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An ulstrastructural study of Acetabularia acetabulum

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An Ultrastructural Study of *Acetabularia acetabulum*

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Approval Page

I certify that I have read this thesis and find that, in scope and quality, it satisfies the requirements for the degree of Master of Science.

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TABLE OF CONTENTS

LIST OF FIGURES

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Master of Science in Biology

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Dr. Roni Kingsley, Thesis Advisor

Abstract

Calcification within the cell and the cell wall of *Acetabularia acetabulum*, a large single cell alga, is examined through an ultrastructural investigation, electron diffraction, and an elemental analysis. These methods suggest that amorphous calcium carbonate is located adjacent to and within the cell wall. The location of the crystals within and directly outside the cell wall suggests the calcium is obtained from the surrounding environment. Fibrillar structures are seen through SEM in areas where calcification is occurring. This fibrillar material appears to serve as the organic matrix where calcification initiates. Intracellularly electron dense material is seen in the chloroplasts. This material is either $CaCl_2$ or $CaCl_2 * 2\ H_2O$ but lacks a strong line through electron diffraction to confirm the compound. The origin and the role the crystals play within the chloroplasts are not clear at this time.

Introduction

Biomineralization is a common occurrence seen in a wide range of organisms. The number of known minerals forms have increased severalfold since 1963 and over 60 mineral types are known. The most common mineral seen in biomineralization is calcium (Simkiss and Wiklbur 1989). Calcification, the deposition of calcium salts, occurs in most algal divisions however only a small percent of the genera in each division calcify. Many environmental factors have played a role in the evolution of calcification. These factors include water movement, ability to take up nutrients and light, and the presence of herbivores (Padilla 1989). Overall, there are more calcifying algae in freshwater than marine environments. However, in the division Chlorophyta the reverse is seen (Pentecost 1991). The extent to which Dasycladales calcify varies. In some species, the whole thallus develops a strong thick calcareous skeleton. Other species develop a thin calcified coat, which under some extreme natural ecological conditions may not form at all. The location of the calcium carbonate within the cell differs in species. The calcium carbonate may be found in the cell wall, in the mucilage surrounding the cell wall, or in the lumen of the cell (Berger and Kaever 1992). All Dasycladales contain mineralization in the mucilage except *Acetabularia*, which contains the calcareous deposits in the cell wall (Frügel 1978).

Calcification in *Acetabularia* serves many functions. It provides structural support for the cell's large frame (Marin and Ros 1992). The stable upright body allows other organisms to attach. The calcification makes the cell more resistant to being torn from the substrate if it becomes damaged (Padilla 1989). Calcification serves as a

structural defense from herbivores in the community. The extent of grazing of the mollusc *Elysia timida* was shown to be inversely proportional to the calcification level seen in *A. acetabulum*. Highly calcified cells were entirely resistant to the grazing by *E. timida* (Marin and Ross 1992). In general, structural defenses are more advantageous than chemical or nutritional defenses. Chemical and nutritional defenses require an organism to taste the plant before rejecting it (Padilla 1989). However, the calcification of algae does not strictly serve as a structural defense, it can also serve as a chemical defense by increasing the pH of the gut in herbivores (Hay et al. 1994).

The skeleton of calcified algae is commonly made up of calcium carbonate and can be arranged as either aragonite or calcite crystal isomorphs (Berger and Kaever 1992). *Acetabularia* deposits have been described as aragonite needles arranged in small bundles layered in the cell wall (Borowitzka 1982). Trace elements of $MgCO_3$ (1%) (Lewin 1962) and $SrCO_3$ (1.3%) (Marszalek 1975) have been found in *Acetabularia*.

Borowitzka (1982) described calcification in plants and algae as either having extracellular or intracellular deposits depending on the location of the carbonate crystals at the initial point of calcification. Extracellular deposits have calcite crystals deposited directly on the outer cell wall surface. An example of an alga with extracellular deposits on the cell wall is *Chara*. The deposits on the cell wall typically form side by side, with slight overlap, at an angle giving a tent like overall appearance.

A second form of extracellular calcification is called sheath formation. The mineralized deposits are layered throughout the cell wall and a $CaCO_3$ free layer, known as a 'sheath' is found outside the cell wall. The sheath is comprised of an unknown

chemical composition. Sheath formation has been used to describe *Acetabularia* calcification. The sheath's function in the calcification process is not clear. It is hypothesized that it may act as a barrier to diffusion of inorganic carbons (Borowitzka 1982). It may also play a role in the regulation and orientation of calcium carbonate crystals (Simkiss and Wilbur 1989). A decrease in the diffusion of inorganic carbons into the cell combined with the removal of photosynthetic carbon dioxide uptake, leads to an increase in pH. The result of an increase in pH is an increase in the rate of $CaCO_3$ precipitation (Borowitzka and Larkum 1976a).

An example of intracellular calcification is seen in coccolithophores where plates of calcite called coccoliths are produced. The coccoliths are initially formed intracellularly and are then expelled outside of the cell. The cell controls the growth of each crystal, eventually forming up to 80 crystalline units depending on the species. With the exception of *Emiliaia huxleyi* the coccoliths are formed on a base plate. The base plates and unmineralized body scales are typically formed in the Golgi vesicles simultaneously. Coccolithosomes are small bodies formed in the coccolith vesicles and determine the shape of the coccolith. As the shape is determined, spaces within the coccoliths become filled with $CaCO_3$. The coccoliths are released from the cell and stick onto the mucilaginous layer around the cell (Borowitzka 1982).

In considering extinct Mesozoic Dasycladales, from which *Acetabularia acetabulum* has evolved, Conrad and Baki (1990) describe four possible patterns of calcification. The first pattern is a calcified hyaline and colorless sheath commonly

found in species that lived in marine or hypersaline environments. The original aragonite needles are completely lost and replaced with a thick calcite.

The second pattern of calcification has calcite as the original precipitation. The sheath has been seen mainly in species that lived in polyhaline habitats suggesting the aragonite precipitation was inhibited (Conrad and Baki 1990).

A third calcification pattern may have occurred over a short period of time in at least four species. The calcification is composed of two layers, a thick interior layer and a thin granular exterior layer. A middle calcite-free portion exists between the two layers. (Conrad and Baki 1990)

The fourth pattern of calcification involves microcrystalline sheaths that are dark in transmitted light. They cover or replace the genuine (hyaline) sheaths and presumably result from an algal/bacterial calcification process (Conrad and Baki 1990).

Two types of calcification were discussed by Flajs (1977a) for extant Dasycladales. Flajs compared calcification in *Cymopolia* and in *Acetabularia*. In *Cymopolia*, calcification occurs in three stages: initially granular crystals are formed, approximately 10 nm in size, and grow until they reach approximately 1 μm in length. Aragonite needles are then formed from the granular crystals, reaching approximately 0.5-2 μm in length, and are arranged loosely in the cell wall. The final stage occurs as the aragonite crystals spread throughout the mucilage throughout the cell wall. In *Cymopolia* species, variability of calcification can be seen within a single thallus. Environmental factors can influence the length, width, and density of the needle arrangement.

As discussed by Flajs (1977a), the size and the arrangement of the calcareous crystals of *Acetabularia* differ from those of *Cymopolia*. In *Acetabularia*, the aragonite aggregates are composed of several parallel needles grown together forming a compact layer on the surface of the caps. There are aggregates up to 5μm in length and 1μm in width. The cap and the stalk differ in the structure of their aragonite needles. Cap calcification consists of thick bundles of aragonite protruding outward and narrow at the end while the stalk appears to have fine granular aragonite throughout the cell wall.

Okazaki and Furuya (1985) described the calcification in *Acetabularia calyculus* as follows. Three regions are distinguished in the cell: the tip of the stalk where no calcification appears, the middle region of the stalk where trace evidence of calcification occurs, and the base of the cell where calcification is complete. Electron dense material is secreted into the wall at the tip of the stalk, where no calcification occurs. The cell wall thickness has been associated with the induction of calcification in *A. calyculus*. When the cell wall reaches 15- 22μm thick, calcium carbonate material is deposited. Small crystalline aggregates form at the inner portion of the cell wall where the electron dense material appears to induce the crystallization. Granular crystals develop from the aggregates in the middle portion of the stalk as development proceeds. The crystals continue to grow and fuse together near the base of the cell, near the rhizoid region, to form crystalline masses and complete calcification. The cell wall is completely calcified except for a thin calcium free layer of the outer portion of the cell wall.

The calcification of *Acetabularia acetabulum* has previously been described using organisms from the wild. Due to this feature, previous studies have been unable to look

at the initial steps of calcification occurring in this species. This study analyzed organisms that were obtained and developed in a laboratory. Due to using organisms from the laboratory, I was able to examine the initiation of calcification. The goal of this study was to compare what was known in other organisms and other species of *Acetabularia* to what was seen in *A. acetabulum*. Through scanning electron microscopy (SEM) and transmission electron microscopy (TEM), calcification within the cell was examined.

Methods and Materials

Young zygotes of *Acetabularia acetabulum*, line Aa0005, were received from Dina Mandoli, University of Washington. The cells were maintained in Ace 27 artificial seawater at room temperature (21° C) and on a 14:10 light cycle (Mandoli personal communication). The stage of development of the cells was recorded by their size. The cells were placed in square petri dishes with the appropriate cell water ratio dependent on the development of the cell. The zygote cells were less than 0.1 mm in length. Approximately 300 cells were placed in 1 ml of Ace 27 artificial seawater. Juvenile cells were less than 1 cm in length and had no more than 6 hair-like whorls per cell. When cells were large enough to be seen by eye, they were thinned out to approximately 100 cells per 50 ml of Ace 27 seawater. The cells were further diluted as they grew and adult cells, cells larger than 1 cm in length with greater than 6 whorls, were placed 20 cells per 50 ml of Ace 27 seawater (Hunt pers. comm.). Sterile techniques were used when transferring the cells and each petri dish was sealed with a thin layer of parafilm to prevent contamination.

