II. The structure and ultrastructure of the silk gland

by H. Akai*

The Sericultural Experiment Station, Yatabe, Ibaraki 305 (Japan)

1. Histology of silk gland

The silk gland of the larva of *Bombyx mori* is a typical exocrine gland secreting large amounts of silk proteins. The gland consists of three divisions (fig. 1). The posterior division secretes fibroin which is the main silk component of a simple sequence of amino acids. The gelatinous silk components, 3 types of sericin which coat the fibroin, are secreted by different regions of the middle division. The anterior division is a mere duct lined with a thick cuticular intima, and does not contribute to the secretion of silk materials. Tracheal branches are distributed along the posterior and middle divisions.

The silk gland is basically a tube made up of huge polyploid cells, each with an extremely ramified nucleus containing numerous nucleoli. Nuclear ramification develops gradually as the larvae grow and reach conspicous size in the 4th and 5th instars (fig. 2). Ramification considerably enlarges the nuclear surface and apparently facilitates the transfer of materials related to the silk synthesis between the nucleus and cytoplasm. The latter shows a basophilic striped structure with a faintly stained layer of fibroin[1].

The 3 types of sericin secreted from different parts of the middle gland division[2,3] are called the inner, middle, and outer layer sericin. Shibukawa[3] reported that the inner layer sericin shows positive reactions for arginine, lipids and polysaccharides. The middle layer sericin also reacts strongly in the histochemical Millon test for xanthoproteins. However, a weak response in histochemical protein tests is obtained with the outer layer sericin which seems to contain predominantly lipids and polysaccharides. The 3 types of sericin can also be distinguished by X-ray analysis[4].

According to the secretory products and other characteristics the middle silk gland division is further divided into a posterior part (piece), distal and proximal portions of a medial part, and an anterior part.

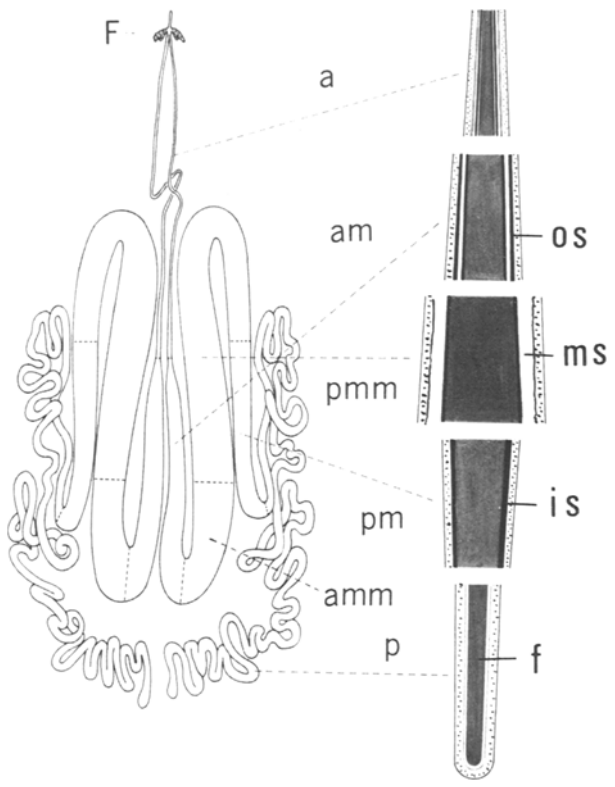

Figure 1. Division and internal structure of *Bombyx* silk gland divided by their secretory materials. a, anterior division (no secretion); am, anterior piece of middle division (secrete outer layer sericin (os)); amm, anterior part of middle piece of middle division (secrete outer (os) and middle layer sericin (ms)); F, Filippi's gland; p, posterior division (secrete fibroin (f)); pm, posterior piece of middle division (secrete inner layer sericin (is)); pmm, posterior part of middle piece of middle division (secrete middle layer sericin (ms)).

