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

III. Ultrastructural Changes of Posterior Silk Gland Cells in the Fourth Larval Instar

TAKASHI MORIMOTO, SHIRO MATSUURA, SUNAO NAGATA, and YUTAKA TASHIRO

From the Department of Physiology, the Kansai Medical School, Moriguchi, Osaka, Japan

ABSTRACT

The development of the cells in the posterior silk gland of the silkworm, *Bombyx mori*, during the fourth larval instar has been studied. In the early stages of this instar, the wet weight of the gland and the amounts of RNA, DNA, and protein per animal increase logarithmically until they reach a stationary state at about 72 hr. At around 96 hr of the fourth instar, the larvae enter the molting state, which lasts for about 24 hr until the fourth ecdysis. Towards the end of the molt stage, the growth of the silk gland is resumed. Electron microscopical observation shows that in the early intermolt stage the cytoplasm is filled with free ribosomes and with rough endoplasmic reticulum (ER), first of the lamellar type (0–6 hr) and then of the vesicular or tubular type. The Golgi apparatus also is well developed. At the beginning of the molt stage (90–96 hr), however, most of the ER becomes lamellar in type, concentric lamellar structures being occasionally observed, and the Golgi vacuoles disappear. Autophagosomes and lysosomes increase markedly and the apical portion of the cytoplasm becomes extensively vacuolated; this suggests that the secretory activities are completely depressed, and pronounced degenerative changes appear during the molt stage. Towards the end of the molt stage, large lamellar ER elements are fragmented into smaller lamellae and there is a pronounced increase in the number of free ribosomes.

INTRODUCTION

Larvae of the silkworm, *Bombyx mori*, like those of some other insects, grow by repeated intermolt (feeding)-molt cycles. It is interesting to study the ultrastructural changes of the posterior silk gland cells which will be referred to as "(the) cells," during these cycles. We have shown in the first of the preceding papers (1) that marked biochemical as well as ultrastructural changes are observed in the posterior silk gland during the last (fifth) instar. The last instar, however, is different from other instars, in that fibroin is extensively synthesized during it. We have studied, therefore, the ultrastructural changes of the cells during the fourth instar, expecting to find out what change is specific for the intermolt-molt cycle and what change is specific for the biosynthesis of fibroin.

Electron microscopical studies on the cells during the fourth instar has been reported by Akai (2). His works, however, cover only ultrastructural changes during the fourth molt stage; no complete ultrastructural observations on the

posterior silk gland during the fourth instar have been published.

MATERIALS AND METHODS

Silkworm

Strains of the silkworm used and the seasons of rearing are the following: Nichi 124 x Shi 124 (autumn), and Shungyoku x Gunpo (spring). The larvae were exclusively fed mulberry leaves and cultivated at 25° ± 1°C. Events in the fourth instar have been precisely timed in hours from the moment of the third eclosion from exuvium, and this event also was used for synchronization by selecting larvae which underwent eclosion within ±3 hr. Under these conditions, the larvae gradually lose their appetite and enter the fourth molt at about 90–96 hr. This molting state lasts for about 24 hr until the larvae carry out the fourth eclosion from the exuvium and become fifth instar larvae. This point marks the end of the fourth instar, which was observed at 120 ± 6 hr under the present experimental conditions. This paper is concerned with the cells of the posterior silk gland from the beginning until the end of the fourth instar. The animals were sacrificed at intervals of 6, 12, or 24 hr, depending on the stage in this larval period.

Biochemistry

15 animals (0–24 hr), nine animals (48 hr), or six animals (72–120 hr) of average body weight were used for each time point. For lipid extraction, more than 20 animals were sacrificed every time. First, the body weight of the larvae was measured; then the posterior silk glands were dissected out and washed briefly in 0.9% NaCl, and the wet weight was measured. The amounts of RNA, DNA, protein, and lipids were determined as described in the first of the preceding papers (1). Lipid analyses were carried out by Saito et al., and the results will be published in a separate paper.[1]

Electron and Light Microscopy

Specimens for light microscopy were prepared as described previously (1). For successful observation of the ultrastructure of the cells during the fourth instar, proper fixation was very important. First, simple fixation with OsO_4 in either phosphate buffer, pH 7.4 or Veronal-acetate buffer, pH 8.6, a procedure which works quite well for the cells of the mature fifth instar larva, was not successful; preservation of neither ribosomes nor intracytoplasmic membrane structures was satisfactory. Glutaraldehyde fixation (3) seems to be essential for good preservation of the ribosomes in the cells and of the membranes. For this purpose, 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, was used exclusively. Fixation was continued for at least several hours or even overnight in the cold, and after several washings with the same buffer the silk gland was postfixed with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for several hours. Block staining with uranyl acetate was frequently used to increase contrast according to the methods of Farquhar and Palade (4). The subsequent procedures were described previously (1).

[1] Saito, K., K. Sato, and M. Gamo. Manuscript in preparation. We thank those authors for allowing us to cite their unpublished data.

