heating 2.7 µmoles of the peptide in 3.0 ml of 2 N NaOH at 90° for 2 hours in a covered, Vycor test tube. The hydrolysate was chromatographed on Sephadex G-25 as illustrated in Fig. 3. Preliminary analyses of the hexose-containing peak (Fractions 48 to 56) indicated that it contained all of the hydroxylysine and that it consisted of a mixture of peptides. Paper electrophoresis of this fraction at pH 3.6 (illustrated as Alk Hyd in Fig. 4) revealed two main components when sprayed with ninhydrin. The two fractions (A and B) were eluted after preparative electrophoresis at pH 3.6 on Whatman No. 40 chromatography paper, and each of the fractions was then rechromatographed on Sephadex G-25. Only Fraction A contained hexose. Fig. 4 illustrates the electrophoresis of these two fractions, and their compositions are given in Table VII.

Fraction A can be accounted for as a mixture of Hyl(Glc, Gal), Met–Hyl(Glc, Gal), and Gly–Met–Hyl(Glc, Gal). The recovery of hydroxylysine in this fraction was 79%. Limited further attempts to separate the peptides in A by paper chromatography or by gel filtration on Sephadex G-10 were unsuccessful. These data, coupled with the information from Pronase digestion of the glycopeptide, are consistent with the sequence Gly–Met–Hyl at the amino-terminal end of the glycopeptide.

Fraction B from partial alkaline hydrolysis and electrophoresis contained equimolar amounts of ornithine and histidine, a small amount of glycine, and no hexose. Its electrophoretic mobility is consistent with this amino acid composition. Since ornithine originated from the arginine residue of the original glycopeptide (18), Fraction B must have originated from the carboxyl-terminal end of the glycopeptide. This information establishes that the carboxyl-terminal amino acid sequence is −Gly–His–Arg− in the glycopeptide of Peak 3. The yield of ornithine in Fraction B was 41% of the original arginine, and the yield of histidine was 45%. The remainder of the ornithine and histidine was present in the free amino acid peak (Fractions 60 to 70) from the Sephadex chromatography (Fig. 9).

Glycopeptides of Peak 1 and Peak 2—The glycopeptides contained in Peaks 1 and 2 from carboxymethyl cellulose chromatography (Fig. 2) were shown to be mixtures of peptides by electrophoresis, by chromatography on larger columns of carboxymethyl cellulose, and by amino acid analyses. These peaks, however, contain significant quantities of hydroxylysine, histidine, and arginine, as well as of lysine, glutamic acid, aspartic acid, proline, glucose, and galactose. The structural relationships of these glycopeptides to the glycopeptide of Peak 3 are, at present, not understood, although the presence of hydroxylysine and histidine, rare amino acids in the over-all collagen structure, suggests the likelihood that similar amino acid regions are involved in the linkage of the remaining hexose and that it too may be attached to hydroxylysine residues.

DISCUSSION

The mode of attachment of carbohydrates to amino acids in glycoproteins and mucoproteins has received much attention in recent years. The linkage in ovalbumin is a glycosylamine bond joining N-acetylgalactosamine to the amide group of a single specific asparagine residue in the peptide chain (18, 19). This
type of linkage was later shown to exist also in ovomucoid (20), ribonuclease B (21), and probably in γ-globulins (22), α-1 glycoprotein (23, 24), fetuin (25), and thyroglobulin (26). The carbohydrate-protein linkages in ovine submaxillary mucins are O-glycosidic bonds to serine and threonine residues (27–29). Heparn and chondroitin sulfates are linked to protein through O-glycosidic linkages of xylose to serine residues (30–32). Ester linkages of carbohydrate to glutamic and aspartic acid in ovine submaxillary mucins have also been suggested (33). The finding that a significant quantity of the hexose of the tropocollagen molecule is linked O-glycosidically to the hydroxy group of hydroxylysine adds still another type of bond shown to link carbohydrate to a glycoprotein.

Blumenfeld et al. (4) found that periodate treatment of ichthyocol destroyed all of the hydroxylysine and concluded that the glycosidic linkages of hexose to this protein do not involve hydroxylysine residues. Hörmann and Fries (34) observed that 50% of the hydroxylysine of acetic acid-soluble collagen could be destroyed by treating the collagen with 0.02 M sodium periodate for 30 hours at 4°C; yet, a small amount of the hydroxylysine was not destroyed by this treatment.

