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HYDRA MESOGLEA: SIMILARITY OF ITS AMINO ACID AND NEUTRAL SUGAR COMPOSITION TO THAT OF VERTEBRATE BASAL LAMINA

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Abstract—1. A method is described for isolating large numbers of mesogleas from hydra.
2. Amino acid analysis shows the mesoglea to be rich in glycine, and to contain significant hydroxyproline, proline and hydroxylysine.
3. The neutral sugars of the mesoglea, shown to be mostly glucose and galactose with some fucose and rhamnose, made up 6·7 per cent of the dry weight of the mesoglea.
4. Some of the glucose and galactose components are present as a dimer stable to hydrolysis by strong alkali.
5. That these and other data support the view that hydra mesoglea is chemically like components of vertebrate basal lamina is presented in the Discussion.

INTRODUCTION

THE MESOGLEA of hydra is a thin (0·5–2·0 μm) acellular material found between the animal's two epithelial cell layers (Haynes *et al.*, 1968). It consists of thin fibrils embedded in an amorphous matrix. Mesogleas from other cnidarians have been shown to contain a collagen-like protein (Piez & Gross, 1959; Gosline & Lenhoff, 1968; Nordwig & Hayduk, 1969; Katzman & Jeanloz, 1970).

In this paper we describe chemical analyses of pure hydra mesoglea that show it to contain the amino acids and neutral sugars normally associated with collagens. In the discussion we note chemical, developmental and structural similarities between cnidarian mesogleas and vertebrate basal lamina.

MATERIALS AND METHODS

Culture of hydra

Cultures of *Pelmatohydra pseudoligactis* were grown according to the method of Lenhoff & Brown (1970). Unless otherwise stated, each day the animals were fed once and washed twice at 1 and 8 hr after feeding. Such animals will be referred to as standard hydra. Other groups of hydra were starved for prescribed times, and were washed once daily.

Preparation of mesogleal fibers from Pelagia

Specimens of the large scyphozoan jellyfish, *Pelagia pacifica*, were collected off the coast of Newport Beach, California, during the summer of 1970 and spring of 1972. Masses of mesoglea were scraped clean of adhering cells and washed several times in distilled water. This material was stored at −80°C until used, at which time the thawed fibers were washed again in distilled water and then lyophilized.

Isolation of mesoglea from hydra

Hydra, after being kept in distilled water for 30 min to remove adhering mucus, were frozen at −25°C for from 3 hr to 2 days in a solution of 0·2% Sarkosyl NL-97 (Geigy). Batches of up to fifty thawed hydra were transferred into 50 ml of distilled water in a Petri dish; the suspension was swirled, using a Pasteur pipet with a fire-polished tip, in order to dislodge the cells from the mesogleas. The intact mesogleas, now free to the eye of cellular contamination, were transferred to a clean Petri dish, swirled and transferred to test-tubes containing 5 ml of 1 mg/ml dithiothreitol (Sigma) at pH 8·8 for 5 min. The tubes were centrifuged for 20 min at 5100 *g*, after which the supernatant was poured off and the pelleted mesogleas were resuspended in 5 ml of distilled water. The resultant clean mesogleas (see Fig. 1) were recentrifuged for 10 min and were either used immediately or lyophilized and stored at −25°C.

Dry weights of whole hydra and isolated mesogleas

Small plastic centrifuge tubes were dried to constant weight at 60°C in an oven. Sets of 25 and 50 whole standard hydra or 100 and 250 isolated mesogleas were then added to the tubes which were again dried to constant weight. The results show that standard specimens of *P. pseudoligactis* had an average dry weight of 97 μg,

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whereas a mesoglea isolated from such individuals weighed about 3 μg.

