Isolation and Structure Determination of Glucosylgalactosylhydroxylysine from Sponge and Sea Anemone Collagen†

Richard L. Katzman,‡ Maurice H. Halford,§ Vernon N. Reinhold, and Roger W. Jeanloz*

ABSTRACT: The major oligosaccharide-amino acid from the collagen of the sponge *Hippospongia gossypina* was isolated in 62% yield, and was shown to have the structure *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-galactopyranosyloxy-(1→5)-lysine. Direct evidence for the assignment of the anomeric configurations was obtained by proton magnetic resonance spectroscopy at 100 MHz. The glucosylgalactosylhydroxylysine compounds isolated from the collagens of the sea anemone *Metridium dianthus*, the sea cucumber *Thyone briareus*, and bovine cornea were found to be identical with the sponge hydroxylysine-disaccharide by mass spectroscopy, optical rotation, retention time on gas–liquid chromatography, elution position on the amino acid analyzer, and rate of oxidation by galactose oxidase. A simple procedure is described for the isolation of large amounts of hydroxylysine-disaccharide from the common household sponge.

A preliminary communication has described the isolation and chemical characterization of glucosylgalactosylhydroxylysine from the body-wall gelatin of the echinoderm *Thyone briareus* (Jeanloz *et al.*, 1969). The structure of this disaccharide-amino acid was established by conventional chemical and biochemical procedures, and it appeared to be very similar or identical with the compound isolated from a number of vertebrate sources (Cunningham *et al.*, 1967; Spiro, 1967, 1969a,b; Kefalides, 1968). It was of interest to establish whether this compound was a component of the collagen of even more primitive animals than the echinoderm. In this paper we describe the isolation of this disaccharide-amino acid from the sponge *Hippospongia gossypina* and from *Metridium dianthus*, a sea anemone. In addition, we show that the compounds from these two animals, as well as the one from *T. briareus* (Jeanloz *et al.*, 1969), are identical with bovine cornea glucosylgalactosylhydroxylysine by optical rotation, cochromatography on the amino acid analyzer, retention time on gas–liquid chromatography, reaction with galactose oxidase, and, finally mass spectroscopy. In addition, this paper describes in detail a simple procedure for the isolation of large amounts of glucosylgalactosylhydroxylysine from the common household sponge.

Experimental Section

Enzymes and Chemicals. α-Glucosidase from *Saccharomyces cereviciae* was a generous gift from Dr. N. A. Kefalides. β-Glucosidase, from almonds, and β-galactosidase, from liver, were products of Sigma Chemical Co. (St. Louis, Mo.). Trypsin was a product of Difco Labs (Difco Standardized 1:250). Crude *Cl. histolyticum* collagenase (125 units/mg) and galactose oxidase (Galactostat) were obtained from Worthington.

Pronase, *p*-nitrophenyl α-D- and -β-D-glucopyranosides, and *o*-nitrophenyl β-D-galactopyranoside were obtained from Calbiochem.

Deuterium oxide (99.8 atom %) was from Mallinckrodt. Deuterium oxide (100.0 atom %, lot no. 690701) was a product of Diaprep, Inc. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate and *myo*-inositol were obtained from Eastman Kodak Co.

Tri-Sil was obtained from Pierce Chemical Co. Gas-Chrom Q gas–liquid chromatography media were obtained from Applied Science Labs. All gel filtration and ion-exchange media were obtained from Bio-Rad Laboratories.

General Methods. Chromatographic fractions were analyzed for fucose by the method of Dische and Shettles (1948), using the 10-min heating treatment, and for total hexose by the anthrone procedure of Hewitt (1958). Ninhydrin-positive material was estimated by the procedure of Rosen (1957). Whatman No. 3MM preparative chromatography paper was washed with 1.0 M hydrochloric acid, water, 1% ammonia, and water. All enzyme digestions were carried out in the presence of chloroform and toluene. Acetic acid solution (1%) was used as eluent in all gel filtrations. Water was removed from solutions by rotary evaporation with a bath temperature of 40°. Methods for amino acid and quantitative carbohydrate analyses have been previously published (Katzman and Oronsky, 1971).

Isolation of Glucosylgalactosylhydroxylysine from the Sea Anemone Body Wall. Insoluble Metridum collagen (6.4 g), prepared as previously described (Katzman and Oronsky, 1971), was suspended in 0.1 M tri(hydroxymethyl)aminomethane chloride buffer (200 ml, pH 7.0) containing calcium chloride (1 mM). The suspension was incubated with crude

† From the Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts 02114, and from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received October 12, 1971.* This is publication IX of the series Invertebrate Connective Tissue and No. 559 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114. This investigation was supported by the U. S. Public Health Service research grants from the National Cancer Institute (Grant CA-18166) to R. L. K., the National Institute of Arthritis and Metabolic Diseases (Grant AM-03564) to R. W. J., and a N. I. H. Special Research Resource for Mass Spectrometry (GM-01523) to Dr. K. Biemann, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

‡ Present address: Department of Biological Chemistry, Harvard Medical School at McLean Hospital Research Laboratory, Belmont, Mass. 02178.

