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Ultrastructure of the Salivary Glands of *Pachycondyla* (=*Neoponera*) *villosa* (Fabricius) (Formicidae: Ponerinae): Functional Changes during the Last Larval Instar

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Summary The cells of secretory region of the salivary glands of *Pachycondyla* (=*Neoponera*) *villosa* at the time of enzyme production presents the basal cellular membranes profusely folded and the intercellular junctional membranes present a few enlarged spaces. The rough endoplasmic reticulum and the Golgi bodies shift from being flat and small vesicular cisternae to enlarged vesicular cisternae according to the cell physiological state and characterize an asynchronic cell cycle. Enzymes are released into the lumen by microapocrine secretion. The stage of silk production is detected after a behavioral act, when the nurse worker separates the mature larva. At this time, the salivary gland cells present only one physiological state (synchronized secretory cycle): this state was characterized by basal cellular membrane poorly folded, intercellular junctions presenting some small spaces, rough endoplasmic reticulum compounded by flat cistenae, enlarged Golgi bodies with fibrous material inside and a few secretory vesicles containing silk, which undergo exocytosis. The silk in the lumen shows 2 forms: tactoid and flocculent material.

Key words Salivary gland, Larvae, *Pachycondyla villosa*, Ultrastructure, Silk.

The ant *Pachycondyla* (=*Neoponera*) *villosa* (Fabricius) is restricted to Neotropical areas (Kempf 1972). Nests of this ponerine ant are usually built on bromeliads of the genus *Aechmea* (Caetano *et al.* 2002). Larvae are white, mainly feeding on prey captured by workers. Some ant species, at the end of the last larval instar, cease feeding and begin to produce cocoon silk for pupation (Wheeler and Wheeler 1976). Although the silk production in silk-spinning ants is related to salivary glands (Wheeler 1918, Athias-Henriot 1947, Lappano 1958, Wheeler and Wheeler 1976), some histological studies present evidence for active secretion of substances other than silk (Athias-Henriot 1947, Valentini 1951, Wang and Happ 1974, Petralia *et al.* 1980) before silk production (Lappano 1958, Zara and Caetanó, in analysis). According to Wheeler (1918) and Athias-Henriot (1947) the salivary glands may also secrete digestive enzymes for extraintestinal digestion of solid food. The activity of digestive enzymes has been determined by biochemical analysis at least for 2 ant species, *Solenopsis invicta* Buren (Petralia *et al.* 1980) and *Acromyrmex octospinosus* (Reich.) (Febvay and Kermarrec 1981). In *S. invicta* the salivary glands of last instar larvae display protease and amylase activity which are related to extraintestinal digestion of solid food (Petralia *et al.* 1980).

The ultrastructure of the secretory region of the salivary glands has been described for 2 ant species, *S. invicta* (Petralia *et al.* 1980) and *Camponotus pennsylvanicus* (DeGeer) (Petralia and Haut 1986). In *S. invicta*, the salivary glands secretion was related to digestive enzymes, but in *C. pennsylvanicus* it was related to silk production for the cocoon.

In this paper we describe the cellular changes in the secretory region of the salivary glands in the last larval instar of *P. villosa*, during which period the secretory cells may be producing digestive enzymes and then, at the end of this instar, change function to silk production.

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Material and methods

Larvae of *P. villosa* were collected from artificial nests maintained in the Departamento de Biologia, Unesp-Rio Claro-S.P. Brazil. The last larval instar was determined according to Zara and Caetano (2001) based on the head capsule size. The silk stage in the last instar is detected after a behavioral act when the nurse worker separates the mature larva form the others moving it to a specific site in the nest ready for pupation. After this, all larvae dissected had silk in the lumen of the salivary glands (Zara *et al.* 2002). The probable digestive enzyme secreting stage was also determined by measuring the head capsule, and also could be assessed because larvae stay together to others instars. When these larvae were removed and dissected, no silk was ever found in the gland lumen.

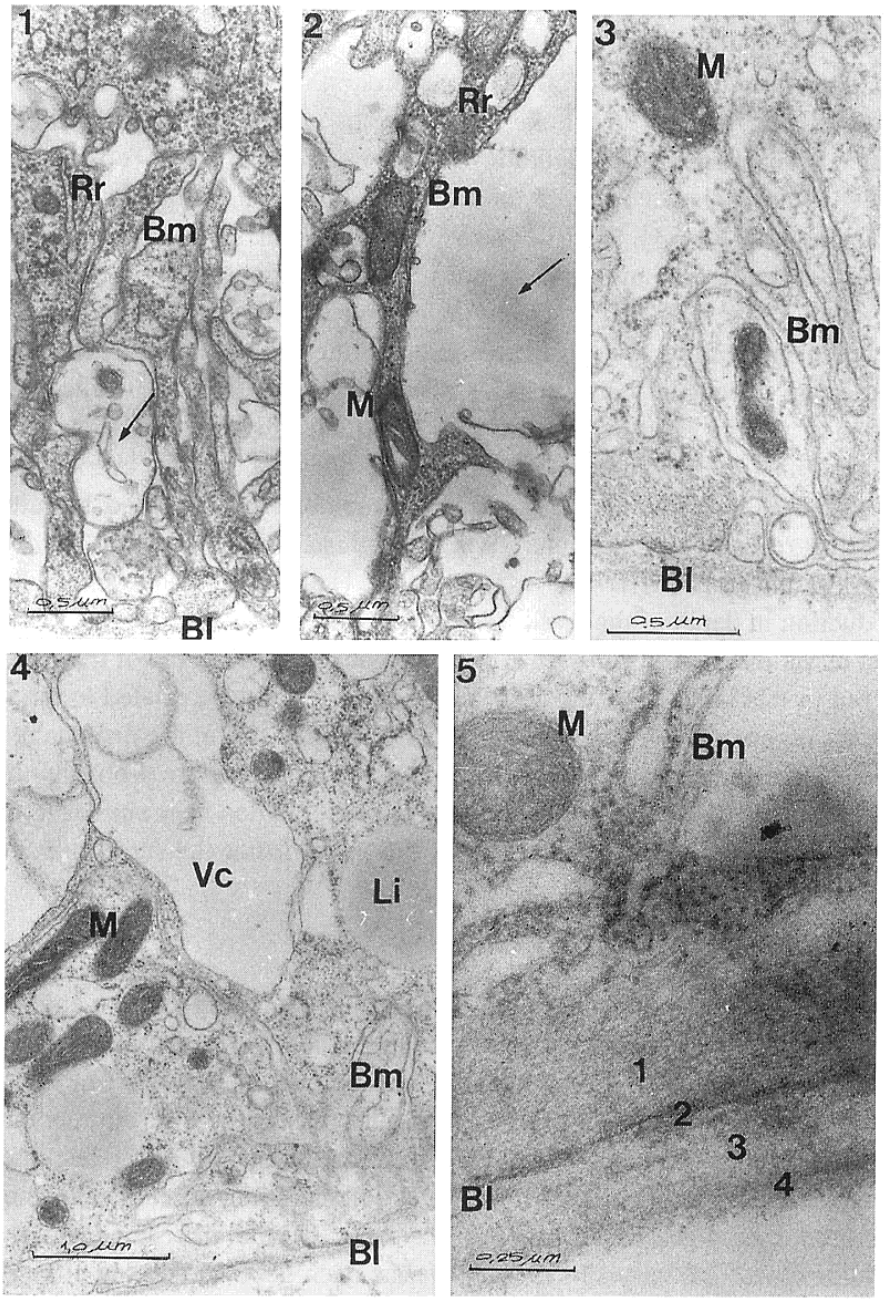
For ultrastructural analysis salivary glands were dissected in fixative (2.5% glutalaldehyde in 0.1 M Na cacodylate buffer at pH 7.2) and kept in this solution for 2 h at room temperature. Samples were then rinsed twice in the same buffer, post-fixed in 1% osmium tetroxide, dehydrated in a series of increasing concentrations of ethyl alcohol, and embedded in Epon-Araldite resin, following usual procedures. Ultrathin sections were obtained with diamond knives, stained with uranyl acetate and lead citrate, and examined in a Philips CM100 transmission electron microscope.

