A FINE STRUCTURE STUDY OF THE ANTHOCODIUM IN *RENILLA MÜLLERI*

Evidence for the Existence of a Bioluminescent Organelle, the Luminelle

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ABSTRACT

A fine structure study of the anthocodium of the sea pansy, *Renilla mülleri*, was undertaken. The anthocodium, a known site of bioluminescence, was selected in order to determine whether a structural entity could be found which would satisfy the biochemical and physiological features associated with the known sites of bioluminescence in this animal. These sites, termed lumisomes, have previously been shown to be small (~0.1–0.2 μm), membrane-enclosed vesicles which contain all the proteins necessary for bioluminescence and its immediate control. One of the lumisomal proteins is an intensely green fluorescent protein and has been used as a probe for the detection of the cellular sites of bioluminescence. This green fluorescence was associated only with gastrodermal cells. We report the identification of a unique morphological entity, restricted to the cells of the gastrodermis, which satisfies the biochemical and physiological requirements for bioluminescence in *Renilla*. It is a large (~4–6 μm), membrane-bounded subcellular organelle comparable in size to a subcellular structure whose green fluorescence is typically associated with the in vivo bioluminescence. Furthermore, it is filled with smaller membrane-bounded vesicles which have the same size and shape as the lumisomes. We suggest that the organelle identified in this study be termed a luminelle.

Renilla (sp), a colonial marine coelenterate commonly called a sea pansy, has been extensively investigated by biochemists to determine the chemical requirements for bioluminescence (1, 2, 7, 11, 27) and by physiologists to probe the nature of its response mechanism (3–6, 18, 19, 21–23). Physiological, histological, and biochemical investigations have provided good evidence that bioluminescence is intracellular (2, 5, 14, 17, 25).

Bioluminescence in *Renilla* is due to the luciferase-catalyzed oxidation of luciferin (11). Anderson and Cormier (2) have isolated a membrane-bounded, subcellular particle containing luciferase, a luciferin-binding protein with its associated luciferin and a green fluorescent protein all of which are necessary for bioluminescence and its control. When subjected to hypotonic solutions of calcium (2), these particles produce a flash of green light

identical to the in vivo luminescence of *Renilla*. These particles, identified as the apparent sites of bioluminescence in *Renilla*, have been termed lumisomes (2).

Biochemical evidence (1, 2) suggests that the organism controls its bioluminescent flash by exerting fine control over the movement of calcium to the luciferin-binding protein sites within the lumisomes. Calcium binding to the luciferin-binding protein results in the release of the bound luciferin which is then oxidized by luciferase to produce light. The light appears green due to energy transfer to a green fluorescent protein (16, 27) which has been isolated and studied by Wampler et al. (28).

As outlined above, biochemical studies show that the sites of bioluminescence in *Renilla* are always associated with presence of the green fluorescent protein. Furthermore, Morin and Reynolds (17) and Reynolds (25), employing image intensification techniques, have shown an exact correspondence between the sites of bioluminescence and the green fluorescent protein in *Renilla kollikeri*. This intensely fluorescent protein exhibits a sharp fluorescence emission that is maximum at 509 nm and is identical to the in vivo bioluminescence emission (27).

The characteristic green fluorescence associated with the light-producing cells (photocytes) in *Renilla* has been used as a probe in these studies in order to identify, by electron microscopy, the location and morphological features of *Renilla* photocytes. In addition, a fine structure study of the anthocodium, a portion of the autozooid that projects above the coenenchyme and one of the known sites of bioluminescence, was undertaken.

MATERIALS AND METHODS

Renilla mülleri obtained from Gulf Specimen Company, Inc., Panacea, Fla. were maintained at room temperature in aquaria with circulating artificial sea water (Instant Ocean). Individual anthocodia were removed by dissection from anesthetized animals (1). The tissues were immediately placed in cold 2% glutaraldehyde/0.1 M cacodylate buffer + 1% CaCl (pH 7.2) for 2 h. Tissues were stored in a buffer wash of 0.1 M cacodylate + 5% sucrose. Individual anthocodia were further dissected to permit separation of the body wall and the tentacle-oral region for subsequent orientation in the embedding procedure. Selected samples were viewed with a fluorescence microscope (excitation max = 470 nm, and emission max = 509 nm) to determine the presence and location of the green fluorescent protein whose importance in the bioluminescence of *Renilla* has been outlined above. Tissues selected for use in the fine structure study were then postfixed in 1% osmium tetroxide/0.1 M cacodylate buffer, dehydrated in a graded series of ethyl alcohol, and embedded in Maraglas (26). Sections were cut on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), stained with lead citrate (24) and viewed in a Philips 200 electron microscope at 80 kv.

RESULTS

R. mülleri is a member of the phylum Cnidaria and the class Anthozoa. As described by Hyman (12), the organism consists of a primary polyp with a distal reniform-shaped rachis and a single proximal peduncle. Numerous secondary polyps are restricted to the superior surface of the rachis and establish a division of labor among the zooids for feeding-reproduction and circulation. The latter is accomplished by numerous clusters of tentacle-less zooids, called siphonozooids, which drive water through the colony. Water is removed from the colony by a single exhalant siphonozooid. The feeding and/or reproductive polyps are called autozooids. These polyps, typical of the subclass Alcyonaria, possess eight hollow pinnate tentacles with eight septa attached to the pharynx. All polyps are embedded in a common tissue mass, the coenenchyme.

The anthocodium of *R. mülleri* follows the general body plan of all Cnidaria: an outer epidermis, an inner gastrodermis and, interposed between the two, a mesoglea. The nerve net and the muscular system, which consists of epidermal longitudinal fibers and gastrodermal circular fibers, will be described below. In order to clearly define modifications in this basic pattern as they relate to specific functional variations, the anthocodium has been divided into two regions: the body wall and the oral-tentacle region. A cross section through the external body wall (Fig. 1) depicts the three distinct layers. The cells of the epidermis appear to have a protective function and are characterized by the presence of numerous short microvilli at the free surface (Fig. 2 *a*). The mesoglea has a highly complex matrix with amoebocytes scattered throughout (Fig. 2 *b*). The gastrodermal cells which line the interior of the body wall have cilia and/or elongated cytoplasmic extensions at their apical surface (Fig. 2 *c*). Fig. 3 is a cross section through a portion of the external body wall at the junction of one of the eight septa which divide the animal into eight distinct chambers. The epidermis of the body wall continues

