The Digestive Diverticula of *Alderia modesta* and *Elysia chlorotica*[1]

(Opisthobranchia : Sacoglossa)

BY

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(3 Plates; 5 Text figures)

INTRODUCTION

THE MAJORITY OF ADULT sacoglossans are green in colour and a number of authors (KAWAGUTI & YAMASU, 1965; TAYLOR, 1968; TRENCH *et al.*, 1969) have shown that this coloration is attributable to ingested chloroplasts. Long-term associations between active algal chloroplasts and the digestive cells of sacoglossans appear to be restricted to the siphonaceous algae (Order Siphonales) and the elysioid sacoglossans (TRENCH, 1975). The eolidiform (cerata-bearing) sacoglossans, on the other hand, apparently retain chloroplasts for shorter time periods, often of less than 24 hours (HINDE & SMITH, 1974; McLEAN, 1976).

Elysia chlorotica (Gould, 1870) has not been examined for intracellularly functional chloroplasts but other species of the genus *Elysia* do retain functional chloroplasts (TAYLOR, 1968; TRENCH *et al.*, 1969; GREENE, 1970; and TRENCH *et al.*, 1973). *Alderia modesta* (Lovén, 1844) has been reported not to retain functional chloroplasts (HINDE & SMITH, 1974). In Nova Scotia the alga consumed by both *E. chlorotica* and *A. modesta* is an undetermined species of *Vaucheria* which forms fuzzy mats upon the salt marsh in summer and in winter occurs as a filamentous aquatic form in the numerous marsh pools. Both slugs occur in both habitats (BAILEY & BLEAKNEY, 1967; BLEAKNEY & BAILEY, 1967). As *Vaucheria* chloroplasts (Order Xanthophyta) have never been reported as establishing symbiotic associations with sacoglossans, the local situation provides an opportunity for a comparative morphological and functional study of two sympatric elysioid and eolidiform sacoglossans feeding on the same algal species. The situation is comparable to European investigations of the elysioid *E. viridis* (Montagu, 1804) and the eolidiform *Placida dendritica* (Alder & Hancock, 1843) feeding on the siphonaceous alga *Codium* (TRENCH, 1975; McLEAN, 1976). Thus, the purpose of this paper is twofold: (1) to provide a description and comparison of the structure of the digesive diverticula of *Alderia modesta* and *Elysia chlorotica* using light and electron microscope techniques, and (2) to assess the photosynthetic capabilities of chloroplasts of *Vaucheria* in *E. chlorotica* and *A. modesta* by studying light-mediated oxygen production.

MATERIALS AND METHODS

Specimens of *Alderia modesta* and *Elysia chlorotica* were collected from salt marshes of the Minas Basin, King's County, Nova Scotia. They were found in trenches and pools dominated by algae of the Cladophorales and on *Vaucheria* sp. mats in damp depressions at the base of *Spartina alterniflora*. Specimens were maintained in refrigerated jars of sea water or in an aerated salt water aquarium.

For histological study, tissues were fixed in Zenker's fixative, paraffin embedded and sectioned at 7 µm. The sections were stained with either Ehrlich's hematoxylin and alcoholic eosin Y (THOMPSON, 1966) or with Mallory's trichrome sequence (LILLIE, 1965).

Several histochemical tests were used. Glycogen was demonstrated in Rossman's fixed, paraffin embedded tissues with the periodic acid Schiff's reaction (PAS) of Hotchkiss as described in THOMPSON (1966). Companion sections were digested with diastase to distinguish the glycogen from other PAS-positive substances. Ribonucleic acid (RNA) was demonstrated in Carnoy's fixed tissues with the methyl green-pyronin Y method of Brachet. To confirm the identification of RNA, control sections were digested with bovine ribonuclease for 2½ h before staining. Lipids were visualized in tissues fixed in either 10% formalin or in Baker's formol calcium, gelatin embedded, and sectioned at 10 µm on a freezing microtome at −20°C.

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Two neutral lipid methods were used, the oil red O method of Lillie and the propylene glycol-Sudan method of Chiffelle & Putt. Acid mucopolysaccharides were investigated in Zenker's fixed and paraffin embedded tissues. The Alcian blue method, staining at pH 2.5 for 30 min., as described by HUMASON (1972) was used. The Gomori method (HUMASON, *op. cit.*) was used to demonstrate acid phosphatase. Tissues were fixed in Baker's formol calcium, gelatin embedded, and sectioned on a freezing microtome at −20°C. An incubation time of 4 h at 37°C was used. Control sections were incubated in a substrate medium containing sodium fluoride to inhibit enzyme activity. Gomori's calcium-cobalt method was used to demonstrate alkaline phosphatase activity. The tissues were fixed in 80% ethanol at 4°C and paraffin embedded. An incubation time of 4 h at 37°C was used. Control sections were prepared in an identical manner except that the sodium glycerophosphatase was omitted from the incubation medium. Calcium was demonstrated in Carnoy's fixed, paraffin embedded tissues with the alizarin red S method of Dahl. Unless other references are given, the above techniques were applied as described by PEARSE (1961).

For electron microscope studies, tissues were fixed in 2.5% glutaraldehyde in filtered sea water, post-fixed in 1% osmium tetroxide in filtered sea water, and embedded in Durcupan. For orientation, thick sections were stained with 1% aqueous toluidine blue – 1% aqueous borax solution. The thin observational sections were stained with uranyl acetate and Reynold's lead citrate (PEASE, 1964) and studied with a Zeiss EM9S electron microscope.

Oxygen production was used as a criterion to measure photosynthetic activity in *Elysia chlorotica* and *Alderia modesta*. A Beckman oxygen macroelectrode attached to a DC Null Voltmeter and a chart recorder was used. Specimens were placed in a 10 mL flask of filtered sea water containing an autonomic stirrer. The tests were conducted in an Econaire Growth Chamber which provided a uniform temperature of 23°C and even illumination on all sides. The specimens were exposed to varying time periods (recorded in Figures 3 and 4) of light and dark and 0.9 g of *Elysia chlorotica* and 0.06 g of *Alderia modesta* were used. As an additional test of photosynthetic activity, inhibition of O_2 production was attempted by adding 3(3,4-dichlorophenol)-1, 1-dimethylurea (DCMU) (4×10^{-5} mol l^{-1}) to the *Elysia chlorotica* flask.