Scanning Electron Microscopy:

Calcified and non calcified cells, at a variety of developmental stages, were fixed overnight in a solution of 2% cetylpyridine chloride in 4% glutaraldehyde and washed with 2M sodium cacodylate seawater buffer. Cells were subsequently placed in 1% OsO_4 for 1 hour and washed with the cacodylate seawater buffer. Specimens were dehydrated in a graded series of ethanol. The specimens were then placed in the critical point drier

and sputter coated using Hummer VII. Images were observed using the Hitachi S2300 scanning electron microscope.

Transmission Electron Microscopy:

Late juvenile cells, ranging in age from 90 to 120 days, were fixed overnight using a fixative of 2% cetylpyridine chloride in 4% glutaraldehyde and washed in 2M sodium cacodylate sea water buffer. Cell were subsequently placed in 4% osmium tetroxide for 2 hours and washed with the cacodylate seawater buffer. Specimens were dehydrated using a graded series of ethanol and embedded in EPON. Ten nm thick sections were cut and placed on 150 mesh copper grids covered with 2% collodion Specimens were examined using a JEM 1010 transmission electron microscope.

Elemental Analysis:

Specimens were preserved as above, sectioned, placed on carbon coated grids and sent to Dr Norimitsu Watabe at the University of South Carolina where electron diffraction and x-ray microanalysis were performed on the specimens. X-ray microanalysis was performed using a Scanning Electron Microscope allowing for point specific analysis. The unique elemental composition of the region traversed by the electron beam was determined from backscattered electrons and x-ray signals. The energy levels of the x-ray signals produced, were recorded and graphed.

Results

Electron dense material (Ca) immediately outside the cell wall was found to be calcareous by elemental analysis. The elemental analysis of the material found outside the cell wall showed several peaks indicating the presence of three chemical components (Figure 2). Only one element, calcium, can be attributed to occurring naturally. The osmium peak represents the use of osmium during fixation while the copper peak corresponds to the specimen grid.

Electron dense material (Ca) within the cell wall was found to be calcareous by elemental analysis. Four elements were detected through elemental analysis (Figure 3). The two peaks of interest are the sulfur and the calcium. The sulfur peak indicates sulfate, part of the organic matrix of the cell wall, and the calcium peak further verifies the presence of calcium. Of the two elements found naturally in the cell wall, 15.1 % was sulfate and 84.9% was calcium (Figure 3). Electron diffraction of this material revealed a non-crystalline, amorphous pattern (not shown). When cells were placed in 6 N HCl, bubbles were instantly generated consistent with the idea that the electron dense material was $CaCO_3$.

Transmission electron microscopy shows the plasma membrane to have a smooth appearance throughout the cell. The extracellular surface of the cell wall has an irregular wavy pattern compared to the straight, smooth plasma membrane (Figure 4). SEM micrographs of an uncalcified cell wall emphasize the uneven pattern of the outer surface of the cell wall (Figure 5).

SEM observations showed the mineralized masses on the cell wall having a round and speckled appearance. The $CaCO_3$ masses range in size and density along the cell wall (Figure 6). Mineralized materials appear individually, in small clusters spread along the cell wall surface and/or as a thoroughly calcified outer cell wall surface (Figure 7). Various levels of calcification can be seen throughout the cell. The rhizoid has light calcification except in areas from which new growth develops (Figure 8). Calcification appears continuous near the base of the stalk (Figure 9) and becomes intermittent as calcification approaches the tip of the stalk. The tip of the stalk shows no calcification (Figure 10).

Through scanning electron microscopy thin fiber-like structures are seen (Figure 11). These structures appear on the stalk connecting and binding large $CaCO_3$ bundles in the less calcified portions of the cell (Figure 12). In sections where heavy calcification is present, the fiber-like structures are less prominent or no longer visible (Figure 7). The non-calcified strand of *Acetabularia* lacks the fibrillar material (Figure 13). The fibrillar material corresponds to the dark material found on the exterior portion of the cell wall seen under TEM (Figure 14). The location of the calcium carbonate on and within this fibrillar material during early stages of calcification is seen throughout the study..

Using TEM, the $CaCO_3$ is seen on, and within the outer surface of the cell wall (Figure 14). Figure 15 shows the $CaCO_3$ penetrating the fibrillar material and protruding directly into the cell wall. No electron dense material was seen excreted into the cell wall from the cytoplasm.

Within the cell wall the $CaCO_3$ initially appears as simple oval or rod- shaped structures. As calcification occurs, thick dense clusters of calcium carbonate are found throughout the cell wall (Figure 16). These aggregate clusters vary in shape, size, and density. Calcification is seen throughout the cell wall but tends to be concentrated towards the outer portion of the cell wall (Figure 17). Within the cell wall, the $CaCO_3$ clusters appear most often near the exterior portion of the cell wall while small clusters of mineral appear closer to the plasma membrane.

A composite micrograph of a cell wall shows the different progressive levels of calcification occurring. Figure 18 shows the variation of calcification in a densely calcified section of the cell. Thick aggregates of $CaCO_3$ are observed at the rhizoidal end of the stalk, while thin, singular rods of less dense $CaCO_3$ is seen toward the axial portion of the stalk. Little calcification is seen near the apical end of the stalk (Figure 19). As progression toward the rhizoidal region occurs, calcium carbonate material appears in the cell wall.

A cross section of the stalk of a cell indicates different degrees of calcification. Figure 20 shows thick calcification on one cell wall and light calcification on the opposite side of the cell.

Electron dense material was seen in the chloroplasts in the cells of *Acetabularia acetabulum* (Figure 21). Electron diffraction analysis indicates that the material was either $CaCl_2$ or $CaCl_2 * 2H_20$, but lacks a strong line required confirming the compound. Elemental analysis indicated that six natural chemicals were found within the chloroplast (Figure 22). The percent composition of each of the elements is illustrated in Figure 22.

Chlorine made up the largest elemental percent at 55.5%, followed by magnesium at 11.0%, calcium at 8.3% and potassium at 1.7%. Silicon made up 20.1% and sulfur consisted of 7.2% of the total composition found within the chloroplast. The analysis of the individual element data resulted in a Ca to Cl ratio of 1:4.21. The computer gave a ratio of 1:5.4. Individual analysis also indicates that some KCl and $MgCl_2$ are also present.

Discussion

In the present study it was found that calcification, including the nature of the calcium carbonate in *Acetabularia acetabulum*, is apparently unique in this species of *Acetabularia*. Previous studies regarding this genus have shown calcified material to be aragonite crystals and calcite (Kerkar 1994). In addition it has been suggested that the calcification process initiates intercellularly in some species of this genus (Okazaki and Furuya 1985). This study examined differences and similarities between *A. acetabulum* and previously examined algae.

The elemental analysis of the material found within the cell wall identified the presence of calcium. The granules seen in the cell wall of *Acetebularia* were of an amorphous nature. Electron diffraction did not show the distinct characteristic pattern for the more stable crystalline calcium carbonate forms indicating that the calcification was amorphous in nature. The biomineralization of calcium carbonate is typically found in a more stable form as aragonite or calcite. Kerkar (1994) reported the calcium carbonate deposits of 17 algal species belonging to 3 algal genera. Organisms in the class Ulvophyceae were found to have their calcium carbonate made up of aragonite. Specifically the calcium carbonate in *Acetabularia* has been found to be aragonite. *Acetabularia kilneri* (Kerkar 1994) and *Acetabularia calyculus* (Okazaki and Furuya 1985) were found to contain mostly aragonite crystals. Flajs (1977b) discussed the skeletal structures of 34 genera in the division Chlorophyta and found that the calcium carbonate crystals were also aragonite needles. The current finding of amorphous calcium carbonate is therefore a significant and unexpected finding. The lack of reports

on the amorphous calcium carbonate precipitation in plant and algal cells indicates that further studies need to be performed.

The lack of fibrillar material on the cell wall of *Acetabularia acetabulum* when no calcification has occurred, such as on the tip of the cell, and the increased abundance of fibrillar material when calcification is present indicates that this material plays a role in the calcification process. The presence of fibrillar material on the surface of *A. acetabulum* is similar to what is seen in *Halimeda*. In *Halimeda*, the initial fibrillar material develops when the crystal granules are approximately 36-48 hours old (Wilbur et al 1969). In *Halimeda*, the location of the $CaCO_3$ outside the cell wall is also similar to what is seen during calcification of *A. acetabulum*. When little calcification is seen in the cell wall, most of the $CaCO_3$ is seen on or in the fibrillar material found outside the cell.

The fibrillar material seen under SEM corresponds to the dark material found on the exterior portion of the cell wall seen under TEM. Although no distinct fibrillar bands are seen in TEM, the locations correspond with each other. This variation may be due to the difference in fixation between SEM and TEM however further examination is warrented.

The exact role of the fibrillar material is unclear in *A. acetabulum*. The presence of the fibrillar material seen when calcification is sporadic indicates the potential of it to serve as an organic matrix. The organic matrix has been shown to have 3 possible roles. The organic matrix in *Halimeda* has been shown to provide nucleation sites for calcium carbonate (Wilbur et al 1969). Additionally, the organic matrix provides a substrate where crystal nuclei are deposited (Watabe 1981). The initial $CaCO_3$ found on

nucleation sites can dissolve and reform in a new region within the cell. Thus, mineral outside the cell wall may dissolve, enter the cell wall and reform in this location. Finally, the organic matrix may aid in isolating the cell wall from the surrounding seawater (Simkiss and Wilbur 1989). In *A. acetabulum*, the organic matrix on the cell wall may provide a combination of all three functions. As calcification continues, the fibrillar layer becomes obscured and only a fully calcified cell wall is visible.

