

STUDIES ON THE POSTERIOR SILK GLAND OF THE SILKWORM *BOMBYX MORI*

VI. Distribution of Microtubules in the Posterior Silk Gland Cells

SADAO SASAKI and YUTAKA TASHIRO

From the Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570, Japan.
Dr. S. Sasaki's present address is the Department of Physiology, Kyoto University School of Medicine, Kyoto 606, Japan

ABSTRACT

There are two microtubule systems in the posterior silk gland cells. One is a radial microtubule system in which the microtubules run radially from the basal to the apical cytoplasm and in which fibroin globules (secretory granules of fibroin) and mitochondria are arranged along these microtubules, thus composing a "canal system" which is assumed to be responsible for the intracellular transport of fibroin globules. The other is a circular microtubule system in the apical cytoplasm which is composed of bundles of microtubules and microfilaments running in a circular arrangement around the glandular lumen at an interval of $\sim 4 \mu\text{m}$ at the end of the fifth instar. This system is presumably concerned with secretion and/or intraluminal transport of fibroin.

It has been suggested by a number of authors that microtubules are involved in the secretion processes of a variety of cells such as the secretion of insulin (9), thyroxine (27, 29), collagen (5, 7), hepatic very low density lipoprotein (10, 23), albumin and other serum proteins (18), salivary gland mucin (19), amylase from parotid (4), and acetylcholine-stimulated release of catecholamine from adrenal medulla (6, 17).

The posterior silk gland cells of *Bombyx mori* are differentiated cells specialized exclusively for synthesis and secretion of a single exportable protein, fibroin (24, 25). They are very large hexagonal cells, each of which extends as much as half the circumference of the gland; for the mature larvae, the cell dimensions are $\sim 1.3 \text{ mm}$ in the direction of the long axis, $\sim 1.6 \text{ mm}$ in half outer circumference of the gland and $\sim 0.17 \text{ mm}$ in depth (15).

The secretory granules in the most basal portion of the cell must travel $\sim 200 \mu\text{m}$ to be secreted at the luminal surface. It is not surprising, therefore, that the special intracellular transport system for fibroin is differentiated in the posterior silk gland cells.

We have already described the existence of two kinds of fibrous structures in the cells, one being the microtubules which appeared to be randomly oriented in the cell cytoplasm, and the other, the bundles of fine filaments found in the cytoplasmic processes on the luminal surface (12, 24). In a recent paper (20) we identified fibroin globules (secretory granules of fibroin) in the cell cytoplasm, and reported that they were sometimes found to be aligned in one direction in close association with microtubules.

The purpose of this paper is to report in greater

detail the distribution of microtubules in the posterior silk gland cells. By sectioning either perpendicularly to the long axis or longitudinally through the center of the gland, it was revealed that the microtubules in the cells are not distributed randomly but are organized into a radial microtubule system which is located between the basal and the apical regions and that fibroin globules and mitochondria are arranged along these microtubules. It was also revealed that the bundles of fine filaments on the luminal surface of the cells are composed of microtubules and microfilaments which run circularly around the glandular surface.

MATERIALS AND METHODS

Silkworm

The strain of silkworm used is a hybrid of Shunrei and Shogetsu. The larvae were cultivated as described previously (24). The same silkworms were also raised under sterile conditions on a synthetic diet generously provided by Dr. M. Kamada, K. Naito and T. Okauchi of Takeda Chemical Industries, LTD., Osaka.

Light Microscopy

The posterior silk gland was fixed with 2.5% glutaraldehyde in 0.2 M cacodylate buffer pH 7.3, containing 0.25 M sucrose, dehydrated with alcohol, and then embedded in paraffin. The specimens for light microscopy were also prepared from the epoxy resin-embedded materials for electron microscopy, and thick sections (~1 μm) were cut on a Porter-Blum MT-2 ultramicrotome and stained with methylene blue or toluidine blue.

Electron Microscopy

For visualization of fibroin globules, the specimens were fixed with 2.5% glutaraldehyde in cold 0.1 M or 0.2 M Na cacodylate buffer containing 0.25 M sucrose and postfixed with 1% OsO_4 as reported previously (20). For visualization of microtubules and microfilaments, however, the same fixative in 0.1 M Na cacodylate buffer containing no sucrose was more useful.