Amino acid analysis

Isolated mesogleas from hydra were hydrolyzed in sealed tubes in 6 N HCl at 110°C for 24 hr; the final amino acid concentration of the hydrolysate was 1 mg/ml. The hydrolysate was evaporated to dryness *in vacuo*. The amino acid analysis was performed on a Beckman Model 121 Automatic Amino Acid Analyzer using a single column system. Standards were also run for the amino sugars glucosamine and galactosamine.

Isolation and identification of neutral sugars

Mesogleal material from either hydra or *Pelagia* was hydrolyzed in 0·25 N sulfuric acid with Bio Rad AG 50W-X8, 200–400 mesh (H^+) according to the method of Kim *et al.* (1967). The neutral sugar fraction, isolated by this procedure, was lyophilized and stored at −25°C until needed.

Total neutral sugar content was analyzed colorimetrically by the phenol–sulfuric acid method of Dubois *et al.* (1956) using glucose as a standard and expressing the results as glucose equivalents.

Thin-layer chromatography was performed on plastic-backed silica gel plates (Eastman Kodak Co., No. 6061) according to the bisulfite method of Adachi (1965). The solvent systems used were ethyl acetate–acetic acid–methanol–water (60 : 15 : 15 : 10 by vol.), isopropanol–ethyl acetate–water (7 : 1 : 2 by vol.), methyl ethyl ketone–acetic acid–water (20 : 5 : 15 by vol.) and *n*-propanol–water (85 : 15 by vol.). For each identification, two to three of these solvent systems were used.

Neutral sugars of the mesogleal fractions were identified by comparing the R_f's with sugar standards run in parallel. Dried chromatograms were sprayed with the diphenylamine–aniline–phosphoric acid reagent prepared according to Stahl (1967); the chromatograms were then heated in an oven at 85°C for 10 min. Methyl pentoses (fucose and rhamnose) stained yellow and hexoses stained gray-green to green. When radioactive sugars were used as standards they were co-chromatographed with the unknown to determine the R_f's of the radioactive standards. The chromatogram was cut into fractions which were placed in a toluene fluor (0·6%, 2,5-phenyloxazole) and analyzed in a Beckman LS-250 Liquid Scintillation Counter.

Labeling of hydra with radioactive material

Individual hydra were labeled with 1-μl aliquots of isotope solutions introduced into the gastrocoel (gut) using a modification of the microinjection technique of Campbell (1965).

The radioactive compounds used were: L-fucose (1,5,6-^{3}H), sp. act. 1–5 Ci/mM, from New England Nuclear (Boston); D-galactose-1-^{14}C, sp. act. 1–10 mCi/mM was obtained from International Chemical and Nuclear Corporation (Irvine, Calif.); D-glucose (UL-^{14}C), sp. act. 11·8 mCi/mM, L-lysine (4,5-^{3}H), sp. act. 41·6 Ci/mM and thymidine (methyl-^{3}H), sp. act. 6 Ci/mM—all obtained from Schwarz/Mann (New Rochelle, New York).

Isolation of a glucose–galactose unit from mesoglea

Mesogleal material from *Pelagia* was hydrolyzed in 2 N NaOH at 105°C for 24 hr according to the method of Spiro (1967). The hydrolysate was passed through a Millipore filter (HA 0·45 μm), adjusted to pH 2·5, passed through a Bio-Rad 50-X8 (H^+) column, eluted with 1·5 N NH_4OH and lyophilized.

The hydrolysate was spotted on Whatman No. 1 filter paper, run in *n*-butanol–acetic acid–water (100 : 22 : 50 by vol.) for 48 hr, dried and sprayed with ninhydrin.

The area corresponding in R_f to the glucose–galactose–hydroxylysine unit isolated using this procedure by Spiro (1967) was eluted with water, lyophilized and analyzed for neutral sugar content.

Because it was difficult to obtain large masses of mesogleal material from hydra, we attempted to identify the glucose–galactose–hydroxylysine unit by means of a double-labeling technique using the unit isolated from *Pelagia* as a standard. Hydra were injected with a 1 : 1 mixture of ^{14}C-D-glucose and ^{3}H-L-lysine. Forty-eight hr later mesogleas were isolated from those hydra and hydrolyzed in 2 N NaOH as described above for *Pelagia*.

