

ULTRASTRUCTURE OF THE ENDOSYMBIOTIC DINOFLAGELLATE
***Symbiodinium microadriaticum* LIVING IN THE SEA ANEMONE**
Anemonia viridis

by

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ABSTRACT

The zooxanthella, *Symbiodinium microadriaticum*, an endosymbiotic dinoflagellate shows variation in its ultrastructure within its population in the sea anemone, *Anemonia viridis*. Such variation included the number of thylakoid, the structure of inclusions and the structure of amphiesma. The string-like structure was also found in the nucleoplasm. Some zooxanthellae have a branching or double pyrenoid with two or three stalks. Under certain condition, which are not clearly understood, two or three zooxanthellae are enclosed within one very thick membrane.

ABSTRAK

Dinoflagellata, *Symbiodinium microadriaticum*, yang bersimbiose dengan sea anemone, *Anemonia viridis*, mempunyai ultrastruktur yang sangat bervariasi. Variasi ultrastruktur meliputi jumlah tilakoid, struktur inklusionis dan struktur amphiesma. Struktur berbentuk seperti benang juga ditemukan di dalam plasma inti. Beberapa zooxanthella juga mempunyai pirenoid kembar atau bercabang dengan dua atau tiga tangkai pirenoid. Pada kondisi tertentu juga ditemukan adanya dua atau tiga zooxanthellae terbungkus dalam satu dinding sel yang sangat tebal.

INTRODUCTION

Endosymbiotic dinoflagellates, commonly called "Zooxantellae", are found in a wide range of marine invertebrates (TRENCH 1987). At least 80 host species, belonging to three phyla have been shown to be associated with zooxanthella (TAYLOR 1974). As well as adopting a wide range of host species, zooxanthellae also possess an extensive geographic range. However, the majority of symbiotic dinoflagellates found in tropical regions are associated with reef building corals.

Endosymbiotic zooxanthellae are unicellular, cocoid in shape, usually brown in colour and are located within host cells. They possess permanently condensed chromosomes visible as oval or circular forms. Chromosomes show no separation during the metaphase stage of division and the chromosomes also lack of centriole (TAYLOR 1979).

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The morphology and life cycle of cultured cells are different from insitu symbionts (FREUDENTHAL 1962, TRENCH 1987). In axenic media, zooxanthellae become motile cells for certain periods of time. The motile cell has two flagella which emerge at the junction between the longitudinal and transverse furrow in the cell surface; one flagellum extends along each furrow (FREUDENTHAL 1962, SMITH and DOUGLAS 1979).

The question of taxonomy of zooxanthellae remains controversial and problematic. There have been several proposals to resolve this problem, but there is no universal agreement. The first major step in zooxanthellae taxonomy was made by McLAUGHLIN and ZAHL (1957) who were successful in isolating algal cells from the Caribbean jellyfish *Cassiopeia*. The symbiotic alga was kept in axenic culture media and was described as *Symbiodinium adriaticum* (McLAUGHLIN and ZAHL 1959). Based on the life cycle and morphology of algae from *Cassiopeia* sp., FREUDENTHAL (1962) proposed a new genus of *Symbiodinium* with the type species *Symbiodinium microadriaticum*. The reason for the new name was that it represents a more logical taxon among the algae group; the dominant stage of this alga appearing to be a vegetative cell or cyst rather than the motile stage. Many authors subsequently have used *Symbyodinium* (= *Gymnodinium microadriaticum* Freudenthal, as a compromise to include endosymbiotic stages or zooxanthellae and motile free-living stages (SCHOEN-BERG and TRENCH 1976, 1980; FITT *et al* 1981). TAYLOR (1971) observing living *Veella* from Florida, found that endosymbiotic algae possessed two thylakoid lamellae instead of usual three or more. Evidence of a girdle lamellae was also recorded in this alga. Subsequently this alga was described as *Enclodinium chattonii* (Hovasse). TAYLOR (1971) also proposed a new generic name *Gymnodinium*, for amended *Symbiodinium microadriaticum* Freudenthal. Further changes were proposed by LOEBUCH and SHERLEY (1979) in which *Symbyodinium* of Freudenthal was placed in synonymy with *zooxanthella* with the species name of *Zooxanthella microadriatica* (Freudenthal). The reason for the proposed changes by LOEBLICH and SHERLEY (1979) was based on the ultrastructure of the theca of the symbiotic alga associated with the red alga, *Chondrus crispus* (L.) from Sippewissett, Massachusetts. They found that *S. microadriaticum* differed slightly from the type species *Zooxanthella nutricula* and it was considered that *Symbiodinium* should be a subjective synonym of *Zooxanthella*.

Many investigators are aware that the wide geographic range of ecological distribution for a single species of *S. microadriaticum* is relatively uncommon (TAYLOR 1969; SCHOENBERG and TRENCH 1976, 1980). The similarity of the morphology and ultrastructure of symbiotic algae might not be sufficient to distinguish between strains or varieties of *S. microadriaticum*. Fundamental differences have been found among algae harboured both by different hosts and by the same host in different geographic locations (BLANK and TRENCH 1985, BLANK 1986),

The aim of this study is to examine variation in the morphology of endosymbiotic

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S. microadriaticum in the sea anemone *Anemonia viridis*, in order to ascertain the possibility of the existence of different strains or varieties of *S. microadriaticum*.

MATERIALS AND METHODS

Intertidal *Anemonia viridis* Forskal were collected from Plymouth on the south west coasts of England. Anemones were maintained in an aquarium with recirculating sea water and kept at 16°C. The anemones were fed with *Artemia* larvae, twice weekly. Anemones were maintained for seven days to acclimatize to laboratory conditions before experimental use.

TRANSMISSION ELECTRON MICROSCOPY

Sea anemones were anaesthetized in a 1 : 1 solution of 0.3 Mg₂, H₂O, and sea water for about 20 minutes. Tentacles and mesenterial filaments were cut into small pieces and fixed in 3% glutaraldehyde, 0.5% tannic acid, and 8.5% sucrose in 0.1 M sodium cacodylate buffer solution for one hour at room temperature, followed by 14 hours at 4°C. Specimens were then rinsed for one hour in sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for one hour, at room temperature. Specimens were subsequently rinsed in cacodylate buffer followed by dehydration in graded series of acetones. Infiltration with Emix resin was performed in several changes and then blocks were stored overnight. The specimens were embedded in Emix resin and polymerization carried out at 60°C for 48 hours. Serial ultrathin section 60-70 nm were cut on an LKB microtome using glass knives. Sections were mounted on copper filmed grids and stained using aqueous uranyl acetate for 30 minutes at 40°C and lead citrate for 20 minutes at 20°C, using an LKB 2168 ultra-stainer. Sections were viewed in a Kraton Cora transmission, electron microscope at an accelerating voltage of 80 KV.