Polarizing Light Microscopy

The posterior silk glands previously fixed with 2.5% glutaraldehyde as described above were immersed overnight in 30% sucrose solution containing 1% gum arabic and frozen. The frozen sections were cut with a Lipshov Cryotome and observed under a Leitz polarizing microscope (Ortholux II Pol-BK) equipped with a rectified strain-free condenser (Nikon, N. A. 0.52) and rectified objective lenses (Nikon, 40 \times , N. A. 0.65 and 10 \times , N. A. 0.3). A Brace-Köhler compensator with a retardation of $\lambda/30$ was used.

RESULTS

The fibroin globules in the cells and the cytoplasmic processes on the glandular surface are definitely larger than the limits of resolution of the light microscope. Therefore, we first attempted to visualize the arrangement of these structures in the cells at the light microscope level. For this purpose, the Epon-embedded glands were either sectioned longitudinally through the center of the glandular lumen (Fig. 1) or sectioned perpendicularly to the long axis of the glands (Fig. 2, inset). These micrographs show that there is a number of parallel linear structures in the cytoplasm which run radially in the basal-apical direction. Each structure is apparently composed of fine granules linked in a necklace-like structure (Fig. 1).

Similar structures seem to exist also in the paraffin sections. As was illustrated in Fig. 5 of reference 24, the striated structures are not observed in the cytoplasm at the beginning of the fifth instar but gradually appear and become apparent at the middle of the fifth instar.

Another interesting finding is that there are zigzag structures on the luminal surface of the cells (Fig. 1, arrows). They are more clearly shown when grazing sections are cut parallel to the luminal surface of the gland as shown in Fig. 5

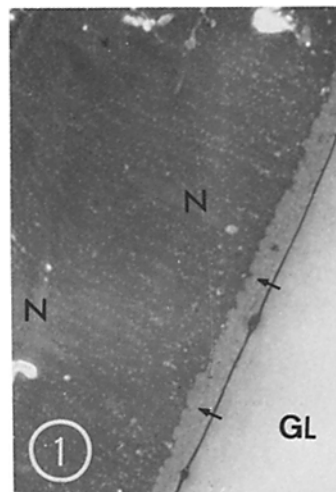
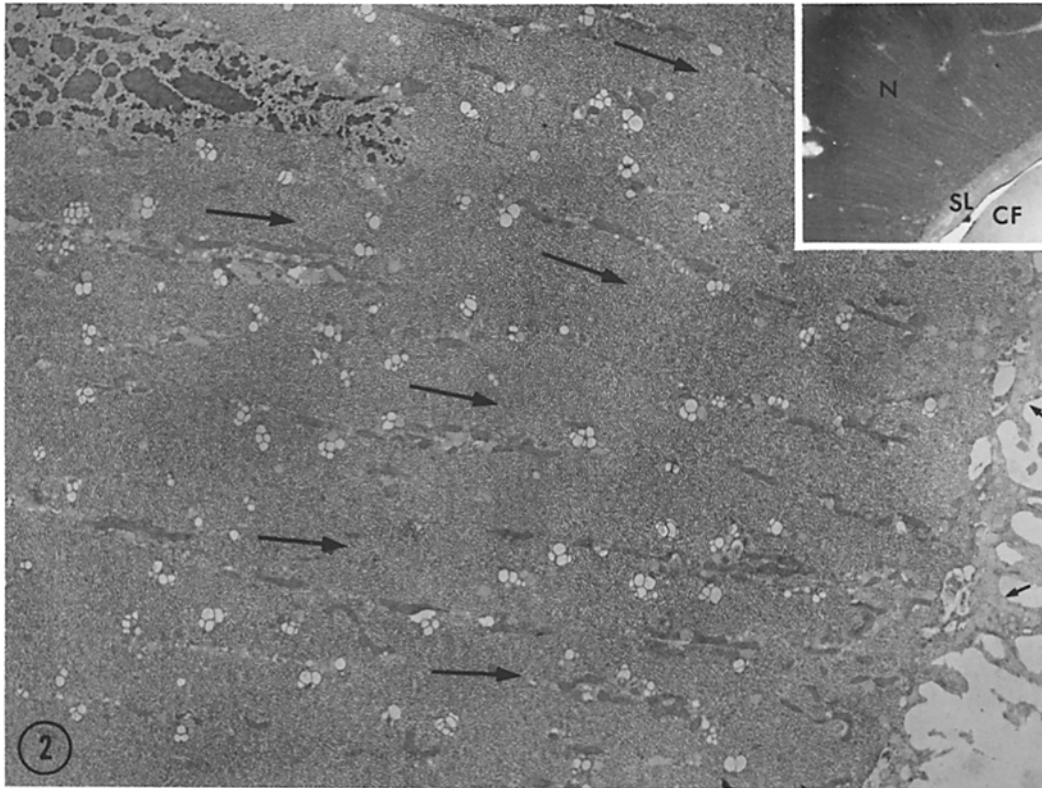


FIGURE 1 Light micrograph of the posterior silk gland cells. Longitudinal section from the epoxy resin-embedded glands. Note necklace-like structures running from basal to apical cytoplasm. Small zigzag structures on the luminal surface of the longitudinally sectioned cells are indicated by arrows. GL, glandular lumen. N, nucleus. $\times 770$.



