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Fine structure, functional morphology and biochemical

aspects of skeletogenesis in <u>Leptogorgia virgulata</u> (Lam.)

(Cnidaria: Gorgonacea)

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Contents

Abstract

The skeletogenic epithelium of the gorgonian coral, Leptogorgia virgulata (Lam.), contains three distinct cell types. At the skeletal branch tips, tall columnar medullocytes secrete a histochemically demonstrable, hydrophobic lipoprotein to increase skeletal length. Another cell type, the corticocyte, secretes the skeletal cortex upon that hydrophobic substratum.

Amino acid analysis shows the organic portion of the skeletal cortex to be collagenous. Electron microscope autoradiography using ^{3}H-proline indicates that procollagen is secreted via corticocytes' flocculent vesicles. Corticocytes also secrete dense-cored vesicles which contain a cytochemically demonstrable hydroquinone. Amino acid analytical identification of dihydroxyphenylalanine (DOPA) in skeletal hydrolysates suggests that the vesiculated hydroquinone is DOPA. Dense-cored vesicles are located near 0.2 μm diameter vesicles which are shown cytochemically to contain DOPA oxidase. It is inferred that the 0.2 μm vesicles contain DOPA oxidase which converts DOPA to a collagen-crosslinking quinone outside the cell.

Amino acid analytical data on young cortical protein and mature cortical protein and morphometric evaluations of dense-cored and flocculent vesicle populations suggest that the skeletal protein is increasingly crosslinked by tyrosine derivatives at sites closer to the skeletal base. The possibility that corticocytes can adapt the skeleton's mechanical properties by modifying rates of collagen and DOPA secretion is discussed.

A third cell type, the desmocyte, has no apparent skeletogenic role and seems to serve in binding soft tissues to the skeleton. Desmocytes spread on the skeleton and develop a pectinate margin facing the mesoglea. A cytochemically demonstrable, sulfhydryl-rich interfacial material found between desmocyte and skeleton is believed to enhance desmocyte adherence. Within the cell, cytoskeletal rods develop perpendicular to the skeleton. The rods are comprised of bundles of 10 nm diameter tonofilaments. Desmocytes are compared to analogous cnidarian cells and to vertebrate desmosome-tonofilament systems. The possibility that desmocyte development is modulated by mechanical stress is discussed.

Abbreviations used

$\overset{o}{A}$ = angstrom

^{o}C = degrees centigrade

Ci. = curie

EM-ARG = electron microscope autoradiography

g = gram or gravity

h = hour

LM-ARG = light microscope autoradiography

M = molar

mg = milligram

min = minute

ml = milliliter

mm = millimeter

mMol = millimolar

N = normal

nm = nanometer

TEM = transmission electron microscopy

μg = microgram

μl = microliter

μm = micrometer

wk = week

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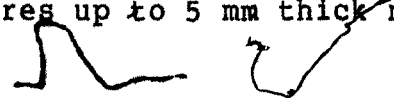
I am also grateful to Tony and Joy who have added much happiness to my stay in Canada.

This thesis is affectionately dedicated to Roxy and the happy times we have together.

Introduction

The goal of this work is to investigate relationships between the functional morphology of skeletogenic cells and the chemistry of the skeletal structures they produce. This study concerns cellular and biochemical aspects of skeletogenesis in the gorgonian coral, Leptogorgia virgulata (Lam.). Leptogorgia was chosen for this study for several reasons. First, the axial skeleton is a collagenous endoskeleton and therefore has analogs throughout the animal kingdom in structures such as tendons, bones and ligaments. Studies of Leptogorgia's skeletogenesis may reveal matters of comparative biological interest. Second, the morphology of the skeleton suggests that patterns of skeletogenesis can be modified by environmental stresses. This is discussed in more detail below. Third, the skeleton is produced by a single layer of epithelial cells and is not subject to remodeling, thereby making it a convenient experimental system.

Leptogorgia's skeletal material, called gorgonin, has been proven to be collagenous by amino acid analytical and x-ray diffraction data (Leversee, 1972). The skeleton is a spindly, branching structure measuring up to one meter in length and attached by a holdfast to the substratum in tropical and semitropical marine habitats. The skeleton measures up to 5 mm thick near the holdfast (Figs. 1, 2).

Leversee (1976) found that branching in _Leptogorgia_ occurs in the plane perpendicular to the direction of prevailing tidal currents. This branching pattern in sessile, colonial cnidarians has been noted in other studies (Théodor, 1963; Théodor and Denizot, 1965; Wainwright and Dillon, 1969; Svoboda, 1970; Riedl, 1971; Grigg, 1972; Rees, 1972; Kinzie, 1973; Velimirov, 1976).

Two sorts of selective advantages in perpendicular orientation of planar, sessile marine organisms to current direction have been identified. The first advantage is the physiological one of optimal orientation for feeding efficiency since perpendicular orientation presents the largest surface area for feeding to the passing water. Leversee (1976) found that colonies of _Leptogorgia_ which were orientated normal to the direction of water movement caught more suspended food organisms than _Leptogorgia_ colonies orientated parallel to water movement. The second advantage is optimal mechanical design for minimizing stresses on the skeleton. Wainwright and Dillon (1969) showed in the gorgonian corals, _Gorgonia flabellum_ and _G. ventalina_, that all colonies were planar and those above a certain height were orientated perpendicular to current direction. These workers postulated that the perpendicular orientation would reduce torque at the base of the skeleton since that orientation is hydrodynamically the most stable. Grigg (1972) upon making similar observations on the gorgonian corals, _Muricea muricata_ and _M. californica_,

suggested that the additional advantage of minimized abrasion between branches would be served by planar branching perpendicular to the direction of water movement.

Gorgonia flabellum and G. ventalina form their skeletal branches in one plane regardless of the colony's age or the pattern of water movement around the colony (Wainwright and Dillon, 1969). This obligatory planar branching is believed to be under genetic control (Leversee, 1976). Leversee's evidence suggested that branching patterns in other gorgonians may be environmentally regulated. Leversee showed that the tendency of Leptogorgia skeletal branches to be formed in one plane increases with the degree of uni- or bidirectionality of the current; in other words, if the water movement direction has no strongly preferred direction, neither does the branching pattern.

Other aspects of skeletal structure seem related to water movement. Riedl (1971) has noted in several sessile cnidarians, including gorgonians, that the length of the skeleton and the degree of branching seem related to water movement around the skeleton since colony skeletons are longer and less branched in areas of relatively little water movement. Grigg (1972) found that gorgonians exposed to great water turbulence had thicker skeletal branch tips than those gorgonians in relatively sheltered waters.

Leversee (1972) observed that Leptogorgia tends to have a thinner skeleton in sheltered water than in more exposed habitats. These observations suggest that mechanical forces due to flow are important in determining gorgonian skeletal morphology. The mechanism regulating skeletal morphology is unknown, although Leversee (1976) hypothesized that the skeletogenic cells may respond to flow forces by modifying their secretory behavior.

The long, thin form of Leptogorgia's skeletal branches is partly responsible for the flexibility of the skeleton. Wainwright and Koehl (1976) have cited some advantages of a flexible skeleton in the severe, turbulent environment many gorgonians inhabit: 1) flexibility allows the colony to bend nearer to the substratum where current velocities are relatively low, 2) bending places the animals in a plane where their surface area is parallel to water movement thereby reducing drag forces, and 3) flexibility increases the amount of energy required to break the skeleton since some of the current's energy goes into bending the skeleton. Flexibility is measured by flexural stiffness which is the product of the elastic modulus, E, and the second moment of area, I (see footnote).

~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Elastic modulus, E = stress/strain, where stress is the force applied to a unit of cross-sectional area of the object and strain is the change of length of the object relative to its original length. In other words, if you have to pull with great force on an object to get it to stretch a tiny amount, the object has a high elastic modulus. The second moment of area, I, is more complicated

I can be modified in the gorgonian skeleton by changing the distribution of skeletal material around the cross-sectional center of the skeleton. Gorgonians may do this by changing skeletal cortex thickness as described in the preceding paragraph. E is related to the molecular organization and chemical nature of the structure. There is evidence suggesting that gorgonians can modify E as well, which will now be discussed.

Goldberg (1974) found that gorgonin fulfills the criteria for aromatic crosslinking (sclerotization) of structural proteins. The criteria listed by Brown (1950), include: 1) the protein contains high levels of aromatic amino acids, 2) the protein changes from light color to dark as it becomes crosslinked, 3) the protein is resistant to dispersion in ordinary proteolytic agents but dissolves readily in sodium hypochlorite and 4) the protein is derived from tissue containing a polyphenol oxidase. Not only did Goldberg find that gorgonin fulfilled these criteria, but he also showed (1978) that the phenol concentration was greater near the skeleton's base. His data suggest that the degree of sclerotization is greater near the skeleton's base than at the skeleton's tip. Goldberg (1978) attributed the change in phenol

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and concerns the distribution of material about an object's central axis. An object with high I is less easily bent than one with low I if other features are equal. For example, a drinking straw has a higher I than a thread because all its material is farther from its central axis.

concentration to a change related to gorgonin "maturation."

However, since those sites near the base are under greatest bending stresses from water movement, the changes in skeletal chemistry could relate to water movement forces. That possibility has not been investigated.

This introductory statement has shown how the morphology and chemistry of the gorgonian axial skeleton is adapted to the environment. Thus far we know the vital importance of the final form of the gorgonian skeleton but not how the skeleton is made. The goal of this work is to understand how skeletogenic cells go about producing a skeleton in a manner which is so well suited to the mechanical and physiological roles it must serve.

The present work is intended to answer the following questions: 1) what cell types are present in the axial epithelium of _Leptogorgia_? 2) which cells produce the gorgonin procollagen? 3) which organelles are involved in procollagen secretion? 4) how does the procollagen secretion rate differ between sites near the skeleton tip and at sites near the skeleton base?

Since another functionally important feature of gorgonian skeletogenesis is collagen sclerotization, the following questions are also addressed: 1) is the collagen sclerotized before or after secretion? 2) can subcellular structures involved in sclerotization be identified? 3) does the degree of sclerotization vary

between locations on the skeleton? Finally, the question
of functional significance will be discussed: how could the
skeletogenic cells of _Leptogorgia_ produce a skeleton with
properties adapted to the mechanical environment of the
animals?

Historical Review

The chemistry of gorgonin has been controversial.
Valenciennes (1855) first chose the term "gorgonin" to name
the structural material of the axial skeleton of _Gorgonia_.
In that study, Valenciennes noted that gorgonin resembled
horn. Gorgonin was first thought to be a keratin (Cook,
1904) because it resembled horn and contained sulfhydryl
compounds. Later, it was called a "pseudokeratin" because
its histidine:lysine:arginine ratio differed from that of
horn (Block and Bolling, 1939). X-ray diffraction data of
Marks _et al_. (1949) showed gorgonin to be, or at least to
contain, a collagen and to not contain keratin. The
mistaken identity of gorgonin as a keratin has not yet been
purged from scientific literature where gorgonin is still
frequently called "horny". The x-ray diffraction data of
Marks _et al_. (1949) was corroborated by Leversee (1972) on
the skeleton of _Leptogorgia virgulata_. Amino acid analysis
has shown gorgonin to have a collagen-like amino acid
composition (Leversee, 1972; Goldberg, 1974). Gorgonin
also contains a minor fraction of lipid and carbohydrate
(Goldberg, 1976).

The identity of the cells which secrete gorgonin is
still controversial. For many years it was debated whether
the gorgonian axial skeleton was a mesogleal or ectodermal
product. Schneider (1905) listed reasons supporting a
mesogleal origin. These reasons included: 1) spicules,

which are products of mesogleal cells, were found in the
axial skeleton, 2) material resembling gorgonin was found
in the mesoglea, 3) the adult skeleton increased in
thickness even in areas where it was covered only with
mesoglea and 4) a model was proposed to show how the axial
epithelial cells could be mesogleal cells. The first two
reasons cited are circumstantial and can not be taken as a
satisfying proof that the axial skeleton is mesogleal. On
the other hand, neither can Chester's assertions (1913)
that he had never seen spicules in gorgonian skeletons or
gorgonin in mesoglea be considered conclusive
counter-evidence. In that same study, Chester states that
he sectioned through branches of the gorgonian
Pseudoplexaura crassa from branch tip to holdfast and found
no sites where axial epithelium did not lie between the
mesoglea and the skeleton. This observation cannot be
considered conclusive, however, since only one specimen was
used. More significant and direct evidence was provided by
Von Koch (1887) and Kinoshita (1910). Von Koch described
the development of a Eunicella larva one week old and found
that the ectoderm was continuous with the axial
epithelium. Kinoshita studied the larval development of
the gorgonian Anthoplexaura and found the pedal disk
epithelium continuous with the axial epithelium. These
observations offer strong support for the ectodermal origin
of the skeleton. In the scheme proposed by Schneider
(1905), mesogleal cells would produce the skeleton by

producing spicules at the tip of the growing skeleton which are later resorbed. This appears to be largely speculative. Most workers have accepted the evidence supporting an ectodermal origin of gorgonin to be the strongest (G. Chapman, 1974).

There have been recent claims based upon light microscopical and electron microscopical observations which revive the old controversy. Goldberg (1973) has proposed that gorgonin is both a mesogleal and ectodermal product. On the basis of electron microscopical evidence, Goldberg suggested that the skeletogenic cells are ectodermal but that some of these cells are infiltrated by mesogleal collagen fibers which are then chemically modified and secreted as gorgonin. Bayer's light microscopical studies (1974) show perforations in the axial epithelium of the gorgonian Plexaura homomalla. He proposes that those holes are the sites where mesogleal collagen meets the skeleton and is presumably transformed into gorgonin.

The cell type responsible for the secretion of gorgonin, regardless of whether it is ectodermal or mesogleal, is also controversial. Chester (1913) identified two cell types in the axial epithelium of Pseudoplexaura: 1) secreting cells which were tall and cylindrical or prismatic and 2) desmocytes which apparently differentiated from secreting cells but were broader and shorter than the secreting cells and contained many

rod-like objects which seemed to be involved in binding the cell to the skeleton. Chester's findings agreed with the previous work by Bourne (1899) on Heliopora which first identified desmocytes. Desmocytes were shown to be located in the regions of slowest skeletal growth thereby suggesting those cells had little, if any, skeletogenic role (Bourne, 1899; Chester, 1913).

Most cnidarian histologists have agreed with Chester's description of two cell types in the gorgonian skeletogenic epithelium except on the crucial question of whether the desmocytes or the taller epithelial cells are the skeletogenic cells. Kinoshita's observations (1910) clearly indicate that the young axial skeleton is produced by tall columnar cells since in the early larval skeletogenesis no desmocytes were observed. Bayer (1954), on the basis of light microscopical study of five gorgonians' histology, suggested that desmocytes may have a "depositional" function. Bouligand (1968) conducted an electron microscopical study of axial epithelial cytology in the gorgonian Lophogorgia sarmentosa and concluded that desmocytes (which he called "striated plate cells") are penetrated by mesogleal collagen fibers and secrete gorgonin. It is unclear whether Bouligand believed the mesogleal collagen to be converted to the gorgonin collagen by the desmocytes as Goldberg (1973) later suggested.

The work of this thesis was begun with the above cited

accounts providing the only information on gorgonian

skeletogenesis.

Figure 1. 'Photograph of an air-dried skeleton of
Leptogorgia. The skeleton is attached to the substratum by
a holdfast (H). This skeleton is a planar form and, in its
natural habitat, the predominant direction of water
movement would be in the plane perpendicular to the page.
Bar = 10 cm.

Figure 2. A diagram of a portion of a Leptogorgia colony's
branch. The central, cut-away part shows the axial
skeleton (AXIS) surrounded by the soft tissues of the
colony. A system of gastrovascular canals (G) runs parallel
to the skeleton and connects the enterons of the polyps (P),
the individual animals of the colony. Bar = 2 mm.

1 H

2 G P AXIS G P P

OUTLINE FOR STUDYING GORGONIAN SKELETOGENESIS

I. WHAT IS THE FINE STRUCTURE OF THE AXIAL EPITHELIUM?

(TEM)

WHAT DIFFERENT CELL TYPES ARE PRESENT IN THE AXIAL EPITHELIUM?

(TEM, histochemistry, cytochemistry)

WHICH CELLS SECRETE PROCOLLAGEN?	WHICH CELLS ARE INVOLVED	OTHER CELLS PRESENT?
(LM-ARG with ^{3}H-proline)	IN SCLEROTIZATION?	(TEM)
	(LM-ARG with ^{3}H-tyrosine,	
	DOPA oxidase cytochemistry,	
WHICH SUBCELLULAR STRUCTURES	hydroquinone cytochemistry)	ROLE OF OTHER CELLS?
CONTAIN PROCOLLAGEN AT THE		(inferences from fine
TIME OF SECRETION?		structure and
(EM-ARG with ^{3}H-proline)		histochemistry)

OUTLINE CONTINUED

II. WHAT IS THE PROTEIN COMPOSITION OF GORGONIN?

(amino acid analysis)

HOW DOES THE GORGONIN PROTEIN DIFFER FROM THE SKELETAL TIPS TO BASE?

(amino acid analysis)

III. WHAT IS THE RELATIONSHIP BETWEEN CHANGES IN SKELETAL PROTEIN COMPOSITION

AND SKELETOGENIC CELL FINE STRUCTURE?

(electron microscopical morphometrics)

Chapter 1: Lengthening of the gorgonian axial skeleton: lipoprotein secretion.

Introduction

This investigation of gorgonian skeletogenesis was begun by studying the axial epithelium's morphology and histochemistry. Early in the study, a morphological difference was observed between cells at the branch tips and cells nearer to the colony's holdfast. That observation was new and suggested that the cells at the skeletal tip, which cause the skeleton to increase in length, and the cells nearer the holdfast, which cause the skeleton to increase in thickness, may secrete different skeletal constituents.

This chapter describes the secretory activity of the cells at the branch tip and speculates on the functional importance of their secretory role.

Materials and Methods

1. Light microscopy and histochemistry

Branch tips 3 cm long were cut from Leptogorgia virgulata (Lam.) colonies freshly collected from the estuarine waters surrounding the Duke Marine Laboratory, Beaufort, NC, USA. The branch tips were placed in seawater until the polyps re-expanded and then the animals were anaesthetized by slowly adding 7.5% $MgCl_2 \cdot 6H_2O$ to the dish. After anaesthetization, the tissue was fixed in 10% buffered formalin (Baker, 1946), decalcified in 2% ascorbic acid in seawater overnight, dehydrated in a graded series of ethanols and infiltrated and embedded with JB-4 plastic (Polysciences Inc., Warrington, Pa., USA). The blocks were polymerized at room temperature under nitrogen and sectioned at 2 μm. Bound lipids were then identified in plastic sections by the acetone-Sudan black technique of Berenbaum (1954). Other tissue samples were embedded in paraffin and sectioned at 7 μm. In these paraffin sections, proteins were identified by the mercury-bromphenol blue technique (Pearse, 1968), non-acidic sugar groups by the periodic acid-Schiff technique of Hotchkiss (1948) and acid glycosaminoglycans by the astra blue technique (Barka and Anderson, 1965).

2. Transmission electron microscopy and cytochemistry

Specimens were collected, sampled and anaesthetized in

the same manner as those used for light microscopy. The tissue was fixed for 2 h at 4 °C in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer containing 0.04% $MgCl_2$ at pH 5.8. The osmotic concentration was 980 milliosmoles. The tissue was osmicated for 1 h and decalcified in either 2% ascorbic acid in seawater overnight or in the phosphate buffer for 48 h at 6 - 10.°C. Dehydration was carried out in a graded series of ethanols. The tissue was infiltrated and embedded in epoxy resin under reduced pressure, thin sectioned and stained with saturated aqueous uranyl acetate and Reynold's lead citrate (1963). The sections were viewed with a Zeiss 10A electron microscope.

Tissue in which sites of osmiophilia were identified was prepared for transmission electron microscopy, thin sectioned and placed on gold, 200-mesh grids. The sections were then treated with thiocarbohydrazide and osmium tetroxide by the technique of Seligman et al. (1966).

3. Scanning electron microscopy

Tissue was fixed as described for transmission electron microscopy, split longitudinally through the axial skeleton with a razor blade and then dehydrated through a graded series of ethanols. The tissue was critical point dried with carbon dioxide, sputter coated with gold and viewed in a Cambridge S150 scanning electron microscope.

4. Electron microscopical morphometrics

The point-counting technique (Weibel, 1973) was used to calculate the percent volume of the secretory end of the skeletogenic cells occupied by the secretory organelles. Points counted were restricted to a 10 μm band at the secretory end of the cells. The percent volume occupied by secretory vesicles was compared to the thickness of the skeletal material over which the cell measurements were made. The relationships between the percent volume of the cell occupied by secretory vesicles and the skeletal thickness over which the cells were found were evaluated by non-linear regression of bivariate data.

5. Extraction of lipids and lipoproteins

Skeletal samples taken from the apical 1 cm of skeleton branches and from basal portions of the skeleton were scraped clean of adhering soft tissues and ground to a powder under liquid nitrogen with a mortar and pestle. The powdered skeletal samples were sonicated for three periods of ten minutes in 100 volumes of 2:1 chloroform-methanol (C:M) and the insoluble material filtered out. The insoluble material was lyophilized and weighed. The C:M solution was partitioned against an aqueous phase by the method of Folch et al. (1957) at 4°C for 12 h. The chloroform phase was collected and dried under a stream of nitrogen and then hydrolyzed with 6 N HCl at 105°C for 24 h. The hydrolysate was then mixed with a ninhydrin

solution made by adding 2.0 g ninhydrin, 0.04 g stannous
chloride, 75 ml methyl cellusolve and 25 ml 4 N sodium
acetate buffer at pH 5.51 $\pm$ 0.03 and 100°C. The production
of a purple color would indicate the presence of amine
groups in the hydrolysate.

Results

The axial skeleton of the gorgonian coral _Leptogorgia_ _virgulata_ is a cylindrical structure consisting of a central core, the medulla, and the surrounding cortex. A third skeletal region, the medullary wall, encloses the medulla and is thus the interface between medulla and cortex (Figs. 1, 2).

The medulla is divided into chambers by horizontal lamellae which extend completely across the medulla (Figs. 2, 3). Within the medullary chambers is a mesh of fibers extending between medullary walls, lamellae or other crossfibers.

Crossfibers, lamellae and medullary walls are produced by tall columnar cells, the medullocytes, found at the distal 2 mm of skeletal branches (Figs. 3, 4, 5). The medullocyte's axis end, i.e. the end nearest the axial skeleton, contains dense vesicles, approximately 1.0 μm in diameter, which are secreted into the medulla of the skeleton. After secretion, some of the secreted material retains its globular form or may coalesce to form bands of skeletal material, the intramedullary crossfibers. The dense vesicles are called lipoprotein vesicles for reasons which will be explained in the following discussion. Medullocytes differ from those cells overlying the cortex, the corticocytes, in morphology and secretory activities (Figs. 4, 6). The corticocytes are shorter, contain a

different population of vesicles, arrays of rough endoplasmic reticula and are joined to one another by septate junctions (Figs. 2, 6, 7).

