1975

The Presence of Acid Polysaccharides in the Mesogloea of Chrysaora quinquecirrha

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THE PRESENCE OF ACID POLYSACCHARIDES IN THE MESOGLOEA OF CHRYSAORA QUINQUECIRRHA

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APPROVAL SHEET

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ABSTRACT

The monosaccharide constituents of hydrolyzed carbohydrate, extracted with trichloroacetic acid from lyophilized Chrysaora quinquecirrha mesogloea, were resoved by thin-layer chromatography and quantitatively estimated by spectrophotometry. The detection of glucosamine, galactosamine, and uronic acids demonstrates the presence of acid polysaccharides. An abundance of neutral sugars, high sulfate content, and inequalities between the hexosamine and uronic acid moieties indicate the simultaneous presence of vertebrate type glycosaminoglycans, novel glycosaminoglycans, and sulfated and/or neutral polysaccharides. Hypothetical structures are provided. The presence of acidic polysaccharides in Chrysaora mesogloea is phylogenetically important and favors the proposition that acid polysaccharides are involved in the stabilization and fibrillogenesis of collagen.

THE PRESENCE OF ACID POLYSACCHARIDES IN THE MESOGLOEA OF *CHRYSAORA QUINQUECIRRHA*

INTRODUCTION

The bilateral animal phyla are triploblastic in that during ontogenesis all of the tissues which will compose the adult organism are derived from three germinal layers: (1) ectoderm, (2) mesoderm, and (3) entoderm. Accordingly, the various connective tissues of these triploblastic phyla, characterized by having a gelatinous, fibrous, or hard intercellular matrix secreted by and containing scattered or loosely associated cells, are chiefly derived from the mesoderm (Balinsky, 1970; Hyman, 1940). In contrast, the three most primitive metazoan phyla: Porifera, Cnidaria, and Ctenophora, have come to be regarded as diploblastic; i.e., lacking a mesoderm. However, these phyla are in fact triploblastic, for they indeed possess a cellular stratum between epidermis (ectoderm) and gastrodermis (entoderm)(Hyman, 1940). This layer, commonly called mesogloea, although variable in its manifestation and composition, is never devoid of cellular elements, and, especially as represented among the Cnidaria, can be classified as a primitive connective tissue (Chapman, 1966; Hyman, 1940).

Several invertebrate connective tissues have been found to resemble vertebrate connective tissue histologically and chemically (e.g., Gross, Sokal and Rougvie, 1956; Maser and Rice, 1963; Nordwig and Hayduk, 1969; Nordwig, Rogall, and Hayduk, 1970; Spiro, 1972a). Such a histological and chemical correspondence between cnidarian mesogloea and vertebrate connective tissue has been established

through histochemical staining techniques, electron microscopy, and amino acid analysis (Chapman, 1953, 1959, 1966; Gross, Dumsha, and Glazier, 1958; Piez and Gross, 1959). For example, fibers in the mesogloea of the scyphozoan Pelagia have been shown by electron microscope examination to be axially banded with a periodicity of 660 Å, approximating that for vertebrate collagen (640 to 700Å) (Chapman, 1959). Similarly, a comparison of the amino acid composition of collagens from Calliactis, Aurelia, Cyanea, Chrysaora, rat tendon, and blood albumen reveals that the relative amounts of amino acids, especially hydroxyproline, in cnidarian collagen agree exceptionally well with that from rat tendon (i.e., vertebrate collagen), but only poorly with blood albumen (Chapman, 1953; Kirchenbaum, 1973). However, as the chemical nexus between cnidarian and vertebrate connective tissues is almost entirely based on collagen, it remains unclear whether or not the more subtle chemical entities and relationships characteristic of vertebrate connective tissue, are present in mesogloea. Since cnidarian mesogloea is a phylogenetically and histologically primitive connective tissue, and therefore may represent the genealogical origin of or a primitive archetype of the connective tissues of the higher Metazoa, it is important to know to what extent a chemical affinity exists between these two tissues, and if cnidarian mesogloea is typical of other invertebrate connective tissues.

In the last ten to fifteen years, the chemical architecture of vertebrate connective tissue has been intensely studied (Castellani, 1968; Hoffman, 1968; Lowther, Toole, and Herrington, 1970; Mathews, 1965, 1968, 1970; Quintarelli and Dellovo, 1970; Serafini-francassini,

Wells, and Smith, 1970; Toole and Harrington, 1970; White, Handler, and Smith, 1973). The outcome of these investigations has been to shift some of the importance in determining the connective tissue motif away from collagen, placing greater emphasis on other chemical constituents, chiefly acid polysaccharides. A considerable body of information has emerged which suggests that acid polysaccharides, especially the acid mucopolysaccharides [glycosaminoglycans (GAG)], are essential for, or in some way facilitate the formation and stabilization of collagen fibrils in vertebrate connective tissue (Hoffman, 1968; Lowther et al., 1970; Mathews, 1968; Weiss, 1962). Moreover, referring to studies of normal and abnormal connective tissues, inequalities in the relative abundance of certain acid polysaccharides appear to be linked with major differences in the fabric of connective tissues (Dorfman and Matalon, 1972; Malmgren and Sylven, 1952; Matalon and Dorfman, 1970; Meyer, Davidson, Linker, and Hoffman, 1956; Robertson and Hinds, 1956). Such inequalities in concentraion of acid polysaccharides may be related to similar variations in invertebrate connective tissue, especially the great differences in flexibility and intercellular structure of mesogloea among the Cnidaria (see Appendix A). Consequently, the detection of acidic polysaccharides in invertebrates may further clarify the relationship between acid polysaccharides and collagen in vertebrates, as well as delimit the extent of the analogy between invertebrate and vertebrate connective tissues. This author believes that if cnidarian mesogloea and vertebrate connective tissues are chemically analogous, then acid polysaccharides (including acid mucopolysaccharides) should be present in

mesogloea. Conversely, the absence of acid polysaccharides may indicate a disparity of phylogenetic significance and/or a gap in the present understanding of connective tissue architecture.

The variability in the manifestation and composition of mesogloea among the three cnidarian classes suggests that the detection of acid polysaccharides is favored in studies involving scyphozoans or anthozoans. It is this writer's opinion that the class Scyphozoa provides the most propitious candidates for a search for vertebrate connective tissue polysaccharides, since the mesogloea of certain scyphozoan medusae is not only close to vertebrate connective tissue in character, e.g., highly fibrous and well-ordered, but also ample in volume. Moreover, the Scyphozoa have not been adequately represented in cnidarian connective tissue studies.

The present investigation for evidence of acid polysaccharides in cnidarian mesogloea was conducted with Chrysaora quinquecirrha (white variety), a scyphozoan medusa abundant in the Chesapeake Bay during the summer months. The mesogloea of Chrysaora (medusa) is exceptionally fibrous and sufficient in volume to simplify its isolation from the remainder of the animal.

Ideal, unequivocal evidence for vertebrate-type acid polysaccharides in Chrysaora mesogloea is the isolation, purification, and specification of conformation of a specific polysaccharide. But this step, in delimiting the extent of a chemical analogy between cnidarian mesogloea and vertebrate connective tissue, is a long way from the preliminary evidence provided by histochemical staining techniques, x-ray diffraction, or amino acid analysis. This study

attempts to form the necessary bridge between these preliminary data and such an elucidation, by determining the identity and relative abundance of the monosaccharide constituents of a hydrolysate of the carbohydrate of Chrysaora mesogloea. More specifically, the acceptance or rejection of the hypothesis that acidic polysaccharides are present in Chrysaora mesogloea (pending confirmation by the isolation of intact polysaccharides with subsequent determination of structure, molecular weight, and glycosidic linkages) is dependent upon the detection of the monosaccharide constituents essential to the structure of acid polysaccharides (glycosaminoglycans), i.e., hexuronic acids and hexosamines. This question is answered in two parts: (1) qualitatively, by thin-layer chromatography, and (2) quantitatively, by spectrophotometric analysis.

ACID MUCOPOLYSACCHARIDES

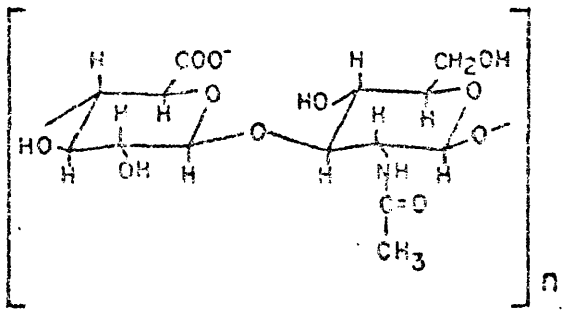
Inasmuch as acid mucopolysaccharides, or more precisely, glycosaminoglycans, are the major acidic polysaccharides in vertebrate connective tissue, it is useful to briefly delineate some of their chemical attributes. The acid mucopolysaccharides of chief importance in vertebrate connective tissue include hyaluronic acid, heparin, chondroitin sulfate A, condroitin sulfate B (dermatan sulfate), and chondroitin sulfate C.

The most ubiquitous of these polysaccharides is hyaluronic acid, having been isolated from umbilical cords, synovial fluid, skin, cockscombs, vitreous and aqueous humors, and a variety of tumors (Laurent, 1970; Meyer, 1956). The high viscosity and

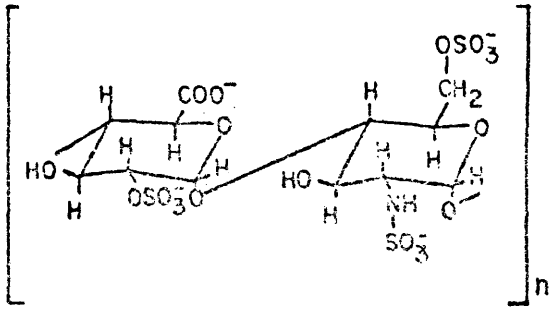
incompressibility of its aqueous solution make hyaluronic acid an essential constituent in watery, jelly-like, intercellular matricies as in the joints where it serves as a lubricant and shock absorber. Hyaluronic acid also has certain adhesive properties, making it an effective intercellular cement and therefore an important factor in maintaining a barrier against infection. But most important, hyaluronic acid forms the basis of the gelatinous, nonstructural portion of the ground substance in vertebrate connective tissue, thus imparting a flexibility to the entire tissue matrix that is commensurate with the stability of collagen fibrils and other structural elements.

Hyaluronic acid is a linear heteropolysaccharide with a minimum molecular weight varying from about 0.93×10^6 in streptococcal cultures to 14×10^6 in bovine synovial fluid, consisting of alternating residues of D-glucuronic acid and N-acetyl-D-glucosamine (Laurent, 1970). The basic repeating unit of hyaluronic acid is a disaccharide composed of the two alternating residues, joined by a $\beta(1\rightarrow3)$ glycosidic linkage, the repeating disaccharide units being joined by $\beta(1\rightarrow4)$ linkages (Laurent, 1970) (see Figure 1).

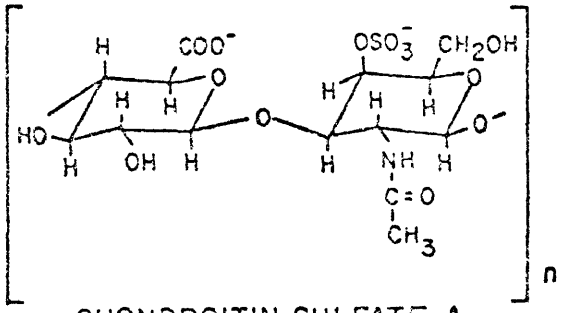
As generally accepted, the structure of the sulfated mucopolysaccharide, heparin, is essentially similar to that of hyaluronic acid. It is a linear heteropolysaccharide made of repeating disaccharide units, each consisting of alternating residues of D-glucuronic acid and a hexosamine. However, heparin differs from hyaluronic acid in three ways: (1) all α-D$(1\rightarrow4)$ glycosidic linkages rather than the alternating β-D$(1\rightarrow3)$ and β-D$(1\rightarrow4)$ of hyaluronic acid; (2) the



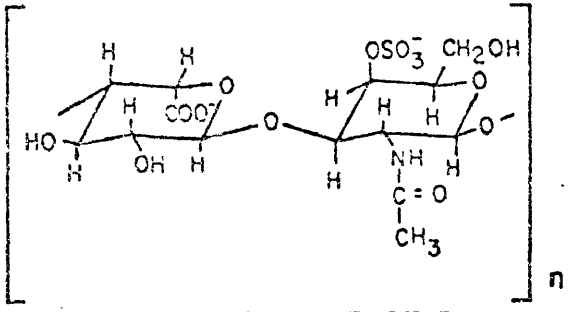
HYALURONIC ACID



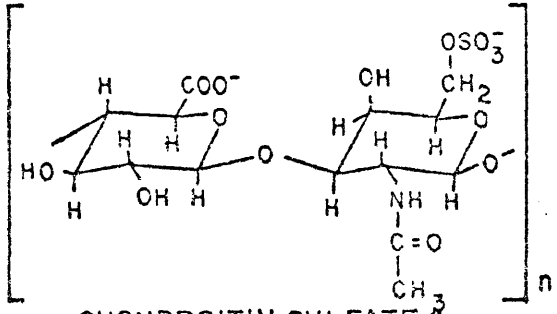
HEPARIN



CHONDROITIN SULFATE A



CHONDROITIN SULFATE B
(DERMATAN SULFATE)



CHONDROITIN SULFATE C

Figure 1. Structures of some acid mucopolysaccharides of chief importance in vertebrate connective tissue.

replacement of N-acetyl-D-glucosamine with an N-sulfated glucosamine; and (3) a sulfate ester group at the sixth carbon position of the N-sulfated hexosamine, and one at the second carbon position of about half of the uronic acid residues (Lindahl, 1970) (see Figure 1).

An acid mucopolysaccharide closely related to heparin is heparan sulfate. As the essential difference between the two polysaccharides appears to be quantitative rather than qualitative, the structure of heparan sulfate is similar or identical to that of heparin (Cifonelli, 1970; Lindahl, 1970). Heparan sulfate is thought to have more N-acetylated and fewer N- and O-sulfated substituents than heparin (Lindahl, 1970). The molecular weight of heparan sulfate and heparin is about 1×10^4 (White et al., 1973).

Although heparin is covalently linked to protein in its native state (Lindahl, 1970; Roden, 1968), its relationship with the structural elements of connective tissue is unclear. Heparin is a major constituent in such tissues as liver, lung, and arterial walls (Whistler and Smart, 1953; White et al., 1973). However, its presence in these tissues is primarily physiological rather than structural since heparin is an excellent anticoagulant (White et al., 1973).

The chondroitin sulfates A, B[1], and C may be described collectively. Through a combination of covalent bonding and electrostatic interaction, the chondroitin sulfates are intimately associated with the structural elements of connective tissue (Quintarelli and Dellovo, 1970). Yet, these sulfated mucopolysaccharides do not occur in connective tissue equivalently. For example, chondroitin sulfate A or A and C have been found to predominate in bond or hard-cartilaginous tissues, chondroitin sulfate C in certain soft-cartilaginous tissues (e.g., spinal disks), and B or B and C in such elastic tissues as skin, tendons, ligaments, and heart valves (Meyer et al., 1956; White et al., 1973). This pattern suggests that the association of the various chondroitin sulfates with the structural elements of connective tissue differs, and that accordingly, chondroitin sulfate A in some way influences the tissue matrix to become fibrous and inflexible, whereas chondroitin sulfates B and C influence the tissue matrix to become less fibrous and more flexible.

The chemical structures of chondroitin sulfates A, B, and C also resemble the structure of hyaluronic acid (see Figure 1 and Table 1). They have the same interdisaccharide glycosidic linkages as has hyaluronic acid, i.e., $\beta(1\rightarrow4)$. Similarly, the

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[1]Due to the degree of structural dissimilarity between chondroitin sulfate B and chondroitin (the parent substance of chondroitin sulfates A and C), some authors (e.g., White et al., 1973) consider the designation chondroitin sulfate B to be a misnomer and prefer instead the term dermatan sulfate. The older name is here retained for simplicity and to emphasize the continuity of the physiological relationship of this compound with chondroitin sulfates A and C in the connective tissue matrix.

TABLE 1

TABLE OF LINKAGES[a]

Polysaccharide	Monosaccharides[b]	Disaccharide linkages	
		Intra	Inter
Hyaluronic acid	β-D-glucuronic acid,		
	β-N-acetyl-D-glucosamine	β(1→3)	β(1→4)
Heparin (and	β-D-glucuronic acid-2-sulfate,		
heparan sulfate)	β-N-sulfate-D-glucosamine-6-sulfate	α(1→4)	α(1→4)
Chondroitin sulfate A	β-D-glucuronic acid,		
	β-N-acetyl-D-galactosamine-4-sulfate	β(1→3)	β(1→4)
Chondroitin sulfate B	β-L-iduronic acid,		
(dermatan sulfate)	β-N-acetyl-D-galactosamine-4-sulfate	β(1→3)	β(1→4)
Chondroitin sulfate C	β-D-glucuronic acid,		
	β-N-acetyl-D-galactosamine-6-sulfate	β(1→3)	β(1→4)

TABLE 1--Continued

[a]Reconstructed from White, Handler, and Smith, 1973.

[b]Monosaccharides are listed in the same order as linkages; e.g., for hyaluric acid, the intra-disaccharide linkage is β(1→3), while the inter-disaccharide likage is β(1→4).

intradisaccharide linkages of chondroitin sulfates A and C are β(1→3), as in hyaluronic acid. The intradisaccharide linkages in chondroitin sulfate B are α(1→3), but have the same absolute configuration[2] as do the intradisaccharide linkages of hyaluronic acid (White et al., 1973).

The chondroitin sulfates differ from hyaluronic acid with respect to monosaccharide constituents: N-acetyl-D-galactosamine, O-sulfated at carbon atom four in chondroitin sulfates A and B, and at carbon atom six in chondroitin sulfate C, replaces the N-acetyl-D-glucosamine of hyaluronic acid. In chondroitin sulfate B, L-iduronic acid is substituted for the D-glucuronic acid of chondroitin sulfates A and C (White et al., 1973).