RESULTS

Body Weight and Wet Weight of Posterior Silk Gland

In the early stage of the fourth instar, the wet weight of the posterior silk gland and the body weight increase logarithmically until they reach a

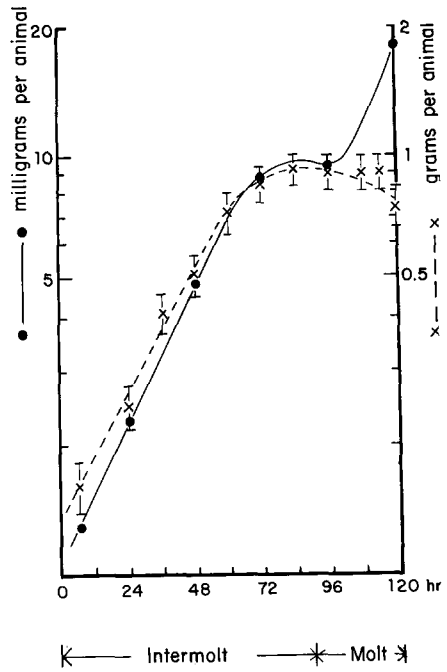


FIGURE 1 Increase in body weight in grams per animal (crosses) and in wet weight of the posterior silk glands in milligrams per animal (solid circles) during the 4th larval instar. At 90–96 hr of this instar the larvae entered molting state.

plateau level at around 72 hr (Fig. 1). This rapid growth is quite similar to the growth in the early stage of fifth instar, except that the increase of the two quantities is proportional; no disproportionately rapid growth of the gland was observed. Corresponding to the gradual loss of appetite at 84–90 hr, both the body weight and the wet weight of posterior silk gland were kept at a constant level. At 90–96 hr the larvae enter the molting state during which the body weight gradually starts to drop because the larvae do not eat. The wet weight of gland, however, increases towards the end of this molting stage. This means that the gland grows at the expense of some other tissues. This increase in growth of the gland during the fourth instar precedes the next logarithmic growth of the gland in the fifth instar which has been described in detail in the first of the preceding papers (1).

Amounts of DNA, RNA, Proteins, and Lipids

Fig. 2 shows that RNA and protein increase in amount rapidly during the early half of the fourth instar. The increase in the amount of DNA, however, is slower than the increase in the amounts of RNA and protein, and the existence of a stationary phase which is clearly seen on the curves for RNA and protein is not so clearly demonstrated From these results, however, it is evident that DNA is synthesized throughout the fourth instar; this is in agreement with the radioautographic studies of Akai and Kobayashi (5) who have shown that radioactive thymidine is incorporated rapidly into the nucleus throughout the fourth instar. The amounts of RNA and protein increase very rapidly at the beginning of the fourth instar (0–72 hr), gradually reach a stationary state (72–96 hr), and then start to increase again towards the end of this instar (96–120 hr). The shape of these curves is very similar to the shape of the curve of the wet weight of the posterior silk gland. Lipids were determined only in the later stage of this instar, because so many larvae were needed to obtain precise values.

All these results show that the posterior silk gland grows logarithmically in the intermolt stage and then arrives at a stationary state. At the end of the fourth instar rapid growth of the gland is triggered again; this precedes the logarithmic growth of the gland in the fifth instar.

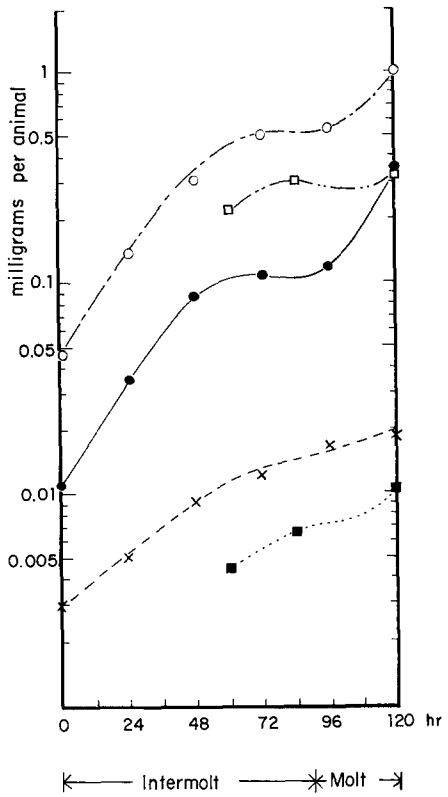


FIGURE 2 Increases in the amounts of DNA (crosses), RNA (solid circles), protein (open circles), total lipids (open squares) and phospholipids P (solid squares) of the posterior silk gland during the 4th larval instar. The ordinate shows the amount of these compounds in milligrams per animal.

Electron and Light Microscopy

Samples for morphological observation were prepared at 0, 6, 12, 24, 36, 48, 60, 72, 78, 84, 90, 96, 102, 108, 114, and 120 hr after the third ecdysis (fourth instar 0 hr). Since the morphological findings in the posterior silk gland in the intermolt stage and in the molt stage are so different, they will be described separately.

INTERMOLT STAGE (0–84 hr)

Figs. 3 *a–d* show light micrographs of the cells in the intermolt stage. From these micrographs it is apparent that the cells grow rapidly and that the nucleus becomes ramified more and more in this stage. No characteristic change in the cytoplasm of the cells has been observed at least at the light microscopical level. In the lumen of the

