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

II. Cytolytic Processes in Posterior Silk Gland Cells During Metamorphosis from Larva to Pupa

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ABSTRACT

Cytolytic processes in posterior silk gland cells of the silkworm, *Bombyx mori*, during metamorphosis from larva to pupa have been studied. During this stage, the wet weight and the amounts of RNA and protein of the gland decrease rapidly and markedly, while the amount of DNA decreases slowly and slightly. The ultrastructural changes observed at the beginning of the prepupal stage consist of the appearance or the increase in the number of autophagosomes containing endoplasmic reticulum (ER), or "early autophagosomes" as we have called them, which seem to be gradually transformed to autolysosomes. A number of usual lysosomes, which frequently contain myelin figures, also appear in the cytoplasm. Sometimes they fuse with each other to form large conglomerates. In the middle of the prepupal stage, a number of smooth membrane-bounded vacuoles appear in cytoplasm. Towards the end of the prepupal stage the partition or sequestration of cytoplasm was observed. Thus large autophagosomes containing cytoplasmic organelles such as rough ER and/or mitochondria are formed. The nucleus is partitioned in a similar way by smooth membranes, and then autophagosomes containing condensed chromatin blocks are formed. These various kinds of autophagosomes, or "late autophagosomes" as we have generally called them, are continuously released into the hemolymph until the gland is completely disintegrated.

INTRODUCTION

The fifth instar larva of the silkworm, soon after arriving at full maturation, starts spinning its cocoon. This spinning continues for 3 days (~72 hr). After about another 24 hr the larva in the cocoon carries out ecdysis to become the pupa (prepupal ecdysis). This metamorphosis from larva to pupa is accompanied by a number of morphological as well as biochemical changes of which the most important is the rapid degenerescence, in the prepupal and early pupal stages, of the silk gland that went through such remarkable development during the preceding fifth instar.

It is quite natural that the histolysis of the silk gland during metamorphosis has attracted a number of light microscopists. Indeed, histological observations of this process already were reported by Helm (1) in 1876, and were confirmed and further extended by Ito in 1915 (2). To our knowledge, however, no electron microscopical observations have been reported except those of

Akai (3), who has not described a number of the characteristic changes described here.

Strictly speaking, the prepupal stage should be included in the fifth larval instar. The morphological and biochemical changes in the posterior silk gland cells which will be referred to as "(the) cells," are described in this paper.

MATERIALS AND METHODS

Silkworm

Strains of the silkworm used and the seasons of rearing are the following: Shi 124 (autumn), Nichi 124 x Shi 124 (spring and autumn), and Shungyoku x Gunpo (spring). The fully matured larvae grown on mulberry leaves were transferred to a special case to induce the larvae to spin silk fiber (this procedure is conventionally called "mounting") and were maintained at 25° ± 1°C. Events in the prepupal stage have been timed in hours from the beginning of the full maturation. In the preceding paper we have indicated how to determine the fully matured state of the larva (4). The total duration of this prepupal stage was 96 ± 6 hr (SD). After prepupal ecdysis, the posterior silk gland degenerates rapidly and disappears almost completely within several days.

Biochemistry

Measurement of the body weight of the larvae and of the wet weight of the posterior silk glands, and determination of DNA, RNA, protein, and lipids were described in the preceding paper (4). Analyses of lipids were carried out by Saito et al.[1]

Biosynthesis and secretion of fibroin in the prepupal stage were studied by sequential determination of the amount of fibroin in the posterior and middle silk glands as well as in the cocoon. The total amount of fibroin extracted from these three sources will show whether synthesis of fibroin continues even in the prepupal stage, while the amount of fibroin in the cocoon will show the time course of spinning of fibroin by the larva. Extraction of fibroin from the middle and posterior silk glands was carried out as described in the preceding paper (4), and the same method was also used for extraction of fibroin from the cocoon.

Light and Electron Microscopy

For light microscopy, the posterior silk gland was fixed either with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, or with Bouin's fixative, dehydrated with alcohol, embedded in paraffin, and observed after staining by Mallory's method. The Feulgen reaction was used to demonstrate the localization of DNA in the prepupal or pupal stage. For electron microscopy, the procedures of fixation were those described previously (4), that is, either glutaradehyde fixation and postfixation with OsO_4 or simple fixation with OsO_4 was used. The former method was effective for the preservation of ribosomes especially in the later prepupal or pupal stage, while the detachment of membranous materials or the formation of myelin figures within the vacuoles or within the intracisternal space of the ER, an artifact frequently produced by glutaraldehyde fixation, was not observed when simple OsO_4 fixation was used. Histochemical demonstration of acid phosphatase at the electron microscope level was carried out according to Miller and Palade (5). Specimens for morphological observations were prepared at intervals of 12 hr from the beginning of the prepupal stage.

