1981

Ultrastructure and development of the female reproductive branches of Polysiphonia harveyi

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ULTRASTRUCTURE AND DEVELOPMENT OF THE FEMALE REPRODUCTIVE BRANCHES OF *POLYSIPHONIA HARVEYI*

A Thesis

Presented To

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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by

Sharon Thompson Broadwater

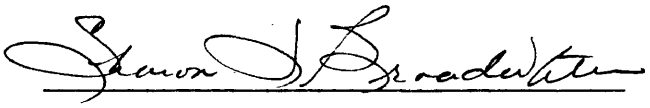
1981

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts



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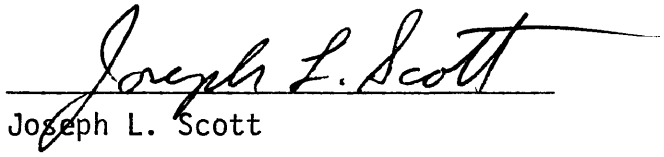
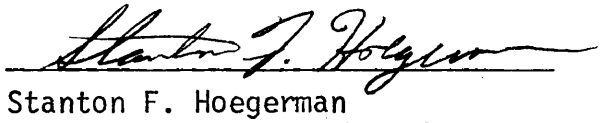
Approved, May 1981



Joseph L. Scott



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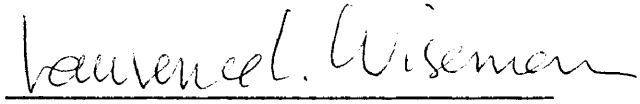

Lawrence L. Wiseman

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Joseph L. Scott for his direction, enthusiasm and friendship during the course of this investigation. I also wish to recognize Dr.s Stanton F. Hoegerman and Lawrence L. Wiseman for critical reading of this manuscript and Kathleen L. Schornstein and Jewel P. Thomas for their companionship and patient instruction.

LIST OF CHARTS AND DIAGRAMS

LIST OF FIGURES

ABSTRACT

Prefertilization and immediate postfertilization development, as well as fertilization, in the female reproductive branch of Polysiphonia harveyi (Rhodomelaceae, Ceramiales) was documented for the first time with electron microscopy. EM results pertaining to prefertilization morphology and development are consistant with those established earlier in light microscopic studies but several new ultrastructural characteristics were discovered.

The mature carpogonium was found to have double membrane-bound vacuoles of nuclear origin and the carpogonial nucleus was found to contain a nucleolus with a distinctive crystalline lattice. Using a number of techniques, trichogynes were determined not to have a separate nucleus. Of greatest interest was the discovery of a highly structured channel of SER which extends uninterrupted, except for pit connections, through the carpogonial branch to the support cell. It is hypothesized that the channel acts to conduct the message of fertilization from the carpogonium to the support cell. Very few observations were made on postfertilization branches but evidence of direct fusion between the carpogonium and auxiliary cell was fairly conclusive.

Special attention was given to the types of pit connections and their role in the development of the female reproductive branch. The name for the type of pit connection previously termed "transfer connection" has been changed to "abbreviated connection" since the results of this investigation do not fully support earlier reports of increased transport through these connections. Instead, abbreviated connections in the procarp appear to be a preformed structural weakness whose disintegration allows rapid development of cytoplasmic continuity between cells.

ULTRASTRUCTURE AND DEVELOPMENT OF THE FEMALE REPRODUCTIVE BRANCHES OF *POLYSIPHONIA HARVEYI*

INTRODUCTION

Development of a natural system of classification for the red algae is complicated by the wide variety of morphological characteristics and complex life histories which they exhibit. Scagel (36) gives a succinct review of the steps leading to the present taxonomic treatment which divides the Rhodophyta into two classes: Bangiophyceae and Floridiophyceae. The more advanced forms belong to the latter class and are divided into five orders according to pre- and post-fertilization development of the female reproductive branch.

Of the five orders, the Ceramiales is not only the most advanced but is also the best delimited (36, 11, 7). It is separated from the other orders on the basis of having the auxiliary cell formed after fertilization (11, 17). This order is composed of four families. The members of the Rhodomelaceae, the largest and most highly evolved of the four families, adhere very closely to a single line of female reproductive development (36).

The present research was begun in order to present an account of female reproductive development in the Rhodomelaceae at the ultrastructural level. A number of light microscopic studies have been conducted previously (46, 36, 17, 26), but this study is the first utilizing the electron microscope. Since female development is pivotal in systematizing the higher red algae, it was felt that any

information might later be valuable in further taxonomic evaluation. In addition, it was hoped that a comparison of results from light microscopy with those from electron microscopy might answer some still debated questions. It was also hoped that the study might illucidate some of the mechanisms responsible for the developing morphology seen at both levels. Finding characteristics at the ultrastructural level not discernable with light microscopy was anticipated, but the results were surprising not only because of the number of unique characteristics exhibited by the female reproductive branch but also by their possible significance.

MATERIALS AND METHODS

Specimens of Polysiphonia harveyi Bailey were collected from sublittoral locations at Yorktown and Cape Charles, Va. Apices were immediately cut from the plant and fixed for 2 hrs in 3-4% glutaraldehyde in 0.1 M PO_4 buffer at pH 6.6 with osmolarity adjusted by addition of sucrose. After several brief buffer rinses, the material was post-fixed for 1 hr in 1% OsO_4, immersed overnight in a 70% acetone - 2% urynal acetate solution at 4°C, then dehydrated in acetone and embedded in Epon 812. To ensure correct orientation, the material was embedded between teflon-coated glass slides. The desired material was excised, glued to a larger block of Epon, sectioned, stained with lead citrate and photographed using a Zeiss EM 9S-2. Photomicrographs were taken with a Zeiss Photomicroscope II equipped with Normarski differential interference optics.

Material for SEM was fixed as for TEM through post-fixation in OsO_4 after which the material was dehydrated in ethanol, dried with a Polaron E 3000 critical point dryer, coated with carbon and gold-palladium, and examined with an AMR 1000 SEM.

Material for fluorescence microscopy was stained with mithramycin using the method outlined by Heath (16) and photographed with a Zeiss Photomicroscope II equipped for epifluorescence.

RESULTS

Polysiphonia harveyi (Fig. 1) is a red alga common to the eastern coast of the United States. It belongs to the family Rhodomelaceae and, like other members of this family, bears the female reproductive structures on trichoblasts, specialized branches of limited growth (Figs. 3, 4). Normally, trichoblasts are uniseriate, but both the basal and suprabasal segment of a fertile trichoblast produce pericentral cells. There are always five pericentrals in the suprabasal segment regardless of the normal number in the vegetative axis of the plant. The fifth and last pericentral enlarges to become the reproductive initial (36, 17).

Subsequent growth and development of the branch consists of a specific series of events which have been summarized in Chart I. This chart was synthesized from my own work and from earlier light microscopic work (46, 36, 17). There has been very little disagreement concerning the sequencing of events prior to fertilization although some postfertilization events are in question.

The prefertilization female branch (Diag. I) in the Rhodomelaceae is characterized by a four-celled carpogonial branch composed of three carpogonial branch cells (CBs) and a carpogonium (CP) with an elongate extension, the trichogyne (TG) (Figs. 2, 9, 13). The TG is the structure which first recieves the spermatium. The carpogonial branch

is borne on the fifth pericentral or support cell (SU) which also produces two sterile cells (STs), cells not directly involved in fertilization.

Prior to fertilization, the above structures together are called a procarp. The procarp is enclosed within the pericarp, an urn-shaped organization of cells formed from pericentrals flanking the SU and the abaxial pericentrals of the basal segment of the trichoblast.

After fertilization, the diploid nucleus of the CP divides mitotically, and the SU produces the auxiliary cell (AU) from its anterior end. One diploid nucleus is passed to the AU which then produces the carposporophyte generation. The carpogonial branch withers, and the entire remaining structure, including pericarp, is called a cystocarp.

Axial and Support Cells

Fertile axial cells (AX) have no characteristics discernable by EM which distinquish them from vegetative axial cells. At maturity, they are typically uninucleate and vacuolated with moderately developed plastids and a normal compliment of other organelles (Figs. 8, 26). Following fertilization, the AX is incorporated into the fusion cell (11), but is one of the last of the cells existing prior to fertilization to fuse (Fig. 38).

The cytoplasm and organelles of the SU are not distinctive. The plastids of the SU are not fully developed, and the SU is always uninucleate as opposed to the multinucleate condition of vegetative pericentrals (Figs. 8, 9, 26, 35). In addition, the SU is characterized by the type of pit connection (PC) which Wetherbee has termed "transfer connection" (42, 43) but which will be called "abbreviated connection"

in this paper since the term abbreviated implies only a structural dissimilarity and not a fuction. The reasoning behind a change in terminology as well as a discription of abbreviated connections will come later in this paper. It should be noted, however, that not all of the connections of the SU are abbreviated, but this, too, will be discussed later.

Upon recieving from the CP an indication that fertilization has occured, the SU divides for the last time to produce the AU. This cell is cut off from the anterior end of the SU into the intervening space between the SU and CP (Fig. 35). After the AU has recieved the diploid nucleus, fusion occurs between the AU and SU (Fig. 38). The resulting cell is the initial of the large fusion cell which supports and nourishes the carposporophyte [See Wetherbee (43) for further information on carposporophyte development].

Pericarp

The Rhodomelaceae have what is considered the most highly developed pericarp of all the families of red algae. The pericarp acts not only to protect the developing procarp but also acts as an assimilatory organ in later developmental stages (36). The pericarp begins development at nearly the same time as the procarp and completely covers the procarp at the time of fertilization. Pericarp development is the result of apical growth of uniaxial, adventitious branches which join by secondary PCs to form an envelope consisting of two halves which fit together much like the valves of a clam (11) (Figs. 3, 4). The pericarp is maintained at this stage of development until fertilization occurs. At that time, each cell divides twice to produce two outer cells. The resulting

structure consists of two layers, the outer one containing twice as many cells as the inner (36). The two valves fuse (11) leaving an opening, the ostiole, through which the carpospores will eventually be released (Figs. 5, 6).

Ultrastructurally, pericarp cells are typical of red algal vegetative cells: proplastids and small vacuoles are found in the newer cells at the apices whereas larger vacuoles and mature plastids are present in older cells where starch accumulation also appears greater (Figs. 7, 35).

Sterile Cells

Prior to fertilization, the first lateral sterile group (ST_1) is composed of two cells and lies in a plane parallel with the carpogonial branch while the second or basal sterile group (ST_2) is one-celled and lies in file behind ST_1 (Diag. I). All three are undifferentiated cells with few organelles and little starch accumulation (Fig. 11). Although somewhat larger than CBs, superficially they appear similiar and are characterized by abbreviated connections, although the connections between the cells of ST_1 are typically wider than those found elsewhere in the procarp (Fig. 11). No differences except cell size could be detected between young and mature STs.

Soon after fertilization, each ST divides once. These divisions result in a four-celled lateral group and a two-celled basal group (Fig. 12). Soon after passage of the diploid nucleus to the AU, the PCs between each ST and between the STs and SU begin breaking down (Fig. 38). Later developmental stages were not seen, but the literature indicates that the STs eventually are incorporated into the fusion cell (46, 26).

Trichogyne

The mature TG measures 30-50 um at maturity although it may continue to elongate if not fertilized (Hommersand, personal communication). The TG is 2-3 um wide, narrowing to a neck region of approximately 1 um where it is attached to the CP. The cytoplasm of the mature TG contains randomly scattered proplastids, mitochondria, ER and a few dictyosomes (Figs. 9, 10). Some microtubules were seen oriented parallel with the longitudinal axis of the TG, but they were few in number and would seemingly not be able to account for producing or maintaining the shape of the TG. TG rigidity may result from a combination of the thick cell wall and the vacuoles located along the length of the TG. The number of vacuoles varies considerably with the age of the TG. My light microscopic investigations, as well as EM analysis of the youngest TGs I was able to section, indicate that young to moderately developed TGs have few and small vacuoles (Fig. 2). These increase in size with age, older TGs becoming extremely vacuolated (Fig. 13).