FIGURES 2-4 Radial microtubule system in the cell. When the section is either perpendicular to the long axis of the gland (Fig. 2 and inset) or longitudinal through the center of the glandular lumen, the fibroin globules and mitochondria in the cell are found to be aligned in one direction (Fig. 2, arrows). Fig. 3 shows at higher magnification a view of such a region where a number of microtubules can be seen running alongside them. Note the close contact between a microtubule and a vacuole (arrow), which changes the direction of the microtubule slightly. Fig. 4 is a gallery of transverse views of the radial microtubule system which suggest close contact between microtubules and fibroin globules or mitochondria. The specimens for Fig. 2 were fixed with 2.5% glutaraldehyde solution in 0.1 M Na cacodylate buffer, pH 7.3, containing 0.25 M sucrose, while the specimens for Figs. 3 and 4 were fixed with the same fixatives without 0.25 M sucrose. The fibroin globules in Figs. 3 and 4 appear less electron transparent than those in Fig. 1 of the previous paper (20) which is a high magnification photograph of Fig. 2. (Fig. 2), $\times 3,500$. (Fig. 3), $\times 30,000$. (Fig. 4), $\times 75,000$. Fig. 2 inset: light micrograph. Cross section from the epoxy resin-embedded gland. Note also radial striae. *N*, nucleus. *SL*, silk layer. *CF*, columnar fibroin. $\times 480$.

(inset). At the upper corner of the glandular lumen, parallel dense lines run across the lumen. This finding suggests that the luminal surface of the cells is covered by the parallel cytoplasmic processes which run circumferentially around the glandular lumen.

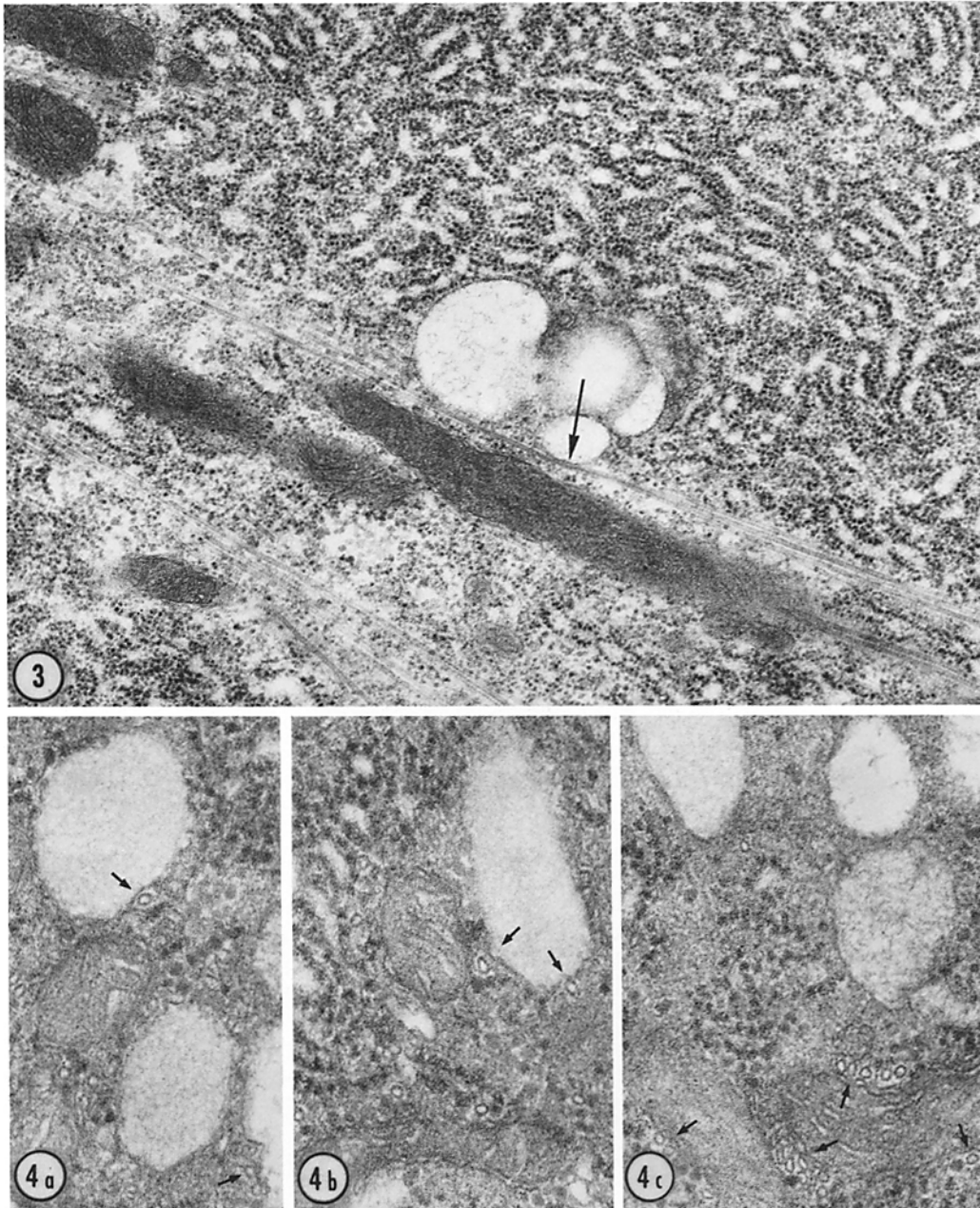
Electron Microscope Observation of Radial Microtubule System