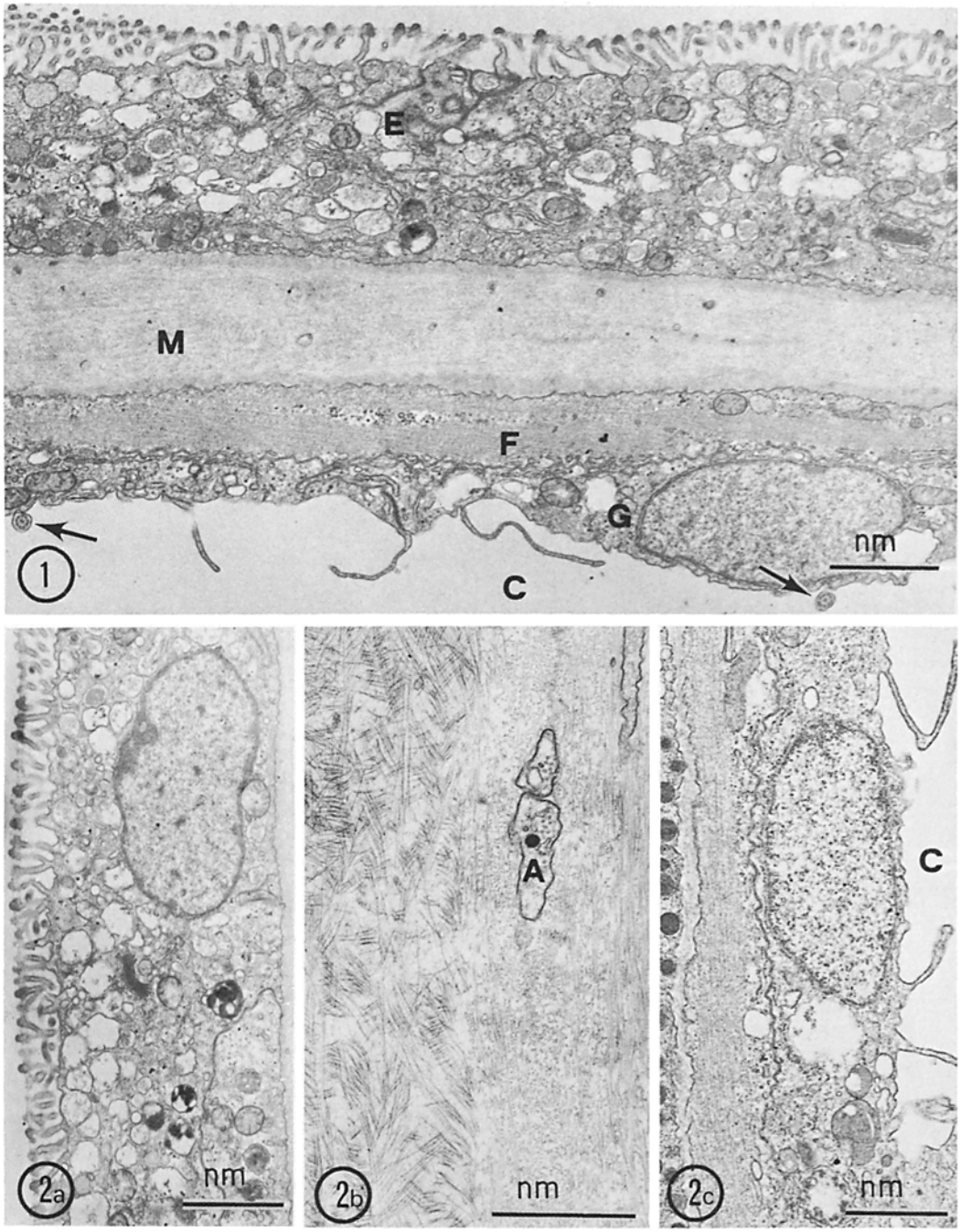


FIGURE 1 A cross section through the body wall of the anthocodium. The epidermis (*E*) is separated from the muscle layer (*F*) by the mesoglea (*M*). The gastrodermis (*G*) lying adjacent to the muscle layer contains many cilia (arrows) and/or cytoplasmic extensions which project medially into the gastrovascular cavity (*C*). × 15,000.

FIGURE 2 (*a*) The epidermal cells of the body wall contain numerous short apical microvilli projecting toward the external surface. × 15,000. (*b*) The mesoglea is made up of numerous small filaments embedded in an electron-transparent matrix that contains amoebocytes (*A*) scattered throughout. × 21,000. (*c*) The apical surface of the gastrodermal cells contains elongated cytoplasmic extensions and/or cilia that extend into the gastrovascular cavity (*C*). × 7,200.