The location of the $CaCO_3$ in *Acetabularia acetabulum* suggests that the origin of the $CaCO_3$ is extracellular; that is, directly from the environment outside the cell. The suggestion that $CaCO_3$ is obtained from the environment is different than what was described in *Acetabularia calyculus*. Okazaki and Furuya (1985) found that electron dense material was produced within the cell and released into the cell wall as bur-like structures. It is unclear where the bur-like structures are created and no association to specific organelles has been noted (Okazaki and Furuya 1985)

Calcification is seen as aggregates within the cell wall of *Acetabularia acetabulum* in TEM micrographs. Aggregates of calcareous material are a common phenomenon seen in many calcifying algae. The $CaCO_3$ pattern seen in *A. acetabulum* appears similar to calcification pattern noted in *Halimeda* (Wilbur et al 1969) which takes place in three stages of calcification. Initially, fine grain aragonite develops in the filament walls to form small granular crystals. The second stage consists of the growth of long aragonite needles in the wall. However, this stage is sometimes found to be lacking. The final stage is the filling in of uncalcified space with aragonite needles shaped irregularly. This study of *A. acetabulum* provided evidence of various stages of

calcification within one cell. Micrographs show diverse amounts of $CaCO_3$ on, in, and near the exterior portion of the cell wall. A distinct pattern is seen in *A. acetabulum* from the tip of the cell to the rhizoid. In regions where little calcification has occurred, towards the apical end of the stalk, single rod shaped structures are seen in the cell wall and large aggregates are seen outside the cell wall within the fibrous layer. The individual mineralized structures appear to aggregate within the cell wall forming large calcified areas near the rhizoidal end of the stalk indicating maturation, as described by Wilbur et al. (1969).

The mineralized clusters seen in *Acetabularia acetabulum* vary in shape, size and density. The size and arrangement of the $CaCO_3$ did not appear as Flajs (1977a) described in *Acetabularia*. Flajs (1977a) stated the aragonite needles form aggregates up to 5 μm long and 1 μm wide where several needles grow together in a parallel manner. The $CaCO_3$ aggregates in *A. acetabulum* did not have this formation. Instead, this study found that the aggregates typically arranged themselves in a loose round shape. *Halimeda monile* and *H. incrassata* also show diversity in the size and shape of the crystals throughout the stalk (Wilbur et. al 1969). This may be due to environmental conditions that play a role in calcification such as pH, light intensity, temperature and salinity. Little research has been done on the effects of environmental conditions on calcification (Flajs 1977a).

The location of $CaCO_3$ in algae varies. Flajs investigated the prevalence of calcification in 16 species belonging to 6 genera of algae. Among them were several species of *Acetabularia* and *Bornetella*. He showed that in each species the cell wall did

not calcify. Instead, calcification only occurred in the extracellular mucilage found between laterals and gametangia. Round (1984) however, reported that numerous species of siphonous algae have $CaCO_3$ deposited in the cell wall. The location of mineralized structures in *A. acetabulum* throughout the entire cell wall more closely corresponds to what was reported by Round (1984). The presence of $CaCO_3$ throughout the cell wall differs in *Acetabularia calyculus* where the outer 10% of the cell wall and a thin inner layer of the cell wall, lacks crystals (Okazaki and Furuya 1985).

The lack of calcification at the tip of the stalk commonly occurs in algae. Two potential explanations include the thinness of the cell wall at the apical end and the growth of the cell from the apical end. The apical end of the cell wall of the stalk is not as thick as the cell wall located at the rhizoidal end of the cell. The cell wall becomes thicker as it progresses down the stalk of the cell. A correlation between the cell wall thickness and the calcification has been seen in *Acetabularia calyculus*. Okazaki and Furuya (1985) determined when the cell wall reaches a thickness of 15-22μm calcification begins. Future studies should examine the correlation between the cell wall thickness and calcification levels.

The lack of calcification at the apical end of the stalk also allows the stalk to continue growing. Due to the development of the stalk at the apical end (Puiseux- Dao 1970), presence of calcium carbonate would inhibit the cells ability to grow. The rhizoid region has continual growth explaining the lack of calcification seen on growing extensions. Calcification in the middle of the stalk and at the base of the cell allows for structural support in the cell (Hay et al 1994). Thorough calcification at the base of the

stalk allows protection from herbivores, support for the large body and a stable body to allow other organisms to attach (Marin and Ros 1992).

Within each cell of *Acetabularia acetabulum*, one side of the cell is more calcified than its opposing side. These different levels of calcification are due to the location of the cell in relation to the light source that was used. Calcification has been shown to be associated with photosynthesis. The side of the cell that is receiving more sunlight will increase the level of calcification due to photosynthesis. The effect of photosynthesis has been examined in *Halimeda tuna* (Borowitzka and Larkum 1979b). It has been noted that continued calcification in the dark occurs at a slower rate than in the light. However, there are many rapidly photosynthesizing algae that do not have the ability to calcify (Simkiss and Wilbur 1989) indicating that more than just photosynthesis is involved in mineral depositing.

Simkiss and Wilbur (1989) described the method of diffusion to allow for most of the Ca^{2+} movement. The fibrillar region on the cell wall of *Acetabularia acetabulum* allows for this diffusion to occur. Bicarbonate ions also enter this space through diffusion as well. The carbon dioxide diffuses in and out of the cell from photosynthesis and respiration. The difference in diffusion rates between carbon dioxide and bicarbonate lead to a change in pH and an increase in carbonate ion concentration due to photosynthesis. The excess carbonate ions will bind to the calcium ions forming a $CaCO_3$ precipitate. Simkiss and Wilbur (1989) use a three phase process to explain the calcium uptake from water. Initially a quick accumulation of Ca^{2+} ions occurs. The accumulation then lags due to the Ca binding to the walls, dissolving of impure crystals

and the recrystalization process. The third phase has $CaCO_3$ accumulating at a constant rate.

Electron dense material was seen in chloroplasts. When analyzed by electron diffraction it was determined that the crystals were likely to be $CaCl_2$ or $CaCl_2 * 2H_20$. However, the sample patterns were lacking a strong line that should have appeared in each of these compounds. Silicon, magnesium, sulfur and potassium were also seen in the chloroplasts in quantities through elemental analysis. There are two possible explanations for the crystals seen in the chloroplasts. The first is that they are artifacts from the fixation process. There is a possibility that fluids in the chloroplasts crystallized during fixation. The second explanation is that crystals are normally present and protected from dissolving during fixation.

Further testing is required to understand the calcification process found in *Acetabularia acetabulum*. This study concentrated on the rhizoid, the base of the stalk and the axial end of the stalk. The stock of cells used in this project did not reach their final cap formation and produce gametes. There has been some research conducted on the localization of calcium in the cap of *Acetabularia* (Harrison et al 1988). An ultrastructural study performed using electron microscopy to understand the cap calcification could assist in further shedding light on *A. acetabulum*'s calcification process.

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Figure 1

A mature *Acetabularia acetabulum* seen with bulging rhizoid (R), a long, narrow stalk (S), and a fully developed cap (C).

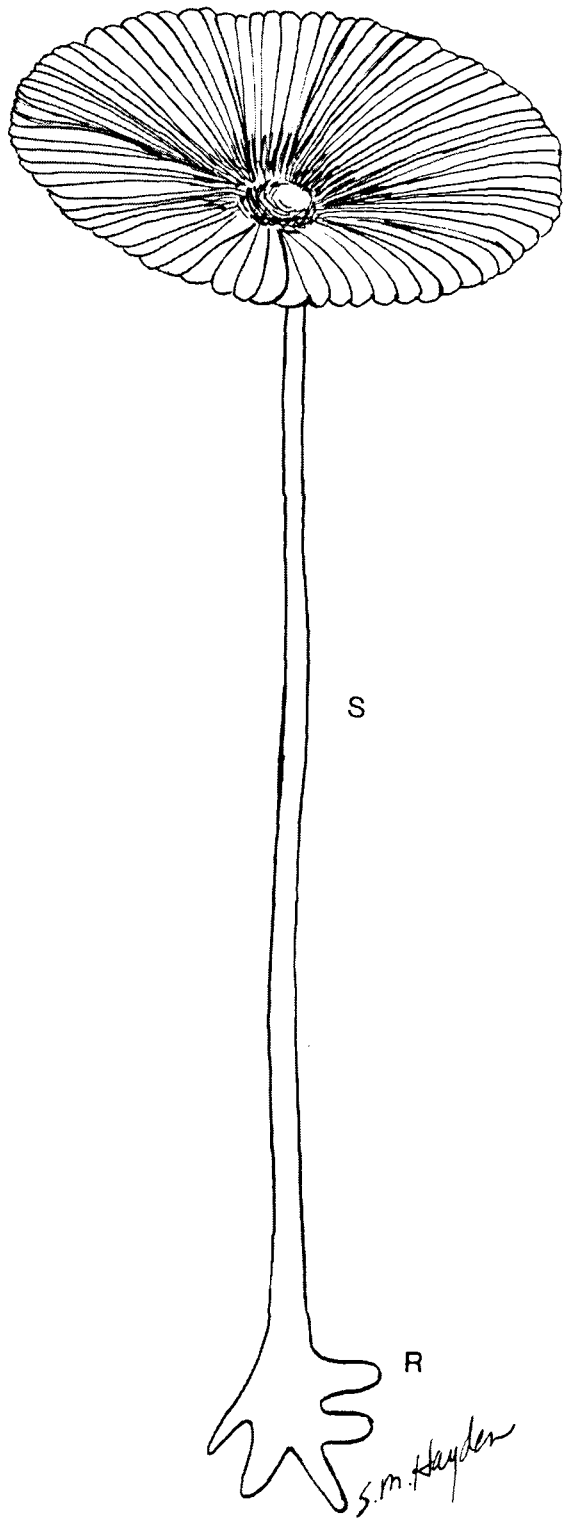
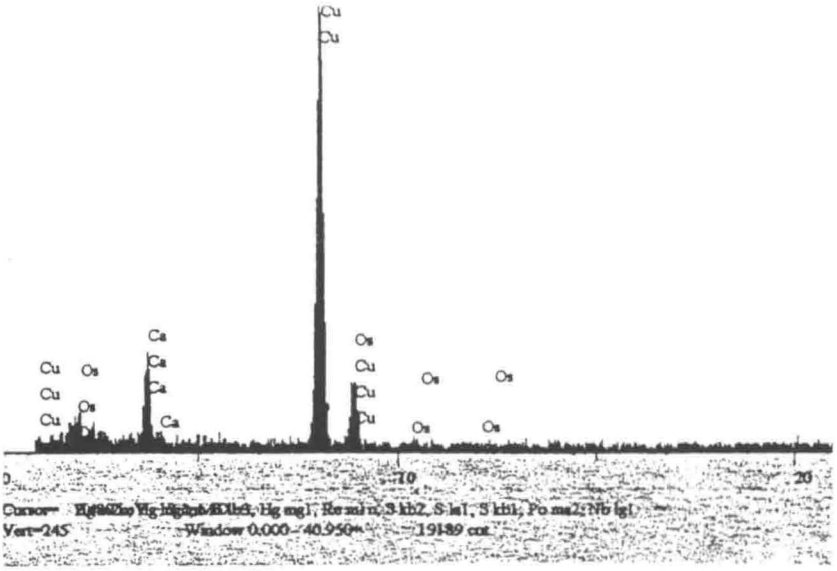

1
C
S
R
s.m. Hayden

Figure 2

Elemental analysis of electron dense material (Ca) found outside the cell wall (CW). Ca is the only naturally occurring element seen. Cu is from the specimen grid, Os occurs from fixation.