A portion of the hydrolysate was passed through a Sephadex G-15 column and was eluted with 0·1 M pyridine acetate buffer, pH 5 (Spiro, 1967). Aliquots (0·1 ml) of each fraction were analyzed for ^{3}H- and ^{14}C radioactivity. Fractions containing both labels were pooled, lyophilized and hydrolyzed; the neutral sugars present in them were isolated and analyzed as described above in *isolation and identification of neutral sugars*.

RESULTS

Purity of mesoglea preparations

Mesoglea isolated as described under Materials and Methods are free of cells, large pieces of debris and visible cellular components such as nuclei and nematocysts (Fig. 1). Those nematocysts adhering to the sticky mesoglea were essentially removed by treating the preparation with dithiothreitol (see Materials and Methods). This reagent dissolves the unusual disulfide-linked collagen which makes up the major part of the nematocyst capsule. As a further indication of purity, mesoglea isolated from hydra that had been injected with ^{3}H-thymidine 2 days earlier were free of radioactivity as determined by autoradiography.

Amino acid composition of hydra mesoglea

Table 1 shows that glycine is the most abundant amino acid present in the mesoglea hydrolysate. Next in quantity are glutamic acid and aspartic acid. Of special significance are the presence of hydroxyproline, proline and hydroxylysine. Hence, from the presence of these latter amino acids and from the large amount of glycine, it can be concluded that the mesoglea contains a protein nelongibg to the collagen group (Spiro, 1970b).

Unlike most vertebrate collagens, however, the mesoglea contains significant tyrosine, phenylalanine

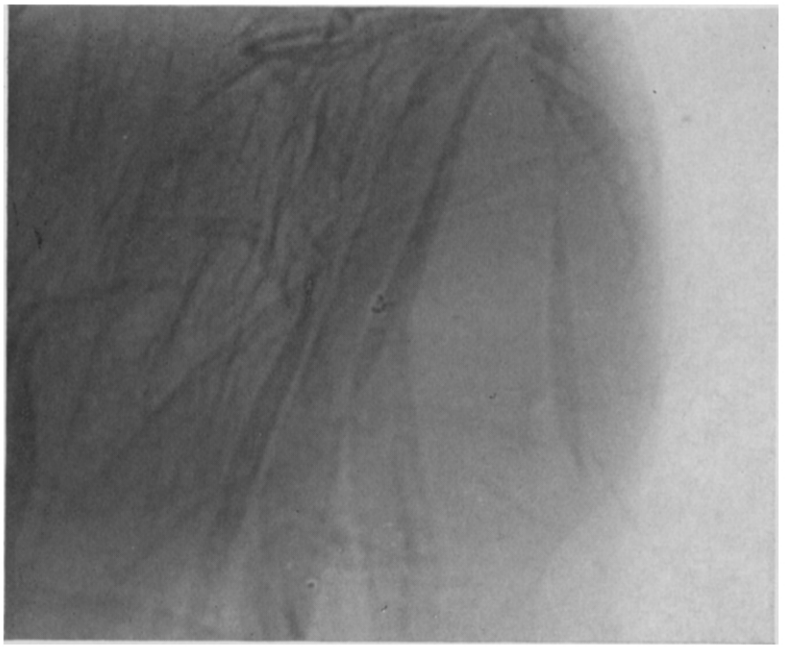

Fig. 1. A portion of an isolated mesoglea stained with periodic acid-Schiff reagent (scale: equals 0·10 mm). At this magnification, any contaminating cells and nematocysts would be easily discernible.