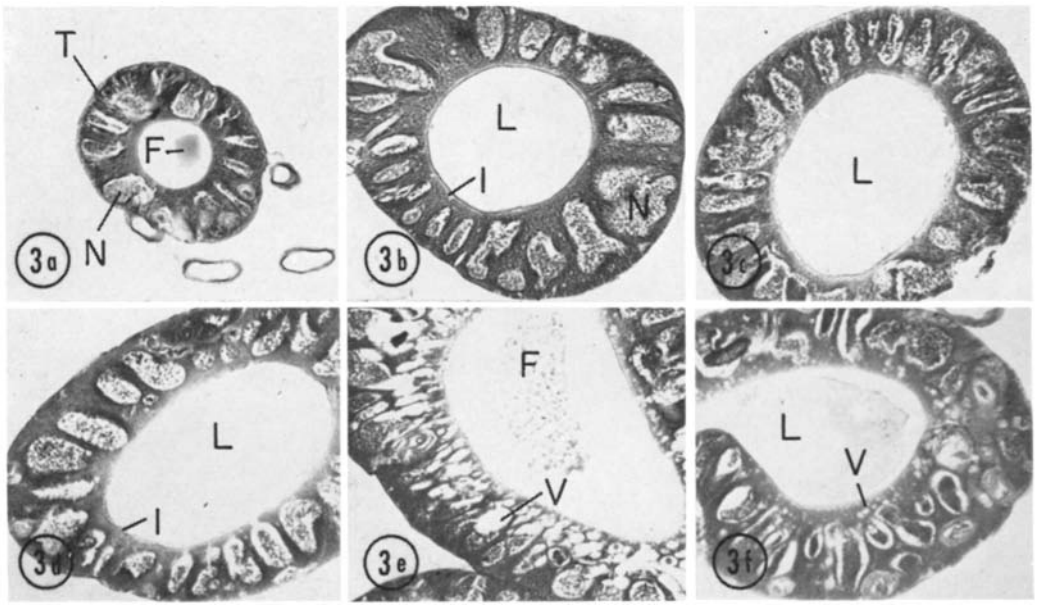


FIGURE 3 Light micrographs of the posterior silk gland in the 4th larval instar. Bouin fixation. *a*, 4th instar 0 hr; *b*, 36 hr; *c*, 60 hr; *d*, 90 hr; *e*, 96 hr; *f*, 114 hr. *T*, tunica propria; *I*, intima; *L*, glandular lumen; *V*, vacuoles; *N*, nucleus (more exactly, branch of nucleus); *F*, intraluminal fibroin. × 256.

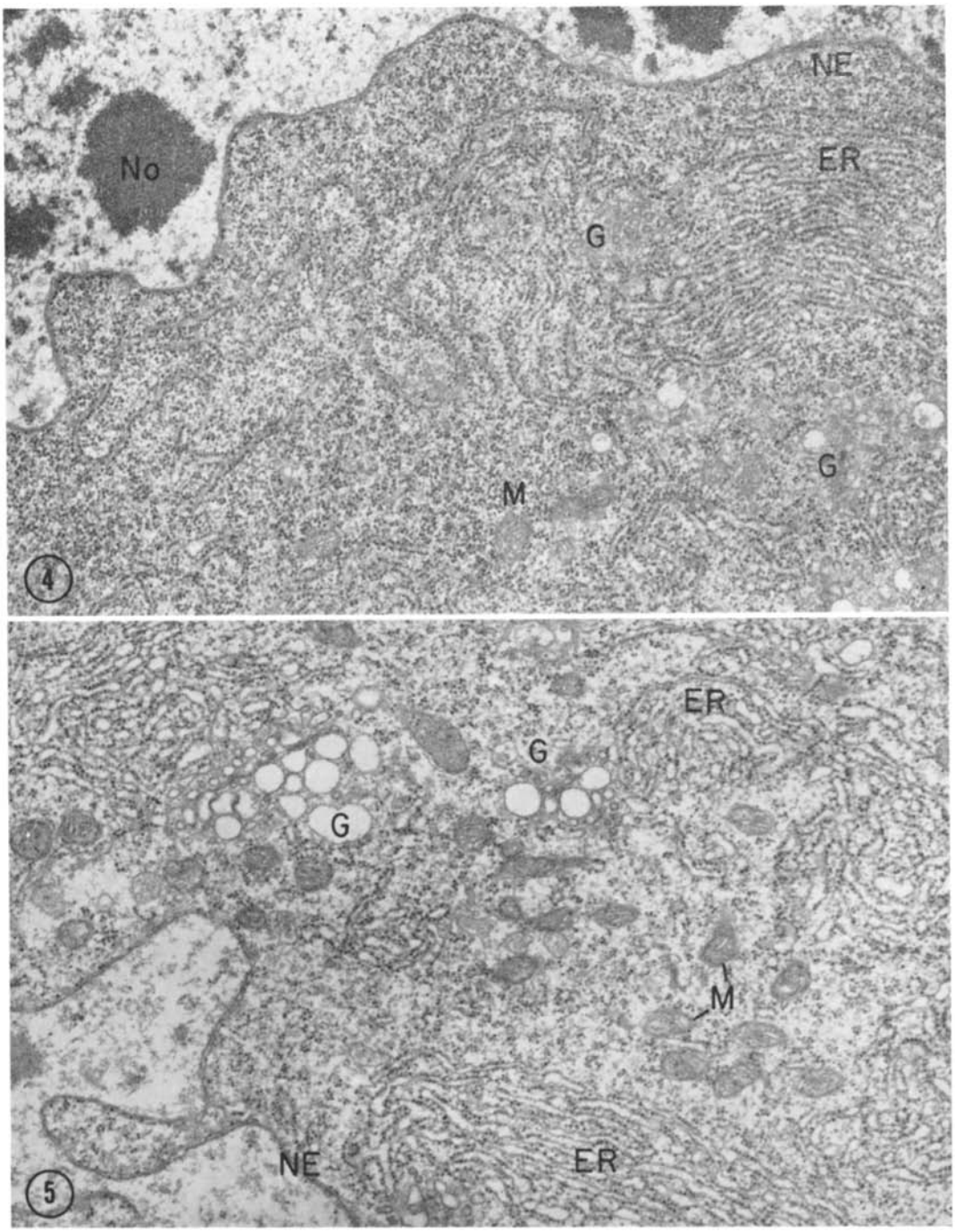
gland, gradual accumulation of fibroin is observed.

