THE CARBOHYDRATE MOIETY OF SEA CUCUMBER (*Thyone briareus*) GELATIN*

GLYN P. ROBERTS†, ANIL BHATTACHARYYA‡, AND ROGER W. JEANLOZ§

Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and the Massachusetts General Hospital, Boston, Massachusetts 02114 (U. S. A.)

(Received July 5th, 1972; accepted July 13th, 1972)

ABSTRACT

A fraction of the gelatinized collagen obtained from the body wall of the echinoderm *Thyone briareus*, and that contained, as the sole sugar components, D-glucose and D-galactose, was degraded with trypsin and Pronase, and by alkaline hydrolysis, to give an oligosaccharide–amino acid. This was studied by partial hydrolysis with acid, enzymic degradation, periodate degradation, methylation, and *N*-(dinitrophenyl)ation, and shown to be 5-[*O*-α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyloxy]-L-lysine.

INTRODUCTION

Vertebrate collagens from such typical sources as calf skin or carp swim-bladder are glycoproteins that contain a small proportion of sugars consisting almost exclusively of glucose and galactose[2]. On the other hand, invertebrate collagens are reported to contain a wide spectrum of firmly bound sugar moieties, including glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, and uronic acids[3]. Recent studies have shown that the gelatin obtained from the collagen of the sea cucumber *Thyone briareus* can be separated into three fractions having different compositions[4,5]. Two of these fractions are collagens, and contain glucose and galactose as the only sugars present. In view of the difference in structure and composition of the polysaccharides found in the amorphous, connective-tissue matrix, it was of interest to ascertain whether the D-galactose and D-glucose residues of invertebrate collagen are linked in a manner similar to that reported for vertebrate

*This is paper No. 11 in the series "Invertebrate Connective Tissue" and is publication No. 584 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital. This study was supported by Grant AM-03564 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service. It was presented in preliminary form at the Symposium on Glycosaminoglycans and Glycoproteins in Bochum (Germany), Sept. 1968 (ref. 1).

†Present address: A.R.C. Institute for Research on Animal Diseases, Compton, Newbury, Berks., England.

‡Present address: Indian Association for the Cultivation of Science, Jadavpur, Calcutta-32, India.

§To whom communications should be addressed.

collagen and for proteins, related to collagen, obtained from vertebrate basement-membranes[6-10], or in a manner similar to that reported for earthworm-cuticle collagen[11,12]. The present communication describes the characterization of the linkage of glucose to galactose, and of this disaccharide to a component of collagen.

RESULTS AND DISCUSSION

Preparation and proteolytic degradation of the gelatin. — The yields, and hexose content, of the gelatin fractions successively isolated from sea-cucumber body-wall by extraction with water are shown in Table I. The hexose content of the first two fractions was high, but, as these fractions were colored by pigments present in the

TABLE I

SUCCESSIVE EXTRACTION OF SEA-CUCUMBER CORIUM WITH WATER[a] FOR 15 MIN AT 123°

Extraction number	*Yield (g)*	*Hexose (%)*[b]
1	0.60	10.1
2	0.62	5.6
3	0.79	4.3
4	2.95	4.2
5	3.29	4.1
6	3.13	3.9
7	1.32	4.0
8	0.09	4.3

[a]Wet corium (162 g) was extracted with 100-ml portions of water. [b]Determined by the anthrone reaction; the value includes the content of fucose; % of dry weight.

epithelium, they were not used in this investigation. The other fractions were combined, and subjected to proteolytic degradation. Incubation with trypsin caused a rapid decrease of the viscosity of the solution and liberated 75 μmoles of amino groups per 100 mg of gelatin, as estimated by the ninhydrin test with leucine as the standard. An acidic heteropolysaccharide that accounted for 11% of the weight of the gelatin and 64% of the hexose content was separated by precipitation with cetylpyridinium chloride. The neutral glycopeptide fraction, isolated from the supernatant liquor of the tryptic digest, contained 3.2% of hexose, accounted for 33% of the hexose in the gelatin and for 41% of the weight of the gelatin, and contained 62 μmoles of amino groups per 100 mg of glycopeptide.

In order to obtain glycopeptides enriched in hexose components, further proteolytic digestion of the glycopeptides was performed with Pronase, which, in 72 h, released 211 μmoles of amino groups per 100 mg of glycopeptide. This material was fractionated on a column of Sephadex G-15 (see Fig. 1) to yield a glycopeptide (Glycopeptide P) which contained 9.7% of hexose consisting of equimolar amounts of glucose and galactose.