Table 1. Amino acid composition of the mesoglea of hydra

Amino acid	Residues/1000 residues
Hydroxyproline	30
Aspartic acid	96
Threonine	38
Serine	61
Glutamic acid	121
Proline	57
Glycine	229
Alanine	57
Valine	27
Half cystine	15
Methionine	12
Isoleucine	24
Leucine	56
Tyrosine	20
Phenylalanine	23
Hydroxylysine	40
Lysine	43
Histidine	13
Arginine	38

The methionine value is corrected for its destruction during hydrolysis.

and half cystine. In addition to the amino acids listed in Table 1, glucosamine was also detected in amounts accounting for 1·4 per cent of the dry weight of the mesoglea.

Presence of neutral sugars in mesoglea

To determine whether or not the mesoglea contained neutral sugars, mesoglea was analyzed by the phenol–sulfuric acid method (Dubois *et al.*, 1969); the results showed that 6·7 per cent of the total dry weight of mesoglea was neutral sugar.

The component neutral sugars were detected in the following manner. Hydra were injected with radioactive glucose, galactose or fucose. Three days later, the mesogleas were isolated and hydrolyzed. The labeled neutral sugars present in the hydrolysates were separated by thin-layer chromatography, the chromatogram was cut into fractions and the radioactivity was analyzed. The labeled sugars were identified by comparing their R_f values with those of standard unlabeled sugars run in parallel and located by staining.

Hydrolysates of mesogleas from animals injected with ^{14}C-glucose showed the presence of large amounts of labeled glucose and galactose, and small amounts of labeled fucose and rhamnose (Fig. 2A). Similar experiments using ^{14}C-galactose showed the presence in the mesoglea of labeled galactose, glucose and fucose (Fig. 2B). On the other hand, the experiments using ^{14}C-fucose, a sugar not normally converted into the other neutral sugars, gave rise only to mesoglea labeled with fucose (Fig. 2C).

Analysis of Pelagia *mesoglea for a glucosylgalactose disaccharide*

Because the amino acid and neutral sugar analyses suggested that hydra mesoglea contains a collagen-like protein, the question arose as to whether the mesoglea contained the "disaccharide unit" of 2-*o*-α-D-glucosylgalactose, recently shown to be bound in collagens by an *o*-glycosidic bond to hydroxylysine (Butler & Cunningham, 1966; Spiro, 1967). To carry out this analysis, we first used the mesoglea of the readily available large scyphozoan jellyfish *P. pacifica* because: (1) the presence of the disaccharide unit would be suggestive that the unit was also present in hydra mesoglea and (2) the disaccharide unit from *Pelagia* could be used as a chromatographic standard in searching for the unit in hydra.