CARBOHYDRATE-PROTEIN INTERACTIONS AND SMALLER CARBOHYDRATE UNITS IN CONNECTIVE TISSUE

By delineating the structures and chief functions of the major vertebrate glycosaminoglycans ("classic" acid mucopolysaccharides), the previous section represents an attempt to describe the major carbohydrate components of vertebrate and advanced invertebrate connective tissues. However, the description is oversimplified in that a number of smaller carbohydrate units, or oligosaccharides certainly play a significant role in effecting and sustaining the superorder of the connective tissue motif. Selected details concerning the

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[2]An α-L glycosidic linkage has the same absolute configuration as a β-D linkage. The intradisaccharide linkage of chondroitin sulfate B is α-L(1→3).

various interactions of these smaller carbohydrate units and the glycosaminoglycans with protein are introduced as they may allow a better interpretation of the results of this investigation.

A numerically minor but structurally important constituent of vertebrate collagen is hydroxylysine (Piez and Gross, 1959, 1970; Spiro, 1972a). Hydroxylysine has been shown to be covalently linked with carbohydrate (Spiro, 1969a, 1972a; Spiro and Fukushi, 1969). In vertebrates, the dominant hydroxylysine-linked carbohydrate of collagen is in the form of single galactose residues, while that of the collagen-like protein of basement membranes is more abundantly in the form of a disaccharide containing glucose and galactose (e.g., glucosylgalactosylhydroxylysine; Sprio, 1972a). A number of invertebrate collagens have been investigated and found to possess hydroxylysine, but at a much higher ratio per 1,000 amino acid residues than that of vertebrate collagen. Moreover, the carbohydrate content of invertebrate collagen is predominantly in the form of hydroxylysine-linked disaccharides identical in structure to that found in basement membranes and certain mammalian collagens (Spiro, 1972a; Spiro and Bhoyroo, 1971). An exception to this is manifest in detailed investigations of collagens obtained from Lumbricus (earthworm) cuticle. Hydroxylysine is absent in this collagen, and therefore, hydroxylysine-linked carbohydrate is not present. Yet di- and tri-saccharides containing galactose were found to be linked to serine and threonine residues [2-O-α-D-glucopyranosyl-D-galactose and O-α-D-galactopyranosyl-(1→2)-O-α-D-galactopyranosyl-(1→2)-D-galactose; Josse and Harrington, 1962; Muir and Lee, 1969; Spiro, 1972a]. With

this exception, hydroxylysine-linked mono- and/or di-, and tri-saccharides of galactose (±glucose) appear to be universally found in both vertebrate and invertebrate collagen (Kelfalides, 1970; Nordwig, Rogall, and Hayduk, 1970; Spiro, 1970a, 1970b, 1972a).

The recent finding by Butler (1970) that there are "holes" in the α_1 chain of rat skin collagen to accomodate disaccharide molecules, underscores the suggestion that hydroxylysine- or other amino acid-linked oligosaccharides may have a direct and highly effective control over the periodicity and fibrillogenesis of collagen, in a manner corresponding to or complimenting that proposed for the acid mucopolysaccharides. It is possible that a small amount of hydroxylysine-linked carbohydrate can be accommodated by the polypeptide chain, resulting in a stabilization or "fixing" of the periodicity characteristic of vertebrate collagen: 640 Å. But when the amino acid-linked mono- or oligo-saccharides are more numerous covalently and sterically speaking than available spaces for the normal assembly of the tropocollagen molecules, the organization of the fibrils is disrupted and a new, often random periodicity, or aperiodicity is established. This would explain, in view of the abundance of carbohydrate, the considerable variation of axial periodicity observed in invertebrate collagens, e.g., 200 Å in Metridium and Physalia, 300-600 Å in collagen from Lumbricus body wall, and no axial periodicity in Lumbricus cuticle cartilage or Ascaris cuticle collagen (Piez and Gross, 1959; Reed and Rudall, 1948; Spiro, 1972a; Watson and Silvester, 1959).

This revelation in no way diminishes the role of the acid

mucopolysaccharides in the stabilization and fibrillogenesis of collagen. Rather, it serves to emphasize the necessity of maintaining a broad view of the possible complexity of mechanisms involved in the ordering of the connective tissue matrix. In this respect, a dichotomous influence of the GAG over the structural elements of connective tissue seems to prevail. Ogston (1970) tends to regard the role of glycosaminoglycans in connective tissue as passive, i.e., they check the proliferation of collagen fibrils in the cellular environment by taking up intercellular space (exclusion effects). In contrast, a number of authors (Meyer, 1956; Quintarelli and Dellovo, 1970; and Weiss, 1962) believe that the evidence from studies of the interaction of the protein and carbohydrate moieties of connective tissue necessitates a more active role for the glycosaminoglycans. Weiss (1962), for example, suggests that electrostatic interactions between collagen and GAG might create physio-chemical conditions in the groundsubstance of connective tissue which would, in effect, stake out acceptor sites for collagen fibers to settle upon. However, the most comprehensive explanation of the observed effects of the glycosaminoglycans in the connective tissue organization recognizes both a passive and active role for the GAG in that steric hindrance of protein (exclusion effects) and electrostatic interaction/covalent bonding with protein are involved (Mathews, 1965, 1970; Quintarelli and Dellovo, 1970).

That the sulfated glycosaminoglycans are covalently bound to protein has been established (Hallen, 1970; Mathews, 1970; White et al., 1970). For example, in cartilage, chondroitin sulfate A is

bound to protein via the trisaccharide galactosylgalactosylxylose (linked to a serine or threonine residue of the polypeptide chain by a xylosidic bond), while in corneal tissue, keratin sulfate is linked by an N-glycosidic bond of the N-acetylglucosamine to asparagine (White et al., 1973). However, it remains unclear whether or not these glycosaminoglycans are covalently linked to collagen, although covalent bonding between chondroitin sulfate and collagen (in bovine nasal cartilage) is strongly suggested (Hoffman and Mashburn, 1970).

It will be recalled from the description of specific vertebrate acid polysaccharides that the relative abundance of the acid mucopolysaccharides in a connective tissue correlates with the overall character of the tissue; e.g., hyaluronic acid and/or chondroitin sulfate B predominate in the more flexible tissues, while chondroitin sulfate A predominates in cartilage and bone. A causal link or dependency between the structural (and functional) features of a connective tissue and its glycosaminoglycan composition is further evidence of a more than passive participation for acid mucopolysaccharides in the fibrillogenesis and stabilization of collagen and thereby, in the ordering of the tissue matrix. The existence of such a causal or dependent relationship is made more apparent by diseases (mucopolysaccharidoses) in which major defects in a connective tissue can be directly tied to an imbalance in the glycosaminoglycan constituents of the tissue (Dorfman and Matalon, 1972; Matalon and Dorfman, 1970; Spiro, 1969b; White et al., 1973).

In addition to the galactose and glucose of oligosaccharides

linked to hydroxylysine and/or other amino acid residues, a variety of sugars has been found intimately associated with invertebrate collagen: mannose, fucose, glucosamine, galactosamine, hexuronic acid, xylose, and arabinose (Katzman and Jeanloz, 1970a, 1970b; Spiro, 1970b, 1972a). It is possible that large polysaccharides other than the glycosaminoglycans, are involved in the stabilization and fibrillogenesis of invertebrate collagen. These monosaccharides may either be (1) components of oligosaccharides that provide a link between unknown larger polysaccharides and the polypeptide chain in a manner analogous to that of galactosylgalactosylxylose with chondroitin sulfate A in cartilage; or (2) may be residual fragments of a large polysaccharide. The presence of most of these sugars in the "protein-free" carbohydrate moiety of invertebrate connective tissues favors the latter suggestion. The possibility of substitutes for GAG is further suggested by the recent finding of a glucuronic acid-mannose dissacharide (in addition to the mono-, di-, and tri-saccharides of galactose) associated with Nereis (clamworm) cuticle collagen (Spiro and Bhoyroo, 1971). This also suggests that unfamiliar associations of uronic acid with other monosaccharide units (e.g., the hexosamines) may occur and that accordingly, glycosaminoglycans which differ structurally and chemically from vertebrate-type GAG may exist. In any event, the occurrance of a novel, uronic acid containing disaccharide unit, demonstrates that by itself, the detection of uronic acids in invertebrate connective tissue is insufficient evidence for the presence of the classic acid mucopolysaccharides such as hyaluronic acid, heparin, or the chondroitin sulfates.

METHODS AND MATERIALS

Isolation of Mesogloea

Chrysaora quinquecirrha (order Semaeostomae, family Pelagidae) manifests a typical life cycle consisting of planula, scyphistoma, strobila, ephrya, and cysts. The tissue structure of the medusa of Chrysaora is typical for the Semaeostomae and can be divided into three layers: (1) an ectoderm, (2) the mesogloea ("mesoderm"), and (3) an entoderm or gastroderm. The semispherical umbrella or bell consists largely of mesogloea, delimited at its outer convex surface by a very thin epithelium (ectoderm). An equally thin endodermal layer sharply separates the bottom or subumbrella side of the mesogloea from the gastrovascular cavity, from which a number of radial canals extend to connect with the sense organs (rhopalia) and tentacles located at the bell margin. The remaining anatomy of the organism consists of four horseshoe-shaped gonads, seated in the floor of the gastrovascular cavity; the manubrium, which is a pendulum-like extension of the mouth; and four frilled oral arms, descending from the manubrium (see figures 2, 3, and 4).

Mature Chrysaora (having a bell diameter of between 13 and 20 cm) were collected from the York River at the Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia. As the mesogloea in the tissue structure of the adult Chrysaora medusa is sharply delimited from the gastrodermal tissues, a surgical isolation is facilitated. Hence, as illustrated by Figures 5 and 6 (A through F),

Figure 2. Chrysaora quinquecirrha, with oral arms extended.

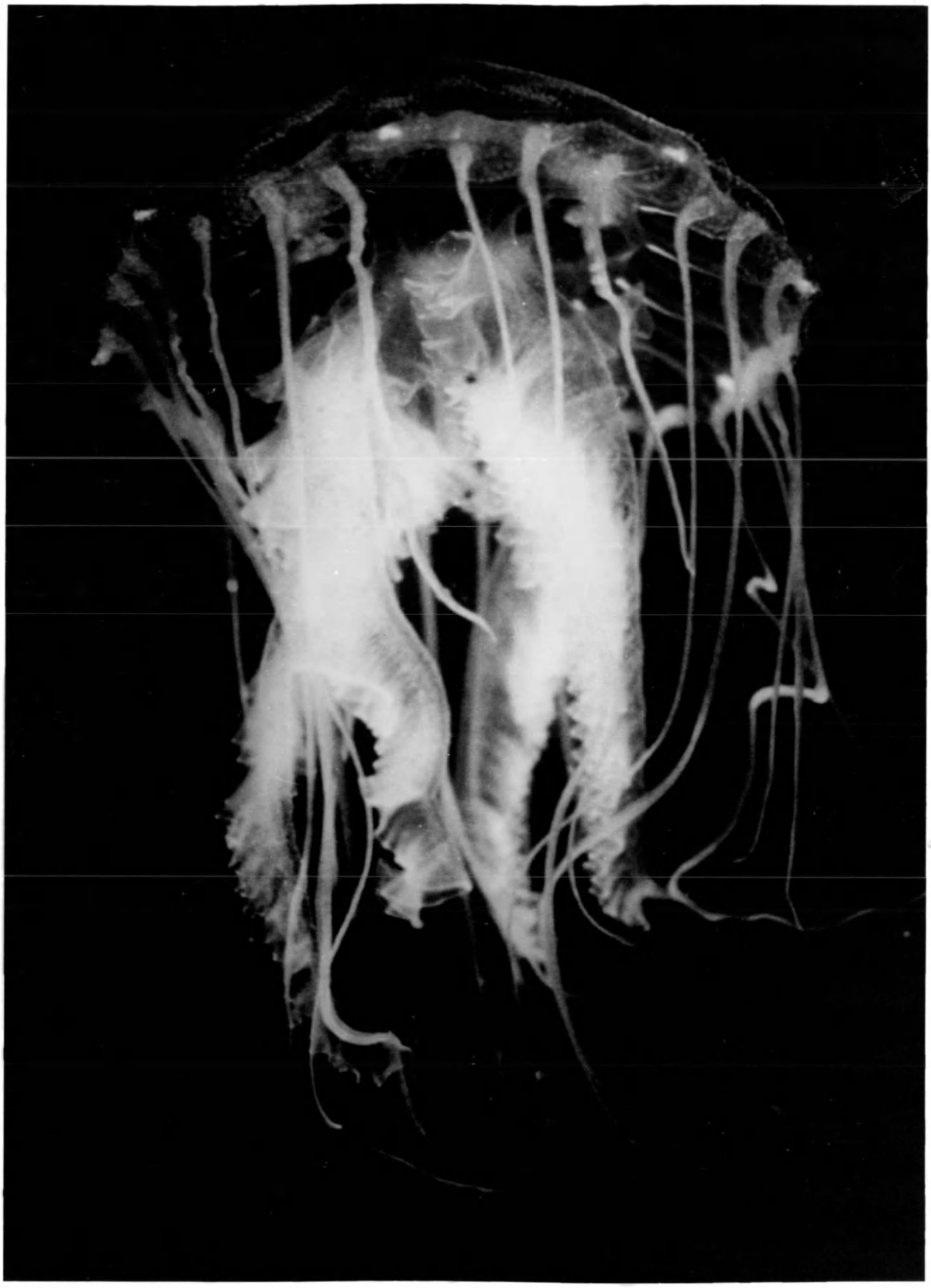


Figure 3. Chrysaora quinquecirrha, showing underside--mouth, opening into gastrovascular cavity.

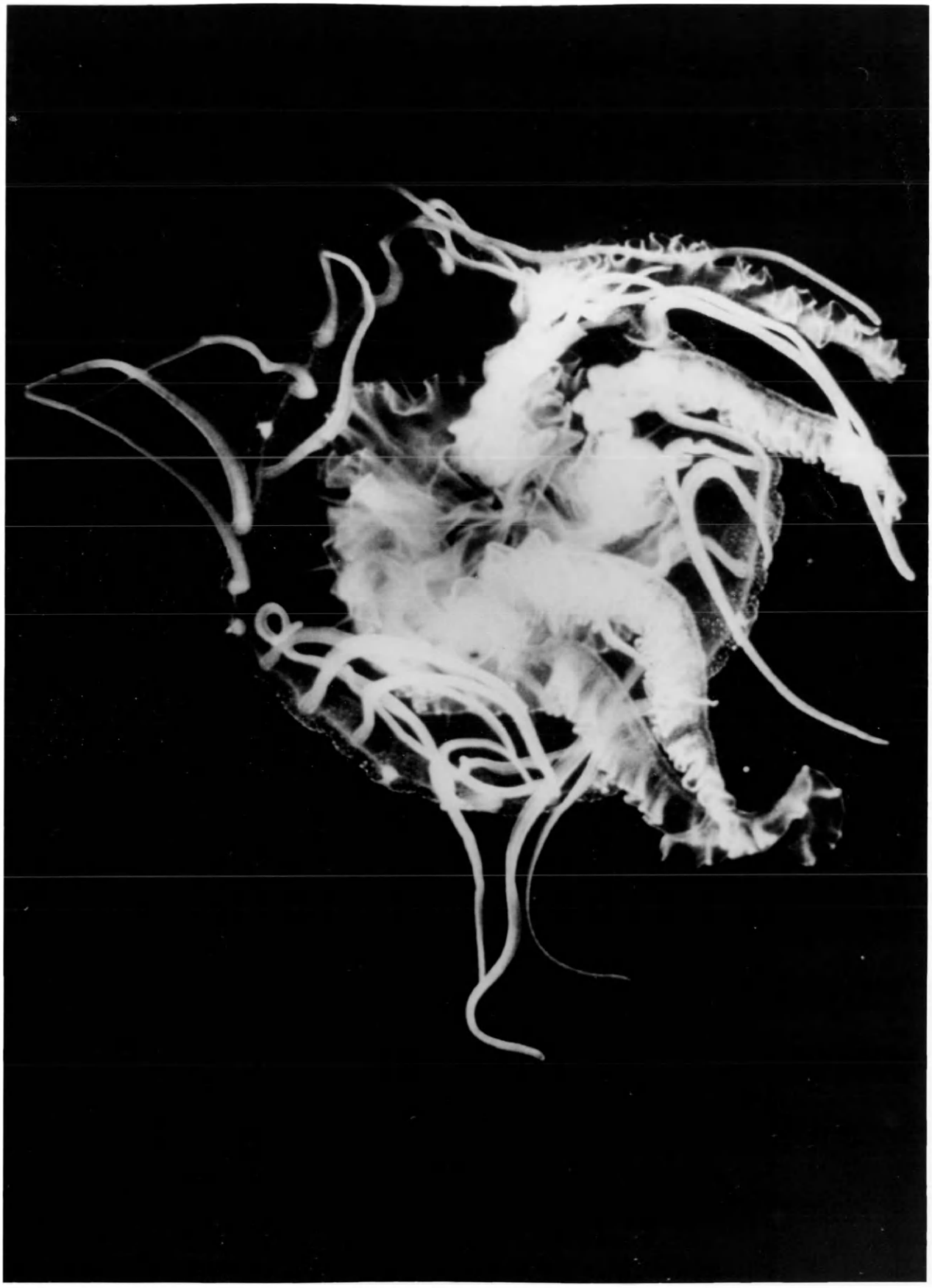


Figure 4. Chrysaora quinquecirrha, illustrating mesogloea in intact organism.