§ Present address: Research Laboratories, Carlton and United Breweries Ltd., Carlton, Victoria 3053, Australia.

* To whom correspondence should be addressed.

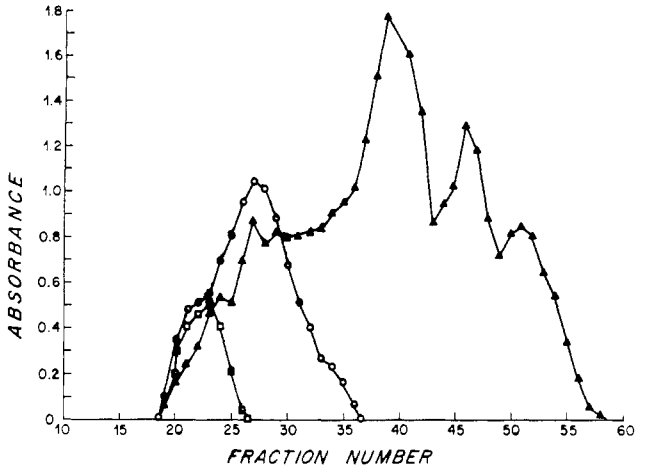

FIGURE 1: Filtration on P-2 gel resin of Metridium body-wall collagenase-Pronase digest. Proteolyzed Metridium body wall (6.4 g in 20 ml of 0.1 M acetic acid) was filtered on a column (13.6 cm^2 × 43 cm) of Bio-Gel P-2 (200–400 mesh) resin. Fractions (12 ml) were collected and aliquots taken for fucose (□), hexose (○), and ninhydrin-positive (▲) determinations. The entire anthrone-positive peak (tubes 18–37) was reduced in volume and refiltered on a column of P-4 resin as shown in Figure 2. See Experimental Section for full details.

Clostridium histolyticum collagenase (50 mg) for 2 days at 37°. The pH of the digest was then adjusted with sodium hydroxide to 8.0 and 100 mg of Pronase was added. The solution was then incubated for 3 days at 45°. The pH of the solution, following the Pronase digestion, was 7.1; the digest was then boiled for 10 min, and the denatured enzymes were removed by centrifugation. The digest was reduced in volume to 20 ml and passed through a column of Bio-Gel P-2 (200–400 mesh) resin as described in Figure 1. The entire anthrone-positive peak, which was quantitatively recovered from the column, was reduced to 12 ml and filtered on a column of Bio-Gel P-4 (100–200 mesh) resin as described in Figure 2. The anthrone-positive material was quantitatively recovered in tubes 14–38. Tubes 24–38 (fraction B) were pooled, the solvent was removed, and the residue was hydrolyzed essentially as described by Spiro (1967), in 2 M sodium hydroxide (13 ml) at 105° for 18 hr in a sealed polypropylene bottle. The recovery of anthrone-positive material was 83%. The hydrolysate was neutralized with hydrochloric acid and chromatographed on Bio-Gel P-2 resin, as described in Figure 3. Tubes 36–44 were pooled and dried to give a pale-brown powder (660 mg). An aliquot (25–50 mg) of this material was further purified by ion-exchange chromatography on a column (1.95 cm^2 × 52 cm) of Dowex 50 (Spinco AA-15 Amino Acid Analyzer resin), using the single-column procedure described by Miller and Piez (1966). The machine employed was a Beckman amino acid analyzer Model 116, equipped with a stream splitter and an LKB fraction collector (Figure 4); the ninhydrin color value for glucosylgalactosylhydroxylysine is 0.947 of the value for leucine, using the Miller and Piez (1966) modi-

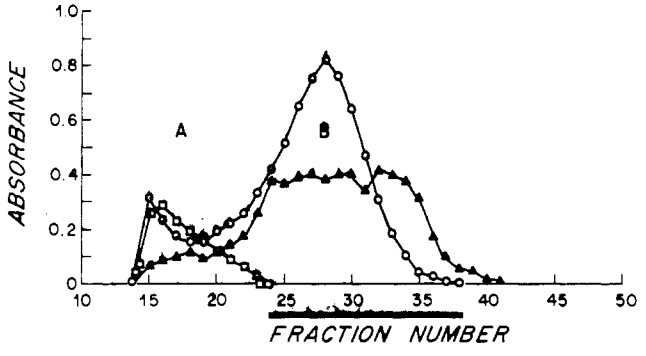

FIGURE 2: Filtration on P-4 gel resin of Metridium glycopeptides. Metridium glycopeptides obtained from the anthrone-positive peak in Figure 1 were filtered on a column (15 cm^2 × 34 cm) of Bio-Gel P-4 (100–200 mesh) resin. Fractions (10 ml) were collected and aliquots analyzed for fucose (□), hexose (○), and ninhydrin-positive material (▲). Fraction B, indicated by the heavy bar, was pooled, lyophilized, and used for the isolation of glucosylgalactosylhydroxylysine as described in the Experimental Section, and in Figures 3 and 4. The amino acid and carbohydrate composition of fraction A (tubes 14–20) has been previously published (Katzman and Jeanloz, 1970b).