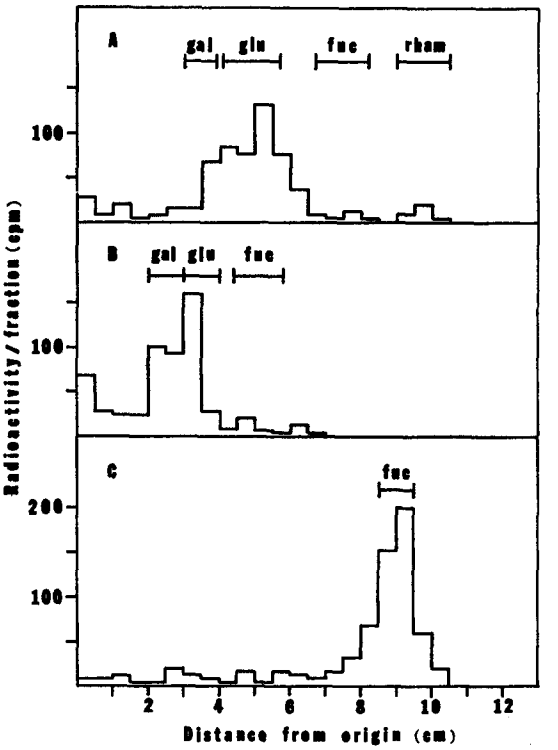

Fig. 2. Thin-layer chromatography of neutral sugars of hydra mesoglea. The sugars were separated by one-dimensional thin-layer chromatography using a propanol–water (85 : 15 by vol.) solvent sytem. Each chromatogram was cut into 0·5 cm strips which were then analysed for radioactivity. The horizontal bars at the top of each figure represent the relative position on each chromatogram of the unlabeled sugar standards. Gal represents galactose; glu, glucose; fuc, fucose; and rham, rhamnose. The figures represent analyses from the following experiments: A. ^{14}C-glucose injected into animals starved 6 days prior to the experiment. B. ^{14}C-galactose injected into standard hydra. C. ^{3}H-fucose injected into standard hydra. See Materials and Methods for details.

Pelagia mesoglea fibers, 3·5% neutral sugar by dry weight, were hydrolyzed, and the hydrolysates co-chromatographed with ^{14}C-glucose (Fig. 3A) and with ^{14}C-galactose (Fig. 3B). The results show that both glucose and galactose are present in *Pelagia* mesoglea.

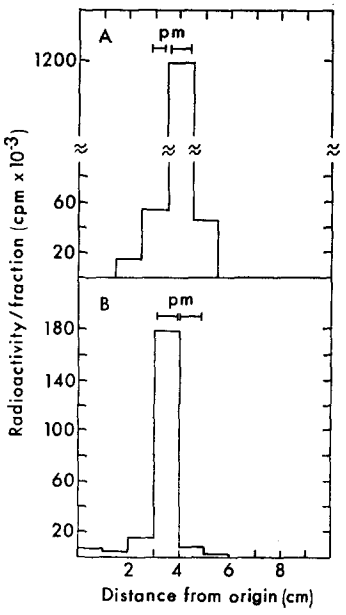

Fig. 3. Co-chromatography of the neutral sugar fraction from *Pelagia* fibers with (A) ^{14}C-glucose and (B) ^{14}C-galactose. The unknown sugars and the radioactive sugar standards were separated by one-dimensional thin-layer chromatography using a proponol–water (85 : 15 by vol.) solvent system. The location of the two unknown neutral sugars were visualized by staining, and are indicated in the figure by the bars lebeled PM (for "*Pelagia* mesoglea"). To analyze for radioactivity, 1-cm fractions of the chromatograms were cut out and analyzed in a scintillation counter to identify the positions of the radioactive standard.

The presence of these two sugars in the disaccharide unit was determined using the strong alkali hydrolysis method of Spiro (1967); such a hydrolysis leaves the glucosylgalactose unit still covalently linked to free hydroxylysine. This is the only type of linkage between carbohydrate and protein known to be stable to strong alkali (Spiro, 1970b).

Paper chromatograms of the desalted alkaline hydrolysate run in *n*-butanol–glacial acetic acid–water (100 : 22 : 50 by vol.) showed an unknown that corresponded in position to the glucose–galactose–hydroxylysine trimer (Spiro, 1967). This spot was eluted, lyophilized and hydrolyzed as described previously to obtain the neutral sugar fraction. The only sugars present in the unknown alkali stable material—as demonstrated by co-chromatography with ^{14}C-glucose and ^{14}C-galactose in *n*-propanol–water (85 : 15 by vol.) (Fig. 4) and isopropanol–ethyl acetate–water (7 : 1 : 2 by vol.)—were glucose and galactose.