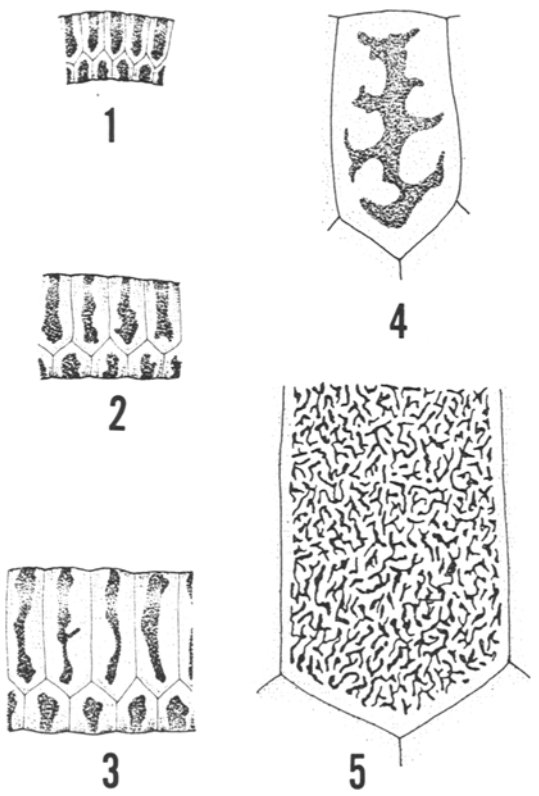

Figure 2. Schematic diagram of nuclear ramification in the gland cells of posterior division. 1, 1st instar; 2, 2nd instar; 3, 3rd instar; 4, 4th instar; 5, 5th instar.

The inner layer sericin is secreted exclusively from the posterior part, middle layer sericin from the medial part (including both the proximal and distal portions), and outer layer sericin from both from proximal portion of the medial part and from the anterior part. The gland cells never divide after the period of late embryonic development, although they increase in weight more than 50,000 times during the larval life. This growth without division leads to enormous cell size: for example, a cell in the posterior division measures in the last larval instar $1400 \times 1500 \times 150$ μm^5.

2. Ultrastructure of silk gland

a) Posterior division

Numerous nucleoli are detected in the ramified nucleus. In early stages of the 5th instar, each nucleolus shows a massive figure of a high electron density fibrous material with many RNP granules on its surface. During the 3rd and 4th days of the instar the nucleoli rapidly develop and produce numerous nucleolonemata[1,6]. Micro-autoradiography has revealed that the most active stage of RNA synthesis coincides with the period when the nucleolonemata appear (fig. 3). Injection of larvae with actinomycin D causes disappearance of the RNP granules[7].

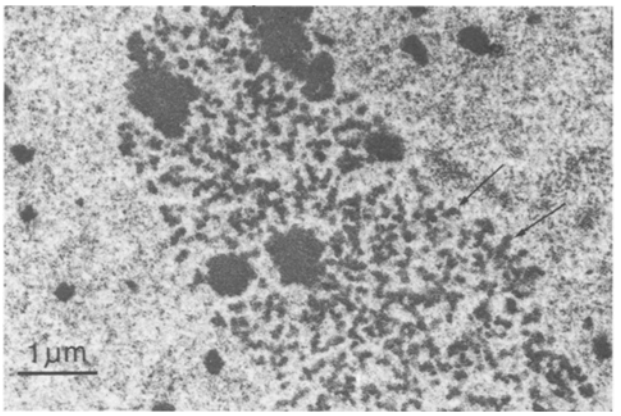

Figure 3. Electron micrograph of developing nucleolus in the posterior division at the 4th day of the 5th instar. Numerous nucleolonemata (arrows) appear during the most active RNA synthetic stage of the 5th instar. × 10,600.