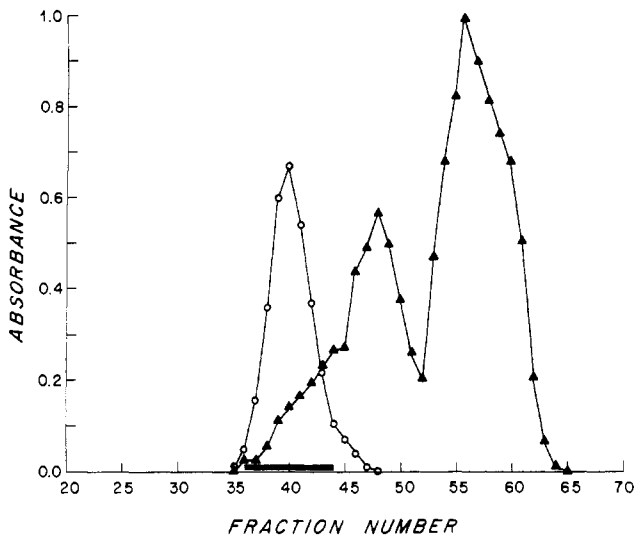

FIGURE 3: Filtration on P-2 gel resin of alkaline hydrolysate of Metridium glycopeptides. Fraction B (tubes 24–38, Figure 2) was hydrolyzed in 2 M sodium hydroxide, neutralized, and filtered on a column (13.6 cm^2 × 43 cm) of Bio-Gel P-2 (200–400 mesh) resin. Fractions (10 ml) were collected and aliquots analyzed for hexose (○) and ninhydrin-positive (▲) material. The tubes containing hexoses (fractions 36–44) were pooled and further purified by ion-exchange chromatography on Dowex-50 resin using the amino acid analyzer, as described in Figure 4. See Experimental Section for full details.

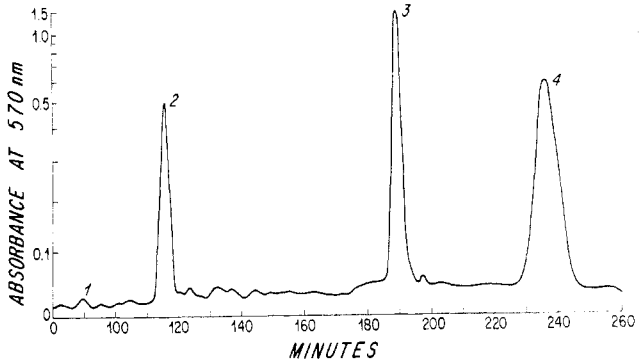

FIGURE 4: Chromatography on Dowex-50 resin of alkaline hydrolysate of Metridium glycopeptides. Approximately 25 mg of crude Metridium glucosylgalactosylhydroxylysine (see Figure 3) was chromatographed on an automatic amino acid analyzer equipped with a steam splitter: (1) glycine, (2) glucosylgalactosylhydroxylysine, (3) ammonia, and (4) post-arginine peak.

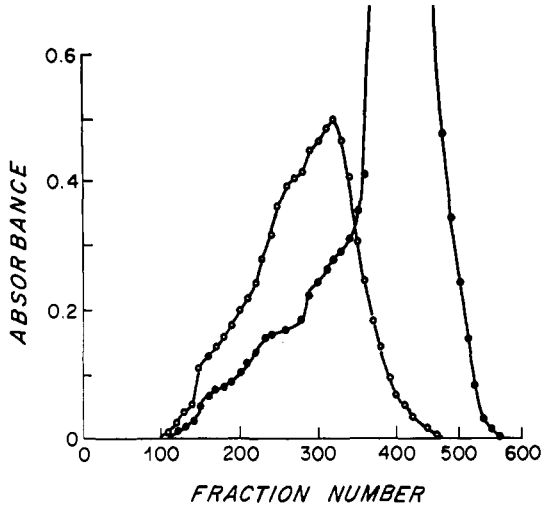

FIGURE 5: Chromatography of alkaline hydrolysate of sponge collagen (spongin B) on Dowex 50-X16 resin. The neutralized, alkaline hydrolysate of spongin B was charged onto a column (30 cm^2 × 150 cm) of Dowex 50-X16 resin, and eluted with 0.96 M ammonia. Fractions (25 ml) were collected and every fifth tube was analyzed for hexose (○) and ninhydrin-positive material (●). (The ammonia was removed by lyophilization from each 5-μl aliquot prior to the ninhydrin treatment.) Tubes 100 to 350 were pooled and lyophilized, yielding 4.85 g of a pale-brown powder containng 32% glucosylgalactosylhydroxylysine. Portions of this powder were purified as described in Figure 4. The balance of the material was purified by preparative paper chromatography as described in the text and used for the proton magnetic resonance studies (Table I and Figure 6).