Results and discussion

The secretory region of *P. villosa* salivary glands shows no ultrastructural differences between proximal and distal gland regions and only 1 cell type was identified. This is in accordance with what has been observed histologically (light microscopy) for several Hymenoptera such as in the bees *Apis mellifera* L. (Nelson 1924), *Melipona quadrifasciata* (Lep.) (Mello and Vidal 1971), *Scaptotrigona postica* Latreille (Cruz-Landim and Mello 1981) and in the ants *Eciton burchelli* West. (Lappano 1958), *Neivamyrmex nigriscens* (Cresson) (Wang and Happ 1974), *S. invicta* (Petralia and Vinson 1980), *P. villosa* (Zara and Caetano, in analysis), and several species recorded by Athias-Henriot (1947) and Valentini (1951). Ultrastructural analyses have been conducted in *Melipona quadrifasciata anthidioides* (Lep.) (Silva de Moraes and Cruz-Landim 1979), *S. invicta* (Petralia *et al.* 1980) and *C. pennsylvanicus* (Petralia and Haut 1986). Although the secretory region presents only 1 cell type, these cells change their function during the last instar.

Basal lamina

Ultrastructurally the salivary glands of *P. villosa* have a very thin basal lamina (Figs. 1, 3, 4) which never penetrates the cells and which at high magnifications shows 4 distinct layers (Fig. 5): the first (more internal) shows fibrilar elements, probably collagen IV (Ashhurst 1982, 1985, Alberts *et al.* 1994, Lane *et al.* 1996); the second is very thin and electrondense; the third and fourth are both composed by granular elements but the last is both more electrondense and thinner. No changes in ultrastructure are observed in the last instar and the presence of thin basal lamina has been observed in some insect salivary glands as Diptera (Jacob and Jurand 1963, Kloetzel and Laufer, 1969, Harrod and Kastritsis 1972, Eeken 1977, Riparbelli *et al.* 1994), Lepidoptera (Akai 1983 1984, Sehnal and Akai 1982, Jochová *et al.* 1997) and Hymenoptera (Silva de Moraes and Cruz-Landim 1979, Petralia *et al.* 1980, Petralia and Haut 1986). However, basal lamina with multiple different layers have not been observed in the larval salivary glands through they do occur in the Malphigian tubes (Martoja and Ballan-Dufrançais 1984, Martinelli 1998) and in the midgut without electrondense layers (Lane *et al.* 1996). The main role of this structure in *P. villosa* may be the same as that described by Ashhurst (1982, 1985) and Alberts *et al.* (1994), where the basal lamina contributes to molecular filtration, avoiding and/or selecting macromolecules that can them be taken into the cells.



Figs. 1–5. 1, 2) Aspect of the secretory cells basal portion during the phase of digestive enzymes production, showing profusely folded basal plasma membrane (Bm) with spaces (arrows) varying from narrowed (1) to enlarged (2). Rr, rough endoplasmatic reticulum. 3) Secretory cells basal portion during the phase of cocoon material production showing reduced spaces between basal plasmatic membrane (Bm) infold. 4) Partial view of the basal portion during the phase of cocoon material production showing no deep basal plasmatic membrane (Bm) infold. Vacuoles (Vc), lipid droplets (Li) and several mitochondria (M) are also observed. Bl, basal lamina. 5) Detail of basal lamina (Bl) composed of 4 distinct layers (1–4). M, mitochondria