Morphometric analysis of the volumetric composition of the skeletogenic cells' axis end indicates that there is a drastic decline in the volume of these cells occupied by lipoprotein vesicles from sites where the cells overlie the medullary wall to sites where the cells overlie a relatively thick layer of skeletal cortex (Fig. 8).

Treatment of longitudinal sections of skeleton by the acetone-Sudan black technique produces a heavy staining of the medullary walls and lamellae and occasionally a perceptible staining of crossfibers in sections prepared for light microscopy (Fig. 9). The acetone-Sudan black positive structures also stain with bromphenol blue for proteins although with neither periodic acid-Schiff reagent for non-acidic sugar groups nor astra blue for acid glycosaminoglycans. In the osmium and thiocarbohydrazide (OTO) treated sections for electron microscopy, no skeletal structures were found to bind osmium (Fig. 10). The OTO technique also indicates that the lipoprotein vesicle contents are not osmiophilic.

Extraction of lipids and lipoproteins from the skeleton shows that samples from the tips of the skeleton where there is little cortex contain 13.9% $\pm$ 0.1 (N=3) C:M soluble material while samples from the skeleton base where

there is a thicker cortex contain $4.4\% \pm 1.8$ (8) soluble material. The C:M soluble material was ninhydrin positive, suggesting that it contains amine groups.

Discussion

The results of the present study provide evidence that the material produced and secreted by _Leptogorgia_ medullocytes is chemically distinct from that secreted in subsequent skeletogenic stages by corticocytes. The following evidence indicates that the medullocytes secrete a lipoprotein.

The acetone-Sudan black technique indicates that medullocytes secrete a bound lipid (Berenbaum, 1954). In this technique, the tissue is washed in water for several hours prior to staining with acetone-Sudan black. The washing is presumed to dissociate protein from lipoprotein thereby allowing the stain to reach the lipid. Berenbaum (1954) has identified protein bound lipids in several tissues including collagenous tissues. Although Berenbaum's (1958) data suggest that the lipids may also be bound to carbohydrates and nucleic acids, the lipid is typically found bound to protein. Since no staining of the medullary wall or intramedullary structures was observed with the periodic acid-Schiff or astra blue techniques for sugars while those same structures stain for protein by the bromphenol blue technique, the lipid appears to be bound to a protein. Further evidence for a lipid bound to a protein was obtained by the extraction of lipids and lipoproteins from the skeleton by a modification of Folch _et al._'s technique (1957). The finding that the non-polar fraction

is ninhydrin positive is also consistent with the presence
of a protein bound lipid or lipoprotein. For these
reasons, the dense vesicles which are secreted to become
the lipoprotein-containing medullary wall, lamellae and
crossfibers are referred to as lipoprotein vesicles. The
OTO technique has shown that the lipoprotein vesicles are
not osmiophilic. Absence of osmiophilia is a
characteristic of saturated lipids since osmium is believed
to be reduced by the oxidation of unsaturated carbon bonds
in lipids (Criegee, 1936). In lipoproteins, osmium was
found to interact almost exclusively with the lipid portion
of the molecule (Hayes et al., 1963) although the amino
acids cysteine, methionine, arginine, ornithine and lysine
can also reduce osmium (Bahr, 1955). The lipoprotein
secreted in early stages of skeletogenesis in Leptogorgia
is therefore believed to contain a saturated or nearly
saturated lipid and to have a low concentration of
osmium-binding amino acids.

Several types of information indicate that the
secretion of lipoprotein is a discrete stage of
skeletogenesis occurring at the skeletal tips. First,
histochemical data show discrete staining with
acetone-Sudan black of the medullary wall and
intramedullary structures. This staining is consistent
with the addition of lipid-free cortical material around
the lipoprotein-containing structures in the medulla and
medullary wall. Second, electron microscopical

morphometrics show that the population of lipoprotein
vesicles drops severely from greater than 20% of the volume
of the secretory end of medullocytes at the skeleton tip to
less than 2% of those cells over thicker areas of the
skeletal cortex. The change in secretory activity also is
consistent with the addition of lipid-free cortical
material around the lipoprotein-containing structures.
Third, extraction of the skeleton with 2:1
chloroform-methanol causes over 3 times more reduction of
weight in the extracted skeleton from the tips than in
extracted skeleton from the base.

.Goldberg (1978) has noted that in two other
gorgonians, Swiftia excerta and Muricea muricata, the
proportion of material extractable from the axial skeleton
by C:M decreases from the tip of the skeleton, where there
is approximately 8% weight loss in the extracted skeleton,
to the base, where there is approximately 1% or less weight
loss. A third gorgonian in Goldberg's study (1978),
Gorgonia ventalina, contained no C:M soluble material.
This indicates that the lipoprotein-secreting stage may not
be present in all gorgonians. Chemical analyses of
gorgonian skeletons suggest that cortical collagen is
aromatically crosslinked (Leversee, 1972; Goldberg, 1974;
1978). The Leptogorgia skeleton therefore contains a
lipoprotein substratum, the medullary wall, upon which a
tanned (i.e. aromatically crosslinked) protein, the cortex,
is secreted.

Layering of lipoprotein and tanned protein has been identified in other structural materials including egg shells of the nematode _Aspiculauris tetraptera_ (Anya, 1964) and of the trematodes _Hexacotyle extensicauda_ (Dawes, 1940) and _Fasciola hepatica_ (Clegg, 1965), and in insect cuticle (Wigglesworth, 1933; 1970; Locke, 1976). In _Hexacotyle_ egg shells and in insect cuticle, the lipoprotein layer has been interpreted to function as a substratum for protein secretion and aromatic crosslinkage. It is proposed here that the lipoprotein of the gorgonian medullary wall serves a similar role. The lipoprotein probably is hydrophobic since it is soluble in non-polar solvents and does not bind osmium, indicating that the lipid portion is saturated. Both non-polarity and saturation are characteristics of hydrophobic lipids.

Production of a water-tight, hydrophobic substratum for collagen secretion would be functionally important in marine gorgonians if gorgonian cortical collagen were not aromatically crosslinked until after secretion. If that were true (which Chapter 2 shows to be probable), gorgonin would be seawater soluble at the time of secretion. Bouligand's observations (1968) suggest that the gorgonian skeletal medulla contains seawater and Kinoshita (1910) has shown that the first medullary walls, crossfibers and lamellae of newly settled gorgonian larvae are secreted into seawater by the pedal disk epithelium. In mammalian collagenous tissues, some of the collagen present has been

found soluble in 0.14 to 2.0 M NaCl solutions (Piez,
1967). That collagen which is soluble in NaCl solutions at
neutral pH includes newly synthesized collagen which has
not yet been covalently crosslinked in the connective
tissue (Monson and Bornstein, 1973). The NaCl
concentration of estuarine water could be nearly as high as
that of sea water (about 0.5 M NaCl) and the pH would vary
between the value for fresh water (about pH 6.7) to that
for seawater (about pH 8.1) (Prosser, 1950). Estuarine
water would presumably solubilize newly-secreted, gorgonian
cortical collagen were there not a lipoprotein layer
interposed.

Figure 1. Diagram of _Leptogorgia_'s morphology. .The
diagram shows a portion of a skeletal branch (arrow) in the
cut-away part of the soft tissue. In the soft tissue,
several gastrovascular canals (G) run parallel to the
skeleton and connect the enterons of the individual
animals, the polyps (P). Bar = 2 mm.

Figure 2. Diagram of a lateral half of a longitudinal
section of the skeleton and overlying epithelium. Bar =
0.2 mm.

A. At the skeletal branch tip, the tall columnar
cells overlie the medullary wall (m) laterally and a
terminal lamella (double arrowheads) at the tip. Between
intramedullary lamellae (arrowheads) are intramedullary
crossfibers.

B. Nearer the base of the skeleton, skeletal cortex
(c) has been secreted onto the medullary wall (m). The
epithelium overlying the cortex is low columnar to cuboidal
and contains a peculiar cell, the desmocyte (d), described
by Bouligand (1968).

1

P

P

G

2A

2B

m

c

d

Figure 3. Scanning electron micrograph of the medulla of an axial skeleton. The medulla, bound laterally by the medullary wall (W), is subdivided by transverse lamellae (L) which divide the medulla's lumen into chambers. Crossfibers (F) lie between lamellae, the medullary wall and other crossfibers. The crossfibers bifurcate within the chambers and ramify on the surfaces of the lamellae. Bar = 30 μm.

F

Figure 4. Transmission electron micrograph of
medullocytes. These cells are producing an intramedullary
lamella (L). The dominant cytoplasmic constituent of many
of these cells are dense, lipoprotein vesicles measuring up
to 1 um in diameter (arrowheads). The abaxial end of these
cells is bound by the loose connective tissue, mesoglea
(M). Bar = 5 μm.

Figure 5. Transmission electron micrograph of the secretory, axis end of the medullocytes. These cells are producing intramedullary crossfibers (F). Most of the globular secretions into the medulla have joined together to form fibers. The electron-lucent material which occupies most of the medulla is believed to be seawater (Bouligand, 1968) although that has not been proved. Bar = 3 µm.

Figure 6. Transmission electron micrograph of corticocytes overlying a thick layer of the"axial skeletal cortex (A). These cells differ from the medullocytes in containing flocculent vesicles (F) and arrays of rough endoplasmic reticula (arrowhead). M = mesoglea, a loose connective tissue. Bar = 3 μm.

Figure 7. Transmission electron micrograph of the axis end
of corticocytes. Flocculent vesicles (F) which are
involved in procollagen secretion are characteristic of
these cells but are rarely seen in medullocytes.
Corticocytes are also characterized by dense-cored vesicles
(arrowhead) and rough endoplasmic reticula (arrows). A =
axial skeleton. Bar = 1 μm.

Figure 8. Results of an electron microscopic morphometric analysis comparing the relative volumes of the skeletogenic cells occupied by lipoprotein vesicles to the cells' location over the skeleton. The abscissa indicates distance from the inner surface of the medullary wall to the outer surface of the cortex over which the cells were observed. The ordinate indicates the lipoprotein vesicles' percent volume of the 10 um of the skeletogenic cells adjacent to the skeleton. An abrupt decline in the proportion of lipoprotein vesicles is apparent from sites near the skeleton tip where the skeleton is thin to thicker sites on the skeleton. The curve $Y = 0.69 + 11.82 (1/X)$ was obtained by non-linear regression of bivariate data. The value $R = -0.90$ is the coefficient of correlation. The high value of $|R|$ where 1.00 is a perfect correlation indicates a significant correlation between the curve and the data. $N = 10$.

MEDULLARY WALL + CORTEX
THICKNESS (uM)

LIPOPROTEIN VESICLES % VOLUME

8

Figure 9A. Light micrograph of a longitudinal section of an axial skeleton stained with acetone-Sudan black. Structures which stain for bound lipids include the medullary wall (W), intramedullary lamellae (L) and crossfibers (arrowheads). The cortex (C) displays little staining. 100 X. Bar = 200 μm. Figure 9B. Scanning electron micrograph of an axial skeleton split longitudinally. Structures which stain for bound lipids shown in Fig. 9A are indicated here: medullary wall (W), lamellae (arrowheads) and crossfibers which lie between the walls and lamellae. Bar = 150 μm.

9A

9B

C

C

C

C

Figure 10A. Transmission electron micrograph of the axis end of medullocytes. This section has been treated by the OTO technique to identify sites of osmiophilia. The least osmiophilic sites are the newly secreted skeletal material (arrowheads) and the lipoprotein vesicles (L).
Bar = 0.2 μm.

10A

Figure 10B. Transmission electron micrograph of a section
adjacent to that shown in Fig. 10A but which has been
stained with uranyl acetate and lead citrate. The
longitudinal striations in the lipoprotein vesicles (L) are
of unknown significance. Lipoprotein vesicles = L. Bar =
0.2 μm.

Chapter 2: Thickening the axial skeleton: secretion and crosslinking of cortical collagen.

Introduction

Chapter 1 provided evidence that medullocytes secrete a lipoprotein. The identity of the procollagen-secreting cells and the way that aromatic crosslinks of skeletal collagen are formed is still unknown. Those aspects of gorgonian skeletogenesis are investigated in this chapter.

Tyrosine derivatives are among the various collagen crosslinkages identified in mammalian tissues (LaBella et al., 1968) where their occurrence increases with the animal's age (Waykole·and Heidimann, 1976). In other structural proteins, such as the human lens protein, tyrosine-derived crosslinkages have also been identified in samples obtained from old individuals (Garcia-Castineiras, 1978). The relationship between tissue age and the presence of tyrosine-derived crosslinking compounds has suggested that the crosslinkages may be formed slowly and spontaneously under the influence of atmospheric oxygen (LaBella et al., 1968). That same investigation has demonstrated that peroxidation of collagen in vitro can cause the formation of dityrosine crosslinkage of collagen. In theory, cells therefore have the potential to influence the degree of tyrosine-derived collagen crosslinking and thereby influence the mechanical properties of connective tissues by peroxidation of

collagen.

A large number of invertebrate structural proteins, including collagen, are believed stabilized by tyrosine-derivatives. These include arthropod resilin (Andersen, 1964), crustacean cuticle (Welinder, 1976), molluscan periostracum (Waite, 1977) and the collagenous axial skeleton of gorgonian corals (Goldberg, 1976; 1978). The proposed mechanism for formation of tyrosine-derived crosslinkages in these invertebrate systems involves the enzymatic hydroxylation of tyrosine aromatic rings to dihydroxy compounds which are subsequently enzymatically oxidized to a quinone (Pryor, 1962), which is the putative crosslinking compound. The enzyme which has the potential to catalyze each of these steps is dihydroxyphenylalanine oxidase (DOPA oxidase) (Burges, 1963). An alternative pathway for tyrosine-derived crosslinkage formation in arthropod cuticle which involves the oxidation of the carbon in the position adjacent to the tyrosine aromatic ring (β-oxidation) has also been proposed (Andersen and Barrett, 1971; Andersen and Roepstorff, 1978).

This portion of this thesis describes the cellular mechanism by which the skeletogenic epithelium of Leptogorgia secretes procollagen and a tyrosine-derived crosslinking compound. A mechanism by which those cells can modify the degree of collagen crosslinkage in gorgonin is proposed.

Materials and Methods

1. Electron microscopy

Three centimeter long branch tips of _Leptogorgia_
virgulata were cut from freshly collected colonies. The
branch tips were anaesthetized, fixed and processed for
transmission electron microscopy using the techniques
described in Chapter 1.

2. Autoradiography

Three centimeter long branch tips were placed in 3 ml
plastic culture dishes containing seawater. After the
retracted animals re-expanded, tritiated proline (115.4
Ci/mMol) (New England Nuclear, Boston, MA, USA) was added
to the dish to a concentration of 10 µl of tritiated
proline per 1 ml of seawater. The proline had a
radiochemical purity greater than 99% as determined by the
supplier four months before use in these experiments. The
animals were removed from the labeled seawater after .1 h
and placed in fresh, unlabeled seawater. At periods of 1,
2, 3, 5, 6, 12, 18 and 24 h total elapsed time since
beginning a 1 h pulse label, specimens were removed from
the dish and processed for electron microscopy as described
above.

Autoradiographs of 0.5 µm plastic sections of labeled
tissue were prepared using Kodak NTB-2 emulsion with
exposure times of 4 wk. Electron microscope

autoradiographs of 60 nm sections were prepared using Kodak
Special Emulsion 129-01 with the dipping technique of Ball
et al. (1981). Sections were exposed to the emulsion for
periods of up to 15 months and developed in phenidon
developer (Lettré and Paweletz, 1966). The autoradiographs
were stained through the emulsion.

A second group of branch tips was collected and
incubated with tritiated tyrosine using an identical
technique. These branch tips were processed for light
microscopy in the same manner as the branch tips exposed to
tritiated proline.

3. Cytochemistry

    a) DOPA oxidase localization

Modifications of and additions to a previously
described technique (Novikoff et al., 1968) were used to
localize sites of DOPA oxidase activity. Tissue was fixed
as for routine electron microscopy except that osmication
was eliminated. The tissue was decalcified in buffer at pH
5.8 for 48 h at 4 - 7 °C. One millimeter thick sections of
the tissue were incubated for 20 h at 6°C and then for 3 h
at 37 C in either 0.1% DOPA (3,4-dihydroxyphenylalanine),
0.1% dopamine (dihydroxytyramine) or 0.1% tyrosine in the
phosphate buffer. All substrates were obtained from Sigma
(St. Louis, MO., USA). After incubation, the tissue was
washed three times (20 min each) in the phosphate buffer.

The tissue was then osmicated for 2 h in 2% osmium tetroxide in distilled water. Routine dehydration and embedding followed. For each of the three substrates tested, each of the following three controls was run: 1) tissue was treated as the experimental tissue except the substrate was not added to the buffer; 2) tissue was incubated in a solution containing the test substrate and a competitive substrate, 0.01% phenylthiourea (PTU) (Sigma); 3) tissue was incubated in a copper chelator, 0.01% diethyldithiocarbamate (DDC) (Sigma) followed by 1 h buffer wash and then incubated with the same test substrate as the experimental tissue. DDC was chosen because DOPA oxidase is a copper-containing enzyme which is blocked by DDC (Okun et al., 1970).

b) Silver methenamine localization of hydroquinones

Tissue was fixed, dehydrated and embedded as for standard electron microscopy but it was not osmicated. Sections were cut and placed on gold, 200-mesh grids. The sections and grids were then immersed in 1.25 M N-ethyl maleimide (NEM) at pH 7.4 for 2 h at room temperature to block sulfhydryl groups (Bennesh and Bennesh, 1961). The grids were then washed in buffer, immersed in 5% sodium metabisulfite for 10 min at room temperature to block aldehyde groups (Locke and Krishnan, 1971) and then washed again in buffer.

The grids were transferred in the dark to an aqueous

solution of methenamine and silver nitrate (Swift, 1968).
After an incubation of 80 min in the dark at 45°C, the
grids were transferred to a 5% sodium thiosulfate solution
for 1 h after which they were finally washed briefly in
three changes of distilled water.

c) Endogenous peroxidase localization

A technique for localizing exogenous peroxidases
(Graham and Karnovsky, 1966) was modified and adapted to
endogenous peroxidase localization. Tissue was aldehyde
fixed as for routine electron microscopy, washed in
phosphate buffer (pH 5.8; 980 mOsm) and then with 0.05 M
Tris buffer at pH 7.6. The tissue was incubated in 10 ml
of 0.03% diaminobenzidine in the same Tris buffer at room
temperature. After 15 min, 0.4 ml of 0.3% H_2O_2 was added
to the solution and the tissue was then incubated for an
additional 30 min. The tissue was finally washed in the
Tris buffer, osmicated, dehydrated and embedded.

d) Morphometrics

The standard morphometric technique of point-counting
(Weibel, 1973) was used to evaluate the percent volume (Vv)
of the axis end of the skeletogenic cells which was
occupied by selected subcellular structures. Micrographs
were taken of the 10 µm at the axis end of the cells and
were printed at 20,000 times total magnification.

Electron microscope autoradiographs of 12 sections

from each of two samples of 24 h elapsed time from initiation of proline label to fixation were evaluated. In electron microscope autoradiographs, silver grains were assigned to a putative source by direct allocation (Kramer and Geuze, 1980). The number of silver grains predicted to lie over a subcellular structure if distribution were random (Ne) was calculated by multiplying the total number of grains lying over tissue by the volume-fraction (Vv) occupied by the selected subcellular structure. Ne was then compared to the number of silver grains actually counted over the selected structure (Ns) by the chi-squared test. Those subcellular sites for which Ns is significantly greater than Ne are considered locations of labeled material.

4. Skeletal DOPA measurements

Skeletal samples were sonicated in three changes of 30 volumes of 2:1 chloroform-methanol for a total of 30 min to remove lipids and lipoproteins. The samples were lyophilized and then hydrolyzed in 6 N HCl under a reduced pressure, nitrogen environment for 24 h at 105 - 110°C. Hydrolyzed samples were analyzed on a Beckman 120 C Amino Acid Analyzer interfaced with a Beckman 125 Integrator. Standard samples of L-β-3,4-DOPA were obtained from Sigma.

Results

1. Electron microscopy

The axial epithelium overlying Leptogorgia's skeletal cortex contains tall columnar cells measuring 15 to 45 µm in height (Fig. 1). The axis end of these cells contains two sorts of vesicles each of which is associated with a separate secretory episode.

One secretory episode involves dense-cored vesicles which are prolate spheroids, measuring about 0.5 µm by 0.4 µm. Clear examples of exocytosis (Fig. 2) show that those vesicles release skeletal constituents into the extracellular space. The dense-cored vesicles are frequently found in close association with smaller, 0.2 µm diameter vesicles.

A second secretory episode involves flocculus-containing vesicles which measure up to 1 µm in diameter (Fig. 3). As the flocculent vesicle nears the plasma membrane at the cell's axis end, the vesicle and plasma membranes become closely apposed (Fig. 4). In some instances, the vesicle and plasma membranes cannot be distinguished from one another (Figs. 4 and 5) and near those sites, the plasma membrane has concavities which suggest locations of previous membrane fusion and exocytosis (Fig. 5). At those sites, the newly secreted material has a more orderly, fibrillar appearence than the

flocculent material within the flocculent vesicles.

On very infrequent occasions the flocculent material has been observed organized into fibrillar bundles within the vesicles (Fig. 6) but typically no organization of the contents is observed. Only a few cells of thousands studied contained vesicles with fibrillar material aggregated into cross-striated bundles (Fig. 7). In the collagenic cells of some other animals, vesicles of similar appearence were believed to be procollagen secretory vesicles (Trelstad, 1971; Weinstock and LeBlond, 1974). These are not thought to be procollagen secretory vesicles in Leptogorgia because they were rarely observed, they were not sighted at the secretory end of the cell and they were striated with a 57 nm periodicity which is dissimilar to the 26 nm periodicity of fiber cross-striations in the skeleton (Fig. 8).

The proposed sequence of secretory events for both flocculent and dense-cored vesicles corresponds to the sequence shown in the classic model of zymogen granule secretion (Caro and Palade, 1964; Jamieson and Palade, 1967; Kern et al., 1979) i.e. membrane apposition, membrane fusion and secretion of vesicle contents. No observations of a flocculent vesicle appearing to have just opened its lumen to the extracellular space have been made, however.