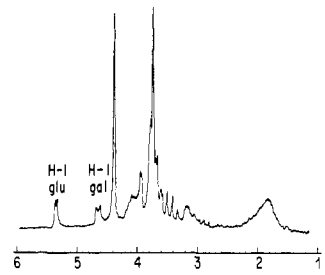

FIGURE 6: Proton magnetic resonance spectrum of sponge glucosylgalactosylhydroxylysine at 100 MHz in deuterium oxide at 70°. Chemical shifts in τ units relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (τ 0.00).

fication of the Rosen *et al.* (1962) procedure (S. Pinnell and S. M. Krane, unpublished data). The glucosylgalactosylhydroxylysine peak was desalted on a column (16 cm^2 × 30 cm) of Bio-Gel P-2 (200–400 mesh) resin.

Isolation of Glucosylgalactosylhydroxylysine from Sponge Collagen (Spongin B). Six live sheepswool sponges were obtained from L. S. Smitzes, Tarpon Springs, Fla., and identified as *Hippospongia gossypina* by Professor W. D. Hartman of the Yale University Peabody Museum. Spongin B, the major morphologically distinct collagen fiber of sponge, was prepared by the trypsinization procedure of Gross *et al.* (1956), as previously described (Katzman *et al.*, 1970). Spongin B from *H. gossypina* has been characterized with respect to its carbohydrate composition (Katzman *et al.*, 1970). The amino acid composition of spongin B from *H. gossypina* was very similar to the values for the spongin B from *S. graminae* (Gross, 1963).

Spongin B (52.3 g) was hydrolyzed in a screw-cap polypropylene bottle with 2 M sodium hydroxide (600 ml) at 105° for 24 hr. The recovery of anthrone-positive material after alkaline hydrolysis was 84%. The pH of the hydrolysate was adjusted to 5.5 with glacial acetic acid (87 ml). A heavy, black sediment (dry weight, 2.1 g) was removed by centrifugation for 20 min at 20,000*g*; this material is apparently inorganic in nature, since there was no weight loss upon combustion at 600° for 2 hr. The clarified Spongin B hydrolysate was placed on a column (30 cm^2 × 150 cm, equilibrated with water) of Bio-Rad AG 50W-X16 resin (H^+, 200–400 mesh), fitted with a medium-porosity, sintered-glass disk. The column was washed overnight with several column volumes of distilled water, and then eluted with 0.96 M ammonium hydroxide, as described in Figure 5. Approximately 5% of the anthrone-positive material, which was mannose rich, was not retained on the column and appeared in the water washings. Tubes 100–466 yielded a quantitative recovery of the remainder of the anthrone-positive material. Tubes 100–350 were pooled and the solvent removed by evaporation. The syrupy residue was dissolved in a small volume of water and lyophilized to give an amorphous pale-brown powder (4.85 g) containing 32% of glucosylgalactosylhydroxylysine. Portions of this material were purified on Dowex-50 resin and desalted on P-2 resin, as described above for the isolation of glucosylgalactosylhydroxylysine from Metridium body wall. Other portions were purified by preparative paper chromatography (Spiro, 1967); approximately a dozen sheets of washed Whatman No. 3MM paper were streaked in the long dimension with 150 mg of crude sponge glucosylgalactosylhydroxylysine and simultaneously developed in a large chromatographic chamber (*e.g.*, Chromatocab chamber, Research Specialties Co., Berkeley, Calif.).

Isolation of Glucosylgalactosylhydroxylysine from Bovine Corneal Collagen. Bovine eyes were obtained from a local slaughterhouse and frozen in liquid nitrogen immediately upon removal from the animal. The corneas and corneal collagen were prepared as described by Freeman *et al.* (1968). Gelatin, obtained from bovine corneal collagen by the method previously described for sea cucumber (Katzman *et al.*, 1969), was digested with collagenase and Pronase. The digest was chromatographed on Bio-Rad P-4 (100–200 mesh) resin, as described by Bosmann and Jackson (1968). The peak II glycopeptide fraction was hydrolyzed with alkali as described for Metridium collagen, and the resulting product, desalted on Bio-Gel P-2 resin, was further purified with the preparative amino acid analyzer and desalted on P-2 resin. The yield of glucosylgalactosylhydroxylysine was 54%, based on the recovery of glucose from the original collagen. The purpose of this isolation was to obtain a glucosylgalactosylhydroxylysine from a mammalian source to be used as a control.

Proton Magnetic Resonance Spectroscopy. Solutions of sponge glucosylgalactosylhydroxylysine (100 mg, purified by preparative paper chromatography) and of methyl α- and β-D-glucopyranosides and methyl α- and β-D-galactopyranosides (30 mg each) in deuterium oxide (*ca.* 1 ml, 99.86 g-atom % D) were evaporated to dryness *in vacuo* at 40°. This process was repeated two more times and finally a fourth time after addition of pure deuterium oxide (100.00 g-atom % D), taking adequate precautions to exclude atmospheric moisture. The residues were dissolved in pure deuterium oxide (*ca.* 0.5 ml) containing 2% of sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and the spectra were determined with a Varian Associates HA-100 spectrometer operating in the "frequency-sweep" mode. The results are shown in Figure 6.