The cell wall of examined TGs was coated by a thick, amorphous substance which decreased in thickness from the tip to the base (Fig. 9). In some specimens (Fig. 10), apparent exocytosis was observed taking place at the tip, possibly in response to further growth or as an avenue for release of the extracellular coat believed to aid in spermatium adherence.

After attachment of the spermatium (Fig. 4), the walls between the spermatium and TG disintegrate, thereby allowing cytoplasmic continuity. The male pronucleus moves into the TG leaving a considerable amount of residual cytoplasm in the spermatium (Fig. 17). The pronucleus is elongate and is enclosed by a double membrane nuclear envelope with

normal appearing nuclear pores (Fig. 18).

As the spermatial nucleus progresses down the TG, the cytoplasm behind the advancing pronucleus becomes disordered and less dense (Fig. 17), a condition previously reported from light microscopic studies (46, 11). Soon after the pronucleus passes into the CP, the TG abscises. The progression of detachment was not clear from previous light microscopic studies (18, 11, 4) nor from my own EM work. Some specimens appeared to have a plug of material sealing the TG from the CP (Fig. 19), and dictyosomes containing an electron-dense substance, possibly the plug material, were seen in some older TGs. Other TGs appeared to be separating from their CPs by vacuolar expansion without a plug-like substance being present (Fig. 20). After detachment, the cytoplasm of the TG soon deteriorates although the TG may be retained for a short time because of the cell wall continuity between the TG and CP.

Carpogonium

The carpogonial branch has a slight curvature so that the CP lies just forward of the SU and further into the interior of the pericarp than the CBs (Diag. I). Prior to fertilization, the mature CP is approximately half the size of the CBs (13, 23). Increase in cytoplasmic volume of all four cells is probably quite similar, the disparity in size between the CP and CBs being due to the fact that much of the cytoplasm of the CP is contained within the structure of the TG.

The major organelles of the CP consist of proplastids, dictyosomes, mitochondria and ER (Figs. 9, 23). The number of vacuoles in mature CBs, as in older TGs, increases with age. In older cells, these

vacuoles may constitute a third of the cytoplasmic volume (Fig. 23).

In addition to these electron-translucent vacuoles, another type of vacuole is sometimes seen in the CP. It is characterized by a marbled, medium electron-dense material surrounded by a double membrane (Fig. 13). In some cases, the membranes of these vacuoles are seen to be continuous with the nuclear envelope (Fig. 14).

The CP nucleus is very distinctive. It increases in size with CP development and is most often spherical but can assume an elongate shape or on ocaasion be irregular or lobed (Fig. 13). The chromatin is generally more thinly dispersed than that of other cells in the branch (Figs. 13, 23), and the nucleolus is strikingly different. In mature carpogonia, the bulk of nucleolar material is present in the form of a crystalline lattice with a center-to-center periodicity of 22.5 nm (Fig. 14). In some sections, this array is seen as parallel rods (Fig. 15); other planes of sectioning show that the rods have hexagonal packing (Fig. 16). The crystalline lattice is not present in very young carpogonia but is always present in the nucleolus of the mature cells. It is never found in any other cells of the procarp or surrounding pericarp.

After fusion of the pronuclei, the PCs between the CP and CBs disintegrate (Fig. 35), and the CP enlarges in size (Figs. 21, 22).

The route whereby the diploid nucleus of the CP is transferred to the AU has been a matter of debate (46, 18, 36, 11, 17). My own light microscopic studies were inconclusive, but at the EM level, rather convincing evidence of direct fusion was twice observed (Figs. 19, 35). Frequently, at the light microscopic level, structures could be seen which appeared to be either small cells produced by the CP or possibly extruded nuclei surrounded by a thin layer of cytoplasm. At no time

were these structures documented with EM, but one CP, already abscised from the AU, had what appeared to be two nuclei in different stages of deterioration (Figs. 21, 22).

After the CP has passed a diploid nucleus to the AU, the CP separates from the AU. At this time the CP appears very electron-translucent and already in the process of deterioration (Figs. 21, 22). Total disintegration follows detachment.

Carpogonial Branch Cells

The carpogonial branch initial is produced by the SU just after formation of the initial of the lateral sterile group and soon divides to produce a three-celled branch (Fig. 8). At maturity, the CBs lie in an arch above the SU, their distal sides lying just beneath the ostiole (Diag. I).

The cytoplasm of both young and nearly mature cells is dense with few and small vacuoles and no starch deposits (Fig. 8). The CBs increase in size, but the number of organelles remains proportionally the same except that the number of dictyosomes appears to decline with maturation (Figs. 9, 13). At maturity, all three cells usually are binucleate and are the only cells of the procarp for which this condition is normal.

The most outstanding characteristic of the mature branch is a channel of closely-meshed, tubular SER (Figs. 23, 24, 25, 26). Serial sections show that this channel extends uninterrupted, except for PCs, from the CP to the SU. In young CBs, it is first seen as a dome-shaped zone of exclusion containing SER abutting and projecting from the area of the PCs (Fig. 25). These dome-shaped areas appear to grow toward each other, finally coalescing to form the complete channel. In each of the CBs,

the channel is located in the portion of the cell proximal to the SU. Frequently, numerous proplastids and particularly mitochondria are lined up along its length (Fig. 25). The heavy concentration of mitochondria may indicate high energy demand due to the production, maintenance or function of the channel.

If fertilization does not occur, the channel remains visible even during disintegration of the CP (Fig. 26). However, if fertilization does occur, the PCs of the carpogonial branch break down and, concomitantly, the number of SER tubules in the channel begins to decrease, finally disappearing before complete deterioration of the branch (Fig. 35).

Cytoplasm and nuclei appear to move from the CBs into the CP through openings left by the dissolved PCs although much cytoplasm remains in the CBs. At this developmental stage, the nuclei of the CBs may be very large (Figs. 22, 36); light microscopy of one cystocarp showed a nucleus wedged through the PC between CB_2 and CB_3, streached across CB_3, and inserted into the CP-CB_3 pit aperture.

Very soon after fertilization, the CBs begin to wither. They abscise from the SU and do not become part of the fusion cell.

Auxiliary Cell

The cytoplasmic appearance of the AU is that of a young undifferentiated cell without any distinguishing characteristics (Fig. 35). The AU appears to receive the diploid nucleus from the CP by direct fusion, but the fate of the haploid nucleus of the AU was undetermined.

After separation of the CP, the anterior end of the AU produces a gonimoblast mother cell which, in turn, produces the first gonimoblast

cell. At this stage, the AU fuses with the SU and the PCs between the STs and between the STs and the SU disintegrate (Fig. 38). This disintegration results in the formation of a large fusion cell.

Pit Connections

PCs found within the female branch of P. harveyi fall into three categories: (1) a standard or vegetative type, (2) an abbreviated or "transfer" type and (3) a "semi-standard" type, a combination of (1) and (2).

The ultrastructure of a standard or vegetative type pit (Fig. 27) consists of a central core of uniformly granular material, previously found to be protienacious (35, 31). The core is bounded on the cytoplasmic ends by a highly electron-dense cap which is adjacent to an electron-translucent layer. This layer, in turn, is bounded by a pit membrane which seems to be structurally different from the plasma membrane (29) but connected to and continuous with the plasma membrane (33, 29, 43). The plasma membrane borders the sides of the pits adjacent to the cell walls and is continuous from cell to cell. This combination of membranes makes the pit, in effect, extracellular (42, 43).

There appear to be two types of abbreviated connections in the procarp, one of which is probably derived from the other. One type is found in young procarp and vegetative cells (Fig. 31) and is the same type previously described by Wetherbee (42, 43). The other is found in older procarp cells (Figs. 28, 30). Both are quite distinct from standard connections. In both, the ends bordering the cytoplasm tend to be more convex than standard connections, and their core is lentiginous or thread-like rather than granular. Both types are bounded on the cytoplasmic

ends by an electron-dense cap. The abbreviated connections described by Wetherbee and found during this investigation in young procarp and vegetative cells have, in addition, a second layer. Adjacent to the cap is an electron-translucent layer with very faint periodic striations perpendicular to the pit cap. There is, however, no detectable pit membrane. The second type of abbreviated connection apparently has neither the translucent layer nor the pit membrane, and it tends to flare more into the cytoplasm. In both, the plasma membrane is continuous from cell to cell and flanks the sides of the pit. The tentative assumption, then, is that abbreviated connections are intracellular.

Abbreviated connections are found at junctures in the procarp as indicated in Diagram II-A. They are present between very young CB cells (Fig. 33). Although these very young connections are smaller and less bowed, they are morphologically abbreviated connections and never develop a pit membrane. However, the connections in young vegetative cells which appear very similar to abbreviated connections will, during maturation, develop into standard PCs with well-developed pit membranes. Figure 32 shows a young vegetative branch which appears to be the process of producing a pit membrane from the outer membrane of the nuclear envelope.

The third major type of PC found in the fertile branch is characterized by having a standard type cap and associated structures on one end and an abbreviated type cap on the other (Fig. 29). For ease of discussion, I have called this a "semi-standard" PC although I am not implying by this nomenclature that this type of connection warrants an entirely different classification. "Semi-standard" pits are concistently found at the SU-AX and SU-CB_1 junctures (Figs. 25, 37).

At sexual maturity, the PCs of the prefertilization female branch

appear as in Diagram II-A. If fertilization does not occur, all PCs remain intact (Fig. 26) and can still be detected in cells in very advanced stages of deterioration.

However, if fertilization occurs, changes take place in the PCs. After passage of the diploid nucleus (Step 14 in Chart I), PC type and location appear as in Diagram II-B. The PC between the SU and AU is extremely variable in appearance. Some sections show very well developed pit membranes whereas other sections of the same cell as well as sections from other cells show discontinuous membranes or no membranes. PC type at all other junctures, however, is very consistent.

As indicated in Diagram II-B, after diploidization of the AU, all abbreviated connections in the carpogonial branch have disintegrated (Fig. 26), and those between the STs have either disintegrated or are in the process of doing so. The physical progress of PC disintegration does not appear specific so that the morphology of deteriorating plugs can be quite different (Figs. 34, 38). In the carpogonial branch, disintegration begins with the CP-CB_3 connection and progresses basipetally to the SU-CB_1 juncture. This connection, which is a "semi-standard" PC, does not disintegrate (Figs. 35, 36); nor, at this stage, has there been a breakdown of any other standard or "semi-standard" PC.

DISCUSSION

The accounts by light microscopists of procarp morphology and development within the order Ceramiales have generally been very consistent. This EM investigation of P. harveyi supports these earlier studies but has also provided some new information which aids in the interpretation of pre- and post-fertilization events.

Sterile Cells

Fritsch (11) suggests that the basal sterile group is homologus with a carpogonial branch whereas the lateral group represents the only vegetative cells of the procarp. The morphology of the PCs between lateral STs (Fig. 11) is very similar to that of vegetative axial cells, an observation which may tend to support Fritsch's hypothesis.

In those orders without a well developed pericarp, the STs sometimes perform an assimilatory role, but this function is inhibited by the pericarp in the Rhodomelaceae. In this order, the STs are thought to either separate the pericarp from the developing gonimoblast (11) or to serve as storage organs (17). They are densly protoplasmic, but they do not have large deposits of starch.