Presence of glucosylgalactose in hydra mesoglea

Having worked out the procedure for isolating the disaccharide–hydroxylysine trimer from *Pelagia* mesoglea, we applied a similar procedure to isolating the trimer from hydra mesoglea. Because of the relatively small amounts of material available from hydra compared with *Pelagia*, we first labeled the

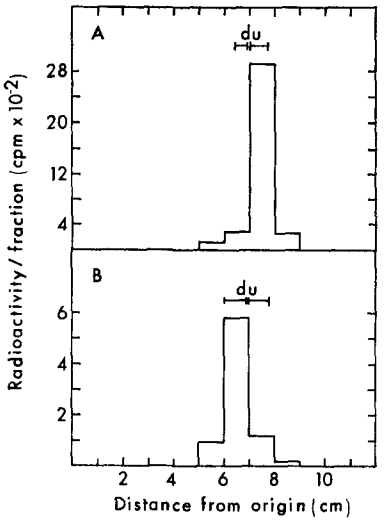

Fig. 4. Co-chromatography of the sugars of the disaccharide unit from *Pelagia* mesoglea fibers with (A) ^{14}C-glucose and (B) ^{14}C-galactose. The locations of the sugars of the disaccharide unit (d.u.) and of the radioactive sugar standards run in a propanol–water (85 : 15 by vol.) solvent system were determined as described in Fig. 3. It should be noted that glucose and galactose standards chromatographed in parallel—had the same R_f values as the radioactive standards co-chromatographed with the d.u. sugars.

hydra with a mixture of ^{14}C-glucose and ^{3}H-L-lysine. After 2 days, the labeled mesogleas were isolated and hydrolyzed in alkali. The presumed glucosylgalactose dimer linked to its amino acid was separated from the other liberated free amino acids by gel chromatography on a Sephadex G-15 column eluted with 0·1 M pyridine acetate buffer, pH 5 (Spiro, 1967).

Figure 5 shows the elution patterns of the ^{14}C- and ^{3}H-labeled material. Fractions 6–9 in particular show a great overlap of the ^{14}C and ^{3}H labels. These fractions were then pooled, lyophilized, hydrolysed and analysed for their content of neutral sugars. Thin-layer chromatography, with *n*-propanol–water (85 : 15 by vol.) as solvent, of this fraction showed 60 per cent of the total ^{14}C activity to be in glucose and 25 per cent to be in galactose. Hence, because

most of the ^{3}H- and ^{14}C-labeled materials were eluted from the Sephadex column in the same fractions, we conclude that hydra mesoglea contains a protein with glucosylgalactose linked to hydroxylysine.

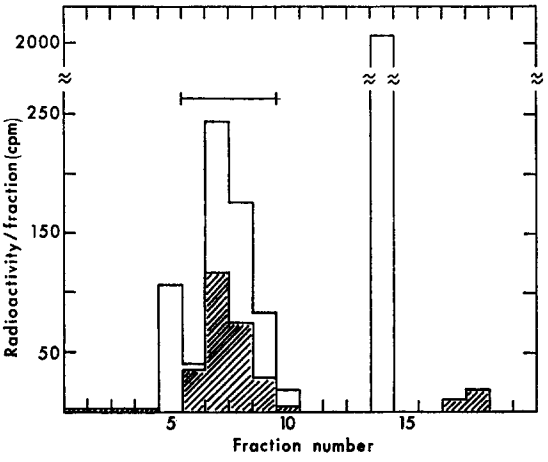

Fig. 5. Gel chromatography of the desalted alkaline hydrolysate of mesogleas from hydra previously labeled with both ^{14}C-glucose (cross hatched bars) and ^{3}H-L-lysine (clear bars). The material was eluted with 0·1 M pyridine–acetate, pH 5, from a Sephadex G-15 column (22·0 × 0·5 cm) and collected in twenty-drop fractions at 6 ml/hr. A 0·1-ml aliquot of each fraction was analyzed for radioactivity. The fractions under the bar were pooled, lyophilized and further analyzed for neutral sugars.

DISCUSSION

In this paper we provide evidence showing that hydra mesoglea contains a protein which has chemical similarities to some vertebrate collagens. For one, the pure mesoglea is rich in glycine, and possesses hydroxyproline, proline and hydroxylysine (Table 1). In addition, it contains the disaccharide unit glucosylgalactose linked to hydroxylysine (Fig. 5).