To compare overall pigment contents, approximately 1 g each of *Alderia modesta, Elysia chlorotica,* and *Vaucheria* sp. was crushed with 10 mL of cold acetone for 15 min. The resulting 3 crude extracts were filtered and the overall absorption spectra determined on a Varian Techtron UV-VIS Spectrophotometer.

OBSERVATIONS

Alderia modesta

The digestive diverticula of *Alderia modesta* branch off the large stomach and pass dorsally into the cerata and ventrally into the foot region. These digestive tubules are lined with epithelium supported by a thin layer of connective tissue. Smooth muscle bundles also envelop the tubules. A basement membrane was not observed. Chloroplasts are frequently present in the lumina of the tubules.

With light microscope techniques, two cell types form the epithelial lining (Figure 6). Cell type AI (*Alderia* I) is large and granular, whereas cell type AII (*Alderia* II) is small and non-granular. Cell AI is the more numerous and forms the lining of much of the tubule. With the hematoxylin and eosin and Mallory's methods, cell AI contains clear vacuoles and acidophilic granules while cell AII stains basophilically. Histochemically, both cells stain positively for glycogen, RNA, neutral lipids and both cells stain negatively for acid mucopolysaccharides and calcium. Acid phosphatase is present along the luminal

Explanation of Figures *6* to *11*

Figure *6*: Digestive tubule of *Alderia modesta* showing cell types AI and AII. Toluidene Blue O. × 1 500

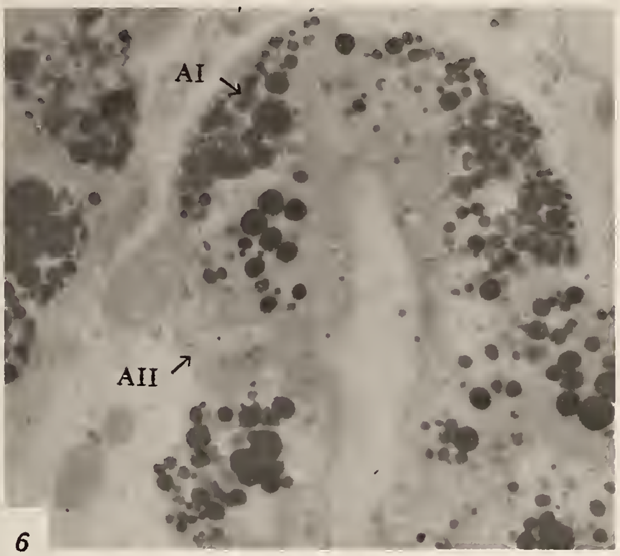
Figure *7*: Digestive tubule of *Elysia chlorotica* showing cell types EI and EII. Toluidene Blue O. × 1 500

Figure *8*: Digestive tubules of *Elysia chlorotica* showing staining reaction for neutral lipid. Oil Red O. × 375

Figure *9*: Digestive tubules of *Elysia chlorotica* showing staining reaction for alkaline phosphatase. Gomori's method. × 375

Figure *10*: Digestive tubules of *Elysia chlorotica* showing staining reaction for alkaline phosphatase along the luminal border. Gomori's method. × 375

Figure *11*: Digestive tubules of *Elysia chlorotica* showing staining reaction for calcium (arrows). Note cell EI is negative and cell EII is positive. Alizarin. × 600



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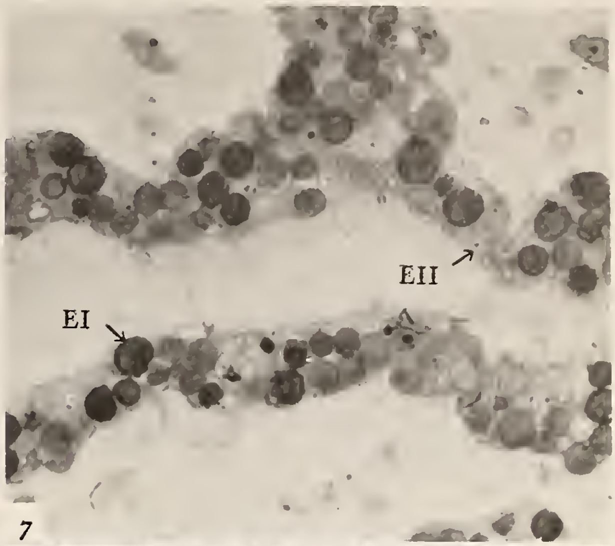

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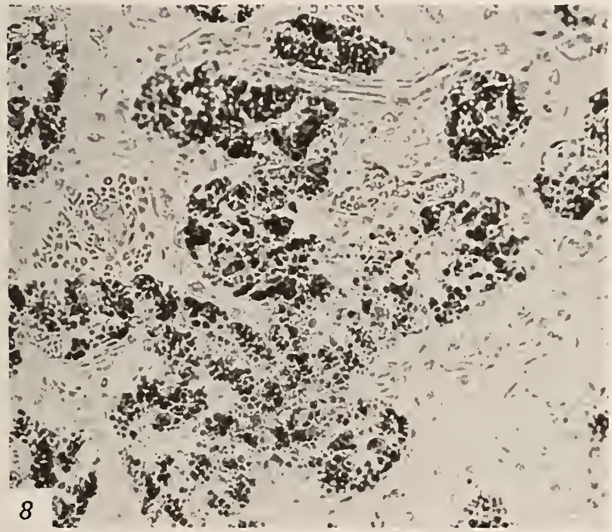

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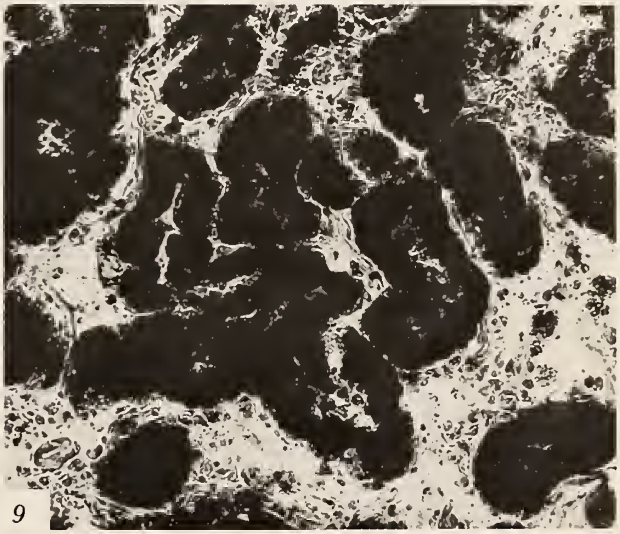