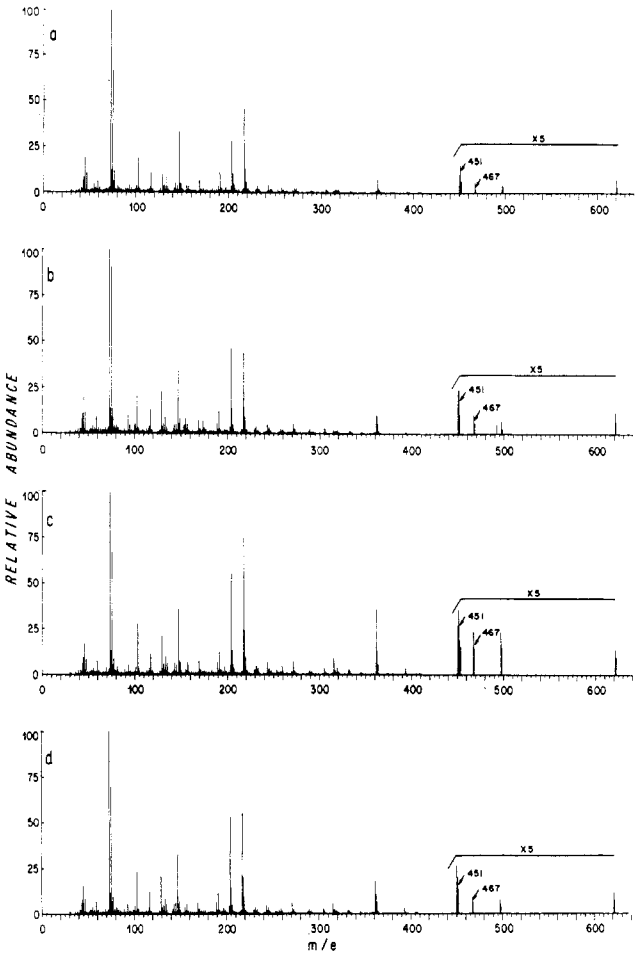

FIGURE 7: Mass spectra of (a) Thyone, (b) Metridium, (c) sponge, and (d) cornea glucosylgalactosylhydroxylysine. These spectra were printed out by an IBM 1800 computer. See Experimental Section for further details.

Gas–Liquid Chromatography–Mass Spectrometry Computer-Assisted Analysis. The analytical system consisted of an IBM-1800 computer which was fed raw data generated by a single-focusing, magnetic scanning mass spectrometer (Hitachi-Perkin Elmer RMU-6), interfaced with a gas chromatograph (Aerograph Hy-FI). Glucosylgalactosylhydroxylysines from sea cucumber collagen (Jeanloz *et al.*, 1969), sea anemone collagen, sponge collagen (spongin B), and bovine corneal collagen (50 μg of each sample) were transferred to screw-top (Teflon-lined) culture tubes (1.3 cm diameter $\times$ 10 cm), and *N,O*-bis(trimethylsilyl)trifluoroacetamide (50 μl) added to each tube. The sealed tubes were heated for 30 min at 100°, and the solutions injected directly into the injection block of the gas chromatograph. Gas chromatography of the per(trimethylsilyl)ated derivative of each sample was performed on a stainless-steel column (0.07 cm^2 $\times$ 300 cm), packed with Chromosorb W coated with 3% OV-16 polymer. The column was conditioned overnight at 300° and equilibrated at 120° in a Perkin-Elmer Model 900 gas chromatograph equipped with a flame-ionization detector. After application of the sample, the temperature of the column was programmed to increase to 300° at a rate of 5° per min. The results of the mass spectrometry are presented in Figure 7.

Galactose Oxidase Treatment. Galactostat was prepared, as described by the manufacturer, according to the procedure of Avigad *et al.* (1962). Duplicate samples of D-galactose, glucosylgalactosylhydroxylysines (sodium salts), and other galactose derivatives were each dissolved in water at a concentration of 50 μg of galactose/ml. These solutions (0.05 ml) were treated with an equal volume of the Galactostat reagent for 10 min at 37°. One-half of the reaction mixture was removed, the reaction was stopped with the glycine buffer (1.5 ml), and the optical density at 425 nm was recorded with a Coleman junior spectrophotometer (light path, 7 mm). The results are given in Table I. The other half of the reaction was incubated for 48 hr at 37°. Additional Galactostat (0.25 ml) was added and, after a total of 120 hr, the samples were lyophilized, methanolyzed with methanolic hydrogen chloride (0.5 M) for 16 hr at 65°, and examined by quantitative gas–liquid chromatography; 90–95% of the D-galactose moiety was destroyed in all four glucosylgalactosylhydroxylysine samples; glucose was quantitatively recovered.

TABLE I: Oxidation Rate of Glucosylgalactosylhydroxylysine and Other D-Galactose Derivatives with Galactose Oxidase.[a]

Substrate	Rel Velocity
D-Galactose	100
Glucosylgalactosylhydroxylysine (Thyone)	23
Glucosylgalactosylhydroxylysine (Metridium)	22
Glucosylgalactosylhydroxylysine (sponge)	23
Glucosylgalactosylhydroxylysine (bovine cornea)	23
D-Galactosamine hydrochloride	69
Lactose	5

[a] See Experimental Section for details of experiment.