Figure 3

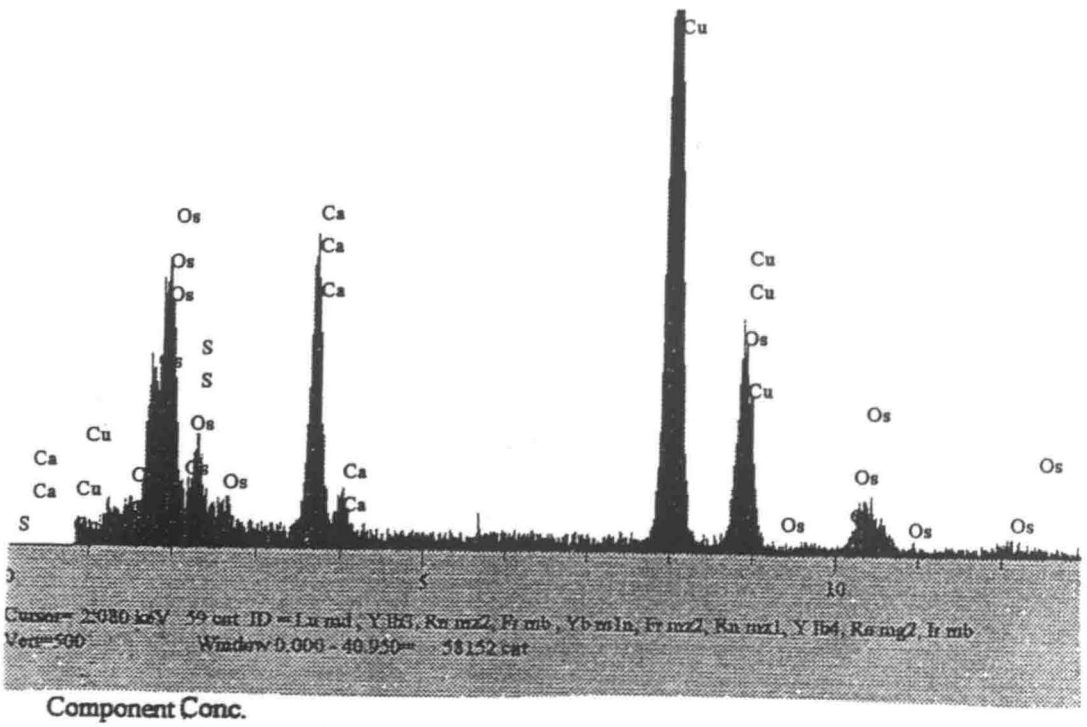
Elemental analysis of electron dense material (Ca) found within the cell wall (CW). S and Ca are the only naturally occurring elements seen in the cell wall and make up 15.1% and 84.9% of the cell wall composition respectively. The strong Os peak is attributed to fixation and the Cu peaks are attributed to the specimen grid.



WALL

Component	Conc.	
Ca	7.244	wt.%
Cu	86.315	wt.%
Os	6.440	wt.%

2



Component	Conc.	
S	15.079	wt.%
Ca	84.921	wt.%

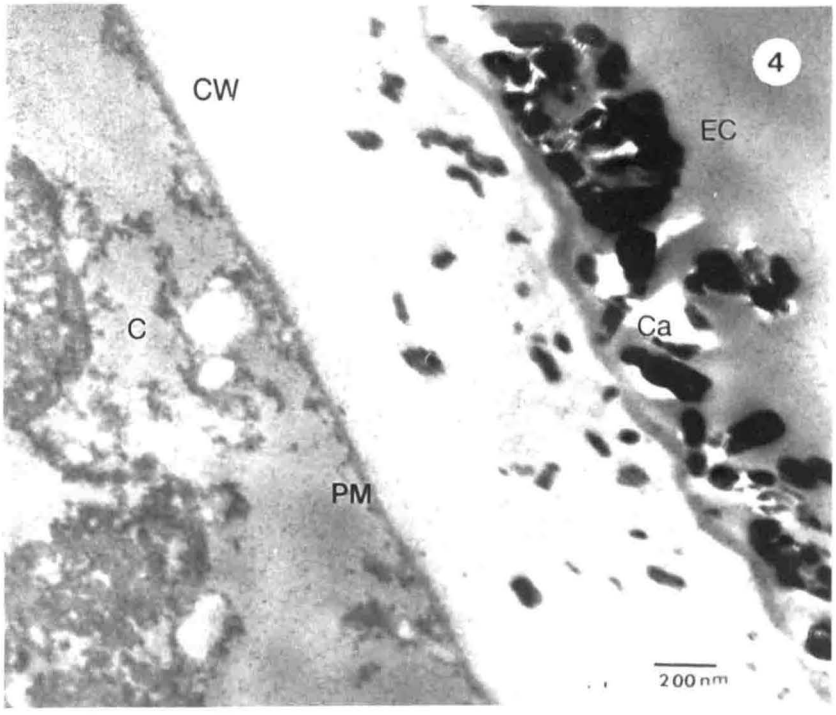
3

Figure 4

Transmission electron microscopy shows the smooth appearance of the plasma membrane (PM) and the irregular wavy pattern of the extracellular surface of the cell wall.

Figure 5

The uneven texture of the stalk (S) of *Acetabularia acetabulum* demonstrated through Scanning Electron Microscopy.


4
CW
EC
C
Ca
PM
200 nm

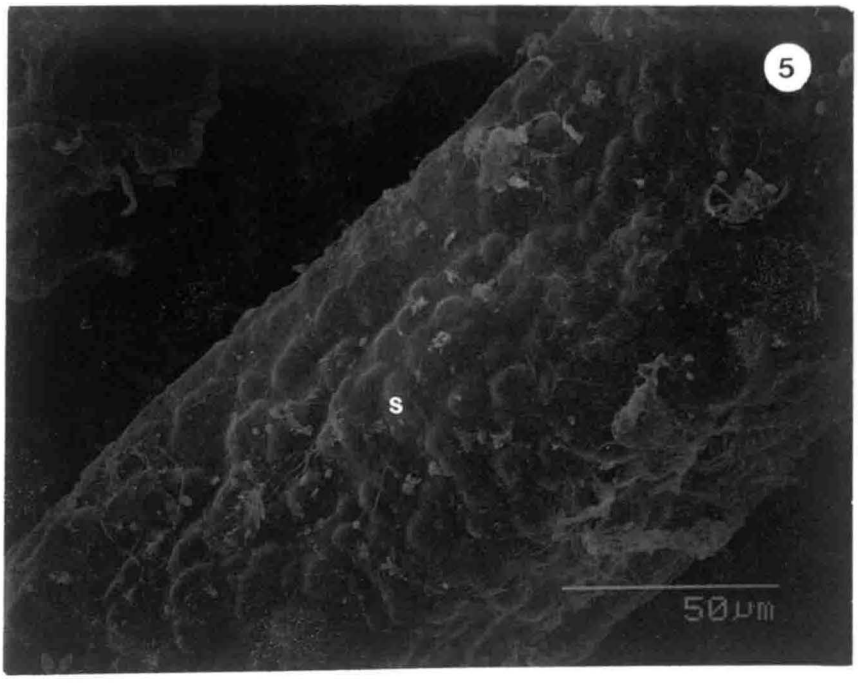
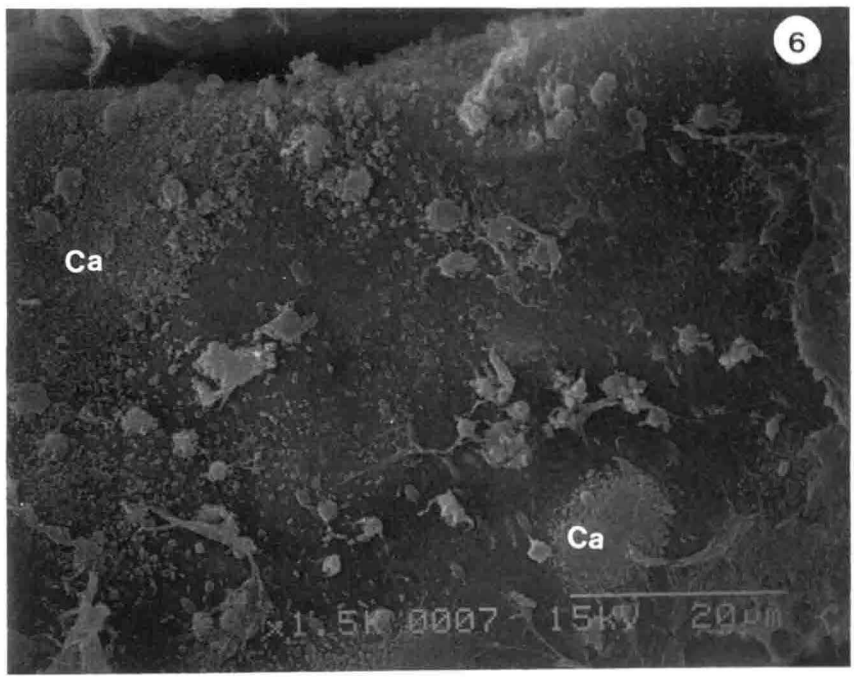

5
S
50µm

Figure 6

As calcification occurs, the amorphous calcium carbonate (Ca) is seen along the cell wall creating a granular appearance. As calcification occurs the shapes and density of the crystals vary, appearing either as round clusters or a singular rod shape.