Basal membrane

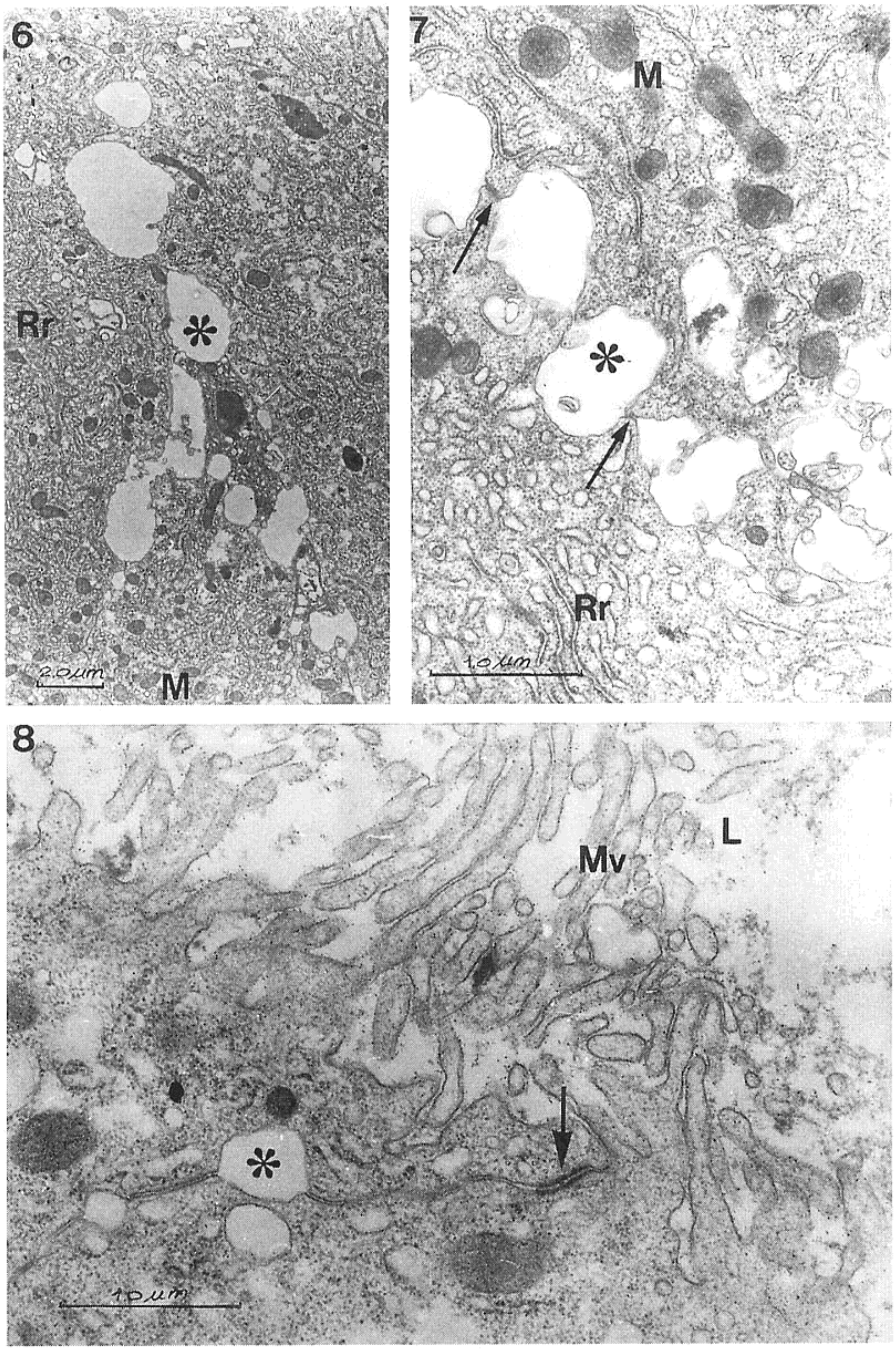
The basal membranes are folded and show some changes between the beginning and end of the last instar. At the beginning, the basal cellular membranes are deeply folded and some spaces between them can be observed. These spaces vary from cell to cell according to their physiological state (Figs. 1, 2). At the end of the last instar, when cells start producing silk, the basal membranes are less deeply folded and without spaces between them (Figs. 3, 4). During this phase numerous

vacuoles can be observed near basal membranes (Fig. 4). Several authors postulated that folded basal membranes may facilitate absorption of amino acids, ions and other precursors of substances from hemolymph used in the synthesis of salivary secretion in, for exemple, Diptera (Jacob and Jurand 1963, Kloetzel and Laufer 1969, Eeken 1977, Curtis *et al.* 1987, Riparbelli *et al.* 1994), Trichoptera (Engster 1976), Lepidoptera (Fukuda *et al.* 1955, Wiley and Lai-Fook 1974, Jochová *et al.* 1997) and Hymenoptera (Silva de Moraes and Cruz-Landim 1979, Petralia *et al.* 1980, Petralia and Haut 1986). Bianchi *et al.* (1973), Bianchi and Terra (1975), Terra and Bianchi (1974, 1975) using biochemical analysis found that amino acids and proteins fractions are absorbed from hemolymph to salivary glands of *Rhynchosciara americana* Wiedemann. This may occur in *P. villosa*, however, products absorbed from hemolymph must be different at the beginning and at the end of the last instar. Early in the last instar, the basal cellular membranes are deep and folded presenting some spaces, similar to *S. invicta* (Petralia *et al.* 1980) of which salivary glands produce digestive enzymes such as proteases and amylases. However this ant does not produce cocoons or silk (Wheeler and Wheeler 1976). At the end of the last instar, basal membranes are folded, but not so deeply and without spaces, thus resembling that found in *M. quadrifasciata anthidioides* (Silva de Moraes and Cruz-Landim 1979) and *C. pennsylvanicus* (Petralia and Haut 1986) whose larvae also produce silk for cocoon. Reduction in depth of the basal folds and loss of spaces between the basal plasma membrane in the last larval instar of *P. villosa* has also been observed in larvae of the lepidopteran, *Manduca sexta* L., but in this latter case, the morphological changes were related to the start of the cellular death process (apoptosis) (Jochová *et al.* 1997). Since all cells at the final stage are engaged in silk production, changes in the basal plasma membranes of *P. villosa* are probably not death related. Cells at the beginning of the last instar thus present the basal region very similar to those producing digestive enzymes, while presenting at the end of the larval instar the same characteristics of silk producing cells.