2. Autoradiography and morphometrics

Light microscope autoradiographs of tissue fixed 12 h after the beginning of a 1 h pulse label with tritiated tyrosine (Fig. 9) or 24 h after beginning a label with tritiated proline (Fig. 10) show bands of silver grains at the corticocytes' axis end and at the interface between skeleton and corticocytes.

In Table 1, the percent volumes of the axial 10 µm of corticocytes which are comprised by dense-cored vesicles, flocculent vesicles and other structures evaluated by electron microscopical morphometrics are tabulated. Based on the percent volume comprised by these structures, the expected random and the actual observed silver grain frequencies counted in electron microscope autoradiographs of tissue exposed to tritiated proline are compared. These data indicate that at 24 h following labeling, only the flocculent vesicles have a grain count significantly greater than that expected from a random grain distribution (Fig. 11).

3. Cytochemical studies

a) Silver methenamine technique for hydroquinones

The silver precipitate of the silver methenamine reaction for hydroquinones was found exclusively over the dense-cored vesicles and infrequently on sites in the skeleton subjacent to the skeletogenic cells (Fig. 12). The precipitate over the core of dense-cored vesicles was

very heavy, while over the peripheral areas of the vesicle much lighter. Treatment of tissue with NEM and sodium metabisulfite eliminated other sites of silver precipitation in the tissue but did not affect the precipitate formed within the dense-cored vesicles.

b) DOPA oxidase localization

The results of the DOPA oxidase localization experiments are presented in Table II. The reaction product at the axis end of the cells was found only in the small, electron-lucent vesicles which are found near the dense-cored vesicles. Not all similar, electron-lucent vesicles were found to produce a reaction product under identical conditions (Fig. 13).

c) Endogenous peroxidase reaction

No sites of endogenous peroxidase activity were identified in any of the cells observed in three repetitions of the experiment.

4. Skeletal DOPA measurements

Measurements of DOPA content in skeletal samples by automated amino acid analysis indicated that DOPA is present within the skeleton in concentrations of 2.2 µg/mg protein.

Discussion

1. Procollagen secretion

Electron microscope autoradiographic evidence indicates that the flocculent vesicles observed within the axis end of corticocytes contain procollagen at the time which procollagen is secreted onto the skeleton of Leptogorgia. Previous investigations (Leversee, 1972; 1980a; 1980b) have shown by chromatographic separation of Leptogorgia branch tip hydrolysates collected 24 hours after commencement of a one hour pulse label with ^{14}C-proline, that 10% of the labeled imino groups were hydroxyproline. Since other than the flocculent vesicles, no subcellular structures evaluated, including the cytoplasm, were found to contain a significant amount of labeled material, these vesicles presumably contain labeled proline and hydroxyproline and therefore procollagen.

The failure to identify examples of flocculent vesicle membranes which have just opened their lumina to the extracellular space even after examination of thousands of skeletogenic cells has three possible explanations: this secretory stage may be very shortlived, animal collection and preparation may interfere with the secretory process or, procollagen may be secreted by a means which does not involve opening the vesicle lumen to the extracellular space. An alternative mechanism would be diffusion of a soluble procollagen across the membranes. A review of the

literature on procollagen secretion shows identification of
this step in collagen production to be a historically
problematic one.

Investigations of the morphology of collagen secretion
have typically involved either simple inferences from
observations on fine structure and comparisons to other,
well described systems (e.g. zymogen granule secretion,
Kern et al., 1979) or autoradiographic analysis using
tritiated proline.

Revel and Hay (1963) studied collagenesis in newt
larvae chondrocytes by using tritiated proline. The
qualitative electron microscope autoradiographic (EM-ARG)
results showed that vesicles about 0.8 μm in diameter
secrete labeled material into the extracellular space.
These vesicles contain a flocculent material and bear close
resemblance to the flocculent vesicles in Leptogorgia.
Revel and Hay found examples of membrane fusion and
exocytosis.

Goldberg and Green (1964) used a different approach to
the same questions in collagenesis. By correlating changes
in cultured murine fibroblasts' fine structure with the
commencement of collagenic activity of the cells as
measured by appearence of hydroxyproline in the culture
medium, they hoped to identify which cell structures were
involved in collagen production. They inferred from these
correlations that vesicles which are derived from smooth

endoplasmic reticulum fuse with the plasma membrane to release collagen. The smooth endoplasmic reticulum in this example contained fibrillar material which resembled collagen fibrils.

Ross and Benditt (1965) provided the first quantitative EM-ARG data on collagenesis. Using tritiated proline, they found that at the time of secretion, procollagen was found in cisternae of rough endoplasmic reticulum. Although they suggest the endoplasmic reticulum to have intermittent communication with the extracellular space, that communication was never observed.

In the chick embryo chondrocytes, Cooper and Prockop (1968) obtained different results by using the same quantitative EM-ARG techniques. In this study, protocollagen hydroxylation was prevented by anaerobic growth conditions. Labeled protocollagen was found to accumulate in the cytoplasm. Oxygen was then added thereby allowing protocollagen hydroxylation to procollagen after which the labeled material passed directly to the extracellular space. Cooper and Prockop concluded that "collagen" (actually procollagen) passes directly from the cytoplasm to the extracellular matrix and vesicular transport is not required.

In quantitative EM-ARG analysis of collagen secretion by newt chondrocytes, Salpeter (1968) also found that

vesicles did not seem required to secrete procollagen and
that the cytoplasm at the secretory pole of the cell
contained most of the labeled proline at the time of
secretion. Salpeter concluded that procollagen was
secreted in a diffusable form across the cell membrane.

Hay and Dodson (1973), using embryonic chick corneal
epithelium, tritiated proline and non-quantitative EM-ARG,
suggested that Golgi-derived vesicles about 1 µm in
diameter and containing a flocculent material transported
procollagen to the cell surface. These investigators did
not observe membrane fusion of the vesicle and plasma
membranes but did see profiles which may have resulted from
previous exocytosis.

Another, more recent attempt to locate procollagen's
intracellular location used ferritin-conjugated antibodies
to procollagen in chick embryo corneal epithelial and
tendon cells (Nist et al., 1975). Electron microscopical
observations showed ferritin in 200 nm "secretory" vesicles
in which procollagen is transported from the Golgi zone to
the cell surface. Proof of secretion by these vesicles was
not provided. Olsen et al. (1975) corroborated these
findings with ferritin-conjugated antibodies of procollagen
in chick embryo tendon fibroblasts.

The above studies show procollagen to be secreted
either from the cytoplasm to the extracellular space or via
Golgi-derived vesicles or possibly by communications of the

endoplasmic reticulum with the extracellular space. Some examples have also been presented in which procollagen is believed to resemble more closely its extracellular form while in secretory vesicles.

Movat and Fernando (1962) studied the fine structure of fibroblasts from a variety of tissues including rabbit tendon and heart valve and rat, human and dog connective tissue. They observed fibrillar, spindle-shaped bodies in the Golgi region which measured 50 - 60 nm by 250 - 300 nm and proposed that the fibrillar material could be collagen. They could not determine whether the collagen had been phagocytosed or was intended for secretion.

In chick embryo corneal cells, Trelstad (1971) observed vesicles which contained fibrils arranged in aggregates with collagen-like cross-striations. These vesicles were found close to the membrane at the secretory surface of the cell although no actual secretion was observed. Trelstad provided evidence that these vesicles were a condensed form of vesicles about 1 μm in diameter containing flocculent material.

Weinstock and LeBlond (1974) conducted and EM-ARG study of procollagen secretion by rat odontoblasts. Their study offers quantitative EM-ARG evidence that cylindrical vesicles derived from the Golgi body contain procollagen and are secretory vesicles to the extracellular space. These vesicles contain "filamentous threads" with

collagen-like 70 nm periodic striations.

If all procollagen secretory vesicles had
collagen-like striations, identification of procollagen
secretory vesicles would be very easy and most of the work
described in this discussion would not have been
necessary. Unfortunately, the observations of
collagen-like striations in intracellular vesiculated
material are exceptional and do not clearly correlate with
biochemical data on collagen production. Procollagen is
not thought capable of striated fiber formation. This has
been shown in the tissues of sheep and cows which suffer
from dermatosparaxis. In this disease, collagenous
tissues, most notably skin, are very fragile and can tear
when touched. Lenaers et al. (1971) found this to be
caused by an inability of the tissue to convert procollagen
to collagen. Dermatosparatic collagen displays fibrous
collagen's periodic striations only infrequently and fibers
observed by electron microscopy appear disorganized
(Bornstein, 1974). Kerwar et al. (1973) have provided
evidence showing that procollagen peptidase, an enzyme
responsible for procollagen to collagen conversion, acts
extracellularly, at least in transformed fibroblasts.

The inability of procollagen to form striated fibers,
the extracellular site of procollagen to collagen
conversion and the observation of striated collagen within
cells seem mutually incompatible. However, as Bornstein

(1974) points out, there is not enough evidence to exclude the possibility of some intracellular, procollagen to collagen conversion. Bornstein speculates that for tissue, such as the cornea, in which a highly ordered array of collagen fibers is required, it may be advantageous for procollagen to undergo some conversion to collagen before secretion.

The purpose of the above description of the variety of mechanisms proposed for procollagen secretion is to show that there seems to be no one mechanism of collagen secretion and that some collagenic activities in Leptogorgia appear to be shared with vertebrate collagenic cells. In Leptogorgia, the procollagen may reach the skeleton by two possible mechanisms: 1) the contents of Leptogorgia's flocculent vesicles may reach the skeleton following membrane fusion and exocytosis as occurs in Revel and Hay's (1963) model of newt chondrocyte collagen secretion and Hay and Dodson's (1973) description of chick corneal epithelium collagenesis, or, 2) the procollagen may reach the skeleton in a form which diffuses across the membranes as occurs in chick embryo chondrocytes (Cooper and Prockop, 1968) and Salpeter's model (1968) of newt chondrocyte collagen secretion. The observations in the present study cannot exclude either of these possibilities.

2. Tyrosine-derived crosslinkage

Light microscopical autoradiography has shown that corticocytes secrete tyrosine or tyrosine-derivatives. The cytochemical evidence presented here indicates that the corticocytes' dense-cored vesicles contain a hydroquinone, DOPA. The silver methenamine reaction used in this study has been found to produce a silver precipitate on sulfhydryl, aldehyde and hydroquinone reducing sites within the tissue, as well as aldehydes and osmium introduced during fixation (Locke and Krishnan, 1971; Swift, 1968). By blocking sulfhydryls with N-ethyl maleimide (Bennesh and Bennesh, 1961), aldehydes with sodium metabisulfite (Locke and Krishnan, 1971) and by eliminating tissue osmication, this reaction can be made quite specific for hydroquinones. The silver methenamine positive reaction of these vesicles following the listed blockades is consistent with the hypothesis outlined here which states that the dense-cored vesicles contain a hydroquinone. The apparent secretion of the dense-cored vesicles in company with 0.2 μm diameter vesicles which have been found in this study to contain DOPA oxidase, an enzyme which acts upon hydroquinone substrates, further supports this proposition. The evidence indicating DOPA oxidase activity includes the precipitation of DOPA and dopamine over the small, electron-lucent vesicles. The failure of that reaction to occur when the tissue is pretreated with DDC, a copper chelator, corroborated the proposed DOPA oxidation

since DOPA oxidase is a copper-containing enzyme (Okun et al., 1970). The observation is further supported by the blocking of the reaction by PTU which is a competitive substrate for DOPA in this enzymatic oxidation (Eppig, 1970). The combined observations that the dense-cored vesicles apparently contain a hydroquinone and that the dense-cored vesicles are joined by DOPA oxidase vesicles near the time of secretion, suggest that the dense-cored vesicles contain the hydroquinone, DOPA. The mechanism which has been proposed to be a general chemical pathway for the conversion of tyrosine to a tyrosine-derived, protein-crosslinking compound (Pryor, 1962) involves the enzymatic conversion of DOPA or a DOPA-like compound to a DOPA quinone. These cytochemical findings are consistent with such a pathway. The amino acid analysis data which identify DOPA within the skeletal protein fraction not only provide further support for this claim but also indicate that the enzymatic conversion of DOPA to the reactive crosslinking compound, DOPA quinone, does not occur until after secretion. The observation of silver methenamine positive material in the skeleton subjacent to the skeletogenic cells is in agreement with this interpretation.

The prototype for histochemical investigations of mechanisms of aromatic crosslinkage of structural proteins was Pryor's study (1940) on cockroach egg case formation. The mechanism showed the egg case to be formed by two

glands. The left gland secretes a white protein and a glucoside of protocatechuic acid. The right gland secretes a glucosidase that removes the glucoside from the protocatechuic acid which can then crosslink the white protein egg case. When crosslinked, the egg case becomes harder and darker and thus "sclerotized" and "tanned" (Pryor, 1962).

Mytilus byssal threads are also aromatically crosslinked. Smyth (1954) presented histochemical evidence to show that an upper or purple gland secretes a polyphenol oxidase while a lower or white gland secretes a phenolic protein. Mixture of the two secretions would thereby produce an aromatically crosslinked protein. Waite and Tanzer (1980) have identified L-DOPA in hydrolysates of Mytilus byssal threads and their attachment discs. Those investigators found about 20 times more catecholamine in the disc than in the threads and suggested that DOPA may aid in adhesion to the substratum as well as in protein crosslinkage.

Histochemical evidence for aromatic protein crosslinkage in other cnidarians has been previously presented. Knight identified a "migratory cell type" in the calyptoblastic hydroid, Laomedea flexuosa, and presented evidence that these cells contain a dihydroxyphenol (1968) and a catecholamine (1970). These data suggested to Knight that the cells could aromatically

crosslink perisarc protein, even though these cells have

not been observed to come in contact with the perisarc.

Laomedea therefore seems to have two separate cells to

produce protein and crosslinking compound while other

animals, such as molluscs and arthropods, employ two

separate glands to reach the same end.

Leptogorgia differs from all of the above examples of

protein and aromatic crosslinking compound production in

achieving both functions with only one cell type. Single

cells serving a variety of functions is a cnidarian trait.

While higher animals-have developed organs, such as glands,

with specialized functions, the cnidarians have a lower

level of cell specialization and no organs (see Hyman,

1940).

3. A proposal for the functional significance of

Leptogorgia's skeletogenic mechanism

In vitro evidence (LaBella et al., 1968) has been

presented which provides a model by which soluble collagen

could be crosslinked by dityrosine groups formed through

the peroxidation of tyrosine. This crosslinkage caused a

collagen solution to gel, thereby changing its mechanical

properties. An increase of collagen crosslinkage in vitro

has been shown to correlate with increased tensile strength

and elasticity in several collagenous systems (e.g. rat

skin, rat tail tendon and rat aorta; Vogel, 1978). Since

collagen is important as a structural material, the

capability of the cell to modify the mechanical characteristics of collagen would be advantageous. Although no endogenous peroxidase activity was found in the skeletogenic cells of _Leptogorgia_, this study provides evidence that collagen secreting cells can influence the formation of non-reducible crosslinkages of collagen by another mechanism. Since procollagen is secreted by way of vesicles which are morphologically distinct and separate from those vesicles within the same cell that contain the putative crosslinking precursor, variations in the relative proportion of these materials could produce a skeletal material of variable mechanical characteristics.

Even though the cellular capability to modify the mechanical characteristics of the gorgonian skeleton is present, the question of whether the skeletogenic cells of _Leptogorgia_ respond to extrinsic or intrinsic stimuli in producing the crosslinking compound is unresolved.

Figure 1. Electron micrograph showing the skeletogenic epithelium bordered by the loose connective tissue called the mesoglea (M) and by the axial skeleton (A). These cells produce the collagen and collagen crosslinking compound which are components of the axial skeleton. Bar = 5.0 μm.

Figure 2. Dense-cored vesicles (arrow) containing a hydroquinoid compound are secreted onto the skeleton and are frequently accompanied by small vesicles (arrowhead) resembling those which precipitate DOPA and dopamine. The skeleton subjacent to the epithelium appears as a finely fibrillar layer at the time of dense-cored vesicle secretion. Bar = 0.3 μm.

Figure 3. An electron micrograph showing the axis end of a corticocyte which contains several flocculent vesicles (F). These vesicles contain variable amounts of a fine fibrillar material at the time of procollagen secretion by the skeletogenic epithelium. Bar = 1.0 μm.

Figure 4. An electron micrograph of the axis end of a
corticocyte. The membrane of one flocculent vesicle (F)
lies near the plasma membrane (arrowheads). A nearby
dense-cored vesicle (d) has also approached the plasma
membrane. Bar = 0.3 μm.

Figure 5. Transmission electron micrograph of the axis end
of a corticocyte. The plasma membrane has concavities
(arrowheads) which may have resulted from sites of previous
vesicle membrane fusion and exocytosis. F = flocculent
vesicle. D = dense-cored vesicle. Bar = 0.3 μm.

Figure 6. Transmission electron micrograph of a flocculent
vesicle containing a bundle of fibrillar material (f).
Vesicles such as this were seen infrequently. Whether they
contain collagenous material is unknown. Bar = 0.5 um.

Figure 7. Transmission electron micrograph of a vesicle
containing cross-striated, fibrillar material in a
corticocyte. The striations (arrowheads) have a 57 nm
periodicity. Bar = 200 nm.

Figure 8. Transmission electron micrograph of a
longitudinal section of an axial skeleton. Some fibrils
show periodic cross-striations (arrowheads) with a 26 nm
repeat. Bar = 200 nm.

Figure 9.   Light microscope autoradiographs showing the
accumulation of labeled material at A. 3h, B. 6h and C. 12h
after beginning a 1h pulse label with tritiated tyrosine.
In A and B, labeled material is seen predominantly in the
corticocytes.   In C., the labeled material is found at the
corticocyte – skeletal interface (arrowheads).   G =
gastrodermis.   E = skeletogenic epithelium.   Axis = axial
skeleton.   Bar = 100 µm.

9A

9B

9C Axis

Figure 10. Light microscope autoradiograph showing the
accumulation of labeled material at the interface between
the axial skeleton and skeletogenic epithelium 24 h after
beginning a 1 h pulse label with tritiated proline. The
objects within the mesoglea (M) which appear as grainy
silhouettes are mineralized spicules. A = axial skeleton.
E = skeletogenic epithelium. Bar = 20 µm.

Figure 11. Electron microscope autoradiograph of the
tissue seen in Figure 4. The black dots are exposed silver
grains indicating the presence of labeled material. The
silver grains here are primarily located over flocculent
vesicles (F) at the axis end of the corticocytes. Bar =
1.0 µm.

M

1

Figure 12. Heavy silver deposits (large arrowheads) are
shown here in a modified silver methenamine reaction to lie
over the core of dense-cored vesicles which are near the
epithelial - axial skeletal interface (E/A). Less heavy
silver deposits lie over the periphery of these vesicles
(outlined with small arrowheads). Small accumulations of
extracellular silver (encircled) suggest that hydroquinones
which have not yet been oxidized may be found within the
skeleton. This tissue was not osmicated or stained. Bar =
0.5 µm.

Figure 13. Incubation with DOPA or dopamine produces a
precipitate on small vesicles (arrow) which lie against or
near dense-cored vesicles. Small vesicles which appear
identical to those which precipitate DOPA or dopamine but
do not produce a precipitate are also present (arrowhead).
Similar small vesicles are found near dense-cored vesicles
at the time of dense-cored vesicle secretion (Fig. 2).
This tissue was osmicated but not stained. Bar = 0.3 µm.

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TABLE I

Percent volume of the axis ends of skeletogenic cells occupied by flocculent vesicles, dense-cored vesicles and other structures. Silver grain counts expected and observed* over these structures were made from electron microscope autoradiographs of tissue Incubated with tritiated proline.

Structure	V_v[†]	N_e[Ψ]	N_a[¶]	χ^2
flocculent vesicles	11.4	124.5	245.5	>.995
other	88.6	967.5	846.5	
total	100.0	1092.0	1092.0	
dense-cored vesicles	1.9	20.7	21.5	<.25
other	98.1	1071.3	1070.5	
total	100.0	1092.0	1092.0	

* = counted by direct allocation to structures lying beneath the grains on electron micrographs.

[†] = percent volume based upon point counting of a total of 7,310 points.

[Ψ] = number of grains expected if grain distribution were random.

[¶] = actual number of grains counted over structure.

χ^2 = confidence limits that N_a differs significantly from N_e.

TABLE II

Occurrence of precipitate over 0.2 µm diameter vesicles at the axis end of skeletogenic cells when incubated with various, possible reducing substrates and controls.

Substrate	Reaction Product
DOPA	+
DOPA + PTU	-
DDC → DOPA	-
Dopamine	+
Dopamine + PTU	-
DDC → Dopamine	-
Tyrosine	-
Tyrosine + PTU	-
DDC → Tyrosine	-
No substrate	-

Chapter 3: Changes in skeletal chemistry and skeletogenic cell fine structure between young and old sites on the skeleton.

Introduction

Chapter 2 of this thesis suggested that the skeletogenic cells of _Leptogorgia_ could change the chemical and mechanical properties of gorgonin by modifying the rates of collagen and crosslinking compound secretion. The goal of this chapter is to describe differences in gorgonin chemistry between the medullary wall, young cortex and mature cortex of the axial skeleton. There are two reasons to propose that such a change occurs: 1) the skeletogenic cells which produce the medullary wall (Chapter 1) have a different fine structure from those that produce the cortex (Chapter 2), and 2) the cortex color changes from light yellow at the branch tips to dark brown near the base.

The color change of the cortex is especially significant since gorgonin fulfills the criteria of an aromatically crosslinked (or sclerotized) protein and color darkening in sclerotized proteins accompanies an increase in aromatic crosslinkage (Pryor, 1962).

Goldberg (1978) has shown that there is an increase in phenolic compounds in the mature cortex of two gorgonians, _Muricea muricata_ and _Swiftia exserta_. Another gorgonian, _Gorgonia ventalina_, showed in that same study a decline in

Phenol concentration from skeletal tip to base. Goldberg proposed that a change in phenolic compound concentration may reflect a change in gorgonin sclerotization related to maturation.

An increase in crosslinkage near the skeleton's base would have functional significance for gorgonians because increased polymer crosslinkage increases tensile strength and hardness of the material while reducing extensibility and solubility (Hanks, 1970). The response of the gorgonian skeleton to physical forces such as those related to water movement would therefore be modified by changes in the degree of crosslinking.

In this chapter, two approaches to investigating changes in the degree of crosslinking will be used. First, amino acid analysis will be used to measure changes in tyrosine concentration between skeletal tip and base. Tyrosine concentrations are presumed to reflect the degree of tyrosine-derived crosslinkage. Second, morphometric techniques will be used to see if the volume fraction of the skeletogenic cells occupied by dense-cored vesicles differs between sites on the skeleton. Dense-cored vesicle populations are evaluated since they contain the tyrosine-derived, putative crosslinking compound (Chapter 2). The changes in skeletal collagen proportion will be similarly analyzed by morphometric evaluation of the procollagen-containing, flocculent vesicle populations and

by amino acid analysis of skeletal samples for changes in hydroxyproline concentration. Hydroxyproline concentrations are presumed to reflect the amount of collagen present because in animal tissue hydroxyproline is found almost exclusively in collagen (Udenfriend, 1966).