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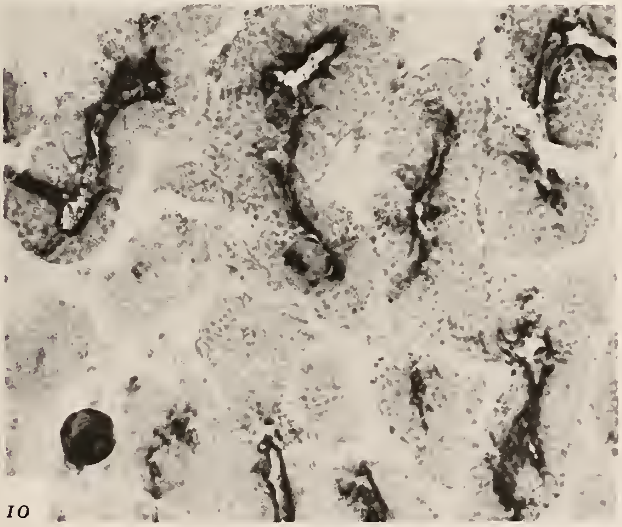

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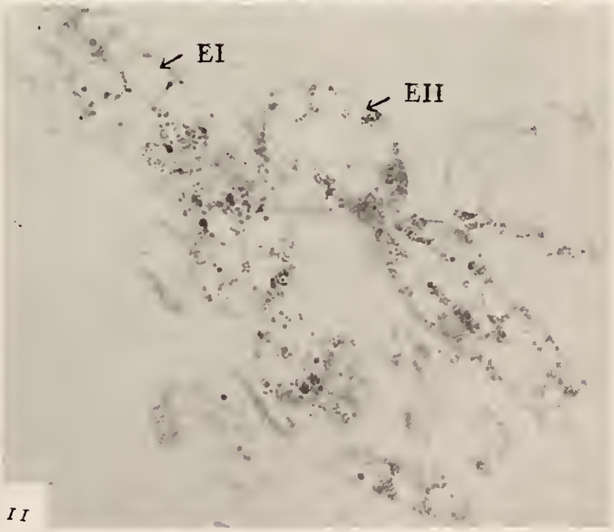

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border of both cell types. These staining reactions are summarized in Table 1.

Table 1

Staining affinities of the cell types in the digestive diverticula of *Alderia modesta* and *Elysia chlorotica.*

Staining Technique	Cell Types			
	Alderia modesta AI	*Alderia modesta* AII	*Elysia chlorotica* EI	*Elysia chlorotica* EII
PAS	*	*	*	*
methyl green-pyronin Y	*	*	*	*
oil red 0	*	*	*	*
propylene glycol	*	*	*	*
Alcian blue	—	—	—	—
alizarin red S	—	—	—	*
acid phosphatase	*	—	**	—
alkaline phosphatase	**	**	**	**

— no reaction
* some reaction
** strong reaction

At the electron microscope level, the most diagnostic components of cell AI (Figures 1 and *12*) are the heterogenous bodies. The heterogenous bodies are generally found in the apical half of the cell, each is encircled by a membrane, and each contains granules which may be loosely to densely packed. Chloroplasts are frequently observed in these cells. When present, they are surrounded by an extrinsic membrane and show varying degrees of degradation. Small vesicles are often found close to the chloroplasts and heterogenous bodies. Lipid inclusions are common, vary in size, and often are surrounded by heterogenous bodies. These cells have long or oval mitochondria, a rough endoplasmic reticulum usually located along the cell periphery and around the cell organelles, and a nucleus which frequently contains a rod-shaped crystal. Microvilli and many small vesicles are found along the luminal border and cilia are occasionally present.

Cell AII (Figures 1 and *13*) is usually observed in the basal half of the epithelial lining. It contains long thin mitochondria, small vesicles, and glycogen. Concentric layers of smooth endoplasmic reticulum are prominently distributed throughout the cell. Lipid inclusions coupled with clear vesicles, vesicles containing material with a dense core, and cytoplasmic lattice-work crystals are present. The nucleus was not observed to contain crystals. Microvilli are present on those cells which extend to the luminal border.

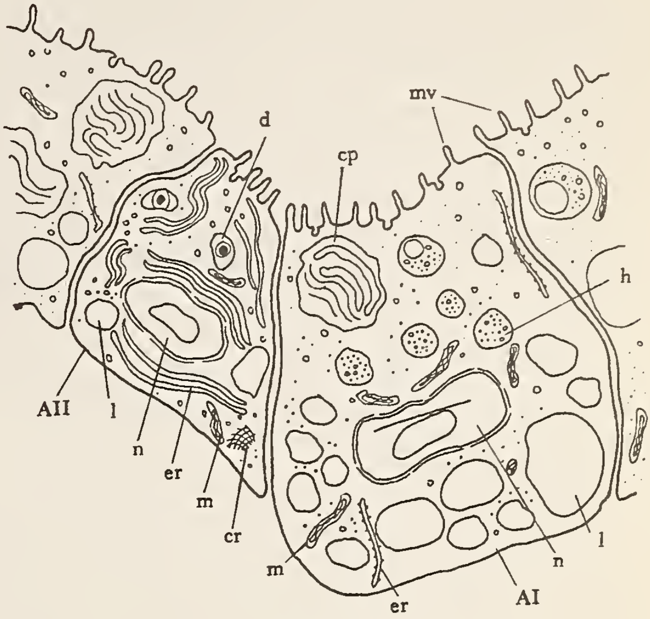


Figure 1

Diagrams of cell types AI and AII lining the digestive diverticula of *Alderia modesta.* cp – chloroplasts; cr – crystals; d – dense vesicles; er – endoplasmic reticulum; h – heterogenous body; l – lipid; ls – lysosome; m – mitochondria; mv – microvillus; n – nucleus

Elysia chlorotica

The digestive diverticula of *Elysia chlorotica* arise from the small stomach as two ducts which immediately subdivide into many small tubules and ramify throughout the entire animal. Unlike *Alderia modesta, E. chlorotica* does not possess cerata. The tubules are lined with epithelium enveloped only with smooth muscle. A subepithelial basement membrane and connective tissue tunic could not be demonstrated. Unlike *A. modesta,* the lumina of the digestive diverticula of *E. chlorotica* are characteristically devoid of chloroplasts.