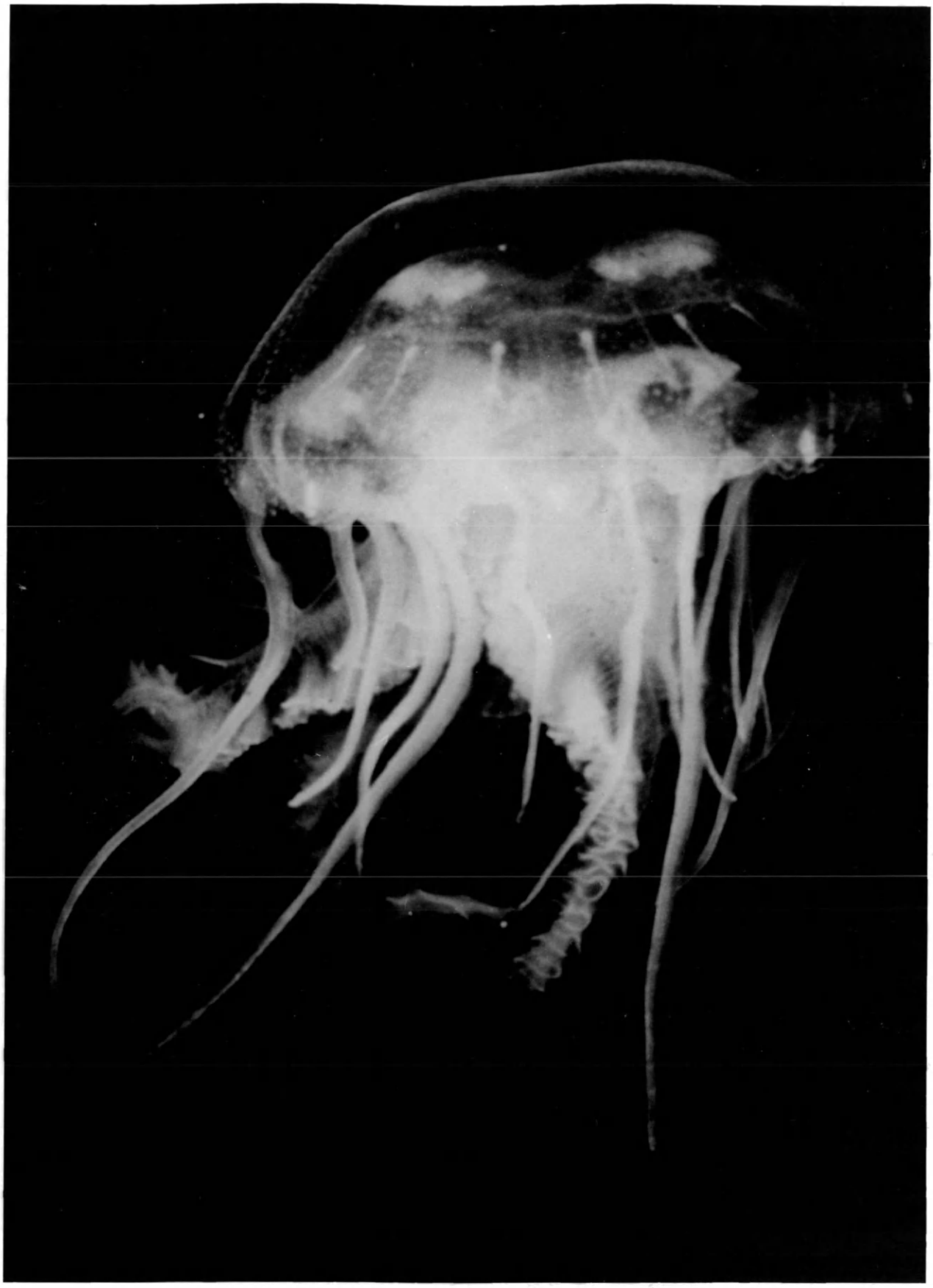
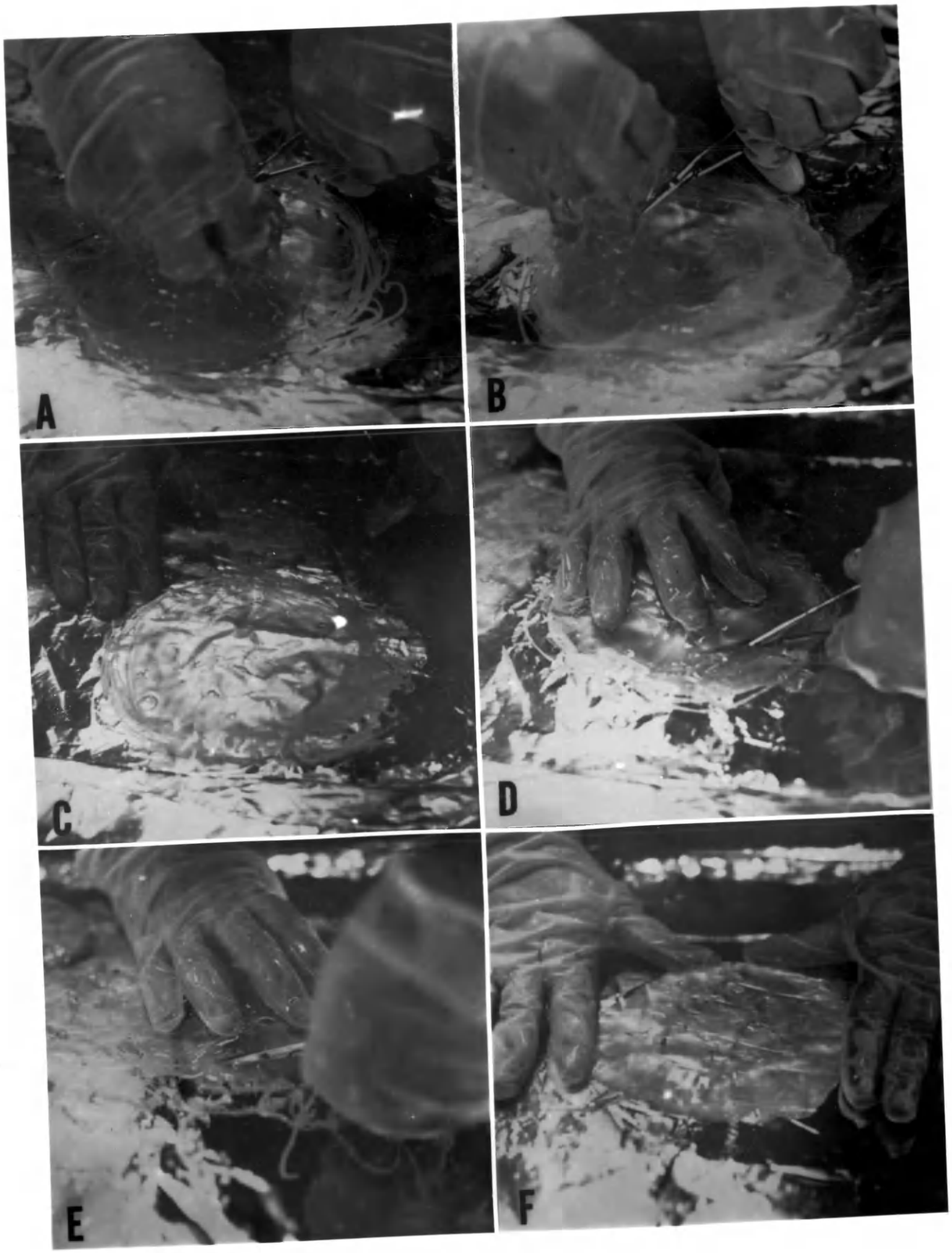


Figure 5. Manubrium, gastrovascular cavity, gonads, oral arms prior to surgical isolation of mesogloea.

Figure 6. Surgical isolation of mesogloea.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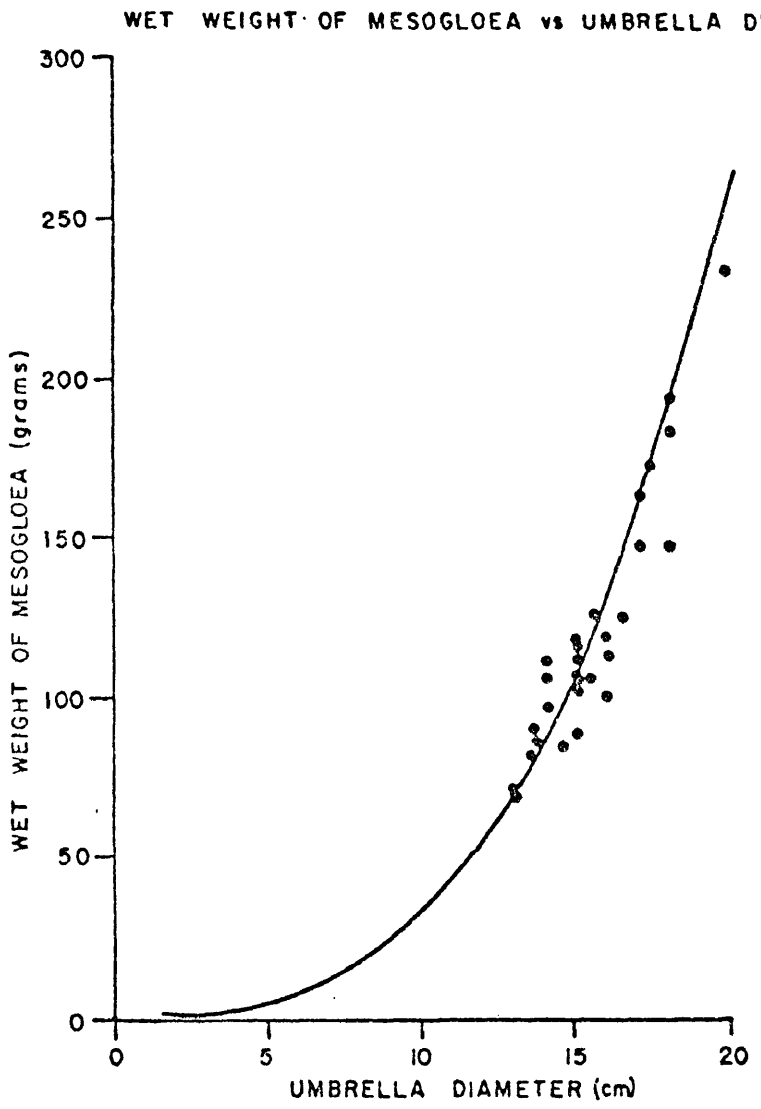
the mesogloea was obtained by the removal of the gastrovascular system (manubrium, gastrovascular cavity, gonads, oral arms), tentacles and approximately ten millimeters of the bell margin. The resulting patties of gelatin were washed in cold, distilled water, blotted dry, weighed, and lyophilized. The percent composition for total solids was found to be 1.834 $\pm$0.002%, i.e., 98.166% water.

To correlate the amount of mesogloea obtained from a single organism with the size of the organism, as determined by the bell diameter, a brief survey was conducted. The bell diameters of thirty randomly selected Chrysaora medusae were measured upon a flat surface, the mesogloea surgically removed as before, and its weight determined. From these data, the mean weight of mesogloea, 119.34 grams, was found to correspond to a mean bell diameter of 15.39 centimeters. A graph of the weight of mesogloea versus bell diameter was plotted, and a regression line for the weight of mesogloea versus bell diameter cubed (see Figure 7) constructed. The slope of the regression line is approximately $\frac{1/6\pi}{16}$. Hence supported by a flat surface, the umbrella contains a volume of mesogloea approximately equivalent to 1/16 of that of a sphere having the same diameter: $V = \frac{1/6\pi d^3}{16}$.

Isolation and Hydrolysis of Carbohydrate

Weighed portions of freeze-dried mesogloea (ten grams each, dried to constant weight) were suspended with ultrasonic agitation in 5% trichloroacetic acid and centrifuged (repeated five times for each sample). Two volumes of 95% ethanol were added to the supernate



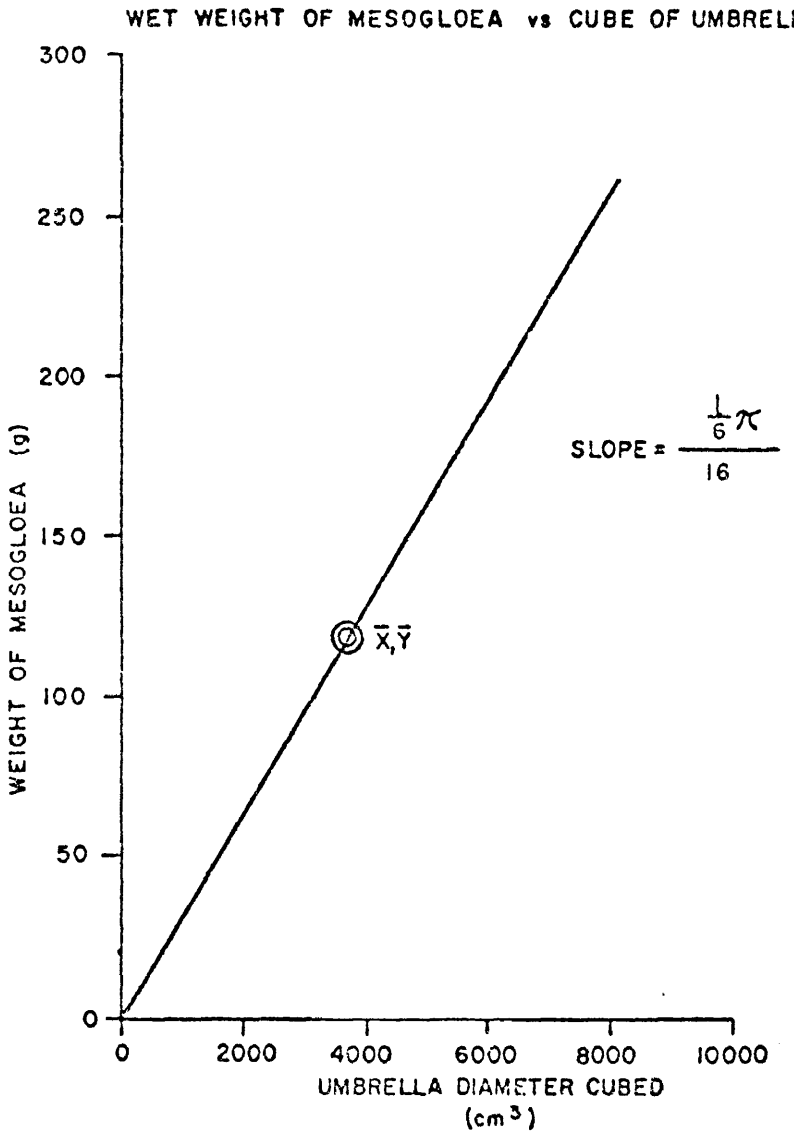


Figure 7. Graphs of wet weight of mesogloea versus umbrella diameter, and versus umbrella diameter cubed.

(acid soluble carbohydrate fraction) and the mixture refrigerated (0°C) for seventy-two hours. The carbohydrate precipitate was recovered by centrifugation, washed three times with 95% ethanol, then with anhydrous ethyl ether and dried to constant weight. The acid insoluble fraction (protein plus trichloroacetic acid insoluble carbohydrate) was washed once with 95% ethanol, followed by anhydrous ethyl ether (three times) to extract the trichloroacetic acid, and dried to constant weight. The percent composition of the mesogloea wet weight for the several moieties was determined (also see Table 2):

0.137%	Protein plus trichloroacetic acid insoluble carbohydrate
0.036%	Trichloroacetic acid soluble carbohydrate
1.661%	Salts
1.834%	Total solids

A portion of the carbohydrate moiety (0.2658 grams) was hydrolyzed for six hours at 100°C in 4N HCl (subjected to ultrasonic agitation after first four hours). The resulting acid mixture was taken to dryness under diminished pressure at 25°C over sodium hydroxide pellets. The dry hydrolysate residue was redissolved in 1.0 ml of distilled water and 0.5 ml of 95% ethanol added to prevent the growth of microorganisms.

Thin-Layer Chromatography

The monosaccharide constituents of the mesogloea carbohydrate hydrolysate were resolved by thin-layer chromatography; one to three µl of the hydrolysate were applied to commercially prepared plates: Silica Gel F-254 on Al (0.25 mm thick; E. Merck, Darmstadt, West

TABLE 2

FRACTIONATION OF 10.0000 GRAMS OF
FREEZE-DRIED MESOGLOEA
(8 Samples)

Sample number	Grams		
	Protein[a]	Carbohydrate[b]	Salts
1	0.7591	0.1681	9.0728
2	0.7587	0.1692	9.0721
3	0.7586	0.1790	9.0624
4	0.7520	0.1924	9.0556
5	0.7472	0.1953	9.0575
6	0.7446	0.2008	9.0546
7	0.7404	0.2307	9.0289
8	0.7274	0.2328	9.0398
Mean	0.74850	0.19604	9.05546
s[c]	±0.01107	±0.02502	±0.01503

[a]Trichloroacetic acid insoluble fraction.

[b]Trichloroacetic acid soluble.

[c]Standard deviation.

Germany). There were four solvents employed (see Figure 8): (1) ethyl acetate-pyridine-acetic acid-water (5:5:1:3), (2) ethyl acetate-2-propanol-water (27:3.5:1), (3) ethyl acetate-1-propanol-water (1:5:1), and (4) 1-butanol-pyridine-0.1 N HCl (5:3:2). The developed plates were dried at 120°C for five minutes, or, if the solvent contained pyridine, at 100°C for forty minutes. The chromatograms were visualized preferentially under ultraviolet light (254 nm, 350 nm) after spraying with 2',7' dichlorofluroescein. (Plates commercially impregnated with a fluorescent indicator were not sprayed.) Iodine was also used as a visualization reagent but with results inferior to that achieved by ultraviolet illumination.

Spectrophotometry

The relative abundance of the monosaccharide constituents represented in the mesogloea carbohydrate moiety was estimated by spectrophotometric analysis. A portion of the mesogloea carbohydrate (0.2041 g) was hydrolyzed for six hours at 100°C in 4N HCl and taken to dryness as before. The dry hydrolysate was redissolved in 10.0 ml of distilled water. The analysis employed four color reactions: (1) Anthrone-Sulfuric acid (hexose), (2) L-Cysteine-Sulfuric acid (pentose), (3) Carbazole-Sulfuric acid (uronic acid), and (4) Elson-Morgan (modification by Blix) (hexosamine) (Dische, 1962a-e).