The ultrastructure of the cell at the beginning of the intermolt stage (0–6 hr) is considerably different from that of the cell in the later intermolt stage (12–84 hr).

FOURTH INSTAR 0 AND 6 HR: As shown in Fig. 4, a number of free ribosomes as well as rough ER of the lamellar type was observed throughout the cytoplasm. In some parts of the cytoplasm, there exists vesicular or tubular ER; the intracisternal space is slightly distended. The Golgi apparatus is poorly developed; minute vesicles, flattened saccules, and/or slightly enlarged vacuoles are observed. A number of mitochondria are present throughout the cytoplasm; their profiles are usually circular or oval and 0.2–0.5 μ in diameter. Autolysosomes and autophagosomes containing ER as described in detail in the preceding paper (6) were occasionally but rarely found at this stage. Structures such as microtubules, septate desmosomes, microvilli, and invaginations of the basal cell membrane are quite similar to the corresponding structures in the fifth instar (1).

The cell has a highly lobated nucleus loaded with large numbers of nucleoli and chromatin blocks. These two kinds of intranuclear bodies were sometimes so similar in electron opacity that it was difficult to tell them apart. A similar situation was found in the fifth instar larvae (1, 2). The nucleoli, however, are associated with ribosome-like particles. The nucleus is usually surrounded by a nuclear envelope (see, however, reference 7). Protrusion of the outer nuclear membrane into the cytoplasm was not observed throughout the fourth instar.

FOURTH INSTAR LARVA 12–84 HR OLD: This period of the fourth instar is characterized by an extensive proliferation of rough ER and by development of Golgi vacuoles (Fig. 5). At the same time, part of the lamellar ER is transformed into a vesicular or tubular form, so that lamellar and vesicular or tubular ER coexist at this stage. Distension of the intracisternal space of the ER and development of Golgi vacuoles, however, are not so pronounced as in the fifth instar larva. In the apical portion of the cells, accumulation of fibroin globules is observed (Fig. 6). This is a reasonable finding, because it is known that a small amount of fibroin is produced even in the fourth instar.



FIGURES 4 and 5 Electron micrographs of posterior silk gland cells in the 4th larval instar at 0 and 36 hr, respectively. Fig. 4 shows that at the beginning of the 4th instar the cytoplasm is filled with a large amount of free ribosomes, rough ER (*ER*), and mitochondria (*M*). The nucleus contains a number of chromatin blocks and of nucleoli (*No*) loaded with ribosome-like particles. The nucleus is completely surrounded by the nuclear envelope (*NE*). By 36 hr (Fig. 5), however, the rough ER has proliferated markedly, and the intracisternal space is slightly distended. In the Golgi apparatus (*G*), several vacuoles are observable. Fig. 4, × 18,000; Fig. 5, × 25,000.

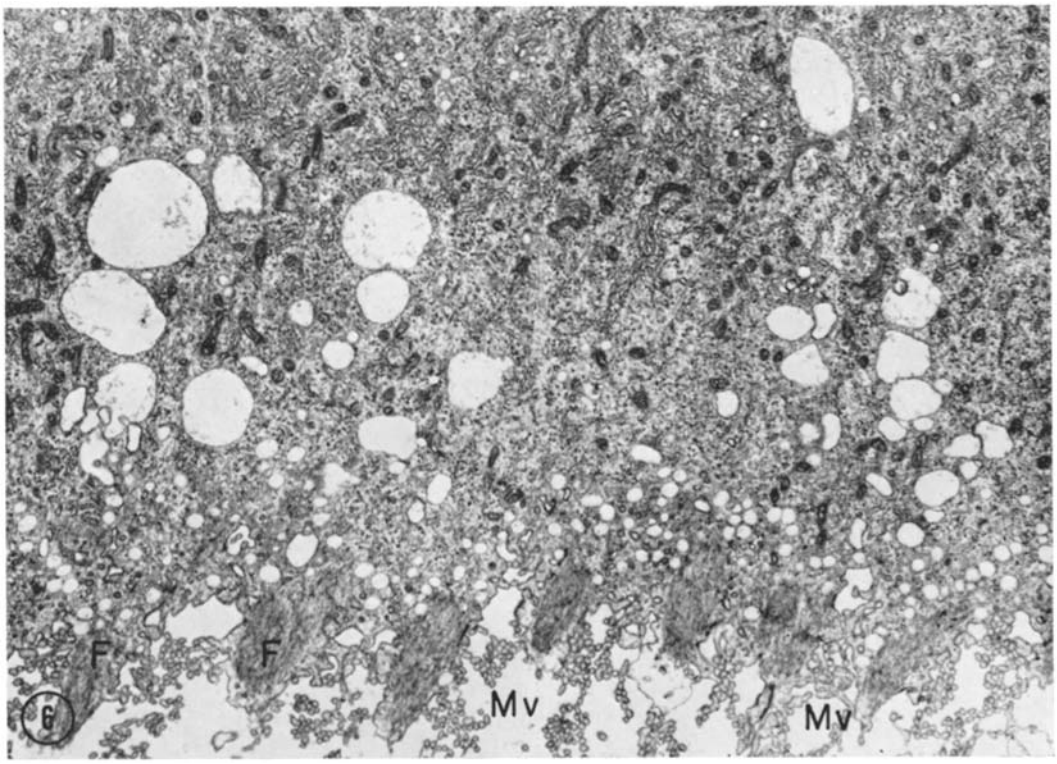


FIGURE 6 Apical cytoplasm of a posterior silk gland cell in the 4th instar, 48 hr. Fibroin globules have accumulated in the apical cytoplasm. Cytoplasmic processes having a number of microvilli (*Mv*) are arranged regularly on the luminal surface of the cell and contain bundles of fine filaments (*F*) similar to those of terminal webs. × 6,300.