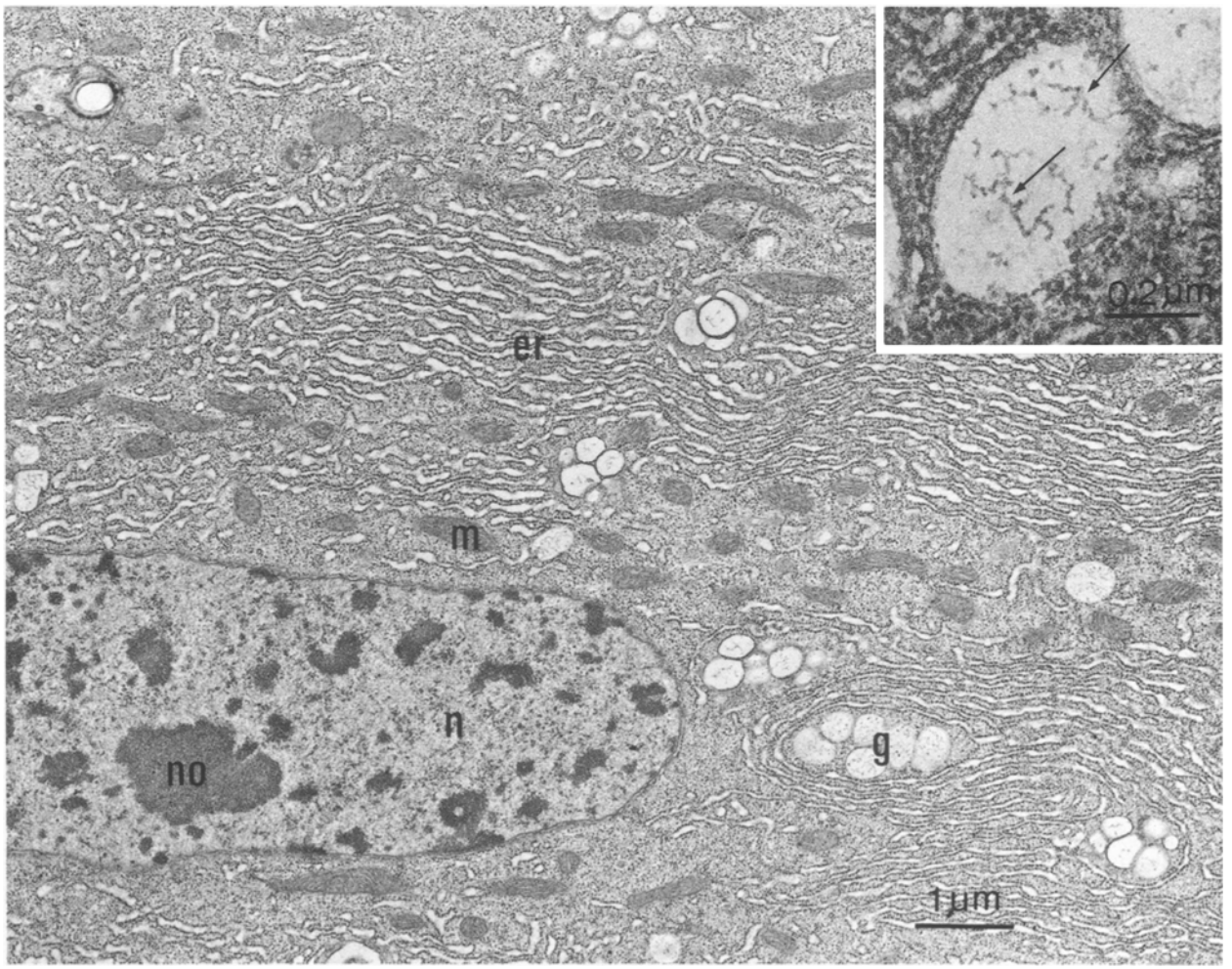

Figure 4. Part of a gland cell of the posterior division showing the developed intracellular organelles. er, Rough endoplasmic reticulum; g, Golgi complex; m, mitochondria; n, part of ramified nucleus; no, nucleolus. - Inset: Enlarged Golgi vacuoles containing the elementary fibroin fibers. Arrows indicate the elementary fibroin fibers.

As shown by both quantitative micro-autoradiography and the electron microscopic autoradiography, tritiated uridine is incorporated into the nucleus within 5–10 min after injection[8], with a maximal grain concentration occurring in 30–60 min. Most grains are located on the nucleolus but some are scattered on diffuse chromatin fibers[1]. Recently, fibroin messenger RNA was isolated from the posterior division of the *Bombyx* silk gland[9,10].

DNA synthesis continues at a considerable level throughout the 4th instar and at a very high level in the early 5th instar but then it decreases gradually until on the 6th day it becomes undetectable. Electron microscopic autoradiography reveals the location of grains on dispersed chromatin but not on the condensed one[8]. As mentioned previously, in larvae the silk gland cells grow by endomitosis without cell divisions. Gage[12] determined that polyploidization of the gland cells involves all genes which replicate to a level at least 4×10^5 times higher than in the haploid sperm nucleus. This is achieved by 17 or more rounds of complete genom replications which occur during larval development. Suzuki and Giza[13] also established that the posterior gland nuclei attain about 4×10^5 ploidy in the late 5th instar.