The results of morphometric and amino acid analyses will then be compared to see to what degree measurable changes in secretory cell fine structure correspond to changes in secretory product chemistry.

Another way to change the mechanical characteristics of an organic, polymeric structure is by adding a material such as a mineral. The occurrence of inorganic crystals with organic polymers together as a composite is a common event seen in bone, cuticle, mollusc shell, etc. (see Brown, 1975 and Wainwright et al., 1976, for many examples).

In this chapter, the inorganic portion of the skeleton will be briefly considered. Although this thesis is concerned primarily with the organic portion of the gorgonian skeleton, Leversee (1972) has found that about 50% of Leptogorgia's skeleton is inorganic. Such a large proportion is surely of functional importance. To identify at least part of the inorganic fraction, calcium, magnesium, phosphate, carbonate and silica were assayed in samples from the skeletal tip and base.

Materials and Methods

1. Amino acid analysis

a) Analysis of apoprotein from skeletal lipoprotein

The method for lipid and lipoprotein extraction is based upon the technique of Folch et al. (1957) with several modifications. Two samples were analyzed in the following procedure.

1) Grind skeleton samples to a powder under liquid nitrogen.

2) Sonicate the powder in 2:1 chloroform-methanol (C:M) for 15 min in a beaker.

3) Place the beaker in the refrigerator (about 10 °C) for 30 min, then pour the supernatant through Whatman #1 filter paper which has been previously washed with C:M. Allow the fitrate to drain into a separatory funnel.

4) Repeat steps #2 and #3 three more times and save the C:M insoluble skeleton in a dessicator.

5) Immediately add to the supernatant 0.3 ml of 0.05 N NaCl (aqueous) per 1 ml of supernatant. Mix well.

6) Allow the supernatant to partition at 4 °C overnight.

7) Drain off the lower fraction (chloroform fraction).

8) Evaporate the chloroform under a stream of nitrogen.

9) Add 1 ml 6 N HCl per mg of chloroform soluble material.

10) Hydrolyze the sample at 105°C for 24 h under partial vacuum and nitrogen environment. Shake the vial frequently during hydrolysis.

11) Evaporate the HCl with a water pump aspirator while the sample is in a water bath at 55 - 70°C.

12) Add 1 ml of 0.2 N Na citrate to the hydrolysate.

13) Filter the sample through a 0.22 μm millipore filter which has been previously washed with citrate buffer.

14) Analyze the sample on a Beckman 120 C automated amino acid analyzer.

b) Young cortex analysis

1) Grind the distal, yellow-colored, 1 cm of skeletal branch tips under liquid nitrogen. The skeleton at these sites was less than 0.3 mm thick. Approximately 38 branch tips are required to collect 10 mg of skeletal material.

2) Extract C:M soluble lipids and lipoproteins as described in "part a" above.

3) Dessicate the C:M insoluble extracted powder to a constant weight and then hydrolyze the sample in 1 ml of 6 N HCl/ mg skeleton for 24 h at 105°C.

4) Evaporate the HCl and analyze the sample in the manner described in "part a" above.

Three samples were analyzed.

c) Mature cortex analysis

Sample preparation and analysis are identical to that for the young cortex except that the skeleton analyzed was dark brown, greater than 1.5 mm thick and more than 7 cm from the branch tip. This skeletal sample would include some young cortical material. Contamination of mature cortex samples with young cortex was minimized by using samples that were thick enough so that young cortex would be a small fraction of the cortical sample analyzed. Three samples were analyzed.

For each of the above samples, most residues were eluted with buffers first at pH 3.25, then pH 4.30 and then at pH 6.25. An exception was hydroxyproline analysis in which the buffer at pH 3.25 was replaced with a buffer at pH 2.82 to separate the hydroxyproline peak from the aspartic acid peak. Using two buffer systems for each analysis was also valuable in confirming the identity of other residues to see if their change in elution time equaled the change in the standard's elution time at a different pH.

2. Morphometrics

Blocks of tissue embedded for transmission electron microscopical study for Chapter 1 and Chapter 2 of this thesis were used for morphometric analysis. Cross sections

of the skeleton and axial epithelium were analyzed in the same manner as described in Chapter 1 under "Morphometrics" except the volume fraction of dense-cored vesicles and flocculent vesicles were evaluated rather than lipoprotein vesicles.

Several technical problems, including tearing of the epithelium from the skeleton when slicing thick pieces of skeleton before fixation and difficulty in sectioning through thick skeleton, made obtaining artifact-free sections of tissue overlying thick skeletal cortex very difficult. For that reason, morphometric data are obtained only for cells overlying layers of cortex 60 um thick or less.

3. The inorganic components

Skeletal samples were ground in a ball mill to a 200-mesh grain size courtesy of Dr. R. Jamieson, Department of Geology, Dalhousie University. The quantitative determinations of calcium, magnesium, silica, phosphates and carbonates were performed at the Nova Scotia Research Foundation Corporation (Dartmouth, N.S., Canada). Calcium, magnesium and silica were all measured by quantitative atomic absorption spectroscopy. The detection limits of this technique are about 0.002 ppm for calcium, 0.003 ppm for magnesium and 0.1 ppm for silica.

Carbonates were determined by acid treatment and then

colorimetrically measuring evolved carbon dioxide by phenolphthalein (Welcher, 1963). This test is sensitive to 4 ug CO_2 in 2 drops of the test solution.

Phosphates were determined as total phosphorus using the method described by Brabson (1963). In this analysis, a dilute, phosphorus-containing sample is treated with acidified molybdovanadate reagent to form blue-violet molybdovanadophosphoric acid. This can be measured spectrophotometrically. Sensitivity is 1.5 µg PO_4 per drop of test solution.

Results

1. Amino acid analysis

   a) Apoprotein analysis

     The extracted lipoprotein comprised approximately 4% of mature skeleton and 16% of young skeleton (Chapter 1). Upon drying, a viscous, dark green oil remained.

     The results of the amino acid analysis of the lipoprotein hydrolysate are shown in Table 1. The peaks' identities were confirmed with two different buffer systems by comparison to elution times of standards (Beckman Instruments, Palo Alto, CA., USA; Sigma, St. Louis, MO., USA).

     The data presented in Table 1 are not an average of the two samples, which varied little, but are from measurements from the analysis giving the most distinct residue peaks, thereby maximizing the accuracy of peak area quantitation.

   b) Young cortex analysis

     The young cortex hydrolysate was light yellow after drying. The total weight of protein identified by amino acid analysis was approximately 41% of the entire sample's weight. These weight calculations are   a minimum possible weight since they are not corrected for hydrolytic losses or take into account the possible occurrence of tryptophan which is destroyed in acid hydrolysis. Just as for the

lipoprotein analysis data, the data presented in
Table I for young cortex are the results of the
analysis producing the most distinct peaks for the
measured residues.

c) Mature cortex analysis

The mature cortex hydrolysate was brown after
drying. The total weight of protein identified by
amino acid analysis was about 37% (uncorrected for
hydrolytic losses) of the entire sample's weight.
The mature cortex data in Table I are also from the
single, most accurately quantified analysis.

2. Morphometrics

The results of the morphometric analysis of ten
samples of approximately 3000 sample points each are
presented graphically for flocculent vesicles (Fig. 1) and
dense-cored vesicles (Fig. 2).

The best fit regression curve found for the flocculent
vesicle data is:

$$Y = 1.42 + 2.3 \ln X.$$

The coefficient of correlation, r, is very high
(0.935) indicating an excellent correlation between the
data and the regression curve.

The best fit regression curve found for the
dense-cored vesicle data is:

$$Y = 0.66 \, X^{0.36}$$

The coefficient of correlation is lower (r = 0.748) than that for the flocculent vesicle data. The data are fit well enough to the curve, however, for a statistically significant correlation within the 95% confidence interval.

The data for both flocculent vesicles and dense-cored vesicles indicate that there is an abrupt increase in the population size of these vesicles after the medullary wall has been produced. When young cortical material is being produced, these vesicles' population sizes increase at a much lower rate. Extrapolation of the curves suggests that the slow rate of population increase continues to skeletogenic sites over mature cortex.

The lower r value for dense-cored vesicle data suggests a greater variability in population size of dense-cored vesicles than of flocculent vesicles. .

3. Inorganic composition

Atomic absor tion spectral data for calcium, magnesium, and silica and quantitative, colorimetric measurements of phosphates and carbonates are presented in Table 2. The empirical molar ratios are:

	Ca	:	Mg	:	PO_4	:	CO_3
Young cortex	117	:	60	:	120	:	1
Mature cortex	101	:	54	:	106	:	1

Disregarding carbonates, which seem unimportant, the empirical molar ratios are:

	Ca	:	Mg	:	PO$_4$
Young cortex	2	:	1	:	2
Mature cortex	2	:	1	:	2

Although these data do not show how or if these inorganic, skeletal constituents are ionically bound to one another, the measurements do suggest that calcium phosphate may serve an important role in the skeleton and that carbonates are unimportant.

Discussion

1. The medullary wall

The medullary wall is defined here as the approximately 3 μm thick part of the axial skeleton which separates the skeletal medulla and cortex (see Chapter 1). Since histochemical data presented in Chapter 1 provide evidence that the medullary wall is rich in lipoproteins while the cortex contains little lipoprotein, it has been assumed for this portion of the present study that the material extracted by 2:1 chloroform-methanol (C:M) is medullary wall material. There is no proof that the C:M soluble fraction contains all of the medullary wall material or only medullary wall material.

Approximately 21% (molar fraction) of the C:M soluble material is comprised of the hydrophobic amino acids alanine, valine, isoleucine, leucine and phenylalanine (Table 1). These amino acids would be found in the C:M soluble fraction if they were either free amino acids in the skeleton or if they were contained in a hydrophobic molecule. It is noteworthy that although proline is also hydrophobic, it does not occur in the C:M soluble fraction which indicates that it does not occur as a free amino acid in gorgonin. This is significant since proline levels have been used to estimate collagen concentration in gorgonin (Leversee, 1972; 1980a; 1980b). Those estimates would be less accurate if some proline had occurred in gorgonin as a

free amino acid.

The high proportion of taurine in the C:M soluble
fraction is interesting and inexplicable. Taurobetaine, a
taurine derivative, has been identified in the gorgonian,
Briareum asbestinum, in which it is more than 0.3% of the
dry weight of entire colonies (Ciereszko et al., 1960). No
functional significance has been identified for gorgonian
taurine or taurobetaine nor has a histological location
been ascribed to either of these compounds in gorgonians
prior to the present identification of taurine in the axial
skeleton of Leptogorgia. The location of taurine in a
skeletal material is anomalous since taurine has not been
identified in a structural molecule.

The large proportion of the C:M soluble fraction that
is neither hydrophobic nor taurine and comprises
approximately 66% of the molar fraction suggests that at
least some of the hydrophilic residues are associated with
a non-polar molecule such as a saturated lipoprotein.
Since the medullary wall is rich in saturated lipoproteins
(Chapter 1) and since the hydrophilic amino acids are
believed to be bound in such a molecule, the C:M soluble
fraction is taken as a rough indication of the protein
composition of the medullary wall.

The near absence of procollagen-containing flocculent
vesicles in cells overlying the thin layers (less than 3 μm
thick) of skeletal material (Fig. 1) and the low proportion

of dense-cored vesicles (less than 0.8% volume) at the axis end of medullocytes (Fig. 2) suggest that the medullary wall is free of sclerotized collagen.

2. The young cortex

Young skeletal cortex is defined here as that skeletal material found at sites where the skeletal cortex and medullary wall have an approximate total thickness of between 3 and 100 μm. The young cortex is a proportionately large skeletal component at the distal 1 to 2 cm of the skeleton branch tip where the skeletal material is light yellow.

Young skeletal cortex from which lipids and lipoproteins have been extracted shows a collagen-like amino acid composition (Table 1). Features which suggest the presence of collagen include the presence of hydroxyproline and that approximately one third of the residues are glycine. There is a 1.28 proline to hydroxyproline (pro:hyp) molar ratio. Leversee (1980b) found the autoclave soluble, non-dialyzable fraction of Leptogorgia axial skeleton to contain a 1.4 pro:hyp ratio by weight (a 1.58 molar ratio). Leversee chose autoclaving to extract collagen since Neuman and Logan (1950) found that collagen differs from other structural proteins in being solubilized by autoclaving. The reason the young cortical protein analyzed in this study contains more hydroxyproline relative to proline concentrations than

Leversee's autoclave soluble fraction is unclear. Possibly some protocollagen was contained in Leversee's analysis.

The morphometric data on flocculent vesicles within the skeletogenic cells (Fig. 1) suggest that the advent of collagen secretion is sudden since the number of procollagen-containing, flocculent vesicles undergoes a rapid increase within cells overlying medullary wall and cortex which have a combined thickness of 5 to 8 μm.

The appearance of tyrosine in the young cortical protein (Table 1) suggests that tyrosine-derived crosslinkages may be occurring in the young cortex. Over the young cortex, the dense-cored vesicles, which have been implicated in crosslinking (see Chapter 2), show an abrupt increase in prevalence in the skeletogenic cells (Fig. 2). The appearance of these vesicles in the skeletogenic cells which overlie the young cortex indicates that at least some of the tyrosine present in the young cortical protein is implicated in protein crosslinking.

3. The mature cortex

Mature cortex is defined here as skeletal material found at sites where the cortex is more than 100 μm thick. The total skeleton thickness at those sites would be greater than 300 μm. The mature cortex would begin approximately 2 to 4 cm from the branch tip although this is a highly variable distance. Mature cortical material is

dark brown unlike the light-yellow medullary wall and young cortex.

Amino acid analysis provides data which show that the mature cortex chemically resembles collagen. Here, as in the young cortex, hydroxyproline is present and about one third of the residues are glycine. The molar ratio of proline to hydroxyproline is 1.65 and in that respect mature cortical protein is more similar to autoclave soluble collagen than young cortical protein. The significance of this and other chemical changes is discussed below.

The largest change in proportion of any amino acid residue is tyrosine which more than doubles from young to mature cortical protein. This occurrence is significant since the model proposed in which tyrosine derivatives increasingly sclerotize skeletal protein at sites nearer the skeleton base requires an increase in the proportion of tyrosine in the skeletal protein.

Changes in proportion of other amino acid residues, especially aspartic acid, valine, alanine and lysine, indicate additional differences between the protein composition of young and mature cortex. Possible explanations for the observed changes in skeletal chemistry are: 1) transcriptional changes in collagen synthesis, 2) post-translational changes in collagen synthesis, or 3) addition of one or more non-collagen peptides.

Difficulties encountered in previous attempts (Goldberg, 1974; 1978; 1980) to solubilize different proteins in gorgonin selectively without degrading the proteins to their constituent amino acids have made it impossible thus far to identify any gorgonin protein other than collagen. However, these amino acid analytical data show that the chemical composition of young and mature cortex are very collagen-like, suggesting that collagen is the major protein constituent of both cortical regions.

A probable source of additional protein or peptides in the cortex is desmocyte cytoskeletal rods buried in the cortex. As discussed in Chapter 5, overgrowth by the secretions of adjacent skeletogenic cells bury these cytoskeletal rods which would thereby be included in crude chemical analyses such as this study. Since essentially nothing is known of cytoskeletal rod chemistry or to what degree they contribute to skeletal protein, correction cannot be made for their presence in the skeletal samples.

A source of another protein or peptide in gorgonin may be the crosslinking compound. The heterogeneous appearance in sectioned material of dense-cored vesicles, which contain the putative crosslinking compound (see Chapter 2), and the size of these vesicles suggest that they contain not only tyrosine derivatives but also other material. That material could account for some of the amino acid residue proportion changes from young to mature cortex if

the relative contribution of dense-cored vesicles and flocculent vesicles to the cortical protein changed between sites.

There are no morphometric data on corticocytes overlying mature cortex since technical difficulties in preparing those tissues prevented obtaining adequately large samples in which cells were not torn or distorted. Qualitative observations of those cells revealed no readily noticeable differences between the fine structure of these cells and those overlying young cortex except corticocytes overlying thick cortex are shorter.

The regression curves obtained from data on cells overlying the medullary wall and the young cortex permit extrapolation of the data to describe cells overlying mature cortex. This extrapolation shows that the proportion of the skeletogenic cell's axis end occupied by dense-cored vesicles and by flocculent vesicles would gradually increase with sampling over thicker and thicker layers of skeleton. Although regression curves are valuable in predicting non-measured data by extrapolation or interpolation of the curves, extrapolation of the curves for these data seems risky since unforseen changes in cell secretory behavior (such as near the colony's holdfast or sites of branching) could introduce new variables into the hypothetical relation between fine structure and skeleton thickness. It is also important to recall that regression

curves, even if they fit the data well, do not prove a causal relationship between the variables. The curves merely describe correlations.

4. Changes in gorgonin sclerotization

In the introduction to this chapter, it was stated that the color change from light yellow to dark brown has been correlated with an increase in aromatic crosslinkage of several diverse proteinaceous, structural materials cited in the literature (see Pryor (1962), Goldberg (1974) and Brown (1975) for many examples). Gorgonin is one such structural material. The skeletal darkening at sites nearer to the gorgonian colony's holdfast suggests that aromatic crosslinkage would be more extensive near the base.

The finding reported in this study that the tyrosine concentration in mature cortical protein is more than twice the tyrosine concentration in young cortical protein is consistent with the proposed increase in aromatic crosslinkage near the holdfast. There is no way to be certain that these tyrosine residues measured by amino acid analysis were involved in crosslinkage. The tyrosine-concentration data are therefore consistent but circumstantial evidence for an increase in tyrosine-derived crosslinkage in mature cortex.

The direct way to measure the number of crosslinkages

requires identifying the crosslinking compound and developing an assay for that compound. Goldberg's recent attempts (1980) at identifying the crosslink have been based upon the assumption that the crosslinking compound would show blue fluorescence in skeletal hydrolysates. Goldberg assumed this because dityrosine and trityrosine are resilin crosslinks which are blue fluorophores (Andersen, 1964; 1971), elastin fluorescence has been attributed to a putative crosslink (LaBella, 1971), and dentin contains a fluorescent compound believed to be involved in crosslinking (Hartles and Leaver, 1953). Examples such as those and Goldberg's own observation that gorgonin hydrolysates contain a fluorophore led him to try to isolate and analyze the gorgonin fluorophore.

In that study, Goldberg separated three fluorescent compounds, each a benzenoid compound with attached carboxyl groups but no attached amines. That these compounds are substituted benzenoid compounds is consistent with a possible tyrosine origin. The absence of an attached amine group indicates either an _in_ _vivo_ modification of tyrosine or that the benzenoid compounds are not tyrosine derivatives. There is no way to be certain of the form of these compounds in gorgonin or to know if these fluorophores actually served as crosslinks.

Since the identity of the gorgonin aromatic crosslink is still eluding Goldberg's persistent and solitary

efforts, the best assay currently available to measure the degree of tyrosine-derived crosslinkage in a sclerotized protein is a measurement of tyrosine concentration. For that reason, these results are interpreted as evidence that gorgonin is more highly sclerotized near the skeleton base than near the branch tips. These results were anticipated by the simple observation of the darkening of the skeletal material near the base.

An increase in crosslinking near the skeleton's base is of functional importance because crosslinking will change the mechanical properties of the skeleton. As mentioned in the introduction to this chapter, increased crosslinking will increase tensile strength and hardness while decreasing extensibility and solubility of the protein (Hanks, 1970). It is inferred that the skeleton's base is under greater stress than the branch tips so the increase in skeletal toughness closer to the base may enable the skeleton to withstand the extreme mechanical stresses of its environment. Whether the change in degree of crosslinking is an age-related occurrence or an active response of the skeletogenic cells to mechanical stress is unknown. Both hypotheses are consistent with these observations and deserve further study.

5. The inorganic component

About 35% of both young and mature cortices are calcium, magnesium, silica, phosphate and carbonate. Those

measurements suggest that Leptogorgia's skeleton does not change its mechanical properties by modifying the skeletal inorganic fraction comprised by compounds included in these assays.

Cook (1904) found by incinerating gorgonian skeletons and weighing the residual ash that the inorganic fractions (estimated by ash weight) vary in proportion between species. Cook's data show Leptogorgia virgulata to contain a higher inorganic proportion, about 41%, than any of the 12 other gorgonian skeletons studied. Muricea and Gorgonia species contained less than 10% ash and Plexaura species contained less than 13% ash. The species with the second largest inorganic fraction was Leptogorgia rigida with 30% ash. Leversee (1972) has since measured ash residues of Leptogorgia virgulata and found the skeleton to leave nearly 50% weight as ash. These findings suggest that the inorganic portion of the gorgonian skeleton varies in structural importance between species.

The measurements performed in this study account for at least 91% of the young cortex (41% protein, 16% lipid, 34% inorganic) and at least 76% of mature cortex (37% protein, 4% lipid, 35% inorganic). Approximately 20% of the mature skeleton has not been accounted for — an omission which may be of compounds important to the animal.

Although these assays alone can provide no conclusive

evidence about the form of the inorganic compounds, the
1:2:1 calcium:magnesium:phosphate ratio suggests these
elements and compound may form an important mineral crystal
in Leptogorgia's skeleton. The small carbonate fraction
shows carbonates are of little importance in Leptogorgia's
gorgonin.

In other anthozoans (e.g.the scleractinian corals
Pocillopora damicornis and Lophelia pertusa (Wainwright,
1964), and the gorgonians Plexaurella nutans (Wainwright et
al., 1976), Eugorgia ampla (Fox et al., 1969), Ellisella
andamanensis, Subergorgiidae suberosa and Melithaea
ochracea (Muzik and Wainwright, 1977)) the skeleton
contains calcium carbonate as calcite or aragonite.
Leptogorgia appears to be unusual in Anthozoa in its
apparent use of mineralized phosphates rather than
carbonates in its axial skeleton.

Table I.   Amino acid composition of the medullary wall
(chloroform-methanol soluble portion of the skeleton),
young cortex and mature cortex.