Light microscopists (36) have noted that, prior to fertilization, the cytoplasm of STs stains more lightly than that of CBs. At the EM level, there is no obvious difference between them with the exception of the double nuclei and the SER channel which occupies a large portion

of the volume of the CBs (Compare Figs. 11 & 23).

Trichogyne

The TG can be initiated at either the three or four celled stage of branch development (17) and is an "ephemeral structrue whose survival must be measured in hours" (7). The fleeting appearance of the TG and its vulnerability to fixation make this stage very difficult to capture, and has led to debate in the literature concerning various aspects of its morphology (46, 18, 11, 7). Much of the disagreement centers around the question of presence of a nucleus in the TG. Yamanouchi (46) was adament in his belief that a nucleus was not only present but also very persistant in Polysiphonia violacea. This observation, as well as the presence of a constriction at the base of the TG, led Yamanouchi to believe the TG to be a fairly autonomous structure.

In their studies of Polysiphonia platycarpa (18), Iyengar and Balakrishnan could find no evidence of a nucleus, and my investigations of P. harveyi whole mounts, thick and thin sections and material labeled with the fluorescent nuclear dye mithramycin never resulted in an indication of a nucleus. I am forced to conclude that, with the exception of the male pronucleus at fertilization, a nucleus is not present in the TG of this species at any stage of development.

In the only other published micrographs of a TG, Chambers (6) found no indication of a TG nucleus in Batrachospermum (although he mistakenly calls the CP nucleus a TG nucleus). Batrachospermum belongs to the Nemaliales, the least advanced order of the Floridiophyceae. Nevertheless, it is significant that there have been conflicting reports concerning this species as well (37, 46, 6).

It is possible that other structures have been mistaken for nuclei. The TGs of Batrachospermum contain double membrane bound sacs (6) which could be confused with nuclei, and the TGs of P. harveyi have large vacuoles which could also be misinterpreted at the light microscopic level (Fig. 4). More EM studies must be conducted, particularly on those genera which are believed to have a TG nucleus, before definitive conclusions can be drawn. However, the absence of a nucleus in two such widely separated genera as Batrachospermum and Polysiphonia, as well as the general disagreement over its presence in other genera, suggest that a TG nucleus is not typical of the Floridiophyceae.

Observations on both young and mature TGs did not indicate significant differences in relative numbers of organelles. Dictyosomes may be involved in the secretion of the extracellular coat since they are frequently involved in similar secretions in red algal cells (25, 20, 38, 39), but rough ER has also been implicated (19). No conclusions could be drawn as to the source of the extracellular coat in this study although exocytosis appeared to be occuring in some specimens.

The means by which the spermatium locates the TG is unknown, but the efficiency of fertilization in the Floridiophyceae is difficult to explain statistically as random contact considering that the TG and spermatium are both nonmotile (7). There appears to be a gradient along the TG with regard to spermatial attraction so that most spermatia attach to the distal end (7).

After adhesion and subsequent dissolution of the intervening walls and plasma membrane, the spermatial nucleus passes into the TG. Light microscopists indicate that all spermatial cytoplasm moves into the TG

(46, 7), but our results show a substantial amount of cytoplasm remaining within the spermatial walls.

In the brown alga Fucus, microtubules were associated with pronuclear movement (3), but no microtubules were detected near the spermatial nucleus in P. harveyi. However, too few observations were made on gamete fusion and subsequent events so that microtubule absence could not be adequately confirmed.

Carpogonium

The mature CP is easily the most distinctive cell in the female branch. Its shape is unique and it is the only cell of the branch that becomes highly vacuolated at maturity. It is not unusual for red algal cells to become vacuolate with enlargement (7), but it is interesting that the CP is the only cell of the branch to do so. This condition may very likely be a continuation of the vacuolization of the TG.

In addition to single membrane bound vacuoles, double membrane bound vacuoles are occasionally seen. These appear to bleb off from the nucleus. Chambers (6) noted double membrane bound sacs in the TG of Batrachospermum. Although much smaller and more numerous, these may be related to the vacuoles of P. harveyi. Wetherbee and Wynn (45) observed double membrane evaginations of the nucleus in carposporangia of Polysiphonia novae-angliae. These contained either fibrous material or an osmiophilic droplet. Both types of material were also found in the cytoplasm and were believed to originate in the nucleus. Superficially, the vacuoles found in the presporangium of the marine protist, Labyrinthula sp. (27) appear similar, but those of Labyrinthula are formed by a protrusion of the outer membrane of the nuclear envelope only.

The nucleus of the CP has two features which distinguish it from other nuclei in the procarp. In mature cells, the chromatin is very finely dispersed and does not appear as clumped as in other nuclei of the procarp, and the bulk of the nucleolar material is in the form of a crystalline lattice with a distinct periodicity. This periodicity is a very constant and persistant feature, having been detected in post-fertilization nucleoli and once in the cytoplasm of a disintegrating CP. To my knowledge, there are no other accounts of comparable nuclei in other species of red algae.

The functional significance of these characteristics is not understood, but they do serve to show that the CP is highly specialized for its role as recipient of the spermatial nucleus.

After fertilization, the CP increases in size, a characteristic previously reported from light microscopic investigations (18, 36). This increase could, in part, be due to movement of cytoplasm from the CBs. The PCs between the CP and all the CBs disintegrate soon after fertilization, and light microscopists have noted movement of cytoplasm from the CBs to the CP (46). In my light microscopic investigations, nuclei from the CBs were observed in the process of moving through the opened connections toward the CP. Movement toward rather than away from the CP was determined from counting the nuclei in each cell; none of the CBs ever had more than two nuclei.

There has been considerable debate in the literature concerning the manner in which the CP passes a diploid nucleus to the AU. The prevailing interpretation for the Rhodomelaceae has been that passage occurs after direct fusion between the AU and CP (46, 36, 11, 17), but a connecting cell has been reported in P. platycarpa (18) and two con-

necting cells have recently been reported in Brongniartella (26). The increase in CP size and the proximity of the CP and SU lend support to the hypothesis of direct fusion as do the EM results from this study. However, my light microscopic studies show what could easily be interpreted as two connecting cells which appear to be produced at or near the site of nuclear exchange between the CP and SU. These entities are very small with most of their volume filled by a body staining like a nucleus. Their small size would argue against their invovement in transfer as well as our fairly conclusive evidence of direct fusion. It is possible that the bodies seen in Brongniartella (26) and in Polysiphonia may actually be nuclei, originally from the CBs which have moved into the CP and been extruded along with a small amount of cytoplasm. This hypothesis is very tentative, however, since no evidence of such structures were observed with the EM. However, movement of nuclei seems certain and one disintegrating CP was found with two nuclei.

Carpogonial Branch Cells

Mature CBs are easily distinguished from other cells both because of their binucleate condition and the SER channel which transverses them. The function of the binucleate condition is undetermined although other orders of red algae are known to have bi- or even multinucleate CBs. Fritsch (11) has suggested that the presence of two or more nuclei may indicate arrested branch formation since some of the more primitive orders have corticating CBs.

The continuous channel of SER is unique to the CBs and was not anticipated from earlier light microscopic work even though the channel can just be distinguished in 1-2 um thick sections of appropriate

material stained by toluidine blue. SER is frequently associated with PCs. It is seen around the PC cap in the outer cortical cells of Nemalion (8) and in the gonimoblast and carpospores of Polysiphonia (42, 43). Lee (23) examined twelve species of red algae in the Bangiales and Nemaliales and typically found SER near the pit cap. However, in no case was the SER as extensive as that of the channel in Polysiphonia nor was it obviously associated with a zone of exclusion.

The only alga studied to date which does have an organization of SER somewhat similar to P. harveyi is Batrachospermum. Brown (4, 5) found associated with the pit caps of this alga a network of SER which extended throughout the cytoplasm, and Aghajanian and Hommersand (1), in a later investigation, found two almost contiguous cones of SER extending from the PCs of the axial cells. Their Fig. 9 looks very much like Fig. 25 in this study of P. harveyi. However, the SER in Batrachospermum never becomes continuous from PC to PC, and more importantly, the investigators are convinced that the SER is present only to participate in the formation of the PC.

PC formation can not explain the presence of the SER channel in Polysiphonia. In young CBs, the abbreviated connections are fully formed, but no SER is present (Fig. 33). Even though the abbreviated connections enlarge after the channel forms, the abbreviated connections of the STs, which appear structurally similar (compare Figs. 28 & 30), also enlarge without the presence of SER. Nor does SER seem necessary for any stage of the development, maintenance or disintegration of the PCs in the STs. We can extrapolate from the situation in the STs that the channel is not involved in morphological alteration of abbreviated

connections. However, the function of the SER may be explained by the unique sequence of events which characteristically follows fertilization in the Ceramiales.

After fertilization, the diploid nucleus of the CP divides mitotically (46, 11). In nearly all Floridiophyceae, one of the nuclei passes into an auxiliary cell which then produces the carposporophyte generation (36, 37). Unlike other orders, the AU in the Ceramiales is produced after fertilization which means that the SU must receive an initiating message from the CP. The SER channel seems the most likely conduit for this information. The circumstantial evidence is compelling. The channel is continuous from the CP to the SU and is located in the region of the cells which results in the shortest route. The channel is formed only by the CBs and at the appropriate time to act in the capacity hypothesized. Finally, it disintegrates soon after the AU is formed although the channel is very persistant in unfertilized branches.

For a membrane system to be involved in the translocation of messages or materials would not be a new concept. Brown (4) suggested an intercellular transport function for the extensive SER found in Batrachospermum, and Goff (13) has hypothesized an endomembrane system in Harveyella mirabilis which acts to conduct photosynthate from its host. Well known examples in mammalian cells are the propagation of a nerve impulse by the sarcoplasmic reticulum and transmission of a hormonal message by the plasma membrane (41). However, I was unable to find anywhere a system of SER so morphologically distinct as the channel in Polysiphonia CB cells.

Pit Connections

Prior to the era of electron microscopy, the most widely accepted view of PCs was that they were open connections between cells (11, 15). When Bouck (2) published the first EM micrographs of PCs, a debate began that still continues concerning PC function.

Ramus (35) suggested that PCs are preformed "weaknesses" which are more labile to chemical or enzymatic degradation than the adjacent septum and their eventual disintegration in certain cell types facilitates transport of materials. He notes that, in the female reproductive structures of Griffithsia pacifica, plugs break down and disappear followed by cellular fusion and probable transfer of large amounts of cytoplasm. Similar patterns occur in other red algae (7, 22), and the results with P. harveyi concur with these observations. However, there are two objections to Ramus' hypothesis. First, in some algae the PCs are persistant in the fusion cell, fusion occuring around the PC (8, 43). Second, the idea of a structural weakness role would apply to specialized areas such as the female reproductive structures and would not be applicable to areas where dissolution of PCs does not normally occur.

Other investigators have proposed that the primary function of PCs is to lend structural support to the thallus. This hypothesis is based on the fact that many red algae are pseudoparenchymatous and their cells can be easily separated except at PCs (22). It has been shown that in different cell types the cytoplasm is more firmly attached to the PC than to the cell walls (11, 15, 8). Also, structures such as the pericarp are not known to be present except in those species capable of forming secondary PCs (17, 15). The hypothesis that PCs offer structural

support seems not to be seriously debated, but until it can be shown that PCs do not perform the functions of material transport and communication, it seems premature to suggest that their primary function is structural.