STAINING FOR CARBOHYDATES

Carbohydrates were stained using a technique developed by COURTOY and SIMAR (1974). Thin sections were mounted on gold grids and exposed to 1% periodic acid for 25 minutes at room temperature. Sections were then washed with distilled water. Sections were subsequently treated with thiosemicarbazide solution for 30-45 minutes at room temperature, washed with an acetic acid series, followed by washing with distilled water. Following this treatment section were stained in silver proteinate at room temperature for 30 minutes in the dark room and were then washed in distilled water and allowed to dry.

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SCANNING ELECTRON MICROSCOPY

Sea anemones were anaesthetized in a 1 : 1 solution of 0.3 M $MgCl_2 \cdot 6H_2O$, and tea water for about 30 minutes. Tentacles were cut into 0.5 cm length and fixed in 5% formaldehyde overnight, followed by dehydration in a graded series of acetones; they were then stored overnight at 4°C. Specimens were subsequently air dried and fractured with a razor blade. They were then coated with gold palladium using an ion coater (Polaron E 5100). Specimens were observed with a scanning electron microscope (Jeol JSM SI) operated at 15 kV.

RESULTS

In situ studies show zooxanthellae in the endodermal lining of the tentacles and mesenterial filaments of *Anemonia viridis*. The algal cells are located intracellularly and are present in individual vacuoles within the cytoplasm at the bases of the endodermal cells (Figure 1). The vacuole membrane of the host cell is usually in close contact with the algal surface/However there is sometimes a gap between them. The typical appearance of zooxanthellae in fixed specimens of *A. viridis* is of a rounded cell with diameter of 5-8 microns. Freshly isolated zooxanthellae are 7—11 microns in diameter. Observations of the surface of zooxanthellae, using scanning electron microscopy; show that the surface of the cell wall is smooth in appearance (Figure 2).

The general appearance of the internal structure of mature zooxanthellae is shown in Figure 3. They possess relatively thin walls, and a nucleus with condensed chromosomes which is typical of the dinoflagellates (TAYLOR 1987). The chloroplast is very obvious and situated near the periphery of the cytoplasm. The most readily identifiable structure is the large flask-like pyrenoid and the accumulation body which appears black and rounded. Starch grains, calcium oxalate crystals and mitochondria are distributed in the cytoplasm.

The nucleus varies from being subspherical to spherical in shape with many irregular indentations of the nuclear membrane. The diameter of the nucleus is about 2.5-4 microns and is dependent on the developmental stage of the zooxanthellae. The location of the nucleus is off-centre in the cytoplasm and occasionally the nucleus appears to be in close contact with the cell plasma membrane (Figure 3). The nucleus is surrounded by a porous double membrane. The thickness of the nuclear membrane is 20-33 nm and the diameter of the nuclear pores is between 83—100 nm. The nucleolus is relatively small and located in close contact with the nuclear membrane. The nucleolus contains numerous granules which are densely distributed in both the periphery and in the center of the organelle. Chromosomes vary in shape from oval to elongated, consisting of compact coiled spiral chromatin : they are randomly distributed (Figure 4). The nucleoplasm contains numerous dark staining granules and fibrous materials which is evenly distributed in the nucleus. In the nucleoplasm, string-like structures with a diameter of 4—7 nm are sometimes found grouped together and

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Are randomly distributed. This string is sometimes found in contact with the chromosom or the nuclear membrane. Amorphous bodies are also present in the nucleoplasm (Figure 4).

The chloroplast is the largest structure in the zooxanthellae cell. It is peripheral in position and entirely surrounded by a triple membrane. Serial sections show that the chloroplast is single and multilobed. The chloroplast contains numerous lamellae which are parallel to the length of the chloroplast. The chloroplast membrane and the nearest lamella membrane are separated by a gap of 0.5 nm width (Figure 5). The thickness of the chloroplast membranes is about 1.3—1.7 nm, the membrane appears as a heavily staining line (Figure 5). One characteristic of zooxanthellae associated with *Anemonia viridis* is that they possess a peripheral lamella tightly underlying the chloroplast membrane. The lamella adheres closely to the shape of the membrane. Each lamella consists of three closely associated thylakoids. However two thylakoids can be found in the middle of the chloroplast when one lamella cross links with another (Figure 5). The thickness of the lamella is 40—50 nm and the thylakoid is 15—17 nm width. In the middle of the chloroplast the thylakoid tends to be tightly packed whereas in the tip of the chloroplast the thylakoids are much less compact (Figure 5).

The pyrenoid is flask-like structure, protruding from the surface of the inner side of the chloroplast by a short stalk (Figure 6). The pyrenoid is surrounded by a continuation of the chloroplast membrane and is also enclosed by a thick starch sheath which is seen as a halo of electron translucent material. The content of the pyrenoid is uniformly granular material which is not penetrated by the chloroplast lamella (Figure 6). The zooxanthellae in *Anemonia* normally possesses a single pyrenoid with a single stalk. However some cells have a branching or double pyrenoid with two or three stalks (Figure 6).

The mitochondria vary in shape and in position. They are ovoid to elongate or branching and are surrounded by a double membrane which is 11—12 nm thick (Figure 7). The internal structure of a mitochondrion consists of microtubular or microvilli-like cristae. The cristae are 33—44 nm in diameter and are attached to the membrane of the mitochondrion. Cristae are not found in the center of the mitochondrion which consists of amorphous or granular material.

The golgi apparatus is relatively small with 3-5 cisternae about 24 nm in diameter and 125 nm length. Small vesicles are scattered close to the Golgi apparatus (Figure 7). There is also a complicated system of microtubules and vesicles which consists of rough or smooth endoplasmic reticulum (Figure 7).

The most easily recognisable feature of the algal cell is the accumulation body, which is large, rounded and possesses a dense outer covering, which is dark and electron dense with some dark patches inside (Figure 8). The remaining inclusions of the cells such as calcium oxalate crystals, starch grains, lipid droplets and microbodies are present in the cytoplasm (Figure 8). Calcium oxalate crystals are clumped and characterized by rectangular shape (Figure 8). Starch grains are randomly distributed and are recognized by a positive reaction when stained with periodic acid thiosemicarbazide silver

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proteinate (Figure 8). Microbodies are also present, they are rounded in shape and enclosed by a single membrane, 300—500 nm in diameter. Small vesicles are found between the microbodies. The contents of the microbodies are homogeneous, consisting of amorphous material (Figure 8).