Our results indicate that there is a small amount of hydroxylysine in citrate soluble guinea pig skin collagen which is resistant to oxidation by periodate. Since the conditions used by Blumenfeld et al. (4) were almost identical with the conditions used by us, it will be of considerable interest to learn if the carbohydrate-protein bond in ichthyocol is different from that found in the collagens of higher species. The extremely long periods of incubation in 8% acetic acid used by Hörmann and Fries may have caused scission of the glycoside bond linking carbohydrate to hydroxylysine, allowing destruction of this amino acid by periodate.

The isolation of a compound containing only hydroxylysine, galactose, and glucose in a 1:1:1 ratio verifies the linkage of a disaccharide to hydroxylysine in tropocollagen. Since the results of Blumenfeld and her co-workers suggest that the hexoses of ichthyocol occur as monosaccharides, it is possible that ichthyocol differs from the collagen of guinea pig skin with regard to its carbohydrate. Additional examinations of glycopeptides from ichthyocol will be necessary to clarify this question.

Degradation studies on the glycopeptide of Peak 3 are consistent with the following sequence of amino acids about the hydroxylysine residue involved in the disaccharide-protein bond:

\[
\text{Gly-Met-Hyl-Gly-His-Arg-} \quad \text{(Glc, Gal)}
\]

Arginine was implicated as the carboxyl-terminal amino acid by knowledge of trypsin specificity and by its release from the peptide by carboxypeptidase B. Isolation from a partial alkaline hydrolysate of the glycopeptide of a fraction (B), containing equimolar amounts of ornithine and histidine and small amounts of glycine, indicates that histidine is the amino acid adjacent to arginine. Since another fraction (A) isolated from the partial alkaline hydrolysate contained residual amounts of methionine, and small amounts of glycine, along with equimolar amounts of hydroxylysine, glucose, and galactose, methionine must be adjacent to hydroxylysine in the glycopeptide. The finding that digestion of the glycopeptide with Pronase resulted in the loss of 1 mole each of glycine and methionine is consistent with this observation, and, since the amino acids on the carboxy-terminal end of the glycopeptide were unaffected by this treatment, locates these amino acids on the amino-terminal end of the glycopeptide.

The occurrence of significant quantities of hydroxylysine, histidine, and arginine in the other two glycopeptide-containing fractions (Peaks 1 and 2) from carboxymethyl cellulose chromatography suggests a structural similarity between other hexose-containing regions of collagen and the region, or regions, from which the glycopeptide of Peak 3 was derived. This comparison is especially impressive when one considers that the amounts of hydroxylysine (5.5 residues/1000 amino acid residues) and histidine (6 residues/1000 amino acid residues) contained in collagen are the lowest of any amino acids present except methionine and tyrosine (see Table I). If hexose is attached to hydroxylysine in the glycopeptide of Peaks 1 and 2, however, it will be necessary to look again at the problem of the quantity of periodate-stable hydroxylysine since the total quantity of this amino acid in all glycopeptide fractions is 22% of the total in collagen, while only 9% is stable toward periodate. Nevertheless, it is apparent that much of the hexose of collagen is attached O-glycosidically to a hydroxylysine residue in a rather unique, highly basic section of the polypeptide chain. Furthermore, in view of the demonstration by Partridge and his associates (37, 38) that the conversion of lysine residues to desmosine and isodesmosine residues accounts for cross-linking of peptide chains in elastin and the later observations (39, 40) that β amino propionitrile prevents cross-linking in elastin as well as collagen, it seems timely and useful to call attention to the possibility that the metabolic fate of the glycosylated hydroxylysine residues of collagen contained in the Peak 3 sequence may be related to cross-linking or "aging" of collagen fibrils. Such participation need not be restricted to a direct interchain hexose residue, but might, for example, involve ε-deamination of the glycosylated hydroxylysine residue, condensation of the resulting enol with a lysine residue of another molecule of tropocollagen, and finally, deglycosylation. The absence of histidine from the peptides reported by Bornstein et al. (41) to contain the sites of intramolecular cross-links appears to rule out the participation of the glycopeptide region in the particular regions of the collagen molecule which those peptides represent. Intramolecular cross-links were not observed in these studies, however, and those or additional intramolecular bridges in peptide regions rich in basic amino acids may have escaped detection. More specific proposals must await elucidation of those areas of the peptide chains of collagen near all points of interchain bridging as well as more direct information concerning the chemical structure of these cross-links.

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Evidence for the Linkage of a Disaccharide to Hydroxylysine in Tropocollagen
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