Intercellular space

Intercellular spaces, also present changes between the beginning and the end of the last instar. At the beginning, intercellular spaces are greatly enlarged and widened extending from the basal to the apical portion of the cells and are punctuated with desmosomes (Figs. 6, 7). At the end of the last instar, intercellular spaces are very reduced and are only present in the medium to the apical portion of the cells (Fig. 8). The intercellular spaces at the beginning of the last instar thus similar to those of *S. invicta* (Petralia *et al.* 1980). This characteristic may therefore be another indication that these cells producing digestive enzymes at this stage. On the other hand, at the end of the last instar, intercellular spaces are very similar to what has been observed during silk production of *M. quadrifasciata anthidioides* (Silva de Moraes and Cruz-Landim 1979) and *C. pennsylvanicus* (Petralia and Haut 1986). Furthermore, according to Silva de Moraes and Cruz-Landim (1979), large intercellular spaces are found in all larval instars except for the last of *M. quadrifasciata anthidioides* when they are greatly reduced, similar to *P. villosa*. This reduction, mainly in the basal region, may indicate a reduction in the transport of substances from hemolymph to salivary glands through paracellular way (Silva de Moraes and Cruz-Landim 1979, 1980). Thus it can be inferred for *P. villosa* that the phase before silk production require a higher level of transport of substances from hemolymph, probably for digestive enzyme production, while during silk production, even though still transporting material from hemolymph, this may be simple predominant compounds which do not need such large spaces for their traffic.

Intercellular junctions of *P. villosa* present no differences between the beginning and the end of the last stage. The apical portion of the secretory region only presents a *zonula adhaerens* (Fig. 8) and, adjacent to this, long septate junctions from the apical to near the basal portion, which is very similar as described for salivary glands of Diptera (Harrod and Kastritsis 1972, Jurand and Pavan 1975, Riparbelli *et al.* 1994), Trichoptera (Engster 1976), Lepidoptera (Akai 1983, Sorour *et al.*

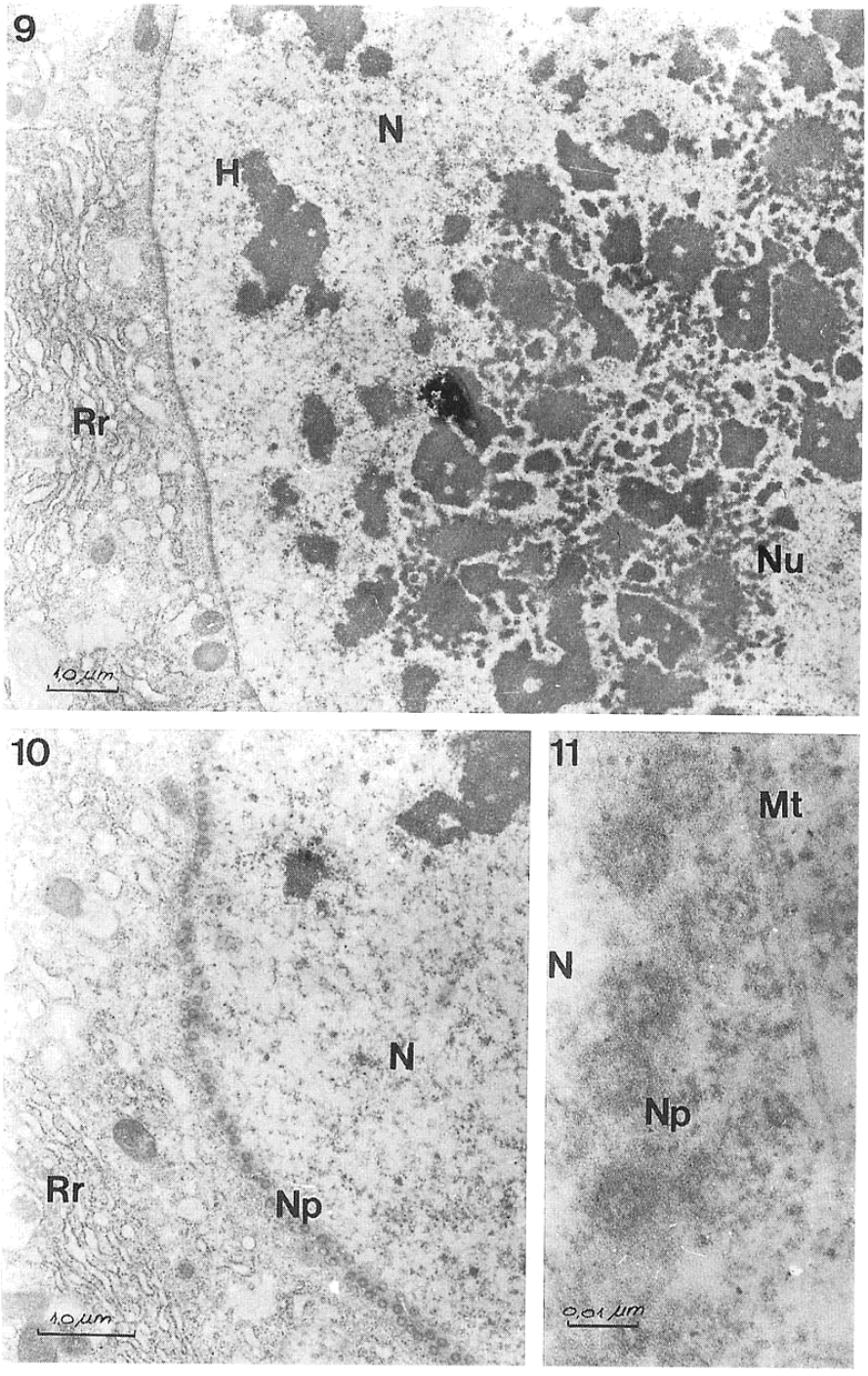


Figs. 6–8. 6) General view of enlarged intercellular spaces (*) in the median portion of cells during the phase before cocoon material production. Rr, rough endoplasmatic reticulum. 7) Detail of enlargement in the intercellular spaces (*) punctuated with desmosomes (arrows) between basal and medium portion, during the probable phase of digestive enzymes production. 8) Apical portion of the secretory cells showing *zonula adhaerens* (arrow). Intercellular spaces are very reduced (*). Mv, microvilli, L, lumen.

1990) and Hymenoptera (Silva de Moraes and Cruz-Landim 1979, Petralia *et al.* 1980, Petralia and Haut 1986).