The classical view is that PCs are an avenue of transport between cells. It has been demonstrated that materials pass from cell to cell (14, 40, 12), but it has not been shown that the passage is through PCs. Mangenot (24) found a direct relationship between the size of the PC and the flow of nutrients in the reproductive cells of Griffithsia and proposed as early as 1924 that a study of reproductive cells might help to elucidate the function of PCs. Two investigators (6, 1) demonstrated acid phosphatase activity in the PCs of Batrachospermum although Ramus (35) was unable to detect such activity in Griffithsia. Bouck (2) saw small papillae on surface membranes of PCs of Lomentaria suggesting that material was either being discharged or taken in. Similar structures were observed by Pueschel in a freeze-etch study of Palmaria (29). However, there is no direct evidence for transport through PCs, and movement through cell walls is an alternate possiblity (19).

Even though investigators had noted different PC morphology within a single thallus (6, 34, 28), Wetherbee (42, 43) was the first to suggest that differences in morphology between PCs in the female reproductive cells and PCs in the vegetative thallus have functional significance. He has proposed that "transfer connections" are found in areas where communication might be expected and in areas which might need supplemental nutrition. The morphological characteristics of "transfer connections" - the lack of a pit membrane and a concomitant increase in plug size with increased need for nutritional support - would intuitively

appear to equip them for such a role. Despite some critism of his theory (30, 19), the significance of these morphological differences needs to be explained.

Studies of parasitic relationships between red algae where food transport is obvious give contradictory evidence for transport through PCs between host and parasite. In some relationships there are PCs (21), others have none (9, 10), and still others appear to have semi-standard PCs (13, 32). In addition to the conflicting evidence, the fact that these are specialized relationships makes them questionable as a source of information on normal PC function within an individual alga.

This research with Polysiphonia has important implications concerning the possible roles of the different types of PCs. There are three types of PCs in the female reproductive branch of this alga although determining which of the three is characteristic of a particular junction has posed some problems. In some cases, the pit membrane appears variable from cell to cell or even from section to section within the same cell. In many cells, the membrane appears fragmented. Some of this uncertainty can result from poor fixation or angle of sectioning or, as Wetherbee and Scott (44) suggest, the membrane may be labile, being produced or disassembled according to the needs of the cell. In addition, younger cells may lack the membrane which is normally present in more mature cells of the same type. Pueschel (29) notes that the cap membrane of Palmaria is formed considerably later than the lateral plug membrane.

It seemed at first as if semi-standard PCs were the result of one of the above factors, but subsequent investigation showed their presence to be consistant at the AX-SU and SU-CB_1 junctures. Interestingly, Peyriere

(28) observed a semi-standard PC at the SU-CB_1 juncture of Griffifthsia.

At first, the results with P. harveyi seemed to support the hypothesis that abbreviated connections facilitate transport, particularly since the SER channel implies that a message is transmitted through PCs. However, the presence of semi-standard PCs is not adequately explained by a hypothesis predicated on the assumption that non-differentiated cells need nutritional support and that the absence of membranes allows increased volume and speed of transport. By inference, any materials arriving from the SU would be slowed and the volume limited by the membrane at the SU-CB_1 juncture.

The presence of a semi-standard PC appears functional and not a simple reflection of the cell's inability to produce other PC types. The SU consistantly has a pit membrane at the SU-CB_1 juncture but, just as consistantly, does not have a membrane at the junctures between itself and the STs. These latter connections are typical abbreviated connections. The STs would seemingly be less important in the overall reproductive effort and less in need of nutritional support than the CBs and, yet, there is no impeding membrane between them and the SU.

These observations indicated a possibly different function for abbreviated connections. The presence of PCs structurally similar to abbreviated connections in young vegetative cells suggests that abbreviated connections in the female branch may be neotic structures. The less developed morphology could have been retained, at least in part, because abbreviated connections are more easily broken-down. Such disintegration within the branch has been established both by light and electron microscopy (46, 35, 20). In this study, all abbreviated

connections disintegrate fairly rapidly after fertilization. This pattern accomplishes several tasks. The breakdown allows materials within the STs to move readily to the SU. It also permits rapid transfer of cytoplasm from the CBs to the CP which is known to enlarge after fertilization (17). The presence of the semi-standard PC between the SU and CB_1 assures that this connection will not dissolve or will not dissolve readily. Therefore, all materials will be directed into the CP and not the SU.

This interpretation of PC function is a revival of the "preformed weakness" hypothesis of Ramus. Though it accounts for results in the young stages of development in the reproductive branch of P. harveyi, it does not explain the results of Wetherbee (42, 43) for later stages of female development in the same genus. He has shown that, during carpospore development, fusion occurs around the abbreviated connections leaving them free in the cytoplasm. PC ability to quickly disintegrate, then, would seem to have no functional significance. It is possible that the membranes of the fusion cell develop a propensity to fuse that is much greater than the tendency of abbreviated connections to dissolve and that abbreviated connections are vestigal and, therefore, not functionally significant.

A much more attractive possibility, however, is one which incorporates Wetherbee's hypothesis. The results of C-14 labeling experiments on Polysiphonia lanosa (40) indicate both that the carposporophyte is not self-sufficient and that organic C-14 moves directionally toward the carposporophyte from the vegetative axis. However, if movement does occur through PCs (by no means proven), then all types of PCs are likely

involved. Possibly, membranes at the ends of PCs function to control entry and exit of materials, but maintenance of these membranes can be assumed to be a metabolic expense. Those cells not capable of self-support may be cast in the role of "beggers-can't-be-choosers", allowing free entry and exit in order to avoid the metabolic costs of a functioning membrane. The end result is that abbreviated connections, by utilizing less energy, would increase the percentage of metabolic materials available for other cellular activities. This savings could be particularly important where large numbers of PCs are involved, such as in a parasite (32). In addition, it is certainly possible that materials may be moved more readily or efficiently within cells where all of the connections are of the abbreviated type.

From this study of P. harveyi as well as the work of other researchers, it has become increasingly apparent that PC function is complex. This complexity is the major objection to Wetherbee's term "transfer connection" since it implies the primary purpose of abbreviated connections, in contrast to standard PCs, to be the transfer of materials. The term "abbreviated connection" has been chosen since it implies only a structural simplicity and does not allude to a still uncertain function. Without doubt, more research is needed to firmly establish the function of all types of PCs, but the present research with P. harveyi indicates a multiplicity of function. Depending on the location in the cell or the condition of the cell, PCs may function primarily as structural mechanisms, as avenues of transport and communication or as structures which allow rapid cytoplasmic continuity between cells.

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Key to Abbreviations

AU	auxiliary cell
AX	axial cell
CB_{1-3}	carpogonial branch cells 1-3
CP	carpogonium
GB	gonimoblast cell
GM	gonimoblast mother cell
PC	pit connection
PR	pericarp cell
PS	pericentral cell
ST_1	lateral sterile group
ST_2	basal sterile group
SU	support cell
TB	trichoblast
TG	trichogyne

Chart I

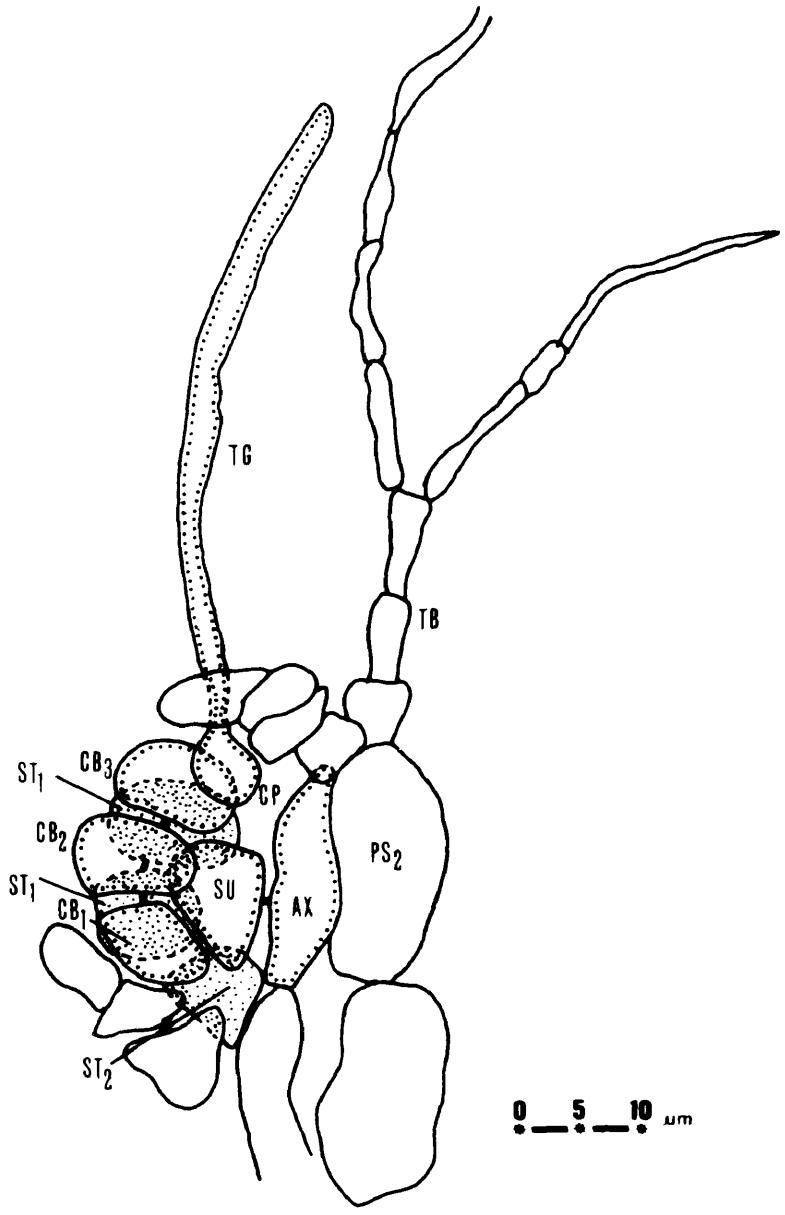
Sequence of developmental events in the female reproductive branch.

Sequence of Developmental Events

1. Development of a trichoblast.
2. Enlargement of fifth pericentral of suprabasal segment of trichoblast.
3. SU cuts off initial of ST_1; pericarp begins formation.
4. SU produces initial of carpogonial branch.
5. Carpogonial initial divides twice to form three-celled branch.
6. SU cuts off ST_2.
7. ST_1 divides to form two-celled branch.
8. CB_3 cuts off CP; pericarp completely surrounds female branch.
9. Elongation of TG (may begin in three-celled stage).
10. Fertilization.
11. CP nucleus undergoes a mitotic division; PCs between CP and CBs breakdown.
12. Cells of ST_1 and ST_2 each divide once to produce a total of six cells; AU cuts off from SU.
13. Continuity develops between CP and AU.
14. Diploid nucleus passes into AU; carpogonial branch begins to wither.
15. PCs between STs disintegrate; AU fuses with SU.
16. Carposporophyte generation initiated.

Diagram I

Camera lucida drawing of sexually mature female branch.