MOLT STAGE (90–120 hr)

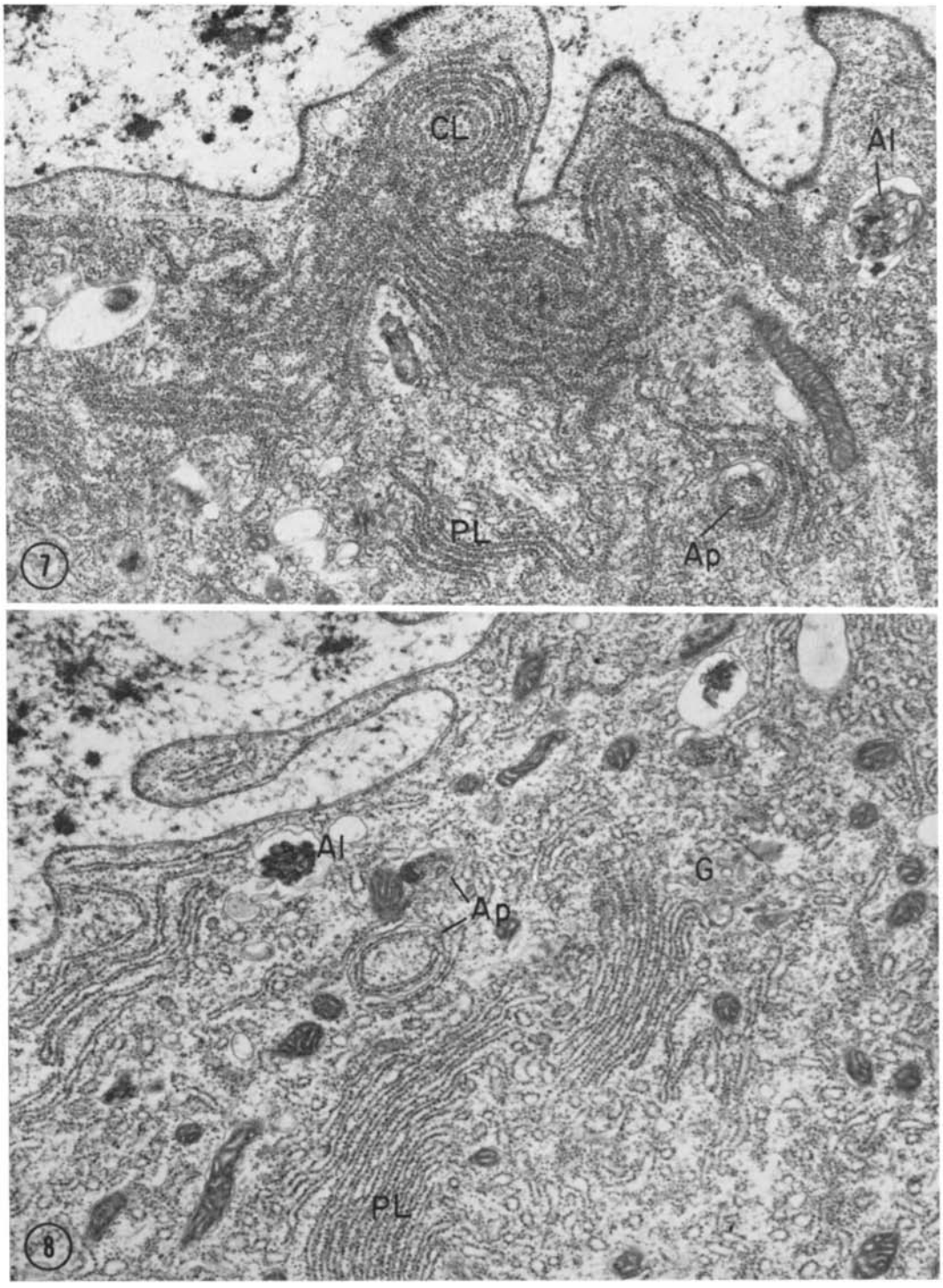
Marked morphological changes appear from the beginning of the fourth molt stage; some of them can be detected even by light microscopy. That is, large numbers of vacuoles appear in the cytoplasm, mainly in the apical portion, as shown in Figs. 3 *e* and *f*, and at the same time the fibroin in the glandular lumen becomes granulated. The vacuolization of the cytoplasm becomes apparent at 96 hr, continues to be observable at 102 and 108 hr, decreases rapidly at 114 hr, and disappears completely at the end of this instar (120 hr).

Electron microscopical observations have also shown several characteristic changes in the ultrastructures of the cells at this stage.

FOURTH INSTAR 90 AND 96 HR: First, the intracisternal space of the ER gradually becomes less distended, and thus the vesicular or tubular ER which is abundantly present in the intermolt stage rapidly decreases in amount and is transformed into the lamellar form; many lamellar ER elements are found at 96 hr of the fourth instar. These lamellae are frequently piled up, to form parallel or concentric lamellar structures as shown in Figs. 7 and 8. The Golgi vacuoles which were numerous in the intermolt stage decrease markedly in size and in number in the premolt stage (90 hr) and become rudimentary at 96 hr, as shown in Fig. 8.