In order to observe the ultrastructure of the necklace-like structure, thin sections were cut

from the same specimens and observed by electron microscopy. Fig. 2 is a low magnification electron micrograph of the cell. It is evident that the necklace-like structure is composed of fibroin globules and elongated mitochondria aligned in the basal-apical direction, the long axis of the mitochondria always remaining parallel to this direction (arrows in Fig. 2). Fig. 1 of previous paper (20) is a high magnification view of the same picture which shows that microtubules always exist in close association with these fibroin globules and mitochondria.

When the cells were fixed in the absence of sucrose, the microtubules were observed more clearly, as shown in Fig. 3. In Fig. 3, several microtubules run parallel to each other, and one microtubule is seen to be in close contact with a fibroin globule at a point where the microtubule

changes its direction slightly (arrow). The topographic relationship between fibroin globules, mitochondria, and microtubules becomes more apparent when the sections are cut perpendicular to these linear structures as shown in Fig. 4. The microtubules, with an average diameter of ~ 24



FIGURES 3-4 See legend under Fig. 2.

nm, are in close contact with the fibroin globules as well as with mitochondria (arrows in Fig. 4). Usually even in the case of the closest contact, the globules and mitochondria are separated from the tubule surface by a space of 80–120 Å as reported by Smith et al. (22).

As the microtubules are always in a radial disposition between the basal and the apical cytoplasm, this microtubule system is termed the “radial microtubule system.” It is interesting to point out here that mitochondria and fibroin globules in the cells are almost exclusively localized in this system, except for the immature or recently matured globules in the Golgi bodies and those mature fibroin globules which accumulate in the apical cytoplasm for secretion (small arrows in Fig. 2).

Electron Microscopy of Circular Microtubule System

A general view of the bundles of fine filaments in the cytoplasmic processes on the luminal surface of the posterior silk gland cells has been included in our previous reports (12, 24) and in Figs. 2 and 3 of a recent paper (20).

Figs. 5 and 6 show high magnification views. The sections for Fig. 5 were cut longitudinally through the center of the glandular lumen so that a cross-sectional view of the cytoplasmic processes is demonstrated. It is apparent that the bundles are composed of microtubules and microfilaments with an average diameter of 24 nm and 7 nm, respectively. In the bundles, these fibers show no regular crystalline arrangement, but appear to be roughly grouped into microtubule-rich and microfilament-rich regions. Some microtubules have central electron-opaque dots (arrows) as described by Tilney and Porter (26).

The section in Fig. 6 was cut perpendicularly to the long axis of the gland so that a longitudinal view of the processes could be visualized. It is again evident that the bundles are composed of microtubules and microfilaments running parallel to each other, although the latter filaments are curled here and there (arrows).