Figure 7

Complete calcification (Ca) progress seen on the stalk of *Acetabularia acetabulum.*


6
Ca
Ca
×1.5K 0007 15KV 20μm

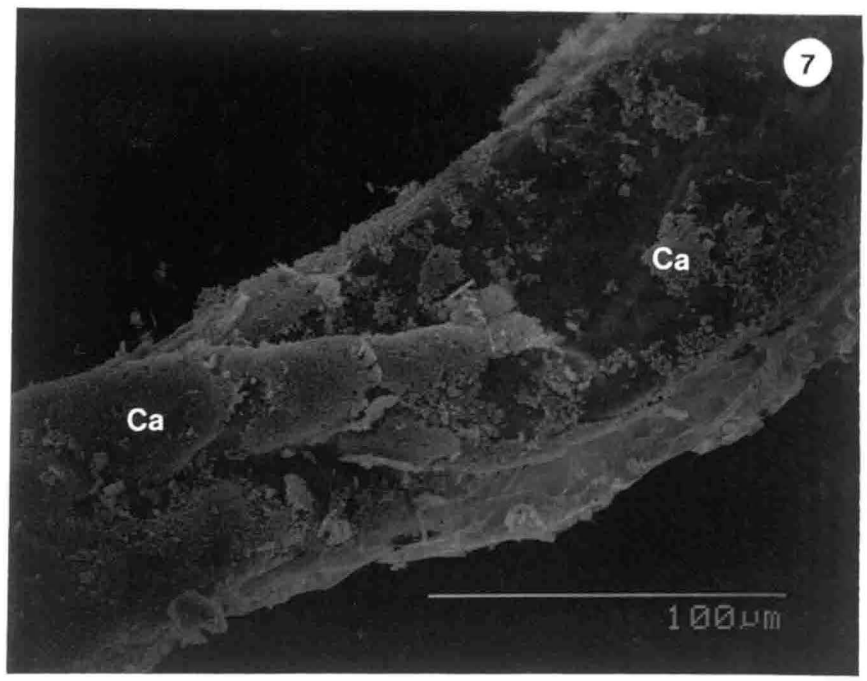
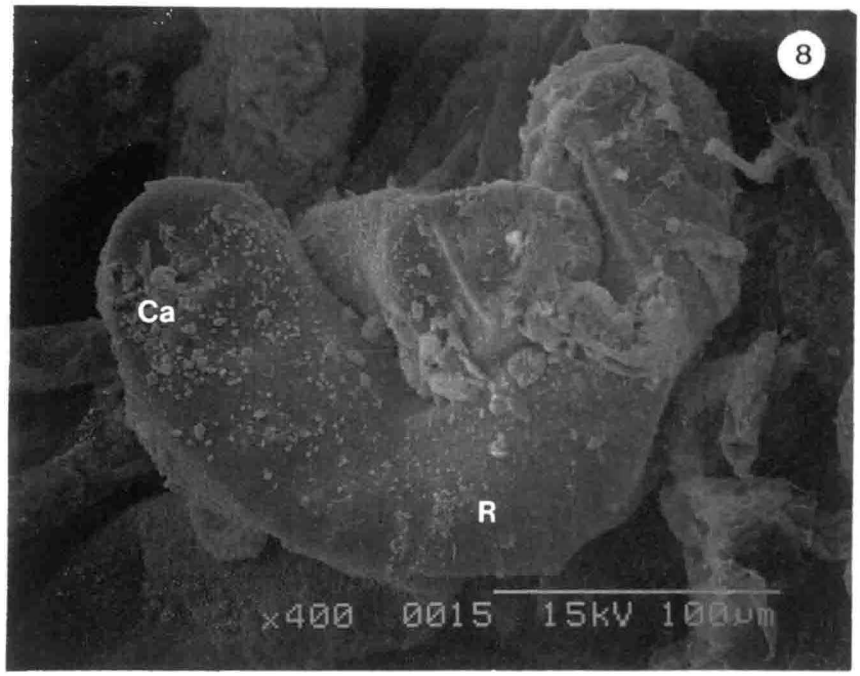

7
Ca
Ca
100μm

Figure 8

The rhizoid (R) shows little calcification (Ca). The calcification appears sporadic and absent in areas from which growth occurs.

Figure 9

A thick layer of calcium carbonate seen on the stalk of *Acetabularia acetabulum*.


8
Ca
R
x400 0015 15kV 100µm

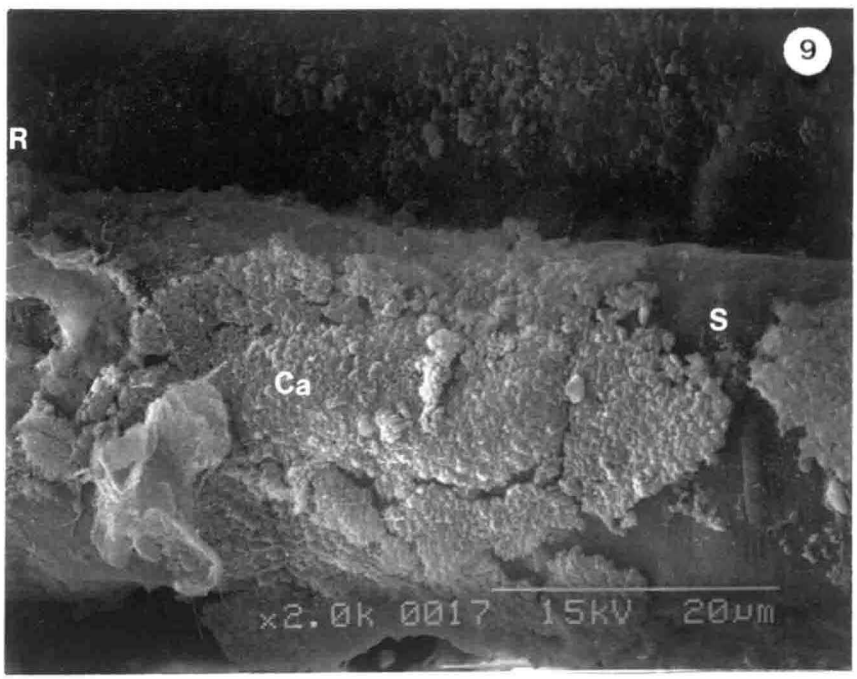
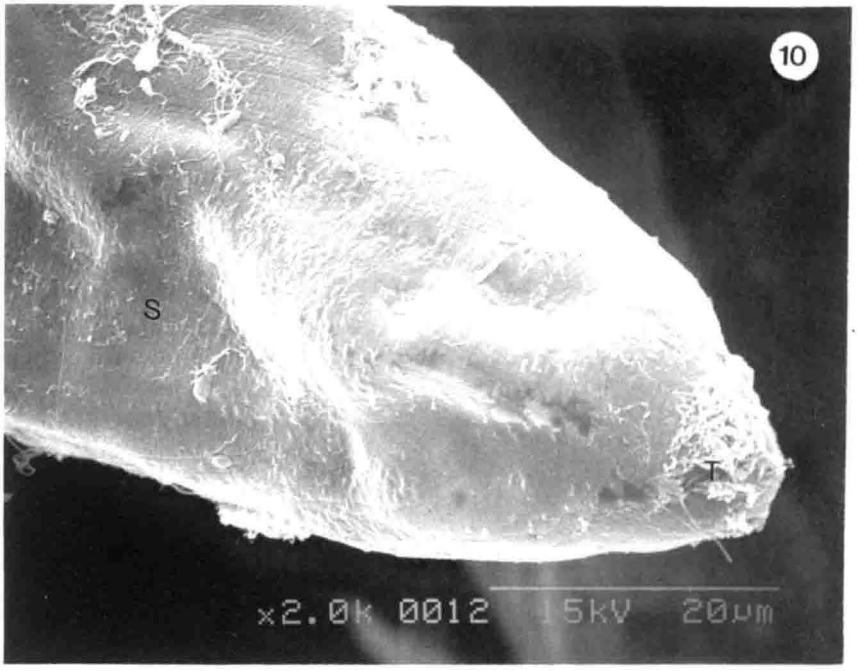

9
R
S
Ca
x2.0k 0017 15kV 20µm

Figure 10

The tip (T) of the stalk (S) shows no crystalline material.