Results

Isolation of Glucosylgalactosylhydroxylysine from Metridium Gelatin. Following proteolytic digestion of Metridium gelatin, the bulk of the ninhydrin-positive material was removed by gel filtration on P-2 resin (Figure 1), and the carbohydrate components were quantitatively recovered. Fractionation of the carbohydrate fraction on P-4 resin resolved it into two peaks (Figure 2). Peak A is a mixture of heterooligosaccharides, some of which are covalently linked to collagen peptides (Katzman and Jeanloz, 1970a; Katzman and Oronsky, 1971). The recovery of galactose in peak B represented approximately 80% of the total galactose of the gelatin. It was not possible to calculate the yields of the recovery of glucose, since fraction A is extremely rich in this hexose. Fraction A contains, however, only negligible quantities of galactose. The glucose in fraction A is present in a connective tissue structural glycoprotein devoid of hydroxyproline and rich in cystine, fucose, glucose, and glucosamine. The ratio of glucose to galactose was close to unity in each tube of fraction B suggesting that little, if any, galactosylhydroxylysine (Spiro, 1969a) was present. The recovery of carbohydrate components after alkaline hydrolysis of fraction B was 83% (average of eight different hydrolyses) and quantitative after purification by ion-exchange chromatography (Figure 4) and desalting by gel filtration for an overall yield of glucosylgalactosylhydroxylysine of 58%.

Isolation of Glucosylgalactosylhydroxylysine from Spongin B. The collagen from the sponge, spongin B, presented

special problems, since it was completely resistant to proteolysis by collagenase, papain, trypsin, or Pronase. Thus the spongin B was hydrolyzed directly in 2 M sodium hydroxide and all of the sodium ions and the bulk of the amino acids were removed by chromatography on a large column of Dowex 50-X16 (Figure 5). The recovery of glucose was 84% after the alkaline hydrolysis, and quantitative after the Dowex 50-X16 chromatography (Figure 5). However, tubes 350–450 were discarded to simplify further purification and thus the overall yield of sponge glucosylgalactosylhydroxylysine, on the basis of the recovery of glucose, was 62%.

The sponge skeleton is an extremely rich and easily accessible source for this substance and therefore the procedure for its isolation from the sponge is given in considerable detail. The results described were for the species *H. gossypina*, but identical results have been obtained with other fresh commercial species, including the hardhead, grass, and mandruka varieties. Apparently any sponge in the order Keratosa may be used. The fresh sponge could be fractionated, after treatment with trypsin, into three components, an acidic polysaccharide fraction and two collagen fractions termed spongins A and B (Gross *et al.*, 1956; Katzman *et al.*, 1970). Spongin B, the skeletal residue, accounts for over 70% of the dry weight of the sponge *H. gossypina* and contains approximately 7% by weight of glucosylgalactosylhydroxylysine (Katzman *et al.*, 1970) but a negligible amount of other sugars (Katzman and Jeanloz, 1970b). The polysaccharide fraction and spongin A are, however, extremely rich in other sugars (Katzman *et al.*, 1970) and should be removed for a successful isolation. We have subsequently found that commercial sponges (purchased at a local hardware store) may also be used. Apparently, the combination of autoproteolysis, beating, and washing used in their manufacture, remove spongin A and the polysaccharide fractions to give a very pure spongin B. After hydrolysis of the spongin B in alkali and chromatography on Dowex 50-X16, as described in Figure 5, a product was obtained which contains 30% to 40% glucosylgalactosylhydroxylysine. Further purification with the amino acid analyzer (followed by desalting on P-2 resin) yields up to 25 mg of a product of 99.8% minimum purity, as determined by amino acid analysis. Larger quantities (up to 500 mg) of less-pure material (90–95%) may be obtained by the preparative paper chromatography technique described by Spiro (1967).

Desalted alkaline hydrolysates of all four collagens gave similar chromatographic patterns when further purified on the amino acid analyzer (Figure 4). The glycosylgalactosylhydroxylysine peak was well resolved from traces of free amino acids and from a large broad peak which was eluted in the vicinity of the arginine peak. This latter peak probably represents small alkali-resistant peptides, since they cochromatograph with glucosylgalactosylhydroxylysine on Bio-Gel P-2 and P-4 resins, and are rich in aspartic and glutamic acids. All four samples of glucosylgalactosylhydroxylysine had identical elution times and peak profiles on the amino acid analyzer. When the four samples were mixed in equal proportions, they cochromatographed on the amino acid analyzer giving a glucosylgalactosylhydroxylysine peak indistinguishable from each of the individual samples.

Galactose Oxidase Reaction and Optical Rotations. Samples of glucosylgalactosylhydroxylysine from the four species reacted with galactose oxidase at the same relative velocity (see Table I). Other derivatives of galactose, including lactose and D-galactosamine hydrochloride reacted at distinctly different rates. These results suggest that the four compounds are very similar or identical. The complete destruction of galactose by extended incubation with galactose oxidase indicates that the galactose units of all four hydroxylysine-disaccharides possess the D-pyranosyl configuration. The values for the optical rotations of the glucosylgalactosylhydroxylysines (see Table II) similarly suggest that all four compounds are either very similar or identical.