As the microtubules in the bundles run circularly around the glandular lumen, this system was termed the “circular microtubule system” or more precisely the “circular microtubule-microfilament system.” This circular microtubule system exists not only at the fifth and fourth instar (12) but also at the beginning of the first instar (not shown), although the distance between the bundles in the

early instar is much smaller than that at the later stage of the fifth instar ($\sim 4 \mu\text{m}$).

It is also to be noted here that the bundles in the molting stage are buried within the apical cytoplasm as shown in Fig. 7. The distance between the bundles at the fourth molting stage was $2.8 \mu\text{m}$, average. The inset of Fig. 7 shows that the bundles are composed of microtubules and microfilaments such as are seen at the feeding stage.

The glandular surface of the cells is relatively smooth at the fourth molting stage or at the beginning of the fifth instar (Fig. 7). As the larvae grow in the fifth instar, the glandular surface becomes complicated by the formation of the cytoplasmic processes which are arranged regularly and encircle the glandular lumen. The circular microtubule system is evident in these processes.

Polarizing Light Microscopy

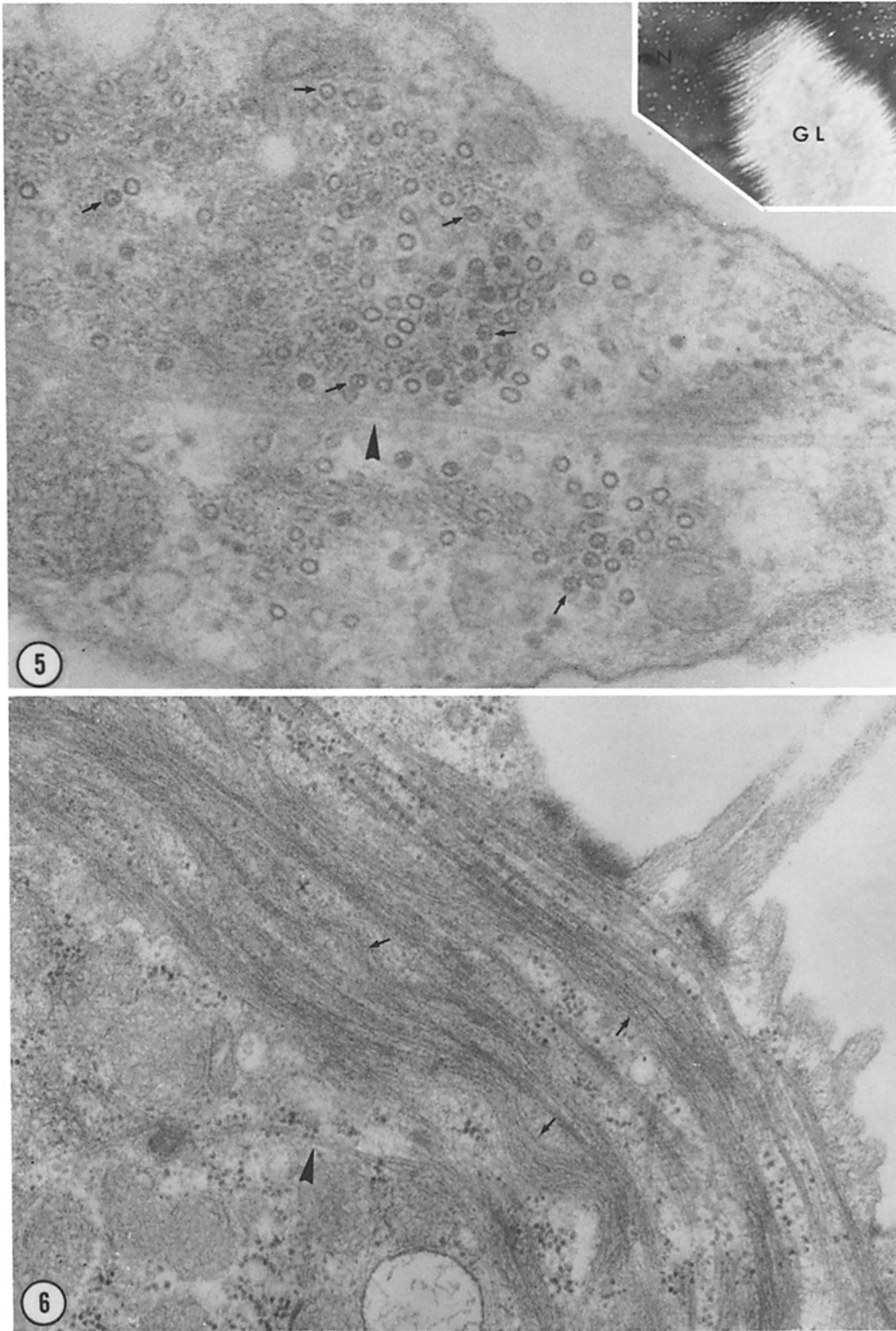
Fig. 8 is a cross-sectional view of the gland in polarized light. In the cytoplasm there is a number of fine positively birefringent lines which run radially from the basal to the apical cytoplasm (arrows). The average distance between the lines is $\sim 4 \mu\text{m}$. Comparing Fig. 8 with Figs. 1–3 of the present paper and Fig. 1 of the previous paper (20), it is certain that the birefringent lines correspond to the radial microtubule system.