Nuclear membrane of the gland cells is composed of

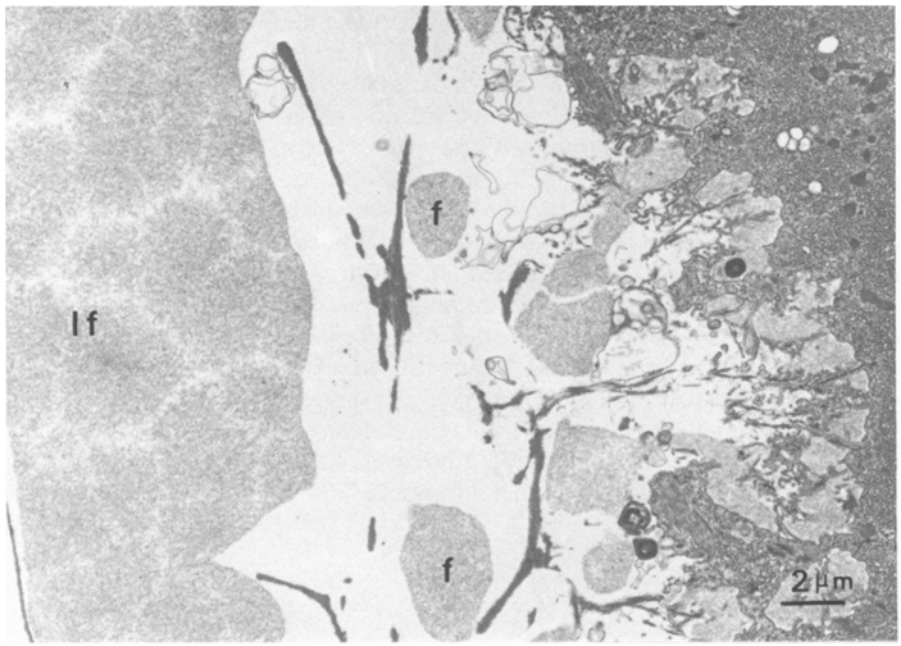

Figure 5. Electron micrograph of silk layer adhering to apical surface of the posterior division. Several spherical masses of fibroin fiber (f) are seen in the silk layer. A large liquid fibroin (lf) accumulated with the spherical masses of fibroin fibers is also seen in the silk layer. × 4080.

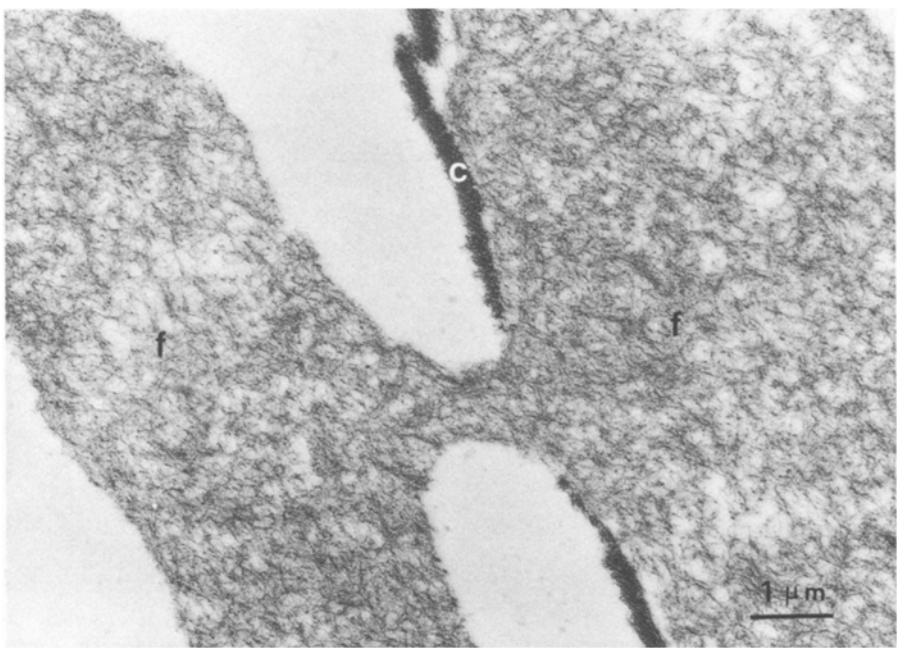

Figure 6. Electron micrograph of the spherical masses of fibroin fiber (f) passing through a cuticular membrane (c). × 11,300.

2 layers. During the active period of RNA synthesis in the 5th instar, nuclear material is seen close to this envelope, indicating transfer of substances from nucleus into the cytoplasm[6]. Early cytologists believed that the entire nucleolus is transferred into the cytoplasm in the silk gland cells of Lepidoptera[14-16]. Extrusion of some nuclear material into the cytoplasm, associated with a partial disappearance of the nuclear membrane, has also been detected by means of the electron microscope[17], but the functional significance of the phenomenon is unclear.