Normally, endosymbiont zooxanthellae possess relatively thin cell walls known as amphiesma (SCHOENBERG and TRENCH 1980). However, in zooxanthellae associated with *A. viridis* the thickness of the amphiesma varies greatly between 100-730 nm (Figure 9). It seems that the thickness correlated with the condition and developmental stage of the zooxanthellae.

Observation on the ultrastructure of the dividing zooxanthellae indicated that the cells undergo cytokinesis within the parent amphiesma. Cytokinesis occurs after caryokinesis (Figure 10). Healthy zooxanthellae divide into equal cells. All organelles in the cell also divide equally between the daughter cells, except for the accumulation body. Serial sections show that only one of the daughter cells has an accumulation body. When the cell undergoes division, it is clear that the membranes of the daughter cells is composed of two layers (Figure 10). Both daughter cells eventually become two individuals after the cells break apart. During further development the cell wall gradually thickens until the algae become mature, after which they discard the thick parent wall. Some algal cells may continue to produce a thick wall and accumulate wastes inside the cytoplasm; such cells subsequently enter a senescent phase which subsequently leads to degeneration.

Figure 11 shows degenerate stages of the zooxanthellae in situ. The first stage of degeneration is characterized by an increase in the amount of calcium oxalate and an enlargement of the accumulation body. This is followed by the disintegration of the chloroplast and nucleus. The chloroplast lamella then becomes less well structured but the cell wall remains unchanged until complete disintegration occurs. In certain conditions, the cell wall breaks down first, followed by the disintegration of cellular inclusions (Figure 11).

DISCUSSION

The location of algae in the host tissue varies among invertebrates. KAWAGUTI (1964) found that zooxanthellae associated with some coral species were intercellular, while algae in the bivalve *Tridacna* were found in blood amoebocytes (FRANKBONER 1971; GOREAU *et al* 1973). Symbiodinium has also been found intercellularly in the octocorallian, *Eunicella* (TRENCH 1979); *Symbiodinium microadriaticum* in the jellyfish, *Cassiopeia* was located in the mesoglea and was enclosed within wandering cells. In the zoanthid, *Protopalythoa* sp., zooxanthellae are found in both endoderm and ectoderm (RYLAND, pers. comm.).

The unusual inclusions sometimes found in the nucleoplasm of *Symbiodinium* associated with *A. viridis* have not been reported previously. A group of string-like structures were also found in the nucleoplasm of the dinoflagellate *Prococentrum*

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micans (SOYER 1981). Soyer suggested that the string-like structures may play a role both in maintenance of nuclear structure and in chromosomal movement during mitosis. STONE and VESK (1982) found a band of dense granular material underlying the nuclear membranes in *Gymnodinium splendens*. Paracrystalline bodies which have a hexagonal structure, were also found in the dinoflagellate *Cyrodinium cohnii* (HEINE 1974).

The lamellar arrangement and the number of thylakoids per lamella in the chloroplast constitute taxonomically important criteria (BLANK 1986). A micrograph by TAYLOR (1968) showed zooxanthellae in *Anemonia sulcata* (= *viridis*) which possessed peripheral thylakoids with three closely packed lamellae. However TAYLOR (1986) does not describe these features in detail. In another paper on the symbionts of *Velella*, TAYLOR (1971) used the characteristic of two thylakoids to establish a new species of dinoflagellate, *Endodinium chotonii*. BLANK (1986) found that zooxanthellae associated with zoanthid, *Zoanthus sociatus* possessed a peripheral thylakoid triplet which entirely surrounded the chloroplast. In the Caribbean jellyfish, *Cassiopeia*, KEVIN *et al* (1969) found chloroplast in *S. microadriaticum* which lacked peripheral thylakoids.

Symbiodinium associated with 27 host species that belong to two phyla has been reported to possess a pyrenoid with one or three stalks (SCHOENBERG and TRENCH 1980), but there are no studies which have described branching pyrenoids or double pyrenoids such as those observed in the present study. The double pyrenoids or branching pyrenoids might be related to early stages of division of this organelle.

The structure of the cell wall varied considerably among zooxanthellae observed in *A. viridis*. SANTORE (1977) investigated the amphiesma of *Cryptomonas* and suggested that the amphiesma structure had taxonomic importance. SCHOENBERG and TRENCH (1988) studied zooxanthella in vitro from different host species found that different strains of algae demonstrated differences in the details of the structure of the amphiesma.

Differences in the ultrastructure of *S. microadriaticum* might be due to phenotypic variability rather than genetic differences within the population. Phenotypic variation in dinoflagellates can vary as the result of a number of factors. Variation may occur in the same geographic region at different times of the year or may be observed between populations of the same species in different regions (TAYLOR 1987). Previous investigators found that there were differences in the morphology of *S. microadriaticum* in different host species and suggested that morphological differences were due to the different stages of development in the dinoflagellate life cycle (TAYLOR 1973; SCHOENBERG and TRENCH 1976; STEELE 1977). SCHOENBERG and TRENCH (1980) compared the ultrastructure of *S. microadriaticum* which were isolated from different hosts and cultured axenically under identical conditions, to determine whether differences in structure were related to genetic differences. They suggested that there were ultrastructural differences between strains from different host species. In the present study, more information has been gathered about *S. microadriaticum* associated

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with *A. viridis*. Results have shown a wide range of variation in zooxanthellae ultra-structure. Direct comparison with type cultures or zooxanthellae cannot be made since, *S. microadriaticum* associated with *A. viridis* from temperate regions has not been successfully cultured in axenic media. Furthermore no free living stages of the algae have been found.

It is also possible that different strains of *S. microadriaticum* may be found in the same host species. Genetic studies might clarify whether this is the case e.g. : indirect measurement of genetic differences can be carried out by measuring protein variations using gel electrophoresis (SCHOENBERG and TRENCH 1980; AYALA and KIGER 1982); and by encoding the nucleotide sequence of the DNA of structural genes.

