The Structure of the Disaccharide Unit of the Renal Glomerular Basement Membrane*

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SUMMARY

The disaccharide unit of the glomerular basement membrane has been studied in regard to its detailed structure and its linkage to the peptide portion.

Graded acid hydrolysis of glycopeptides containing this unit resulted in a more rapid release of glucose than galactose and indicated that glucose was in the external position. Isolation of a disaccharide consisting of glucose and galactose with galactose in the reducing position confirmed the sequence of the unit.

Studies by methylation, periodate oxidation, and galactose oxidase treatment of the isolated disaccharide, as well as of glycopeptides containing it, indicated that the glucose is linked glycosidically to C-2 of the galactose. The anomeric configuration of this linkage was shown to be α on the basis of studies with α- and β-glucosidases. These findings indicated that the isolated disaccharide was 2-O-α-D-glucopyranosyl-D-galactose.

Alkaline hydrolysis of glycopeptides or of the entire basement membrane permitted isolation of glucosylgalactosylhydroxylysine in 80 to 90% yield. Upon mild acid hydrolysis of this compound, glucose was released preferentially, and galactosylhydroxylysine was obtained in about 80% yield. Studies of N-acetylated glycopeptides indicated that the stability to acid of the galactosylhydroxylysine bond is markedly influenced by the charge on the α-amino group of the hydroxylysine.

Both α- and β-galactosidases were used to study the anomeric configuration of the galactosylhydroxylysine linkage. The β-galactosidase was effective in cleaving this bond, but only when the galactosylhydroxylysine was in its N-acetylated form.

These studies are consistent with the following structure for the disaccharide unit and its peptide attachment: 2-O-α-D-glucopyranosyl-0-β-D-galactopyranosylhydroxylysine.

Measurement of the glucosylgalactosylhydroxylysine content of the basement membrane could be performed directly on the amino acid analyzer after alkaline hydrolysis of the basement membrane. Such analyses indicated that there are 14.5 disaccharide units per 100 mg of the basement membrane (17.3/1000 amino acid residues). All of the glucose of the basement membrane occurs in the form of this hydroxylysine-linked disaccharide unit. The disaccharide units could be removed from the peptide chain by a single Smith degradation.

Studies reported in a previous communication indicated that the carbohydrate of the bovine renal glomerular basement membrane is about equally distributed between two types of carbohydrate units (1). One of these is a branched heteropolysaccharide unit which is attached to the peptide chain through asparagine. The other is a disaccharide consisting of glucose and galactose, which is linked glycosidically to the hydroxyl group of hydroxylysine.

It was the purpose of the present investigation to determine the detailed structure of the disaccharide unit in regard to sequence and linkages. This information was obtained from studies involving graded acid hydrolysis, methylation, periodate oxidation, and enzymatic degradation.

Important to the elucidation of the structure of this unit was the characterization of three fragments isolated from the basement membrane glycopeptides. These compounds were shown to be a glucosylactoate disaccharide (2-O-α-D glucopyranosyl-β-D-galacto- pyranosylhydroxylysine), the internal sugar of this disaccharide attached to hydroxylysine (2-O-α-D glucopyranosyl-β-D-galactopyranosylhydroxylysine), and the internal sugar of this disaccharide attached to hydroxylysine (0-β-D galactopyranosylhydroxylysine).

EXPERIMENTAL PROCEDURE

Preparation of Glomerular Basement Membrane—Glomerular basement membranes were prepared from bovine renal cortex as previously described (2).
Preparation of Glycopeptides Containing Disaccharide Unit—The glycopeptides were obtained by collagenase and Pronase digestion of the glomerular basement membrane, followed by gel filtration of Sephadex G-25, as previously reported (1). The glycopeptide fraction which was studied contained glucose, galactose, and hydroxylysine in the molar ratios of 1:1:1.

N-Acetylation of Glycopeptides and Basement Membrane—For N-acetylation, the glycopeptides were dissolved in 4.5 mM sodium acetate at a concentration of about 3 μmoles per ml. Acetic anhydride was added to the sample at room temperature in five equal portions over a period of 1 hour. This reagent was used in a 25-fold excess over the available amino and hydroxyl groups present. The reaction was terminated by diluting the sample with 20 volumes of water and heating in a boiling water bath for 10 min. The samples were freed of sodium ions by passage through columns of Dowex 50-X16, 20 to 50 mesh, H+ form, used in 4-fold excess. The effluent and washed from these columns were lyophilized. Hexose determinations indicated full recovery of the glycopeptides. The completeness of N-acetylation was shown by the absence of color in the ninhydrin reaction (3).

For the N-acetylation of the intact basement membrane, 15 mg of the membrane were suspended per ml of the 4.5 mM sodium acetate. A 50-fold excess of acetic anhydride was added, and the samples were shaken during the period of the reaction. The excess reagent was removed by extensive washing with water by centrifugation. The samples were dried by lyophilization.

Graded Acid Hydrolysis—To determine the rate of release of the glucose and galactose, the glycopeptides (0.25 μ mole per ml) were hydrolyzed in 0.1 N sulfuric acid in sealed tubes at 10os for varying periods of time. The hydrolysates were passed through coupled columns of Dowex 50-H+ and Dowex 1-formate (4). The effluent from these columns contained the released sugars and was analyzed by quantitative paper chromatography (4).