Several intracellular organelles, such as rough-surfaced endoplasmic reticulum (rough ER), free ribosomes, Golgi complexes, fibroin globules, mitochondria, microvilli, bandles of microfilaments and microtubules are distributed in the cytoplasm of cells in the posterior gland division[6,8,18-21]. Changes in the cell ultrastructure were studied in the course of the 4th and 5th instars (fig. 4) and it was demonstrated that rough ER is the site of fibroin synthesis. In an early period of the 5th instar only a small number of tubular rough ER is scattered in the cytoplasm which contains a large amount of widely distributed free ribosomes. The rough ER develops rapidly during the first half of the 5th instar and in spinning larvae it occupies the entire cytoplasm while the free ribosomes disappear[6,8]. After spinning the rough ER regresses rapidly both in size and the density of ribosomes[6]. Lamellated rough ER showing a decline of functional activity is frequently observed during larval molts as well as during larval-pupal metamorphosis[19,21,22]. Similar morphological changes are also detected when the glands are cultured in vitro[23].

Golgi complexes consisting of several vacuoles and vesicles devoid of the typical Golgi membranes are found scattered in the areas containing the rough ER. The vesicles seem to be derived by budding from the rough ER[6]. At the beginning of a larval instar, no material is visible inside the Golgi vacuoles but coiled fibers can be detected there in increasing quantities as the instar progresses. Elementary fibroin fibers are clearly discernible at higher magnifications[24]. They form helicoidal bandles about 130 Å thick (fig. 4). Elementary fibroin fibers are also seen in fibroin globules near the cell membrane, in the primary silk layer between the cell membrane and the extracellular bordering membrane, and in the columnar fibroin in the lumen of silk gland[1,25]. McKnight et al.[26], who examined silk gland cells with methods for genome preparation, detected polysomes with attached fibrils of a distinctly banded appearance, each of a maximum length of 0.1 µm. These fibrils seem to represent nascent silk fibroin.

Mature Golgi vacuoles containing a high concentration of the elementary fibroin fibers leave the Golgi complex as fibroin globules and move to the apical cytoplasmic reagion where they are discharged as spherical masses into the silk layer (fig. 5). The latter is separated from the gland lumen by a discontinuous bordering membrane[1]. The spherical fibroin masses pass through spaces in this membrane and accumulate as columnar fibroin in the gland lumen (figs 5

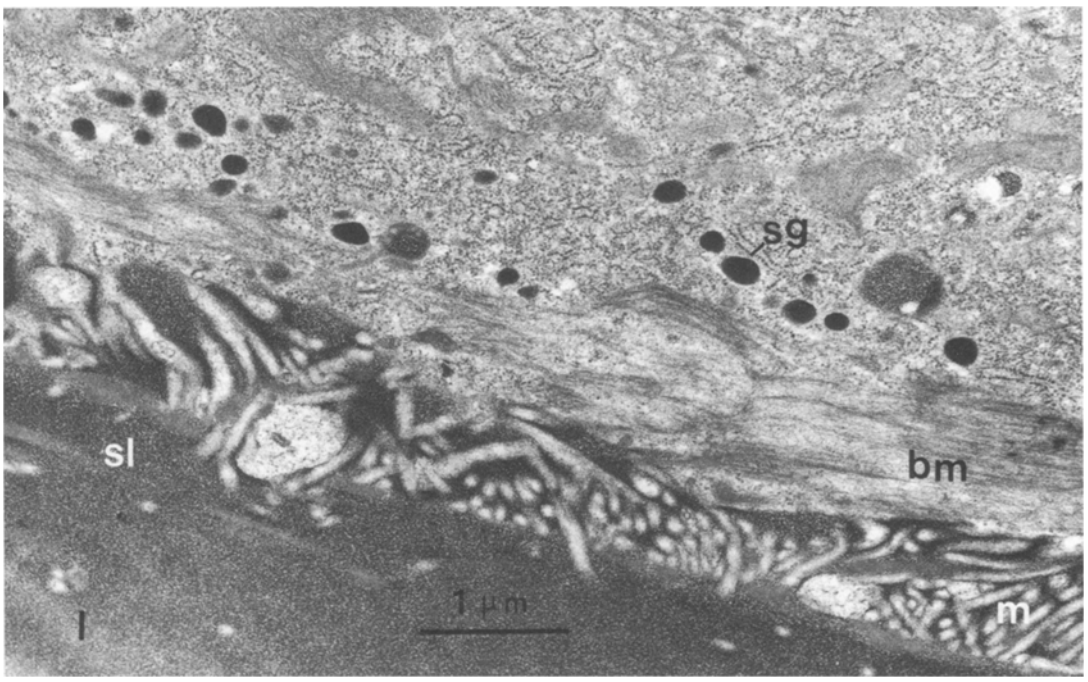

Figure 7. Apical region of a gland cell of middle division secreting the middle layer sericin into the lumen through the silk layer. bm, Bundle of microfilaments; l, lumen filled with sericin; m, microvilli; sg, sericin globules; sl, silk layer. × 20,000.