Glycopeptide P was not homogeneous, as shown by chromatography on *O*-

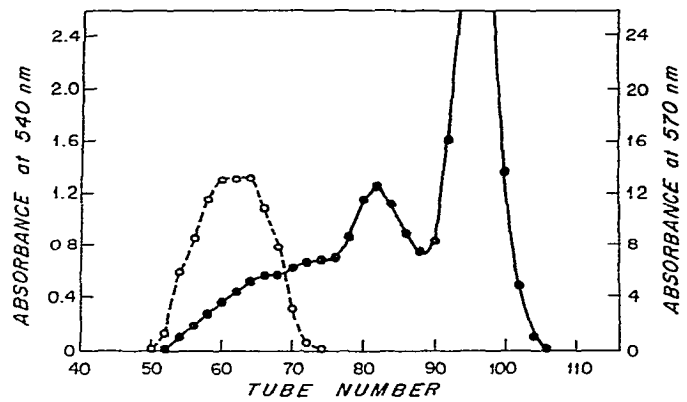

Fig. 1. Fractionation, on Sephadex G-15, of a Pronase digest of a fraction obtained by tryptic degradation of sea-cucumber gelatin (9 g), followed by precipitation with cetylpyridinium chloride. A solution of the digest in 0.1M pyridine acetate buffer (pH 5.0) was passed through a column (93 × 3.0 cm diam.), and 3.1-ml fractions were collected. Aliquots (0.1 ml) were analyzed at 540 nm by the orcinol (○) reagent, and at 570 nm by the ninhydrin (●) reagent.

(carboxymethyl)cellulose followed by examination of the fractions by paper electrophoresis, or by paper electrophoresis of Glycopeptide P itself.

Characterization of the carbohydrate–peptide linkage in Glycopeptide P. — In view of the difficulty of obtaining large amounts of a homogeneous glycopeptide, characterization of the hexose–hydroxylysine unit was performed both on Glycopep-

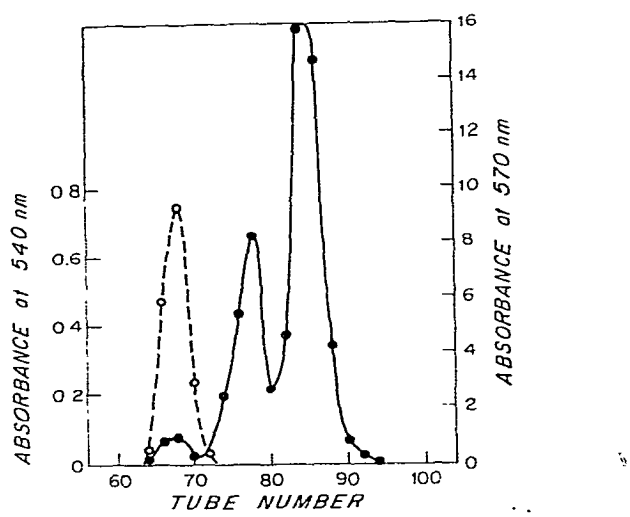

Fig. 2. Fractionation, on Sephadex G-15, of an alkaline hydrolyzate (2M sodium hydroxide, for 24 h at 105°) of Glycopeptide P (176 mg). A solution of the hydrolyzate in 0.1M pyridine acetate (pH 5) was passed through a column (93 × 3.0 cm diam.), and 3.1-ml fractions were collected. Aliquots (0.1 ml) were analyzed at 540 nm by the orcinol (○) reagent, and at 570 nm by the ninhydrin (●) reagent.

tide P and on a glucosylgalactosyl derivative of hydroxylysine obtained by alkaline degradation of this glycopeptide.

Alkaline hydrolysis[6] of Glycopeptide P, followed by gel filtration on Sephadex G-15, separated the hexose-containing material from free amino acids (see Fig. 2). Analysis of this material showed that glucose, galactose, and hydroxylysine were present in equimolar amounts, with negligible proportions of any other amino acid. The recovery of hexoses after this treatment was 80%, which is similar to that (85%) reported by Spiro[7] for the basement-membrane glycopeptide; further treatment with alkali under the same conditions removed a further 36% of the hexose content.

Treatment of the glucosylgalactosyl derivative of hydroxylysine with 1-fluoro-2,4-dinitrobenzene, followed by hydrolysis and chromatographic separation on Silica Gel, revealed the presence of a component that, in two solvent systems, migrated identically with 2,6-bis-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine, but differently from 6-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine. This result indicated that the sugars were linked glycosidically to the hydroxyl group at C-5 of 5-hydroxylysine, because an ester linkage would not be expected to survive the alkaline hydrolytic conditions used.

Partial hydrolysis of Glycopeptide P with acid showed a favored release of glucose, indicating that this sugar is at the nonreducing end of the oligosaccharide chain; the rate of release of glucose and galactose was similar to that reported by Spiro[7] for the glucosylgalactosyl derivative of hydroxylysine isolated from glomerular basement-membrane. Isolation of galactosyloxylysine from the partial acid hydrolyzate of the glucosylgalactosyl derivative of hydroxylysine proved conclusively that galactose was the sugar linked directly to the 5-hydroxylysine.

Characterization of the disaccharide unit. — Partial hydrolysis of *N*-acetylated Glycopeptide P with acid resulted in the release of a small proportion of a disaccharide [which was isolated by gel filtration on Sephadex G-15 (Peak II, Fig. 3)], besides unhydrolyzed peptides (fraction I) and a fraction (III) containing glucose.