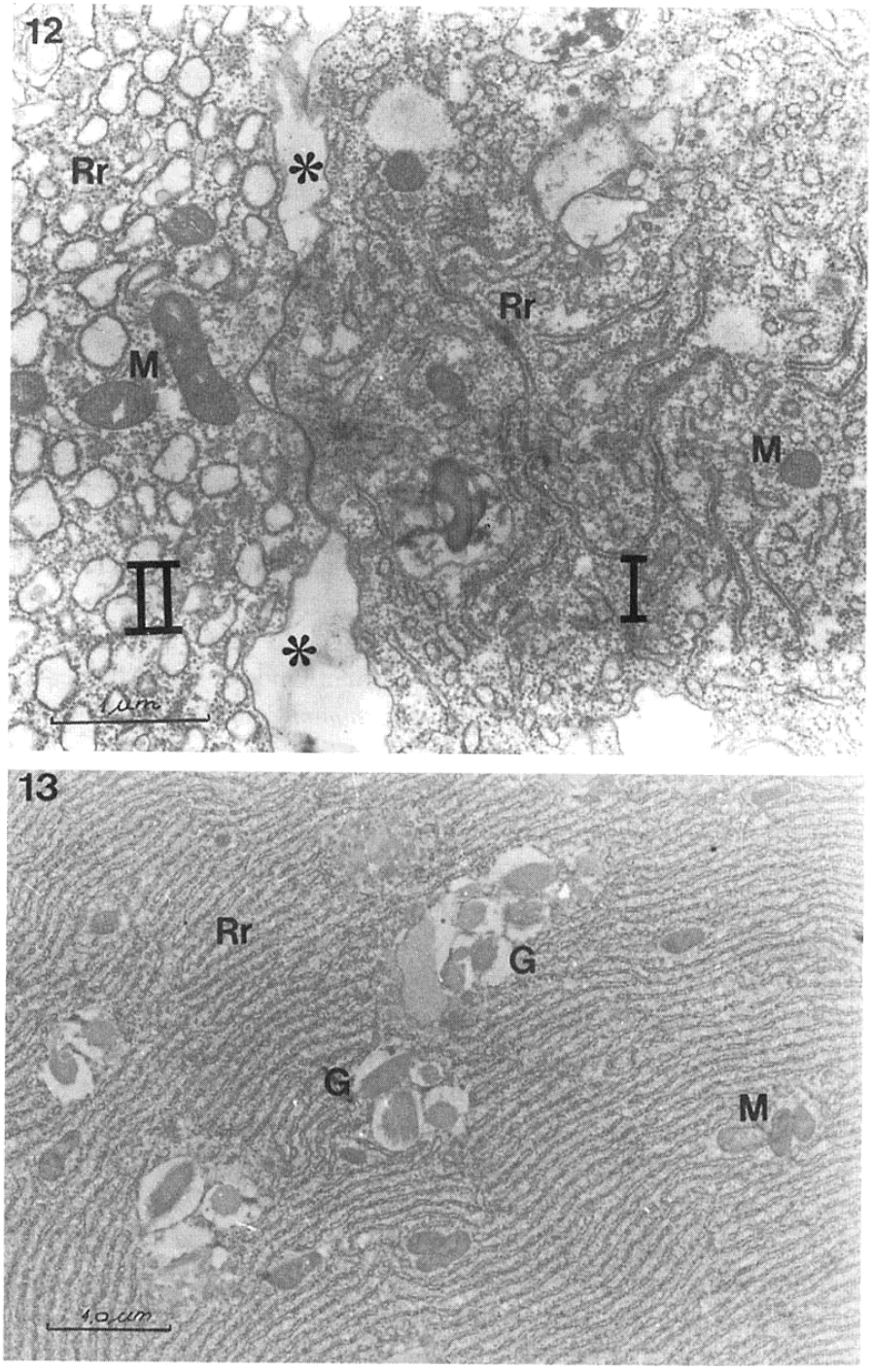
Cell nuclei

Nuclei of the salivary glands of the secretory region of *P. villosa* are large and spherical (Fig. 9). The nuclear envelope possess several pores indicating high nuclear activity during this instar (Figs. 10, 11). Salivary gland cells of *Bombyx mori* L. larvae do not undergo cellular division after



Figs. 9–11. Partial view of nucleus showing a great amount of nucleolar material (Nu), heterochromatin (H). In (10) a great amount of nuclear pores (Np) are observed, which are in detail in (11). Mt, microtubules.

the embryonic period but the nuclei undergo endomitosis during larval development producing polyploid nuclei at the end of the last instar (Ono 1951). This feature seems to be common in Lepidoptera and in other groups (Wiley and Lai-Fook 1974, Sehnal and Akai 1982, Tamura *et al.* 1982). Polyploid nuclei may occur in *P. villosa* as the large amount of heterocromatin and nucleolar material present could be indicate, and as has been suggested for salivary glands of both *N. nigriscens* (Wang and Happ 1974) and *S. invicta* (Petralia *et al.* 1980). Confirmation of polyploidy wold require cytomorphometric investigation.



Figs. 12, 13. 12) Electron micrograph of 2 cells during the phase of digestive enzymes production presenting rough endoplasmatic reticulum (Rr) in different physiological states. In I RER is composed of flat and small vesicular cisternae type , while in II totally composed by enlarged vesicular cisternae. *, intercellular spaces, M, mitochondria. 13) Aspect of RER (Rr) composed of flat cisternae during the phase of cocoon material production. G, Golgi bodies.

Rough endoplasmatic reticulum

The rough endoplasmic reticulum (RER) is well developed and changes in morphology the last instar. Before cocoon material production, the RER varies from cell to cell, with 3 different RER forms occurring in adjacent cells: the first, characterized by RER arranged in flat cisternae; an intermediate form, composed of flat and small vesicular cisternae; the last composed only of enlarged vesicular cisternae (Fig. 12). Variation in the RER morphology has also been observed in the