At the light microscope level, the epithelial lining contains 2 cell types, cell EI (*Elysia* I) and cell EII (*Elysia* II). With the histological techniques used, cell EI is cuboidal and contains acidophilic granules and cell EII is narrow, non-granular and stains basophilically (Figure *7*).

Histochemically, both cells stain positively for glycogen, RNA, and neutral lipids (Figure *8*). Acid mucopolysaccharides could not be demonstrated. Acid phosphatase is present in cell EI (Figure *9*). Alkaline phosphatase occurs along the luminal border of both cells (Figure *10*). The alizarin red S method demonstrates calcium granules in cell EII (Figure *11*) and the Gomori method for alkaline phosphatase demonstrates extracellular calcium spherules throughout the animal. These staining reactions are summarized in Table 1.

At the electron microscope level, cell EI (Figures 2, *14* and *15*) contains chloroplasts and cell EII does not. The chloroplasts are distributed throughout the cell and have an average diameter of $5.4\,\mu m$. They are bounded by an intrinsic double membrane, are composed of parallel lamellae and a homogenous matrix containing a few strands of lamellae. Small bodies, possibly oil droplets, may be present between the lamellae. Occasionally, this lamellar structure is not well defined and the chloroplasts appear to be degrading. Lipid inclusions, glycogen, mitochondria, and smooth endoplasmic reticulum are distributed throughout the cell. Large, often irregular shaped, clear or granular vesicles are occasionally present. The nucleus, often triangular in shape, is located in the apical half of the cell. The luminal border has many microvilli and a few cilia. Numerous small round vesicles, probably pinocytotic vesicles, many containing granules, are distributed throughout the apical cytoplasm and may also be located deeper in the cell.

Cell EII (Figures 2 and *15*) occurs with the same frequency as cell EI. It is a narrow cell, having the nucleus situated in the apical half. The cytoplasm contains lightly stained granular material. Glycogen, small mitochondria, smooth endoplasmic reticulum, and granular vesicles which are presumably calcium spherules are found through the cell. Occasionally, Golgi bodies are observed. Lipid inclusions are found in the basal region of the cell. The apical border has microvilli, a few cilia, and subjacent small pinocytotic vesicles.

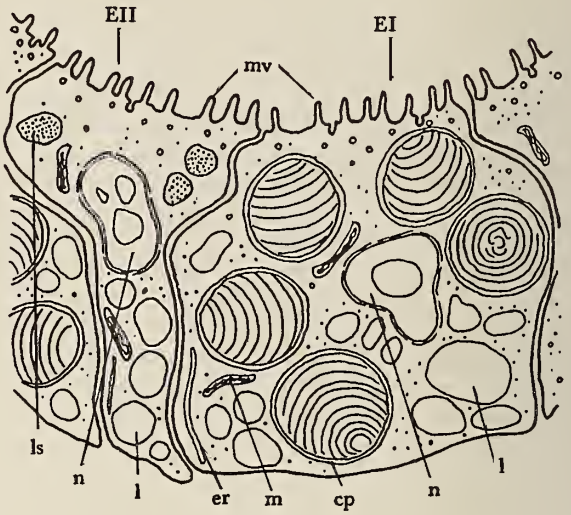


Figure 2

Diagrams of cell types EI and EII lining the digestive diverticula of *Elysia chlorotica*. Abbreviations as in Figure 1

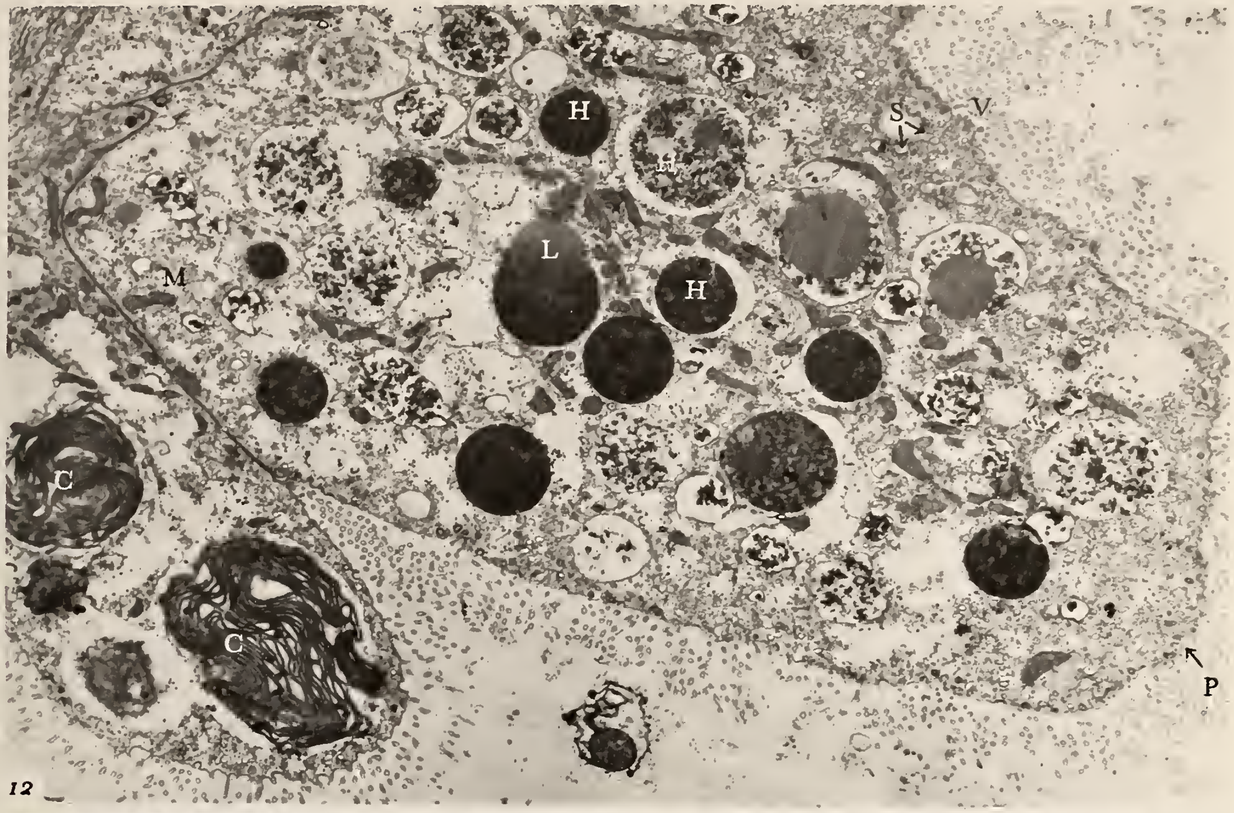
Photosynthesis

The tests for photosynthetic activity with ***Elysia chlorotica*** show an increase in PO_2 when the flask was illuminated and a PO_2 decrease when the flask was darkened (Figure 3). The addition of DCMU to the flask containing ***E. chlorotica*** caused a decrease in PO_2 similar to that caused by the absence of light. Tests using ***Alderia modesta*** demonstrated that the presence of light did not influence the PO_2 level in the flask (Figure 4).