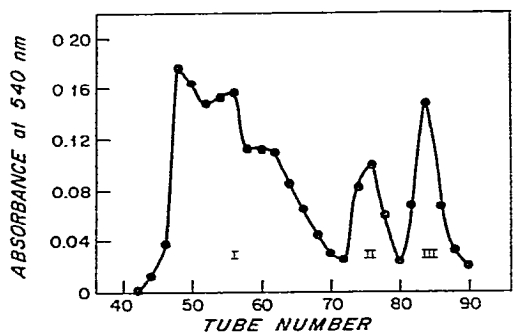

Fig. 3. Fractionation, on Sephadex G-15, of an acid hydrolyzate (0.1M hydrochloric acid, for 5 h at 100°) of *N*-acetylated Glycopeptide P (191 mg). The hydrolyzate in water was adsorbed on a column (93 × 3.0 cm diam.), and elution was performed with 50mM acetic acid. Fractions (4 ml) were collected, and aliquots (0.4 ml) were analyzed by use of the orcinol reaction. Peak I, unhydrolyzed peptides; peak II, disaccharide; peak III, glucose.

On cellulose, the disaccharide migrated slightly faster than maltose, and it was composed of approximately equimolar proportions of glucose (47%) and galactose (48.5%). On reduction of the disaccharide with sodium borohydride, all of the galactose residue was converted into an equimolar amount of galactitol residue, whereas the glucose residue was unaffected.

Periodate oxidation of the glucosylgalactosyloxylysine showed the uptake of 2.9 moles of oxidant per mole of oligosaccharide–amino acid, with oxidation of both the glucose and the galactose residues. This result eliminated the possibility of a (1→3)-linkage for the glucosylgalactosyl disaccharide. After reduction of the oxidized mixture with sodium borohydride, followed by mild hydrolysis with acid, glycerol, but no erythritol or threitol, was detected by g.l.c. This result indicated that the linkage in the glucosylgalactosyl group was either (1→6) or (1→2).

Confirmation that the linkage was indeed (1→2) was obtained by methylation, followed by acid hydrolysis, of the *N*-acetylated glucosylgalactosyloxylysine and of the *N*-acetylated Glycopeptide P. 2,3,4,6-Tetra-*O*-methylglucose and 3,4,6-tri-*O*-methylgalactose were identified by paper electrophoresis, t.l.c., and g.l.c. (see Table II); this result established that both hexose residues were in the pyranose form. This assignment of ring structures was of interest, as glucofuranose residues have been found in glycolipids isolated from the sea urchin (*Stronglylocentrolus intermedius*), an animal closely related to the sea cucumber[13].

Incubation of the disaccharide with an α-D-glucosidase isolated from *Saccharomyces cerevisiae* (see Table III) resulted in cleavage of the disaccharide into glucose

TABLE II

METHYLATED MONOSACCHARIDES OBTAINED BY HYDROLYSIS OF PERMETHYLATED GLYCOPEPTIDE P AND *N*-ACETYLATED GLUCOSYLGALACTOSYLOXYLYSINE[a]

Compounds	*Method of identification*		
	T.l.c.[b] R_F	*Paper electroph.*[c] M_T	*G.l.c. (min)*[d]
2,3,4-Tri-*O*-methyl-D-galactose	0.52	1.0	3.67
2,3,6-Tri-*O*-methyl-D-galactose	0.70	1.0	2.70
2,4,6-Tri-*O*-methyl-D-galactose	0.60	1.0	2.40
3,4,6-Tri-*O*-methyl-D-galactose	0.45	0.5	2.70
2,3,4,6-Tetra-*O*-methyl-D-glucose	0.87	1.0	1.05
Hydrolyzate of methylated Glycopeptide P	0.45; 0.87	0.5; 1.0	1.10; 2.70
Hydrolyzate of methylated glucosyl-galactosyloxylysine	0.45; 0.87	0.5; 1.0	1.10; 2.72

[a]See conditions of methylation, hydrolysis, and reduction in the Experimental section. [b]On Silica Gel G containing 16.7% (w/w) of Silica Gel GF_{254}. [c]In 20mM $Na_2B_4O_7$ buffer (pH 9–10) at 20 $V.cm^{-1}$; the M_T values are given relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose as unity. [d]Of alditol acetate on column *B*; after reduction with sodium borohydride and acetylation, as described in the Experimental section.