The mesoglea, however, differs in the relative amounts of those substances from extractable vertebrate collagens as defined by Ramachandran (1963). Instead, the chemical composition of hydra mesoglea, as well as that of other mesogleas, is more closely akin to that of epithelial basal lamina. For example, whereas hydroxylysine makes up about 7 residues per 1000 in extractable vertebrate collagens (Piez & Gross, 1959; Nordwig *et al.*, 1970), vertebrate basal lamina contain from 20 to 35 hydroxylysine residues per 1000 (Kefalides, 1970). In coelenterate mesogleas or purified cnidarian collagens, hydroxylysine makes up from 23 to 40 residues per 1000 (Piez & Gross, 1959; Nordwig & Hayduk, 1969; Katzman & Jeanloz, 1970; Table 1, this report).

On the protein structure level, it has been reported elsewhere that some cnidarian mesogleas and vertebrate basal lamina contain collagen molecules having three identical alpha chains (Nordwig *et al.*, 1970; Kefalides, 1971). Such is also the case with collagen synthesized by embryonic spinal cord epithelium (Trelstad *et al.*, 1972). In vertebrate connective tissue, however, only one of the collagens from cartilage shows three identical alpha chains (Miller, 1971); other collagens are of the type $[\alpha_1(I)_2\alpha_2]$.

Other evidence for chemical similarities between vertebrate basal lamina and coelenterate mesogleas comes from comparison of their neutral sugar compositions. First, it should be noted that vertebrate basal lamina contains 6·8–11·8 g of neutral sugar per 100 g of material (Kefalides & Denduchis, 1969; Kefalides, 1970) whereas vertebrate collagens contain less than 2 g of neutral sugar per 100 g of protein (Spiro, 1970a). Cnidarian mesogleas, for the most part, are like vertebrate basal lamina in that they contain from 5·7 to 14 g neutral sugar per 100 g of material (Gross *et al.*, 1958; Nordwig & Hayduk, 1969; this paper). Hydra in particular contains 6·7 g of neutral sugar per 100 g of mesoglea. The one possible exception is our measurement showing the mesoglea from the large scyphozoan jellyfish *Pelagia pacifica* contains 3·5 g of neutral sugar per 100 g of material; this difference in neutral sugar content may be due to extraction of some components during isolation of the fibers.

Not only is the absolute amount of neutral sugar found in cnidarian mesogleas similar to that found in vertebrate basal lamina, but the same sugars are found in both. We have identified glucose, galactose, fucose and rhamnose in hydra mesoglea. These sugars are also found in other cnidarian mesogleas (Gross *et al.*, 1958; Katzman & Kang, 1972) and some have been reported in vertebrate basal lamina (Spiro, 1970a; Kefalides, 1973). In contrast, extractable vertebrate collagens contain only glucose and galactose in the neutral sugar fraction (Spiro, 1970a).

Recently, Katzman & Kang (1972) reported that collagen isolated from the mesoglea of the sea anemone *Metridium dianthus* contains fucose, mannose and N-acetylglucosamine as well as glucose and galactose. Their report is the first of a purified collagen containing a saccharide moiety other than the glucose–galactose dimer. It is not known at present whether or not the fucose, rhamnose (Fig. 2) and glucosamine reported herein to be components of hydra mesoglea are actually linked to a collagen.

We believe that the chemical, developmental and morphological similarities between hydra mesoglea and vertebrate basal lamina may mirror some functional similarities as well. A role for epithelial collagens in vertebrate morphogenesis has been proposed (Hay & Dodson, 1973). A similar role in morphogenesis for hydra mesoglea as a "model" basal lamina is presently being explored.

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Key Word Index—Cnidarian mesoglea; basal lamina and collagen; neutral sugars; amino acid composition; invertebrate collagens; *Pelmatohydra pseudoligactis*.