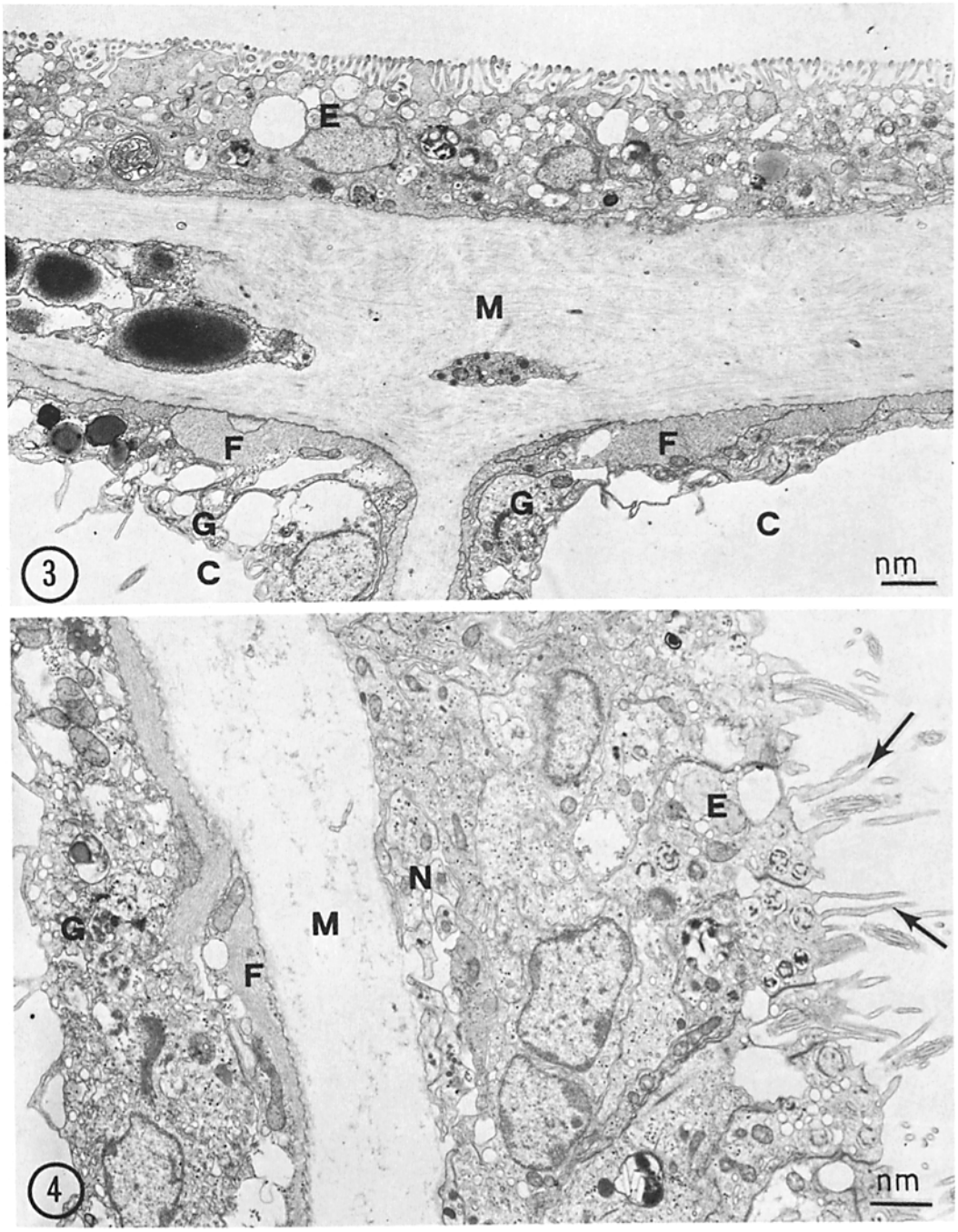


FIGURE 3 A cross section of the body wall at a septum. The septum, which extends medially to the pharynx and separates adjacent gastrovascular cavities (*C*), is composed of two layers of gastrodermis (*G*) separated by the mesoglea (*M*). × 7,200.

FIGURE 4 A cross section through the pharynx. The epidermis (*E*) contains numerous flagella that are surrounded by characteristic cytoplasmic extensions (arrows). In the subepithelial region structures resembling neurites (*N*) are seen.

uninterrupted across the septal junction and the two visible adjacent chambers. Gastrodermal muscle fibers of the body wall, and retractor muscles of the septum are seen. The mesoglea and the gastrodermal cells of the body wall are contiguous with complimentary cells of the septa. Body symmetry is established by the eight septa which, with their mesoglea, extend inward from the body wall to become continuous with the mesoglea, middle layer, of the pharynx. The gastrodermal cells also follow the path of the mesoglea and, upon reaching the pharynx, continue their role as endodermal cells lining the entire coelenteron, or gastrovascular cavity. The tubular pharynx, lined by epidermal cells, extends downward from the mouth into the gastrovascular cavity and serves chiefly in the ingestion of food. The mesoglea and gastrodermis invest the pharynx to complete the basic three-layer plan. The pattern described above, three distinct separate layers, persists throughout the anthocodium. To simplify the identification of fine structural variations associated with functional changes, the entire epidermal surface will be described first, followed by a description of the gastrodermis. The following cell types are present in the epidermal layer: epidermal, mucous, interstitial, and cnidoblast cells. The mesoglea, muscle fibers, and nerve net will be described only when they vary morphologically.

Epidermis

The epidermal cells of the body wall and pharynx are continuous but have been modified in the latter to perform a new function, ingestion (Fig. 4). There is a significant increase in the thickness of the epidermis in the pharyngeal region. This is attributed to an increase in the total number of mucous, granular, interstitial and flagellated epidermal cells (Fig. 5). The presence of flagellated cells and their morphological similarity to the choanocytes, or collar cells (Fig. 6), long thought to be restricted to the Porifera, is of interest. Norrevang and Wingstrand (20) reported the presence of choanocyte-like cells in Echinoderms and cite the occurrence of similar cell types in a variety of other animals.

The epidermal cells of the oral region are active in capturing prey. The predominant cell type in this area, which is also diagnostic of the phylum, is the cnidoblast (Fig. 7). The cnidoblast identified as atrichous isorhiza (29) is the only type present in Alcyonaria (12). The oral epidermis is slightly thickened due to the increased number of cnidoblasts, mucous, and interstitial cells. The surfaces of these cells exhibit microvilli as noted in the cells of the body wall. Neurites and well developed epidermal muscle fibers are present. The cells of the nerve net (Fig. 8) are conspicuous in this area and are similar to the nerves identified in other coelenterates (8, 13). The large, clear nerve cells have characteristic neurofilaments and/or neurosecretory droplets scattered throughout their cytoplasm, and the nucleus has a distinct nucleolus. We have observed neurites only on the epidermal side of the mesoglea and, in addition, no synaptic junctions have been detected in this material. The epidermis of the tentacles becomes extremely thin, containing few cnidoblasts but numerous mucous cells. The number of Golgi apparatuses present in these cells is significantly increased (Fig. 9) when compared to the mucous cells of the body wall. The greater number of mucous cells is probably related to the production of mucus which entraps prey after it has been immobilized by the nematocysts. The apices of the epidermal cells have microvilli which are longer and, in some areas, thickened and blunt when compared to the microvilli observed in similar cells of the body wall.

Gastrodermis

The gastrodermis lines the inner surfaces of the tentacles and the body (12). A cross section through the gastrodermis, shown in Fig. 10, depicts cells of this layer in apposition to highly developed circular gastrodermal muscle fibers. The gastrodermal cells are responsible for the movement of nutrients throughout the colony. Their apical surfaces reflect this function and consist of elongated cytoplasmic extensions and/or cilia. The cytoplasmic constituents vary according to the cell's function, i.e., secretory production (Fig. 11 *a*), phagocytosis (Fig. 11 *b*), or the production of presumed digestive enzymes (Fig. 11 *c*). No cnidoblasts or neurites were observed in the gastroderm. The only deviation from the basic three-layer plan was found in the septa (Figs. 3 and 12) where gastrodermal cells line both sides of the structure. Retractor muscles are also present on the septa. Fig. 13 is a cross section through the body wall, exposing all three layers previously described. The most prominent feature observed in this micrograph is a gastrodermal cell containing a large membrane-bounded, cytoplasmic structure which encases a number of smaller membrane-