TABLE II: Optical Rotations of Glucosylgalactosylhydroxylysines.[a]

Source	$[\alpha]_D^{20}$ (water, *c* 1.2), deg
Sponge	+42
Metridium	+41
Thyone	+42
Bovine cornea	+40.5

[a] As the sodium salt. The determinations were performed with a Perkin-Elmer polarimeter No. 141 in a 1-dm tube.

Proton Magnetic Resonance Spectroscopy. Although the linkage between the glucose and galactose components of sponge glucosylgalactosylhydroxylysine could be easily cleaved with α-glucosidase, using the procedure described by Spiro (1967), very poor yields were obtained when sponge galactosylhydroxylysine or N-acetylated galactosylhydroxylysine was treated with either α- or β-galactosidases, even after incubations of up to 2 weeks, and with frequent additions of enzyme. Therefore, assignment of the β configuration to the anomeric center of the D-galactose residue was made on the basis of proton magnetic resonance spectroscopy at 100 MHz; the same technique confirmed that the configuration of the D-glucose residue was α. Hydroxyl and amino protons were replaced by deuterium and the material examined in deuterated dimethyl sulfoxide and in deuterium oxide (D_2O). Spectra obtained using deuterated dimethyl sulfoxide solutions were characterized by low signal-to-noise ratios, due to limited solubility, and by poor resolution, due to high viscosity The material was much more soluble in deuterium oxide, but high viscosity with increasing concentration, resulting in broadened signals, was again a problem. Optimum results for resolution and signal-to-noise ratio were finally obtained with a solution of approximately 200 mg/ml at a probe temperature of 70°; sodium 2,2-dimethyl-2-silapentane-5-sulfonate, a water-soluble compound whose main resonance is at the same frequency as that of tetramethysilane (Tiers and Coon, 1961), was used as internal reference.

Examination of the chemical shifts and doublet spacings and their comparison with the published spectra of the methyl α- and β-glycosides of D-glucose and D-galactose (Williams and Bhacca, 1964; Hall, 1964; Coxon, 1965) lead to the assignment of the anomeric protons and their configuration.

The spectrum (Figure 6) shows two one-proton signals at low field, well separated from the DOH resonance which is conveniently shifted upfield by operating at high temperatures. The D-glucose residue of glucosylgalactosylhydroxylysine undoubtedly assumes the C-1 (D) configuration (Reeves, 1951, 1954), in which all groups, other than the anomeric substituent, are equatorial. Proton magnetic resonance studies have suggested the C-1 conformation for the D-glucose units of maltose, cellobiose, the α- and β-Schardinger dextrins, and other glucosans (Rao and Foster, 1963; Mackie and Perlin,

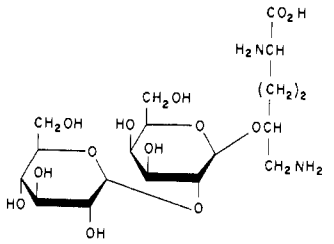

FIGURE 8: *O*-α-D-Glucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-oxy-(1→5)-L-lysine. Hydroxylysine has been assigned the L-erythro configuration (Witkop, 1956).

1965). Although the signal attributable to the anomeric proton of the D-galactose residue in glucosylgalactosylhydroxylysine is not well resolved (due either to long-range coupling or to nonaveraging of environment with respect to time), the observed doublet spacing of 7.5 Hz is sufficiently large to establish a trans-diaxial arrangement for the H-1–H-2 protons; such an arrangement is realized only in the β-D-glycoside in which the pyranose ring has the C-1 (D) conformation.

The proton magnetic resonance spectrum does not, in itself, define which hexose residue possesses the α-D or β-D configuration. However, the α-D configuration is well established for the glucose residue in glucosylgalactosylhydroxylysine from a number of invertebrate and vertebrate sources (Jeanloz *et al.*, 1969; Spiro, 1967, 1969a,b), so that the β-D configuration can be assigned to the galactose residue.

Gas–Liquid Chromatography and Mass Spectrometry. The hydroxylysine-disaccharides from the four species was treated with bis(trimethylsilyl)trifluoroacetamide in acetonitrile and introduced into the gas chromatographic inlet of a single-focusing, low-resolution mass spectrometer. The four derivatives were eluted 960 sec after injection and their retention time relative to that of the per(trimethylsilyl) derivative of *myo*-inositol was 2.76. When cochromatographed, they showed only one symmetrical peak.

Figure 7 shows a comparison of the mass intensity plots for the low-mass fragments of the four derivatives. The major fragments in this region compare favorably with that of a hexopyranoside-linked residue (DeJongh *et al.*, 1969). The ion at *m/e* 451 corresponds to a terminal hexose residue cleaved between C-1 and the glycosidic oxygen. Cleavage of the terminal residue between the glycosidic oxygen and the penultimate residue is indicated by the occurrence of the ion at *m/e* 467. A detailed interpretation of the fragmentation of the glycoprotein linkage moiety by high-resolution mass spectrometry will appear in a subsequent publication (V. N. Reinhold, K. Biemann, and R. W. Jeanloz, unpublished data).