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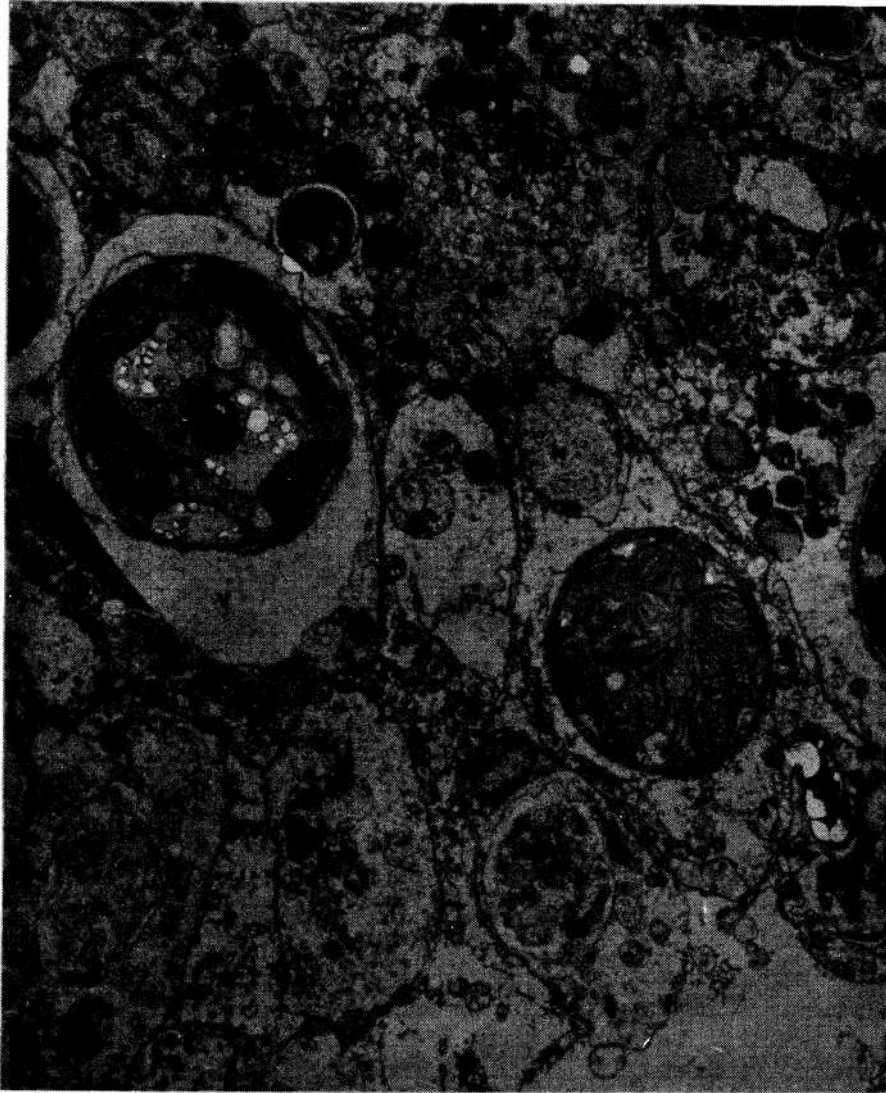


Figure 1. Electron micrograph of a section of the endoderm of *Anemonia viridis* showing cell features of *Symbiodinium microadriaticum* enclosed in vacuoles within host cells.

N = nucleus; V = vacuole; Z = zooxanthellae.

Scale bar is in microns.

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Figure 2

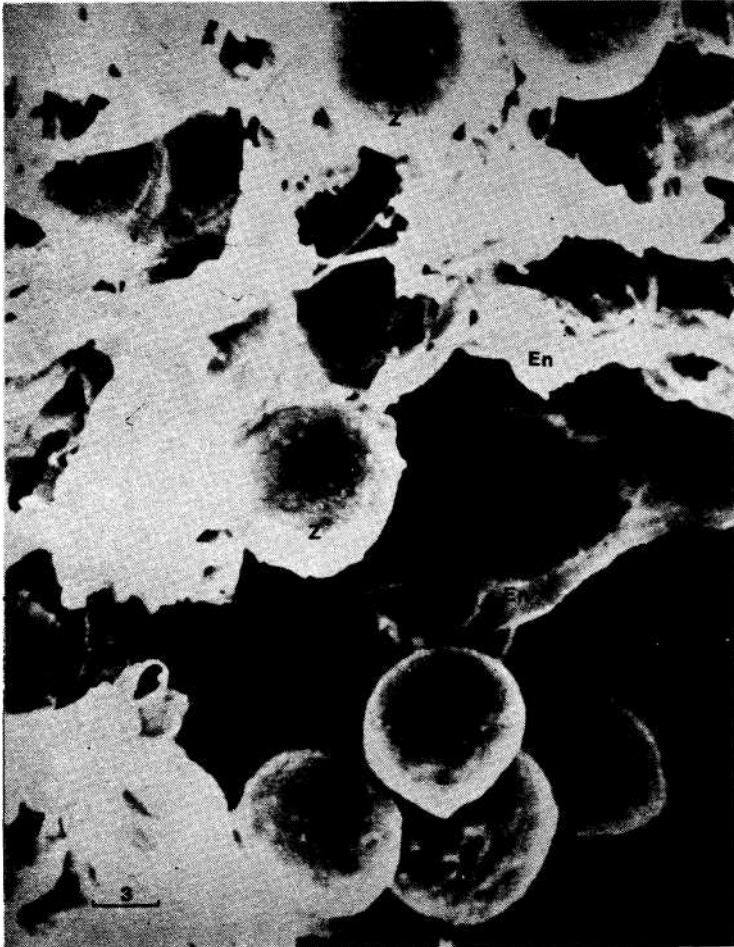


Figure 2. Scanning electron micrograph showing the smooth, surface of the cell wall of *Symbiodinium microadriaticum* in the endoderm of *Anemonia viridis*. Z = zooxanthellae; En = endodermal tissue. Scale bar is in microns.