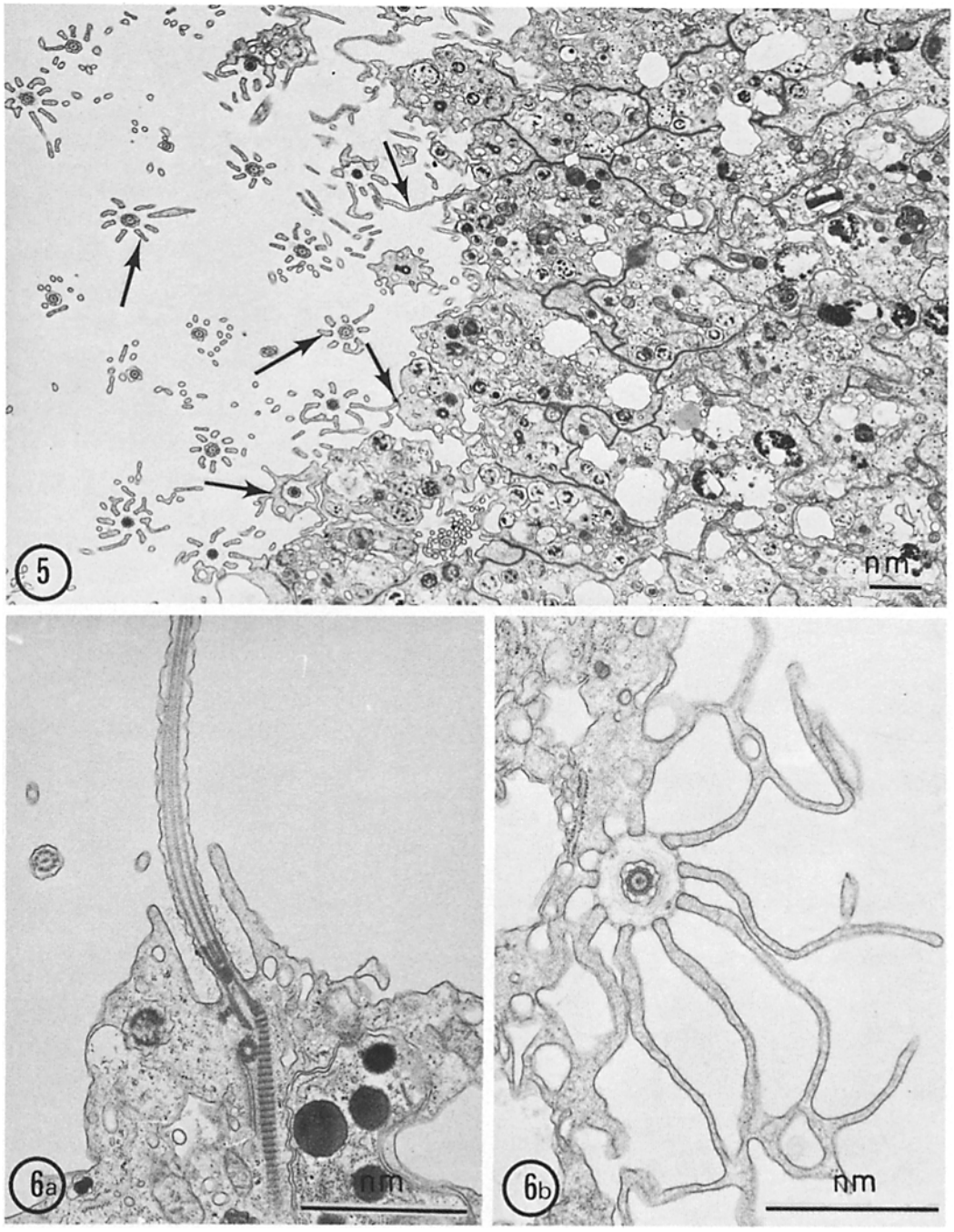


FIGURE 5 Pharyngeal epidermis containing numerous flagella surrounded by cytoplasmic extensions (arrows). × 7,500.

FIGURE 6 The pharyngeal epidermis showing a flagellum and cytoplasmic extensions at higher magnification in longitudinal view, 6 *a*, × 23,600; and in cross section, 6 *b*, × 25,000.

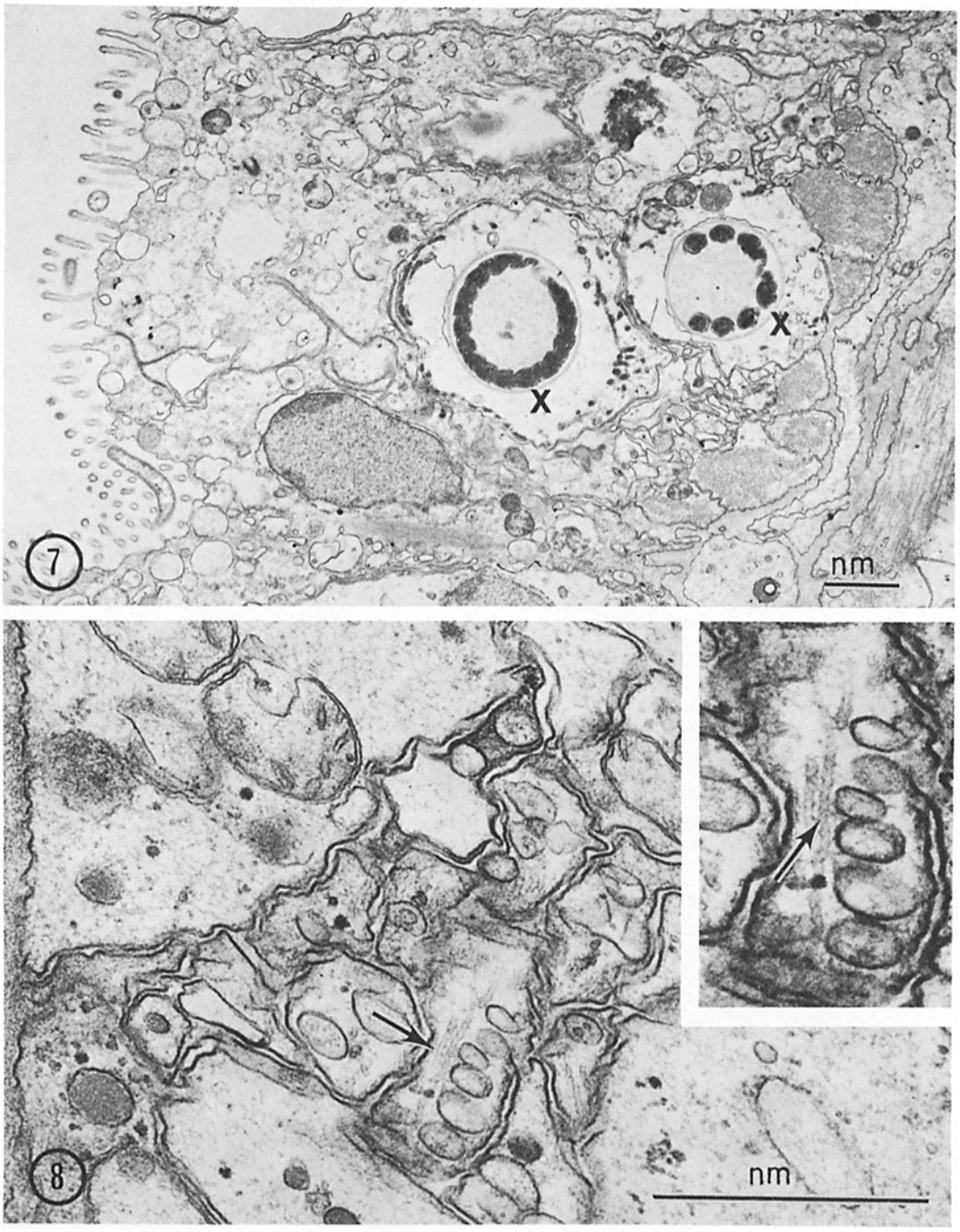
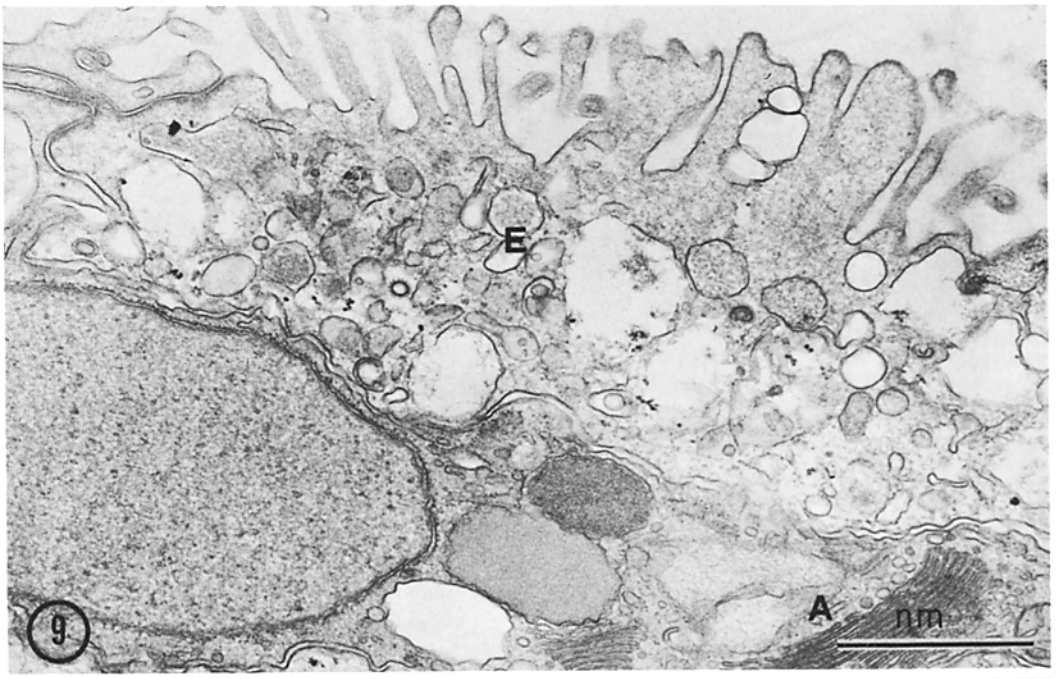