Another interesting finding is the appearance or increase in number of autophagosomes at 90–96 hr of the fourth instar. As shown in Figs. 7 and 8, they usually contain rough ER and occasionally, but rarely, mitochondria and seem to be rapidly transformed into autolysosomes as in the prepupal stage (6).

The vacuoles which are numerous especially in the apical portion of the cytoplasm are bounded by smooth membranes, and their contents usually are quite transparent except for a small amount of



FIGURES 7 and 8 Electron micrographs of posterior silk gland cells in the 4th instar, 96 hr. Lamellar ER predominates in the cytoplasm at this molt stage, and concentric (*CL*) or parallel lamellar structures (*PL*) are frequently observed. A number of autophagosomes (*Ap*) and autolysosomes (*Al*) also appear at this stage. The nuclear matrix is coarse. *G*, Golgi region. Figs. 7, 8, × 15,000.

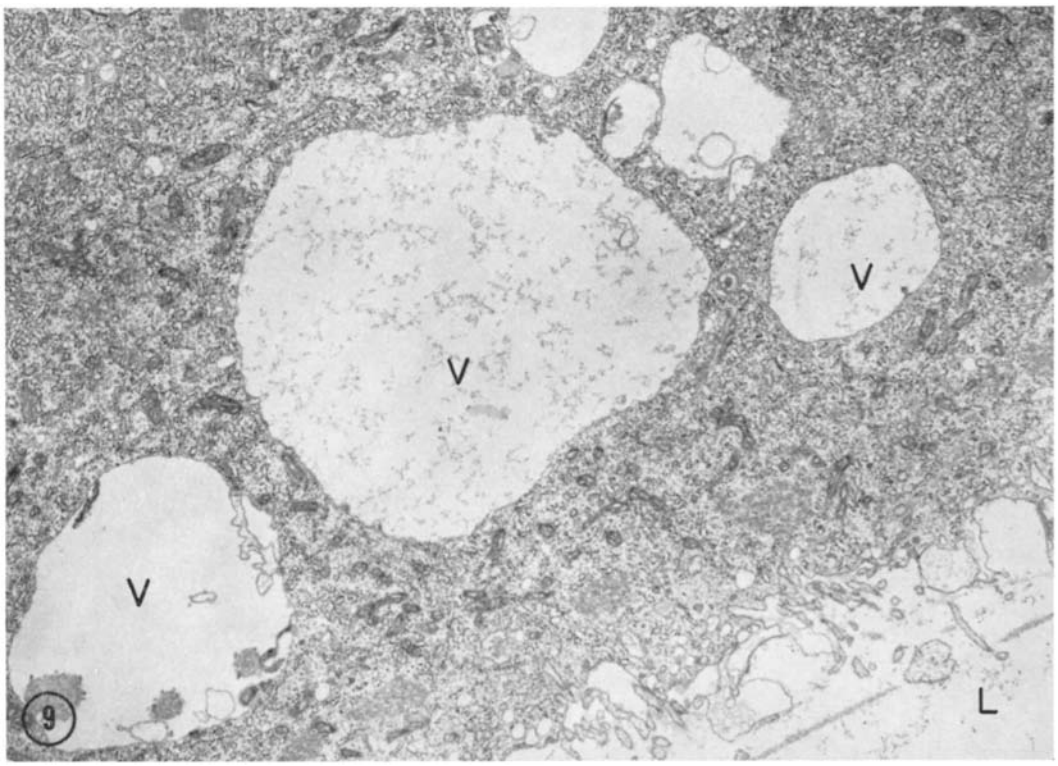


FIGURE 9 Marked vacuolization of the apical cytoplasm in posterior silk gland cells at 96 hr of the 4th larval instar. Vacuoles (*V*) are limited by a smooth membrane, and contain small amounts of filamentous material. *L*, glandular lumen. × 7,500.

filamentous material as shown in Fig. 9. Large vacuoles probably are formed by fusion of smaller ones. The cytoplasm surrounding the vacuoles is similar to the other parts of the cytoplasm, no special degenerative changes being apparent.