TABLE III

DEGRADATION[a], WITH α-D-GLUCOSIDASE OF *Saccharomyces cerevisiae*, OF GLYCOPEPTIDE P, *N*-ACETYLATED GLYCOPEPTIDE P, AND ISOLATED GLUCOSYLGALACTOSE, AT pH 6.8 FOR 18 H AT 37°

Substrates and standards	*Retention time (min)*
D-Glucose	8.7, 10.5, 14.5
D-Galactose	7.9, 9.3, 10.7
Enzyme-treated	
Glucosylgalactose	7.9, 8.6, 9.3, 10.5, 14.5
Glycopeptide P	8.6, 10.4, 14.3
N-Acetylated Glycopeptide P	8.6, 10.4, 14.4

[a]The de-ionized solution was lyophilized, the residue was per(trimethylsilyl)ated, and the product was examined by g.l.c. on column *D*, as described in the Experimental section. The per(trimethylsilyl)ated disaccharide and glycopeptides were retained on the column under the conditions used. No peak was observed in the controls not treated with α-D-glucosidase.

and galactose. This result indicated the α-D configuration for the D-glucosyl group, and that the disaccharide is 2-*O*-α-D-glucopyranosyl-D-galactose.

Finally, the glucosylgalactosyloxylysine isolated from Thyone has been shown to be identical with the glucosylgalactosyloxylysine isolated from sponge, Metridium, and bovine cornea; this was achieved by comparison of the optical rotations, the results of oxidation with galactose oxidase, the gas–liquid chromatograms of the per(trimethylsilyl) derivatives, and the mass spectrograms[14]. Thus, it may be concluded that the carbohydrate–amino acid unit of *Thyone briareus* collagen is 5-[*O*-α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyloxy]-L-lysine, identical with that found in vertebrate collagens and collagen-like proteins isolated from basement membranes, and different from that of the earthworm-cuticle collagen.

Despite the large proportion of carbohydrate components in the native Thyone collagen, the proportion of glucosylgalactosyloxylysine is only slightly higher (about 3%) than that found in vertebrate skin or tendon collagens (less than 1%). In contrast, the proportion of galactosyloxylysine is, however, small, and, in this respect, Thyone collagen resembles the basement-membrane proteins.

Spiro[10] made the observation that those collagens that have the least organization and that show no fibrillar structure on examination with the electron microscope have the largest number of hydroxylysine-linked carbohydrate units. Thyone collagen does show a periodic striation in electron micrographs, which is in accord with its relatively low content of glucosylgalactosyloxylysine. Kefalides[8] has suggested that the collagen molecules may be prevented from aggregating into fibers either by association of the collagen molecules with a non-collagen type of protein, or by their containing an excess of carbohydrate components. This structure would confer more elasticity and flexibility on the collagen molecule, with the probability of more selective permeability for such a membrane as the glomerular basement-membrane. It is probable that the intermediate value for the hexose content of Thyone collagen would confer some flexibility on the collagen molecule, without making the corium permeable to larger molecules, as is the case with the basement membrane.

EXPERIMENTAL

General methods. — Evaporation of volatile solvents was performed *in vacuo* (bath temp. <45°). Small volumes (<20 ml) were evaporated under a stream of dry nitrogen. Aqueous solutions were lyophilized.

Analytical methods. — Total neutral hexoses were determined by use of the anthrone[15] or the orcinol[16] reagents. The individual sugars were identified and estimated after hydrolysis of the glycopeptides in 2M hydrochloric acid for 4 h at 100° followed by neutralization of the hydrolyzate on a column of Dowex 1 X-8 (AcO^-) (200–400 mesh). Amino acids and peptides were detected in column eluates with the ninhydrin reagent[17].

Thin-layer chromatography on cellulose and silica gel, and paper chromatography. — T.l.c. was performed on Cellulose MN 300 (Brinkman Instruments Inc., Westbury, New York) and on Silica Gel G containing 16.7% (w/w) of Silica Gel GF_{254} Merck (mesh 0.05–0.2 mm, or ASTM 20-325, Brinkman), and paper chromatography on Whatman No. 1 paper in the following solvent systems: (*A*) 3:1:1 (v/v) ethyl acetate–acetic acid–water, (*B*) 70:30:0.3 (v/v) chloroform–2-methyl-2-butanol–acetic acid, (*C*) 40:10:1 (v/v) benzene–pyridine–acetic acid (4 developments), (*D*) 500:6:3 (v/v) acetone–water–conc. ammonia, (*E*) 7:3 (v/v) phenol–water, (*F*) 7:3 (v/v) ethanol–water, (*G*) 4:1:1 (v/v) butyl alcohol–acetic acid–water, and (*H*) 4:1:5 (v/v) butyl alcohol–acetic acid–water. The sugars were detected by staining with the aniline hydrogen phthalate[18] or silver nitrate[19] reagents.

Paper electrophoresis. — Paper electrophoresis was performed on Whatman No. 1 or No. 3 MM paper placed on a cooling surface at 20 $V.cm^{-1}$ in (*A*) 20mM sodium tetraborate (pH 9–10), and (*B*) 50mM pyridine acetate (pH 5). The peptides were detected by spraying with a 0.2% solution of ninhydrin in acetone, and heating for 10 min at 100°, and the glycopeptides, by spraying with a solution of sodium periodate and then with a solution of potassium permanganate[20].