FIGURE 7 A section through the epidermis of the oral region. Note the microvillous surface and the undischarged nematocyst (*X*). × 8,000.

FIGURE 8. A section through the base of the pharyngeal epidermis showing nerve elements. × 42,000. Neurosecretory granules and neurofilaments (arrows) are present. (See *inset*.)



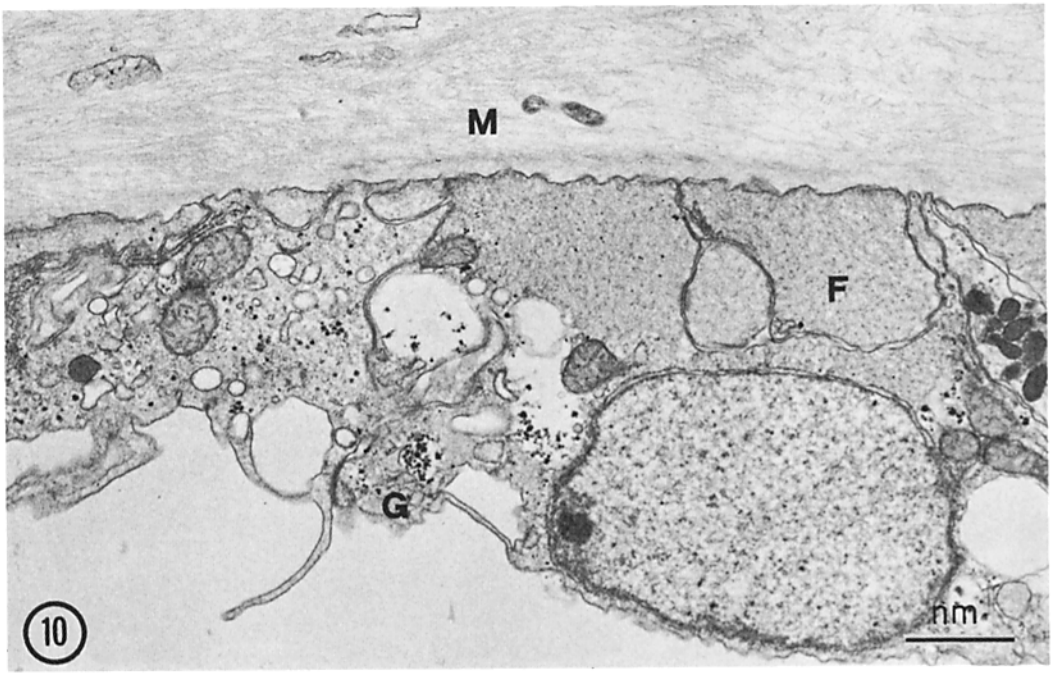
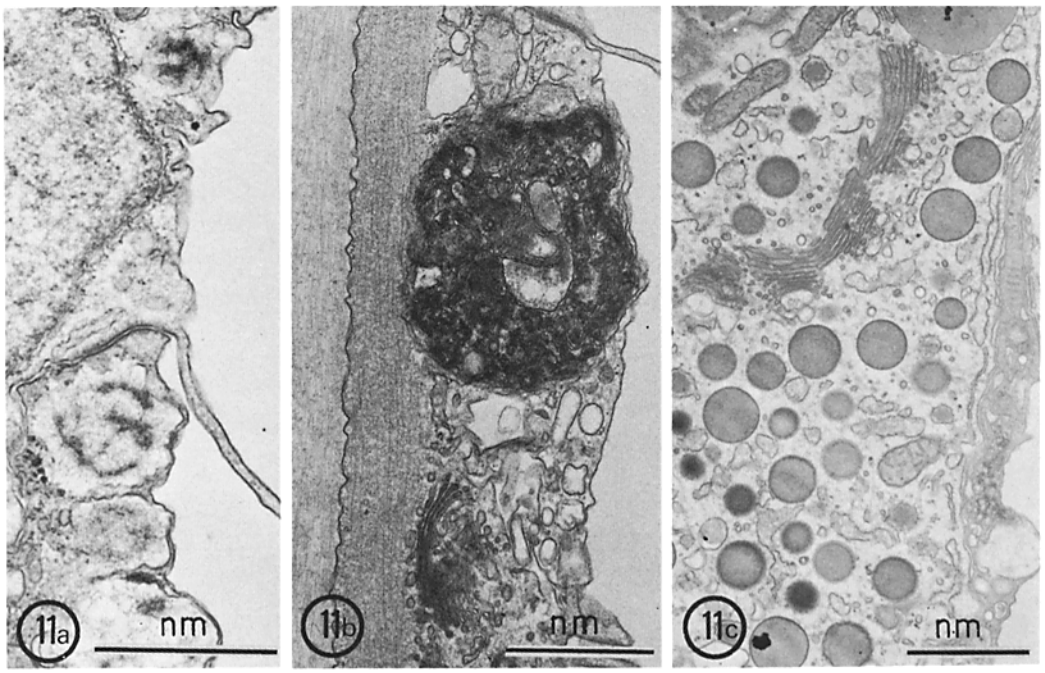


FIGURE 9 A section through a mucous cell in the epidermis of a tentacle showing a well developed Golgi apparatus (*A*) and blunt microvilli. × 26,000.