Figure 11

Fibrillar material (F) appear on the stalk (S) of *Acetabularia acetabulum.*


10
S
T
×2.0K 0012 5kV 20µm

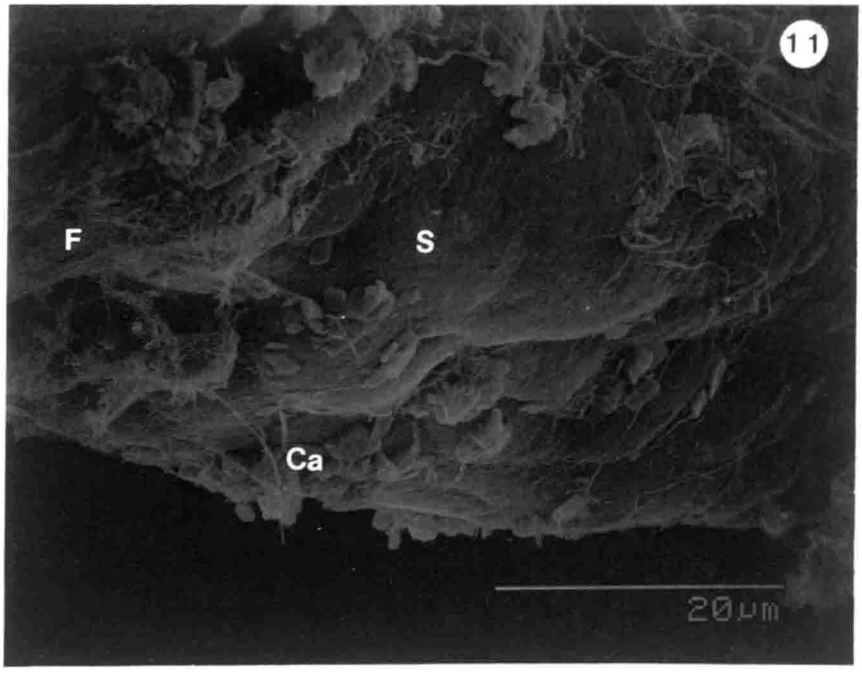
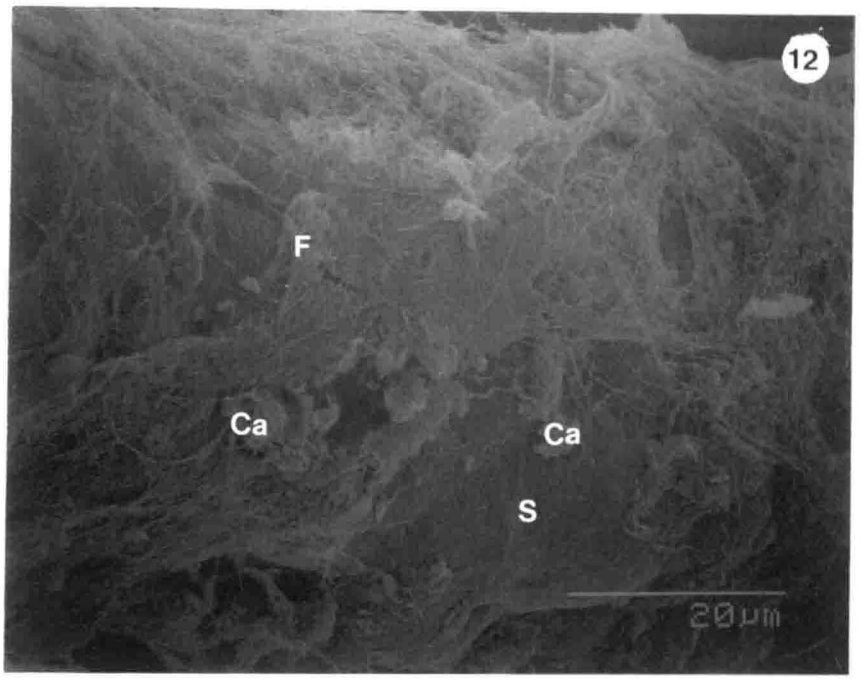

11
F
S
Ca
20µm

Figure 12

Fibrillar material (F) appears when calcification is occurring and absent in areas where no calcification is occurring.

Figure 13

A non-calcified cell of *Acetabularia acetabulum* indicates no calcification or fibrillar material located on the stalk (S), apical end of the stalk (A) or rhizoid (R).


12
F
Ca
Ca
S
20µm

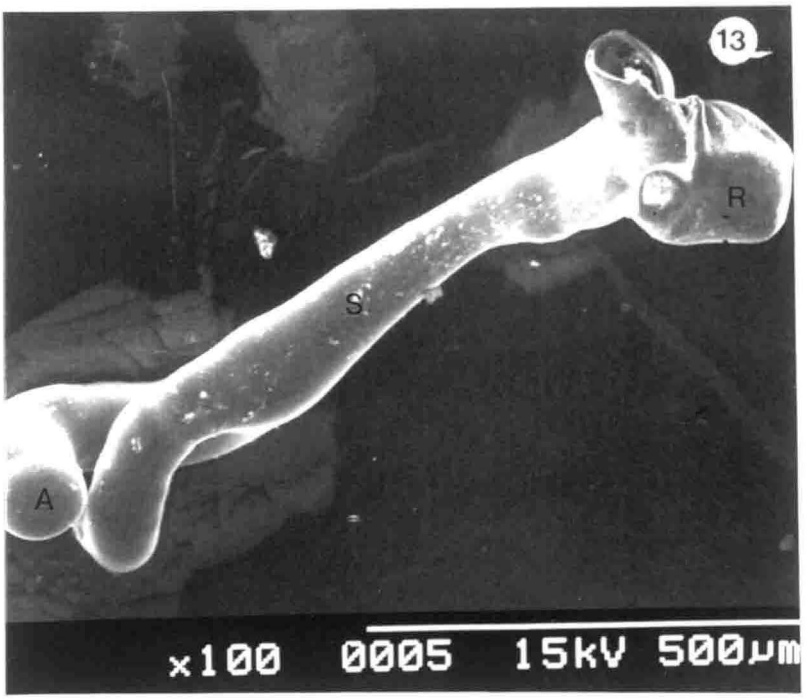
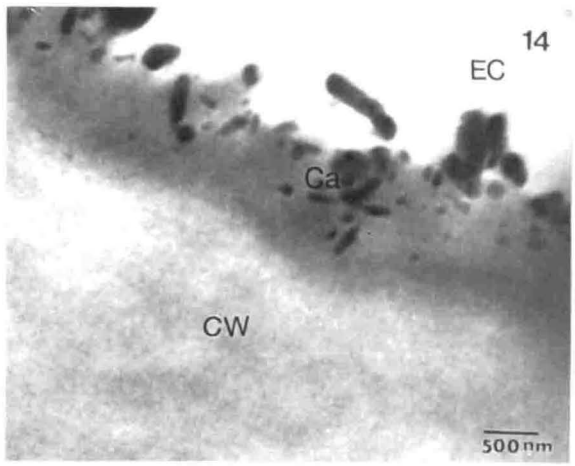

13
R
S
A
x100 0005 15kV 500µm

Figure 14

The gray matter outside the cell wall (CW) corresponds to the fibrillar material seen through Scanning Electron Microscopy.

Figure 15

Amorphous calcium carbonate is seen in the organic matrix (OM) and penetrating into the cell wall (CW).


14
EC
Ca
CW
500nm

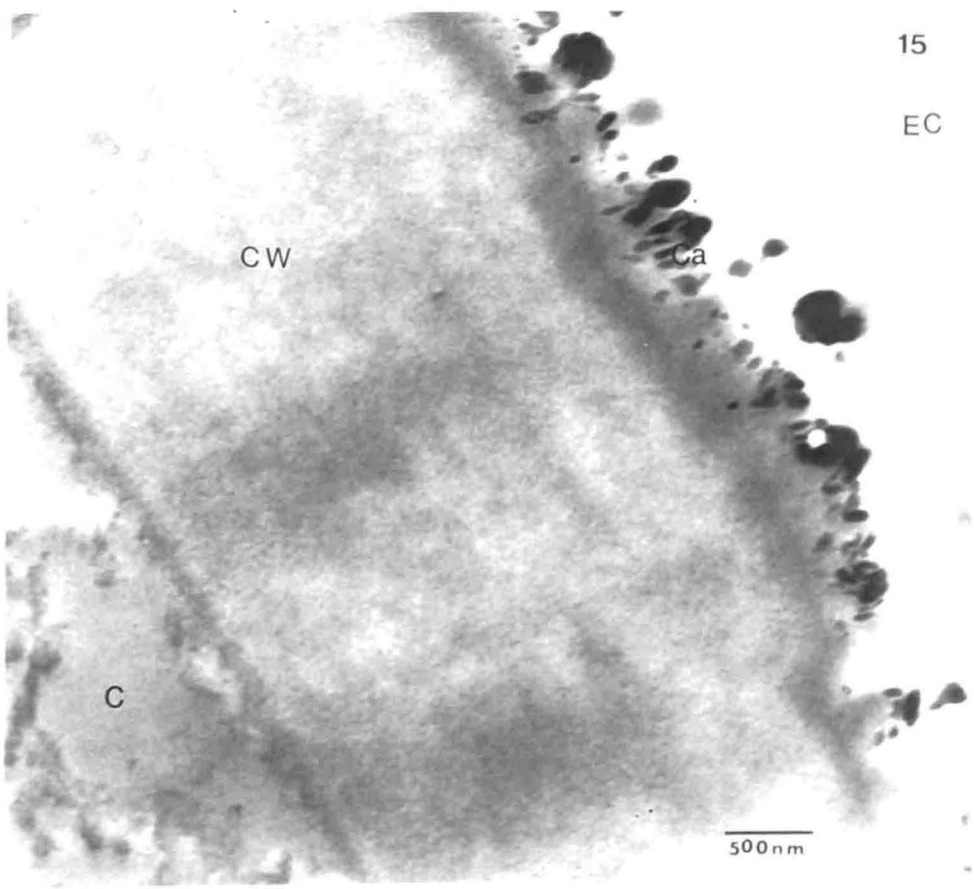
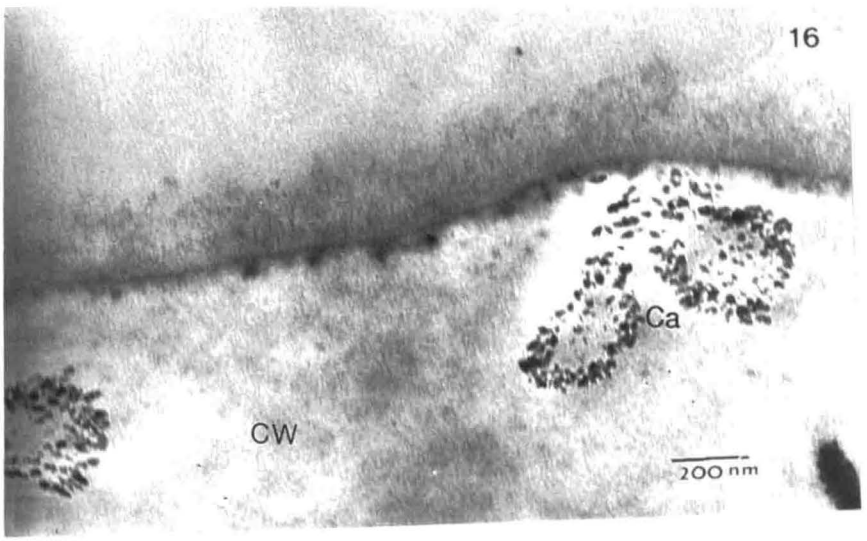

15
EC
CW
Ca
C
500nm

Figure 16

Loosely compacted groups of amorphous calcium carbonate (Ca) are seen in the cell wall (CW).