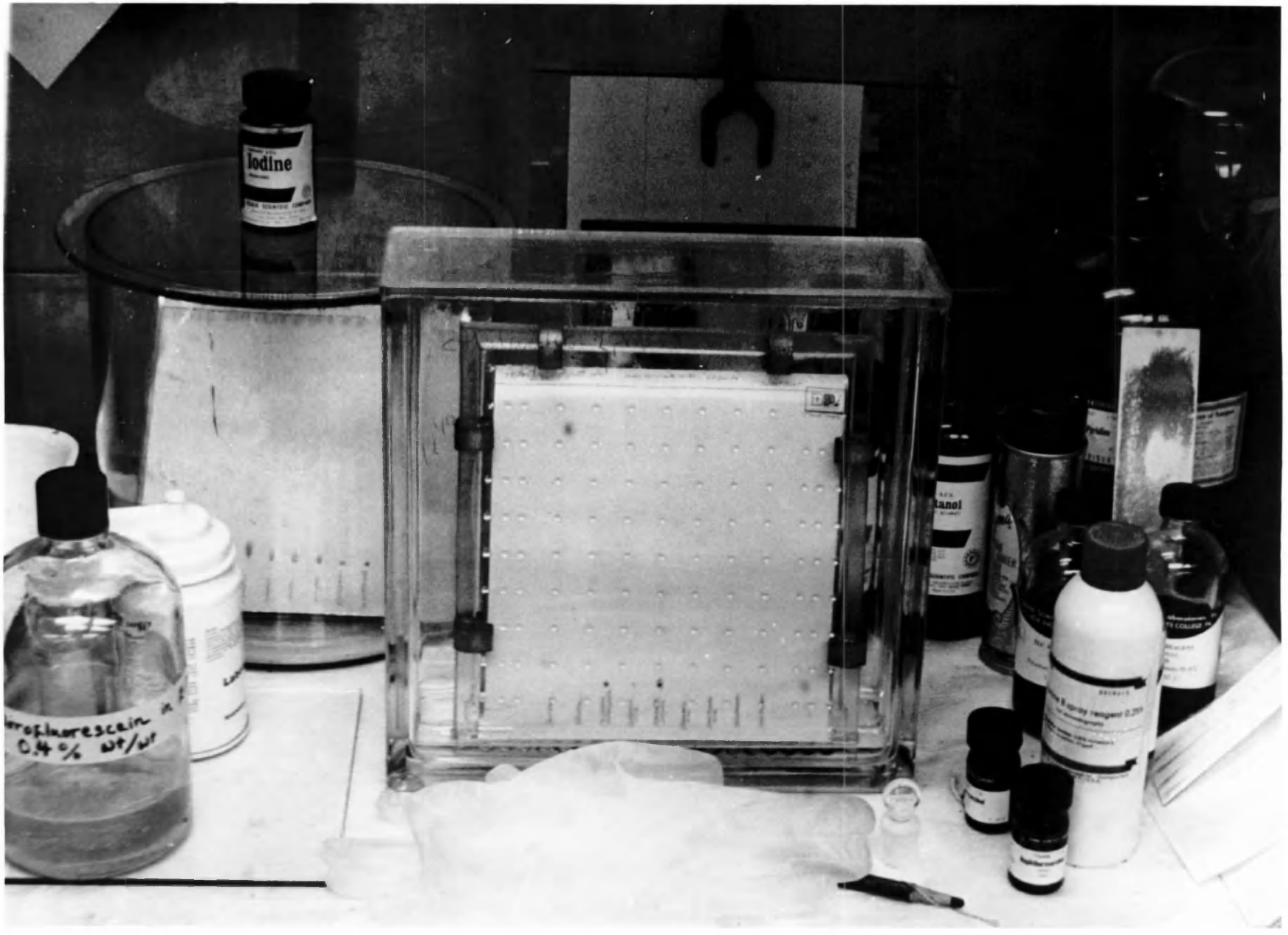
Anthrone

Approximately 0.004 g of the hydrolyzed carbohydrate (0.2 ml) was dissolved in 10.0 ml of distilled water. To 1.0 ml of this solution, 10.0 ml of freshly prepared anthrone reagent were added, the

Figure 8. Solvent System 2: ethyl acetate-2-propanol-water (27:3.5:1) with Silica Gel F-254 on Al.

A plate, which has been spotted with the mesogloea hydrolysate and several known sugars, is placed in a developing tank (foreground) saturated with solvent. By removing the plate, the movement of the solvent--from the lower to the upper end of the plate--is arrested. To enhance the reproducibility of the chromatograms, the limit of the solvent front has been preselected by scoring a line across the upper part of the silica gel layer. From left to right, the sugars applied to this plate are: fucose, glucose, the mesogloea carbohydrate hydrolysate, hyaluronic acid hydrolysate, chondroitin sulfate (mixed isomers A, B, C) hydrolysate, glucuronic acid, galacturonic acid, glucosamine, and galactosamine. Running time for this solvent system is approximately two hours.

After the plate is dried, spots are visualized under ultraviolet light with 2',7' dichlorofluorescein spray or without if an UV fluorescent indicator has been commercially added to the plate. The tank at the left-rear of the photograph contains iodine crystals, an excellent general visualization reagent but lacking the revsolving power of ultraviolet light.


Iodine
0.4 % wt/vt

mixture chilled in a water bath to approximately 15°C. The mixture was agitated, warmed to room temperature, placed in a boiling water bath for fifteen minutes, and cooled to room temperature. Xylose, fucose, and glucose, comprising three sets of serially diluted standards, were run.

The approximate concentrations of xylose, fucose, and glucose were obtained by solving three simultaneous equations with absorbance values taken at 503, 590, and 620 nm (see Appendix C). (A blue color with an absorption maximum at approximately 620 nm is produced with glucose.) Spectrophotometric measurements were made immediately following the completion of the reaction.

L-Cysteine

Approximately 0.002 g (0.1 ml) of the hydrolyzed carbohydrate moiety was dissolved in 10.0 ml of distilled water. To 1.0 ml of this solution, 4.0 ml of concentrated sulfuric acid was added. The mixture was shaken, cooled to room temperature under tap water, and allowed to stand for one hour with frequent agitation. To this, 0.1 ml of 3% L-cysteine hydrochloride monohydride solution (3 g/100 ml distilled water) were added with shaking. Fucose, xylose, and arabinose, comprising three sets of serially diluted standards, were run.

Spectrophotometric measurements were made about twenty minutes after the addition of the cysteine solution. The approximate concentrations of xylose, fucose, and arabinose were determined by the method of three simultaneous equations using absorbance values taken

at 397, 390, and 327 nm. (The absorption maximum for xylose occurs at about 390 nm.)

Carbazole

Approximately 0.001 g (0.05 ml) of the hydrolyzed carbohydrate was dissolved in 1.0 ml of distilled water, and 6.0 ml of concentrated sulfuric acid added. The mixture was heated for twenty minutes at 100°C in a water bath and cooled to room temperature under tap water. After cooling, 0.2 ml of 0.1% carbazole in ethanol (0.1 g/100 ml) was added, the mixture shaken and allowed to stand for about two hours. (A purple color appeared with an absorption maximum at about 535 nm.) Xylose, fucose, and glucuronic acid, comprising three sets of serially diluted standards, were run.

The approximate concentrations of hexuronic acid (glucuronic acid plus iduronic acid), xylose, and fucose were determined by the method of three simultaneous equations using absorbance values taken at 440, 515, and 560 nm.

Elson-Morgan

Approximately 0.001 g (0.05 ml) of the hydrolyzed carbohydrate was dissolved in 4.0 ml of distilled water, and 2.0 ml of 4% acetyl acetone in 1.25 N sodium carbonate (4 ml/100 ml) added. The mixture was heated in a water bath at approximately 90°C for one hour. After heating, 16.0 ml of ethanol and 2.0 ml of N,N-dimethyl-p-aminobenzaldehyde reagent (1.6 g of N,N-dimethyl-p-amino-benzaldehyde: 30 ml 95% ethanol:30 ml concentrated HCl) were added with agitation. (A red color with an absorption maximum at about 530 nm developed

rapidly.) Glucosamine and galactosamine, comprising two sets of serially diluted standards, were run.

Spectrophotometric measurements were made immediately following the addition of the N,N-dimethyl-p-aminobenzaldehyde reagent. The approximate concentrations of glucosamine and galactosamine were obtained by solving two simultaneous equations with absorbance values taken at 529 and 539 nm, respectively.

RESULTS

Thin-Layer Chromatography

Analysis of the Chrysaora mesogloea carbohydrate hydrolysate by thin-layer chromatography revealed the presence of hexosamines and hexuronic acids, in addition to an abundance of neutral sugars. In order of their relative abundance suggested by inference from the size and intensity of the spots, the following monosaccharides were detected in the hydrolysate: xylose, fucose, glucosamine, galactosamine, "iduronic acid," glucuronic acid, arabinose, mannose, and glucose.

Rf Values

Each monosaccharide has a characteristic Rf value when run with a given chromatographic system [= solvent system (i.e., solvent + thin-layer support, e.g., silica gel) + physiochemical conditions in which the chromatography takes place, including chamber saturation, ambient humidity, and temperature]. The Rf value for a particular sugar is determined by measuring the distance from the center of the visualized spot to the origin, and dividing this value by the distance from the origin to the solvent front. A tabulation of Rf values (Table 3) precedes the following description of results obtained with the employed solvent systems.

Changes in humidity, chamber saturation, and temperature can cause substantial variations in Rf values between runs of the same

TABLE 3

R_f VALUES (X 100) of MONOSACCHARIDES USING THIN-LAYER CHROMATOGRAPHY WITH SILICA GEL F-254 ON AL

Solvent system							
1	Ratio	2	Ratio	3	Ratio	4	Ratio
Ethyl acetate	5	Ethyl acetate	27	Ethyl acetate	1	n-Butanol	5
Pyridine	5	2-Propanol	3.5	n-Propanol	5	Pyridine	3
Acetic acid	1	Water	1	Water	1	0.1N HCl	2
Water	3						
Development time (hours)							
5		2		7		10	
Resolution							
Neutral	good	retarded		good		good	
Acidic	poor	excellent		good		fair	
Amine	good	retarded		retarded		poor	

	Reference	Cq[a]	Reference	Cq	HA[b]	CS[c]	Reference	Cq	Reference	Cq
Uronic acids										
glucuronic	95,86,17	96,85,17	70,41,31	71,41,31	71,42,32	72,41,32	88,80,76,6	88,80,75	93,89,82,13	93,90,82,12
"iduronic"	-[d]	90	-	58	*[e]	58	-	93		
galacturonic	82,9	*	19	*	*	*	69,3	*	85,6	*
Neutrals										
fucose	69	69	10				62	62	73	73
xylose	66	66	5				59	59	70	70
arabinose	58	60	4				48	48	59	59
mannose	56	56	0				50	50	63	63
glucose	51	51	0				43	43	51	51
galactose	47	*	0				34	*	45	*
Amines										
glucosamine	29	30	0				0		7	7
galactosamine	25	25	0				0		4	4
Unidentified	-	11					-	9		

[a] Cq = hydrolyzed *C. quinqueciirrha* mesoglea carbohydrate.

[b] HA = hydrolyzed hyaluronic acid.

[c] CS = hydrolyzed chondroitin sulfate (mixed isomers A, B, and C).

[d] - = not applied.

[e] * = not detected

solvent system. The illustrations and reported Rf values (hRf = Rf X 100), supplementing the description of the four solvent systems, were taken from chromatograms chosen as being most representative of the observed results.

Solvent System 1:
Ethyl acetate-pyridine-acetic acid-water [5:5:1:3];
Silica Gel F-254 on Al

To the nearest half-hour, the running time for this solvent system was five hours. The resolution attained was very good for hexosamines and neutral sugars, but only fair to poor for the uronic acids.

When visualized under ultraviolet illumination (using plates commercially impregnated with a fluorescent indicator), alternating between short wave length (254 nm) [dark spots against a brightly fluorescing background] and long wave (350 nm) [brightly fluorescing spots against a dark background], the most intense spots in hydrolysate are xylose, fucose, glucosamine, galactosamine, and glucuronic acid, respectively. A tracing of a typical chromatogram for this system is provided in Figure 9 (see Table 3 for the corresponding Rf values).

Solvent System 2:
Ethyl acetate-2-propanol-water [27:3.5:1];
Silica Gel F-254 on Al

The running time for this solvent system was approximately two hours. In contrast to a partial or complete retardation of the neutral sugars and hexosamines, the resolution of the hexuronic acids was extremely good. As a result, two hexuronic acids were

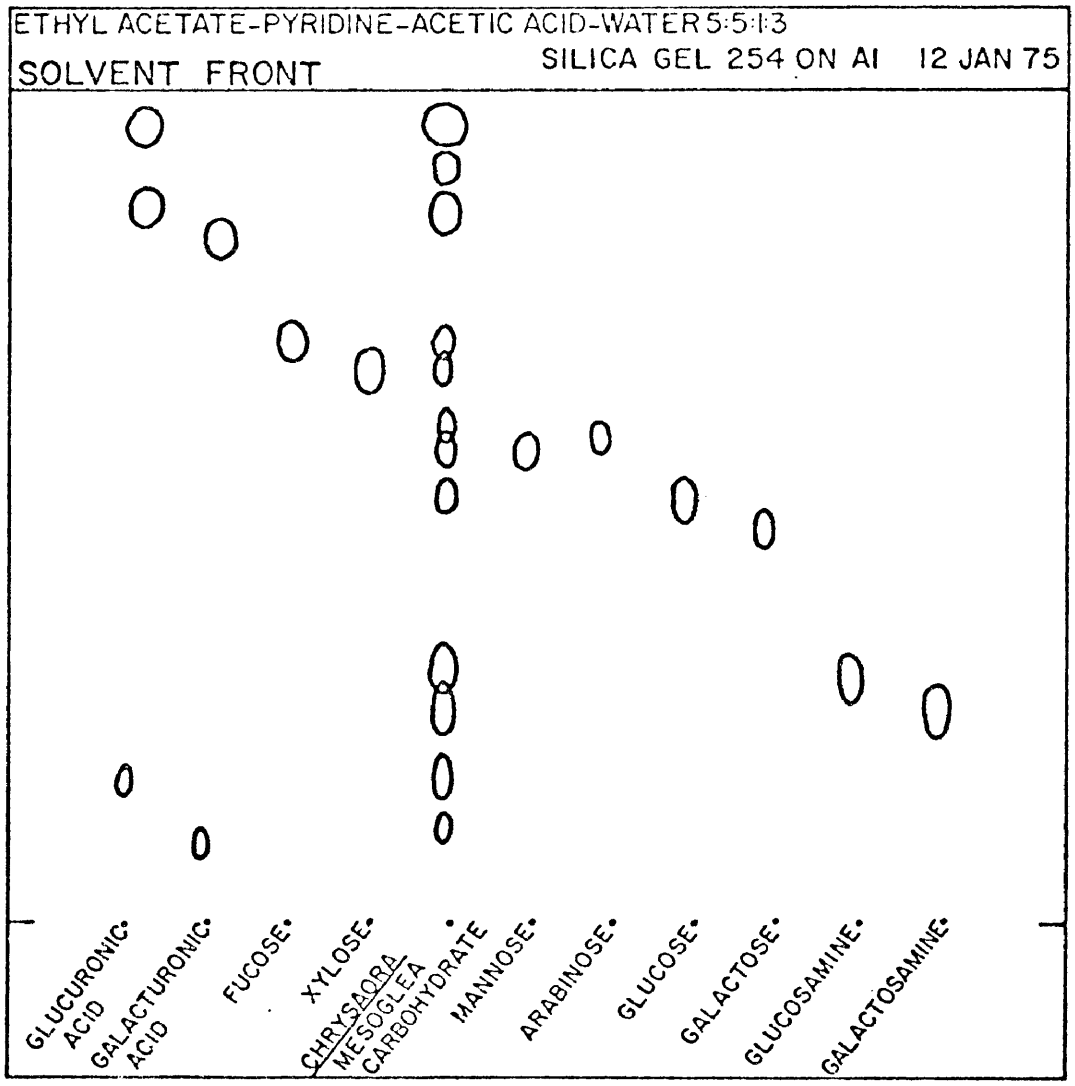


Figure 9. Thin-layer chromatogram from Solvent System 1.

detected in the mesogloea carbohydrate hydrolysate.

Examining the tracing of the chromatogram in Figure 10, three spots (hRf 70, 41, and 31) are manifest by glucuronic acid. (The multiplicity of spots results from the interaction of the glucuronic acid with the solvent, rather than from impurities.) The same spots (hRf 71, 41, and 31) are manifest in the mesogloea carbohydrate hydrolysate. Moreover, an additional spot (hRf 58) is detected in the mesogloea hydrolysate which--turning to a tracing of a second chromatogram (Figure 11)--matches a similar spot in the hydrolysate of chondroitin sulfate (a mixture of isomers A, B, and C). As chondroitin sulfate B is a polymer of alternating units of N-acetyl-galactosamine[3] and iduronic acid, and chondroitin sulfates A and C, of N-acetyl-galactosamine and glucuronic acid, the hRf 58 spot in the chondroitin sulfate hydrolysate is most probably iduronic acid. [To sustain this tentative identification, a number of other substances, including several uronic acids, were run, but their Rf values were much lower than hRf 58 (e.g., mannuronic acid, hRf = 36; galacturonic acid, hRf = 19; guluronic acid, hRf = 15).]

Solvent System 3:
Ethyl acetate-N-propanol-water [1:5:1];
with Silica Gel F-254 on Al

The running time for this system was approximately seven hours. The resolution of hexuronic acids and neutral sugars was good, and good to fair, respectively, with hexosamines completely retarded.

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[3]In acid hydrolysis, the N-acetyl group is split from the hexosamine.