Gas–liquid chromatography. — G.l.c. of the alditol acetates and trimethylsilyl derivatives of sugars was performed on a Perkin–Elmer No. 226 or 900 gas–liquid chromatograph with a hydrogen-flame ionization detector and the following columns: (*A*) 3% ECNSS M on Gas Chrom Q (100–200 mesh; Applied Science Labs., State College, Pennsylvania), 2.5 m × 3 mm o.d.[21,22] at 190° for the alditol acetates; (*B*) 0.2% Hi-EFF-2BP, 0.2% Hi-EFF-2AP, and 0.4% X-F1150 on Gas-Chrom P (100–120 mesh; Applied Science Labs.), 1.5 m × 3 mm o.d.[23], at 160° for the alditol acetates and 140° for the methylated alditol acetates; (*C*) 3% SE-30 on Chromosorb W-DMCS (60–80 mesh; Perkin–Elmer), 3.6 m × 3 mm o.d.[24] at a temperature programmed from 180° to 190° at 0.5°/min; and (*D*) 3% SE-52 on Chromosorb W-DMCS (60–80 mesh; Perkin–Elmer), 2.7 m × 3 mm o.d. at 200° for the trimethylsilyl derivatives of sugars. The quantitative determination was obtained by adding D-arabinose as the internal standard before reduction, and then measuring the areas under the peak with a planimeter.

The sugars and their methylated derivatives (1–3 mg) in neutralized hydroly-

zates (0.2 ml) were reduced with an equal volume of a 4% solution of sodium borohydride for 4 h at room temperature. After decomposition of the excess of sodium borohydride with acetic acid, the sodium ions were removed by passage through a column (4×0.6 cm) of Dowex 50 X-8 (H^+) (200–400 mesh). After lyophilization of the eluate, the boric acid was removed as methyl borate by five additions of methanol (0.5 ml) containing a trace of hydrogen chloride, followed by evaporation under a stream of nitrogen after each addition.

The alditols were acetylated with 100 μl of 1:1 (v/v) pyridine–acetic anhydride in a sealed tube for 5 h at 100°. The solution was evaporated to dryness, the residue dissolved in chloroform, and the solution injected into the gas chromatograph. The trimethylsilylation of the sugars and their derivatives was performed as described by Sweeley *et al.*[24] with TRI-SIL (Pierce Chemical Co., Rockford, Illinois).

Amino acid analysis. — The amino acid analyses were kindly performed by Dr. G. Mechanic of the Department of Orthopedics at Massachusetts General Hospital by using a Phoenix Amino Acid Analyzer on samples that had been hydrolyzed with 6M hydrochloric acid under an atmosphere of nitrogen for 24 h at 105°.

Preparation of the gelatin. — The sea cucumbers (*Thyone briareus*) were obtained alive from the Marine Biological Laboratories (Woods Hole, Massachussetts). The body-wall connective tissue was scraped free of most of the epidermal layers, cut into small pieces (162 g), washed three times with 0.15M disodium hydrogen phosphate (pH 8.0) at 4°, and dialyzed against distilled water for 4 days at 4°, with frequent changes of water. The residue was filtered through cheesecloth, water (100 ml) was added, and the suspension was heated in an autoclave under a pressure of 1 atm. for 15 min at 123°. The undissolved residue was isolated by centrifugation at 8,000 r.p.m. in a Sorval centrifuge for 30 min, and re-extracted in the same way with further quantities of water (100 ml) until it had all been solubilized; this normally required 5 to 8 extractions. The supernatant liquors were dialyzed against distilled water for 7 days at 4°, with frequent changes of water, and then lyophilized (total yield, 12–15 g). The yields from the successive extractions are reported in Table I.

Proteolytic degradation of the gelatin. — The pH of the solution of gelatin (Fractions 4–6, Table I; 9.3 g) in water (500 ml) was adjusted to 8.4 with M sodium hydroxide, and crystalline trypsin (100 mg; Worthington, Freehold, New Jersey) was added. Toluene was added to inhibit bacterial growth, and the solution was kept for 19 h at 37°, the pH being maintained at 8.4 by addition of M sodium hydroxide. The degradation was complete within 19 h as shown by a ninhydrin test, and the solution was then boiled for 10 min, cooled, and lyophilized. The residue was dissolved in water (50 ml), and the solution was treated with a 2% solution of cetylpyridinium chloride (120 ml). The precipitate, which contained a heteropolysaccharide fraction[4], was centrifuged off, and material in the supernatant liquor was precipitated with ethanol (5 vol.). The precipitate was centrifuged off, washed twice with ethanol, dissolved in water, and lyophilized (3.6 g). To a solution of the material (precipitated by ethanol) in 0.1M Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] acetate buffer (pH 7.8) containing 5mM calcium chloride (50 ml) was added Pronase (20 mg,

45,000 puK/mg; CalBioChem, Los Angeles, California). The mixture was incubated at 37° in the presence of toluene, and further additions of Pronase (5 mg) were made at 18 and 47 h. After 72 h, the ninhydrin test indicated that the proteolysis was complete, and the solution was lyophilized. The residue was dissolved in 0.1M pyridine acetate buffer (pH 5.0), and fractionated in two separate lots on a column (3.0 × 93 cm) of Sephadex G-15. Aliquots of the eluate were analyzed by the ninhydrin and anthrone tests (see Fig. 1). The hexose-containing fractions from both experiments (Tubes 51–71) were combined and lyophilized (1.5 g, Glycopeptide P).