Isolation of Disaccharide from Partial Acid Hydrolysates—A disaccharide which migrated with an Rf value of 1.35 on paper chromatography in 1-butanol-ethanol-water (10:1:2) was observed in hydrolysates of N-acetylated glycopeptides. For the purpose of isolating this compound, N-acetylated glycopeptides containing the disaccharide unit were hydrolyzed for 6 hours at a concentration of 0.4 μ mole per ml in 0.1 N sulfuric acid at 100o in sealed tubes. The hydrolysates were passed through coupled columns of Dowex 50-H+ and Dowex 1-formate (4). The effluent and wash from these columns were lyophilized and then chromatographed as a streak on washed Whatman No. 1 paper in 1-butanol-ethanol-water (10:1:2) for 120 hours. The area of the disaccharide was located by stained guide strips and eluted with water.

Digestion with Glycosidases—Several glycosidases were used in this study, and they were incubated under the following conditions.

α-Glucosidase (amyloglucosidase) from Aspergillus niger (5) was a gift of Dr. J. H. Pazur. Incubations with this enzyme were carried out in 0.2 M sodium acetate buffer at pH 5.0 at 37o. The concentration of the substrate was 1.5 μ moles per ml, and the enzyme was present at 0.5 mg per ml.

β-Glucosidase from almond (Mann) was incubated with the substrate under the same conditions as described for the α-glucohydrolase.

β-Galactosidase from Escherichia coli (6) was kindly made available by Dr. F. J. Reithel. The substrate was incubated with this enzyme at 37o in 0.05 M potassium phosphate at pH 7.0 in the presence of 0.01 M MgSO4. The concentration of the substrate was 1.5 μ moles per ml, and the enzyme was present at 0.7 mg per ml.

α-Galactosidase was prepared from green coffee beans (Santos) (7). About 1.5 μ moles of substrate per ml were incubated with this enzyme in 0.2 M sodium acetate buffer at pH 5.0 at 37o, and the enzyme was present at a concentration of 1 mg per ml.

All of the enzymatic digestions were accompanied by controls containing the enzyme or the substrate separately. Small amounts of toluene were added to prevent bacterial growth.

The anomeric specificity of the glucosidase was tested with kijibiose (2-O-α-D-glucopyranosyl-β-D-glucose), a gift of Dr. K. Matsuda, and sophorose (2-O-β-D-glucopyranosyl-β-D-glucose), kindly made available by Dr. W. L. Wolfgram.

The galactosidases were tested with melibiose (6-O-α-D-galactopyranosyl-β-D-glucose) and lactose (4-O-β-D-galactopyranosyl-β-D-glucose).

At the end of the incubations, the released mono-saccharides were separated from the enzyme and the remainder of the substrate by passage through small columns of charcoal-Celite, and the samples were lyophilized.

The concentration of the substrate was 1.5 μ moles per ml.

The galactose oxidase was present at a concentration of 0.8 mg per ml, and the catalase at 0.04 mg per ml. Digestions were accompanied by controls containing either the enzyme or the substrate separately. At the end of the incubation, the digest was hydrolyzed in 2 N sulfuric acid at 100o for 4 hours, and the hydrolysate was passed through coupled columns of Dowex 50-H+ and Dowex 1-formate (5). The effluent and wash from these columns were analyzed for galactose by quantitative paper chromatography.

Alternatively, the amount of galactose destroyed was estimated by measuring the content of Glc-Gal-Hyl present in the sample after hydrolysis in 2 N sodium hydroxide at 105o for 24 hours.

Periodate Oxidation—Periodate oxidation followed by sodium borohydride reduction of the oxidized product was carried out as previously described (1). The reagents were removed from the oxidized and reduced basement membrane by dialysis against 0.1 N NaCl, followed by distilled water. The oxidized and reduced glycopeptides were desalted by adsorption on columns of Dowex 50-X2, 200 to 400 mesh, H+ form. After washing with water, they were eluted from the columns with 1.5 N NH4OH and the ammonia was removed by lyophilization. Alternatively, the iodate was removed by passage through Dowex 1-X8, 20 to 50 mesh, formate form, and the boric acid was volatilized as methyl borate (1).

For the serial periodate oxidation, two Smith modifications were performed (9, 10). The oxidized and reduced basement membrane or glycopeptides were hydrolyzed in 0.1 N HCl at 50o for 2 hours. The hydrolysate was neutralized with sodium hydroxide, and the periodate oxidation and sodium borohydride reduction were repeated.

The abbreviations used are: Glc-Gal-Hyl, glucosylgalactosylhydroxylysine; Gal-Hyl, galactosylhydroxylysine.
The oxidized and reduced samples were analyzed for neutral sugars and alcohols after hydrolysis in 2 N sulfuric acid at 100° for 4 hours (10). The Glc-Gal-Hyl content of the treated samples was measured after alkaline hydrolysis.