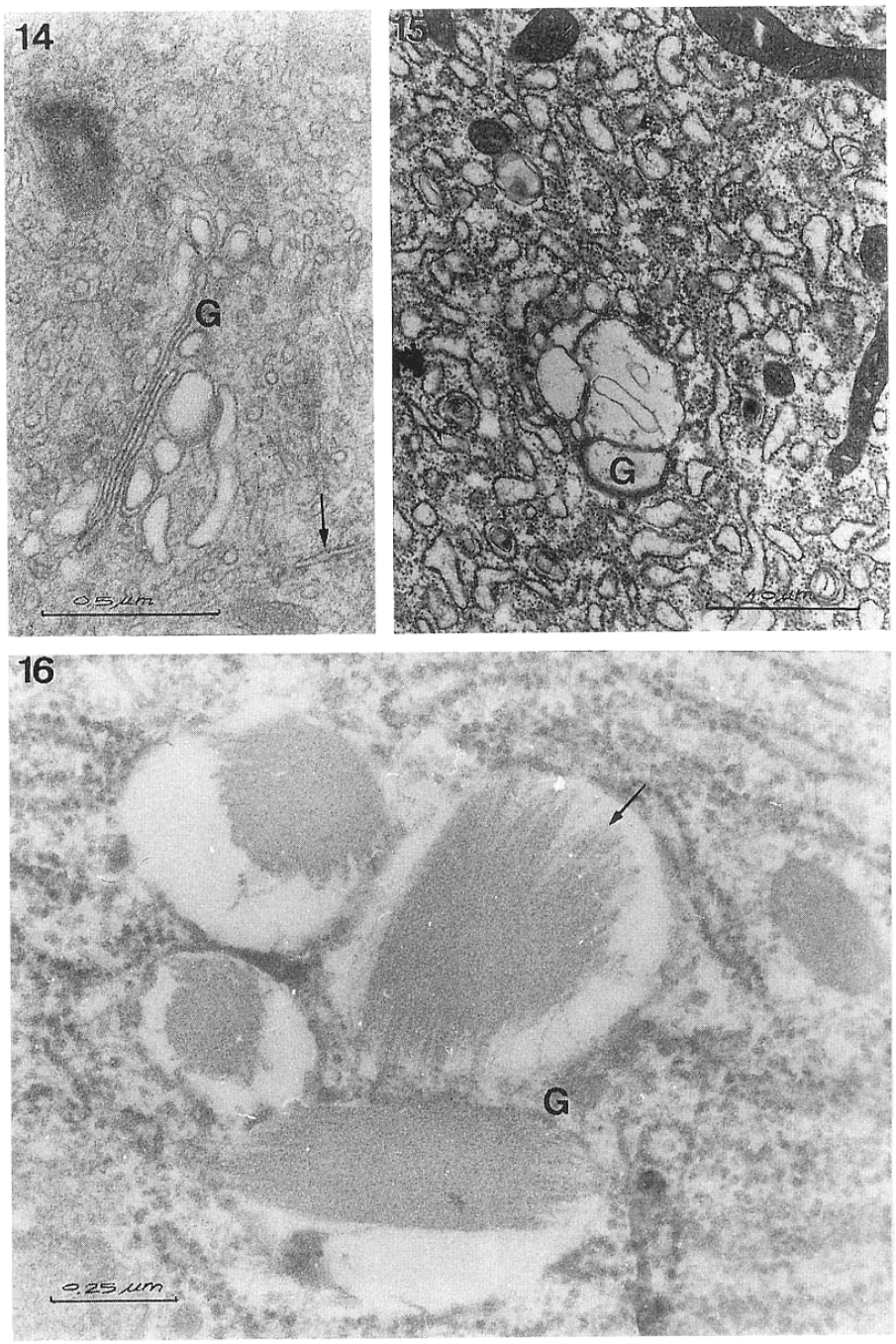
digestive enzyme producing salivary glands of *S. invicta*, however, in that case the variation always occurs with in the same cell (Petralia *et al.* 1980). Different RER morphologies in adjacent cells has been observed in some Diptera and can be indicative of different cellular physiological states as postulated by Jurand and Pavan (1975) and Simões *et al.* (1977), and this seems to be occurring in *P. villosa*. The secretory region cells of *P. villosa* in the last instar are morphologically the same and the RER is arranged in flat cisternae (Fig. 13). This arrangement is similar to that found in the silk producing glands of *B. mori* (Akai 1983, Sehnal and Akai 1990) and *Calpodes ethlius* (Stoll) (Wiley and Lai-Fook 1974). However, it seems to differ from Hymenoptera, in which the RER is arranged in both flat and vesicular cisternae in the same cell, especially in either the apical (Silva de Moraes and Cruz-Landim 1979) or basal (Petralia and Haut 1986) portion.

Golgi apparatus

The Golgi bodies are evident during the entire secretory cycle of *P. villosa*. The Golgi bodies also display morphological changes during the last instar, which occur at same time as changes in the RER. Thus, when the RER is present as flat and small vesicular cisternae (Fig. 12), the Golgi bodies are of a typical shape, composed of flattened sacs and small vesicles (Fig. 14) and they are numerically rather few. However, when RER changes to expanded vesicular cisternae (vesicular RER) the Golgi bodies also change to expanded vesicles (Fig. 15) and are more abundant and have with fibrous like material inside (Figs 13, 16). Two distinct kinds of Golgi bodies in the same salivary gland region have been observed in *Chironomus thummi* Kieffer, but in that specie they were arranged differently to *P. villosa* in that they occurred in the same cell (Kloetzel and Laufer 1969, 1970). Differences become more conspicuous when comparing *P. villosa* to *S. invicta*, in which, according to Petralia *et al.* (1980), the Golgi bodies are not evident, and probably occur only at specific times during the secretory cycle and thus secretion would be concentrated and transported *via* expanded RER cisternae. This description of the Golgi bodies is the same as that observed in several species of Lepidoptera such as *C. ethlius* (Wiley and Lai-Fook 1974), *B. mori* (Akai 1983, 1984, Sehnal and Akai 1990), *Galleria mellonella* (L.) (Sehnal and Akai 1982), *Antheraea yamamai* (Guerin-Meneville) and *A. pernyi* (Guerin-Meneville) (Tamura *et al.* 1982), *Spodoptera littoralis* Boisd. (Sorour *et al.* 1990), *Diatraea saccharalis* (Fabricius) (Victoriano 1998). According to Akai (1983, 1984) and Sehnal and Akai (1990), in *B. mori* vesicles of the complex bud from the RER, and during the development of the instar they accumulate elementary fibroine fibers inside them. After maturation they contain a high concentration of elementary fibers and the vesicles leave the Golgi bodies and become secretory vesicles named fibroin globules. Material observed inside the Golgi bodies of *P. villosa* may thus be elementary fibers which will be come part of the silk which are released from the Golgi bodies to form secretory vesicles after a period of storage. However, Silva de Moraes and Cruz-Landim (1979) argue that such structures, named by them the Golgi bodies, can be groupings of vesicles from RER that had lost their ribosomes. In *P. villosa* these structures must be named Golgi bodies since the vesicle membranes were stained by osmium tetroxide impregnation for Golgi bodies of Hayat (1972) (Zara and Caetano in prep.).