In the apical cytoplasm there are two positively birefringent zones which run circumferentially around the glandular lumen. The outer zone shown by arrowheads is dark in Fig. 8a and bright in Fig. 8b, indicating that the birefringent fibers in this zone run perpendicular to the radial microtubule system. This fact and the comparison of Fig. 8 with Fig. 1, 5, and 6 of the present paper and Fig. 3 of the previous paper (20) indicate that the birefringence in this zone is most probably due to the circular microtubule system.

The inner birefringent zone in the apical cytoplasm (stars in Fig. 8) is bright in Fig. 8a and dark in Fig. 8b and is broader than the outer zone. It is in this region that the radial microtubule system converges and fibroin globules accumulate before secretion (Fig. 2). The birefringence here could be explained by such structural features in this region. In the glandular lumen, the central columnar fibroin and the silk layer are also birefringent as shown in Fig. 8.

DISCUSSION

Our light and electron microscope observations have revealed two microtubule systems in the pos-



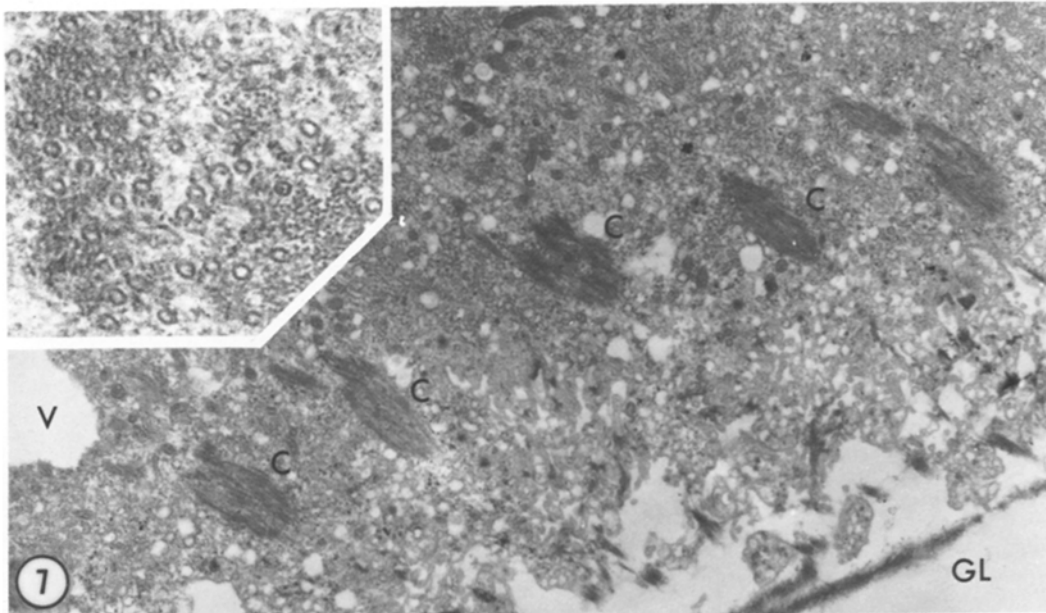


FIGURE 7 Circular microtubule system (C) in the cells at the molting stage of the fourth instar (96 h). This system is found buried in the apical cytoplasm. GL, glandular lumen. V, vacuole. $\times 10,000$. Inset: a cross-sectional view of the circular microtubule system at a higher magnification. $\times 72,000$.

terior silk gland cells; a radial and a circular microtubule system.