Methylation—N-Acetylated glycopeptides containing the disaccharide unit were treated with methyl iodide in the presence of methylsulfanyl carbamion by the method of Hakamori (11), performed in the following manner. Approximately 5 μmoles of dry glycopeptides were dissolved in 0.5 ml of dimethylsulfoxide and stirred at room temperature under nitrogen. The methylsulfanyl carbamion was prepared by the method of Corey and Chaykovsky (12), and 0.4 ml of a freshly prepared 1 mM solution was rapidly added to the sample. The mixture was stirred at room temperature under nitrogen for 1 hour. Methyl iodide (0.5 ml) was then added to the reaction mixture, and stirring was continued at room temperature for 2 hours. The reaction was terminated by dilution with water. After acidification with HCl and the addition of NaCl to a concentration of 5 M, the methylated product was extracted into chloroform. The pooled chloroform extracts were washed with small quantities of water and then evaporated to dryness.

The methylated glycopeptides were hydrolyzed in 2 N sulfuric acid for 4 hours at 100°, and the hydrolysate was passed through coupled columns of Dowex 50-H⁺ and Dowex 1-formate. These columns were washed with 10 column volumes of 20% methanol, and the effluent and wash which contained the methylated hexose were taken to dryness in a vacuum rotator.

For the identification of the methylated sugars, the following methods of separation were used: paper chromatography in 1-butanol saturated with water; thin layer chromatography on silica gel in acetone-water-concentrated ammonia (250:3:1.5) in the system of Stoffyn (13); and paper electrophoresis in 0.2 M borate buffer, pH 10, at 750 volts in a water-cooled horizontal strip electrophoresis apparatus (14). The methylated sugars were located with the aniline hydrogen phthalate reagent (15). The trimethyl ether derivatives of galactose used as standard were kindly made available by Dr. P. J. Stoffyn; the 2,3,6-tri-O-methylgalactose was a preparation of Dr. R. W. Jeanloz.

Preparation of Glc-Gal-Hyl—This compound was isolated from alkaline hydrolysates of either glycopeptides or the entire basement membrane. The glycopeptides were hydrolyzed in 2 N NaOH at a concentration of about 5 μmoles per ml at 105° for 20 hours in capped polypropylene tubes. The hydrolysate was neutralized with an equivalent amount of HCl and subjected to gel filtration on Sephadex G-15. This gel was packed to a height of 80 cm in glass columns 2.1 cm in diameter fitted with sintered glass plates, and was equilibrated with 0.1 M pyridine-acetate buffer, pH 5.0. Samples containing 13 to 20 μmoles of the original glycopeptides were placed on the column in approximately 6 ml. Elution was achieved with the same buffer at the rate of 0 ml per hour, and fractions of 5 ml were collected. Aliquots of the fractions were analyzed by the ninhydrin (3) and anthrone (4) reactions. The tubes making up a peak were pooled, and the pyridine-acetate was removed by lyophilization.

The intact basement membrane was hydrolyzed in 2 N NaOH at 105° for 24 hours at a concentration of 15 mg per ml. The hydrolysates were acidified and placed on columns of Dowex 50 X4, 300 to 400 mesh, H⁺ form, containing 8 times the equivalents of the sodium hydroxide used in the hydrolysis. After extensive washing of the column with water, elution was carried out with 10 to 12 column volumes of 1.5 N NH₄OH. The ammonia was removed by lyophilization. This ammonia eluate was salt-free and contained all of the amino acids, as well as the hydroxylysine-bound carbohydrate. The Glc-Gal-Hyl was separated from the amino acids by gel filtration on Sephadex G-15 as described for the alkaline hydrolysate of the glycopeptides.

Preparation of Gal-Hyl—The isolated Glc-Gal-Hyl was hydrolyzed with 0.1 N sulfuric acid for 28 hours in sealed tubes at 100° at a concentration of 0.3 μmole per ml. The Glc-Hyl was separated from the released glucose by adsorption on Dowex 50 and eluted from the column with ammonia, as described for the preparation of the Glc-Gal-Hyl.

Carbohydrate Analyses—The monosaccharides were identified, separated, and estimated as previously described (2, 4).

Amino Acid Analyses—Amino acid analyses were performed with the Technicon amino acid analyzer after hydrolysis in constant boiling HCl in sealed tubes at 105° for 28 hours.

Measurement of Glc-Gal-Hyl—For the estimation of Glc-Gal-Hyl and unsubstituted hydroxylysine, alkaline hydrolysates were placed on the amino acid analyzer after neutralization with HCl.

Hydrolysis in 2 N sodium hydroxide at 105° for 24 hours of an amino acid mixture, as well as of isolated Glc-Gal-Hyl, indicated that the extent of destruction of leucine, Glc-Gal-Hyl, and hydroxylysine was similar and that under these conditions approximately 85% of these compounds was recovered. For this reason measurements of Glc-Gal-Hyl and unsubstituted hydroxylysine were related to the weight of original basement membrane on the basis of the amount of leucine in the sample and the known content of that amino acid in the basement membrane.

Sodium Borohydride Reduction of Disaccharide—For the determination of the reducing sugar of the isolated disaccharide, sodium borohydride reduction was performed, followed by actid hydrolysis and paper chromatography, as previously described (16).