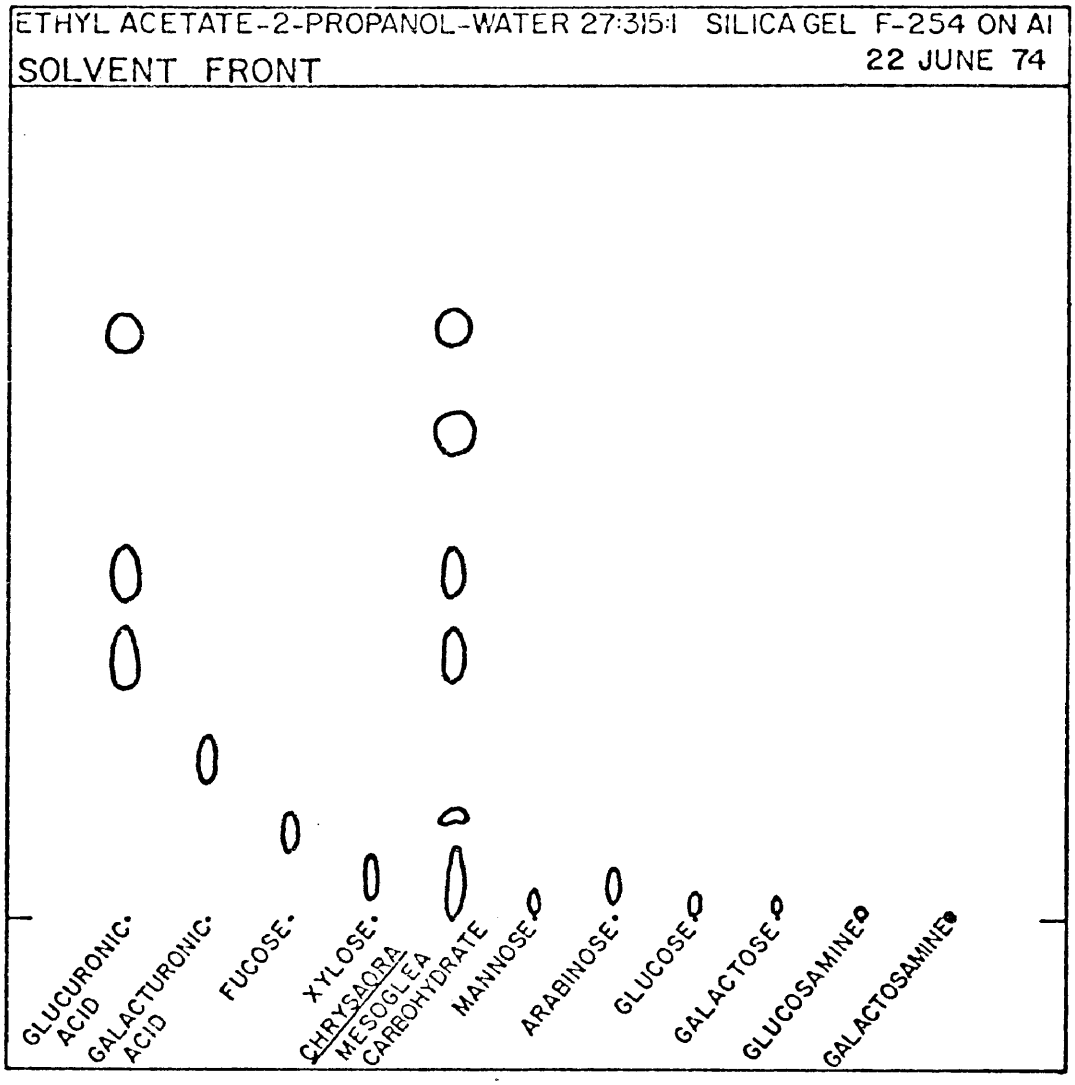


Figure 10. Thin-layer chromatogram from Solvent System 2.

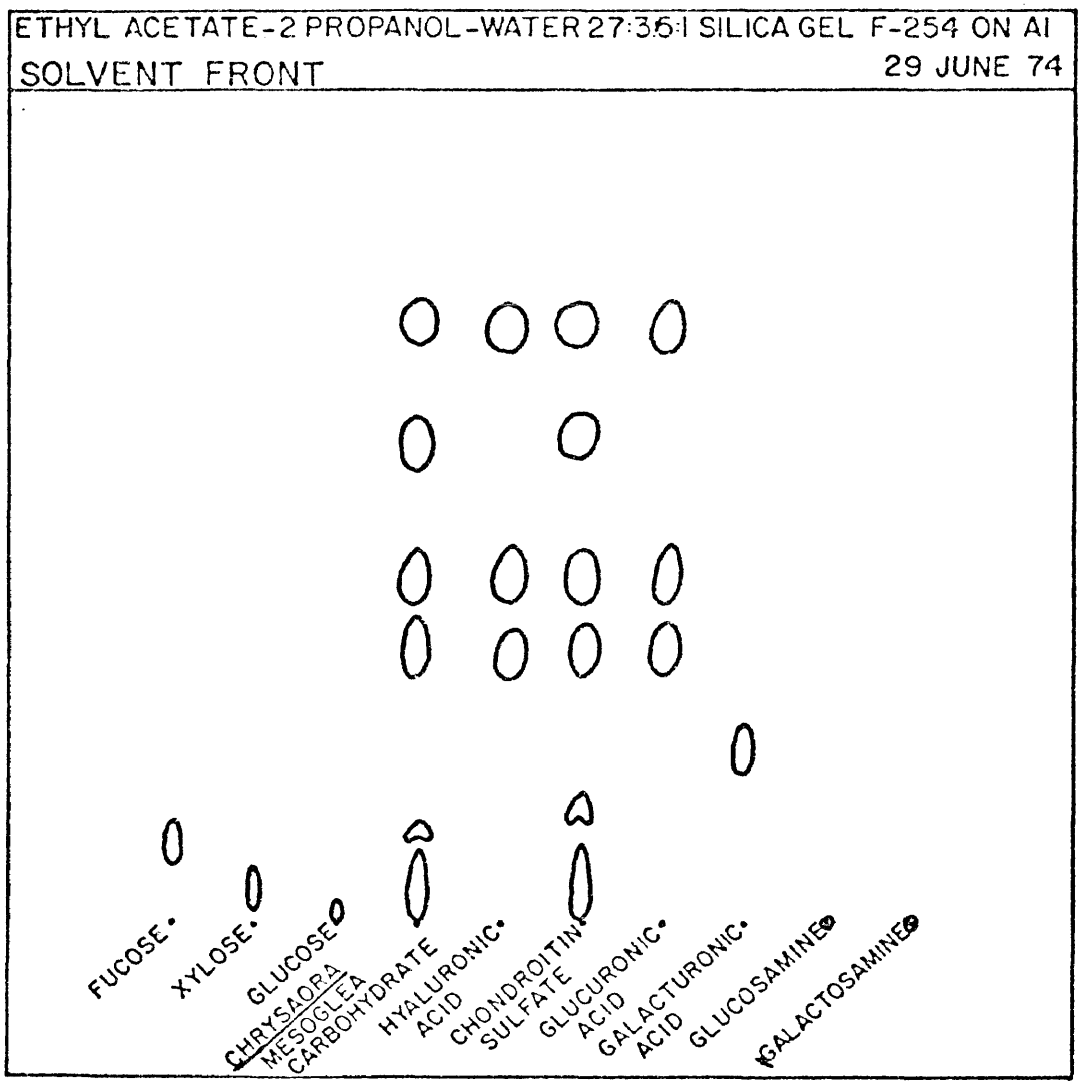


Figure 11. Thin-layer chromatogram from Solvent System 2, showing separation of monosaccharide constituents of chondroitin sulfate (mixed isomers A, B, C) and hyaluronic acid for comparison with the Chrysaora mesogloea hydrolysate.

Glucuronic acid manifests three spots (hRf 88, 80, 76; disregarding hRf 6) which are clearly detected in the mesogloea carbohydrate hydrolysate (hRf 88, 80, 75). An additional spot (hRf 93) is detected and tentatively identified as iduronic acid. (Although difficult to visualize using this system, a corresponding spot was detected in the hydrolysate of chondroitin sulfate, mixed isomers A, B, and C; not shown).

Figure 12 is a tracing of a typical chromatogram (see Table 3 for the Rf values). Alternating between short and long wave ultraviolet light, the most intense spots detected in the hydrolysate are xylose, fucose, glucuronic acid, "iduronic acid," glucose, arabinose, and mannose, respectively. (Although their Rf values are too low to be included, in practice galactosamine and glucosamine can be detected with this system.) A photograph of a chromatogram, visualized under short wave (254 nm) ultraviolet, is provided in Figure 13.

Solvent System 4:
1-butanol-pyridine-0.1 N HCl [5:3:2];
Silica Gel F-254 on Al

The running time for this system was about ten hours. The resolution of neutral sugars and hexuronic acids was good and fair, respectively; hexosamines, poor or retarded.

Figure 14 is a tracing of a typical chromatogram produced with this solvent system (see Table 3 for corresponding Rf values). Under short and long wave ultraviolet light, the dominant substances detected in the mesogloea hydrolysate are xylose, fucose, glucosamine, galactosamine, glucuronic acid, arabinose, mannose, and glucose,

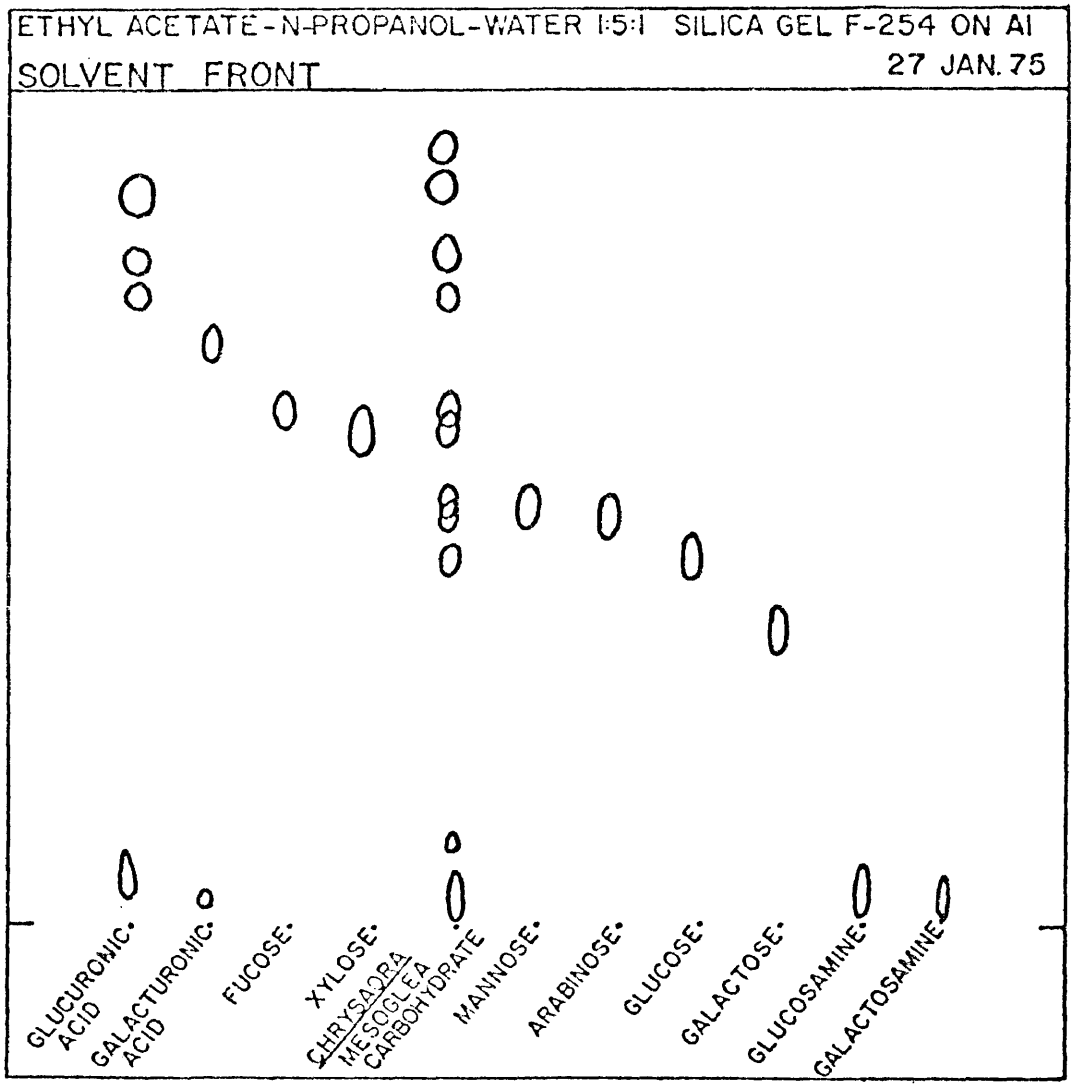
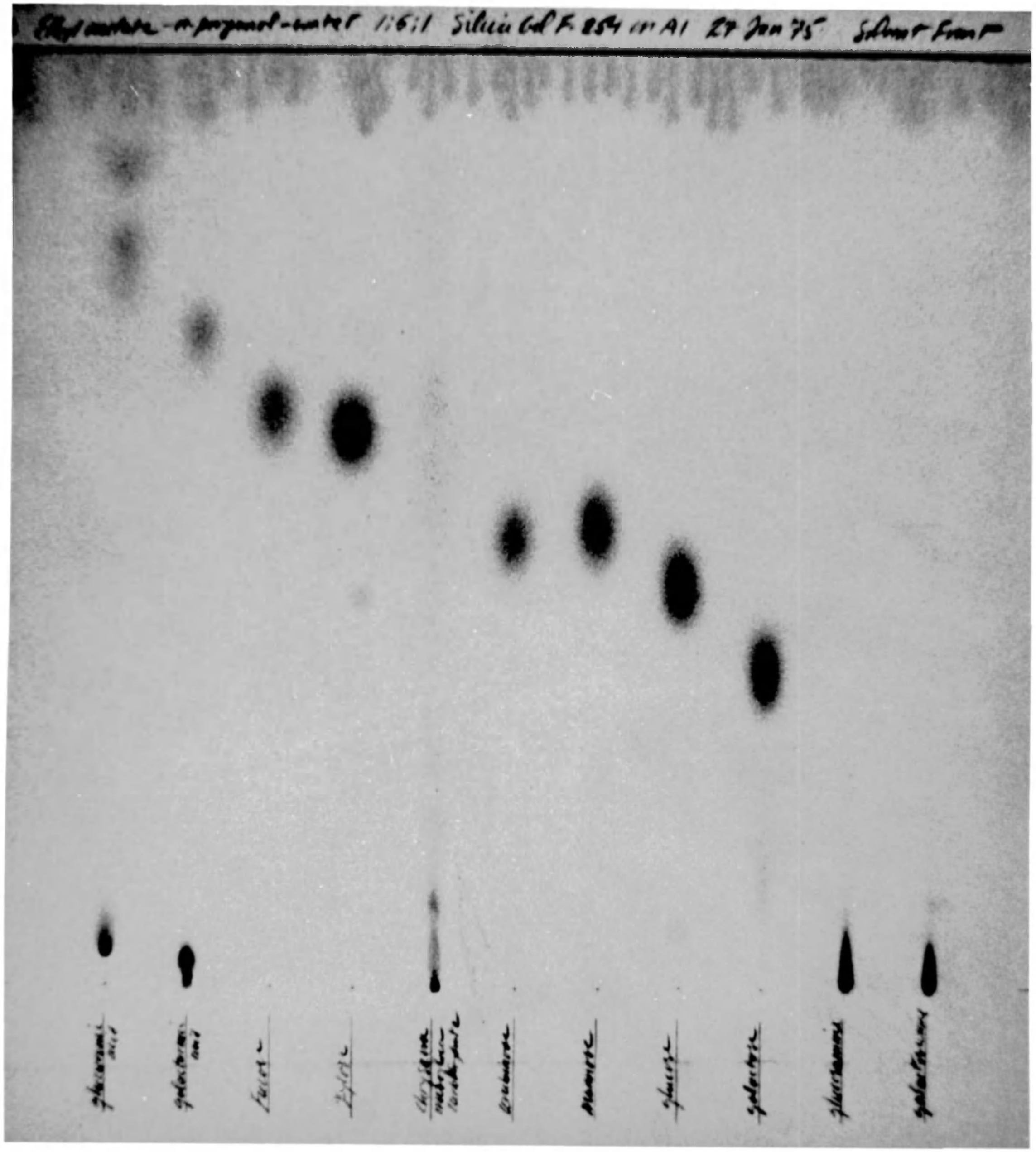


Figure 12. Thin-layer chromatogram from Solvent System 3.

Figure 13. Photograph of a chromatogram from Solvent System 3 under short wave (254 nm) ultraviolet light.