and 6)[25]. Scanning and transmission electron microscopic studies indicate that bordering membranes are composed of fine fibrous material. It has been proposed that they represent degenerated cuticular intimas which are produced by the gland cells at each larval molt (fig. 9)[27].

Bundles of microfilaments are located in the apical region of the gland cells. Their regular arrangement along the cell membrane indicates that they may have a function as a mechanical cell skeleton[1]. A large number of microtubules, which are distributed throughout the cytoplasm, apparently plays a role in the intracellular transport of fibroin globules[28] but also serves to strengthen the cell mechanically. Discharge of fibroin globules is probably an energy-dependent process because reaction products of an ATPase test were found concentrated on the microvilli of the apical cell surface[29].

Tritiated glycine can be detected both by the light and electron microscopic autoradiography in the rough ER within 10 min after its injection. Within 15 min the silver grains appear in Golgi complexes and within 45–60 min in the fibroin globules accumulated in the apical cytoplasm. Finally the grains move to the spherical fibroin masses in the silk layer and eventually to the columnar fibroin in the gland lumen (fig. 10)[1].

Numerous fine tracheal branches are distributed in the posterior as well as in the middle cell divisions. Tracheal end cells can be detected in the basal region of the gland cells during larval molts.

b) Middle division

Cells of the posterior part of the middle gland division are similar to those of the posterior division: in the last larval instar they possess richly ramified nucleus with numerous nucleoli and highly evolved rough ER, Golgi complexes, and sericin globules distributed throughout the cytoplasm[1]. Golgi complexes display increased functional activity during the spinning period. Sericin globules usually appear as dense spherules, 200–300 nm in diameter, and enclose strands (110–130 Å thick) of material derived from Golgi vesicles. Globules move to the apical cell surface and release their contents into the silk layer. Discontinuous membranes separate silk layer from the lumen of the gland. Sericin is pushed through the holes in the membranes and accumulates as the inner sericin layer on the columnar fibroin which moves down from the posterior gland division.

Middle layer sericin is secreted by the cells in the posterior section of the middle part of the middle gland division. In their ultrastructure these cells closely resemble those of the posterior part but differ by the appearance of their secretion (figs 7 and 8). The sericin globules show a granular texture and are less dense than those seen in the cells of the posterior part of the middle gland division. Globules are formed by condensation of synthetic products within the Golgi membranes. Sericin fibers floating in the globules are released into the silk layer and later pass through the bordering discontinuous membranes onto the surface of the previously deposited inner layer sericin.