TABLE I

RESIDUE	% MOLAR		
	CHLOROFORM:METHANOL SOLUBLE	YOUNG CORTEX	MATURE CORTEX
TAU	12.2	0	0.1
HYP	0	3.9	3.1
ASP	10.1	9.2	8.2
THR	4.2	3.4	3.9
SER	8.3	2.8	3.3
GLU	6.7	4.2	3.4
PRO	0	5.0	5.1
GLY	34.4	32.8	32.3
ALA	2.8	11.4	10.4
CYS	2.0	1.4	1.0
VAL	10.8	3.0	4.2
MET	0	0.7	0.7
ILE	1.8	2.1	2.0
LEU	3.0	2.7	2.5
TYR	0	1.4	2.9
PHE	2.2	2.3	2.0
HYL	0	1.5	1.3
LYS	0.2	3.9	5.9
HIS	0	1.9	1.1
ARG	1.3	6.3	6.7

TABLE II

SAMPLE	Ca	Mg	PO₄	CO₂	SiO₂	Total
Young cortex	8.9	2.9	22.1	0.13	<0.05	34.03
Mature cortex	9.4	2.9	22.7	0.12	<0.05	35.12

The measurements are the percent of the total skeletal weight.

Figure 1. Graph of the volume fraction of the skeletogenic cell's axis end occupied by flocculent vesicles shown as a function of the thickness of skeletal material over which the cells lay. "Cortex + Medullary Wall Thickness" refers to the distance from the outer surface of the skeletal cortex to the inner surface of the medullary wall. The correlation coefficient for the regression curve is much higher than required for the 95% confidence interval (t-test).

FLOCCULUS VESICLES
%VOLUME

MEDULLARY WALL + CORTEX THICKNESS (µM)

Figure 2. Graph of the volume fraction of the skeletogenic
cell's axis end occupied by dense-cored vesicles shown as a
function of the thickness of the skeletal material over
which the cells lay. "Cortex + Medullary Wall Thickness"
refers to the distance from the outer surface of the
skeletal cortex to the inner surface of the medullary
wall. The correlation coefficient for the regression curve
places the function within the 95% confidence interval
(t-test).

2

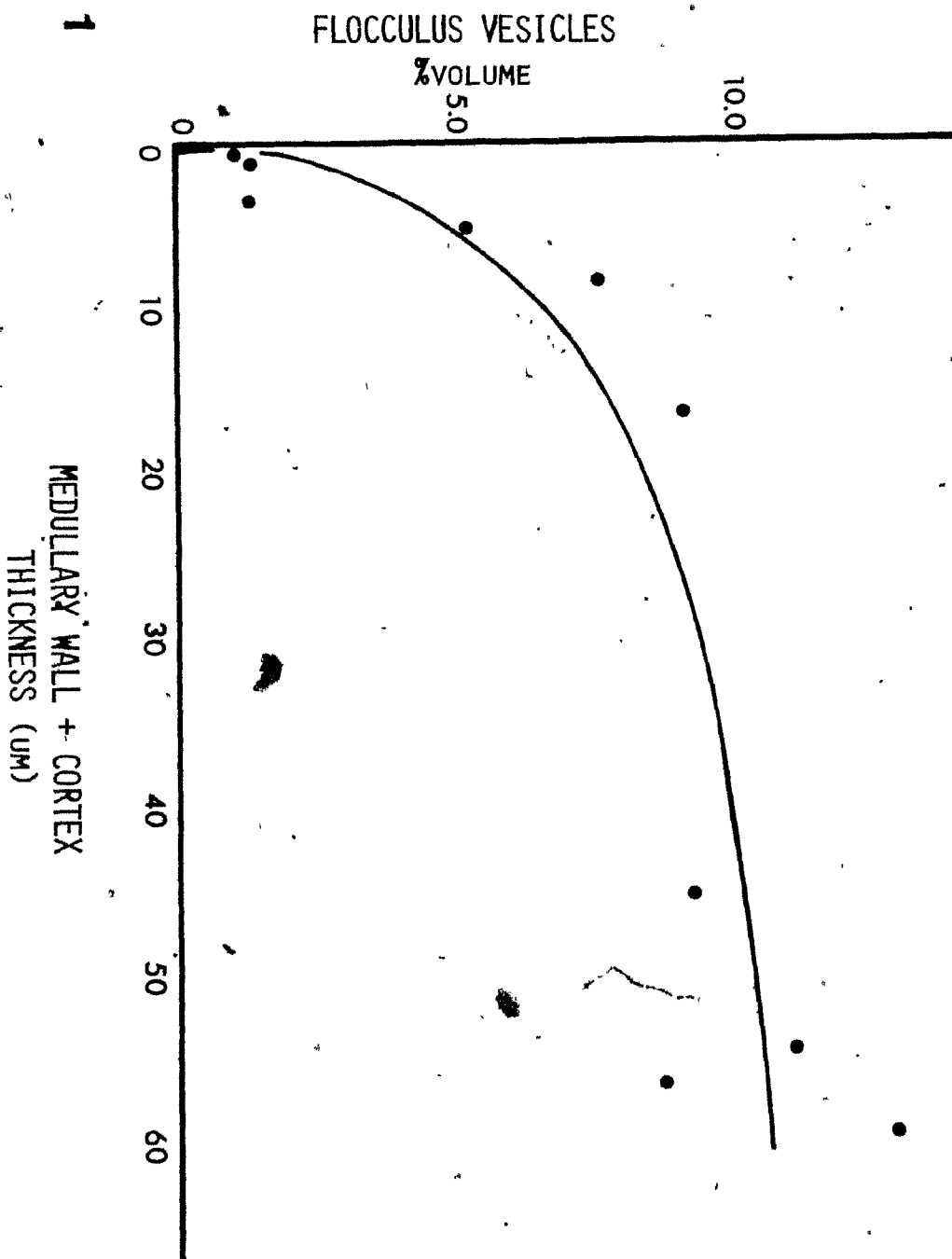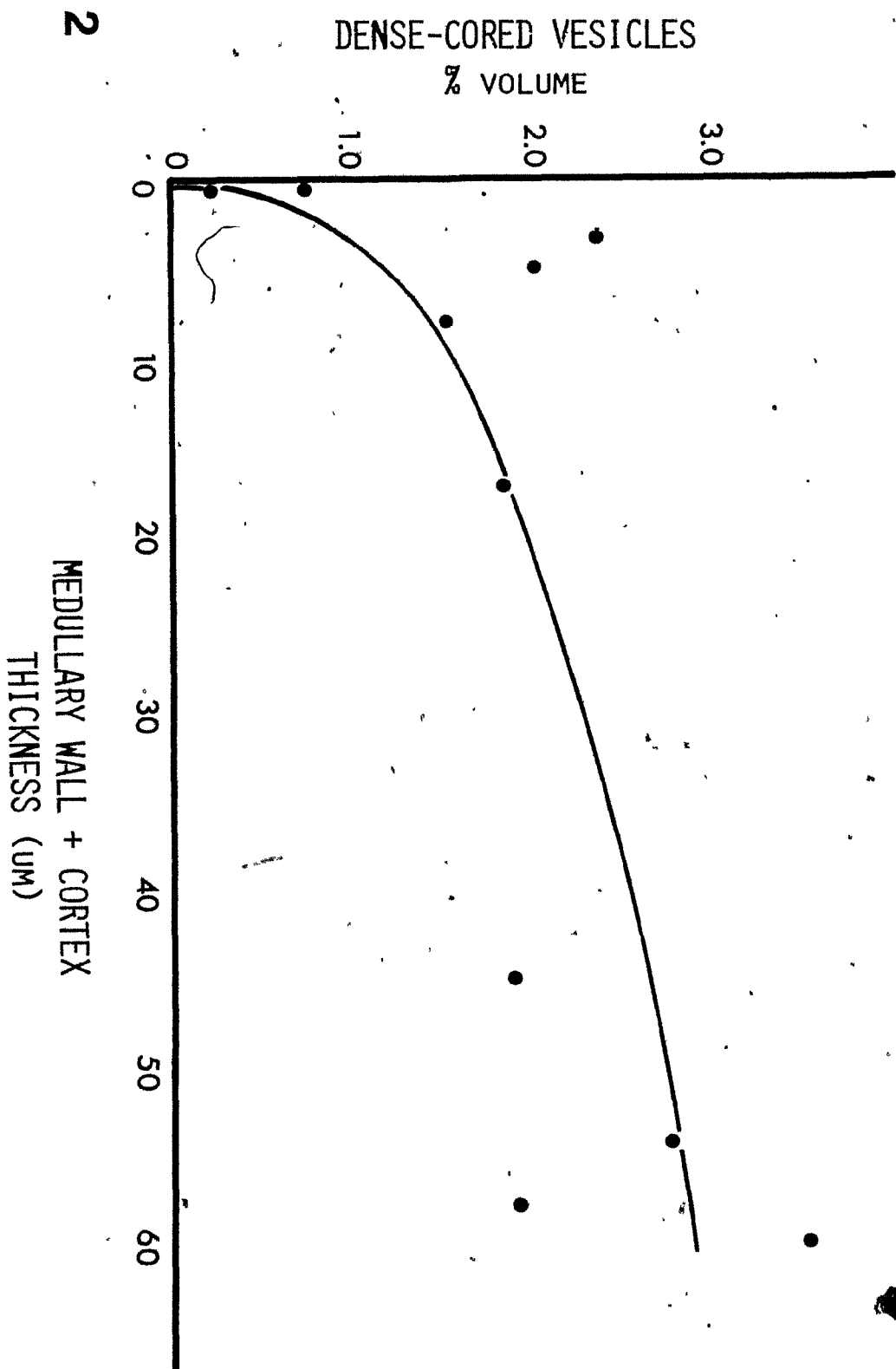DENSE-CORED VESICLES
% VOLUME

MEDULLARY WALL + CORTEX
THICKNESS (UM)

Chapter 4: Gorgonin collagen fiber organization.

Introduction

This chapter concerns chemical and molecular events in gorgonin collagen formation. Vertebrate collagen fiber formation has been described and reviewed many times (see Bornstein, 1974; Kivirikko and Risteli, 1976; Grant et al., 1979; Prockop et al., 1979 for recent bibliographies). Although most chemical changes associated with transformation of gorgonin procollagen molecules to collagen fibers are beyond the scope of this work, an attempt has been made to investigate various stages in formation of gorgonin collagen fibers by studying the fine structure of gorgonin microfibrils, fibrils and fibers and then comparing their structure to the structure of vertebrate collagen microfibrils, fibrils and fibers.

When vertebrate procollagen is secreted, it is a triple helix about 300 nm long and 1.5 nm wide with a non-helical, terminal extension at each end. A portion of each extension is then enzymatically cleaved from the molecule, enabling the molecules, which are now tropocollagen, to aggregate into microfibrils (about 3 to 20 nm diameter) and then into fibrils (20 to 120 nm or greater diameter). The fibrils aggregate to form fibers (usually 1 μm or greater diameter). Fibrils and fibers frequently display cross-striations with 64 nm periodicity (see Gustavson, 1956; Morse and Low, 1974; Prockop and

Guzman, 1977; Eyre, 1980 for discussions on general aspects of collagen fiber formation).

The extensive literature on extracellular modifications of vertebrate collagen and the evidence which suggests collagen is the major gorgonin protein served as the basis for this study of extracellular modifications of gorgonin collagen.

Materials and Methods

1. Transmission electron microscopy

Samples of _Leptogorgia_ colonies were prepared for electron microscopical observation by a technique identical to the technique described in Chapter 1.

2. Scanning electron microscopy

Skeletons studied by scanning electron microscopy were fixed as described for transmission electron microscopy (Chapter 1). The soft tissues were stripped from the skeleton with forceps. The skeletons were dehydrated through a graded series of ethanols and critical point dried in isoamyl acetate with carbon dioxide. The skeletons were coated with gold and viewed in a Cambridge S150 scanning electron microscope.

3. Fractionation of gorgonin fibrillar component

Acid-soluble gorgonin collagen was isolated by the following technique.

a) Grind an air-dried skeletal sample under liquid nitrogen and then extract with 2:1 chloroform-methanol (see Chapter 1).

b) Store the extracted sample at 4°C in the dark for 3 months in 0.5 M acetic acid. Shake the solution occasionally.

c) Filter the sample through Whatman #1 paper.

d) Dialyze the supernatant against distilled water for 20 h (dialysis tubing permeable below 12,000

molecular weight).

e) Precipitate the non-dialyzable fraction by titration against 0.5 M NaOH to pH 6.5.

f) Centrifuge the sample at 1720 g to pellet the precipitate.

g) Pour off the supernatant and resuspend the pellet in 0.5 M acetic acid.

h). Repeat steps "e" to "g" two more times.

i) Place a drop of the suspension on a Parlodion-coated grid at 4°C and then rinse with water.

j) Negatively stain with 2% phosphotungstic acid at pH 7.0 (adjust pH with 0.1 N NaOH).

k) Observe the sample by transmission electron microscopy.

4. Replication of gorgonin fibers .

Gorgonin fibers were separated and replicated with platinum by the following technique.

a) Grind formaldehyde-fixed skeleton samples under liquid nitrogen.

b) Suspend the skeletal powder in distilled water.

c) Centrifuge the suspension at 1720 g for 40 min to pellet the precipitate.

d) Pour off supernatant and resuspend the top, light-colored band of the pellet in water.

e) Pipette the suspension onto a sheet of cleaved mica and allow it to air dry.

f) Replicate with platinum-carbon in an Edwards vacuum

evaporator at 2×10^{-5} torr.

g) Strip the replica from the mica sheet by slowly immersing the sheet in water.

h) Pick up the replica on a Parlodion-coated grid.

i) Observe the sample by transmission electron microscopy.

Results

Subjacent to corticocytes with procollagen-containing, flocculent vesicles (see Chapter 2) is a loose, filamentous mesh (Fig. 1a). The filamentous structures are about 3 nm in diameter and unbranched. These structures are called microfibrils because their size is identical to collagen microfibrils' (Morse and Low, 1974). They have not been observed deeper in the gorgonian axial skeleton. Some of the microfibrils appear to be in contact with the plasma membrane (Fig. 1b).

Dialysis of acid-soluble collagen against distilled water causes tropocollagen molecules to link end-to-end to form long filaments (Hodge et al., 1960). Collagen can be precipitated from these solutions by neutralization (Piez, 1968). After dialysis against distilled water, acid-soluble gorgonin precipitates at pH 6.5 or above. The precipitate is a white, flocculent material. Negatively stained preparations of precipitated material show parallel aggregates of long filamentous structures. The structures in this preparation measure about 3 - 4 nm in diameter (Fig. 2) and resemble the unbranched, 3 nm diameter microfibrils seen subjacent to corticocytes in sectioned tissue. No 3 - 4 nm structures with axial striations were observed.

Platinum replicas of powdered skeletal samples show fibrils measuring up to 70 nm in diameter (Figs. 3 and 4). The fibrils are wound in right-hand helices to form

branching fibers measuring up to 1 um in diameter
(Fig. 4). Longitudinally sectioned fibrils show
cross-striations with a 26 nm periodicity (Fig. 5).

Crystals shaped like rectangular parallelapipeds (or
boxes) are embedded in the fibers. The crystals measure
about 370 nm X 260 nm X 120 nm or smaller (Fig. 4).
Lacunae observed between fibrils in decalcified, sectioned
material (Fig. 5) may represent former sites of crystals.

Scanning electron microscopical observations of fibrous
areas of the skeletal surface show that gorgonin fibers are
aligned with the longitudinal skeletal axis (Fig. 6).
Fibers appear to aggregate into groups in the same
circumaxial plane and then separate from one another to
join other fiber groups. Occasionally fibrils are seen
passing from one fiber to another (Fig. 6).

Discussion

1. Gorgonin collagen fiber formation

The conclusions based upon these observations rely on the assumption that the structures observed were collagen. The microfibrils' location subjacent to collagenic cells, the chemical data suggesting gorgonin is largely collagen and the cross-striated fibrils characteristic of collagen, all support that assumption.

Leversee (1972) found that dried Leptogorgia skeletons produce a 12.3 Å equatorial arc in wide angle, x-ray diffraction patterns. In vertebrate collagen diffraction patterns, a 12 to 17 Å equatorial spot represents the distance between the backbones of tropocollagen chains that comprise the microfibril. If the sample is dry, the chains are closer together and the spacing is closer to 12 Å. If the specimen is fully hydrated the spacing is nearer 17 Å (Ramachandran, 1967). Leversee's (1972) diffraction patterns show that gorgonin tropocollagen molecules are the same diameter as vertebrate tropocollagen. The 3 nm microfibril diameter observed in this study is attributed to the first stages of tropocollagen aggregation. A trimer or tetrad of 1.5 nm diameter tropocollagen molecules would appear 3 nm in diameter in sectioned or negatively-stained material (Fig. 7). Tropocollagen monomers were not observed but their small size may place them beyond the resolution limits of this technique.

Ross (1968) suggested that an aggregate of tropocollagen

molecules would need to be at least 7 nm or, more likely, greater than 10 nm thick before banding would be perceptible (Fig. 6) with 300 nm, quarter-staggered molecules (see Eyre, 1980, for a current discussion of lateral packing of collagen molecules).

When tropocollagen aggregates are thick enough to display banding, they are called fibrils (Morse and Low, 1974). The theoretical, 7 to 10 nm value for minimum striated fibril diameter (Ross, 1968) is considerably less than the minimum diameter of striated fibrils observed in the laboratory (e.g. 20 nm, Frederickson and Low, 1971; 25 nm, Hay and Dodson, 1973). Gorgonin collagen fibrils clearly display banding when 50 to 70 nm in diameter or greater.

After mammalian collagen is secreted and fibril formation has begun, covalent intermolecular crosslinks are formed. The first crosslinkages are reducible in dilute acetic acid. Collagen crosslinked by those reducible, covalent bonds can be solubilized in 0.5 M acetic acid (Bailey et al., 1974). Covalent bonds which are not reducible in dilute acetic acid are formed later (Bailey et al., 1974). Dityrosine is one example of a non-reducible, covalent crosslink of collagen (Malanik and Ledvina, 1979).

The microfibrillar appearence of newly-secreted gorgonin collagen and the extractability of some gorgonin in 0.5 M acetic acid suggest that non-reducible intermolecular bonds

are formed in gorgonin collagen after secretion.

The difference between Leptogorgia's gorgonin collagen fiber striation periodicity (about 26 nm) and mammalian collagen fiber periodicities (about 64 nm) may indicate different chemical compositions. In other gorgonian genera, different gorgonin collagen striation periodicities have been observed, e.g. about 34 nm (Marks et al, 1949; Goldberg, 1974) and 43 nm (Szmant-Froelich, 1974). The tropocollagen aggregation pattern may be determined to a large but not clearly understood degree by tropocollagen's terminal peptides, the telopeptides. The telopeptides differ from the rest of the tropocollagen molecule by being non-helical, poor in hydroxyproline and rich in tyrosine, aspartate and glutamate (Rubin et al., 1963). Hodge et al., (1960) had previously discovered that collagen purification techniques which removed tyrosine-containing terminal peptides would destroy tropocollagen's fiber-forming ability. The dense bands of striated collagen seen by transmission electron microscopy are believed to be sites where telopeptides are linked to neighboring tropocollagen molecules (Cox et al., 1967). The dense striations would therefore be rich in telopeptides. The difference between gorgonin collagen fiber and vertebrate collagen fiber cross-striation periodicities may be a difference in telopeptide chemistry. Whether or not gorgonin collagen's cross-striations result from a lateral, quarter-staggering

of tropocollagen as proposed for vertebrate collagen (Eyre, 1980) would depend on the molecule's length. That length is currently unknown. A biochemical study could provide interesting comparative data.

2. The fiber's crystals

Although this thesis does not deal with the inorganic portion of gorgonin, the serendipitous finding of crystals embedded in gorgonin collagen fibers is interesting.

The box-shaped crystals embedded in gorgonin fibers are presumably the major inorganic portion (and probably the calcium phosphate containing portion) of the skeleton. Comparable scanning electron microscopical observations have been made by Ledger and Franc (1978) on the anthozoan pennatulid, _Veretillum cynomorium_. Those investigators also found box-shaped crystals in a collagenous axial skeleton. Those crystals differed, however, in being calcite and in forming a "distinct phase" separate from collagen fibrils. In those respects, the gorgonian skeleton's crystals and collagen more closely resemble bone's collagen fibrils which are impregnated with calcium phosphate crystals (Cameron, 1972). The crystals in bone are needle-shaped rather than box-shaped, however.

These fine structural observations show there are structural similarities between the formation of gorgonin collagen fibers and vertebrate collagen fibers. Similarities between calcification mechanisms may also

exist. The next and important step in comparing these collagens is chemically confirming that the acid-soluble skeletal fraction is collagen and determining its chemical composition. The probable extraction of intact, unsclerotized gorgonin collagen microfibrils reported in this study is the first successful attempt to separate a gorgonin protein constituent without degrading the entire skeleton to amino acids. This separation procedure may allow the future determination of the size of the gorgonin collagen molecule and the composition of certain interesting molecular regions such as the telopeptides.

Figure 1. Transmission electron micrograph of the axis end of a corticocyte.

A. This corticocyte contains several procollagen-containing, flocculent vesicles and overlies a loose meshwork of gorgonin microfibrils. Bar = 200 nm.

B. The cell-skeleton interface shown in Fig. 1A is shown here at higher magnification. Between the brackets, a few 3 nm microfibrils appear to contact the plasma membrane. The plasma membrane shows a trilaminar profile which suggests it is cut transversely. Bar = 200 nm. F = flocculent vesicles. E = extracellular space.

Figure 2. Transmission electron micrograph of an aggregate
of microfibrils. These microfibrils were extracted from
powdered gorgonin with 0.5 M acetic acid, precipitated and
negatively stained. Distinct microfibrils (e,g. arrows)
measure 3 to 4 nm in diameter. Bar = 100 nm.

2

Figure 3. Platinum replica of powdered gorgonin. Rectangular parallelapiped crystals (arrows) are shown here. Bar = 1 μm.

Figure 4. Platinum replica of gorgonin fiber. Several box-shaped crystals are embedded in this fiber. On the fiber's surface is evidence of fibrils wound in right-handed helices to form the fiber. One of these fibrils is shown between the arrows. Bar = 1 μm.

Figure 5. Transmission electron micrograph of thin-sectioned gorgonin. Cross-striations (arrows) of several fibrils are labeled and have a 26 nm periodicity. The short period makes identifying striations in fibrils over long distances difficult since slight tilts of the fibril out of the plane of section cause the bands to overlap. Bar = 200 nm.

Figure 6. Scanning electron micrograph of a fibrous region
of the skeletal surface. The fibers run along or at small
angles to the skeleton's long axis (arrow). They form
bundles and then ramify to reform into other bundles.
Fibrils occasionally pass from one fiber to another
(arrowheads). Bar = 4 µm.

Figure 7. Schematic representation of microfibrils in cross-section to show possible types of tropocollagen packing. The circles represent cross-sections of tropocollagen molecules.

A. The 3 nm microfibrils observed in sectioned and negatively stained preparations could be accounted for by a trimer or tetrad of tropocollagen chains. A trimer would be the beginning of a hexagonal packing and a tetrad the beginning of orthogonal packing.