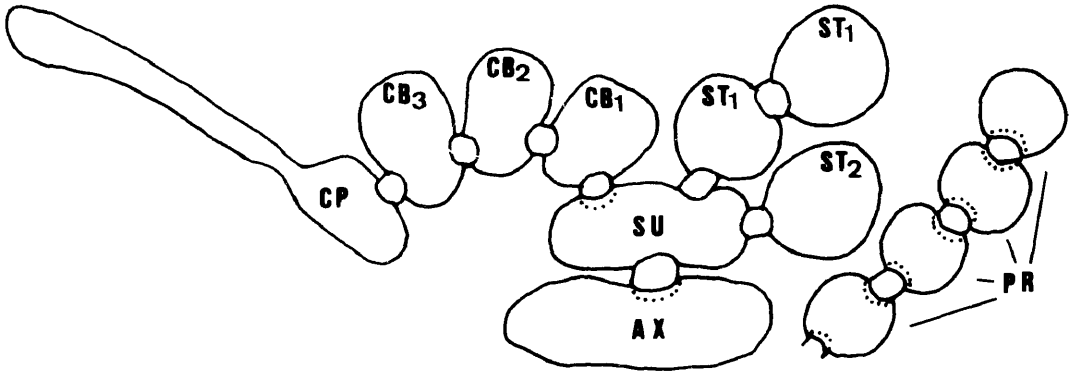
POLYSIPHONIA HARVEYI

Diagram II

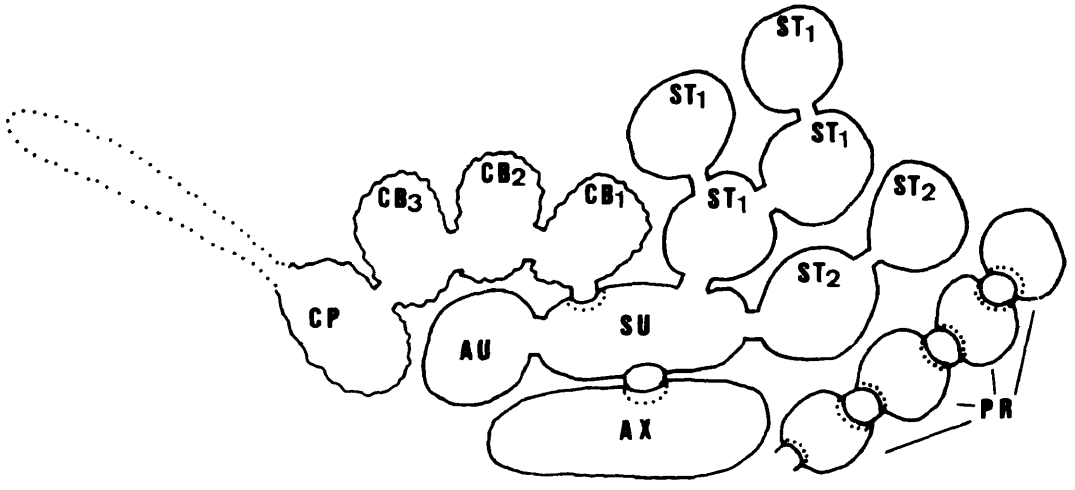
II-A Prefertilization: diagramatic view of the different types of pit connections and their locations in the prefertilization female branch.

II-B Postfertilization: stage after the TG has detached and disintegrated. The carpogonial branch is deteriorating and the AU has fused with the SU. All abbreviated connections have or are in the process of disintegrating, but standard and semi-standard connections are intact.

PREFERTILIZATION



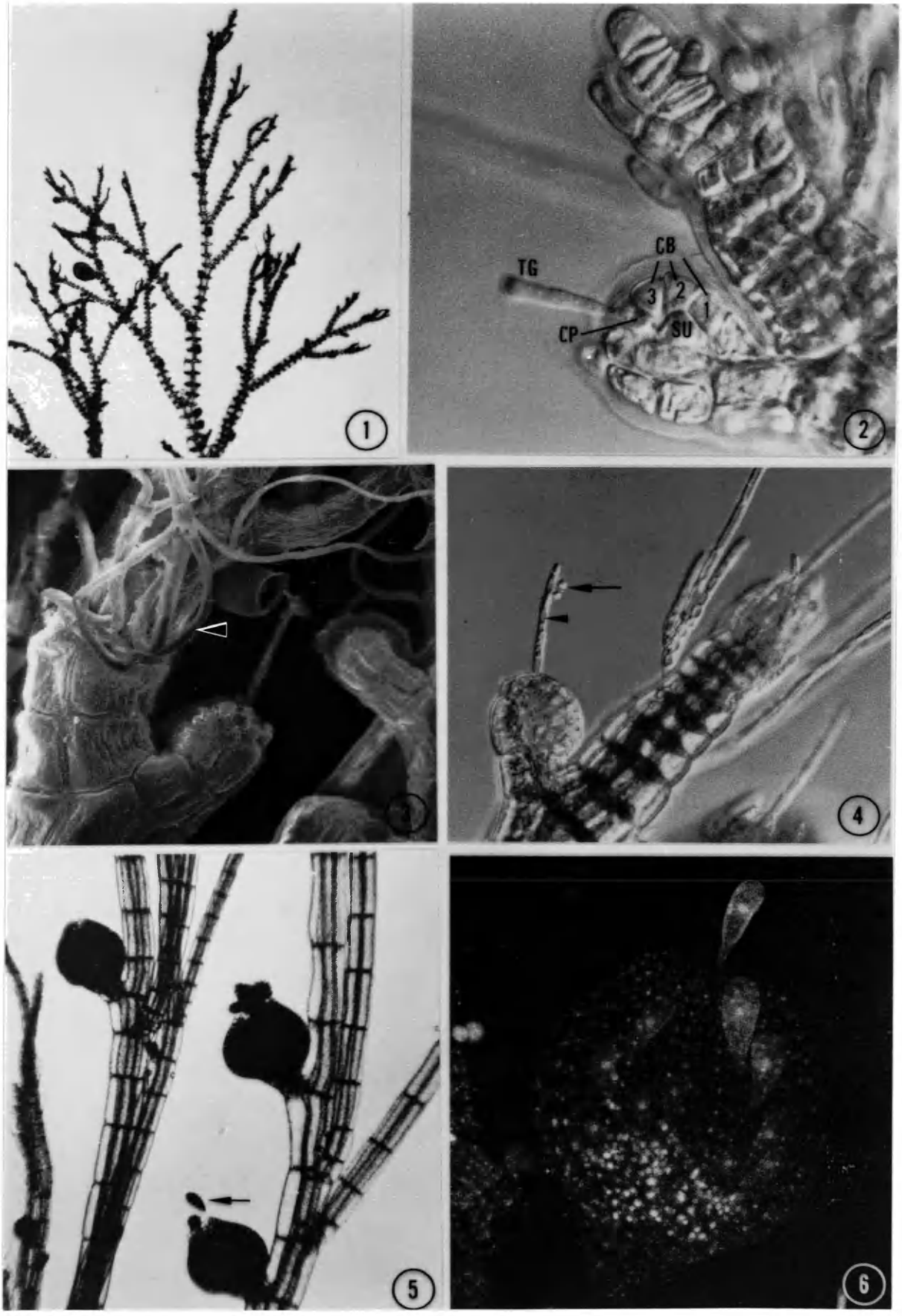
POSTFERTILIZATION



..... Standard PC

..... Abbreviated PC

..... Semi-standard PC


TG
CB
3
2
1
CP
SU
1
2
3
4
5
6

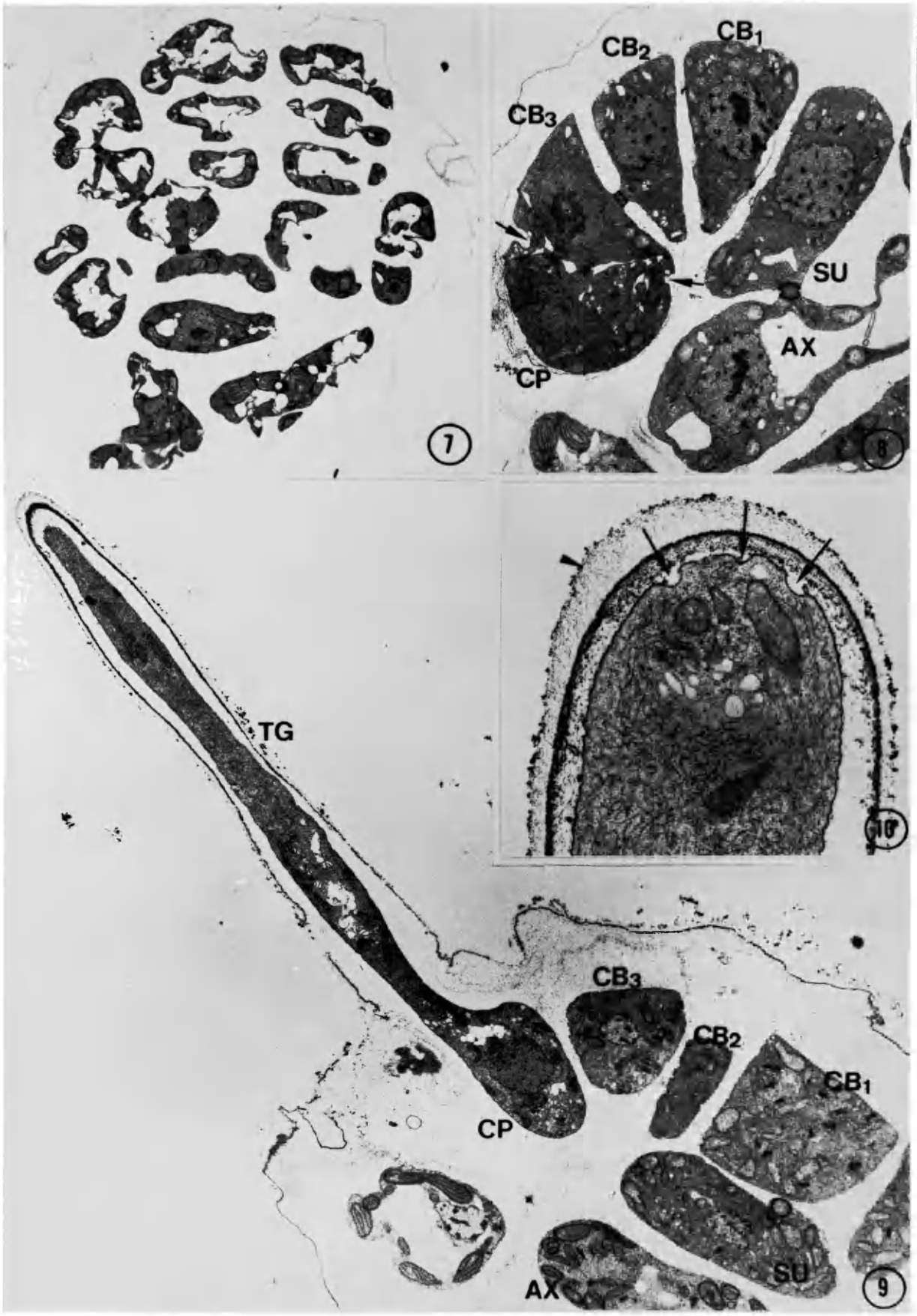

CB2
CB1
CB3
SU
AX
CP
7
8
TG
10
CB3
CB2
CB1
CP
SU
AX
9

Plate III

Figure 11. Prefertilization lateral sterile group. The abbreviated connection (arrow) is longer and more narrow than those found elsewhere in the procarp. The cells are densly protoplasmic with little to no starch accumulation. X 4000.

Figure 12. Postfertilization lateral sterile group. Each of the original two cells has divided to produce a total of four cells. X 3400.

Figure 13. Carpogonial branch past optimum fertilization age. The TG is extremely vacuolate. The CP nucleus is elongated and the heterochromatin is finely dispersed. A double membrane-bound vacuole can be seen at the arrow. A similar vacuole is enlarged in Fig. 14. X 5350.

Figure 14. CP with large vacuole enclosed by double membrane continuous with the nuclear envelope (arrow). Note also the crystalline appearance of the nucleolus (double arrows). X 13,000.

Figure 15. Longitudinal section through crystalline structure of nucleolus. The rods show a 22.5 nm periodicity. X 74,000.

Figure 16. Cross section of nucleolus showing hexagonal packing of rods. X 74,000.