In the former system, microtubules run in a radial direction between the basal and the apical cytoplasm, and fibroin globules and mitochondria are aligned along these microtubules. This system penetrates deeply into the cytoplasm, dividing it into two regions; one rich in microtubules, mitochondria and fibroin globules, and the other rich in rough endoplasmic reticulum (ER) (Fig. 2). The Golgi bodies in the rough ER region appear to be in close association with the radial microtubule system, thus linking the two regions.

It has been suggested (24) that fibroin accumulated within the intracisternal space of the ER is transported to the Golgi vacuoles via Golgi vesicles or transitional elements, as in the case of the

pancreatic exocrine cells (8). In the previous paper (20), we remarked that some of the vacuoles in Golgi bodies appear to be functioning as condensing vacuoles. The topographic relationships of the rough ER, Golgi bodies, and the radial microtubule system strongly suggest that these structures compose a very efficient and elaborate system for intracellular transport of fibroin. After condensation in the Golgi bodies, the mature Golgi vacuoles or fibroin globules seem to slide laterally, enter the radial microtubule system, and are transported rapidly to the apical region; thus, the radial microtubule system is functioning as a "canal system" for the intracellular transport of fibroin globules. Such a hypothesis is supported by the marked accumulation of fibroin globules in the Golgi region after treatment with colchicine and

FIGURES 5 and 6 Transverse (Fig. 5) and longitudinal view (Fig. 6) of the circular microtubule system, respectively. This system appears to be composed of microtubules and microfilaments. Some microtubules have central electron-opaque dots (Fig. 5, arrows). The bundles of microfilaments are curved in some places (Fig. 6, arrows). Occasionally, microtubules which probably belong to the radial microtubule system penetrate deeply into the circular microtubule system (arrowheads), suggesting the connection of these two systems in the apical cytoplasm. (Fig. 5), $\times 85,000$. (Fig. 6), $\times 44,000$. Fig. 5 inset: light micrograph. Grazing section (parallel to the luminal surface of the cells) from the paraffin-embedded gland. Note zigzag structures on the luminal surface of the cells. GL, glandular lumen, N, nucleus. $\times 740$.