B. Larger aggregates of tropocollagen at the size which Ross (1968) believes cross-striations would begin to be perceptible i.e. when 7 tropocollagen molecules would lie in a row.

7A

3 NM   3 NM

7B

10.5 NM   10.5 NM

Chapter 5. The fine structure, development and function of desmocytes.

Introduction

Recent investigators have mistakenly identified desmocytes as the skeletogenic cells of gorgonians (Bouligand, 1968; Goldberg, 1973; Bayer, 1974). The preceding chapters of this thesis show that the columnar cells of the axial epithelium are the only cells in which skeletogenic activity is demonstrable. The desmocytes, therefore, seem to serve only the role originally proposed by Bourne (1899), that is, binding soft tissues to extracellular skeletal structures.

Several cells and remnants of dead cells which have the specialized mechanical function of binding soft tissues to extracellular skeletal structures have been described in the Phylum Cnidaria (Bourne, 1899; Chester, 1913; Bouligand, 1968; Chapman, 1969; Bouillon and Lévi, 1971; Goldberg, 1973; Bayer, 1974; Vandermeulen, 1974; Van-Praët, 1974; Ledger and Franc, 1978; Marcum and Diehl, 1978). Therefore, these binding cells and cell remnants, usually called desmocytes, are analogs. Questions concerning desmocytes which are of general cytological interest are: 1) how do the cells develop their intricate shape, 2) what is the stimulus for that development, and 3) how is that shape appropriate for serving a mechanical role? The

present study is directed toward providing answers to these questions.

Desmocyte morphogenesis has been studied previously in each of the three cnidarian classes: Anthozoa (Bourne, 1899; Chester, 1913), Scyphozoa (Chapman, 1969), and Hydrozoa (Marcum and Diehl, 1978). Each of these studies dealt primarily with cell shape changes and with the accumulation of intracellular filamentous structures. Subcellular mechanisms involved in the cell shape changes and tonofilamentous organization are investigated in this work. Data from those previous studies of desmocyte development suggest that differences in structure and development of cnidarian desmocytes exist among the classes. The electron microscopical data obtained from this study, which is the first available for anthozoan desmocyte development, will be compared to that of the development of hydrozoan desmocytes (Marcum and Diehl, 1978) and the somewhat speculative development of scyphozoan desmocytes (Chapman, 1969).

Observations on the fine structure, distribution and cytochemistry of desmocytes and related structures will be used as the basis for inferences on the possible stimuli for desmocyte development and on the manner in which desmocytes serve their binding function. These data will then be compared to the fine structural and chemical data obtained from the vertebrate analog of desmocytes, the

tonofilament-desmosome system. The possibility of homology
between these cnidarian and vertebrate cell-binding
structures will be discussed.

Materials and Methods

Tissue samples used for light and transmission electron microscopy were prepared in the manner described in Chapter 1 of this thesis.

Tissue studied by scanning electron microscopy was fixed as described for transmission electron microscopy (Chapter 1). The soft tissues were stripped from the skeleton by using fingers and forceps. The skeletons were then dehydrated through a graded series of ethanols and critical point dried from isoamyl acetate with carbon dioxide. The skeletons were sputter coated with gold and viewed in a Cambridge S150 scanning electron microscope.

Tissue used for cytochemical studies was prepared as described in Chapter 2. The method for tissue treated with the periodic acid – silver methenamine technique for non-acidic sugars follows.

1. Place thin sections on 200-mesh gold grids.
2. Treat sections for 10 min with 5% sodium metabisulfite to block aldehydes.
3. Rinse briefly in water.
4. Treat sections with 1% periodic acid (aqueous) for 20 to 25 min at room temperature.
5. Wash in two rapid changes of water, then in water for 10 min.
6. Proceed with the silver methenamine reaction

described in Chapter 2 including blocks for
sulfhydryl groups.

For controls, sections were treated identically except
Step 4 is omitted.

This reaction is believed moderately specific for
sugar groups which have a pair of free hydroxyl groups on
adjacent carbons (Pearse, 1968). Pearse reports the
reaction to occur with polysaccharides, glycoproteins, and
non-acidic mucopolysaccharides. Acidic sugars and sugars
in nucleic acids do not react.

The method for cytochemical localization of sulfhydryl
groups in sections of tissue prepared in the manner
described under "Cytochemistry" in Chapter 2 follows.

1. Place sections on 200-mesh gold grids.

2. Treat the sections in 5% sodium metabisulfite for
   10 min at room temperature.

3. Wash in water briefly.

4. Immerse in 1.25 M N-ethyl maleimide for 4 h at room
   temperature.

-or-

Immerse in a solution of 23% iodoacetate in 1 N
NaOH for 2 h at room temperature.

5. Wash in two changes of water for 5 min each.

6. Continue with the silver methenamine reaction.

The above method produces sections in which sulfhydryl

groups are blocked. By preparing other sections by the same method except eliminating Step 4, sites with sulfhydryl groups will bind silver. Comparison of the two preparations permits identification of sulfhydryl sites (Knight and Lewis, 1977).

Disulfides can be identified by the above preparation if two additional steps are inserted between Steps 5 and 6 above. The additional steps are:

5b. Immerse sections in 0.05 M sodium thioglycollate

   for 4 h at room temperature.

5c. Wash in water for 5 min.

Sodium thioglycollate will reduce disulfide bonds and produce two sulfhydryls which will then bind silver in the silver methenamine reaction (Blackburn, 1970).

Results

1. Desmocyte development

In the Class Anthozoa, desmocytes are easily
recognized by light microscopy as sites where the loose
connective tissue, called mesoglea, dips deep into the
skeletogenic epithelium almost to the axial skeleton (Fig.
1). Electron microscopical observations show that these
sites harbor a cell, the desmocyte, which has a pectinate,
or comb-like, margin facing the mesoglea. The "teeth"
contain dense, intracellular, cytoskeletal rods (Fig. 2)
which are believed to function in binding soft tissue to
the extracellular skeleton because when the soft tissue is
mechanically stripped from the axial skeleton, the
cytoskeletal rods can still adhere (Fig. 3). In this
study, the sequence of changes in skeletogenic cells
leading to formation of desmocytes has been determined by
using the presence of cytoskeletal rods as the hallmark of
desmocyte formation and assuming those cells with the
fewest cytoskeletal rods to be in the earliest stages of
desmocyte formation.

Desmocyte formation in the skeletogenic epithelium
begins with an increase in surface area of the lateral
plasma membrane resulting in numerous membrane folds (Fig.
4). These cells then invaginate from the center of the
mesoglea end of the cell toward the axis end, i.e. the end
nearest the axial skeleton (Fig. 5). Invagination is

apparently achieved by the action of microfilaments about 5 nm in diameter. During invagination of the cell, there is a loss of subcellular constituents so that the cell becomes largely electron lucent (Fig. 6). Cisternae of rough and smooth endoplasmic reticula and large lipid droplets which are present in the skeletogenic cells disappear in early stages of desmocyte formation. Organelles which persist include a nucleus with heterochromatin, Golgi bodies, polysomes and flocculent vesicles 1 µm in diameter. Also present are numerous vesicles 0.2 µm in diameter which appear cup-shaped or at times seem to contain smaller vesicles. These vesicles have been previously noted in the desmocytes of _Lophogorgia sarmentosa_ (Bouligand, 1968) in which they were called a "système de deux vésicules." They will be referred to here as double vesicles.

Double vesicles appear to be Golgi derivatives and to acquire their more complex, folded morphology while still near the Golgi complex (Fig. 7). The double vesicles become arranged along the axis end of the cell where they are connected to one another by microfilaments (Fig. 8). The center of the double vesicles may become electron dense, a change which precedes the formation of the cytoskeletal rods characteristic of desmocytes (Figs. 9, 12).

The cytoskeletal rods are about 2.4 µm long, 0.2 µm wide, electron dense, filamentous structures arranged at

the axis end of the desmocyte approximately perpendicular to the axial skeleton's surface. The formation of the cytoskeletal rods precedes the completion of cell invagination (Fig. 9). During cytoskeletal rod formation, microtubules are arranged either parallel or at small angles to the forming rods (Fig. 10).

Both longitudinal (Fig. 11) and transverse (Fig. 12) sections of the developing desmocyte show double vesicles between the folds of invaginating plasma membrane where the cytoskeletal rods will form. The central region of the double vesicle becomes more electron dense as development progresses. The outer membrane of the vesicle then becomes closely apposed to the plasma membrane (Fig. 12).

Microfilaments appear to draw the invaginating plasma membrane laterally until this invaginating membrane nearly meets the membrane forming the lateral margin of the cell (Fig. 13). At this stage, the axis end of the cell becomes totally occupied by an array of cytoskeletal rods and the plasma membrane is drawn into the spaces between the radial rows of rods (Fig. 14). A cross section of a mature desmocyte shows that the ridges formed by the rods maintain an extracellular (mesogleal) space of about 0.27 μm between the neighboring ridges. When two ridges diverge, another ridge is interposed, thereby keeping the spacing nearly constant. This radial pattern is not seen on the skeleton's surface (Fig. 15) since each rod splays out into

several rounded ends (Fig. 16) which fit into pits on the skeletal surface.

The collagen fibers of the mesoglea extend into the interstices of the pectinate portion of the desmocyte (between the comb's teeth) yet the fibers are not directly attached to the plasma membrane by the mesogleal collagen fibers. A fine fibrillar coat is found between the plasma membrane and the collagen fibers (Fig. 16). The collagen fibers seem attached to this fibrillar coat.

The fibrils of the fibrillar coat make an angle of 55° ± 20 with the long axis of the cytoskeletal rods at the desmocyte membrane. The fibrillar coat is found on the cell surface across the membrane from which lie either cytoskeletal rods or double vesicles (Figs. 2, 11, 12, 13, 16).

As the numbers of cytoskeletal rods increase, the numbers of other desmocyte organelles diminish. Eventually the desmocyte becomes entombed in the skeleton by overgrowth of secretions of neighboring skeletogenic cells (Fig. 17).

2. Cytoskeletal rod structure

Cytoskeletal rods seen in longitudinal sections show longitudinal striations (Fig. 16). In cross-section, the rods appear to consist of tubular structures about 10 nm in diameter with a dense, central core (Fig. 18). The length

of the 10 nm diameter structures has not been determined.

3. Desmocyte distribution

Scanning electron microscopical observations show that desmocytes' distribution on the skeletal surface is variable; they may occur either singly or in groups (Fig. 19). Serial transverse 1 μm sections of branch tips show that desmocytes are not found at the extreme distal end of branches where lipoprotein-secreting medullocytes are located. Desmocytes are seen to occur singly within about 2 mm of the skeletal branch tips and then in larger clusters closer to the colony's holdfast.

4. Cytochemistry

The small size of the cytoskeletal rods and their close relationship to the desmocyte membrane's fibrillar coat and the extracellular mesoglea fibers made conclusive histochemical data on the rods difficult to obtain. Electron microscopical cytochemical investigations were conducted instead.

The cytoskeletal rods did not bind silver in the silver methenamine tests for hydroquinones, sulfhydryls, disulfides or non-acidic carbohydrates. These cytochemical tests did, however, reveal positive reactions for certain structures associated with the cytoskeletal rods.

The collagen fibers of the mesogleal extension present

a beaded appearence when treated with periodic acid –
silver methenamine (PA-silver) (Fig. 20). The fibrillar
coat lying between these collagen fibers and the desmocyte
membrane did not bind silver in this test. The collagen
fibers are therefore chemically distinct from the fibrillar
coat.

The thin layer of material found between that part of
the desmocyte which contains cytoskeletal rods and the
skeleton reduces silver in the silver methenamine reaction
(Fig. 21). This reaction did not occur if the sections
were first treated with the sulfhydryl-blocking compounds,
NEM or iodoacetate. The reaction did not occur if the
sections were first treated with iodoacetate followed by
sodium thioglycollate treatment for disulfide reduction.
The material subjacent to the skeletogenic cells in the
axial epithelium did not reduce silver in any of these
treatments. These cytochemical test results indicate that
the desmocyte-skeleton interfacial material is the only
cell-skeleton interfacial material rich in sulfhydryl
groups.

Discussion

1. Desmocyte development

The skeletogenic epithelium of _Leptogorgia virgulata_ is initially comprised of medullocytes which secrete a lipoprotein to form intramedullary structures and the medullary wall (see Chapter 1). Corticocytes then secrete cortical collagen and a tyrosine-derived crosslinking compound onto the medullary wall (see Chapter 2). The present portion of this study reports that desmocytes develop from cells which are indistinguishable from corticocytes. Those cells which clearly display an ultrastructure suggestive of desmocyte formation, e.g. plasma membrane invagination and cytoskeletal rods, have reduced numbers of the organelles found in corticocytes. The disappearence of rough endoplasmic reticulum and dense-cored vesicles (which contain a collagen-crosslinking compound) suggest that desmocytes lose their skeletogenic role as they form.

The developing desmocytes contain flocculent vesicles which in the skeletogenic cells of _Leptogorgia_ have been found by electron microscope autoradiography to contain procollagen (Chapter 2). This flocculent material may be destined to form the mesogleal extension which is attached to the surface coat of the pectinate margin of the desmocyte; both Bourne (1899) and Chester (1913) suggest that cells adjacent to the mesogleal extension secrete

mesogleal material during desmocyte formation. Bourne suggests that the desmocyte invaginates and then neighboring cells secrete a mesogleal process which fuses with the desmocyte and rest of the mesoglea. The autoradiographic study of collagen secretion which is described in Chapter 2 showed no obvious proline secretion and, therefore, no obvious collagen secretion by any cells into the mesogleal extension. There is no experimental evidence to indicate the origin of the mesogleal extension.

A predominant feature of developing desmocytes is the Golgi body which persists into late developmental stages. This occurrence is consistent with the Golgi body's probable role in producing the double vesicles prevalent in developing desmocytes and with its generally accepted role in membrane formation. An increase in plasma membrane surface area would be required to accommodate the broadening and flattening of the desmocyte during development and the formation of the highly convoluted pectinate margin of the cell. A distinct population of vesicles responsible for increasing membrane surface area has not been identified. The increase in plasma membrane precedes invagination of the developing desmocyte.

Observations on the arrangement of double vesicles and changes in their appearence during desmocyte formation indicate that these structures play a primary role in

formation of cytoskeletal rods. The sequence of events in
cytoskeletal rod formation inferred from electron
microscopical observations is: 1) microfilaments arrange
double vesicles in an array at the plasma membrane of the
cell's axis end, 2) more double vesicles come to form rows
perpendicular to the skeletal surface above those double
vesicles which already lie at the cell membrane, 3) the
central region of each double vesicle becomes increasingly
electron dense while the outer vesicle membrane becomes
closely apposed to the invaginating plasma membrane, and 4)
the vesicle membrane disappears and its dense contents join
with those of the vesicles in the same row to form a
cytoskeletal rod (Fig. 22).

A comparison of the changes occurring during desmocyte
development in Leptogorgia to those cytological changes
observed in other developing anthozoan desmocytes suggests
a similar developmental mechanism throughout the class.
Light microscopical observations of desmocyte formation in
the anthozoan, Heliopora (Bourne, 1899), indicate that
cells indistinguishable from skeletogenic cells become
"much vacuolated" and display internal striations after
which each modified cell becomes a shallow cup with
irregular margins. These observations are consistent with
those reported here on desmocyte formation in Leptogorgia.
Chester (1913) found in the gorgonian coral, Pseudoplexaura
crassa, that desmocyte formation resembled that reported
for Heliopora.

Chester also noted that an early change in the transformation to the desmocyte involved a spreading of the cell's axis end thus pushing aside neighboring cells. The spreading of the desmocyte during development also occurs in _Leptogorgia_. Electron microscopical observations suggest that the initiation of cell margin spreading precedes the formation of cytoskeletal rods. The final pattern and the order of rod formation in the desmocytes (Fig. 14) indicate that the spreading occurs radially.

2. Desmocyte distribution and speculations on the stimuli for desmocyte development

The only evidence which provides insight into the nature of the stimulus or stimuli for desmocyte development is the observation that desmocytes are not found at the extreme distal end of branch tips but are found in increasing numbers at sites nearer the holdfast. This observation suggests at least two possible stimuli for development: mechanical stress and age of the cells.

Since desmocytes are believed to serve a mechanical function, the hypothesis that they develop in response to mechanical stress is appealing. This hypothesis was first proposed by Bourne (1899) who made the only recorded observation to suggest that mechanical stress may be an important factor in initiating the development of these cells. In Bourne's study of _Heliopora_, he noted that desmocytes were most frequently located in large numbers in

the skeletogenic epithelium where the mesenteries of polyps met the corallum, the calcified skeleton. Bourne assumed the mesenteries would pull on the tissue overlying the corallum at these sites. He inferred that this preferential desmocyte distribution indicated both a mechanical function for these cells and a role for mechanical stress in stimulating their development.

The distribution of gorgonian desmocytes has been studied twice: by Chester (1913) and in this study. Chester found no correlation between distribution and hypothetical stimuli such as the locations of polyps. The present work on Leptogorgia shows that the frequency of desmocyte occurrence increases in sampling from branch tip to skeletal base. Mechanical stress on the skeleton is believed to be greater near the skeletal base. Therefore, the observed desmocyte distribution is consistent with the hypothesis that mechanical stress is the stimulus for desmocyte development. These observations are not conclusive proof, however, because other factors such as the cells' age may be co-variables with stress along the skeleton.

Age of the cells which are to become desmocytes may be an important factor. The occasional occurrence of desmocytes within 2 mm of the branch tip makes it seem unlikely that mechanical stress is the sole stimulus since it is not expected that the stresses placed upon the cells

1 mm or less apart at the branch tip vary greatly.
Desmocyte development may be a regularly occurring event in
skeletogenic cell differentiation which occurs independent
of external stimuli.

There is evidence that other varieties of desmocytes
may develop in response to a different sort of stimulus.
Chapman (1969) observed that _Aurelia_ scyphistoma desmocytes
could form on the stalk ectoderm at sites where that
ectoderm touches the substrate or other foreign matter.
Normally, _Aurelia_ desmocytes are formed only on the pedal
disk. Chapman's observations suggest that physical contact
or perhaps a chemical influence could affect desmocyte
development.

No conclusive evidence for the developmental stimulus
of any desmocyte type has been presented. It is postulated
here that different desmocyte types develop in response to
different stimuli, that is, axial epithelium desmocytes and
pedal disc desmocytes would develop under different
stimuli.

3. Mechanical design of desmocytes

If it is assumed that desmocytes function in binding
soft tissues to skeletal structures, then several
characteristics of desmocytes can be explained as
specializations for a binding function. Some of these
desmocyte characteristics which probably have a mechanical

role are: a) adhesion and spreading, b) pectinate margin formation, and c) cytoskeletal rod formation. Each of these characteristics will be discussed separately and compared to other cells with an analogous function.

a. Adhesion and spreading

For this discussion, adhesive mechanisms will be divided into two types: specific and non-specific. Specific adhesion mechanisms would involve binding sites on the cell membrane which could interact only with substrata with a special chemical nature. Non-specific adhesion mechanisms would involve cell-substratum interactions such as Van der Waals forces or electrostatic forces. The cell and substratum surface charges would be important in determining these non-specific mechanisms.

The cytochemical data presented in this study indicate that the desmocyte-skeleton interface is sulfhydryl rich while the interface between other axial epithelial cells and the skeleton is not. The evidence for the presence of sulfhydryls is a layer of interfacial material which reacts with silver in the silver methenamine reaction unless the sections are pre-treated with the sulfhydryl blocking compounds, NEM or iodoacetate.

Sulfhydryls have been implicated in other cell adhesion systems. The adhesion of platelets to collagen can be inhibited _in vitro_ by the addition of NEM to the

culture medium (Al-Mondhiry and Spael, 1970). Baby hamster kidney cells are similarly inhibited from adhering to polystyrene following NEM treatment and the normal increase in adhesive strength following attachment in that experiment was slowed (Grinnell et al, 1973). The same study showed that platelet adhesion was similarly affected by trypsin treatment which led those investigators to propose that the adhesive, sulfhydryl-containing compound is a protein or protein complex.

A model which was proposed for sulfhydryl-mediated adhesion involves unattached cells with sulfhydryl groups distributed uniformly on their surfaces. Once contact with the substratum has been made, more sulfhydryls approach the attachment site and "interact" with the substratum (Grinnell et al., 1973). This model would account for an increase in adhesive strength over time. Experimental data collected in other studies agree with the proposed migration of adhesion sites to the attachment surface. For example, the apical surface of attached epithelial cells becomes non-adhesive to other cells when the basal surface is attached to a substratum (Middletown, 1973).

Three rôles for sulfhydryls in cell-substratum adhesion have been proposed (Grinnell and Srere, 1971): 1) sulfhydryls may form a direct bond between cell and substratum, 2) they may be an essential part of a bridge between cell and substratum, or 3) they may be an essential

part of an enzyme or other molecule which mediates bond formation.

The presence of carbohydrates on cell surfaces is a universal feature and has been studied morphologically by Rambourg and Leblond (1967). These workers found that "nearly all cells" in a variety of rat tissues are coated with a thin layer of material which binds silver in the PA-silver reaction. They also observed that the PA-silver material was continuous with the middle plate and terminal bar of desmosomes. These observations led them to propose that surface carbohydrates are involved in cell adhesion.

Although the desmocyte-skeleton interface was found PA-silver negative in this study, that observation only shows that the adhesive material is different from that which is present on the rat's cell surface. The PA-silver test does not react with acidic sugar groups; therefore, the desmocyte adhesive material could contain acidic sugars and still go undetected by this method.

One sugar-protein complex which has been subject to a great deal of study in the past few years is the glycoprotein, fibronectin. A variety of evidence, some of which is rather circumstantial, indicates that fibronectin is a fibroblast surface glycoprotein involved in adhesion to collagen. The evidence is from studies which include the following.

1) fibronectin is present at the fibroblast surface

(Mautner and Hynes, 1977; Hedman _et al._, 1978),

2) the same cell surface sites are rich in
   procollagen (Bornstein and Ash, 1977),

3) fibronectin and collagen display an affinity _in_
   vitro (Engvall and Ruoslahti, 1977), and

4) the presence of fibronectin enhances the
   spreading of cells on collagen (Grinnell and
   Minter, 1978).

Fibronectin is found on the cell surface as a
disulfide-linked dimer or polymer (Yamada _et al._, 1977),
although the disulfide bonds are not involved in binding
fibronectin to other surface proteins or to substratum
proteins (Olden _et al._, 1980). My cytochemical data show
that disulfide reduction does not produce additional
sulfhydryl groups at the desmocyte-skeleton junction. This
observation is taken as evidence that fibronectin is not
involved in desmocyte adhesion.