Alkaline degradation of Glycopeptide P to glucosylgalactosyloxylysine. — A solution of Glycopeptide P (176 mg) in 2M sodium hydroxide (18 ml) was heated for 24 h at 105° in stoppered polypropylene tubes, after the air had been removed by bubbling nitrogen. After neutralization with conc. hydrochloric acid, the hydrolyzate was lyophilized. The residue was dissolved in 0.1M pyridine acetate (pH 5.0, 7 ml) and fractionated on a column (3.0 × 93 cm) of Sephadex G-15 by use of the same buffer for elution (see Fig. 2). The fractions containing hexose components (Tubes 65–70) were combined, lyophilized (15.7 mg), and analyzed for carbohydrate and amino acid components. A similar degradation was performed with a saturated solution of barium hydroxide (4 ml) for 60 h at 110°, followed by neutralization with carbon dioxide.

Anal. Calc. (% of dry wt. based on barium salt): Glc, 29.3; Gal, 29.3; Hyl, 28.9; Glu, 0.0; Gly, 0.0. Found: Glc, 29.8; Gal, 27.2; Hyl, 30.1; Glu, 1.0; Gly, 0.4.

Effect of alkali on Glycopeptide P and glucosylgalactosyloxylysine. — In order to ascertain the extent of degradation of the hexose moiety by alkali, Glycopeptide P (1.5 mg) and glucosylgalactosyloxylysine (0.55 mg) were separately treated with 2M sodium hydroxide (1.5 ml) for 24 h at 105–110°. The loss of carbohydrate components in Glycopeptide P was 80%, and that in glucosylgalactosyloxylysine, 64%. Control solutions were kept for the same length of time at −20°; no loss of carbohydrate components was observed.

N-*Acetylation of Glycopeptide P and glucosylgalactosyloxylysine.* — Glycopeptide P in 4.5M sodium acetate was *N*-acetylated by treatment with five equal additions of a 25-fold excess of acetic anhydride during 1 h at room temp., as described by Spiro[7]. The reaction was terminated by addition, to the mixture, of water (20 vol.) and boiling for 10 min, followed by removal of the sodium ions on a column of Dowex 50 X-8 (H^+) (200–400 mesh), and lyophilization of the eluate. Glucosylgalactosyloxylysine was similarly treated.

Isolation and characterization of a disaccharide from a partial hydrolyzate of the N-*acetylated glycopeptides.* — A solution of *N*-acetylated Glycopeptide P (191 mg) was hydrolyzed with 0.1M hydrochloric acid (6 ml) in a sealed tube for 5 h at 100°. The hydrolyzate was neutralized with Dowex 1 X-8 (CO_3^{2-}) (200–400 mesh), and lyophilized. A solution of the residue in water (2 ml) was fractionated on a column (3.0 × 93 cm) of Sephadex G-15 with 50mM acetic acid as the eluant (see Fig. 3). The fractions were examined for peptide and hexose content, and each peak was examined by cellulose t.l.c. in solvent *A*. Peak II (Nos. 73–79), containing a disaccharide and

traces of peptide, was lyophilized (3.5 mg). A solution of an aliquot (2/5) of this disaccharide in water (10 μl) was reduced with 4% sodium borohydride (50 μl) for 4 h at room temp., and the boric acid was removed as previously described. The reduced disaccharide and an equivalent aliquot of the original disaccharide were hydrolyzed with M hydrochloric acid (0.1 ml) in sealed tubes for 4 h at 100°. The hydrolyzates were neutralized, per(trimethylsilyl)ated, and examined by g.l.c. on column *C*. The following retention times (min) were observed: D-glucose, 8.3, 9.8, 14.0; D-galactose, 7.7, 8.9, 10.4; D-glucitol, 12.6; D-galactitol, 12.8; hydrolyzate of disaccharide, 7.6, 8.3, 8.8, 9.8, 10.3, 13.9; and hydrolyzate of reduced disaccharide, 8.4, 9.9, 12.8, 14.2.

Graded hydrolysis of glucosylgalactosyloxylysine with acid. — The rates of release of glucose and galactose from glucosylgalactosyloxylysine were determined by hydrolysis of a 0.025% solution with 0.1M hydrochloric acid in sealed tubes for various periods of time at 100° (*cf.*, Spiro[7]). The sugars released were determined by g.l.c. of the alditol acetate derivatives on columns *A* and *B*. The following proportions (%) of sugars released were observed.