Figure 17

Bundles of amorphous calcium carbonate are located in the outer portion of the cell wall (CW).


16
Ca
CW
200 nm

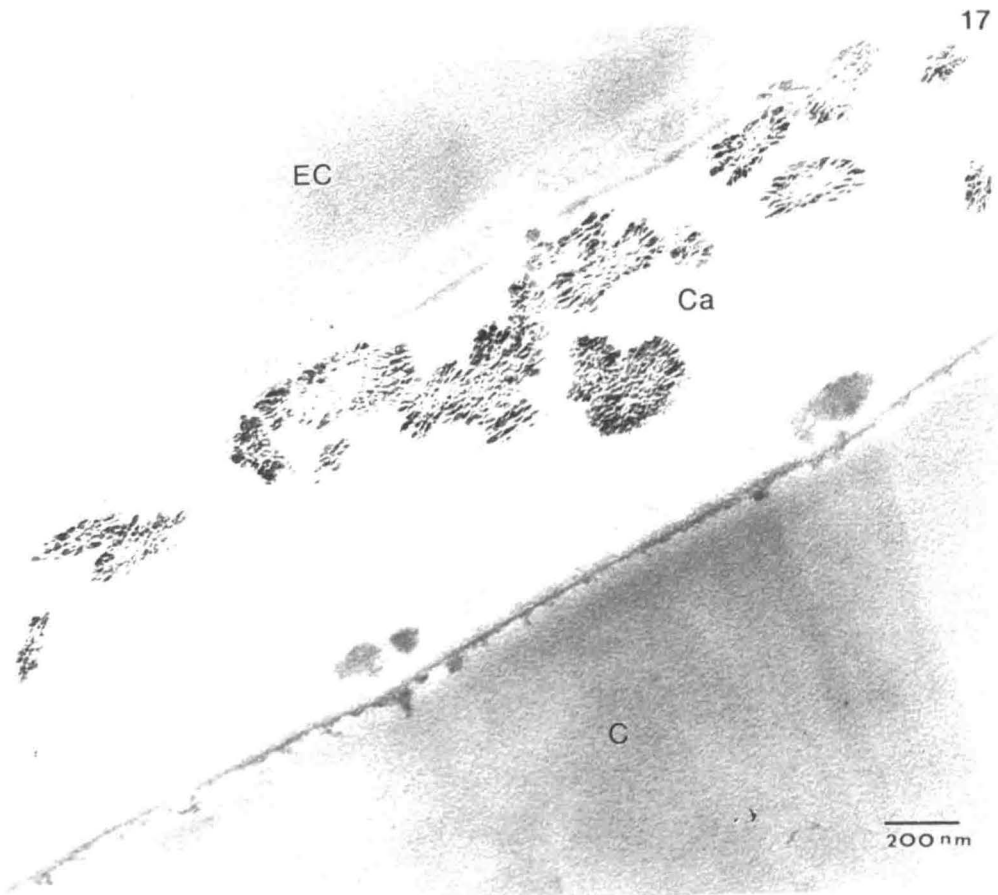

17
EC
Ca
C
200 nm

Figure 18

In the cell wall (CW) of more developed portions of the stalk, large, condensed bundles of amorphous calcium carbonate (Ca) appear. As the calcification continues towards the apical end of the stalk, singular calcium carbonate rods are seen in close proximity to one another.

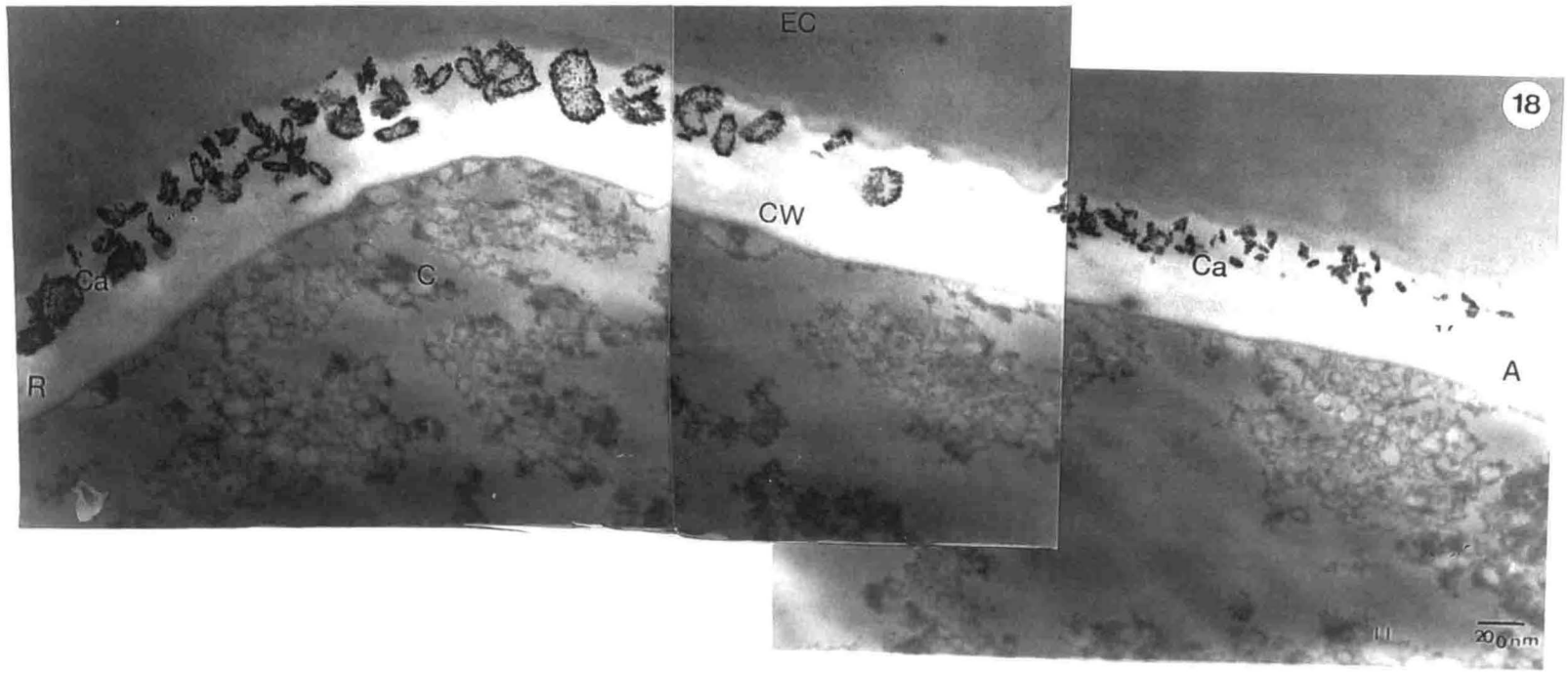
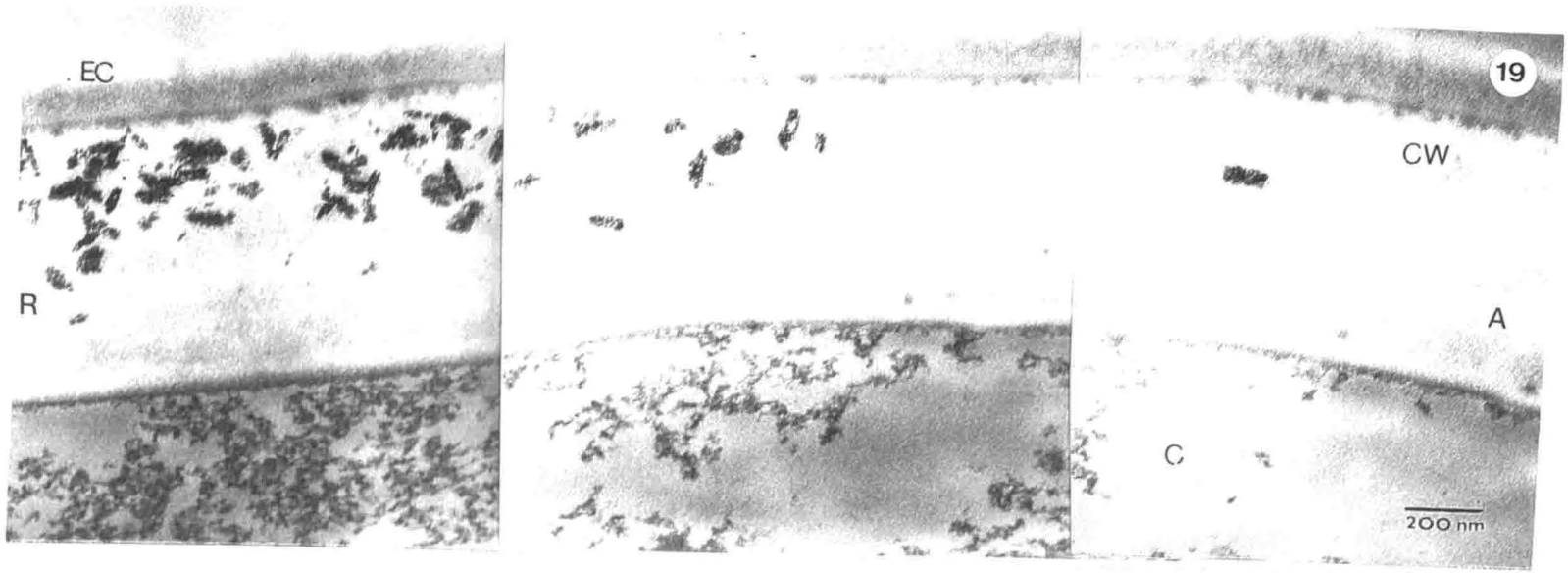

EC
CW
Ca
C
R
Ca
A
18
200nm

Figure 19

A series figure illustrates the extent that calcification occurs. Towards the rhizoid end of the stalk, the cell wall (CW) singular calcium carbonate (Ca) rods. As calcification progress towards the apical end of the stalk, the cell wall shows little or no calcification occurring.



19

Figure 20

A cross section of a stalk shows the large discrepancy between the calcification (Ca) that occurs in the cell wall (CW) of both sides of the cell.