Ethyl acetate–n-propanol–water 1:6:1 Silica Gel F 254 on Al 27 Jan 75 Solvent Front

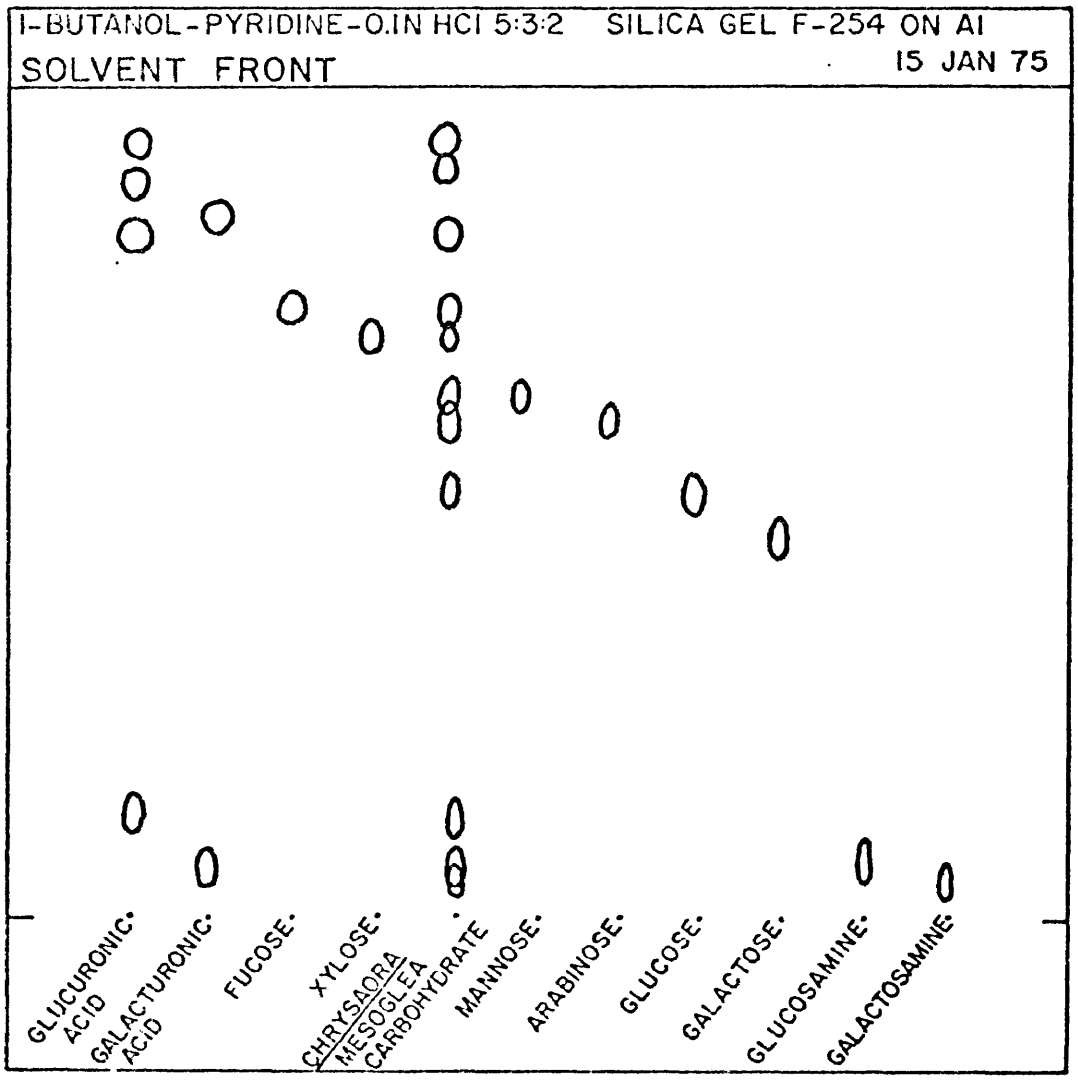


Figure 14. Thin-layer chromatogram from Solvent System 4.

respectively.

Xylose or Lyxose?

In addition to the sugars identified in the Chrysaora mesogloea carbohydrate hydrolysate (plus galacturonic acid and galactose), a number of substances were run with the four solvent systems (e.g., 2-dexoy-glucose, mannosamine, N-acetyl-mannosamine, N-acetyl-glucosamine, rhamnose, raffinose); but these were excluded when their Rf values did not correlate with those of the mesogloea hydrolysate in one or more solvent systems. The Rf values of one substance, however, lyxose, matched those of xylose sufficiently in all solvent systems to necessitate further consideration. An examination of the literature regarding neutral sugars commonly associated with invertebrates, with lyxose appearing only as a somewhat exotic possibility, leads this writer to accept the identification of the corresponding spot as xylose. However, reservations should be underscored since additional experimentation reveals that xylose and lyxose are truly cochromatographic with the four solvent systems employed in this investigation. It is emphasized, therefore, that while an identification as xylose is made, the possibility of lyxose (or xylose and lyxose) being present in the Chrysaora hydrolysate is significant.

Spectrophotometry

All spectrophotometric measurements were made with the Cary Spectrophotometer, Model 15. The form of Bouguer-Beer's Law employed is $A = abc$, where absorbance (A) equals the $\log [P_o/P]$ (P_o and P =

incident and transmitted light, respectively). Absorptivity (a) is defined as A/bc, where b is the length in centimeters (in this case 1.0 cm) of the path of light transmitted through the absorbing medium, and c the concentration of the monosaccharide being measured in grams/liter or milligrams/milliliter. The absorptivities for the monosaccharide constituents of the Chrysaora mesogloea hydrolysate were determined from the slope of the graph of absorbance versus concentration for the corresponding serially diluted reference sugars. A tabulation of wavelengths of maximum absorbance of the reference sugars for the four color reactions is provided in Table 4. Similarly, a tabulation of concentration of Chrysaora mesogloea carbohydrate hydrolysate (mg/ml), absorbance, absorptivity, and concentration (mg/ml) of monosaccharide moieties is provided in Table 5. The quantities (micromoles per 100 µg of mesogloea carbohydrate) of the monosaccharide constituents of the Chrysaora mesogloea hydrolysate are listed in Table 6. These values are treated as approximations of the absolute amounts of the respective sugar moieties in the hydrolysate. An example of the absorption spectra of glucose, fucose, xylose, mannose, arabinose (0.1 mg/ml each), and Chrysaora carbohydrate hydrolysate (0.2 mg/ml), between 450 and 720 nm for the Anthrone reaction is shown in Figure 15. The method of three simultaneous equations employed in determining by the Anthrone reaction the concentration of xylose, fucose, and glucose in the mesogloea hydrolysate (0.4 mg/ml) is demonstrated in Appendix C.

TABLE 4

WAVELENGTHS OF MAXIMUM ABSORBANCE

	Anthrone (nm)	L-Cysteine (nm)	Carbazole (nm)	Elson-Morgan (nm)
Xylose	640, 503	390	515	
Fucose	620	327, 397	440	
Uronic acid			535	
Arabinose	503, 640	390, 327	515	
Mannose	610, 490	327		
Glucose	620, 490	327	515, 432	
Glucosamine				529
Galactosamine				539

TABLE 5

SPECTROPHOTOMETRIC ANALYSIS OF HYDROLYZED MESOGLOEA CARBOHYDRATE

	Chrysaora carbohydrate (mg/ml)	Wavelength (nm)	(nm)	(nm)	Sugar composition[a] (mg/ml)
Anthrone reaction:		620	590	503	
	0.40 Absorbance =	0.477	0.420	0.572	
Absorptivity[b]					
Reference sugars					
xylose		1.254	0.586	2.801	0.125
fucose		1.527	1.853	1.091	0.129
glucose		3.304	2.879	2.192	0.037
L-Cysteine reaction:		397	390	327	
	0.20 Absorbance =	0.691	0.830	0.421	
Absorptivity					
Reference sugars					
xylose		6.737	10.190	0.410	0.062
fucose		3.218	1.865	4.988	0.064
arabinose		4.009	4.682	4.704	0.016
Carbazole reaction:		560	515	440	
	1.00 Absorbance =	0.067	0.326	0.394	
Absorptivity					
Reference sugars					
xylose		0.000	0.430	0.380	0.312
fucose		0.000	0.000	0.800	0.323
uronic acid		0.630	1.810	0.370	0.166
Elson-Morgan reaction:		539	529		
	0.25 Absorbance =	0.085	0.085		
Absorptivity					
Reference sugars					
glucosamine		3.530	3.752		0.012
galactosamine		4.645	4.351		0.009

[a] Concentrations of monosaccharides in Chrysaora hydrolysate as determined via simultaneous equations.

[b] $a = \frac{A}{bc}$.

TABLE 6

QUANTIFICATION OF MONOSACCHARIDE CONSTITUENTS OF CHRYSAORA MESOGLOEA CARBOHYDRATE

Sugar	Micromoles (10^{-6} mole) per 100 micrograms of mesogloea carbohydrate	Spectrophotometric reaction
Xylose	0.208	Anthrone L-Cysteine
Fucose	0.196	Anthrone L-Cysteine
Uronic acid	0.055	Carbazole
Arabinose	0.054	L-Cysteine
Mannose	+ [a]	
Glucose	0.052	Anthrone
Glucosamine	0.027	Elson-Morgan
Galactosamine	0.020	Elson-Morgan

[a]Unable to be quantified satisfactorly with any of the four reactions.

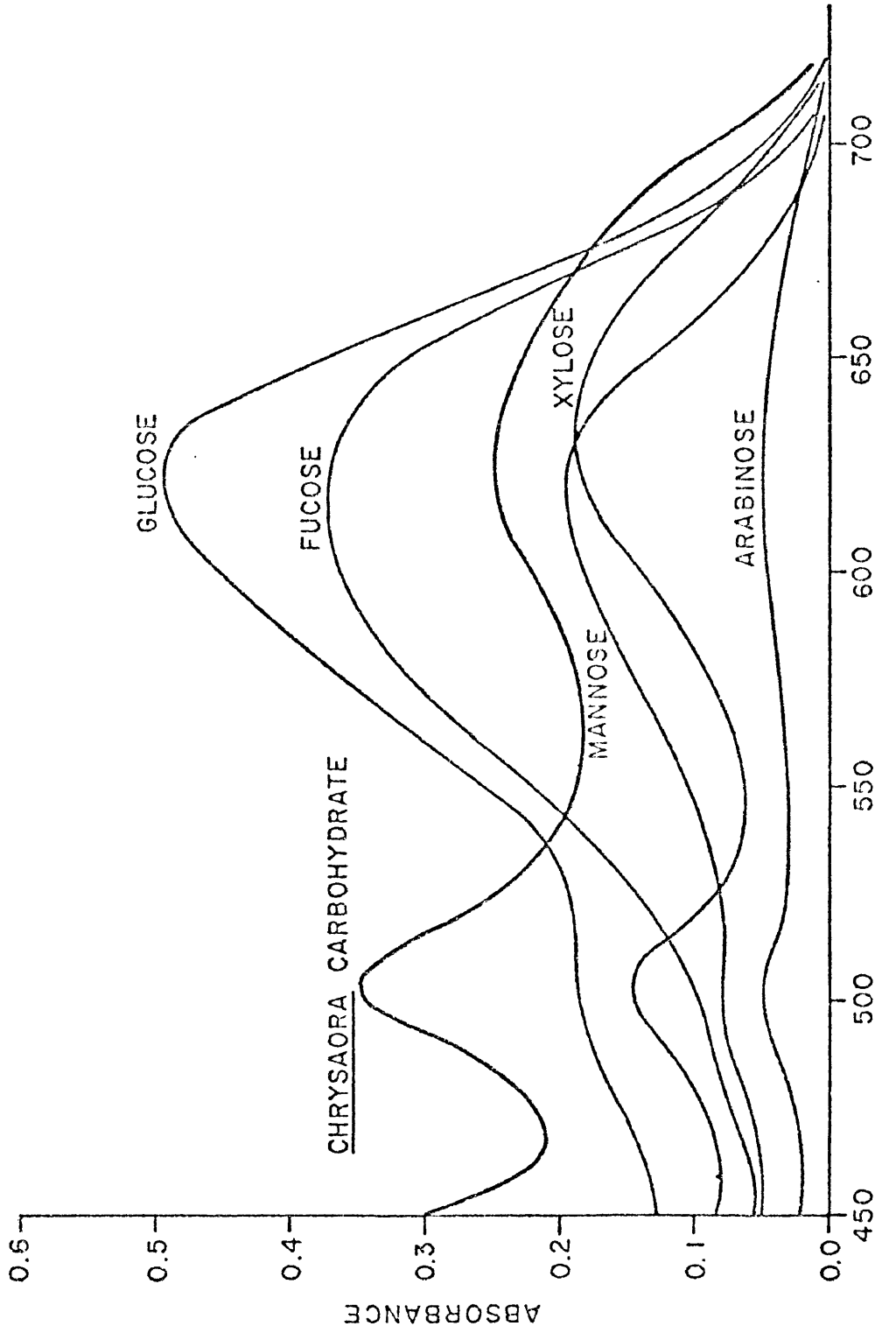


Figure 15. Example of the absorption spectra of glucose, fucose, xylose, mannose, arabinose (0.1 mg/ml each) and the Chrysaora carbohydrate hydrolysate (0.2 mg/ml between 450 and 720 nanometers, for the Anthrone reaction.

DISCUSSION

ANALYSIS

This investigation reveals the presence of xylose, fucose, arabinose, mannose, glucose, glucosamine, galactosamine, glucuronic acid, and "iduronic acid" in the Chrysaora mesogloea hydrolysate. The detection of glucosamine, galactosamine, glucuronic acid, and "iduronic acid" in the hydrolysate necessitates acceptance of the hypothesis that acidic polysaccharides are present in Chrysaora mesogloea. More specifically, the detection of these monosaccharide moieties strongly suggests the presence of vertebrate-type acid mucopolysaccharides. In this context, the presence of hyaluronic acid and chondroitin sulfate B is favored. But the presence of highly sulfated mucopolysaccharides, such as heparin or heparan sulfate, is preferred since the total amount of sulfate in the Chrysaora carbohydrate is very high; i.e., more consistent with the sulfate content of heparin or heparan sulfate than with that of chondroitin sulfate B. [A total sulfate determination was conducted on hyaluronic acid, chondroitin sulfate (mixed isomers A, B, and C), and dry Chrysaora mesogloea carbohydrate. The total sulfate in the dry Crysaora mesogloea carbohydrate was found to 39.1%; for hyaluronic acid, 1.2%; and for chondroitin sulfate, 22% weight/weight (Zubkoff, Gardner, and Enwright, 1975).] Moreover, (contrary to the accepted structure for heparin provided in the Introduction) a

significant amount of iduronic acid has been detected in heparin (Lindahl, 1970).

It may be pointed out that galactose was not detected in the Chrysaora mesogloea hydrolysate. In view of the ubiquity of galactose in invertebrate and vertebrate connective tissues, the absence of galactose in the hydrolysate is surprising, but should not be interpreted as absolute in Chrysaora mesogloea. Since hydroxylysine has been detected in Chrysaora mesogloea collagen (Quensen, 1975), and is very likely linked to mono-, di-, and/or tri-saccharides of galactose, it may be interpreted that the absence of galactose in the mesogloea hydrolysate indicates that carbohydrate containing galactose has remained attached to protein after chemical fractionation and that the hydrolysate is virtually free of protein, i.e., free of the collagen moiety of Chrysaora mesogloea. This conclusion is in agreement with the techniques employed to extract the polysaccharide or carbohydrate moiety. It was anticipated that a trichloroacetic acid extraction would yield: (1) polysaccharides which are not intimately associated with protein; and (2) polysaccharide fragments broken from larger polysaccharides that are covalently attached to protein, but not dissacharides or small oligosaccharides so attached. Hence, the absence of galactose in the mesogloea hydrolysate indicates that this monosaccharide is not represented, other than in the form of galactosamine, in the "protein-free" carbohydrate moiety of Chrysaora mesogloea.

The detection of hexuronic acids and hexosamines characteristic of the classic glycosaminoglycans, does not exclude the

possibility that other, totally unfamiliar, acid polysaccharides may be present in Chrysaora mesogloea. Indeed, novel acid polysaccharides, perhaps important phylogenetic precursors to vertebrate GAG, may be the only glycosaminoglycans present. Hence, there are three possible alternatives which fit the results of this investigation:

1. That the classic glycosaminoglycans, such as chondroitin sulfate or heparan sulfate, and large neutral and/or sulfated polysaccharides, but no novel GAG are present.

2. That classic GAG, novel GAG, and neutral and/or sulfated polysaccharides are present.

3. That no classic GAG are present, but only novel GAG and neutral and/or sulfated polysaccharides.

The second alternative best describes the carbohydrate moiety of Chrysaora mesogloea, i.e., that classic GAG, novel GAG, and other neutral and/or sulfated polysaccharides are present. With the exception of the hexosamines, the results of the spectrophotometric analysis (molar quantities) of the mesogloea carbohydrate are in agreement with the relative abundance of the monosaccharides detected by thin-layer chromatography as inferred from the appearance of the chromatograms. This indicates the presence of a large polysaccharide or a large number of oligosaccharides in which xylose and fucose are major constituents, with arabinose, mannose, and glucose, respectively, as possible minor constituents. Moreover, the excessive amount of sulfate in the mesogloea carbohydrate increases the possibility that this polysaccharide is sulfated, or that few, "neutral" oligosaccharides are present. This conclusion is further supported by analogy,

as polyfucose sulfate has been isolated from the connective tissue of the echinoderm Thyone briareus (Katzman and Jeanloz, 1969).

As previously stated, the presence of sulfated classic acid mucopolysaccharides (especially heparan sulfate, heparin, and perhaps chondroitin sulfate B) in Chrysaora mesogloea is consistent with the results of this investigation. Yet, the spectrophotometric analysis of the mesogloea hydrolysate reveals a disparity between the molar concentrations of total hexosamine and total hexuronic acid such that the exclusive presence of these acid polysaccharides is contradicted. This disparity--specifically a surplus of hexuronic acid over hexosamine--indicates that: (1) novel GAG, incorporating some neutral sugar residues, or (2) novel acid polysaccharides lacking hexosamine, but incorporating hexuronic acid residues linked with a neutral sugar, are present. This conclusion is strengthened if one considers that this disparity is opposite to that anticipated from the method of hydrolysis, since an acid hydrolysis is often destructive to uronic acids (i.e., the disparity should be even greater in unhydrolyzed carbohydrate).

To summarize, this investigation clearly demonstrates the presence of acid polysaccharides and/or glycosaminoglycans in the acid extractable (trichloroacetic acid) fraction of Chrysaora mesogloea. Moreover, the interpretation best fitting the analysis by thin-layer chromatography and spectrophotometry strongly indicates the presence of at least three polysaccharide components: classic GAG, novel GAG, and sulfated and/or neutral polysaccharides. However, in the absence of isolated chemical species, the exact nature of this

carbohydrate, in terms of specific structures and chemical composition, remains unknown.