Hours	0.5	1	5	12.5	26
Glucose	3	5.4	25	51	64
Galactose	0	0	1.1	5.5	14

Isolation of galactosyloxylysine after partial hydrolysis with acid. — Glucosylgalactosyloxylysine (20 mg) was hydrolyzed with 0.1M sulfuric acid (40 ml) in a sealed tube for 26 h at 100°. The solution was passed through a column (4.5 × 1.9 cm) of Dowex 50 X-8 (H^+) (200–400 mesh). The column was washed with water (120 ml), the adsorbed product eluted with 1.5M ammonium hydroxide (100 ml), and the eluate evaporated to dryness. The product (16 mg) obtained in this way was slightly contaminated with hydroxylysine and with starting material.

Periodate oxidation of glucosylgalactosyloxylysine. — A 0.25% solution of glucosylgalactosyloxylysine was oxidized with 50mM sodium periodate at room temp. in the dark. At intervals, aliquots were withdrawn and diluted with water, and the amount of periodate consumed was determined by measuring[25] the absorbance at 223 nm. The proportions of periodate consumed (moles of periodate/mole of glucosylgalactosyloxylysine) were as follows.

Minutes	5	30	60	120	300	500
Periodate	1.1	2.1	2.5	2.7	2.9	3.2

After 9 h, the excess of periodate ions was removed by passing the solution (0.25 ml) through a column (4 × 0.6 cm) of Dowex 1 X-8 (AcO^-) (200–400 mesh),

followed by washing with water (3 ml). The material in the eluate was reduced by treatment with 4% sodium borohydride (0.1 ml) for 3 h at room temp. and, after acidification with acetic acid, the boric acid was removed as methyl borate. Aliquots of the residue were hydrolyzed with 0.1M hydrochloric acid in sealed tubes for 1 h at 80°, and with 2M hydrochloric acid for 4 h at 100°. The hydrolyzates were neutralized, reduced, and acetylated as previously described. The alditol acetates were identified by g.l.c. On column *A*, neither galactose nor glucose was detected, and, on column *B*, the only peak present had a retention time of 1.7 min, which corresponds to that of glycerol and is very different from that of erythritol (6.7 min).

N-(*Dinitrophenyl*)*ation of glucosylgalactosyloxylysine.* — To a solution of glucosylgalactosyloxylysine (500 μg) in water (200 μl) was added, with shaking, triethylamine (50 μl) and a 5% solution of 1-fluoro-2,4-dinitrobenzene in ethanol (800 μl). After 2 h at room temp. in the dark, the mixture was acidified, diluted with water, washed three times with ether (2 vol.), evaporated to dryness, and the residue hydrolyzed with M sulfuric acid in a sealed tube for 4 h at 100°. The hydrolyzate was diluted with water, and extracted with ether, and the organic and aqueous phases were separately evaporated to dryness under a stream of nitrogen. The product was examined by t.l.c. on Silica Gel in solvents *B* and *C*, in the presence of 2,6-bis-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine, prepared by the method of Sanger and Thompson[26], and of 6-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine[27]. The product was identical with the first compound.

Methylation procedure. — The *N*-acetylated Glycopeptide P (30 mg) was treated with methyl iodide (0.7 ml) in dimethyl sulfoxide (1 ml) in the presence of 1.18M methylsulfinyl carbanion (0.4 ml) according to the procedure of Hakomori[28]. After 20 h, the reaction was stopped by addition of water (3 ml) and a few drops of acetic acid, the solution was lyophilized, and the residue was extracted with chloroform. The extracts were evaporated to dryness, and purified on a column (3.0 × 90 cm) of Sephadex G-15, with 50mM acetic acid as the eluant. The fractions containing the methylated glycopeptide were combined and lyophilized, and the residue was hydrolyzed with M sulfuric acid for 4 h at 100°. After neutralization with Dowex 1 X-8 (CO_3^{2-}) (200–400 mesh), the solution was divided into two portions.

One portion was examined by t.l.c. on Silica Gel G in solvent *D* (described by Stoffyn[29]), and by paper electrophoresis in 20mM sodium tetraborate buffer (pH 9–10) at 20 $V.cm^{-1}$. The sugars were detected on the Silica Gel by spraying lightly with conc. sulfuric acid and heating; and on paper, with the aniline hydrogen phthalate reagent. The results are given in Table II.

The second portion was reduced with sodium borohydride, and the product acetylated and examined by g.l.c. on column *B*. 2,3,6- (ref. 30), 2,3,4-, 2,4,6-, and 3,4,6-tri-*O*-methyl-D-galactose[31,32], and 2,3,4,6-tetra-*O*-methyl-D-glucose[33] were also tested (as standards) after similar treatment and under similar conditions. A similar procedure was applied to *N*-acetylated glucosylgalactosyloxylysine. For the results, see Table II.