Discussion

In a preceding investigation (Roberts *et al.*, 1972) the major oligosaccharide-amino acid from the collagen of the sea cucumber *T. briareus* was isolated and shown to consist of a 2-*O*-α-D-glucopyranosyl-D-galactopyranosyl unit linked glycosidically to the hydroxyl group of hydroxylysine (Figure 8). This carbohydrate group is apparently identical with that which occurs in a number of collagens from vertebrate sources (Cunningham *et al.*, 1967; Spiro, 1967, 1969a,b; Kefalides, 1968), and we became interested in the extent of its phylogenetic distribution. In this present paper we have shown that this disaccharide-amino acid occurs in the most primitive metazoan, the sponge, and in another primitive animal, the sea anemone.[1] By a number of criteria, including retention time on gas–liquid chromatography, elution time on the amino acid analyzer, optical rotation, rate of oxidation with galactose oxidase, and, most importantly, mass spectroscopy, we have provided evidence that the disaccharide-hydroxylysine units from these primitive animals are identical with those isolated from the sea cucumber (Jeanloz *et al.*, 1969), an echinoderm, and from a mammalian source, bovine cornea.

Additionally, we have confirmed the assignment of the anomeric configurations of the disaccharide by proton magnetic resonance spectroscopy. This technique demonstrated the presence of both α-D and β-D anomeric configurations in the disaccharide. We have also described in detail a simple procedure for the isolation of relatively large amounts of glucosylgalactosylhydroxylysine from the common household sponge.

Gross (1963) has pointed out that invertebrate collagens have greater amounts of carbohydrate than have vertebrate collagens. These observations, made almost a decade ago, were based on a relatively small number of species of which the carbohydrate components of the collagens were not fully characterized. In at least two of these examples, it has subsequently been shown that the majority of the carbohydrate components associated with the collagen were in the form of sulfated polysaccharides which probably are not covalently attached to the collagen molecule (Katzman and Jeanloz, 1970a; Katzman *et al.*, 1969, 1970). More information is at present available on the composition and structure of the carbohydrate moieties of both vertebrate and invertebrate collagens, and it may be worthwhile to briefly review these data.

Spongin B from *H. Gossypina* contains 6% of carbohydrates, consisting mainly of glucose and galactose (Katzman *et al.*, 1970). This is, however, a maximal value since the minor components, mannose and glucosamine, may not be part of the collagen molecule. As shown in the present paper, at least 62% of the glucose is a component of glucosylgalactosylhydroxylysine. Collagen from the coelenterate *Metridium dianthus*, prepared by pepsin solubilization and precipitation with 10% KCl, contains 8% of carbohydrates, consisting predominantly of glucose and galactose, although fucose, mannose, and glucosamine have also been demonstrated to be part of the collagen molecule (Katzman amd Jeanloz, 1970a; Katzman and Oronsky, 1971). This pepsin-solubilized material contains, however, a glycoprotein contaminant (rich in fucose, glucose, and glucosamine) which contributes about 1% to the carbohydrate value, so that 7% would be a more accurate figure for the carbohydrate content of Metridium collagen. Gelatin from the echinoderm *T. briareus*, freed of acidic polysaccharides and glycoproteins by DEAE-cellulose chromatography, contains only 1.5% of carbohydrates (Katzman *et al.*, 1969) consisting exclusively of glucose and galactose. As previously shown (Jeanloz *et al.*, 1969), glucosylgalactosylhydroxylysine is a component of Thyone gelatin.

Collagen from the cuticle of the common earthworm *Lumbricus terrestria* contains 12–14% of galactose, in the form of α-D-(1→2)-linked di- and trisaccharides linked to the collagen *via* alkali-labile linkages to serine and threonine (Lee and Lang, 1968; Muir and Lee, 1969). The collagen from the cuticle of the pig intestinal worm *Ascaris lumbricoides* has been reported (Spiro, 1967) to contain only 0.5% of carbohy-

[1] The sea anemone, a member of the coelenterates, occupies a disputed phylogenetic position (Blackwelder, 1963).

drates. However, a collagen isolated from the muscle layer of Ascaris contains 12.6% of carbohydrates (Fujimoto, 1968). Mammalian glomerular basement-membrane collagens contain 10–12% carbohydrate (Kefalides, 1968; Fujimoto, 1968) from which glucosylgalactosylhydroxylysine can be isolated in high yield (Spiro, 1967). In contrast, mammalian-skin collagens contain only about 0.5% of carbohydrate (Gross, 1963), mainly in the form of glucosylgalactosylhydroxylysine (Butler and Cunningham, 1966). We conclude, therefore, that there is no apparent relationship between the phylogenetic position of a species and the carbohydrate content of its collagen. It is also becoming apparent that collagens having very high and very low carbohydrate contents may be found in the same animal.

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