The role of sulfhydryls in desmocyte-skeleton adhesion
is completely unknown. There is no evidence to show
whether the presence of sulfhydryls on the desmocyte
surface is essential to adhesion other than the
circumstantial evidence which shows that this adhesive
region is sulfhydryl-rich while other, less adhesive,
neighboring cells contain no cytochemically demonstrable
surface sulfhydryls.

Another non-specific adhesive mechanism which is

probably involved in desmocyte-skeleton interaction is interlocking. Interlocking in biological adhesion has been discussed recently by Emerson and Diehl (1980) who have shown its involvement in the adhesion of the tree frog toe pad cells to inclined substrata.

Interlocking can be intuitively understood by considering the greater traction that a tire with good tread has than a smooth-surfaced tire. This traction is caused to some degree by interlocking of the tire with the substratum, the road. The SEM observation of the pitted skeletal surface subjacent to desmocytes (Fig. 15) and the TEM observation which shows how the cytoskeletal rods fit into those pits (Fig. 16) indicate that interlocking . between desmocyte and skeletal surface occurs. The limitation on interlocking mechanisms is that they offer no sticking to resist forces which would pull up the desmocyte (or tire) in the direction perpendicular to the skeleton's surface (or road).

This study suggests that several mechanisms may be involved in desmocyte-skeleton adhesion. These proposed mechanisms include specific chemical interactions, electrostatic interaction and interlocking. Adhesion resulting from each of these mechanisms would be increased if cell surface contact area were increased. This could be achieved by cell spreading.

Cell spreading requires metabolic energy (Michaelis.

and Dalgano, 1971) and that the cell adhere to the substratum (Gingell and Vince, 1980). Microfilaments are the effectors in many cell shape changes which are associated with spreading (e.g., Nath and Srere, 1977; Grinnell, 1978).

Desmocytes contain a few mitochondria and may thereby fulfill the required metabolic requirements for cell spreading. Cell adhesion has been inferred from the previously discussed evidence. However, microfilament bundles do not appear to be involved in desmocyte spreading. The mechanism of spreading of the lateral cell margin is completely unknown in these cells and this study provides no data upon which speculation could be based regarding that mechanism. It is doubtful that microfilament bundles were overlooked and doubtful that they were not preserved in desmocytes since individual microfilaments were observed. I propose that desmocyte spreading involves a different mechanism which does not require microfilament bundles.

b. Pectinate margin formation

If the function of desmocytes is the mechanical one of binding tissue to the skeleton, then the formation of the pectinate margin of this cell may have functional significance in that role. This portion of the discussion will present speculations on the relationships of

mechanical stresses to the form of the desmocyte pectinate margin.

The direction and magnitude of forces which act upon the desmocyte's membrane are modified by the presence of the fibrillar desmocyte coat between mesogleal collagen fibers and plasma membrane. The fibrils of the fibrillar coat lie at an angle of $55° \pm 20°$ to a line perpendicular to the surface of the skeleton. In Fig. 23, the angle λ is approximately $55°$. This means the force, F_T, transmitted through the collagen fiber would be divided into a y-axis component ($\cos \lambda = 0.57\ F_T$) and an x-axis component ($\sin \lambda = 0.82\ F_T$). Therefore, the force transmitted in the line of the collagen fiber would be nearly halved while that force perpendicular to that direction would be over 80% of the original force. This, then, would be the force acting upon the plasma membrane if the desmocyte coat fibrils do not move when stressed. There is evidence, however, that the fibrils may move when stressed, which will now be discussed.

The forces predicted to be acting on the plasma membrane will be diminished since the desmocyte coat's fibrils probably move in a hinge-like action at their junction with the membrane. This movement is inferred from the variety of orientations that the fibrillar material is seen to make with respect to the plasma membrane. By moving, the fibrils reduce the amount of force transmitted

because the motion consumes part of the force's energy.
The hinge motion would also change the fibril orientation
so that when stressed, the fibrils would lie at a more
acute angle to the y-axis thereby decreasing the angle λ,
increasing the y-axis component of the force and decreasing
the x-axis component. The longitudinal orientation of the
cytoskeletal rod's tonofilaments suggest that the rods are
placed under tension along the y-axis.

Aside from the benefit derived from the absorption of
some of the force by the interposed fibrillar layer and the
division of the force into x-axis and y-axis components,
the increase in membrane surface area which accompanies the
described arrangement would be beneficial. By increasing
surface area, stress per unit area of membrane would be
decreased. This same rationale for increase in cell
surface area may apply to other systems such as
muscle-tendon junctions in which the muscle cells acquire a
convoluted margin where their basement membrane joins the
tendon's collagen fibers (Mackey et al., 1969).

The frequent observation of folded membranes of cells
involved in cell-extracellular matrix contacts suggests
this membrane morphology has a functional significance.
Although there are no data providing proof of a mechanical
role for this membrane form, that speculative role is
usually inferred (e.g. in aortic tunica media, Cliff, 1967;
portal vein wall, Komuro and Burnstock, 1980).

A source of interesting speculation is the sort of changes in the membrane structure and chemistry which may occur during desmocyte development. Are proteins inserted into the membrane to stiffen it? Does the degree of lipid saturation of the phospholipid chains change and thereby change membrane fluidity? It seems possible that specializations of these cells would appear at the level of membrane chemistry, as well as the morphological ways cited in this work.

c. Tonofilaments

The filamentous appearence of the desmocyte cytoskeletal rods and their probable role as tensile elements means that these filaments are, in a literal sense, tonofilaments (Gr. tonos, tension). Comparison of the tonofilamentous cytoskeletal rods to other tonofilamentous systems reveals some interesting similarities, dissimilarities and areas for further study.

Vertebrate tonofilaments are about 8 - 10 nm in diameter (Steinert, 1975; Skerrow and Skerrow, 1980). The morphological classification of intracellular structural proteins contains three types: 1) microtubules, which are about 25 nm in diameter, 2) actin-containing microfilaments, which are 5 - 7 nm in diameter and 3) intermediate filaments, which are 7 - 12 nm in diameter (Steinert et al., 1978). Vertebrate tonofilaments are

therefore morphologically classified as intermediate filaments.

Vertebrate tonofilaments are the protein prekeratin (Skerrow and Skerrow, 1980). The most extensively studied prekeratins are obtained from bovine snout epidermis (Matoltsy, 1965; Skerrow, 1972; Baden et al., 1971). Although amino acid analyses of tonofilaments obtained by various investigators exhibit some slight variability in composition even when samples are all obtained from cow's snout epidermis (Matoltsy, 1975), in all cases proline values are low (about 1.5%) as are cysteine concentrations (about 0.9%).

Tonofilaments are attached to electron-dense material at cell membranes at desmosomes (sites of intercellular adhesion) and hemidesmosomes (sites of cell-substratum adhesion). The adhesive capabilities of the desmosomes and hemidesmosomes have been attributed to calcium ion bridges (Benedetti and Emmelot, 1968), linking proteins (Borysenko and Revel, 1973; Grinnell, 1980) and adhesive sugar groups (Rambourg and Leblond, 1967). The desmosome-tonofilament system is believed to serve in strengthening the cell membrane (Skerrow and Skerrow, 1980) and in stress distribution throughout the cell (McNutt and Weinstein, 1973).

The functional similarities between vertebrate hemidesmosome-tonofilament systems and gorgonian desmocyte

cytoskeletal rods are obvious. The probable role of
cytoskeletal rods as tensile elements has been mentioned
and the adhesive capabilities of the membrane subjacent to
the rods has been discussed. Cytoskeletal rods and
vertebrate tonofilaments therefore appear to serve similar
functions.

The fine structure of cytoskeletal rod tonofilaments
also shows similarities to vertebrate tonofilaments. The
cytoskeletal rod's filaments are about 10 nm in diameter
which places them in the intermediate filament category.
Cross sections of the cytoskeletal rod tonofilaments show
them to be cylindrical, a feature shared with vertebrate
intermediate filaments (Franke et al., 1979a).
Longitudinal sections of the cytoskeletal rods show no
branching of the filaments, a trait also shared with
vertebrate intermediate filaments (Franke et al., 1979a).

The dense core seen in desmocyte tonofilaments in
cross-section (Fig.18) is similar to the core seen in
keratin microfibrils (Fraser, 1969). Fraser (1969)
suggested that keratin microfibrils contain protofibrils
with a "9 + 2" arrangement. This has not been
substantiated, however. The intracellular precursor of
keratin microfibrils is thought to be prekeratinous
intermediate filaments (Steinert et al., 1978).

Cytochemical tests provide the only chemical data on
the cytoskeletal rod tonofilaments. The desmocyte

tonofilaments do not bind silver in either the test for
sulfhydryls or for disulfides. Although these data are
consistent with the low sulfhydryl levels in vertebrate
tonofilaments (Matoltsy, 1975), and the absense of
disulfide bonding in tonofilament prekeratin (Skerrow,
1974), they certainly offer no substantiative evidence that
cytoskeletal rod tonofilaments are prekeratin.

Immunocytochemistry and biochemical techniques show
intermediate filaments to be somewhat chemically
heterogeneous between vertebrate taxa. Anderton (1980)
points out that intermediate filaments show much more
tissue specificity than microtubule tubulin or
microfilament actin. In other words, microtubules and
microfilaments differ little between tissues while
intermediate filaments have easily observed differences.

Intermediate filaments have been divided into five
groups on the basis of immunologically and
electrophoretically demonstrable differences (e.g.,
Borenfreund et al., 1980; Anderton, 1980). The groups are:

    1) Tonofilament-type filaments are also called
       prekeratin-like proteins and cytokeratins. They
       are distributed throughout vertebrate epithelia
       (Franke et al., 1979a; Osborn et al., 1979) and
       react with antisera to bovine prekeratin (Osborn
       et al.,1979) and keratin (Borenfreund et al.,
       1980).

2) Vimentin may be the only intermediate filament in mesenchymal cells although it is also found in some epithelial cells (Osborn et al., 1979).

3) Desmin, also called skeletin, is a chemically distinct, 10 nm filament found in smooth, cardiac and skeletal muscle.

4) Neurofilaments are also chemically distinct and appear in neurons and neuroblastoma cells.

5) Glial filaments are found in astrocytes but have not been demonstrated in other glial cells.

Leptogorgia cytoskeletal rod tonofilaments fit into the above list of vertebrate intermediate filaments in the tonofilament-type group. In addition to the fine structural and functional similarities between cytoskeletal rod tonofilaments and vertebrate tonofilaments is the epithelial location of the structures. There have been few attempts at investigating homology and analogy for intermediate filaments; exceptions are the attempts of Steinert et al. (1978) and Franke et al. (1979b). I have found no comparative biochemical studies of intermediate filaments in invertebrates although this would be an interesting test of the tissue specificities of various vertebrate filament types.

Possibly the largest known difference between gorgonian desmocyte tonofilaments and vertebrate tonofilaments relates to the extent of their intracellular

distribution. Vertebrate tonofilaments diverge from dense bundles attached to the the cell membrane at desmosomes or hemidesmosomes and spread throughout the cell (e.g., Earnst, 1973; Skerrow and Skerrow, 1980). Immunocytochemistry shows that tonofilaments are tethered either to other cytoskeletal structures or to the plasma membrane at other sites in the cell (e.g. Borenfreund et al., 1980). Leptogorgia desmocyte tonofilaments are, instead, gathered into discrete cytoskeletal rods. Rather than the tonofilaments diverging to course through the cell and attach to the cell membrane at the opposite end of the cell, the cell membrane is drawn down to become attached to the cytoskeletal rods. This solution to the same mechanical problem might be the most conservative of material since far less tonofilament production is required. Of course, more membrane must be produced instead but this may have additional advantages in stress distribution discussed in the previous section of this Discussion (part 3b).

The vertebrate tonofilament disposition is more closely resembled in cnidarians other than Leptogorgia. In Cordylophora, the tensile elements are filaments 7 nm in diameter (Marcum and Diehl, 1978). In this example, the filaments begin in rods which resemble the cytoskeletal rods of desmocytes but then diverge from one another to pass within the cell and attach at the plasma membrane of the cell's mesoglea end. Tubular invaginations formed at

the cell's mesoglea end give it a convoluted appearence but the cell is not as deeply invaginated as the gorgonian desmocyte margin (Fig. 23). Cordylophora desmocytes therefore seem to present features of both gorgonian desmocytes and vertebrate tonofilament systems.

The scyphistoma stage of the cnidarian, Aurelia, also contains desmocytes but these have a far different form from other tonofilamentous structures and in their functional state they are dead remnants of cells (Chapman, 1969). Chapman calls the tensile elements in Aurelia desmocytes "tonofibrillae." The Aurelia desmocyte tonofibrillae span the entire 10 - 30 μm length of the parent cell from a flared end embedded in the cuticle to a rounded head at the mesoglea end (Fig. 24). It is unknown whether any adhesion occurs at the cuticle-desmocyte junction or whether this desmocyte is effective in binding only because the flared end is embedded in the cuticle as Chapman has shown (1969). A lucent center was not noted in these tonofibrillae nor was fibrilla diameter measured.

Chapman's histochemical studies of Aurelia desmocyte tonofibrillae (1969) show these fibrillae to be a basic protein containing no demonstrable arginine or cystine. Vertebrate tonofilaments contain about 6% arginine (Matolsky, 1975) and differ chemically from Aurelia desmocyte tonofibrillae in at least this respect.

Van-Praët (1977) has noted desmocytes in the

scleractinian coral, _Hoplangia durotrix_. In that study, she observed that desmocytes and muscle cells both stain orange with Masson's trichrome technique while mesoglea stains blue. Van-Praët did not specify the exact staining procedure, but on the basis of the histochemical results she concludes that desmocytes are myofibrillar ectodermal cells. However, in at least some formulations of Masson's trichrome, "cytoplasmic elements," keratin and muscle all stain similarly with Masson's trichrome (Humason, 1962). Concluding that desmocytes are myofibrillar therefore seems unsupported. Van-Praët's micrographs showing desmocytes resemble those of _Leptogorgia_ desmocytes presented here, although the relatively low magnification of Van-Praët's micrographs prevents a detailed comparison.

From a comparison of the above studies concerning a variety of tonofilament systems, the appealing hypothesis presents itself that the cytoskeletal rods of _Leptogorgia_ desmocytes are not only functional analogs to the tonofilament-hemidesmosome systems in vertebrates, but that they may be homologs, as well, i.e. share a common ancestral cell.

Grimstone (1951) has warned against speculating on homology of cellular, subcellular and molecular structures, however. He explains that if one of these structures is faced with a problem, there is only a limited number of solutions within physical and chemical possibility that may

be reached. For example, if a cell is to withstand tensile stress, then one of a limited number of tensile, subcellular elements can be employed. This means that similar solutions may arise independently so that similarities need not be taken as proof of descent from a common ancestral state, i.e. homology.

Since Grimstone's objections to the use of fine structural observations in taxonomy, electron microscopy has been used convincingly in investigating homology and analogy of microscopic structures, e.g. by Rieger and Tyler (1979). These investigators have listed guidelines for microscopical approaches to systematics. They propose that traits which indicate structural homology are: 1) the structures maintain a similar spatial relationship to surrounding structures, 2) the structures share similarities at many levels of organization (i.e., microscopical to molecular), 3) the structures are linked by an ontogenetic sequence and 4) the occurrence of the structures coincides with the occurrence of other homologs. Rieger and Tyler then suggest weighing the "homology theorem" with an "analogy theorem" in which the following features are appraised: 1) the structures are under similar selective pressure, 2) the structures are composed of similar materials which are exposed to similar environmental influences, 3) the structures are the only (or one of the few) possible ways by which the required function could be fulfilled, and 4) the structures differ

ontogenetically.

The similarities of the mechanical requirements placed on cnidarian tonofilamentous systems, the few appropriate subcellular structures available to meet that mechanical stress and the observed differences in ontogeny (Fig. 24), at least between _Aurelia_ desmocytes and the other desmocytes, all support analogy rather than homology of cnidarian desmocytes. However, the data are too scanty to discuss satisfactorily Rieger and Tyler's (1979) criteria for the homology versus analogy question between various cnidarian tonofilament systems. More thorough studies on more cnidarians are required before conclusive comments can be made.

Nevertheless, the morphological and functional similarities between the tonofilaments of _Leptogorgia_ and vertebrate tonofilaments suggest this to be an area for interesting comparative biochemical investigation.

Figure 1. Light micrograph of a cross-section of the axial skeleton (AXIS) and surrounding soft tissues of Leptogorgia virgulata . The loose connective tissue, the mesoglea (M), lies between the skeletogenic epithelium (E) and the cells lining the gut (G). The mesogleal extension (arrow) invades the skeletogenic epithelium to almost reach the skeleton. The pectinate portion of a desmocyte (not seen at this low magnification) separates the mesogleal extension and skeleton. Bar = 40 um.

Figure 2. Transmission electron micrograph of the pectinate portion of a desmocyte separating mesoglea (M) and axial skeleton (AXIS). The dense, cytoskeletal rods (arrowheads) are characteristic of desmocytes. The non-pectinate portion of the desmocyte (D) contains a loose meshwork of microfilaments and ribosomes (arrow). Bar = 1 um.

Figure 3. Scanning electron micrograph of the axial skeleton's surface from which soft tissue has been stripped. Still adherent to the skeleton (AXIS) are groups of desmocyte cytoskeletal rods (arrowheads). Bar = 2 um.
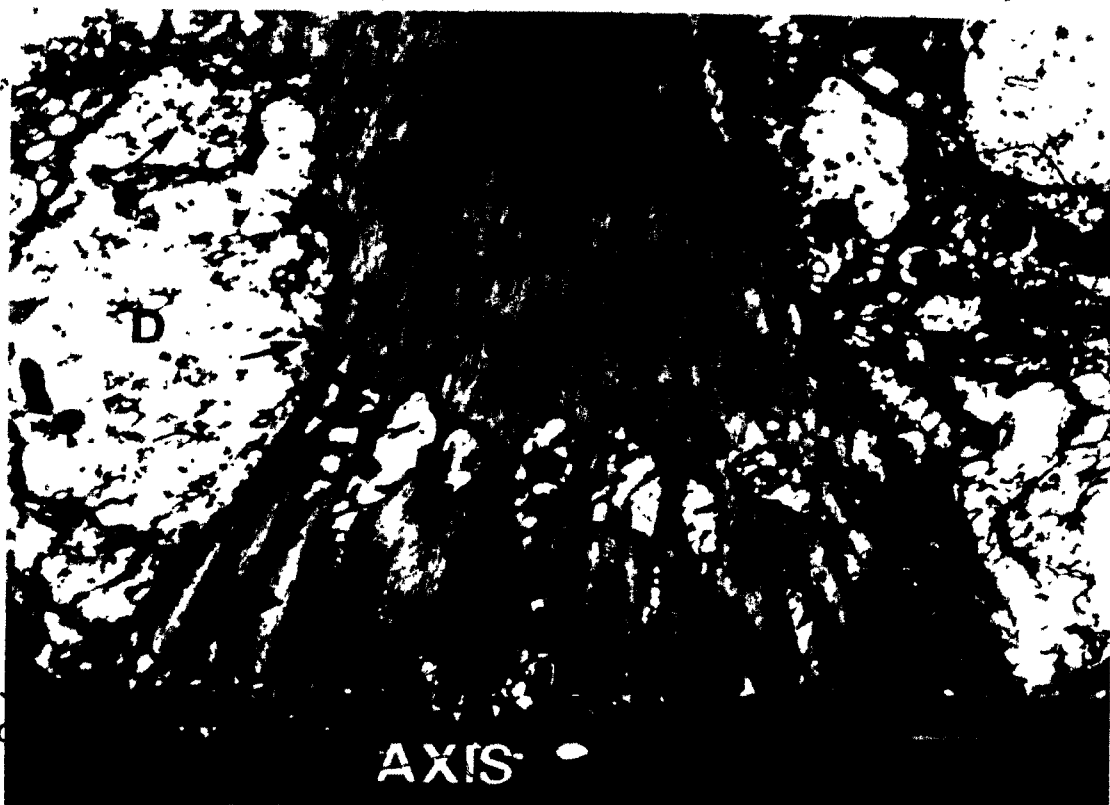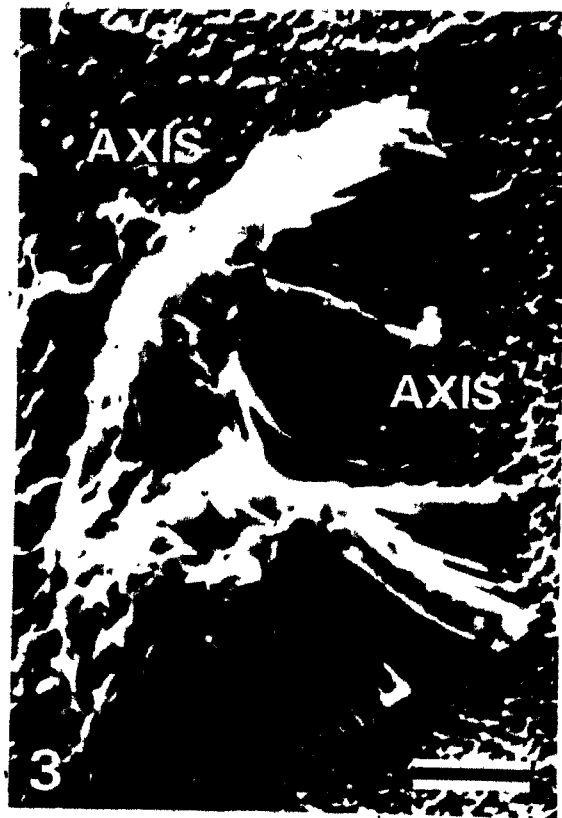
Figure 4. Transmission electron micrograph of skeletogenic epithelial cells shown in cross-section in a region where cells are starting to display changes characteristic of desmocyte formation. These cells display multiple folds in the plasma membrane and an increasingly vacuolated cytoplasm (V). Bar = 2.5 μm.

Figure 5. Transmission electron micrograph of a cross-section of the skeletogenic epithelium in a region where cells are nearing completion of desmocyte formation. The cells have invaginated so that an extension of the mesoglea (M) passes down into the center of the cell. The membrane folding is reduced as the cell invaginates (arrows). Few structures other than nuclei (N) are apparent in the cytoplasm. A part of a mature desmocyte is also shown (D). Bar = 5 μm.

Figure 6. Transmission electron micrograph of a longitudinal section of the skeletogenic epithelium with developing desmocytes. There is a marked difference in the lucent cytoplasm of the developing desmocyte (D) displaying a loose mesh of microfilaments and the dense cytoplasm of a skeletogenic cell (S). M = mesoglea. AXIS = axial skeleton. Bar = 5 μm.