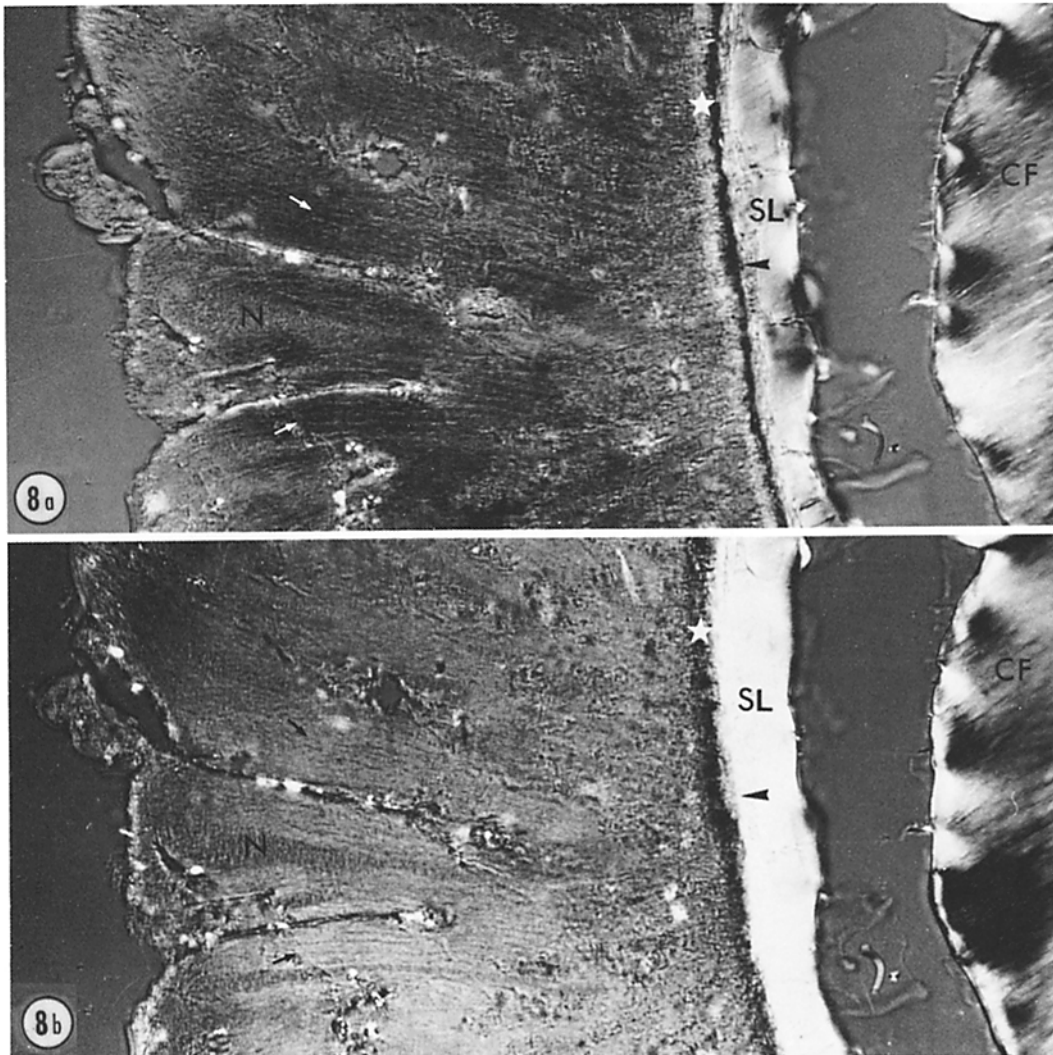


FIGURE 8 Polarizing microscope view in a frozen section of the posterior silk gland cells, cut almost perpendicular to the long axis of the gland. Fig. 8*b* shows same field as Fig. 8*a*, reverse compensation. The fine positively birefringent lines running radially from basal to apical cytoplasm in the cells (arrows) represent the radial microtubule system. Two positively birefringent zones run circumferentially around the glandular lumen. The outer zone (arrowheads) is dark in Fig. 8*a* and bright in Fig. 8*b*. This zone represents the circular microtubule system. The inner birefringent zone (stars) is bright in Fig. 8*a* and dark in Fig. 8*b*. It is broader than the outer zone and is probably the region where the radial microtubule system converges and the fibroin globules accumulate before secretion. *CF*, columnar fibroin. *N*, nucleus. *SL*, silk layer. $\times 800$.

vinblastin by which the microtubular paracrystal reported by Bensch and Malawista (3) was also observed in the cell (Sasaki, S., Y. Kuriyama and Y. Tashiro, manuscript in preparation) and is consistent with the findings of Redman et al. that colchicine causes accumulation of secretory protein in Golgi-derived secretory vesicles (18).

There is electron microscope evidence of the association of microtubules with membrane-bounded vesicles in a variety of cells, such as synaptic axoplasm (22), melanophores (13), and *Paramecium caudatum* (1). The present studies provide additional evidence for the close apposition of microtubules with the secretory granules.

Whether the microtubules in the radial microtubule system are working simply as a supporting framework for the maintenance of a "canal system" or are contributing more directly to the transport of the fibroin globules as suggested by Schmitt (21) remains to be clarified.

Another system is the circular microtubule system which is composed of microtubules and microfilaments and exists in the very apical portion of the cells, that is, in the cytoplasmic processes at the later stage of fifth instar, thus enclosing the luminal space circumferentially.

The function of the "circular microtubule system" is not clear. One possibility is that this system simply maintains the structure of the glandular lumen. Another possibility is that it plays some role in the secretion of fibroin at the luminal surface and/or in the intraluminal transport of fibroin. The circular microtubule system contains microfilaments, which may be contractile, and the posterior silk gland cells are connected by septate desmosomes (11). It is possible that the luminal fibroin moves from the posterior to the anterior portion of the posterior silk gland by peristaltic contraction of the circular microtubule system.

The role of microfilaments in secretion has been frequently assessed, e.g. in the endocrine pancreas (9, 16), the thyroid (14, 28), the parotid (4), and the exocrine pancreas (2). It was suggested that they may play a role in the last step in the sequence of the cellular events leading to the exocytosis of secretion granules (16). As the circular microtubule system is composed of microtubules and microfilaments, organized to a higher degree than in the other systems such as exocrine pancreatic cells (2), the posterior silk gland cells may afford an appropriate system for detailed studies on role of microfilaments in protein secretion.

The interrelationship of the two microtubule systems is now under investigation. The distance between the microtubule bundles in the radial microtubule system is similar to that in the circular microtubule system: $\sim 4 \mu\text{m}$ in average at the end of the fifth instar. Moreover, we have occasionally observed that the microtubules of the two systems are intermingled in the apical cytoplasm (Figs. 5 and 6). It is suggested that the two systems are connected in this portion of the cytoplasm.

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