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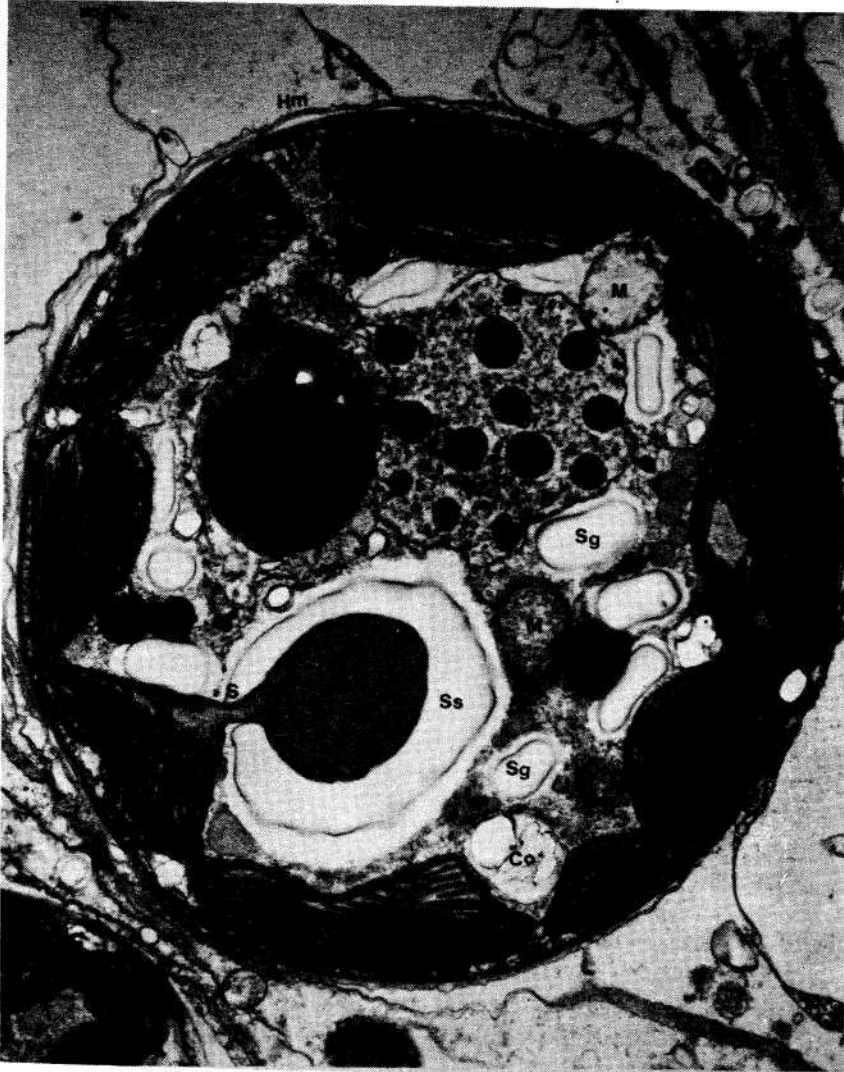


Figure 3. Electron micrograph of *Symbiodinium microadriaticum* showing the characteristic appearance of the healthy cell.

Ab = accumulation body; Cl = chloroplast; Co = calcium oxalate crystal;
Cr = chromatin; Hm = host cell membrane; L = pyrenoid; S = stalk;
Sg = starch grain; Ss = starch sheat; Zm = zooxanthellae membrane.
Scale bar is in microns.

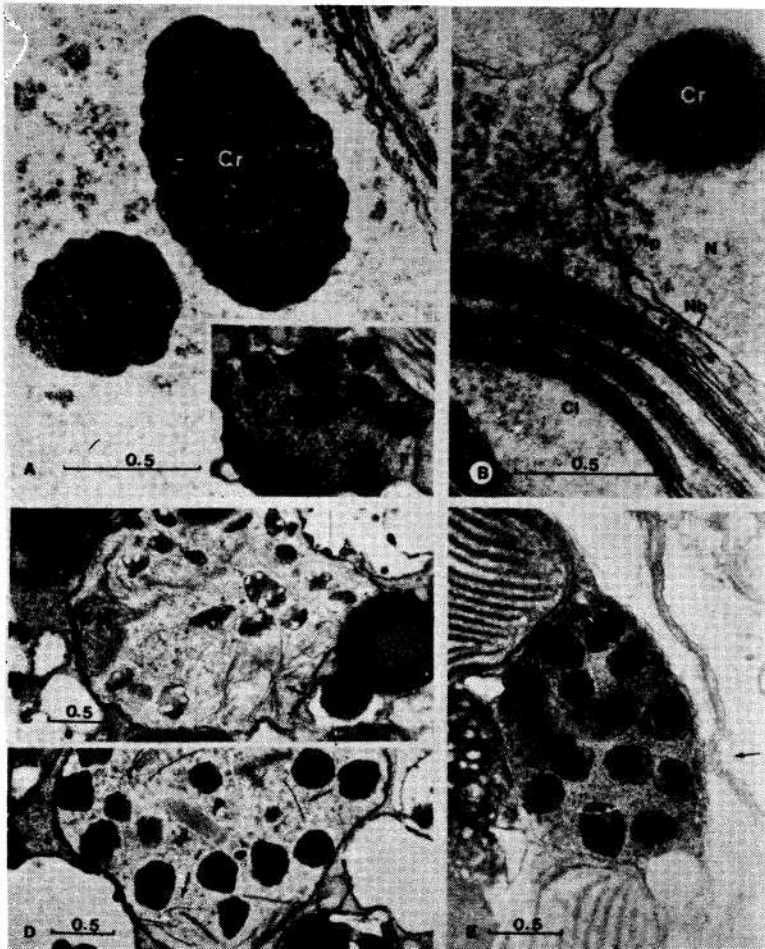


Figure 4. Electron micrograph showing the nucleus of *Symbiodinium microadriaticum* displaying : A. Densely coiled chromatin and the nucleolus (insert) in close contact with the nuclear membrane; B. A double nuclear membrane indicated by arrow (Nb) and pores of the nucleus envelope also indicated by arrow (Np); C. A bundle of string-like structure indicated by arrow; D. A string-like structure indicated by arrow and arrow head showing amorphous body; E. Nucleus of zooxanthellae in close contact with algal cell wall which is indicated by an arrow.

Cl = Chloroplast; Cr = chromosome; Cw = cell wall; N = nucleus; Nb = nuclear pore.

Scale bar is in microns.

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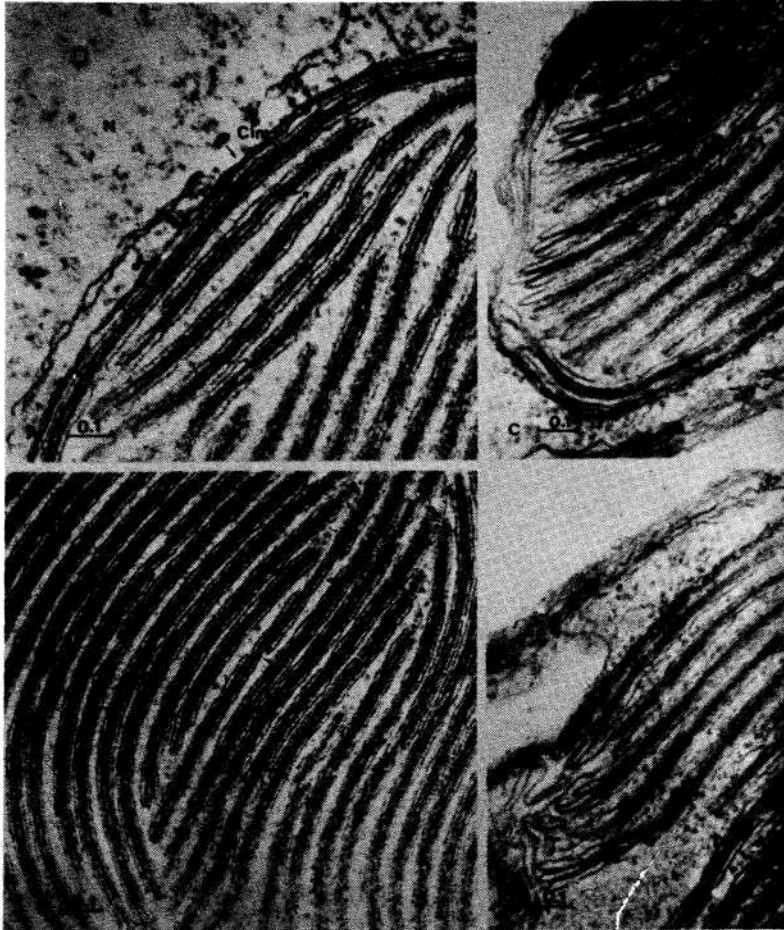