Explanation of Figures *12* and *13*

Figure *12*: Digestive tubule of *Alderia modesta* showing portions of two AI cells containing loosely and densely packed heterogenous bodies (H), degraded chloroplasts (C), lipid (L) within heterogenous body, mitochondria (M), microvilli (V), small vesicles (S), and pinocytotic activity (P). Uranyl acetate and lead citrate. × 6 120

Figure *13*: Digestive tubule of *Alderia modesta* showing AII cell. Mitochondria (M); endoplasmic reticulum (E); lipid (L) inclusions coupled with clear vesicles; vesicles containing material with a dense core (D); lattice-work crystals (W); and nucleus (N). Uranyl acetate and lead citrate. ×7 600



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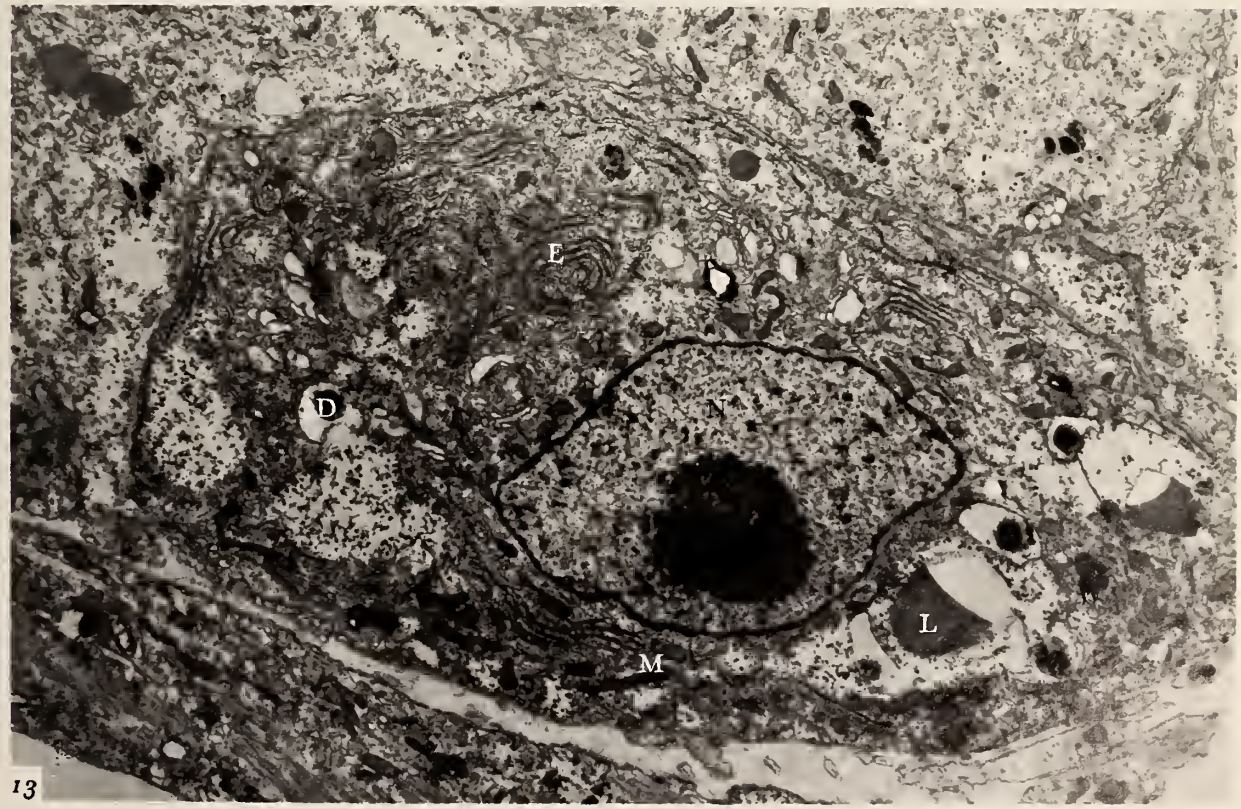

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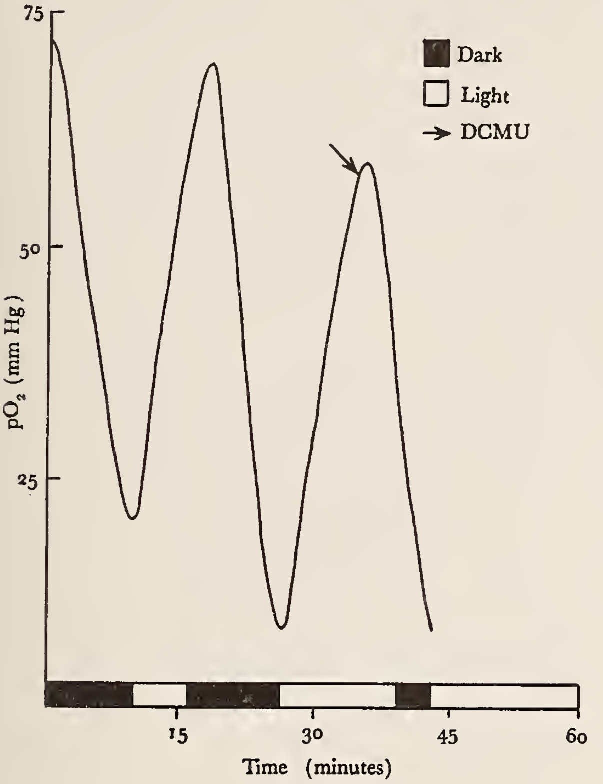


Figure 3

Graph showing oxygen release by *Elysia chlorotica* during light and dark periods and following administration of DCMU