Mild Alkaline Treatment of Disaccharide—The isolated disaccharide, as well as various disaccharide standards, was treated with 0.1 N NaOH at 37° at a concentration of 0.5 μmole per ml for various periods of time. The hydrolysate was neutralized with an equivalent amount of acetic acid and desalted by passage through Dowex 50-H⁺. After lyophilization of the effluent and wash, the extent of destruction of the disaccharide was evaluated by paper chromatography.

RESULTS

Graded Acid Hydrolysis—The release of glucose and galactose from glycopeptides containing the disaccharide unit during hydrolysis in 0.1 N sulfuric acid at 100° is shown in Fig. 1. It may be noted that glucose was released at a considerably more rapid rate than galactose, suggesting that it is the sugar in the external position. After 24 hours 79% of the galactose had been liberated. After 24 hours 79% of the glucose had been released, while only 8% of the galactose was liberated.

In order to evaluate the influence of the α-amino group of the hydroxylysine on the hydrolysis by acid of the glycosidic bond of this disaccharide unit, N-acetylated glycopeptides were hydrolyzed under the same conditions as the native glycopeptides. It may be noted from Fig. 2 that there was a markedly increased rate of release of galactose from the N-acetylated glycopeptides. At 11 hours, for example, 48% of the galactose had been liberated from the N-acetylated glycopeptides, compared to only 4% from the native compounds. Even glucose was released at a somewhat more rapid rate from the acetylated glycopeptides. These
findings would suggest that the presence of a positive charge on
the ε-amino group of the hydroxylysine hinders the cleavage by
acid of the O-glycosidic bond adjacent to it. However, both the
glucose and galactose could be completely released by hydrolysis
of the native glycopeptides in 2 N sulfuric acid at 100° for 4 hours.

Isolation of Disaccharide—Paper chromatography of the
neutral sugar fraction from graded acid hydrolysis of the N-
acetylated glycopeptides indicated the presence, in addition to
the glucose and galactose, of a compound migrating to the re-
gion of a disaccharide (R_Lac 1.35) in butanol-ethanol-water
(10:1:2) (Fig. 3). This compound was present in maximal
amounts after hydrolysis of the N-acetylated glycopeptides in
0.1 N sulfuric acid for 6 hours, and was isolated in an average
yield of 14% after that length of hydrolysis.

Only trace amounts of this disaccharide were evident at any
time during graded acid hydrolysis of the native glycopeptides
(Fig. 3).

Characterization of Disaccharide—Chromatography of the
isolated disaccharide revealed a single spot in butanol-ethanol-
water (10:1:2) (R_Lac 1.35, R_Glc 0.29) and in pyridine-ethyl
acetate-water-acetic acid (5:5:3:1) (R_Lac 1.08, R_Glc 0.68).
Paper electrophoresis in 0.2 M borate buffer at pH 10 revealed a
single component which migrated at 0.24 the rate of glucose.

Acid hydrolysis of the isolated compound (2 N sulfuric acid,
4 hours, 100°) produced equimolar amounts of glucose and galac-
tose.

After reduction of the compound with sodium borohydride
followed by acid hydrolysis, the galactose was no longer present,
and only glucose and galactitol were detected by paper chromatography. This indicated that galactose is on the reducing end of this compound, which is therefore a glucosylgalactose disaccharide.

Reaction of the disaccharide (0.50 μmole) with the triphenyltetrazolium reagent (17) gave no color, suggesting that the linkage of the glucose is to C-2 of galactose. The (1 → 2)-linked disaccharides, koihibiose and sophorose, also failed to give color in this reaction, whereas substances in which the linkage is to C-3, C-4, or C-6 of the reducing sugar gave strong color when tested in the amounts of 0.1 to 0.2 μmole.

It was also noted that the disaccharide from the basement membrane stained relatively weakly on paper with the silver reagent (18) when compared to disaccharides in which the linkages were to C-4 or C-6 of the reducing sugar. Its staining intensity was comparable to that of koihibiose and sophorose.

Treatment of the disaccharide from the basement membrane for 16 hours in 0.1 N NaOH at 37° resulted in a 90% recovery of the intact disaccharide. Under similar conditions, 75% of the koihibiose remained undegraded. In contrast, 3-O-methylglucose, lactose, and melibiose were completely destroyed. The resistance of the basement membrane disaccharide to this treatment is consistent with a (1 → 2) linkage, in view of the known greater stability of this type of glycosidic bond to alkali (19).

These findings on the disaccharide are consistent with information obtained from periodate, galactose oxidase, and methylation studies, to be reported in later sections of this paper, which indicated that the linkage of glucose to galactose is through a (1 → 2)-glycosidic bond.

**Determination of Anomeric Configuration of Glucosylgalactose Linkage**—Incubation of the isolated glucosylgalactose disaccharide from the basement membrane with α-glucosidase from A. niger for 36 hours resulted in its complete cleavage into glucose and galactose. Under the same conditions, the α-linked koihibiose was 90% cleaved, in contrast to only 3% of the β-linked sophorose. While this enzyme worked effectively on the free disaccharide, it did not release any glucose from the disaccharide linked to peptide or to hydroxylsine alone, even after prolonged incubation. The following compounds were not acted upon by this α-glucosidase: the intact basement membrane; N-acetylated basement membrane; basement membrane solubilized with mild alkali; glycopeptides containing the disaccharide; N-acetylated glycopeptides containing the disaccharide; Glc-Gal-Hyl; and N-acetylated Glc-Gal-Hyl.