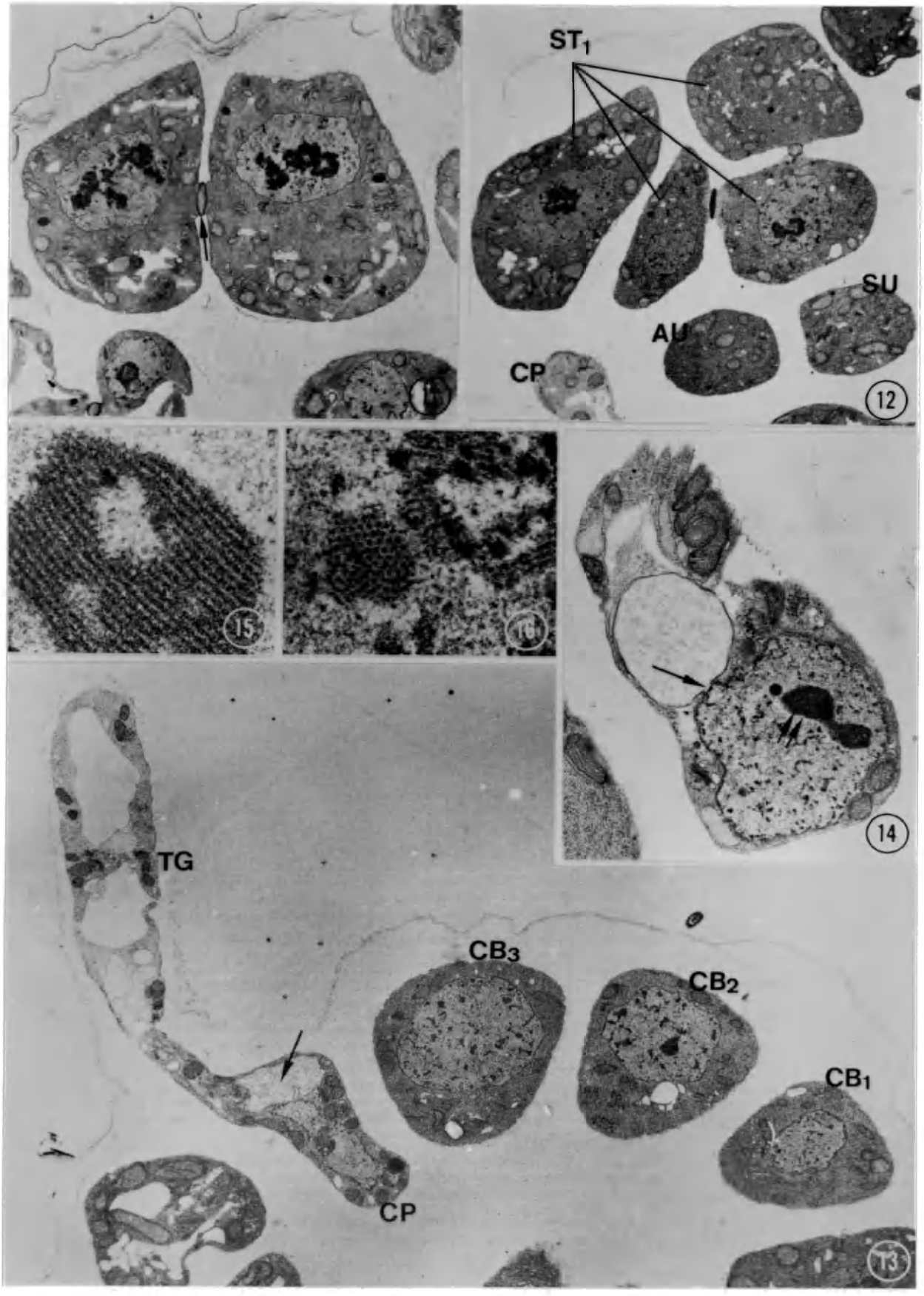

ST1
SU
AU
CP
12
15
16
14
TG
CB3
CB2
CB1
CP
13

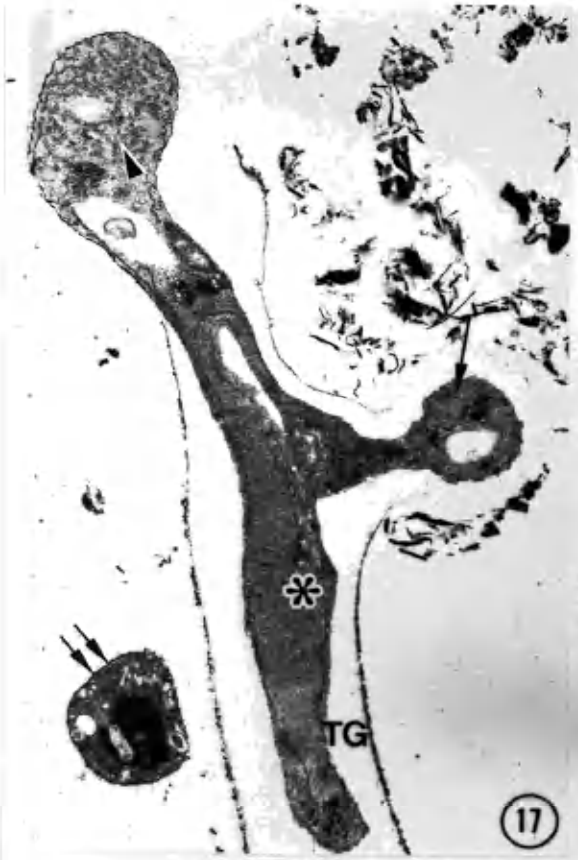
Plate IV

Figure 17. TG with attached spermatium (arrow). A wide cytoplasmic channel exists between the spermatium and the TG, but the spermatium retains a high percentage of cytoplasm. The TG cytoplasm behind the advancing spermatial nucleus becomes disordered as evidenced at the TG tip (arrowhead). The asterisk marks the position of the spermatial nucleus as seen in a later section (Fig. 18). A second spermatium which was unable to effect fertilization and is disintegrating is designated by double arrows. X 5350.

Figure 18. Spermatial nucleus inside TG (section following that in Fig. 17). The chromatin of the male pronucleus is very dense and is surrounded by a double membrane in which nuclear pores are visible. X 28,000.

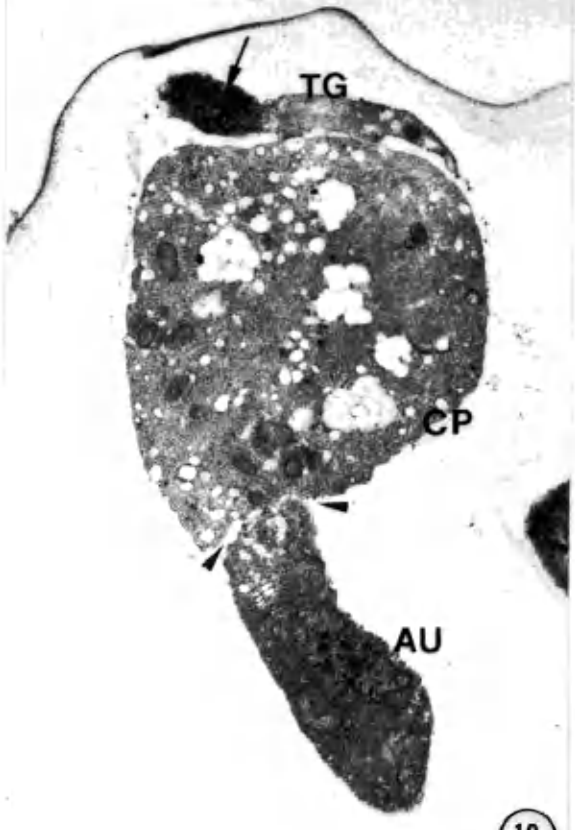
Figure 19. Fertilized CP with detaching, disintegrated TG. A mass of electron-dense material (arrow) appears to plug the base of the TG which is almost completely detached from the CP possibly by the formation of a series of vacuoles. The AU also appears to be in the process of abscissing from the CP after direct fusion (arrowheads). X 7000.

Figure 20. CP and detaching TG without plug of material. As in Fig. 19, separation appears to occur by the development of a vacuole (arrow). Note the finely dispersed heterochromatin of the CP and the crystallinity of the nucleolus (arrowhead). X 22,000.



17

18



19

20

Plate V

Figure 21. Postfertilization CP with detached and disintegrating TG. One of two nuclei found in this CP is visible at the arrow. Another nucleus from a different section can be seen in Fig. 22. X 3250.

Figure 22. Later section of the CP seen in Fig. 21. A second, deteriorating nucleus is visible at the arrow. One of the nuclei may have moved into the CP from the CBs. Note the enlarged nucleus of CB_3 and the cytoplasmic connection between the CP and CB_3 (arrowhead). X 4500.

Figure 23. Carpogonial branch with channel of SER (arrowheads). Serial sections show that the SER extends uninterrupted through the cytoplasm from the CP to the SU, breaking continuity only at the pit connections. X 6300.

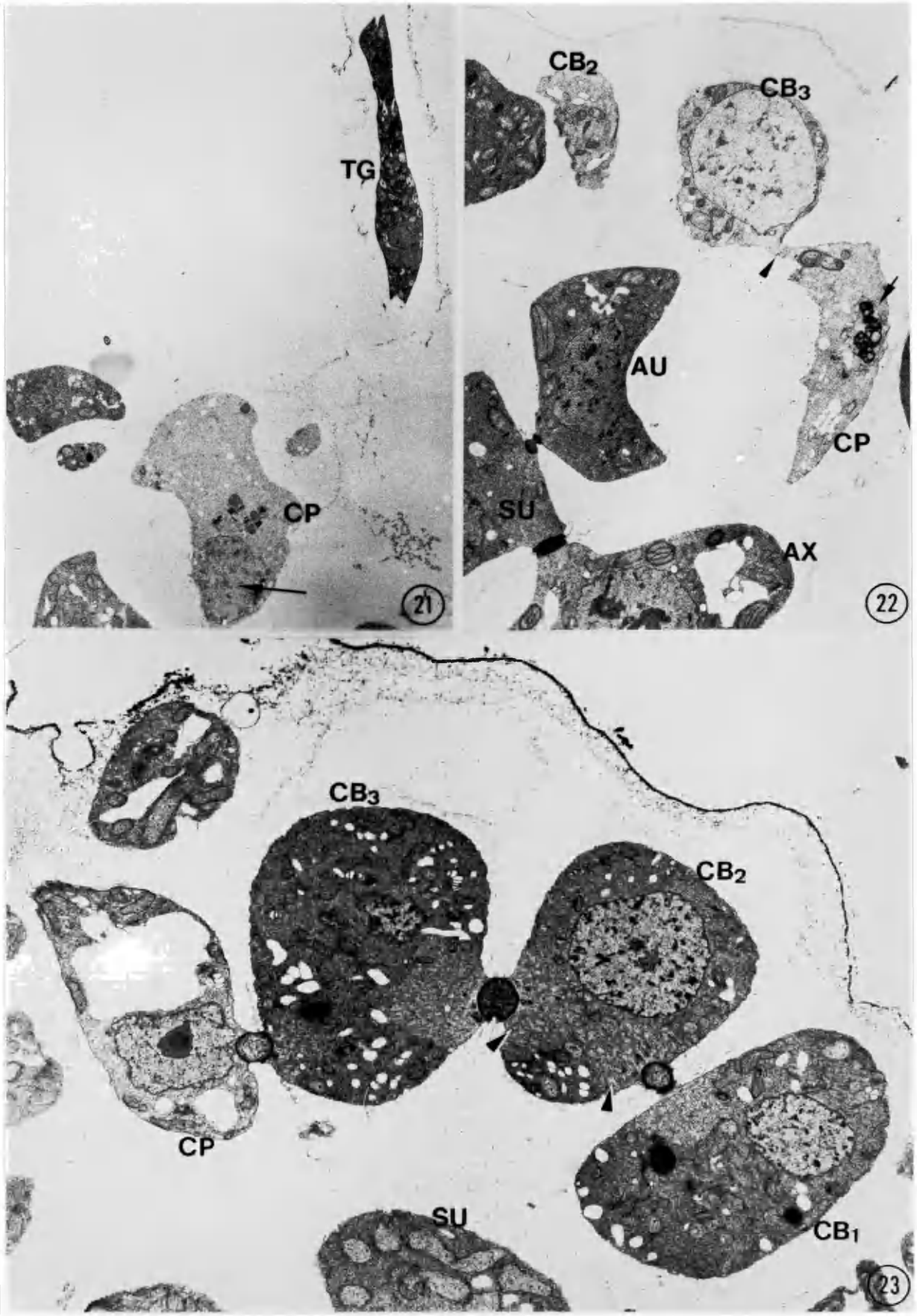

TG
CP
21
CB2
CB3
AU
CP
SU
AX
22
CB3
CB2
CP
SU
CB1
23

Plate VI

Figure 24. Channel of SER continuous across CB_2 in a pre-fertilization procarp. The channel (arrowheads) appears to be a zone from which all other organelles are excluded. The nucleus in CB_2 is not visible in this plane of sectioning. X 10,950.