Figure 5. Electron micrograph of chloroplast of *S. microadriaticum* showing : A The structure of the peripheral lamella which indicates that each lamella consists of three thylakoids; B. Arrow showing lamella which consists of two thylakoids; C and D. Lamellae in the tip of Chloroplast are loosely packed. L = lamella; N = nucleus; T = thylakoid; Cm = chloroplast membrane. Scale bar is in microns.

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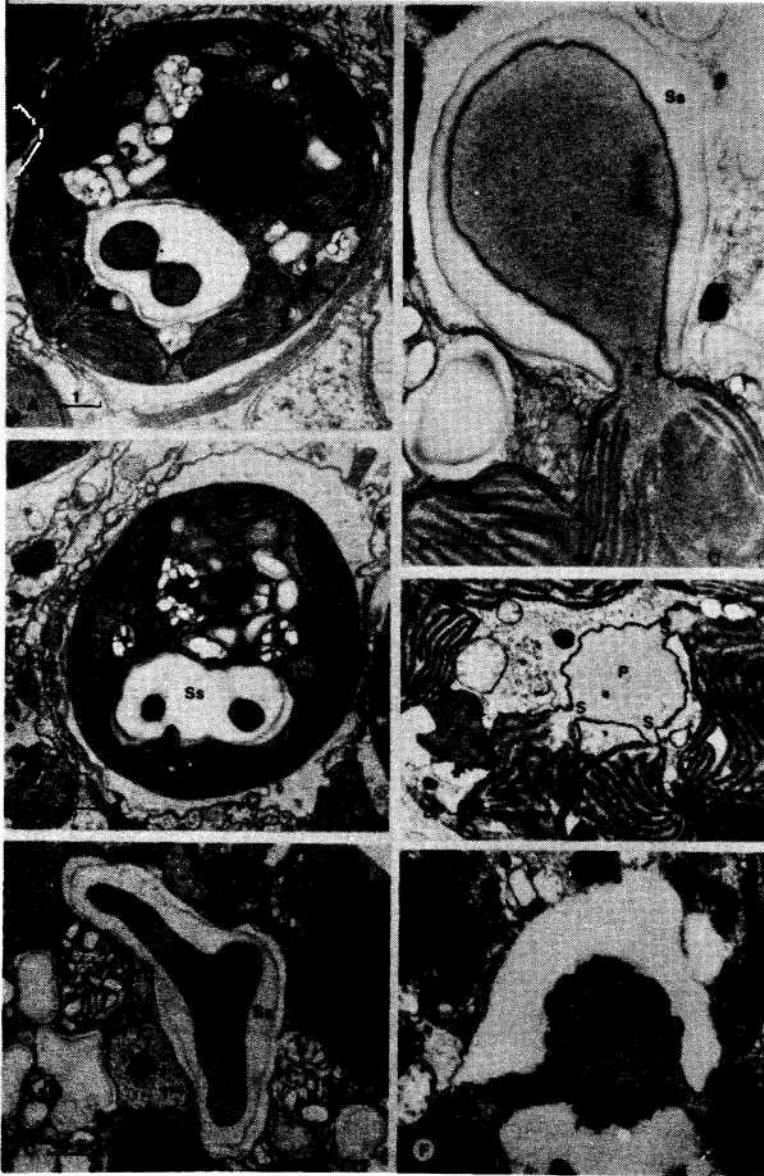


Figure 6. Electron micrograph of the pyrenoid of *S. microadriaticum* showing the variation of the pyrenoid and the stalk. A and B. The double pyrenoid; C. The branching pyrenoid; D. The fine structure of the pyrenoid and the stalk. The lamella does not penetrate to the pyrenoid; E. Pyrenoid with triplet stalk; F. Pyrenoid with doublestalk. P = pyrenoid; S = stalk; Ss = starch sheat. Scale bar is in microns.

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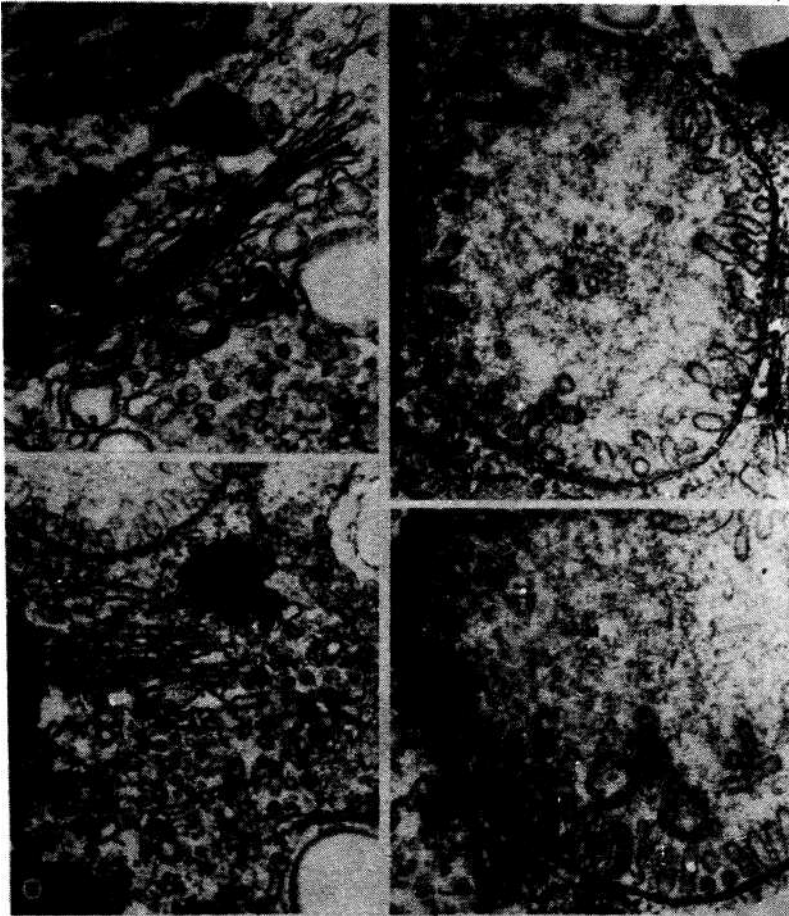


Figure 7. Electron micrograph of the cell organelles of *S. microadriaticum* showing :
A and B. Golgi apparatus with cisternae, vesicles and complex microtubules system indicated by arrow; C and D. The fine structure of double membranes of the mitochondria. Cl = chloroplast; Ct = cristae; Ga = Golgi apparatus; M = mitochondrion; V = vesicle.
Scale bar is in microns.