Degradation of Glycopeptide P, N-acetylated glucosylgalactosyloxylysine, and

isolated glucosylgalactose with α-D-glucosidase. — The substrate (5.4 mg of glycopeptides, or 2.4 mg of disaccharide) was dissolved in 0.2 ml of 66mM dipotassium hydrogen phosphate buffer (pH 6.8), and incubated, in the presence of toluene, for 18 h at 37° with 50 μl of a solution of α-D-glucosidase, isolated from *Saccharomyces cerevisiae*[34]. Inositol was added as the internal standard, and the solution was then passed through columns (0.6 cm × 3 cm) of Dowex 50 X-8 (H^+) (200–400 mesh) and Dowex 1 X-8 (AcO^-) (200–400 mesh). The deionized eluate was lyophilized, per(trimethysilyl)ated, and examined by g.l.c. on column *D*. Controls from which the enzyme or the substrate had been omitted were treated similarly. The results are reported in Table III.

ACKNOWLEDGMENTS

The authors are grateful to Dr. P. J. Stoffyn for providing the 3,4,6-tri-*O*-methyl-D-galactose; Dr. G. Mechanic, the 6-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine; and Dr. N. A. Kefalides, the α-D-glucosidase. They also thank Miss E. Zollinger and Mr. L. Siever for technical assistance.

REFERENCES

1 R. W. Jeanloz, A. K. Bhattacharyya, and G. P. Roberts, *Z. Physiol. Chem.*, 350 (1969) 663.
2 K. Kuhn, W. Grassmann, and U. Hofmann, *Z. Naturforsch.*, 14b (1959) 436.
3 J. Gross, in M. Florkin and H. S. Mason (Eds.), *Comparative Biochemistry*, Vol. 5, Academic Press, New York, 1963, p. 307; S. Tekman and N. Öner, *Nature*, 200 (1963) 77.
4 R. L. Katzman, A. K. Bhattacharyya, and R. W. Jeanloz, *Biochim. Biophys. Acta*, 184 (1969) 523.
5 R. L. Katzman and R. W. Jeanloz, in E. A. Balazs (Ed.), *The Chemistry and Molecular Biology of the Intercellular Matrix*, Academic Press, New York, 1970, p. 217.
6 W. T. Butler and L. W. Cunningham, *J. Biol. Chem.*, 241 (1966) 3882.
7 R. G. Spiro, *J. Biol. Chem.*, 242 (1967) 4813.
8 N. A. Kefalides, *Proc. 6th Cong. Intl. Diabet. Fedn.*, Stockholm, July 20–Aug. 4, 1968; *Excerpta Med. Intl. Ser.* No. 172; *Biochemistry*, 7 (1968) 3103.
9 R. G. Spiro and S. Fukushi, *J. Biol. Chem.*, 244 (1969) 2049.
10 R. G. Spiro, *J. Biol. Chem.*, 244 (1969) 602.
11 Y. C. Lee and D. Lang, *J. Biol. Chem.*, 243 (1968) 677.
12 L. Muir and Y. C. Lee, *J. Biol. Chem.*, 244 (1969) 2343.
13 I. G. Zhukova and G. P. Smirnova, *Carbohyd. Res.*, 9 (1969) 366.
14 R. L. Katzman, M. H. Halford, V. Reinhold, and R. W. Jeanloz, *Biochemistry*, 11 (1972) 1161.
15 S. Seifter, S. Dayton, B. Novic, and E. Muntwyler, *Arch. Biochem. Biophys.*, 25 (1950) 191.
16 C. François, R. D. Marshall, and A. Neuberger, *Biochem. J.*, 83 (1962) 335.
17 S. Moore and W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367.
18 S. M. Partridge, *Nature*, 164 (1949) 443.
19 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature*, 166 (1950) 444.
20 M. L. Wolfrom and J. B. Miller, *Anal. Chem.*, 28 (1956) 1037.
21 J. S. Sawardeker, J. H. Sloneker, and A. Jeanes, *Anal. Chem.*, 37 (1965) 1602.
22 H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 21 (1967) 1801.
23 P. Albersheim, D. J. Nevins, P. D. English, and A. Karr, *Carbohyd. Res.*, 5 (1967) 340.
24 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497,
25 J. S. Dixon and D. Lipkin, *Anal. Chem.*, 26 (1954) 1092.
26 F. Sanger and E. O. P. Thompson, *Biochem. J.*, 53 (1953) 353.

27 G. MECHANIC, unpublished results.
28 S. HAKOMORI, *J. Biochem.* (Tokyo), 55 (1964) 205.
29 P. J. STOFFYN, *J. Amer. Oil Chem. Soc.*, 43 (1966) 69.
30 N. R. WILLIAMS AND R. W. JEANLOZ, *J. Org. Chem.*, 29 (1964) 3434.
31 D. J. BELL, *Advan. Carbohyd. Chem.*, 6 (1951) 11.
32 G. M. MAHER, *Advan. Carbohyd. Chem.*, 10 (1955) 273.
33 E. J. BOURNE AND S. PEAT, *Advan. Carbohyd. Chem.*, 5 (1950) 145.
34 H. HALVORSON, *Methods Enzymol.*, 8 (1966) 559.