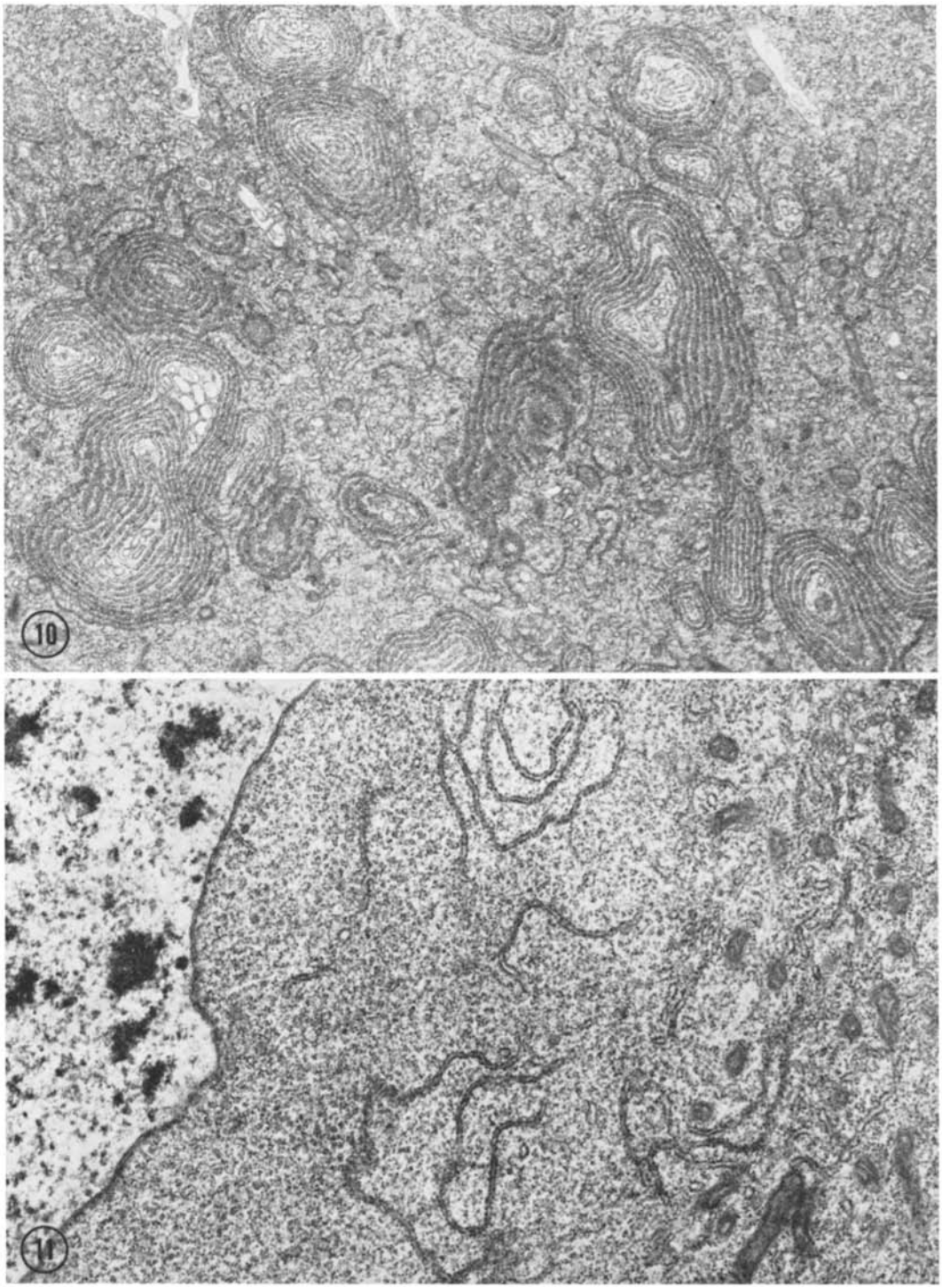
The nucleus shows a coarse matrix at this stage, and the association of ribosome-like particles with nucleoli is hardly visible.

FOURTH INSTAR 102–120 HR: The ultrastructure of the cells at 102 and 108 hr is quite similar to the ultrastructure of the cells at 96 hr; occasionally a number of parallel or concentric lamellar structures were observed, especially in the basal portion of the cytoplasm (Fig. 10). Towards the end of the fourth instar, however, these structures seem to be degraded rapidly and, at 114 or 120 hr, neither concentric nor parallel lamellar structures were any longer observable, only short portions of lamellar ER being found scattered here and there. It is not clear how the whorled structures are degraded. One possibility is that the large lamellar ER structures are fragmented into smaller lamellae, and another is that the membrane component of the ER is selectively degraded; this releases attached ribosomes as free ribosomes. The Golgi vacuoles are poorly developed throughout the molt stage. Autophagosomes and lysosomes also decrease rapidly in number and disappear almost completely at the end of the fourth instar. At the same time, free ribosomes start to accumulate especially in the perinuclear zone as shown in Fig. 11, and ribosome-like particles appear again in the nucleoli; this suggests that the cells have resumed the rapid growth which precedes the logarithmic growth in the early stage of the fifth instar.

DISCUSSION

In the two preceding papers, we have described the biochemical and morphological changes of the posterior silk gland in the fifth larval instar (1) as well as in the prepupal and early pupal stages (6). It is interesting to compare those results with the sequential changes in the gland in the fourth instar.

The logarithmic growth of the gland at the



FIGURES 10 and 11 Electron micrographs of posterior silk gland cells in the 4th instar, 108 and 114 hr, respectively. In Fig. 10, numerous concentric lamellar structures are observed in the basal portion of cytoplasm. In Fig. 11, large lamellar structures are no longer observable and the perinuclear region is again filled with a large number of free ribosomes. Fig. 10, × 8,400; Fig. 11, × 15,000.

beginning of the fourth larval instar is quite similar to that in the early stage of the fifth larval instar except that in the former the growth is approximately proportional to the increase in body weight and that the duration of the former (~72 hr) is shorter than that of the latter (~96 hr).

The logarithmic growth of the gland is followed by a stationary state in either case. Quite marked differences, however, appear at this stage. That is, in the fifth instar fibroin is extensively synthesized until the larva reaches the fully mature state (fifth instar, 192 hr), whereas in the fourth instar biosynthesis of fibroin is probably soon suppressed and marked degenerative changes appear. The inhibition of biosynthesis of fibroin is strongly suggested by the disappearance of the distension of the intracisternal space of the ER as well as by the regression of Golgi vacuoles and the disappearance of the intraluminal fibroin. The first change probably is induced by the decrease in the accumulation of intracisternal fibroin and probably is responsible for the transformation of vesicular or tubular ER into lamellar ER. The degenerative changes occur concomitantly as shown by the appearance or increase in number of autophagosomes and lysosomes, as well as by the marked vacuolization in the apical portion of cytoplasm.