FIGURE 10 A section through a gastrodermal cell (*G*) illustrating the well developed gastrodermal muscle fibers (*F*) abutting the mesoglea (*M*). × 14,500.



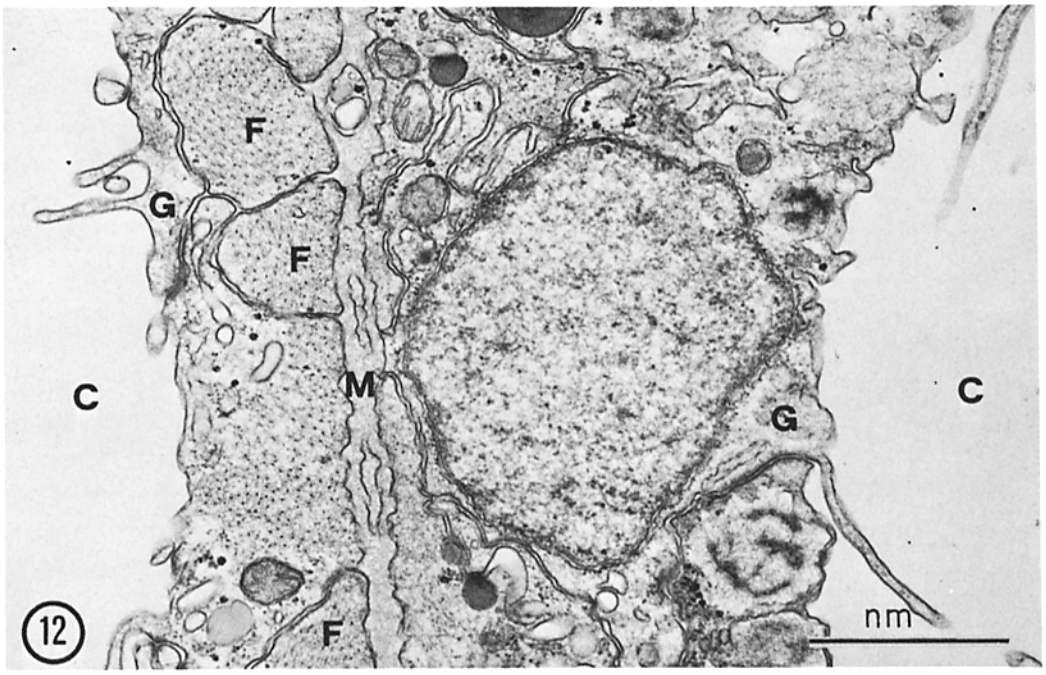


FIGURE 11 Sections through three cell types commonly found in the gastrodermis. (*a*) Mucous cell. × 27,500. (*b*) Phagocyte. × 19,750. (*c*) Secretory cell. × 13,500.

FIGURE 12 A section through the septal wall which separates two adjacent gastrovascular cavities (*C*). The gastrodermal cells (*G*) line both sides of the mesoglea (*M*). Prominent muscle fibers (*F*) are present. × 26,000.