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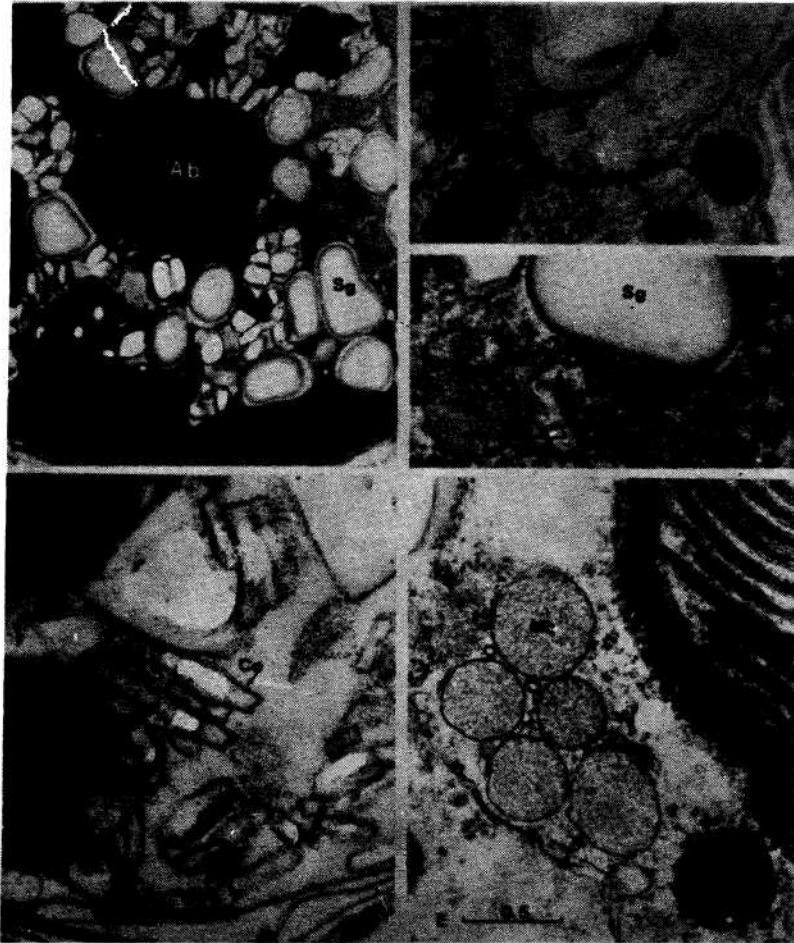


Figure 8. Electron micrograph section of *S. microadriaticum* showing : A. The accumulation body with darkened areas; B. Periodic acid thiocarbohydrazide silver proteinate staining demonstrating starch grains identified by arrows; C. A complex of microtubules with vesicles around the starch grain indicated by arrows; D. The fine structure of crystals of calcium oxalate indicated by arrow; E. microbodies, each enclosed by a single membrane.
Ab = accumulation body; Co = calcium oxalate crystals; M = mitochondrion;
Mb = microbody; Mt = microtubules; Sg = starch grains.
Scale bar is in microns.

Endosymbiotic Dinoflagellate

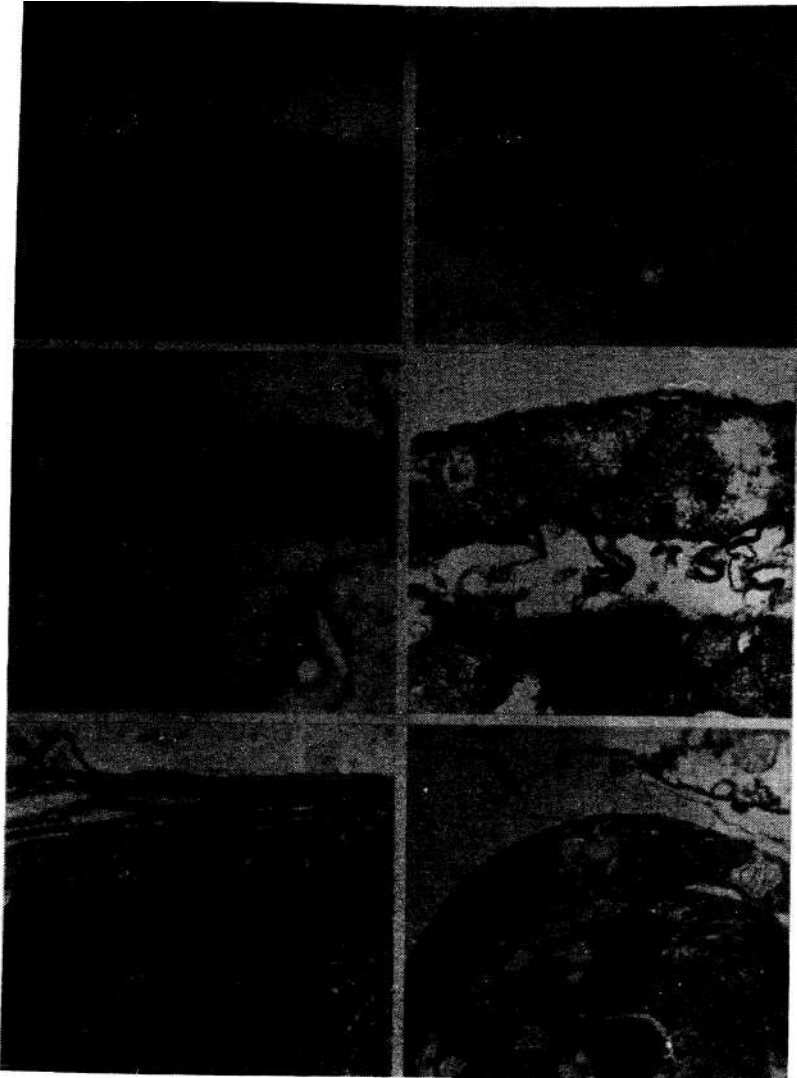


Figure 9. Electron micrograph of the cell walls of *S. microadriaticum* showing variations in the thickness of the cell Wall. A. The cell wall is composed of outer membrane pellicle, inner pellicle and plasma membrane; B. The plasma membrane is composed of at least two membranes, arrow shows plasma membrane which consist of a continuation of the chloroplast membrane; C. The cell wall consists of a series of cell membranes; D. A thick pellicle composed of plate-like structure with a suture; E. A normal cell wall with an infolding membrane deposited outside the outer membrane; F. The unusual appearance of the cell membrane.
clm = chloroplast membrane; Ipm = inner pellicle membrane; Om = outer membrane; P = pellicle; Pm = pellicle membrane; S = suture.
Scale bar is in microns.