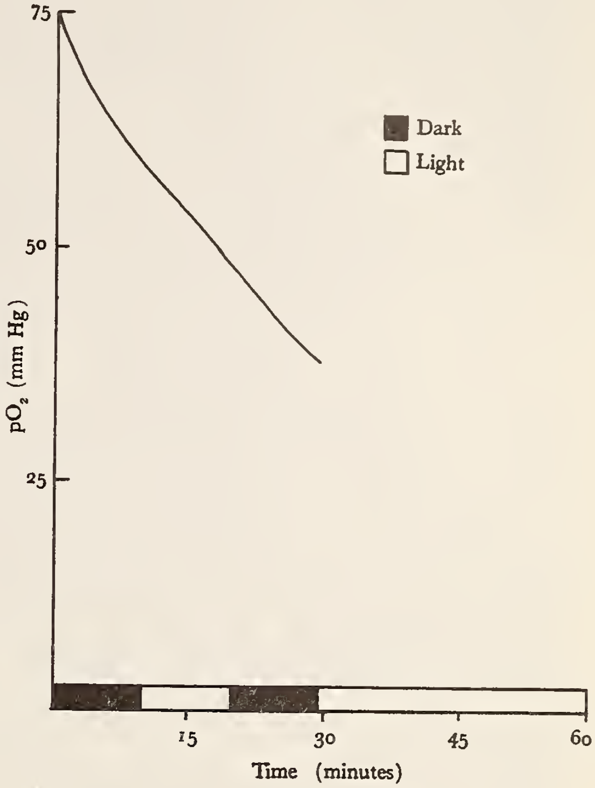


Figure 4

Graph showing oxygen release by *Alderia modesta* during light and dark periods

Pigment Analysis

As shown in Figure 5, the crude acetone extracts of *Elysia chlorotica* and *Vaucheria* sp. show absorption maxima at 370, 410, 425, 470, 570, 610 and 660 nm. The maxima for *Alderia modesta* are at 408, 442, 470, and 660 nm, but are not as high nor as distinct as those of *E. chlorotica* and *Vaucheria* sp.

DISCUSSION

Histology

The digestive tubules of *Alderia modesta* are lined with epithelium supported by connective tissue and smooth muscle layers. A basement membrane was not observed. The epithelial lining of *Elysia chlorotica* is surrounded by a smooth muscle layer, but no basement membrane or connective tissue layer was observed. The subepithelial layer of *E. viridis* contains a thick basement membrane and connective tissue layer (Taylor, 1968), while that of *E. atroviridis* has a thick basement membrane and both connective tissue and muscle layers (Kawaguti & Yamasu, 1965). The lumina of the diverticula of *E. chlorotica* are devoid of chloroplasts. A similar observation has been reported for *E. viridis* (Trench *et al.*, 1973). The lumina of *Alderia modesta,* on the other hand, characteristically contains chloroplasts. This suggests that *A. modesta* feeds regularly, whereas *E. chlorotica* feeds only sporadically, obtaining energy from the photosynthetic activity of its intracellular chloroplasts.

The digestive diverticula of *Alderia modesta* are lined by 2 epithelial cell types. Cell AI is the larger, is more