Figure 7. Transmission electron micrograph of a Golgi body in a developing desmocyte. The cup-shaped vesicles and double vesicles surrounding the Golgi body suggest these vesicle types are Golgi body derivatives. Bar = 0.33 μm.

Figure 8. Transmission electron micrograph of an almost tangential section of the axial skeleton (A) and a developing desmocyte. Double vesicles are joined by 5 nm microfilaments (arrowheads). Bar = 0.5 μm.
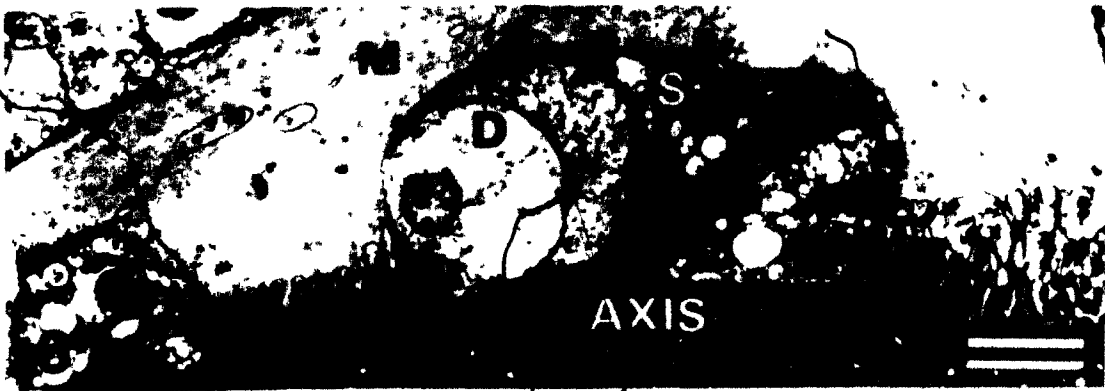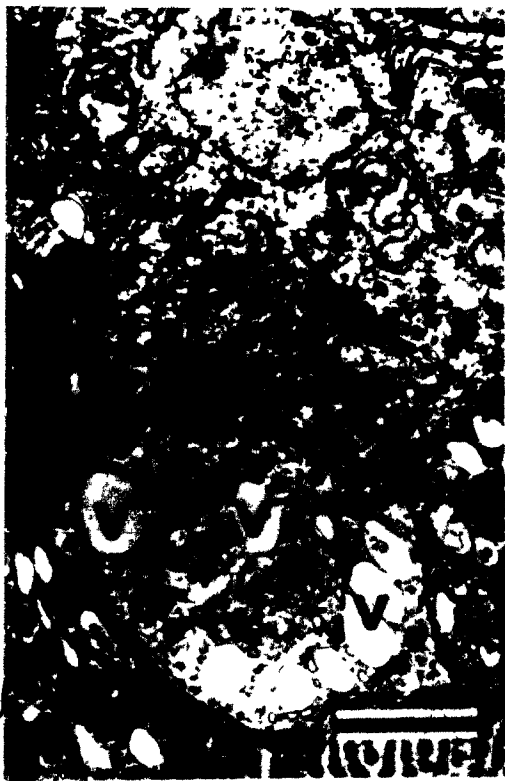
AXIS

Figure 9. Transmission electron micrograph of a longitudinal section of a developing desmocyte. The plasma membrane along the mesogleal extension (M) appears to be drawn (arrowheads) toward the axis end of the cell. At the axis end, cytoskeletal rod formation preceeds completion of invagination. Flocculent vesicles (F) are still a prominent feature of the cytoplasm at this stage. The central portion of one double vesicle shown has become dense (arrow). Bar = 1.0 µm.

The area between the the brackets is shown at higher magnification in Figure 10.

Figure 10. Higher magnification of the area between the brackets between the Fig. 9. Near the developing cytoskeletal rods are double vesicles (V), ribosomes (arrowheads) and microtubules (arrows). Notice that the rods are splayed at their axis ends. A = axial skeleton. Bar = 0.3 µm.

Figure 11. Transmission electron micrograph of a longitudinal section of a developing desmocyte. A row of double vesicles (arrowheads) is between folds of invaginated plasma membrane where cytoskeletal rod formation takes place. Cytoskeletal rod formation is nearly complete in examples to the left. The invaginated membrane at the mesogleal surface of the cell nearly meets the membrane at the skeletal surface of the cell at sites

between the cytoskeletal rods (arrows). * = extracellular,
mesogleal space. Bar = 1 μm.

Figure 12. Transmission electron micrograph of a
cross-section of a developing desmocyte. The central
portion of the double vesicles has become dense (arrowhead)
while the outer limiting membrane of the vesicles becomes
closely applied to the plasma membrane. * = extracellular,
mesogleal space. Bar = 1 μm.

Figure 13. Transmission electron micrograph of a cross-section of the lateral margins of two adjacent, mature desmocytes. Note that the filamentous cell coat is heaviest over sites where cytoskeletal rods are found. The invaginated membrane appears to be pulled or anchored by microfilaments (arrows) which extend to the lateral portion of the cell membrane. Note the heavy coat of the inner leaflet of the lateral plasma membrane (arrowhead). * = extracellular space. Bar = 0.5 μm.

Figure 14. Transmission electron micrograph of a cross-section of skeletogenic epithelium near the axial skeleton's surface. The radial array of cytoskeletal rods is shown in the discoid desmocyte (D). The electron dense areas are cytoplasmic rods in cross-section and the intervening light areas are extracellular regions continuous with the mesoglea. Notice the greater surface area of the skeleton covered by the desmocyte than by an adjacent skeletogenic cell (S). Bar = 3.3 μm.

Figure 15. Scanning electron micrograph of the skeletal surface from which a desmocyte (D) has been removed. The ends of the cytoskeletal rods make imprints on the skeletal surface. The reticulate surface of the skeleton beneath the desmocyte does not display the same fibrous appearance as the skeleton beneath the skeletogenic cells (F). Bar = 3.3 μm.

Figure 16. Transmission electron micrograph of a longitudinal section of the pectinate portion of a desmocyte. The mesogleal fibers (arrows) are not in direct contact with the desmocyte membrane but are joined to the fibrillar cell coat (arrowheads). The longitudinal striations of the cytoskeletal rods are evident. Bar = 0.5 µm.

Figure 17. Transmission electron micrograph of longitudinal section of a desmocyte's cytoskeletal rods buried deep in the skeleton. The rods are still attached to the skeleton (arrows) and it appears that some of the fibrillar coat (arrowheads) may still be intact. Bar = 1 µm.
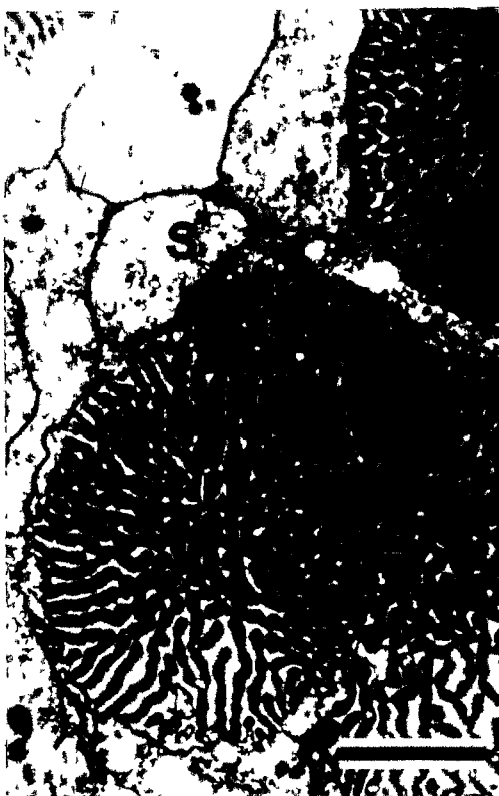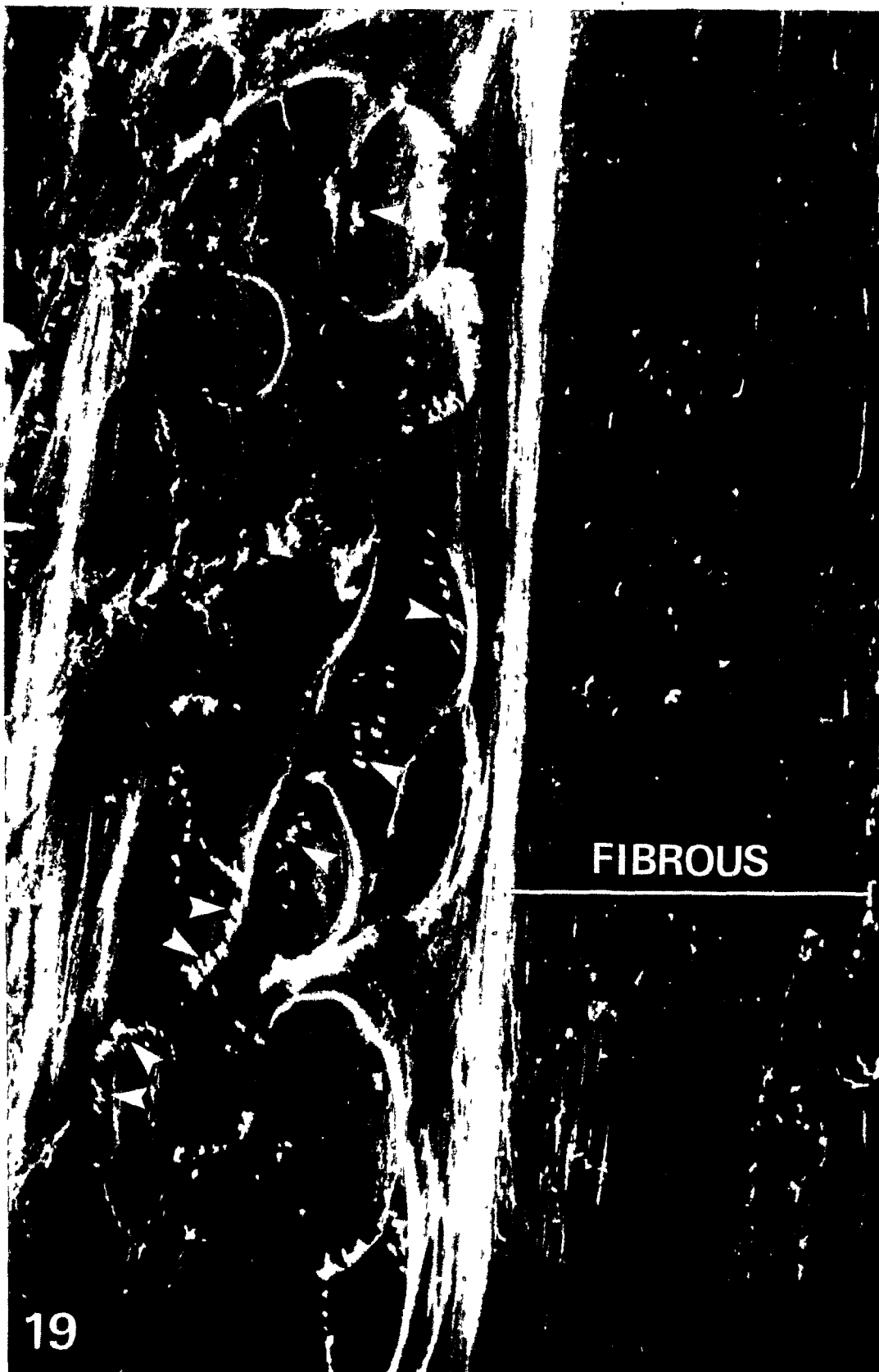
Figure 18.   Transmission electron micrograph of a
cross-section of a cytoskeletal rod.   Some of the
constituent tonofilaments are cut in cross-section so that
their circular profile surrounding a less dense central
region containing a dense, central core can be seen (white
boxes).   Between the black brackets many tonofilaments can
be seen in cross-section.   Bar = 100 nm.

Figure 19. Scanning electron micrograph of axial skeletal
surface. This site is several centimeters from the branch
tip. The oval depressions on the skeletal surface are
locations from which desmocytes were stripped. The small
white rods protruding from these depressions (arrows) are
cytoskeletal rods. The desmocytes occur in clusters at
this distance from the branch tip. An adjacent region of
the skeletal surface is fibrous and shows no desmocyte
depressions. Bar = 20 μm.

FIBROUS

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Figure 20. Transmission electron micrograph of desmocyte
cytoskeletal rods (D) and associated structures in a
section treated with the periodic acid - silver methenamine
technique for non-acidic glycosaminoglycans. The mesogleal
fibers which lie between the rods of the desmocyte's
pectinate portion have obtained a beaded appearence from
the silver grains (arrows). The desmocyte's fibrillar coat
and desmocyte-skeleton interface (I) bind little, if any,
silver. Bar = 240 nm.

Figure 21. Transmission electron micrograph of a desmocyte and subjacent axial skeleton (AXIS) after treatment with the silver methenamine reaction. A. The desmocyte appears denser than the skeleton in unstained tissue. The arrowheads indicate the desmocyte-skeleton interface. Bar = 1 um. B. The same section as Fig. 21A except showing the desmocyte-skeleton interface (arrowheads) at a higher magnification. The silver grains seen at the interface are not observed in tissue pre-treated with sulfhydryl blocking reagents. The interfacial material is therefore thought to be sulfhydryl rich. The other silver grains which appear in Fig. 21A are unaffected by sulfhydryl blocks. Bar = 250 nm.

AXIS

DESMOCYTE

2la

AXIS

TE

2lb

Figure 22. Diagram showing several steps in desmocyte
development. A. Double vesicles joined by microfilaments
are arranged at the axis end of the cell. B. More double
vesicles come to lie in rows over each of the vesicles at
the axis end (only one of the rows is shown here).
Microtubules (arrows) lie nearly parallel to the rows of
double vesicles. The center of some vesicles becomes more
electron dense. The plasma membrane at the mesoglea end
nears the axis. C. Cytoskeletal rods (r) have replaced the
rows of vesicles. Some of the rods are splayed at the axis'
end. The membrane at the mesoglea end is now closer to the
axis. D. Cytoskeletal rod formation and cell invagination
are complete. One cytoskeletal rod (R) is shown. The
plasma membrane has a fibrillar coat and a layer of
interfacial material (*) lies between the skeleton and the
desmocyte. The membrane (arrowheads) at the mesoglea and
axis ends of the cell are separated by a small distance. A
= axial skeleton.

mesoglea

22a   b   c

r

R

d   A

Figure 23. Model of a cytoskeletal rod and associated structures. The rod (r) is surrounded by the plasma membrane bilayer (M). Two of the fibrils of the fibrillar coat (C) are shown attached to the membrane at an angle λ, which is about 55° from the y-axis. The fibrils are shown disproportionately long. The collagen fibers transmit the force, F_T, to the fibrils in a direction parallel to the y-axis.

Y-AXIS

Ft

C   C

γ

X-AXIS

M→

axis

23

Figure 24. Diagram showing the cytological changes associated with desmocyte formation in: Class Hydrozoa (Cordylophora; Marcum and Diehl, 1978), Class Scyphozoa (Aurelia; Chapman, 1969) and Class Anthozoa (Leptogorgia; this study). The sequence of changes in each diagram occurs from left to right.

A. Class Hydrozoa

    1) secretory cell

    2) cytoskeletal rods appear with 7 nm fibers attached,

    3) channels form in the cell's mesoglea end,

    4) the cell becomes embedded in secretory products of adjacent cells.

B. Class Scyphozoa (some stages speculative)

    1) secretory cell

    2) tonofibrillae appear at the core of the cell,

    3) the tonofibrillae become a dense rivet, pits form in the head of the rivet and the margins of the cell are pulled up as the adjacent cells build up secretory material around the embedded rivet,

    4) the rivet is deeply embedded until only a dead remnant of the parent cell remains.

C. Class Anthozoa

    1) secretory cell

    2) multiple folds form in the plasma membrane,

    3) the cell begins to invaginate near the center

of its mesoglea end while a few cytoskeletal
rods form at the axis end of the cell,

4) invagination has resulted in the membrane being
drawn between the cytoskeletal rods until the
membrane at the mesoglea end of the cell lies
near the membrane over the skeleton,

5) the cytoskeletal rods dominate the cell and the
mesoglea fibers are found in the interstices of
the pectinate margin of the cell.

mesoglea

axis

24a

mesoglea

axis

b

mesoglea

axis

c

Conclusion

In the introduction to this thesis the relationships
between gorgonian skeletal growth patterns and the animal's
ecological niche were discussed. There it was mentioned
that although the significance of the final form is
appreciated, the mechanism of skeletal growth was poorly
understood and was based upon generally unsubstantiated and
contradictory observations. This study has shown
experimentally many cellular aspects of gorgonian
skeletogenesis (Table I) which will now be summarized
briefly.

Tall columnar, axial epithelial cells, called
medullocytes, are found at Leptogorgia's skeletal branch
tips. These cells secrete a lipoprotein to form
intramedullary crossfibers, lamellae and the medullary
wall. The production of these structures lengthens the
skeleton.

Shorter cells, called corticocytes, then secrete
procollagen onto the medullary wall via flocculent vesicles
to thicken the skeletal cortex. Whether an ontogenetic
relationship exists between medullocytes and corticocytes
is unproven.

Subjacent to the corticocytes, newly secreted collagen
appears as 3 nm diameter, unbranched microfibrils which
aggregate to form 50 to 70 nm diameter fibrils displaying a

26 nm axial periodicity. Fibrils wound in right handed helices form 0.5 to 1 μm diameter fibers which are aligned at small angles to the long axis of the skeleton. Rectangular parallelipiped crystals are embedded in the fibers.

Corticocytes also secrete dense-cored vesicles containing a hydroquinoid, collagen-crosslinking compound. The dense-cored vesicles are accompanied by smaller, DOPA oxidase-containing vesicles which oxidize the hydroquinone extracellularly to form the crosslinking compound.

The degree of collagen crosslinking, as estimated by skeletal tyrosine concentrations, is more than twice as great at sites near the skeletal base than at the skeletal tip. Collagen concentration, as estimated by hydroxyproline measurements, is slightly less in mature cortex than in young cortex. The inorganic skeletal fraction, comprised almost entirely of calcium, magnesium and phosphates, is constantly about 35% of total skeletal weight from skeletal tip to base.

Desmocytes are differentiated from corticocytes. Desmocytes develop by spreading on the skeleton to form discoid cells with pectinate margins facing the mesoglea. As desmocytes develop, an array of cytoskeletal rods forms perpendicular to the skeletal surface; a layer of sulfhydryl-rich interfacial material is found between the desmocyte and skeleton; and, a fibrillar surface coat is

added to the plasma membrane of the cell's pectinate margin. Desmocytes are more prevalent nearer the skeleton's base than at the tip.

Although these findings provide insight into the process of gorgonian skeletogenesis and thereby provide a mechanism for the observed growth patterns, the regulatory mechanism for different aspects of skeletogenesis is still unknown.

Leptogorgia skeletogenesis provides a system to explore some basic questions in cell biology. One question is: what factors influence the changes in the type and amount of structural proteins synthesized? Genetically determined differentiation may explain the change in synthesis of cytoskeletal proteins (desmocyte tonofilaments) and extracellular skeletal proteins (collagen and crosslinking compound). An alternative explanation is that the mechanical environment may influence the pattern of protein synthesis. If a cell could modify its synthetic activities in response to applied force, cells and structural materials adapted to a particular stressful environment could be produced.

The proposal that the mechanical environment can influence the production of structural proteins is appealing. If this were to occur, the cell or nearby extracellular material could act as a transducer of mechanical energy to chemical energy.

Piezoelectricity may be involved in the mechanical
environment's regulation of cell behavior. Although there
is absolutely no evidence to indicate a piezoelectric
effect in Leptogorgia's skeleton, it has been believed to
be a "fundamental property of biological tissues" by some
investigators (Shamos and Lavine, 1967). In organic
material, piezoelectricity is thought derived from shear
stress placed on long chain, fibrous molecules. Shamos and
Lavine (1967) have proposed that deformation of the
crosslinkages in such a molecule, e.g. collagen, can cause
a displacement of charge on the molecule. The resulting
potential difference may effect the division (Rinaldi
et al., 1974), migration (Harrington et al., 1974),
orientation (Katzberg, 1974)and cAMP production (Norton et
al., 1977) of cells on the substratum containing the
deformed molecules. The way that potential differences
affect these cellular functions is not known.

Recent, exciting work by Ben-Ze'ev, Farmer and Penman
(1980) has begun to show another possible way that the
mechanical environment can influence macromolecule
synthesis. These investigators found that when
anchorage-dependent murine fibroblasts were suspended in
methyl cellulose, synthesis of protein, DNA, mRNA and rRNA
stopped. By allowing the cells to contact the substratum,
protein synthesis would begin again but nucleic acid
synthesis would begin only if the cells were allowed to
spread. They inferred from these findings that these cells

are sensitive to surface contact and to shape changes and respond differently to each stimulus.

Since the studies of Ben-Ze'ev and his colleagues (1980) used haploid, contact-dependent fibroblasts in vitro, extrapolation of the results to normal, in vivo cells' activities could be inaccurate. However, in that same paper, they report tantalizing personal communications saying the "spectrum of synthesized proteins" by epithelial cells (E. Griepp and D. Sabitini) and chondrocytes (J. Folkman) can be "radically changed" by cell-shape changes. Hopefully, the next few months will see the publication of Griepp, Sabitini and Folkman's studies and they may provide stronger evidence for a relationship between a cell's shape and its synthetic activities.

The possibility that cell-shape changes caused by stresses placed on Leptogorgia's axial skeleton could affect skeletogenesis and desmocyte differentiation is intriguing. The value of this study on Leptogorgia's skeletogenic epithelium is that distinct, quantifiable, subcellular structures have been associated with the synthesis of several functionally-defined structural materials. The results of this thesis provide a means for studying and measuring morphologically the effects of mechanical stresses on macromolecular synthesis in vivo. As stated by Ben-Ze'ev and his colleagues: "it is quite possible that shape and contact signals regulating

macromolecular metabolism are a fundamental part of cell

behaviour in the formation of metazoan tissue."

TABLE I

Summary of changes in <u>Leptogorgia</u>'s skeletogenic, epithelial cell behavior and skeletal chemistry from sites at the skeletal tip to sites nearer the skeletal base.

	Skeletal tip	Nearer skeletal base
<u>Epithelium</u>		
1. Lipoprotein secretion	+	-
2. Collagen secretion	-	+
3. DOPA secretion	-	+
4. Desmocyte differentiation	-	+
<u>Skeletal chemistry</u>		
1. Lipoproteins	+	-
2. Collagen (Hyp.)	-	+
3. Crosslinking (Tyr.)	-	+
4. Crosslinking (DOPA)	-	+

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