Incubation of the glucosylgalactose disaccharide from the basement membrane with almond β-glucosidase for 8 hours failed to cleave this compound. Under the same conditions, the β-linked sophorose was completely cleaved, while the α-linked koihibiose was not hydrolyzed at all. None of the above mentioned substrates containing the basement membrane disaccharide attached to peptide material was acted on by the β-glucosidase.

**Periodate Oxidation**—When the glycopeptides containing the disaccharide unit were oxidized with periodate, there was a rapid and complete destruction of both the glucose and galactose (Table I). After reduction of the oxidized product with sodium borohydride, glycerol was the only alcohol obtained upon acid hydrolysis. The complete destruction of the galactose with the formation of glycerol but no threitol indicated that the glucose is linked to the galactose at either C-2 or C-6.

Periodate oxidation of the isolated glucosylgalactose disaccharide for 20 hours followed by sodium borohydride reduction also showed the complete destruction of glucose and galactose, with the formation of glycerol.

Although the intact basement membrane contains heteropoly-saccharide units in addition to the disaccharides (1), the effect of periodate oxidation on the latter could be specifically evaluated by measuring the amount of Glc-Gal-Hyl obtained after alkaline hydrolysis of the periodate-oxidized membrane.

**Table I**

<table>
<thead>
<tr>
<th>Step*</th>
<th>Length of oxidation</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Hydroxylysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>1</td>
<td>38</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>21</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>27</td>
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<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Second</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

* Each step consists of a Smith degradation performed as described in the text.

**Table II**

Effect of serial periodate oxidation on hydroxylsine and hydroxylsine-bound glucosylgalactose units of intact glomerular basement membrane

<table>
<thead>
<tr>
<th>Step*</th>
<th>Length of oxidation</th>
<th>Glucosylgalactose unitsa</th>
<th>Hydroxylysine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
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<td>13.8</td>
<td>18.7</td>
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<tr>
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<td>27</td>
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<td></td>
<td>46</td>
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<td>72</td>
<td>0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Each step consists of a Smith degradation performed as described in the text.

a Determined on the amino analyzer after alkaline hydrolysis.

b Determined on the amino analyzer after acid hydrolysis.
Disaccharide Unit of Glomerular Basement Membrane

Vol. 242, No. 20

FIG. 4. Paper chromatography of alkaline hydrolysate (2 N NaOH, 105°, 20 hours) of glycopeptides from the glomerular basement membrane after one Smith degradation (Spot 3). 2, unoxidized glycopeptides; 1 and 4, standards. The solvent system was 1-butanol-acetic acid-water (4:1:5) run for 100 hours, and the paper was stained with ninhydrin. The spot moving between Hyl and Lys in Samples 2 and 3 is ornithine.

Controls containing unoxidized glycopeptides subjected to this mild acid hydrolysis did not show such destruction of hydroxylysine on subsequent oxidation.

After mild acid hydrolysis of the periodate-oxidized, borohydride-reduced basement membrane, most of the remaining hydroxylysine residues became susceptible to periodate oxidation. Only about 18% of the hydroxylysine (3.3 amoles/100 mg) was recovered after the second step of the degradation performed on the intact membrane (Table II).

Galactose Oxidase Treatment—Incubation with galactose oxidase was performed in order to determine whether C-6 of the galactose in the disaccharide unit is unsubstituted. This enzyme has been shown to oxidize galactose at C-6 (20).

Incubation of galactose oxidase for 24 hours with the isolated glucosylgalactose disaccharide of the basement membrane resulted in complete destruction of the galactose but full recovery of the glucose. This indicated that C-6 of the galactose is unsubstituted and suggested, in view of the results of the periodate oxidation, that the linkage of the glucose is to C-2 of the galactose.

The ability of various other galactose-containing fragments from the disaccharide unit to react with galactose oxidase was also tested. After 48 hours of incubation, the galactose in the following substrates was destroyed to the extent of 80 to 100%: N-acetylated glycopeptides containing the disaccharide unit; Glc-Gal-Hyl, as well as its N-acetylated derivative; and Gal-Hyl and its N-acetylated derivative. The galactose of the intact basement membrane or the basement membrane solubilized by mild alkaline treatment was oxidized to a very small extent (15% destruction); there was, however, a greater degree of destruction of the galactose when the N-acetylated basement membrane was incubated (40% destruction).

Methylation—After methylation of the N-acetylated glycopeptides containing the disaccharide unit, essentially all of the glycopeptides could be extracted into chloroform. On paper chromatography of the neutral sugar fraction of the acid-hydrolyzed, methylated glycopeptides in 1-butanol saturated with water, two components were seen. One corresponded to 2,3,4,6-tetra-O-methylglucose, while the other had a migration of 0.625 that of the tetramethylglucose. No other components, such as unmethylated hexoses, could be detected. Thin layer chromatography of the methylated sugars, performed in the system of Stoffyn (13), showed the presence of two components, which corresponded to 2,3,4,6-tetra-O-methylglucose and either 2,3,4-tri-O-methyl- or 3,4,6-tri-O-methylgalactose (Fig. 5). Further identification of the galactose derivatives by paper electrophoresis indicated that the methylated basement membrane glycopeptides contained, in addition to the tetramethylglucose, a trimethylgalactose corresponding to the 3,4,6 derivative.