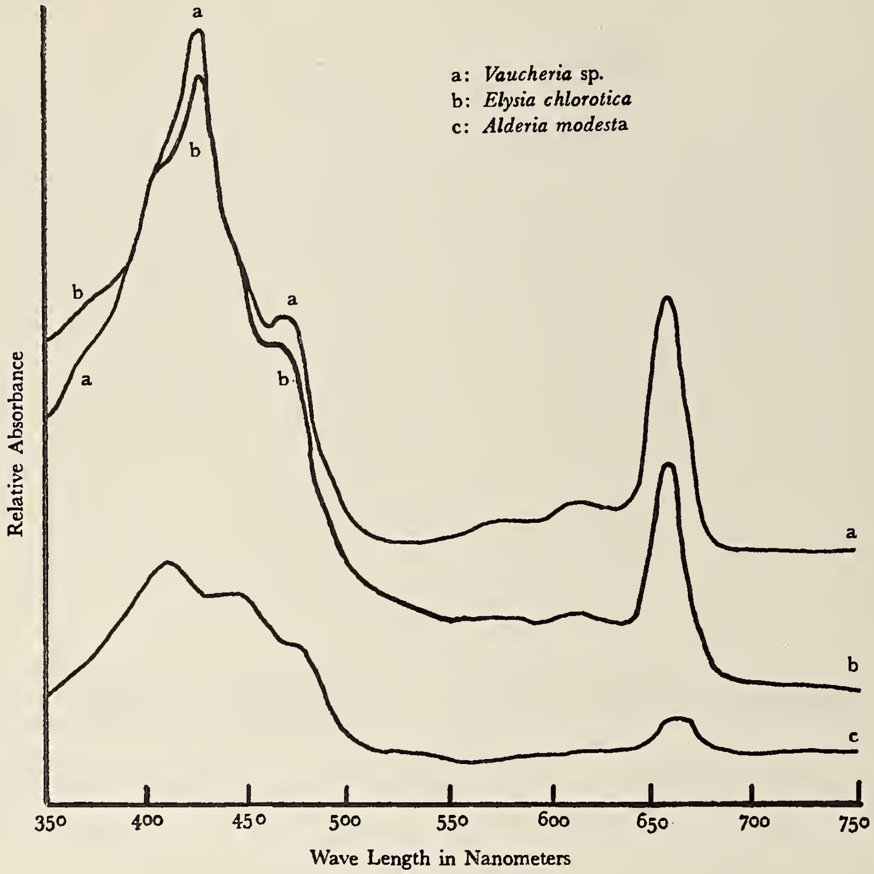


Figure 5

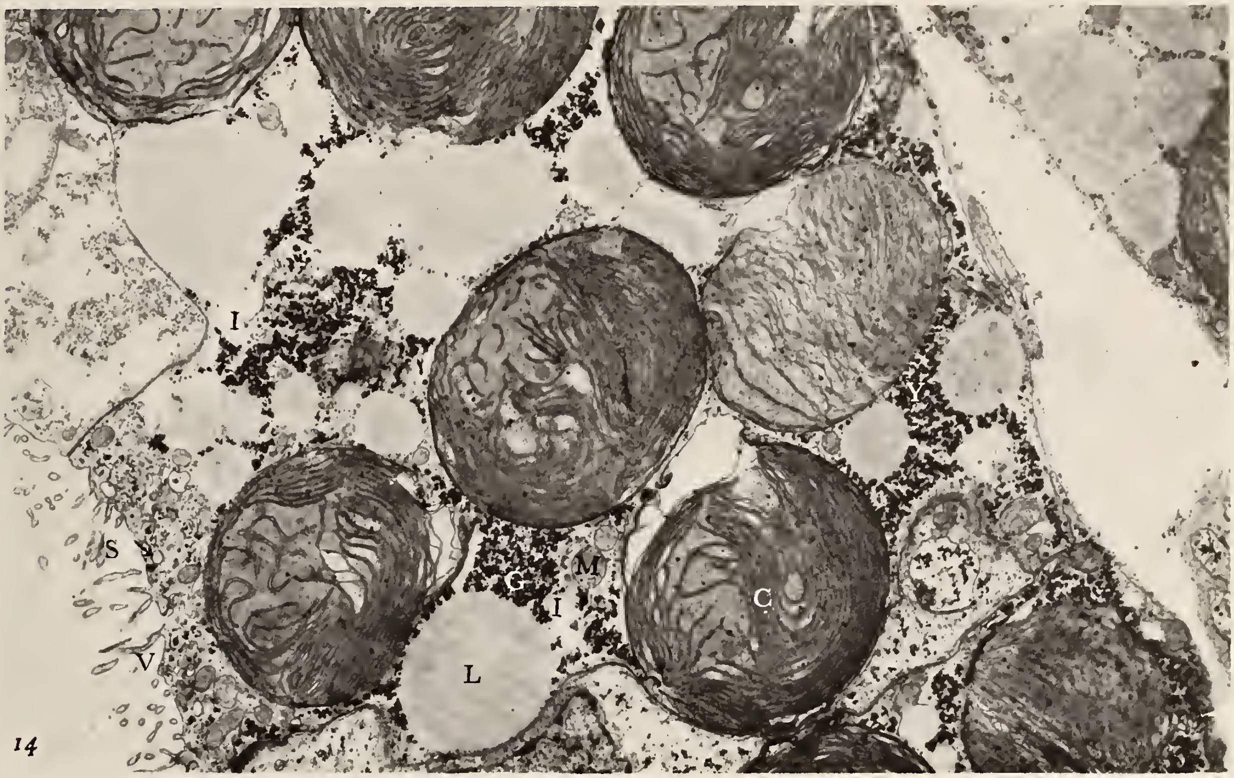
The absorption spectra of crude acetone extracts from tissues of *Vaucheria* sp., *Elysia chlorotica* and *Alderia modesta*

numerous, and possesses a granular cytoplasm. It is suggested that this cell has a digestive function because it stains positively for acid phosphatase, an enzyme associated with lysosomes. Also, the luminal border stains intensely for alkaline phosphatase, indicating the presence of microvilli and suggesting a border which is active in transport. Ultrastructurally, this cell contains chloroplasts in various stages of degradation surrounded by an animal cell membrane, presumably a phagosome. MUSCATINE *et al.* (1975) proposed that chloroplasts within vacuolar membranes are subject to hydrolysis by lysosomes, whereas symbiotic chloroplasts occur free in the sacoglossan

Explanation of Figures *14* and *15*

Figure *14*: Digestive tubule of *Elysia chlorotica* showing EI cell with intact chloroplasts (C); glycogen granules (G); lipid inclusions (L); mitochondria (M); irregularly shaped vesicles (I); microvilli (V); and small vesicles along luminal border (S). Uranyl acetate and lead citrate. × 6 120

Figure *15*: Digestive tubule of *Elysia chlorotica* showing EII cell between two EI cells. Glycogen (G); mitochondria (M); granular vesicles or lime spherules (R); lipid (L); microvilli (V); and small vesicles along luminal border (S). Uranyl acetate and lead citrate. ×7 200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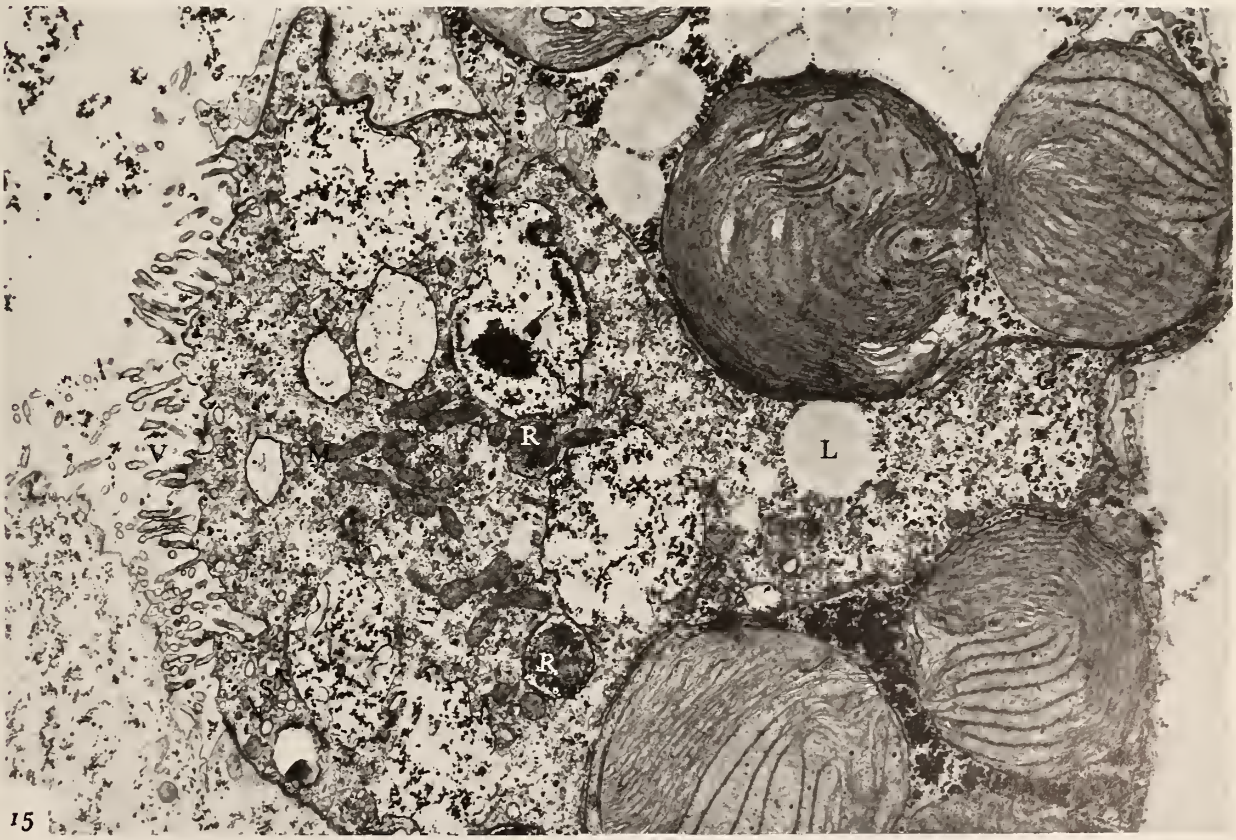
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digestive cell cytoplasm and are thereby protected from enzymatic degradation. During degradation, chloroplasts of *A. modesta* lose their original shape and the lamellae become indistinct. Often small vesicles are associated with the chloroplasts. The heterogenous bodies, typical of the AI cell, are possibly vesicles of chloroplast digestion. Some of the small vesicles associated with both the chloroplasts and the heterogenous bodies may be lysosomes. Others may be pinocytotic vesicles from the luminal border. Presumably, phagocytosis also occurs as chloroplasts are often observed under the luminal border. However, the process of phagocytosis as described by McLean (1976) for another eolidiform sacoglossan, *Placida dendritica,* was not seen.