POLYSACCHARIDES

Hypothetical Structures, Chemical Composition, and the Literature

The results of this investigation, interpreted with the corelative studies of Spiro and Bhoyroo (1971), and Katzman and Jeanloz (1969) provide a basis for conceiving specific polysaccharides as highly probable components, or at least analogs of such components of Chrysaora mesogloea. The structure and chemical composition of the substances most likely comprising the first polysaccharide component (classic glycosaminoglycans) as inferred from the relative abundance of galactosamine (N-acetyl-D-galactosamine) and sulfate, are those of heparan sulfate, heparin, and less likely chondroitin sulfate B. With regard to the second component (novel glycosaminoglycans), an acid polysaccharide conceived as a surrogate for or compliment to vertebrate-type GAG in Chrysaora mesogloea is provided in Figure 16. It is a polymer of α-L-iduronic acid, β-D-mannose, and β-N-acetyl-D-glucosamine; more specifically:

$$\{\ \alpha\text{-L-iduronic acid}\ [\ \beta(1\rightarrow3)\ ]\ \beta\text{-D-mannose}\ [\ \beta(1\rightarrow3)\ ]\ \alpha\text{-L-iduronic acid}\ [\ \beta(1\rightarrow3)\ ]\ \beta\text{-D-mannose}\ [\ \beta(1\rightarrow3)\ ]\ \beta\text{-N-acetyl-D-glucosamine}\ [\ \beta(1\rightarrow4)\ ]\ \}_n$$

The possible existence of such a polysaccharide in Chrysaora mesogloea is extrapolated from the detection of the mannose-glucuronic acid disaccharide unit in the clamworm Nereis by Spiro and Bhoyroo (1971), and the quantitative disparities among the

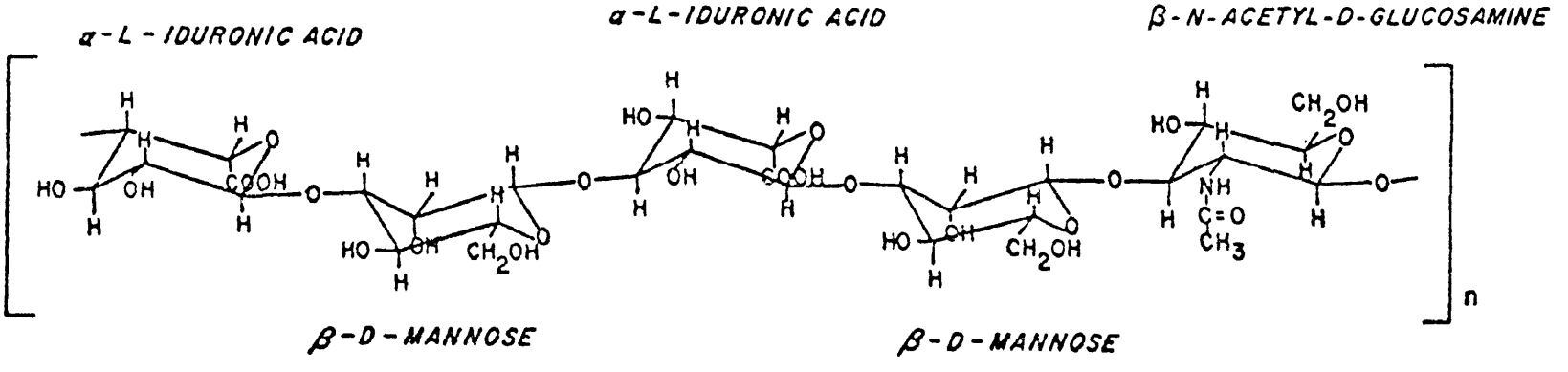


Figure 16. Structure of hypothetical novel glycosaminoglycan.

monosaccharide residues in the mesogloea hydrolysate. This polysaccharide explains, for example, the surplus of uronic acid--predominantly iduronic acid--and the abundance of glucosamine in the hydrolyzed mesogloea carbohydrate (not accounted for by the presence of heparan sulfate, heparin, or chondroitin sulfate B), and is not in disagreement with the amount of mannose present as interpreted from the results of thin-layer chromatography.

Excluding the glycosaminoglycans, perhaps the remaining most significant feature of the Chrysaora mesogloea carbohydrate revealed in this study, is the apparent presence of a sulfated or neutral polysaccharide. With respect to this third component, a sulfated polysaccharide which may be present in Chrysaora mesogloea is a form of α(1→2)-linked polyxylosylfucose sulfate with residues of arabinose occurring after every fourth repeating disaccharide unit, more specifically (see Figure 17):

$$\{\{\beta\text{-D-xylose-4-sulfate } [\ \alpha(1\rightarrow2)\ ]\ \beta\text{-L-fucose-3-sulfate } [\ \alpha(1\rightarrow2)\ ]\ \}_4\ [\ \alpha(1\rightarrow2)\ ]\ \beta\text{-L-arabinose}\}_n$$

The criterion for suggesting the presence of this or a similar polysaccharide in Chrysaora mesogloea is derived from the predominance of xylose and fucose in the mesogloea hydrolysate, the abundance of sulfate, and the isolation of α(1→2)-linked polyfucose sulfate (sulfated at the third or fourth carbon atoms) from the connective tissue of the echinoderm, Thyone briareus by Katzman and Jeanloz (1969).

The results of this investigation can be phylogenetically compared with the literature. The presence of acid polysaccharides

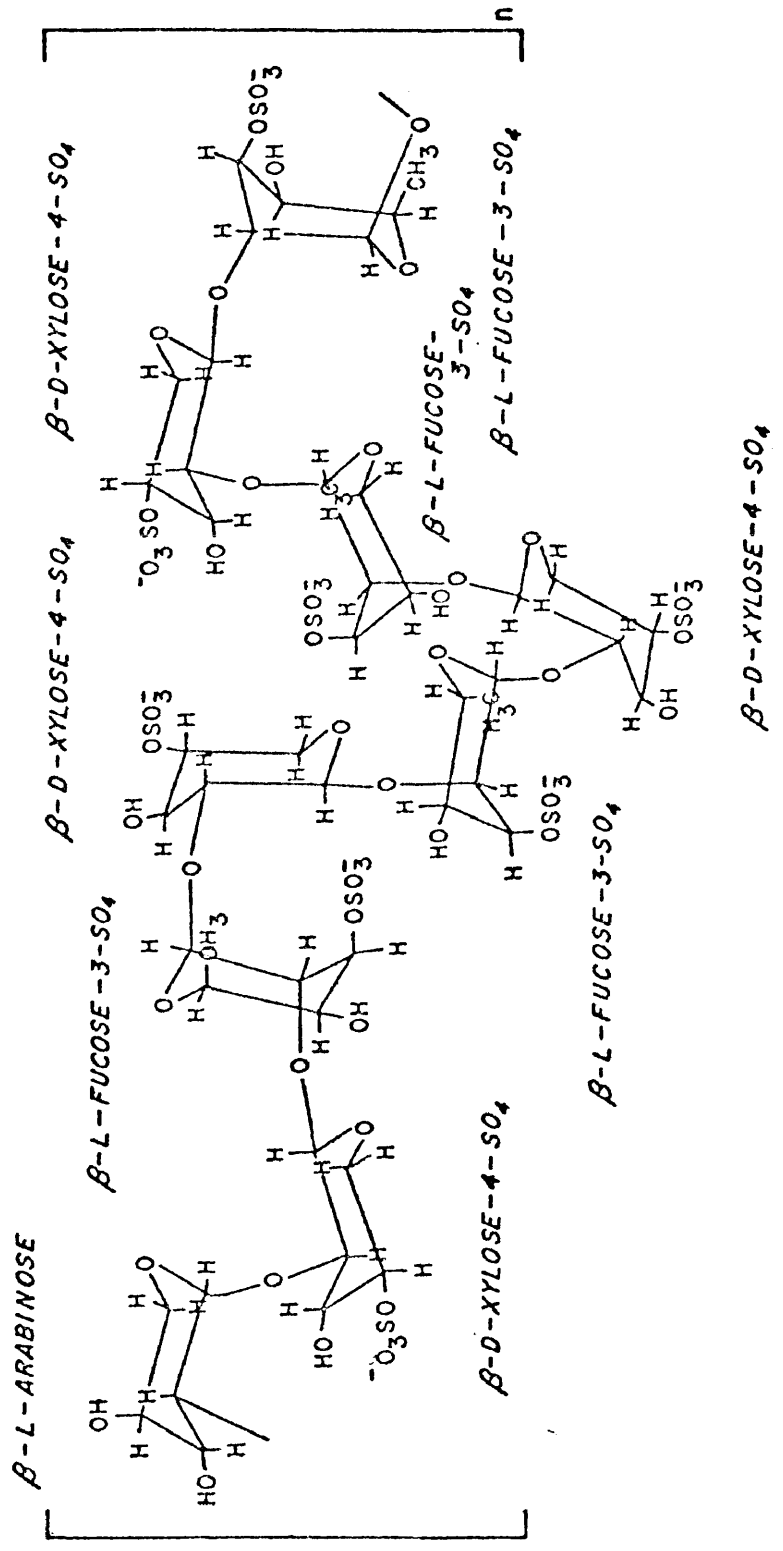


Figure 17. Structure of hypothetical polysaccharide, polyxylosylfucose sulfate with arabinose residues occurring after every fourth disaccharide unit.

or acid mucopolysaccharides in Chrysaora mesogloea is consistent with their presence in the sponges Spongia graminea (Gross et al., 1956), and Hippospongia gossypina (Katzman and Jeanloz, 1970a).[4] Most of the monosaccharide residues detected in hydrolyzed Chrysaora mesogloea carbohydrate, including those which are essential constituents of vertebrate-type acid mucopolysaccharides [e.g., galactosamine (N-acetyl-D-galactosamine), glucosamine (N-acetyl-D-glucosamine), and hexuronic acid], were detected in these organisms. (Galactose was also detected in Spongia and Hippospongia, further suggesting that it will be found in Chrysaora mesogloea in carbohydrate-protein conjugates involving hydroxylysine.) These monosaccharide residues were also detected by Gross et al. (1958), and Piez and Gross (1959) in the float of the hydrozoan coelenterate, Physalia physalis, and in the echinoderm, Thyone. Moreover, Katzman and Jeanloz (1969) have reported the isolation of chondroitin sulfate and polyfucose sulfate from Thyone. Similarly, chondroitin sulfate has been isolated from the connective tissues of the phyla Annelida, Arthropoda (Arachnida), and Mollusca (Hunt, 1970). Molluscan connective tissues have also been found to contain chondroitin, keratosulfate, heparin, and certain glucan sulfates (Hunt, 1970).

Perhaps because very few coelenterates have been examined for the chemical constituents of acidic polysaccharides, including

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[4] In addition to uronic acid containing acid polysaccharides in H. gossypina, Katzman and Jeanloz (1969, 1970a) report the presence of a highly sulfated polysaccharide with a large proportion of arabinose.

notably the anthozoan Metridium dianthus (Katzman and Jeanloz, 1971), the search for acid polysaccharides in the connective tissue of coelenterates has not been generally successful. Katzman and Jeanloz (1969, 1971) found ribose, fucose, mannose, galactose, glucose, and glucosamine but were unable to detect galactosamine, hexuronic acid, or any sulfate (or arabinose and xylose) in the gelatin of Metridium. They concluded (1971) that acid polysaccharides do not have an essential role in the stabilization and fibrillogenesis of sea anemone collagen, and, therefore, are not likely involved in the stabilization and fibrillogenesis of mammalian collagen. This conclusion is contrary to the evidence already presented which indicates that acid polysaccharides do play a role, albeit an essential one, in the fibrillogenesis and stabilization of collagen in most vertebrate connective tissues, and in some invertebrate tissues. More specifically, while it is clear that acid polysaccharides cannot have an essential role in the fibril formation and stabilization of Metridium collagen, the possibility that they may be important to the fibril formation and stabilization of collagen in other connective tissues is not precluded. In this respect, this author agrees with the contention of Katzman and Jeanloz that, because of the structural similarity of Metridium collagen to mammalian collagen, the anemone might be useful in studying those processes in which acid polysaccharides are ostensibly involved, e.g., wound healing and calcification; however, it is suggested that a search should be made for nonacidic polysaccharides in Metridium which might be able to substitute for glycosaminoglycans or other acidic

polysaccharides generally implicated in a causal relationship with the fibrillogenesis and stabilization of collagen. Similarly, using paper chromatography, Bocquet, Pujol, Rolland, Bouillon, and Coppois (1972) found xylose, fucose, arabinose, glucose, and glucosamine, but were unable to detect galactosamine and hexuronic acids (or galactose and mannose) in the (trichloroacetic acid soluble) mesogloea carbohydrate of the scyphozoan Rhizostoma pulmo. However, Bocquet et al. (1972) were able to detect hexuronic acid in Rhizostoma mesogloea carbohydrate by spectrophotometry. Hence, the inability to detect these monosaccharides by paper chromatography may be more a reflection of the lower resolving power of paper chromatography (compared to thin-layer chromatography) than a literal absence of acid polysaccharides. Yet, the apparent absence of acid polysaccharides in Rhizostoma mesogloea may be phylogenetically significant, in that it suggests that, at least chemically, the order Rhizostomae may be more closely affiliated with the class Anthozoa than with the order Semaeostomae of which Chrysaora is a member.

Phylogenetic Interpretation

With reference to the phylogenetic significance of the absence of acid polysaccharides in Rhizostoma mesogloea, it should be noted that one outcome of the present investigation of cnidarian polysaccharides is a possible contribution to the explication of the phylogeny and evolution of the Metazoa. As the Porifera are less a reflection of metazoan ancestry than a separate protozoan offshoot, the phylum Cnidaria is commonly recognized as the most

primitive of the Metazoa or, more precisely, of the Eumetazoa. Owing to Haeckel's "Gastraea" theory of metazoan origin, based on the premise that a hydromedusan polyp is essentially an elongated gastrula (Dodson, 1960), the higher Metazoa were thought of as having evolved more or less directly from an ancestral cnidarian polyp. The Gastraea theory has since been revised by the viewpoint promulgated by Hyman (1940), Hand (1959, 1963), Dodson (1960), and Rees (1966) which derives the Eumetazoa from a planuloid descendant of the protozoan class Flagellata (see Figure 18). More specifically, according to the chief proponent, Hyman (1940), the pre-Cambrian ancestral cnidarian--from which the three classes of the Cnidaria have risen--evolved from a planula-like, diploblastic precursor to the phylum Platyhelminthes (or "primitive acoel flatworms"), the bilaterally symmetrical gateway to the higher Metazoa. In this account, the Hydrozoa are seen to be the most primitive of the Cnidaria, the Scyphozoa as medusoid transitions from the Hydrozoa, and the Anthozoa as evolutionary adaptions to an increasingly sessile existence via the scyphozoan order Rhizostomae. Alternatively, Hadzi (1953, 1963), Hanson (1958), Steinböck (1963), deriving the platyhelminthian class Tubellaria (order Acoela) from the primitive polynuclear Ciliata or "Plasmodial ciliates" (secondarily removed from the Flagellata), have proposed that the Anthozoa have evolved from the tubellarians, and that the class Anthozoa is the most primitive cnidarian class with the classes Scyphozoa and Hydrozoa being derived from it, respectively (see Figure 19). Although the first theory, that deriving the Cnidaria from a planuloid descendant of the colonial flagellates (with

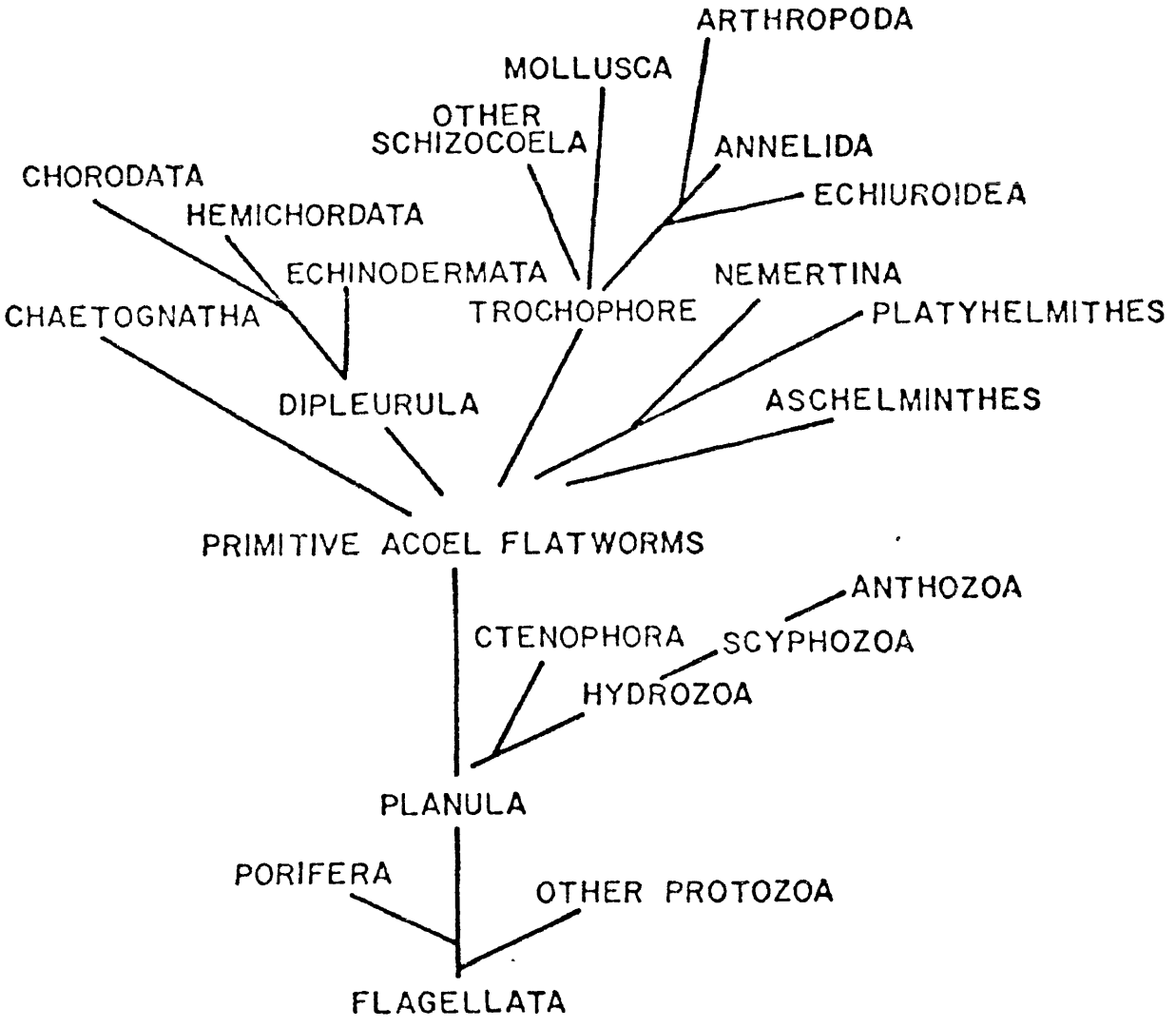

Figure 18. Genealogical tree of the animal kingdom after Hyman.