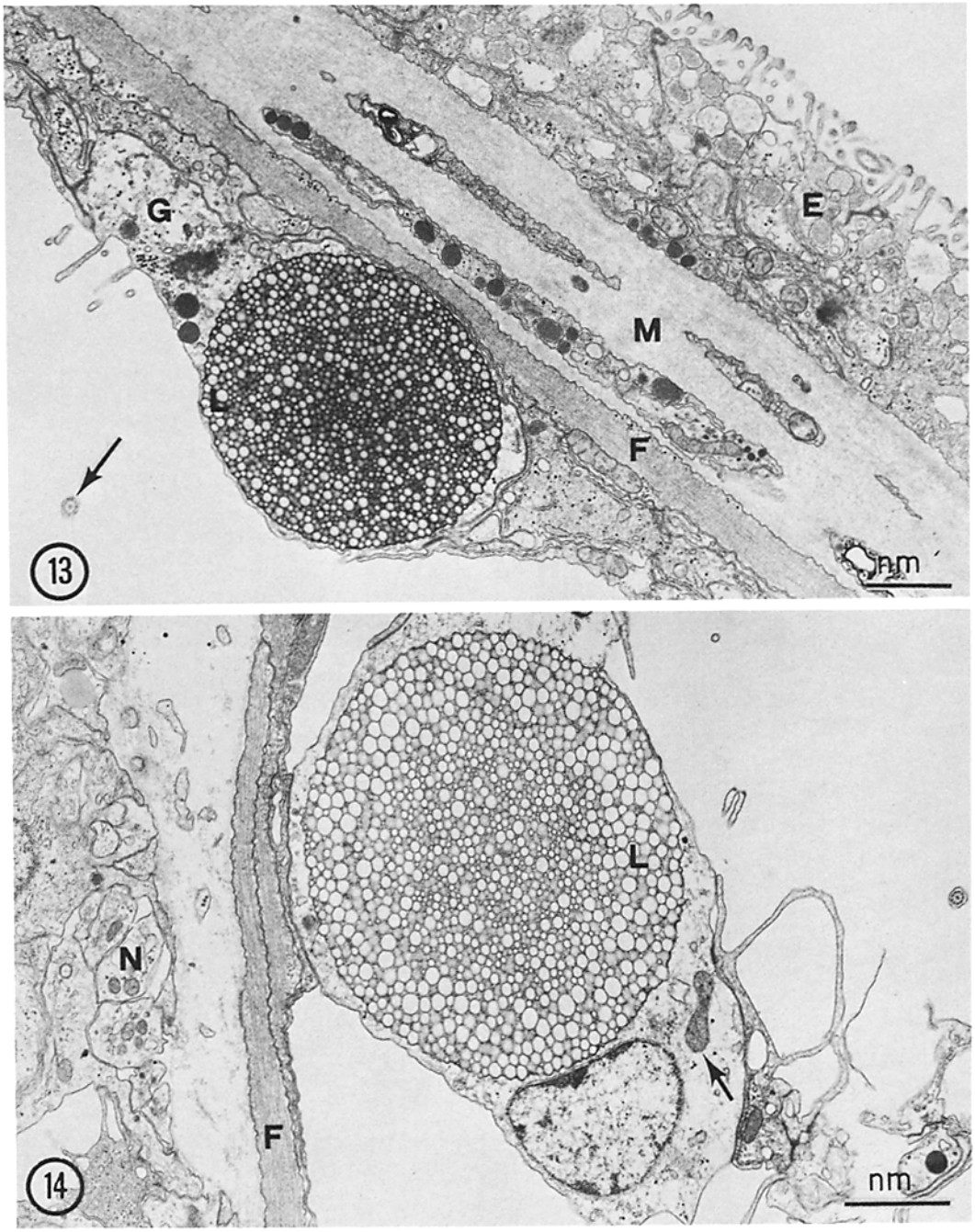


FIGURE 13 A cross section through the body wall similar to that in Fig. 1. Note the presence of a large intracellular structure, the luminelle (*L*), in a gastrodermal cell (*G*). Cilia (arrow). × 12,500.

FIGURE 14 A section through a gastrodermal cell in the pharyngeal region. Compare the size of the luminelle (*L*) to the nucleus and the mitochondria (arrow) present in the same cell. Nerve element (*N*). × 13,500.

bounded vesicles. A gastrodermal cell containing a similar structure, but found in the pharyngeal gastroderm, is seen in Fig. 14. A comparison of this structure to the nucleus and mitochondrion present in the cell provides a better appreciation of its enormous size. Note that this cell type is always appressed to a well developed muscle fiber. On the far side of the mesoglea in this micrograph is a structure resembling a neurite. Fig. 15 shows a gastrodermal cell of the septa containing a similar structure at an apparently earlier stage of development, and illustrates an active Golgi apparatus. Fig. 16 depicts another view of this structure in the tentacle. The material inside the structure has changed shape and becomes electron dense, but the membrane surrounding it and the cell membrane remain intact.

As outlined in the introduction, the sites of bioluminescence in *Renilla* are always associated with the presence of a green fluorescent protein. Using appropriate interference filters, this site, which always shows an intense green fluorescence with an emission maximum at 509 nm, can be located. Employing this method, we selected only tissues that exhibited green fluorescence, and then we determined by bright field and phase microscopy the size and location of the fluorescent material. Fluorescent structures were restricted to the gastroderm; they were apparently intracellular and significantly larger than the nuclei present in the same field. Furthermore, crude subcellular preparations of large, green, fluorescent, membrane-bounded structures have recently been prepared which produce flashes of green light when exposed to hypotonic calcium (Anderson and Cormier, unpublished results). The structures observed in Figs. 13–16 correspond in size and location to the green fluorescent structures observed by fluorescence microscopy.

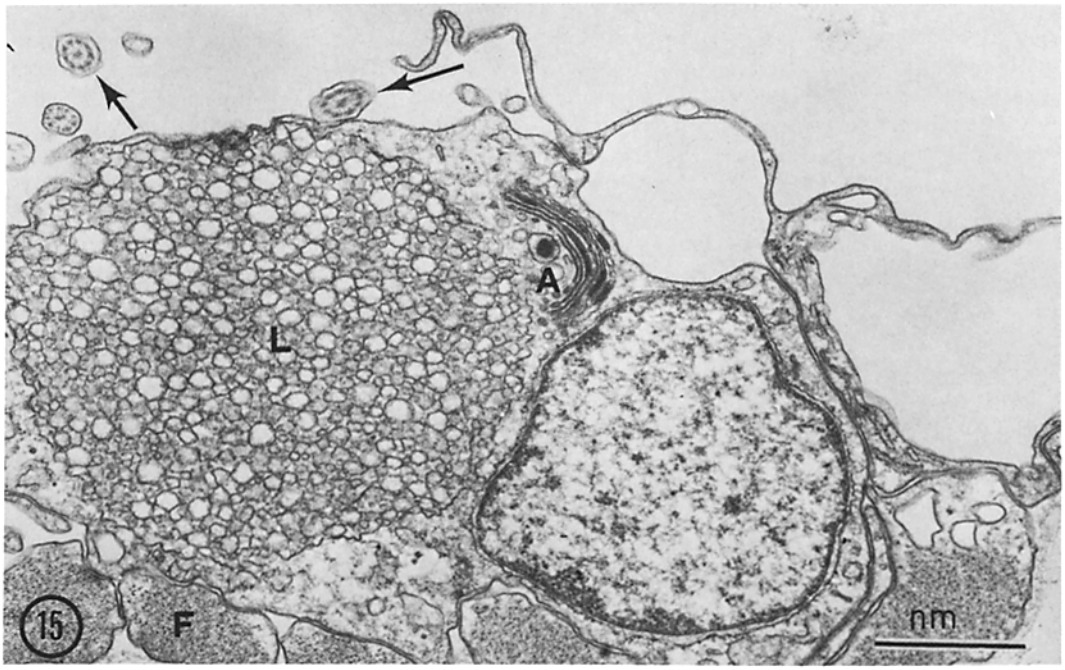
The muscular system of the anthocodium consists of longitudinal epidermal fibers, circular gastrodermal fibers, and the retractor muscles of the septa. The epidermal muscle fibers are poorly developed in the body wall. When specimens were oriented to provide longitudinal views of this area, only scant myomeres of the epithelio-muscular cells were detected. In the oral-tentacle region the number of epidermal muscle fibers are significantly increased. Numerous epitheliomuscular cells are also present. The gastrodermal muscle fibers, on the other hand, are very well developed throughout the entire anthocodium. The significance of this highly developed gastrodermal muscle system with respect to its possible involvement in the phenomenon of bioluminescence will be discussed below.

The nervous system in coelenterates is often referred to as a nerve net. Our findings suggest that the nervous tissue in the anthocodium is restricted to the oral disk region. The ultrastructural findings were similar to those reported in other coelenterates (8, 13). Neurofilaments and neurosecretory granules were observed (Fig. 8), but no synapses were detected. It should be noted that we were able to identify nervous tissue only on the epidermal side of the mesoglea and never on the gastrodermal side. We were unable to make a definitive identification of nervous tissue in any of the sections through the body wall.

DISCUSSION

A number of observations on the physiology of *Renilla* bioluminescence were made in the eighteenth and nineteenth centuries (10). Since then, numerous investigators have sought to determine the molecular basis for the luminosity of this colonial form. Most of the studies have involved defining the chemical basis for bioluminescence and its control, as well as the physiological aspects of light emission. However, there has been little or no emphasis on the morphological features associated with this phenomenon. We, therefore, made a fine structural study of a known bioluminescent area in an attempt to correlate some of the known physiology and biochemistry with specific structural entities.