Figure 25. Pit connection between SU and CB_1 in early stage of development. The SER channel begins as a dome-shaped mass of SER adjacent to the pit connection. Bordering the SER are aggregations of proplastids and mitochondria (arrowheads). X 20,000.

Figure 26. Unfertilized, deteriorating procarp. The CP has almost completely disintegrated, but the pit connections (arrows) are still present and the SER channel still obvious. X 5400.

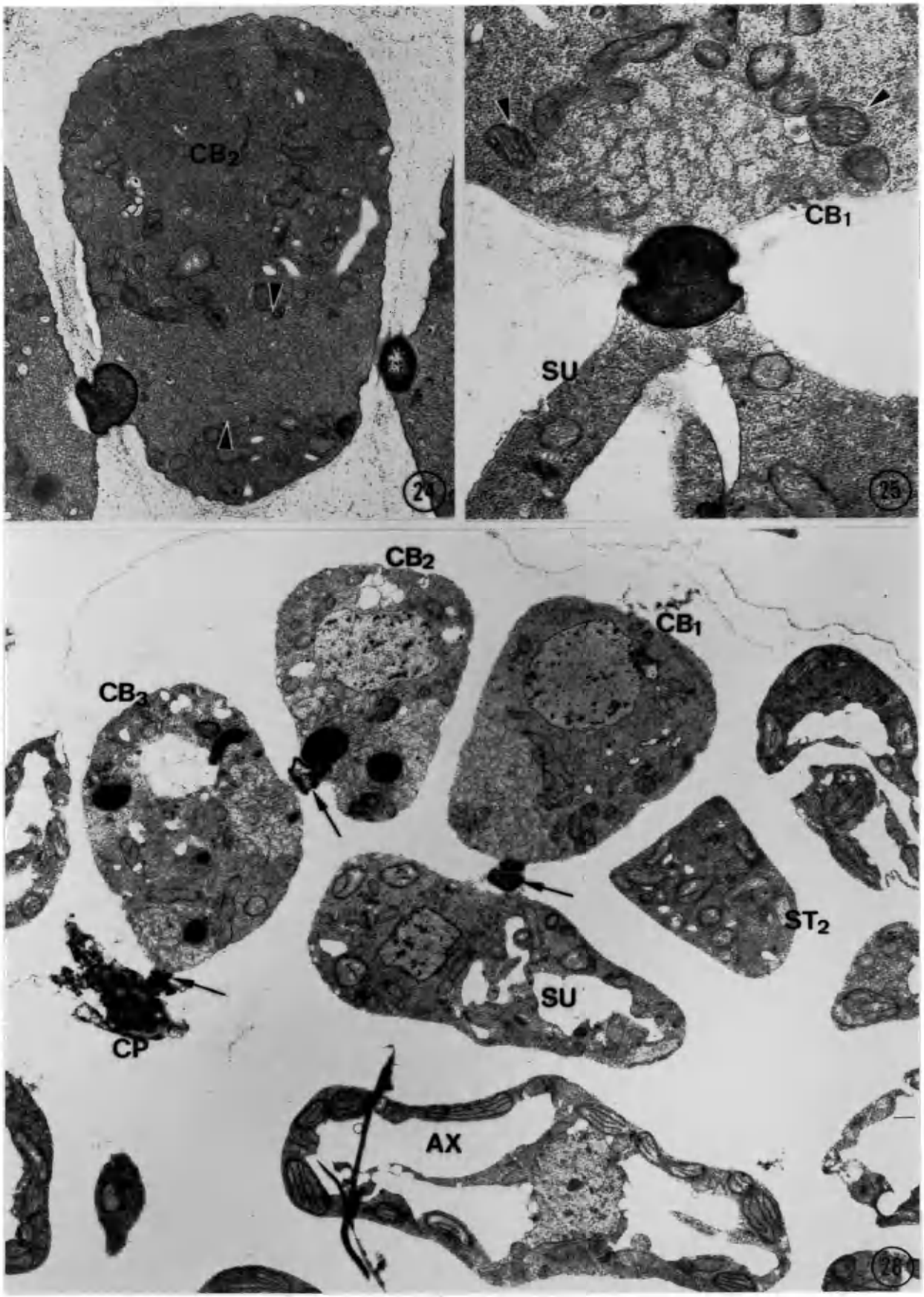

CB2
24
CB1
SU
25
CB2
CB1
CB3
ST2
SU
CP
AX
26

Plate VII

Figure 27. Standard pit connection between two cells of the vegetative thallus. The core is bounded on the cytoplasmic ends by pit caps (arrows). The pit membrane is denoted by large arrowheads and the plasma membrane by small arrowheads. X 42,000.

Figure 28. Abbreviated connection between CB_2 and CB_3. An electron-dense cap is present (arrowheads), but neither the electron-translucent layer nor the pit membrane can be detected. SER from the channel appears to abut the pit cap. X 42,000.

Figure 29. Semi-standard connection between AX and SU. The end of the plug facing the AX has a cap and membrane system similiar to a standard connection ([]) whereas the end facing the SU appears to have only a plug cap (arrowhead). X 42,000.

Figure 30. Abbreviated connection between SU and ST_2. This connection appears structurally identical to the pit connection in Fig. 28, but there is no SER associated with it. X 42,000.

Figure 31. Pit connection between young vegetative cells. The connection is morphologically similiar to an abbreviated connection except that adjacent to the cap is a layer containing periodic striations perpendicular to the pit cap (arrowhead). X 42,000.

Figure 32. Standard pit connection of vegetative branch showing possible pit membrane deposition. The nucleus which is generally located in the center of the cell has moved close to the pit connection, and the nuclear envelope seems to be providing the pit membrane (arrow). X 42,000.

Figure 33. Young pit connection between CB_2 and CB_3. The pit connection does not flare into the cells as far as a mature abbreviated connection, but no other structural differences are discernable. The absence of SER indicates that SER is not necessary for formation of the abbreviated connection. X 42,000.

Figure 34. Disintegrating pit connection between cells of basal sterile group. Pit connections do not seem to have a standard appearance during disintegration. X 51,600.

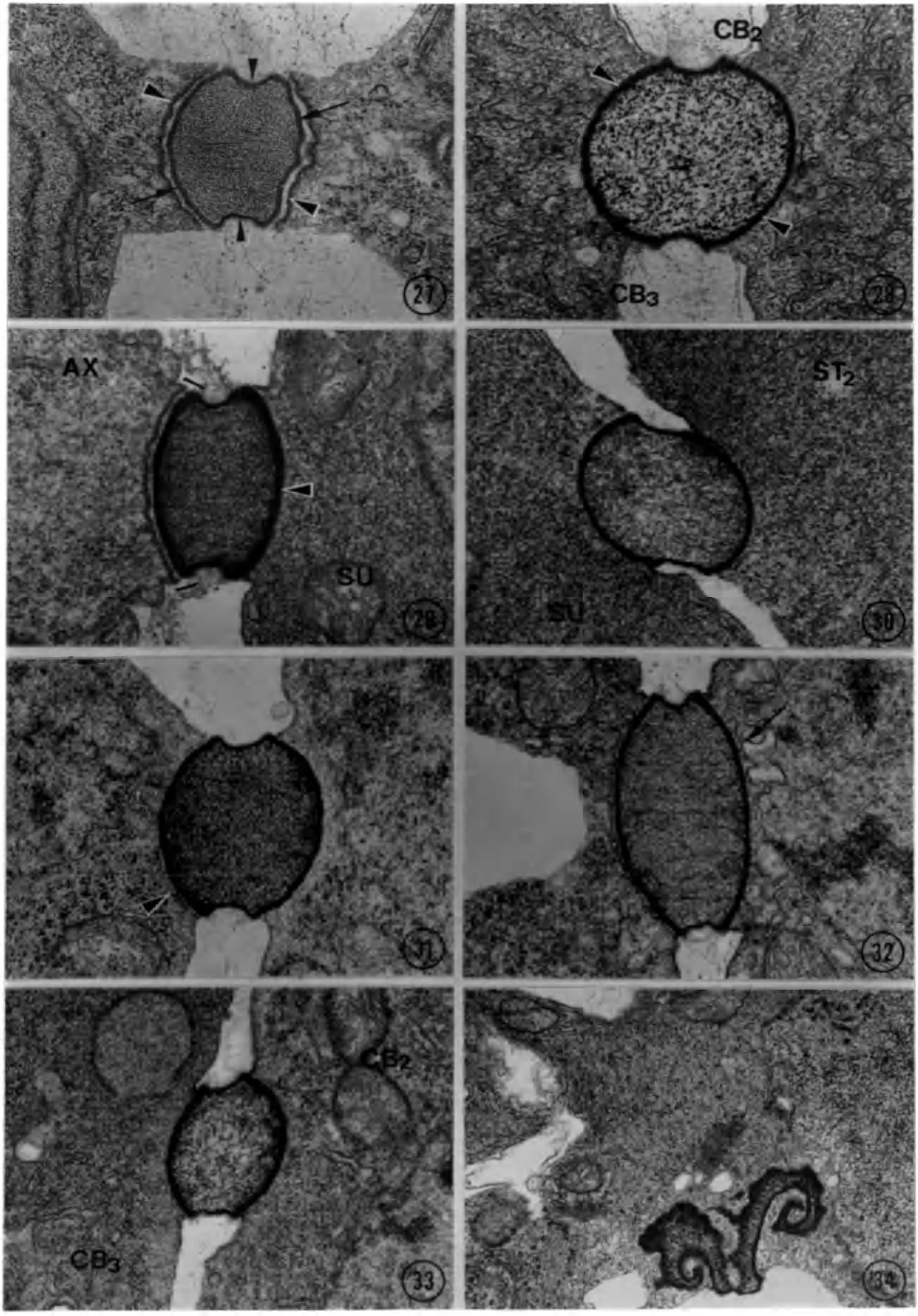
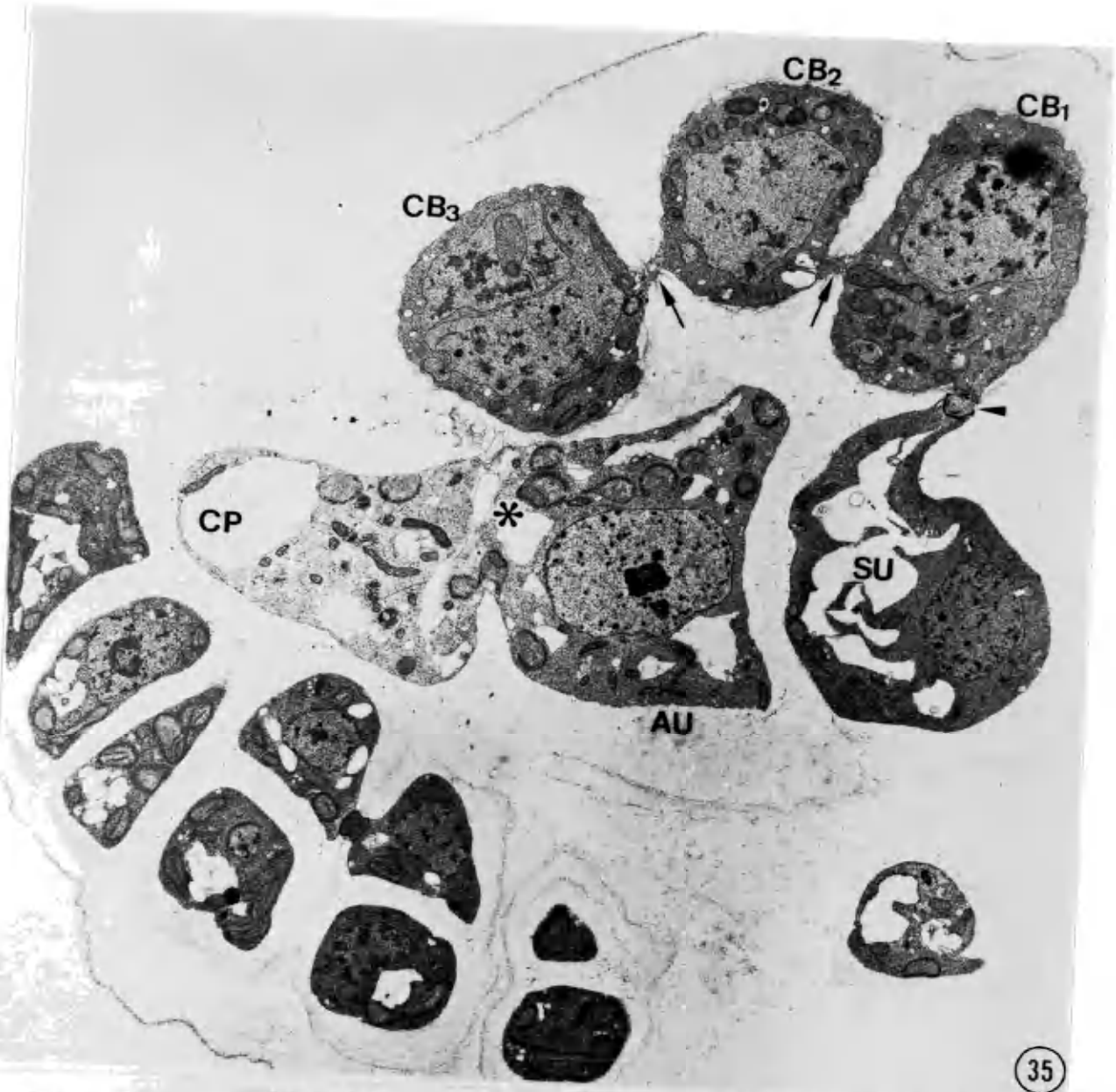