Cell AII is smaller and less numerous than cell AI and, histochemically, it differs from AI in that it does not exhibit a positive reaction for acid phosphatase. At the electron microscope level, cell AII contains some distinct inclusions: vesicles having a dense core, lattice-work crystals, and an extensive endoplasmic reticulum. The function of this cell is unknown. However, the extensive endoplasmic reticulum and the pinocytotic or endocytotic activity of the luminal border indicate a very active cell. Also, the intense alkaline phosphatase activity along the luminal border indicates active transport. Thus, it appears that this cell is either secreting into the lumen or sequestering substances from the lumen.

The epithelial lining of the digestive diverticula of *Elysia chlorotica* also has 2 cell types, cell EI and cell EII. Cell EI is cuboidal in shape and contains acidophilic granules. Fretter (1940) reported a cell showing a similar histology in *E. viridis* except that the cell she described contained vacuoles of yellowish or brown material which she suggested were excretory masses derived from the digestion of food. Taylor (1968) also described the granular cells in *E. viridis* which contained the excretory masses. The cell in *E. viridis* contains acid mucopolysaccharides, otherwise it is histochemically similar to the one reported in the present study. It is suggested that cell EI is digestive in function as it exhibits an intense acid phosphatase reaction indicating the presence of lysosomes, and an intense alkaline phosphatase reaction along the luminal border indicating active transport. The presence of microvilli and pinocytotic vesicles supports this suggestion. Fretter and Taylor also refer to this cell type in *E. viridis* as a digestive cell.

At the electron microscope level EI of *Elysia chlorotica* is comparable to the digestive cell of *E. viridis* as described by Taylor (1968). However, certain differences are evident. The chloroplasts of *E. chlorotica* differ in size and structure from those of *E. viridis*. Those in *E. chlorotica* possess a lamellar structure and a region of homogenous matrix containing a few strands of lamellae. They have a mean diameter of 5.4 μm. Only the chloroplast envelope was observed, which supports the conclusions of Muscatine *et al.* (1975) that active chloroplasts are in direct contact with the host's cytoplasm and that only defunct chloroplasts in *Elysia* spp. become enveloped by autophagic host vacuoles. The chloroplasts of *E. viridis,* from the siphonaceous alga *Codium fragile,* are 2 - 3 μm in diameter and possess a lamellar structure of double disc bands, oil droplets and a discoidal starch grain. The large clear digestive vacuoles of *E. viridis* are not present in *E. chlorotica*. However, numerous lipid inclusions are present and are large and lightly stained.

Cell EII is a long narrow cell, less common than cell EI, and stains basophilically. This cell stains positively for calcium and appears to be similar to the lime cell in *Elysia viridis* described by Taylor (1968) which is also positive for calcium. Both Fretter (1940) and Taylor (*op. cit.*) suggest that this cell type may have a buffering function. At the electron microscope level, the lime cells of *E. chlorotica* and *E. viridis* appear to be similar. Both cells lack chloroplasts and contain electron dense lime vesicles. The large lipid inclusions found in the basal region of the *E. chlorotica* lime cell are comparable to the large vacuoles often found in the lime cell of *E. viridis*. Pinocytotic or endocytotic activity is present along the luminal border of the lime cells in both species.

Thus, at the electron microscope level, the 2 cell types in *Elysia chlorotica* correspond closely with the digestive and lime cells of *E. viridis* as reported by Taylor (1968).

Photosynthesis

Studies of O_2 production undertaken to test *Elysia chlorotica*'s capacity for photosynthesis showed that these animals are capable of producing an increase in the PO_2 of the surrounding water when supplied with sufficient light. This major increase in the water PO_2 to the level of saturation of the chamber and consequent formation of O_2 bubbles, could only be caused by photosynthesis of chloroplasts in the digestive cells. The PO_2 levels in the flask containing *Alderia modesta* were not influenced by light, indicating that the chloroplasts of *A. modesta* are not photosynthetically active. This is in agreement with Hinde & Smith (1974).

Pigment Analysis

Spectral analysis of the acetone-extracted pigments reveals that the absorption maxima of *Elysia chlorotica* and the alga *Vaucheria* sp. are the same, indicating that *Vaucheria* sp. is the food plant of *E. chlorotica*. Although *Al-*

deria modesta is known to eat *Vaucheria* sp. (Evans, 1953) and is found on *Vaucheria* sp. in the Minas Basin region, the absorption maxima of *A. modesta* are similar but not identical to those of *Vaucheria* sp. This indicates that chloroplast pigments are broken down soon after ingestion. Hinde & Smith (1974) reported that chlorophyll degradation is probably rapid in *A. modesta*.

SUMMARY

Although *Alderia modesta* and *Elysia chlorotica* occupy similar habitats and feed on the same alga, they differ with respect to the structure of their digestive diverticula and their mode of nutrition. The majority of the cells lining the diverticula of *A. modesta* are involved with rapid degradation of the algal cell contents, including chloroplasts, whereas much of the epithelial lining of the diverticula of *E. chlorotica* is involved with the long term retention of photosynthetically active chloroplasts.

This discovery of active chloroplasts of *Vaucheria* sp. in the digestive cells of *Elysia chlorotica* is the first evidence of a symbiotic relationship between an elysioid sacoglossan and an alga of the Order Xanthophyta.

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