Our electron microscope observations on the anthocodium revealed a general body plan consistent with that reported in other members of the phylum with minor modifications. The epidermal cells are characterized by numerous short microvilli at the apical surfaces. In the oral region, where these cells invaginate to form the pharynx, there is an abrupt change from a microvillous surface to a flagellated one. This morphologic change reflects a possible modification in physiological activity from a protective function to an ingestion function. This change becomes more significant in view of the similarity between these flagellated cells and choanocytes, or collar cells, long thought to be restricted to the phylum Porifera. Lyons (15)



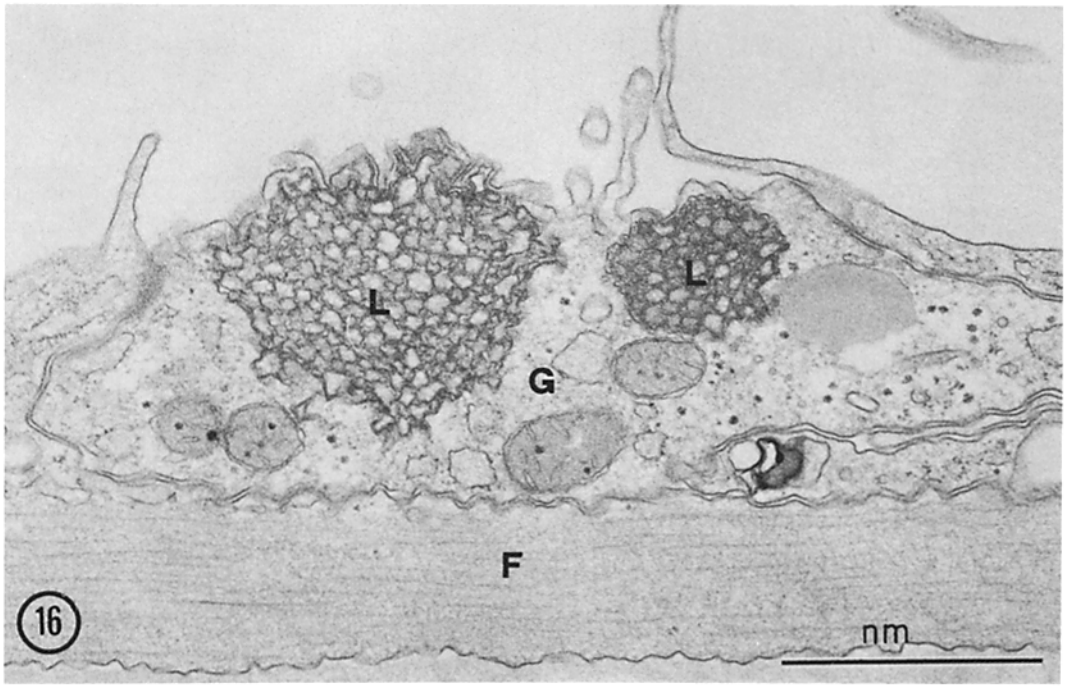


FIGURE 15 A section through a gastrodermal cell of the septum containing another luminelle (*L*). Note the muscle fiber (*F*) in cross section, flagella (arrow), and a Golgi apparatus (*A*). × 20,000.

FIGURE 16 A section through the tentacle showing the relationship of the gastrodermis (*G*) to the muscle (*F*). Note that the luminelle (*L*) is smaller, more dense, but remains intracellular. × 33,000.

recently reported the presence of choanocytes in the planula of *Balanophyllia regia*, a member of the class Anthozoa. In view of the location of the cells described here, the frequency with which they occur and their ultrastructural similarity to other known collar cells, we consider these cells to be functional choanocytes.

The most striking feature of this electron microscopy study was the presence within certain gastrodermal cells of a large membrane-bounded, intracellular structure containing smaller membrane-bounded vesicles (see Figs. 13 and 14). These vesicles correspond in size and shape to the lumisomes isolated by Anderson and Cormier (2) and identified by electron microscopy as having an average diameter of 0.2 μm. We suggest that these vesicles are indeed lumisomes. We further suggest that the unique structure observed in the cells of the gastroderm by electron microscopy is the same green fluorescent structure identified by fluorescence microscopy (see Results). We propose that this structure be termed a luminelle since such nomenclature would be compatible with the term lumisome. The luminelle can thus be viewed as a highly specialized organelle containing numerous smaller vesicles, the lumisomes. The identity and location of the luminelle is consistent with the histological studies of Lyke (14) who reported the presence of "unusual gastrodermal cells associated with the bioluminescent regions of the autozooids and siphonozooids."

While our findings do not provide an exact mechanism for eliciting a luminous response, they do afford an opportunity to suggest possibilities. The luminous waves in *Renilla*, which move in a concentric fashion, have long been assumed to mirror the path of excitation of the nerve net (10). The sparsity of nerve tissue observed in the anthocodium in this study poses some difficulties in accepting this interpretation. An alternative hypothesis could involve the musculature. We have noted that in every instance in which a luminelle was observed, the cell containing the structure abutted a highly developed muscle fiber. It is possible that the source of calcium required to initiate a bioluminescent response could arise from the muscle fiber as a result of excitation-contraction coupling. Alternatively, calcium release could arise from within the photocyte itself as a result of events which are secondary to the excitation-contraction coupling process. In either event, the membranes of both the luminelle and the lumisome probably participate in controlling the flow of calcium ions necessary to trigger the release of luciferin from the luciferin-binding protein. It thus appears likely that this conspicuous, well developed muscle system may be more intimately involved in the primary excitation of the luminelle than the nervous system. The nerve net is probably involved in the control process via neuromuscular connections.

Although there has been a recent and very interesting study on the location of photocytes within bioluminescent Ctenophores (9), the present report represents the first identification of an intracellular structure suggested to be involved in the bioluminescent process in *R. mülleri*. This suggestion is based on the following observations on the luminelle: it is restricted to the cells of the gastroderm; it is comparable in size to a subcellular structure whose green fluorescence is typically associated with the in vivo bioluminescence; it is intracellular; and its substructural elements have the same size and form as the lumisome. Finally, to our knowledge there have been no reports of a similar highly specialized structure in any electron microscope studies.

The authors express their appreciation to Dr. W. J. Humphreys, director of the Central Electron Microscopy Laboratory, University of Georgia, Athens, Georgia, for his support and encouragement during this project and to Dr. J. J. Paulin, Department of Zoology, University of Georgia, Athens, Georgia, for his suggestions during the preparation of this manuscript.

This work was supported in part by grants from the National Science Foundation (GB-28559X) and the United States Atomic Energy Commission (AT-(38-1)-635). Milton J. Cormier is a Career Development Awardee (5-KO3-GM-03331-09) of the United States Public Health Service.

This is contribution no. 279 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

Received for publication 8 April 1974, and in revised form 16 September 1974.

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