The formation of vacuoles is quite characteristic in the molt stage. Their origin, however, is not always clear. Akai (2) has suggested that the vacuoles are formed by fusion of intracellular fibroin globules (secretion granules of fibroin). Our electron microscopical observation and the localization of the vacuoles in the apical cytoplasm also support this possibility. Since most of the intracellular fibroin which is synthesized during the fourth instar is not secreted, it may be digested to the constituent amino acids within these vacuoles during the molt stage. It is also possible that some of these vacuoles are formed by reabsorption of the intraluminal fibroin which undergoes autodigestion during the molt stage as suggested by Akai (2).

These changes, in many respects, are similar to the degenerative changes of the posterior silk gland in the prepupal stage (6), except that the vacuolization of cytoplasm is much more pronounced in the molt stage than in the prepupal stage. For example, the degenerative changes in both cases seem to be preceded by the appearance of autophagosomes containing ER, or ER bodies described by Locke and Collins (8), and concentric lamellar structures appear occasionally in either case. However, there is a big difference between these two cases. In the prepupal stage the degenerative changes continue until the gland is completely degraded and disappears, whereas in the fourth instar, the posterior silk gland has to resume explosive growth for the fifth instar. Thus, towards the end of the fourth instar, some biogenetic processes already are initiated, as clearly shown by the increase in the number of free ribosomes especially in the perinuclear region and by the reappearance of ribosome-like particles in the nucleoli or in the nucleoplasm.

In spite of this difference, it seems that the cells grow by repeated changes in accordance with the molt-intermolt cycle. This cycle is known to be under hormonal control, and it may be interesting to try to correlate the hormone action more directly with the ultrastructural changes in the cells.

We have shown in this series of papers (1, 6) that typical concentric lamellar structures or whorls appear frequently in the cell. Similar structures have been described in the exocrine pancreas cells by Weiss (9) and by Herman and Fitzgerald (10) and in other cells (11), and it has been suggested that they are centers of proliferation of ER. In the posterior silk gland these structures appear at three different stages: in the molt stage of the fourth instar probably when the biosynthesis of fibroin as well as the biogenesis of ER is completely inhibited; at 96 hr of the fifth instar when the cells are in transition from the logarithmic growth to a stationary state; in the prepupal stage when the biosynthesis of fibroin is slowed down and the biogenesis of ER is completely inhibited. It is suggested, therefore, that in the posterior silk gland the concentric lamellar structures are not centers of proliferation of ER but rather a piling up of the ER which exists in excess in cytoplasm without synthesizing exportable protein. The occasional appearance of these structures at 96–108 hr of the fourth instar and at 96 hr of the fifth instar may indicate a decrease in the function and an overproduction of ER, respectively, or both. These structures may be an arrangement for storage of ER in the most efficient way, because whorled structures or parallel lamellar structures may occupy least volume per unit weight of ER.

Our observations, in many respects, have con-

firmed the pioneering works by Akai (2). For example, the inhibition of biosynthesis of fibroin and the vacuolization of the apical portion of the cytoplasm of the cells in the fourth molt stage have already been described by him. Akai has also reported the disappearance of the membrane component of rough ER during this fourth molting stage. By careful fixation with glutaraldehyde and postfixation with OsO_4, we have shown that all membrane structures are preserved during the molt stage. We conclude, therefore, that the apparent disappearance of membrane is probably an artifact produced by insufficient fixation, though we do not deny the possibility that during the molt stage the membrane structures become difficult to fix with these fixatives.

We should like to thank Professor A. Inouye of Kyoto University for his encouragement, Doctors T. Matsumoto and K. Hayashiya of Kyoto University of Industrial Art and Textile Fibers for supplying us silkworms and for their helpful discussion and advice. We are greatly indebted to Miss H. Ueda for her technical assistance.

This work was supported by grants from the Ministry of Education of Japan.

Received for publication 28 November 1967, and in revised form 6 May 1968.

REFERENCES

1. TASHIRO, Y., T. MORIMOTO, S. MATSUURA, and S. NAGATA. *J. Cell Biol.* **38**:574.
2. AKAI, H. 1965. *Sanshi Shikensho Hokoku.* **19**:375.
3. SABATINI, D. D., K. BENSCH, and R. J. BARRNETT. 1963. *J. Cell Biol.* **17**:19.
4. FARQUHAR, M. G., and G. E. PALADE. 1965. *J. Cell Biol.* **26**:263.
5. AKAI, H., and M. KOBAYASHI. 1966. *Symp. Cell Chem. (Japan).* **17**:131.
6. MATSUURA, S., T. MORIMOTO, S. NAGATA, and Y. TASHIRO. *J. Cell Biol.* **38**:589.
7. TASHIRO, Y., S. MATSUURA, T. MORIMOTO, and S. NAGATA. 1968. *J. Cell Biol.* **36**:C5.
8. LOCKE, M., and J. V. COLLINS. 1965. *J. Cell Biol.* **26**:857.
9. WEISS, J. M. 1953. *J. Exptl. Med.* **98**:607.
10. HERMAN, L., and P. FITZGERALD. 1962. *J. Cell Biol.* **12**:297.
11. FAWCETT, D. W. 1964. *In* Intracellular Membrane Structure. S. Seno and E. V. Cowdry, editors. Japan Society for Cell Biology, 15.