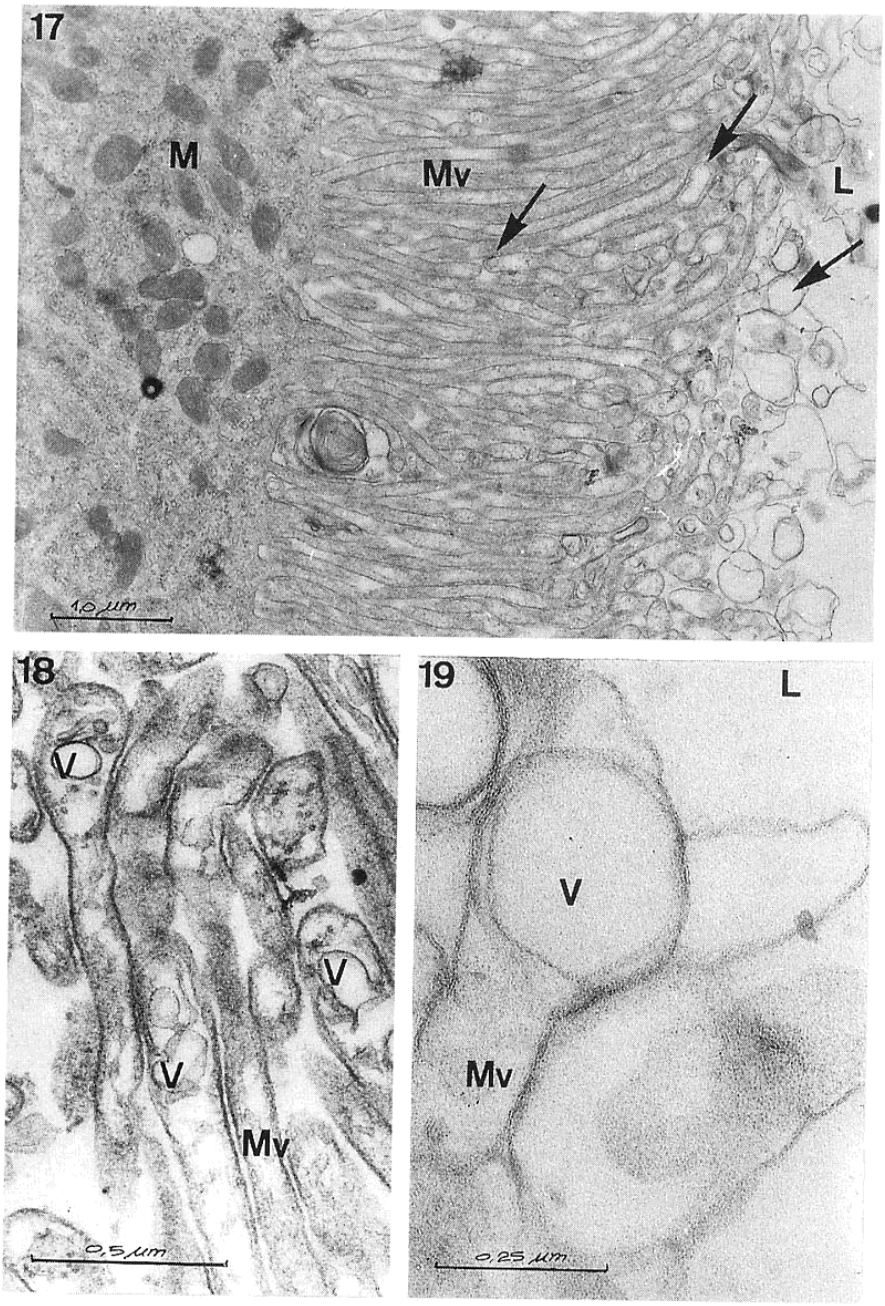
General discussion

In relation to release of secretion, great differences are found between the beginning, before production of cocoon material, and at the end of the last instar. Microvilli are large, continuous and regular (Fig. 17) during the phase before cocoon material production. Vesicles can be observed inside the microvilli, and being released on tip of microvilli by pinching-off, resulting in a single membrane vesicle into the lumen (Figs. 17–19). This mechanism is similar to that found in the salivary glands of *Drosophila lebanonensis* Casteeli (Eeken 1977) in which the secretory process starts before the production of membrane-bound secretory vesicles which are released upon fusion with the apical plasma membrane. For *P. villosa* it can be supposed that the secretions are digestive en-