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Methylation—After methylation of the N-acetylated glycopeptides containing the disaccharide unit, essentially all of the glycopeptides could be extracted into chloroform. On paper chromatography of the neutral sugar fraction of the acid-hydrolyzed, methylated glycopeptides in 1-butanol saturated with water, two components were seen. One corresponded to 2,3,4,6-tetra-O-methylglucose, while the other had a migration of 0.625 that of the tetramethylglucose. No other components, such as unmethylated hexoses, could be detected. Thin layer chromatography of the methylated sugars, performed in the system of Stoffyn (13), showed the presence of two components, which corresponded to 2,3,4,6-tetra-O-methylglucose and either 2,3,4-tri-O-methyl- or 3,4,6-tri-O-methylgalactose (Fig. 5). Further identification of the galactose derivatives by paper electrophoresis indicated that the methylated basement membrane glycopeptides contained, in addition to the tetramethylglucose, a trimethylgalactose corresponding to the 3,4,6 derivative.
FIG. 6. Paper electrophoresis of sugars from methylated glycopeptides of basement membrane (B-M). Electrophoresis was performed in 0.2 M borate buffer at pH 10 at 20 volts per cm for 24 hours. Me-, methyl.

Fig. 7. Gel filtration on Sephadex G-15 of alkaline hydrolysate (2 N NaOH, 105°, 20 hours) of glycopeptides from basement membrane containing 14 moles of disaccharide unit. The elution diagram shows hexose determined by the anthrone method, and peptide and amino acids by ninhydrin. Column size was 2.1 x 80 cm. Elution was performed with 0.1 M pyridine-acetate buffer, pH 5.0. Tubes 29 to 32 were pooled for the isolation of Glc-Gal-Hyl.

(Fig. 6). These findings are consistent with the glycosidic linkage of the glucose to position 2 of the galactose, as was indicated by the periodate and galactose oxidase studies.

Isolation of Glc-Gal-Hyl from Glycopeptides—Because of the stability to alkali of the O-glycosidic linkage to hydroxylysine, it was possible to isolate in very high yield the disaccharide unit still attached to this amino acid after alkaline hydrolysis of the glycopeptides. Gel filtration of such a hydrolysate on Sephadex G-15 (Fig. 7) clearly separated the carbohydrate from the free amino acids. An average of 90% of the hexoses present in the original glycopeptide was obtained in the carbohydrate peak (tubes 29 through 32). Chromatography of an aliquot of the carbohydrate peak on the amino acid analyzer (Fig. 8, upper frame) indicated the presence of one major component, which emerged in this system in a position corresponding to that just following the methionine in a standard amino acid run. Small amounts (less than 0.05 mole per mole of Glc-Gal-Hyl) of Gal-Hyl and hydroxylysine were also present in this Sephadex peak.

Paper chromatography in butanol-acetic acid-water (4:1:5) (Fig. 9, Number 9) showed the presence of one component migrating with an R_H value of 0.32. Chromatography in pyridine-ethyl acetate-acetic acid-water (5:5:3:1) showed one spot with an R_H value of 0.65. Electrophoresis at pH 3.5 indicated a component migrating to the cathode at a rate 0.60 that of hydroxylysine.

Analysis, after acid hydrolysis, of the material in the Sephadex G-15 peak gave the following results, expressed as molar ratios to hydroxylysine: glucose, 1.02, and galactose, 1.08. In addition, trace amounts of lysine and ornithine were present. These
FIG. 9. Migration on paper chromatography of Glc-Gal-Hyl and Gal-Hyl. 1, 2, and 3, standards; 4, aliquot of carbohydrate-containing peak (tubes 29 to 32) from Sephadex G-15 column (Fig. 7); 4, Glc-Gal-Hyl after partial acid hydrolysis (0.1 N H₂SO₄, 100°C, 28 hours). The solvent system was 1-butanol-acetic acid-water (4:1:5), run for 100 hours. The paper was stained with ninhydrin.

Preparation of Gal-Hyl—When the isolated Glc-Gal-Hyl was hydrolyzed with 0.1 N sulfuric acid for 28 hours at 100°C, the glucose was released preferentially and Gal-Hyl was obtained in high yield by adsorption on and elution from Dowex 50. Fig. 8 (lower frame) shows the position of elution on the amino acid analyzer of the component obtained after this acid hydrolysis. It was well separated from the Glc-Gal-Hyl, and emerged in a position corresponding to that between tyrosine and phenylalanine.

This column chromatography indicated the presence, in addition to the Gal-Hyl, of 0.08 mole of Glc-Gal-Hyl and 0.15 mole of free hydroxylysine per mole of Gal-Hyl. This indicated that about 80% of the Glc-Gal-Hyl had been converted to Gal-Hyl by the mild acid treatment.