Figure 21

Electron dense material is seen intracellularly in the chloroplasts and in some cytoplasmic material close to chloroplasts that have ruptured.


20
Ca
CW
C
CW
Ca
500 nm

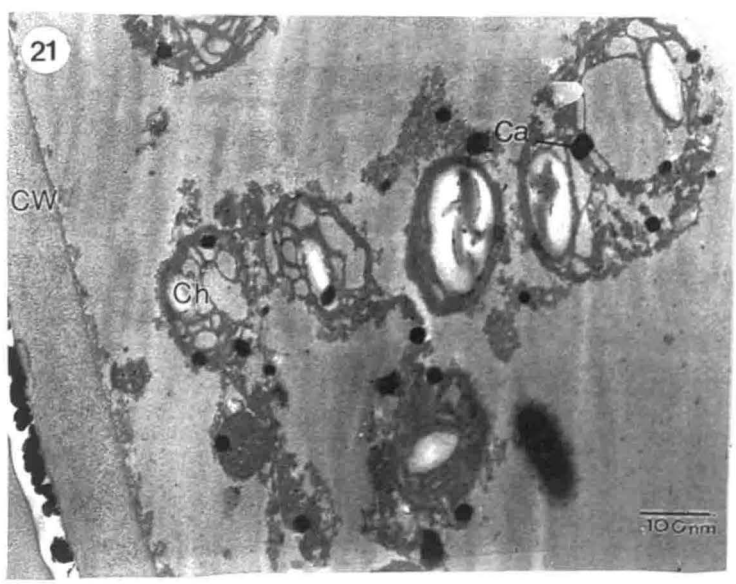
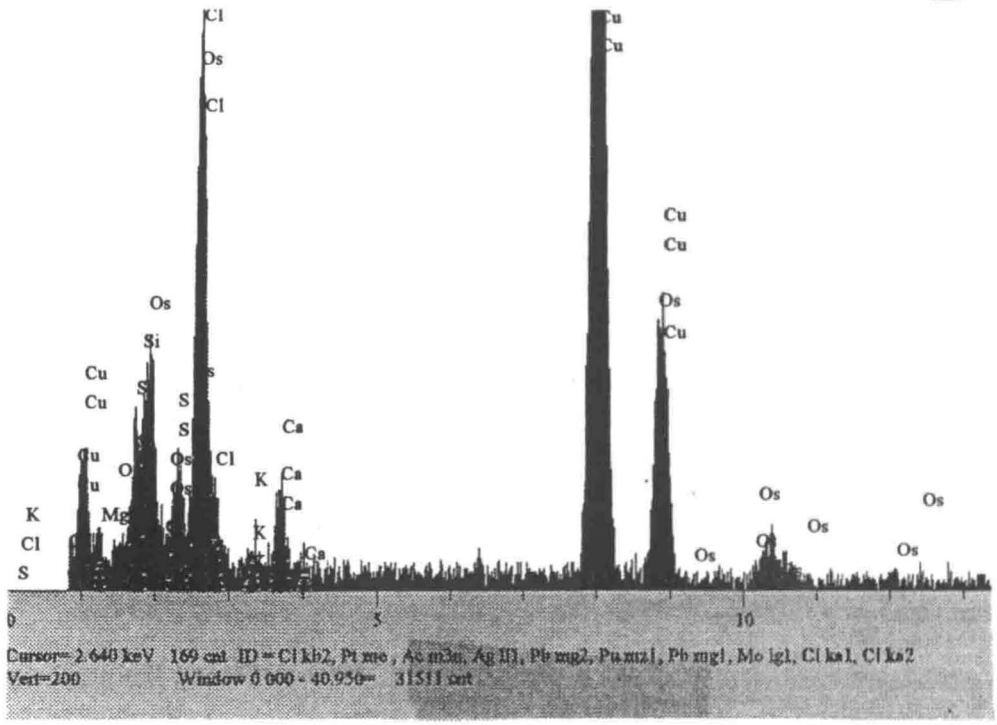

21
Ca
CW
Ch
100nm

Figure 22

Six elemental peaks are seen in the chloroplasts using elemental analysis. These elements and percent of composition they made up are Cl (55%), Si (17%), Ca (10%), Mg (8%), S (7%) and K (2%).



Component	Conc.	
Mg	8.123	wt.%
Si	17.169	wt.%
S	7.038	wt.%
Cl	55.453	wt.%
K	2.114	wt.%
Ca	10.103	wt.%

Appendix

As early as 1640, sketches of *Acetabularia acetabulum* existed and the interest in this alga has continued for nearly 360 years. In the early 1900's, Joachim Hämmerling discovered that *Acetabularia* has a single nucleus located in the rhizoidal region of the cell. Hämmerling found that anucleated *Acetabularia* could form structures such as stalks, sterile whorls, and species-specific reproductive caps indicating that the cytoplasm controls morphological events in the absence of a nucleus. It was at this time that an interest in the cell biology of *A. acetabulum* began (Bonotto 1979). While this alga has been used extensively in research and as a teaching tool, little information regarding its calcification exits.

Acetabularia acetabulum is classified in the division Chlorophyta, class Ulvophyceae and order Dasycladales. The Dasycladales are single cell organisms known for their large size, the smallest species growing one to two mm and the largest species growing up to 20 cm (Berger and Kaever 1992). Eight genera are found in the family Dasycladaceae and *Acetabularia* has 22 species. *A. acetabulum* can reach up to 4 cm in length (Harrison et al.1988).

Acetabularia acetabulum is most commonly found in the Mediterranean Sea. Outside the Mediterranean, it has been seen in the Suez Canal. It is found in calm waters and at depths up to a few meters. It is typically seen in sunny environments with little temperature change throughout the year and it is tolerant of great fluctuations in salinity. It is most often found in bays, coastal lagoons, tidewaters, and rock-pools (Cinelli 1979). In its natural environment, it is frequently found grouped in large clusters but it has also

been seen growing individually in favorable conditions (Berger and Kaever 1992). Individual and colonial algae are found to adhere to solid substrates such as rocks, shells, and artificial material (Cinelli 1979).

The cell is divided into three sections: the rhizoid region, the stalk, and the cap (Figure 1). The rhizoid region is found at the base of the cell and consists of many extensions, varying in length, up to several mm, protruding outward. The rhizoid region from multiple cells is often seen intertwined (Puiseux-Dao 1970). The rhizoid region has a thick cell wall and the large single nucleus of the cell is typically found just below the beginning of the stalk in the upper portion of the rhizoid region (Berger and Kaever 1992). The nucleus is the dominant structure seen in this region although there also are many mitochondria and chloroplasts filled with carbohydrate reserves (Menzel 1994; Puiseux-Dao 1970). The rhizoid region functions to connect the individual cells together to form a cluster and to hold the cell to the substrate (Berger and Kaever 1992)

As in other algae, size and thickness of the stalk can vary depending on environmental conditions (Berger and Kaever 1992). The stalk contains a large central vacuole, mitochondria, chloroplasts, and lipid droplets. Stalk chloroplasts differ from those of the rhizoid by having a longer shape, smaller carbohydrate reserves, and being positioned linearly within the cytoplasm. Mitochondria are more abundant in the stalk than in the rhizoid region. As the cell grows, the stalk elongates at the apical end (Puiseux-Dao 1970).

The small apical zone, or cap, is the reproductive structure of the cell. It has few chloroplasts and mitochondria. The cytoplasm is denser in the cap than in the stalk or

rhizoid region (Puiseux-Dao 1970). The cap is formed at the growing tip of the stalk. The formation of the cap has been examined extensively. In preparation for cap formation the stalk develops a round bud. The bud flattens and tip growth begins (Harrison et al. 1988). Cap rays, which signify the beginning of cap formation, are seen on the flattened head at the tip of the stalk. The number of rays that ultimately bind and form the cap vary between 55-90. Secondary whorls develop at the end of the rays and bind, giving the cap it's final appearance. Rays are cone-shaped and joined together along their lateral margins. Together the bound rays form the two portions of the cap: the corona superior and the corona inferior. The corona superior has rounded distal ends, which form a continuous crown when the segments are joined. The corona inferior is slightly rounded and laterally joined with neighboring segments to complete its formation. The cap reaches it's final shape when the cap rays are fully extended. The hairs, which provide protection for the developing cap fall off as the cap reaches its final shape and size (Berger and Kaever 1992). The broad shape of the cap aids in the main function of reproduction and light absorption (Menzel 1994).

The cell wall, composed mainly of mannose, glucose, and galactose, gradually becomes thicker at the rhizoid end. At the tip of the stalk, the youngest portion of the cell, the cell wall is thinnest. Chemical composition of the cell wall between the cap and the stalk differ. The stalk lacks rhamnose, which is found in the cap. In addition, the cell wall of the cap contains a higher proportion of glucose and galactose than the cell wall of the stalk (Puiseux-Dao 1970).

Development of *Acetabularia acetabulum* has been studied extensively. Two

biflagellate gametes unite beginning the life cycle of the cell (Menzel 1994). Two basal body rootlets combine to form the microtubule cytoskeleton. The rootlets overlap at the distal cell pole and run along the edge of the cell. The location of the nucleus when branching begins determines the polarity of the cell. The beginnings of a rhizoid region can be seen by the tenth day of development. The length of time required for cells to develop varies between 4 months to 24 months (Zeller and Mandoli 1993). The cell becomes elongated and the final vegetative morphogenesis occurs with the formation of the segmented cap. The nucleus undergoes meiosis and the daughter nuclei divide mitotically until approximately 1000 secondary nuclei have formed. The secondary nuclei travel through the stalk into the cap rays where they transform the surrounding cytoplasm into cysts. Gametes form in the cysts and escape from the lid of the cyst wall into seawater where they can fuse with other gametes (Menzel 1994).

Vita

Rachel VanGilder was born November 3, 1970 in New Haven, Connecticut and was raised in Iowa City, IA. After obtaining a B.A. degree in biology from Augustana College in Rock Island IL in 1992, she began her graduate work at the University of Richmond in 1994. She is continuing her education in the field of epidemiology at the University of Iowa in Iowa City, IA.