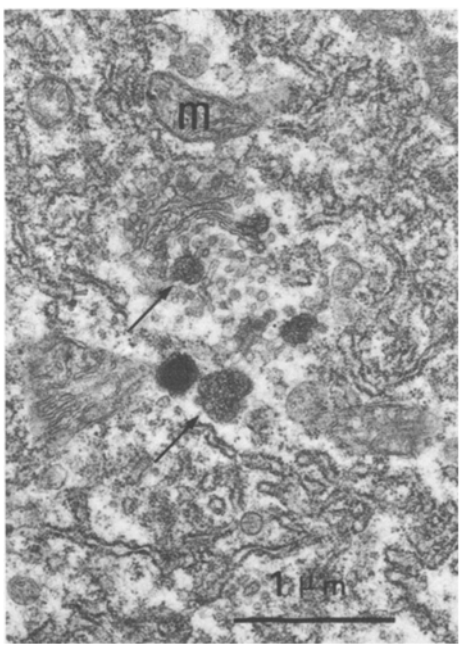

Fig. 8

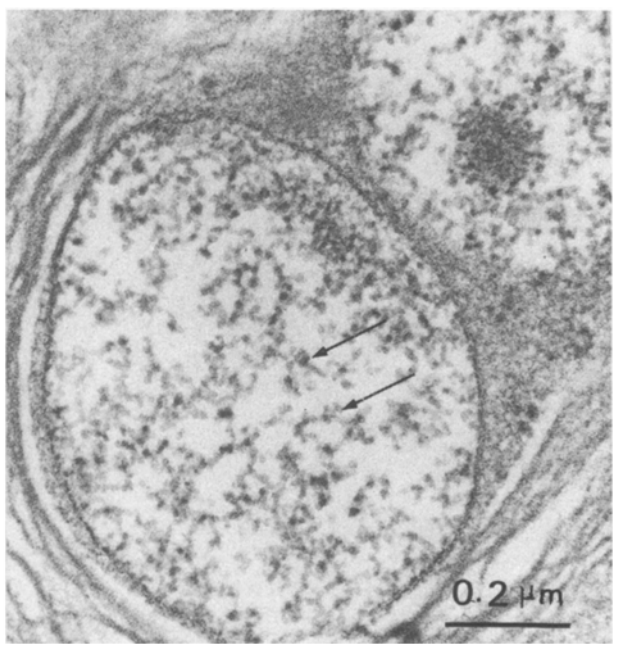

Fig. 9

Figure 8. Part of a gland cell of the posterior part in the middle piece of middle division, showing the formation of sericin globules (arrows) in Golgi complex. m, Mitochondria. × 21,000.

Figure 9. Enlarged sericin globules in the gland cell of anterior piece of middle division. Arrows indicate sericin fibers. × 80,000.

Cells in the proximal portion of the medial part of the middle gland division differ from those just described by the appearance of Golgi complexes and the sericin globules. Golgi vacuoles and the globules contain numerous fine fibers whose diameter is 150–200 Å.

Outer layer sericin is secreted in the anterior part of the middle gland division. Cells of this part are very large and are characterized by the presence of lipidic bodies which are arranged in characteristic figures. Vacuoles of Golgi complexes and sericin globules in the apical cytoplasm contain freely floating sericin strands 100–120 Å thick (fig. 9). These sericins also pass through the silk layer and then accumulate on the middle layer sericin to form the outer sericin layer of the silk.

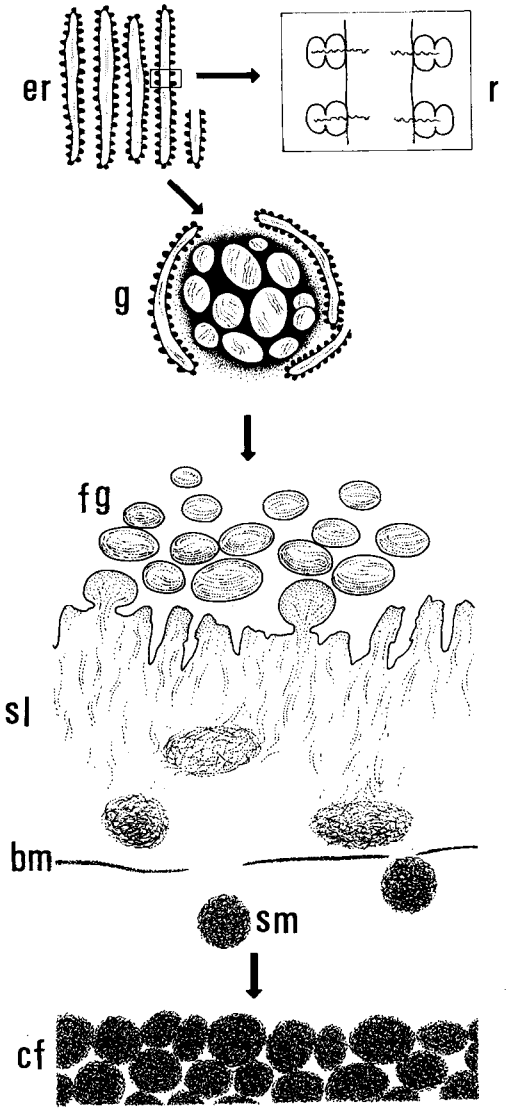

Figure 10. Schematic drawing of the process of fibroin secretion in posterior division. bm, Boundary membrane of cuticular intima between the silk layer and the lumen; cf, columnar fibroin accumulated with spherical masses of fibroin fiber in the lumen; er, rough endoplasmic reticulum; fg, fibroin globules; G, Golgi complex; r, ribosome; sl, silk layer; sm, spherical masses of fibroin fiber.