Paper chromatography in 1-butanol-acetic acid-water (4:1:5) (Fig. 9, Number 4) showed a component migrating with an R₅₆ value of 0.69. After further purification of the Gal-Hyl by preparative paper chromatography in the 1-butanol-acetic acid-water system, it contained only galactose and hydroxylysine in equimolar amounts.

The Gal-Hyl, when chromatographed in pyridine-ethyl acetate-water-acetic acid (5:5:3:1), gave a single spot with an R₅₆ value of 0.72, while on paper electrophoresis at pH 3.5 it moved toward the cathode ahead of the Glc-Gal-Hyl at 0.73 the rate of hydroxylysine.

Anomeric Configuration of Gal-Galhydroxylysine Linkage—Incubation of the Gal-Hyl with α-galactosidase for as long as 130 hours failed to release any galactose, although melibiose was completely and rapidly cleaved by this enzyme. Nor could release of galactose be achieved when N-acetylated Gal-Hyl was incubated with the enzyme. The enzyme was also inactive on peptides containing Gal-Hyl, whether their amino groups were free or in the N-acetylated form.

When Gal-Hyl or peptides containing Gal-Hyl were incubated with β-galactosidase for 96 hours, no release of galactose was observed. However, a slow release of galactose by this enzyme was achieved from N-acetylated Gal-Hyl or N-acetylated peptides containing Gal-Hyl. After 96 hours approximately 20% of the galactose was released, while at 144 hours 40 to 45% of this sugar was liberated. This β-galactosidase cleaved lactose rapidly but did not cleave melibiose, even after prolonged incubation.

Measurement and Isolation of Glc-Gal-Hyl from Intact Basement Membrane—After alkaline hydrolysis it was possible to measure and isolate the Glc-Gal-Hyl present in the entire basement membrane without the necessity of prior proteolytic digestion to obtain glycopeptides. Fig. 10 shows the elution diagram from the amino acid analyzer of the alkaline hydrolysate of the entire basement membrane. The large component appearing between the methionine and alloisoleucine was identified as Glc-Gal-Hyl. This compound could also be demonstrated by paper chromatography of the desalted alkaline hydrolysate of the entire basement membrane. The large amount of

![Fig. 10. Segment of an elution diagram from an alkaline hydrolysate (2 N NaOH, 24 hours, 105°C) of entire glomerular basement membrane on the amino acid analyzer (Technicon). The component appearing between the methionine and alloisoleucine was identified as Glc-Gal-Hyl.](http://www.jbc.org/)
Glc-Gal-Hyl and the much smaller amount of unsubstituted hydroxylysine present in the basement membrane are evident from this chromatogram.

Quantitative estimation on the amino acid analyzer of the Glc-Gal-Hyl in the basement membrane gave an average value of 14.5 ± 0.1 µmoles/100 mg in four analyses. The average free hydroxylysine measured in the alkaline hydrolysate was 4.4 µmoles/100 mg. This represents a total hydroxylysine content of 18.9 µmoles/100 mg, which is close to the value of 18.7 µmoles/100 mg determined after acid hydrolysis (2).

For the isolation of Glc-Gal-Hyl from the alkaline hydrolysate of the entire basement membrane, the material adsorbed on and eluted from Dowex 50 was subjected to gel filtration on Sephadex G-15 (Fig. 12). The major hexose peak (tubes 27 to 31) was well separated from the large amount of amino acids present in this hydrolysate. The material in this peak represented hexoses equivalent to 11.5 µmoles of disaccharide units per 100 mg of hydrolyzed basement membrane. Analyses indicated that 97% of this carbohydrate was present as Glc-Gal-Hyl, with the remainder occurring as Gal-Hyl. Only trace amounts of free amino acids were present.

In the hydroxylysine-bound carbohydrate obtained by gel filtration from either the glycopeptides or the entire basement membrane after alkaline hydrolysis, small amounts of Gal-Hyl were present (Fig. 8, upper frame). In no case did the Gal-Hyl represent more than 5% of the total hydroxylysine-bound carbohydrate units. It is possible that this small amount of Gal-Hyl was the result of the degradation of Glc-Gal-Hyl during alkaline hydrolysis. When purified Glc-Gal-Hyl was hydrolyzed with 2 N NaOH at 105° for 24 hours, a total of 15% of this compound was destroyed, with about 5% of the original appearing as Gal-Hyl.

**DISCUSSION**

The results of this investigation on the disaccharide unit of the glomerular basement membrane and its peptide attachment are consistent with the structure 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine, shown in Fig. 13. This carbohydrate unit consists of a glucosylgalactose disaccharide in which a glucose residue is linked by an α-glycosidic linkage to C-2 of the galactose. This sugar in turn is attached by a β-glycosidic bond to the hydroxyl group of hydroxylysine.

This structural formulation was arrived at by studies carried out on the disaccharide unit attached to the peptide chain, as well as by the isolation and characterization of several fragments. These fragments were the disaccharide itself, the disaccharide attached to the amino acid involved in the glycopeptide bond, and the internal sugar of the disaccharide linked to this amino acid.