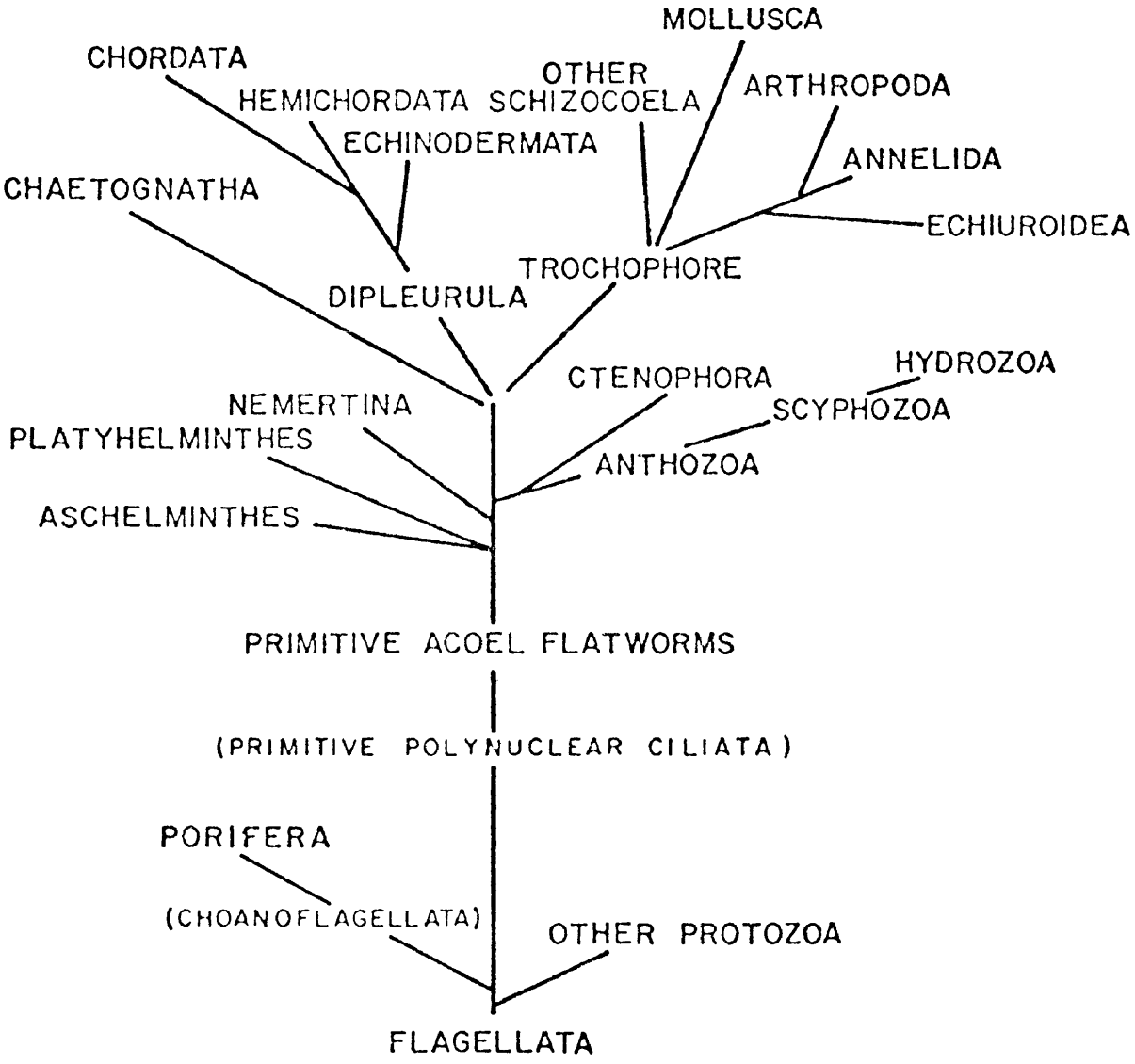

Figure 19. Genealogical tree of the animal kingdom reconstructed from Hadzi.

the class Hydrozoa being the most primitive), seems to be better supported by morphological, ontological, and serological evidence (and the fossil record) (Hand, 1959, 1963), the relative phylogenetic merits of the two contrasting points of view continue to be argued. In this respect, the results of this investigation and others involving the presence of acid polysaccharides among the Cnidaria are more in agreement with Hyman's theory of cnidarian evolution than with the theory, chiefly promulgated by Hadzi, deriving the Cnidaria from the Tubellaria. In addition to being ubiquitous among the higher Metazoa (i.e., the deuterostomous and protostomous phyla), acid polysaccharides appear to be very ancient, as they have been detected in bacteria [polyfucose sulfate in Escherichia coli, Barry (1957); hyaluronic acid in groups A and C streptococci, Kendall, Heidelberger, and Dawson (1937), Stoomiller and Dorfman (1970)], in Protozoa (Katzman and Jeanloz, 1969), and in the phylum Porifera. Hence, the presence of acid polysaccharides in the cnidarian class Hydrozoa (Gross, et al., 1958; Piez and Gross, 1959; and Wineera, 1972), in the scyphozoan order Semaeostomae (Chrysaora), but not in the order Rhizostomae (Bocquet et al., 1972), favors the interpretation that the class Hydrozoa is the most primitive cnidarian class, that the Scyphozoa are derived from the Hydrozoa, and that the class Anthozoa--having lost the genetic information necessary to produce the enzymes involved in the synthesis of acid polysaccharides--is a terminal group. Moreover, the carbohydrate moiety of Chrysaora mesogloea, as revealed by this investigation, most closely resembles that of the sponge Spongia (Gross, et al., 1956), further suggesting that the ancestral

cnidarian is not far removed phylogenetically from the origins of the Porifera, presumably the flagellates. This conclusion is further strengthened if one considers that there are major differences between the carbohydrate moieties of the Platyhelminthes and the cnidarian class Anthozoa, especially with respect to sialic acid,[5] which is absent in the Anthozoa and other lower metazoan groups, but present in the acoel tubellarians, e.g., Polychoerus carmelensis (Warren, 1963).

It has been suggested that the detection of acid polysaccharides in cnidarian mesogloea might further delineate the relationship between acid polysaccharides and collagen in vertebrate connective tissue, and delimit the extent of the analogy between vertebrate and invertebrate connective tissues. Accordingly, this investigation and others (e.g., Gross, et al., 1956, 1958; Katzman and Jeanloz, 1969, 1970a, 1970b, 1971; Katzman, Lisowska, and Jeanloz, 1970; Piez and Gross, 1959; Spiro and Bhoyroo, 1971; Spiro, 1972a) are consistent in that they reveal significant differences between the connective tissue of invertebrates and the established vertebrate connective tissue motif. Some of these differences are: (1) the greater abundance of carbohydrate associated with the collagen moiety of invertebrate tissues relative to that of vertebrates; (2) the very high sulfate content of most invertebrate tissues;

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[5] A nine-carbon monosaccharide found throughout the vertebrata, and to a limited extent, among the invertebrata, as glycosidically-linked structural units of polysaccharides, glycoproteins, and glycolipids (Tuppy and Gottschalk, 1972; Hunt, 1970).

(3) the presence of a large variety of novel acidic and neutral polysaccharides; and (4) the absence of certain characteristically vertebrate glycosaminoglycans, e.g., hyaluronic acid and chondroitin sulfate B (Hunt, 1970), in nearly all of the invertebrate phyla. Nevertheless, there are certain important similarities which strengthen the nexus between vertebrate and invertebrate connective tissues. The most outstanding of these is the presence of hexosamine and/or uronic acid containing oligosaccharides or polysaccharides, i.e., apparent homologues of vertebrate glycosaminoglycans, in most of the invertebrate groups. These substances seem to imitate, in invertebrate connective tissue, the attributes of acid mucopolysaccharides in vertebrate connective tissue, thereby sustaining the argument that they are involved in the stabilization and fibrillogenesis of invertebrate collagen and at least partially responsible for the ordering of the invertebrate connective tissue motif. Unfortunately, salient investigations of invertebrate connective tissues remain too sparse to allow one to make definitive statements about the evolution of the relationship, in vertebrate connective tissue, between collagen and acid polysaccharides or to delimit the nature of this relationship on the basis of comparison. However, the considerable variation in the carbohydrate composition of invertebrate connective tissues, especially with respect to acid polysaccharides, certainly suggests that the interaction between acid polysaccharides and collagen in vertebrate connective tissue is more complex than currently perceived.

CRITICAL ASSESSMENT

This investigation transcends those studies which have relied on histochemical and cytochemical techniques to detect acid polysaccharides in the mesogloea of scyphozoans, and establishes--by demonstrating the presence of the monosaccharide constituents of acidic polysaccharides--a basis for further biochemical research. However, the limitations of this study are readily apparent. The carbohydrate preparation employed must be characterized as intrinsically crude. Moreover, the techniques for hydrolysis suffer from the possibility of destruction of monosaccharides or rearrangement of the polysaccharides, allowing misinterpretation of identity and in situ relationship with protein. Other difficulties manifest in this investigation arise from the employment of thin-layer chromatography and spectrophotometry in the analysis of hydrolyzed carbohydrate. While thin-layer chromatography is capable of detecting smaller quantities of substances than paper chromatography and very useful in situations where rapid analysis is advantageous, it is in both respects inferior to gas-liquid chromatography. (However, gas-liquid and thin-layer chromatography can yield complimentary results, achieving a more reliable and efficient system of detection than either method alone.) Similarly, the spectrophotometric analysis of the Chrysaora carbohydrate is subject to misinterpretation in that the respective color reactions are somewhat nonspecific for individual monosaccharide species. Further biochemical analysis will ultimately center upon the isolation and characterization of specific acid

polysaccharides in the connective tissue of Chrysaora quinquecirrha. It is suggested that a prelude to this step should be a more sophisticated analysis of the constituents of the carbohydrate moiety of Chrysaora mesogloea by the purification of the carbohydrate via ion-exchange resins (or ion-exchange celluloses), followed by a partial and complete enzymatic degradation with subsequent analysis by gas-liquid chromatography.

SUMMARY

1. Mature Chrysaora quinquecirrha were collected from the York river, the mesogloea surgically isolated, weighed, and lyophilized. From wet and freeze-dried weights, it was established that the mesogloea is 98.166% water and 1.834% solids (=salts, protein, and carbohydrate).

2. The freeze-dried mesogloea was chemically fractionated, in 5% trichloroacetic acid and subsequently in 95% ethanol, into (1) acid soluble carbohydrate, (2) acid insoluble protein, and (3) salts. These fractions, comprising the total solids (i.e., 1.834% of the wet mesogloea) individually represent 0.036%, 0.137%, and 1.661%, respectively of the mesogloea wet weight.

3. A portion of the acid soluble carbohydrate (0.2658 g) was hydrolyzed for six hours at 100°C in 4N HCl, taken to dryness under diminished pressure over sodium hydroxide pellets, and redissolved in 0.1 ml of distilled water with 0.5 ml of 95% ethanol added to prevent microbial contamination.

4. The monosaccharide constituents of the hydrolysate were detected by thin-layer chromatography using four solvent systems in order of relative abundance: xylose, fucose, glucosamine, galactosamine, "iduronic acid," glucuronic acid, arabinose, mannose, and glucose.

5. The approximate concentrations of the monosaccharides were determined by spectrophotometry. A second portion of the acid soluble carbohydrate (0.2041 g) was hydrolyzed and taken to dryness as before. The hydrolysate was redissolved in 10.0 ml of distilled water, aliquots of which were subsequently analyzed by four reactions: (1) Anthrone (hexose), (2) L-cysteine (pentose), (3) Carbazole (uronic acid), and (4) Elson-Morgan (hexosamine). The number of micromoles per 100 µg of mesogloea carbohydrate are: xylose, 0.208; fucose, 0.196; uronic acid, 0.055; arabinose, 0.054; mannose, not determined; glucose, 0.052; glucosamine, 0.027; and galactosamine, 0.020.

6. The detection of uronic acid and hexosamine demonstrates the presence of acidic polysaccharides in Chrysaora mesogloea. The high sulfate content, abundance of neutral sugars, and inequalities between uronic acid and hexosamine moieties indicate the simultaneous presence of three polysaccharide components: (1) "classic" or vertebrate-type glycosaminoglycans, (2) novel glycosaminoglycans, and (3) sulfated and/or neutral polysaccharides.

7. Hypothetical structures are suggested for the novel glycosaminoglycan, and sulfated and/or neutral polysaccharide components, respectively:

(a) { α-L-iduronic acid [β(1→3)] β-D-mannose [β(1→3)]
α-L-iduronic acid [β(1→3)] β-D-mannose [β(1→3)]
β-N-acetyl-D-glucosamine [β(1→4)] }$_n$; and

(b) α(1→2)-linked polyxylosylfucose sulfate.

APPENDIX

APPENDIX A

VARIATION IN STRUCTURE AND COMPOSITION OF MESOGLOEA AMONG THE CNIDARIA

That cnidarian mesogloea is intrinsically variable with respect to volume, structure, and composition is illustrated by Hyman (1940) who applies several names to it depending on class: Hydrozoa, mesogloea; Scyphozoa, collenchyme; Anthozoa, mesenchyme. [According to Chapman (1966) the term mesogloea should be used when referring to this layer in all three classes, as it appropriately satisfies the need to underscore the homologous origin of this intermediate cellular stratum among the Cnidaria; i.e., it is formed by the inward migration of the ectoderm.] The variation in structure is demonstrated by, at one extreme, the very thin, order-less, almost obscure, interstitial stratum of such hydrozoans as Aequorea to the relatively thick, well-defined, well-ordered and highly fibrous "mesoderm" of scyphozoans like Aurelia, Pelagia, Chrysaora, or anthozoans like Calliactis and Metridium. Similar variation is reflected in the composition of mesogloea. For example, the quantity of organic material in the mesogloea of Metridium and Physalia (Gross et al., 1958) versus Chrysaora (Chapman, 1953) differs by a factor of about three to one. Variation in volume of mesogloea among the three cnidarian classes is as great as or greater than the variation in structure and composition. If one compares the volume

of mesogloea per body-volume of the three classes: Hydrozoa, Scyphozoa, and Anthozoa, using Chapman's (1966) diagram which illustrates the proportional volume of mesogloea in typical cnidarians (i.e., a hydra, Pelagia, and Metridium), a ratio of 1:35:12, respectively, can be calculated.

APPENDIX B

COMMON AND CHEMICAL NAMES FOR SUGARS

COMMON NAME	CHEMICAL NAME	SOURCE
arabinose	D(+)arabinose	Sigma[a] A 3256[b]
chondroitin sulfate	chondroitin sulfate (mixed isomers A,B,C) Grade III (from whale and shark cartilage)	Sigma C 3254
chondroitin sulfate B	chondroitin sulfate, type B: sodium salt (from pig skin)	Sigma C 4259
fucose	L(-)fucose	Sigma F 2252
galactosamine	D(+)galactosamine HCl	Sigma G 0500
galactose	D(+)galactose	Sigma G 0750
glucosamine	D(+)glucosamine HCl	Sigma G 4875
glucose	D(+)glucose	Sigma G 5000
glucuronic acid	D-glucuronic acid (Grade I)	Sigma G 9000
hyaluronic acid	hyaluronic acid: Grade I (from human umbilical cord)	Sigma H 1751
iduronic acid	L-iduronic acid[c]	
mannose	D(+)mannose	Sigma M 4625
xylose	D(+)xylose	Sigma X 1500

[a]Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178

[b]Sigma product number (1973 catalog)

[c]This monosaccharide was obtained by the hydrolysis of chondroitin sulfate B (dermatan sulfate), Sigma product number C 4259 (and C 3254, which contains the B isomer).

APPENDIX C: Solving for the Concentrations of Xylose (C_x), Fucose (C_f), and Glucose (C_g) [Anthrone Reaction] by the Method of Three Simultaneous Equations.

I. 1. $A_{620} = a_{x620}{}^{b}\, c_x + a_{f620}{}^{b}\, c_f + a_{g620}{}^{b}\, c_g$

$0.4771 = 1.254c_x + 1.527c_f + 3.304c_g$

2. $A_{590} = a_{x590}{}^{b}\, c_x + a_{f590}{}^{b}\, c_f + a_{g590}{}^{b}\, c_g$

$0.4200 = 0.586c_x + 1.853c_f + 2.879c_g$

3. $A_{503} = a_{x503}{}^{b}\, c_x + a_{f503}{}^{b}\, c_f + a_{g503}{}^{b}\, c_g$

$0.5720 = 2.801c_x + 1.091c_f + 2.192c_g$

$$c_x = \frac{A_{503} - a_{f503}c_f - a_{g503}\, c_g}{a_{x503}}$$

$c_x = 0.20421 - 0.38950c_f - 0.78258c_g$

II. 1. $0.4771 = 1.254(0.20421-0.38950c_f-0.78258c_g)+1.527c_f+3.304c_g$

$0.22102 = 1.03857c_f + 2.32264c_g$

2. $0.4200 = 0.586(0.20421-0.38950c_f-0.78258c_g) + 1.853c_f + 2.879c_g$

$0.30033 = 1.62475c_f + 2.42041c_g$

$$c_f = \frac{0.22102 - 2.32264c_g}{1.03857}$$

$c_f = 0.21281 - 2.23638c_g$

III. $0.30033 = 1.62475\,(0.21281 - 2.23638c_g) + 2.42041c_g$

$$c_g = \frac{0.04544}{1.21315} = 0.03746 \text{ mg/ml}$$

$$= 37 \text{ µg/ml glucose}$$

$$c_f = 0.21281 - 2.23638\,(0.03746) = 0.21281 - 0.08377$$

$$= 0.12904 \text{ mg/ml}$$

$$= 129 \text{ µg/ml fucose}$$

$$c_x = 0.20421 - 0.38950\,(0.12904) - 0.78258\,(0.03746)$$

$$c_x = 0.20421 - 0.07958$$

$$= 0.12463 \text{ mg/ml}$$

$$= 125 \text{ µg/ml xylose}$$

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