3. Silk materials in the gland lumen

It has already been mentioned that the columnar fibroin, which moves through the gland lumen, consists of packed masses of fibroin fibers. In the 4th instar larvae, about 200 sperical masses can be counted in a cross section through the posterior gland division[30]. Each sphere within the posterior division measures about 1 μm but this size is reduced as the spheres descend down the gland; the reduction is due to dehydration which takes place in the middle division. Elementary fibroin fibers inside the spheres are not oriented, not even in the most proximal region of the anterior gland division. Hence, it seems that the fibers get aligned only when passing through the spinneret.

The relationship between the position of gland cells and the progress of their secretory products through the gland lumen has been elucidated by means of autoradiography[31]. Currents of fibroin in the lumen of posterior gland division are shown in figure 12. It can be seen that fibroin secreted in the distal part of posterior division moves more rapidly than the fibroin secretion from a more anterior region.

Large amounts of columnar fibroin stored in the posterior division, and of both fibroin and sericins accumulated in the middle division, are dissolved when the larva molts. Drastic changes occur also in the gland cells[8]. Nuclear material concentrates in the basal part of the nucleus and the entire nucleus moves towards the cell base; this movement is associated with, and perhaps driven by, the formation of numerous vacuoles in the apical cell region. Rough ER and Golgi complexes decrease in concentration and cessa-

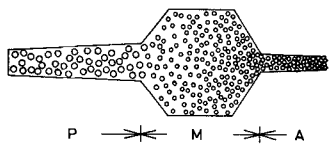

Figure 11. Schematic diagram of histological changes of the spherical masses of fibroin fiber in the silk gland. P, Posterior division; M, middle division; A, anterior division.

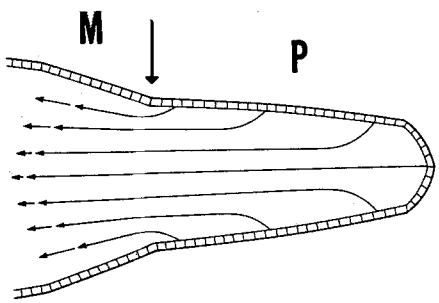

Figure 12. Schematic diagram showing the relationship between the secretory parts and their progress rutes of fibroin in the lumen in *Bombyx* silk gland. Arrows and length indicate the progress rutes and speeds respectively.

tion of protein synthesis is indicated by the formation of lamellated ER. Disappearance of fibroin globules is accompanied by formation of many lysosomes and large vacuoles. Protein synthesis is promptly resumed after ecdysis and within a few hours newly secreted silk materials appear already in the gland lumen.

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III. The physico-chemical properties of silk fibers and the fiber spinning process

by E. Iizuka

Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386 (Japan)

1. Species specificity of the conformation of silk fibroins

Arthropods produce every variety of silk fibers. Amino acid compositions and the primary structure of silk fibroins have been extensively studied and found specific to the species they belong. The molecular conformation of polypeptides including proteins is dependent on physical (shape, size, rigidity) and chemical (hydrophobic, hydrophilic, etc.) properties of the side-chains of the constituent residues that characterize the amino acids. Therefore, the conformation of silk fibroins is species specific.

a) Fiber

Glycine is the only amino acid having no geometrical isomers and proline (and hydroxyproline) is the only one having no peptide group, $-NH \cdot CO-$. A sequence of glycine residues prescribes a special helix called polyglycine II and that of poly-L-proline residues, poly-L-proline I and poly-L-proline II. Most of the other sequences of L-residues prescribe a helical conformation called α-helix, of the right-handed type[1] and the rest, an extended conformation called β-form. Those ordered conformations except poly-L-proline I and II can be transformed to the β-form, like the unordered conformation, when stretched as in fiber.

So far, the α-helix, the β-form, polyglycine II and collagen-like coiled coil have been distinguished in the structure of silk fibers[2]. It should be noted, however, that the principal conformation is that of (parallel) β silk even when specified as others. In the β-form, each extended chain is hydrogen-bonded, through $-C=O$ and $-NH$ radicals, between 2 neighboring chains to form a pleated sheet which is piled up one by one forming a so-called antiparallel-chain pleated sheet structure[3]. The side-chains are accomodated between the sheets. Thus, the c-dimension of a unit cell, the minimum unit of the crystal structure, becomes smaller with more glycine residues, which have the shortest side chain, $-H$, in the molecular chains. The β silk is further classified into 6 groups based on this c-dimension[2,4]. These are summarized in table 1.

b) Aqueous silk

It would appear that there is no felicitous experimental means to study the conformation of silk proteins as they are found in the silk gland. Silk fibroins are