Graded acid hydrolysis, as well as the isolation of the glucosylgalactose disaccharide, clearly established the sequence of the sugars in this unit. The periodate oxidation studies limited the possible attachment of the glucose to galactose to either C-2.
Galactose oxidase treatment resulted in the destruction of the galactose, indicating that C-6 of the galactose was unsubstituted and that C-2 must be involved in the linkage.

A (1 → 2)-glycosidic linkage between the glucose and the galactose was further suggested by the results of methylation, which yielded only two products, which were shown to be 2,3,4,6-tetra-O-methylglucose and 3,4,6-tri-O-methylgalactose. The linkage of the internal sugar of this disaccharide to hydroxylysine was demonstrated by the isolation of Gal-Hyl in high yield. This is consistent with previous studies with dinitrophenylation and periodate oxidation, which had indicated that the disaccharide units are linked to the hydroxyl groups of hydroxylysine by an alkali-stable bond (1).

The occurrence of a glycosidic linkage adjacent to a free amino group, as in the attachment of this unit to the peptide chain, is an unusual occurrence in nature. The amino groups of hexosamines in glycoproteins, mucopolysaccharides, and glycolipids are believed to be always substituted. The graded acid hydrolysis studies of the native and N-acetylated glycopeptides showed the pronounced influence that this charged amino group has on the stability of the glycosidic bond adjacent to it. While the glycosidic linkage of the galactose to the hydroxylysine was considerably more resistant to acid hydrolysis than that of a more conventional glycosidic bond, such as the glucosylgalactose bond of this unit, it did not exhibit the very great stability to acid which has been reported for hexosaminides (21, 22), in which the charged amino group is on the glycosyl moiety itself, rather than on the aglycone. It is of interest that when the glycosidic bond between the galactose and the hydroxylysine had been converted to an acetal during a Smith degradation, the linkage to this amino acid was cleaved under conditions of very mild acid hydrolysis.

The isolation of 2-O-α-D-glucopyranosyl-1-D-galactose adds a new disaccharide to those which have been obtained from natural sources. Chemical synthesis of this compound has been reported (23). It is of interest that in glycolipids and milk oligosaccharides, where glucose and galactose also occur together (24), their sequence is the reverse of that found to exist in the basement membrane disaccharide.

Convincing demonstration of a carbohydrate-peptide linkage depends to a large extent on the isolation of a complex containing only the sugar and amino acid involved in the bond. This has been achieved in only a few cases (25, 26). From the basement membrane, it was possible to obtain such a complex, that is, Gal-Hyl, in very high yield. This was due to the fact that the bond between galactose and hydroxylysine was more stable to alkali than the peptide bonds. In addition, it was more resistant to cleavage by acid than the bond linking the external glucose to the galactose.

Several sugar-amino acid complexes have previously been reported. These include N-acetylglucosamine linked to asparagine (25), xylose linked to serine (26), and N-acetylgalactosamine attached to either serine or threonine (27). The finding of galactose linked to hydroxylysine therefore represents another such sugar-amino acid combination.

Serial periodate oxidation with four Smith degradations has previously been performed on the relatively large carbohydrate units of fetuin (10), leaving only 2 sugar residues still attached to the peptide chain. When this technique was applied to the disaccharide units of the basement membrane, it resulted after one degradation in the release of the entire carbohydrate unit, with the unentwining of the hydroxylysine to which it was attached.

The good separation on the amino acid analyzer of the Glc-Gal Hyl from the amino acide present in alkaline hydrolysates has made possible a convenient method for obtaining quantitative data on the number of glucosylgalactosyl units present in the entire basement membrane. These measurements indicated that there are 14.5 μmoles of Glc-Gal-Hyl per 100 mg of basement membrane (17.3/1000 amino acid residues), which represent approximately 77% of the hydroxylysine residues of the membranes. This is in agreement with the periodate oxidation studies, which indicated that about 70% of the hydroxylysine (13.1 μmoles/100 mg of basement membrane) are involved in this linkage (1). Moreover, since there are 13.7 μmoles of glucose per 100 mg of basement membrane (2), these analyses again show that this sugar occurs in the membrane uniquely as a component of the disaccharide units.

Butler and Cunningham (28) have isolated from the alkaline hydrolysate of guinea pig skin tropocollagen a disaccharide attached to hydroxylysine. Although the structure of this compound has not yet been reported, it is likely that it is similar to the disaccharide present in the basement membrane.

Studies carried out in our laboratory on several vertebrate collagens, including calf skin tropocollagen, Ichtiochoc, and bovine tendon collagen, have indicated that all these proteins also contain glucosylgalactosyl disaccharide units and, in addition, substantial amounts of single galactose residues linked to hydroxylysine (Reference 29 and footnote to title). Small amounts of Gal-Hyl were also shown to be present in the glomerular basement membrane, which represented a maximum of 5% of the total hydroxylysine-bound carbohydrate units. Control studies indicated, however, that this small amount of Gal-Hyl could have been due to degradation during alkaline hydrolysis of Glc-Gal-Hyl.

The presence of essentially all of the hydroxylysine-bound carbohydrate in the form of glucosylgalactosyl disaccharide units is consistent with the previous report that all of the hydroxylysine-containing glycopeptides obtained from the basement membrane had glucose and galactose in a molar ratio of unity (1).
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