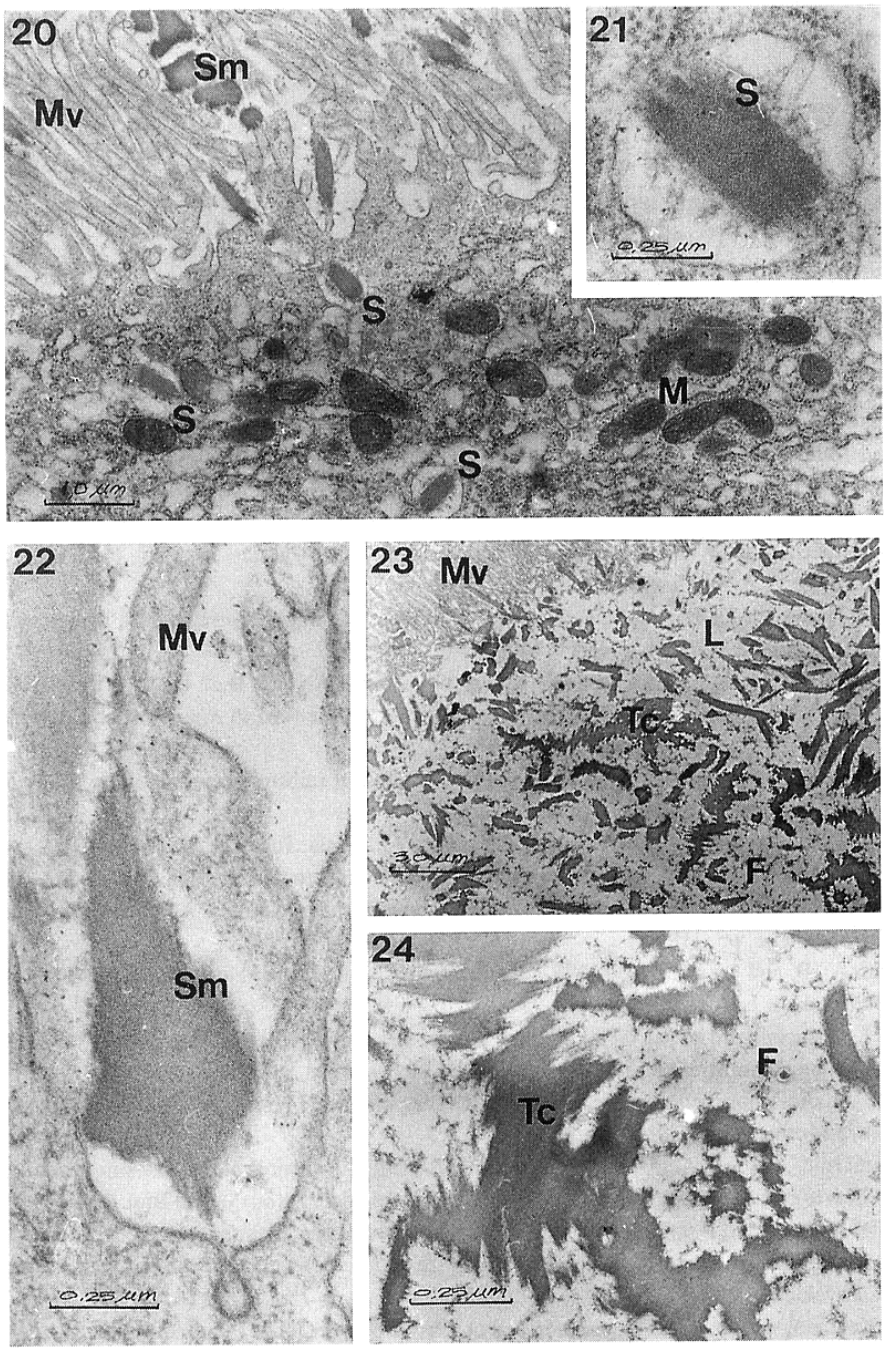
Figs. 14–16. 14, 15) Golgi bodies (G) in detail during the phase before cocoon material production. 14) This morphology is observed when RER is arranged in flat (arrow) and small vesicular cisternae. 15) Such morphology is observed when cells of the secretory region present RER composed by enlarged vesicular cisternae. 16) Aspect of Golgi bodies (G) during the phase of cocoon material production. In this phase Golgi bodies present no morphological changes, being always composed by enlarged Golgi vesicles and presenting inside fibrous material (arrow).

zymes that were still being produced at the beginning of the fourth stage. Support for such digestive enzyme secretion is given by studies of the midgut of *Spodoptera frugiperda* (Smith) (Ferreira *et al.* 1994, Jordão *et al.* 1999). Release of digestive enzymes in this organ occurs through a mechanism similar to that observed at the begining of the fourth larval instar of *P. villosa*. Trypsin is released in the midgut of *S. frugiperda* by pinching-off of the tip of the microvilli, and this type of release is called microapocrine secretion (Ferreira *et al.* 1994, Jordão *et al.* 1999). Digestive enzymes in the salivary glands have been recorded for larvae of the ant *S. invicta* (Petralia *et al.* 1980)



Figs. 17–19. 17) General view of the apical portion of the secretory cells during the probable phase of digestive enzymes production with long and continuous microvilli (Mv). Vesicles are observed inside microvilli (arrows) and in the lumen (L). A great amount of mitochondria are observed in this portion. 18) Detail of microvilli (Mv) inside which vesicles (V) of different sizes are observed. 19) Detail of the microvilli (Mv) tip showing the pinching-off to the lumen of a secretory vesicle (V).

with protease and amylase, and for *A. octospinosus* (Febvay and Kermarrec 1981) which produces sterase-lipase. That cells presenting both RER and enlarged Golgi bodies produce secretion, indicates that the secretions are assynchronically produced. The last larval instar of *P. villosa* can thus be divided in 2 phases: one producing digestive enzymes with microapocrine secretions and the other producing silk. When the secretory cells produce cocoon material, all show the same ultrastructural morphology, characterizing a synchronic cycle, and numerous vesicles containing fibrous material inside can be observed in the apical cytoplasm (Fig. 20). Those are very similar to those found in the enlarged Golgi bodies (Fig. 21) and their membranes are also stained by osmium tetroxide impregnation for Golgi bodies (Hayat 1972) indicating their Golgean origin (Zara and



Figs. 20–24. 20) Aspect of the apical portion of the gland secretory cells during the phase of cocoon material release. This micrograph shows a great amount of mitochondria (M), secretory vesicles (S) and microvilli (Mv) which are shorter than in the anterior phase and between them small masses of secretion are observed. 21) Secretory vesicle (S) in detail presenting inside fibrous materials. 22) Detail of release of a secretory vesicle to the lumen by exocitosis forming a small fibrous mass (Sm) between microvilli (Mv). 23) General view of lumen (L) where small fibrous masses are observed forming larger fibrous masses, the tactoids (Tc). Flocculent material (F) are also observed. 24) Detail of a tactoid (Tc) and flocculent material in the lumen of the salivary gland secretory region.

Caetano, in prep.). Such vesicles are very similar to those observed in Lepidoptera and Trichoptera at the time of silk production (Wiley and Lai-Fook 1974, Akai 1983, 1984, Sehnal and Akai 1990, Tamura *et al.* 1982, Sorour *et al.* 1990, Victoriano 1998, Engster 1976) and are identical to those found in another Hymenoptera *M. quadrifasciata anthidioides* (Silva de Moraes and Cruz-Landim 1979). In the lumen of the gland, small masses of fibers can be observed between the microvilli

(Figs. 20, 22, 23) and these seem to fuse to form large masses of fibers called tactoids (Flower and Kenchington 1967) (Figs. 23, 24).

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