CB2
CB3
AX
SU
ST2
SU
CB2
CB3

Plate VIII

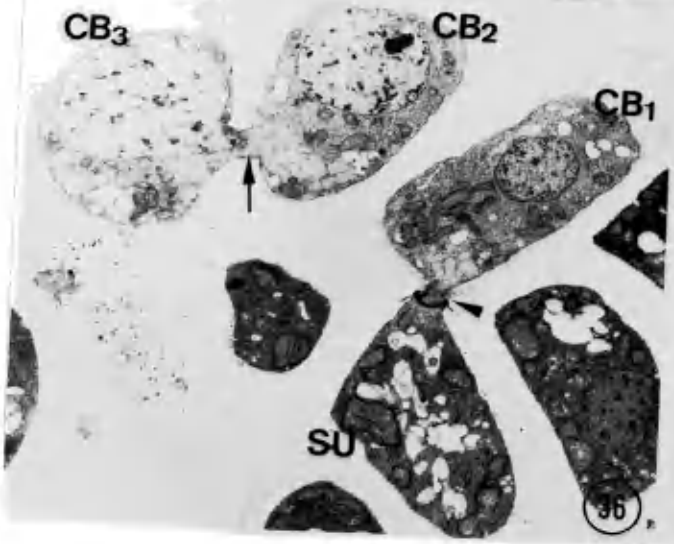
Figure 35. Postfertilization female branch above portion of pericarp. The pit connections between the CBs have disintegrated allowing cytoplasmic continuity, and the SER channel has disbanded (arrows). The semi-standard connection between the SU and CB_1 has not deteriorated (arrowhead). The CP is interpreted as abscissing from the AU following apparent direct fusion (*). X 4600.

Figure 36. Postfertilization female branch. Although the pit connections between the CBs have disintegrated, some SER remains (arrow). In the semi-standard connection between the SU and CB_1, the side of the pit connection originally lacking a pit membrane is dissolved, but the side with the membrane is intact (arrowhead). Fig. 37 is a magnification of this connection. X 3550.

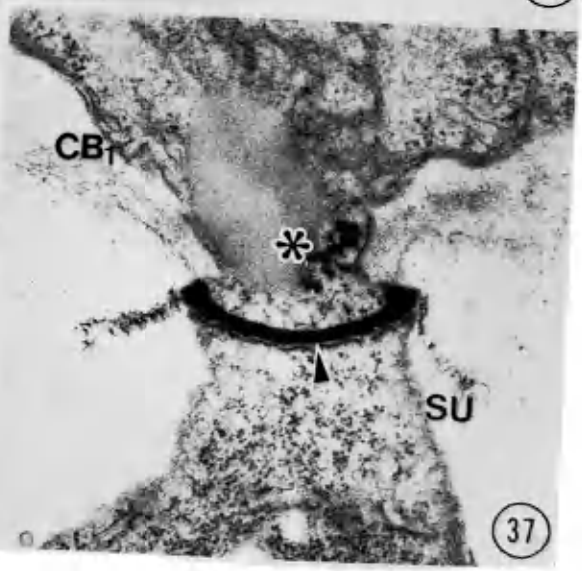
Figure 37. Enlargement of the semi-standard connection in Fig. 36. Note the absence of the pit core and the pit cap in CB_1 (*). Yet, the pit cap and pit membrane adjacent to the SU are clearly visible (arrowhead). X 41,000.



35



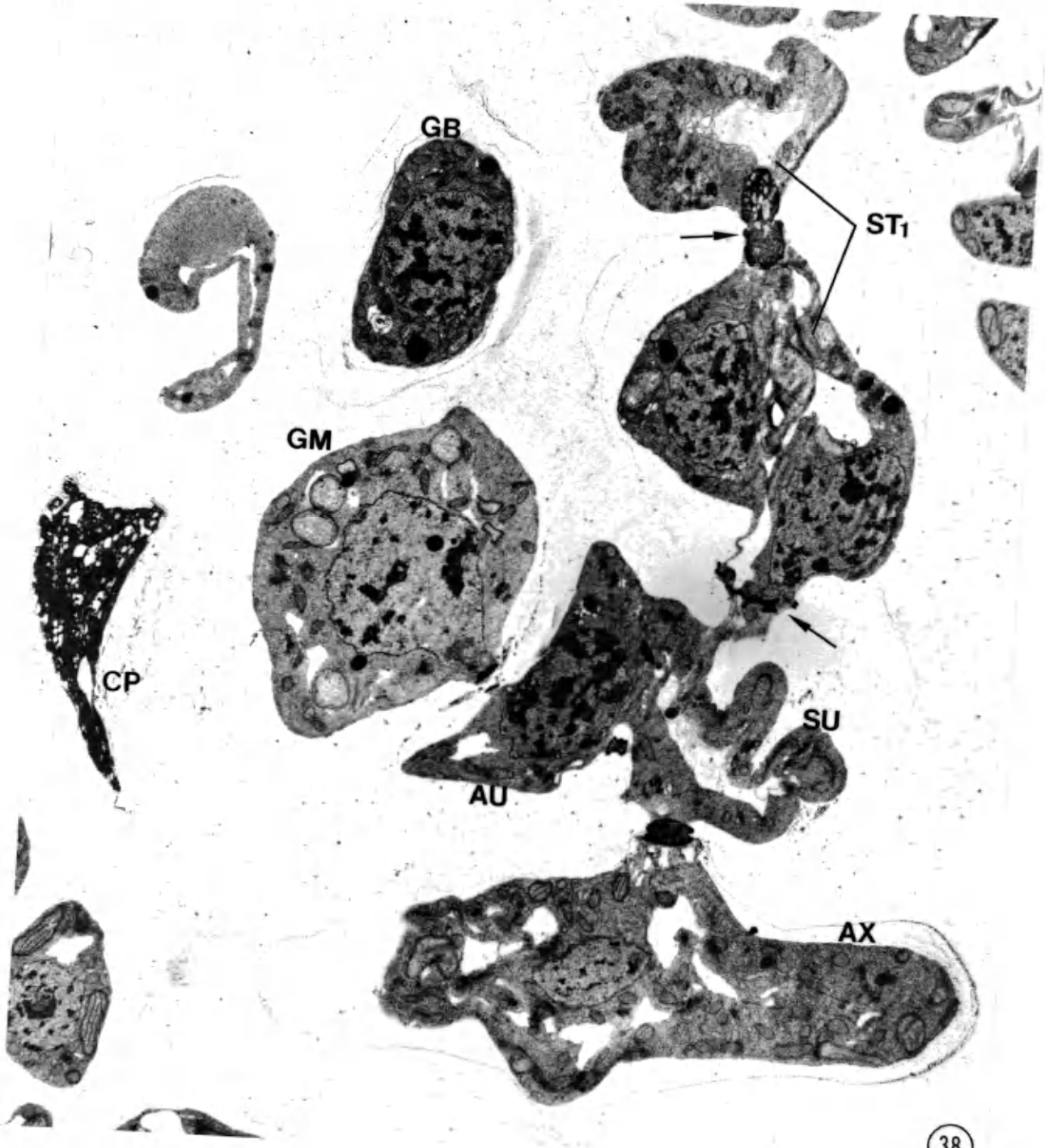
36



37

Plate IX

Figure 38. Development of the gonimoblast filaments and fusion cell. The CP and CBs are disintegrating. The AU has fused with the SU adjacent to, but not including, the intact SU-AU pit connection. The pit connections between the STs are disintegrating (arrows). The gonimoblast mother cell and the first gonimoblast cell are already present. X 5350.


GB
ST1
GM
CP
SU
AU
AX

VITA

Sharon Thompson Broadwater

Born in Middlesboro, Kentucky, November 26, 1944. Graduated from Middlesboro High School, Middlesboro, Kentucky in June, 1962. Began initial undergraduate studies at Berea College, Berea, Kentucky, 1962-64. Attended the University of Kentucky, Lexington, Kentucky, 1964-66, recieving a B.A. in Zoology from this institution in December, 1966. Taught biology and general science at Brooklawn Junior High School, Parsippany, New Jersey, 1967-69 and at The Hebrew Day School, Richmond, Virginia, 1976-77. Teaching assistantship in biology at the College of William and Mary, 1978-80. Candidate for M.A. at the College of William and Mary, 1978-81. Presently enrolled, under a National Science Foundation grant, in the Ph. D. program at the Virginia Institute of Marine Science of the College of William and Mary, Gloucester Point, Virginia.