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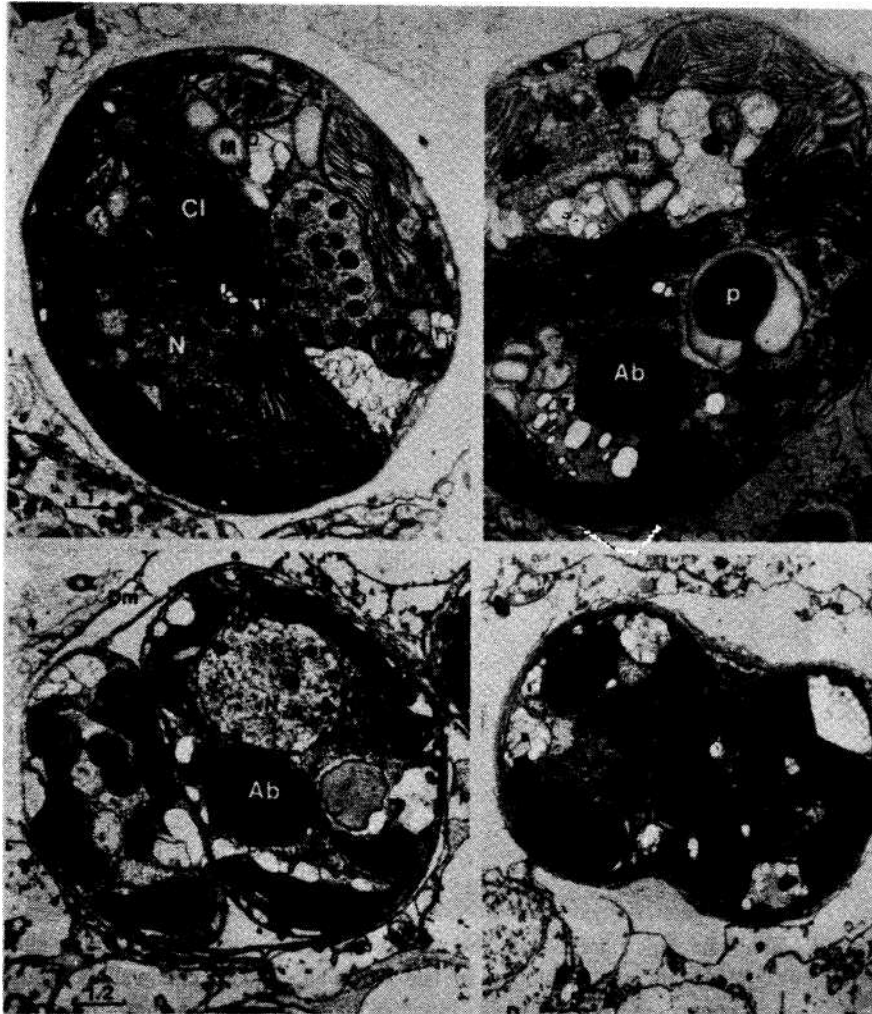


Figure 10. Electron micrograph section of a divided cell of *S. microadriaticum* showing:
A. The first stage of the division. Cytokinesis occurs after caryokinesis;
B. The accumulation body remain in the parent cell; C. Discarded membranes of the parent cell; D. Two newly formed daughter cells remain cemented in the old cell wall. Ab = accumulation body; Cl = chloroplast; Dm = discarded membranes; M^r = mitochondrion; N = nucleus; P = pyrenoid. Scale bar is in microns.

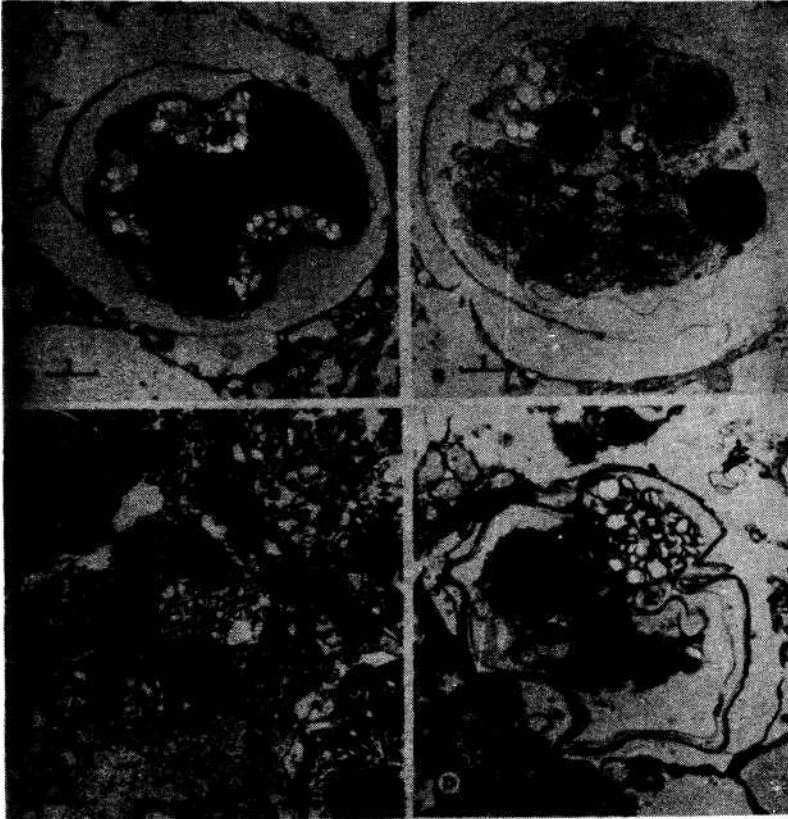


Figure 11. Electron micrograph of the degenerate cell showing : A. The cell wall breaking down in early stage of the degeneration; B. The normal appearance of the degenerate cell, the cell wall breaks down after the disintegration of the content of the cell; C and D. The latest stages of degeneration showing the intact cell wall. Cl = chloroplast; Co = calcium oxalate crystal; N = nucleus; Z = zooxanthellae. Scale bar is in microns.