RESULTS

Biochemistry

The wet weight, RNA, and protein of the posterior silk gland decreased rapidly from the beginning of the prepupal stage so that at the end of the prepupal stage the amount of RNA and protein was only 6–7% of the initial values (Figs. 1 and 2); DNA decreased much more slowly, to only 76% of the initial level. From these data, the amounts of RNA and DNA per unit wet weight of the silk gland were calculated and plotted in Fig. 3, which clearly shows that the amount of RNA per gram posterior silk gland decreases rapidly while the amount of DNA per gram increases markedly.

Biosynthesis and Secretion of Fibroin

In Fig. 4, solid circles show the total amount of fibroin (fibroin in the posterior silk gland plus middle silk gland plus cocoon) synthesized by the cells. At the fully mature state (or prepupal larva 0 hr old), most of the fibroin has already been synthesized; only approximately 10–20% were synthesized after full maturation, especially on the first and second days of the prepupal stage. Open circles show the amount of fibroin spun from the larva into the cocoon in the prepupal stage and illustrate that the spinning proceeds rapidly on the second and the third day and terminates at around 72 hr of the prepupal stage.

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

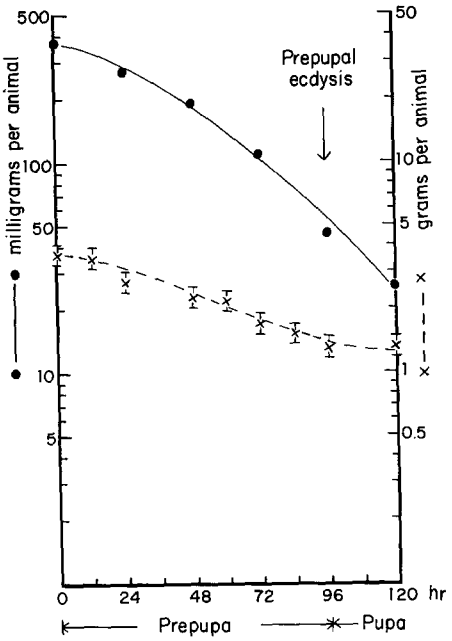


FIGURE 1 Decrease in the body weight (crosses) and in the wet weight of the posterior silk gland (solid circles) in the prepupal and the early pupal stage.

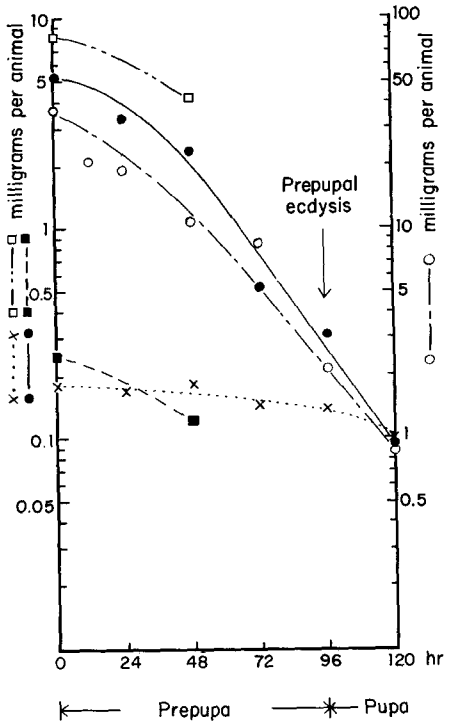


FIGURE 2 Decrease in the amount of DNA (crosses), RNA (solid circles), protein (open circles), total lipids (open squares) and phospholipids P (solid squares) of the posterior silk gland in the prepupal and early pupal stages.

Morphological Observations

Light micrographs of the cells in the prepupal and in the early pupal stages are shown in Fig. 5. Since our observations are generally in good agreement with those of Ito (2), detailed description will be unnecessary. Therefore, the light microscopical observations will be explained briefly together with the electron microscopical observations.

EARLY PREPUPAL STAGE (PREPUPA 0–36 HR OLD)

In the early half of the prepupal stage, when silkworms are spinning vigorously, hardly any morphological change is observed by light microscopy except that the radial diameter of the cells decreases gradually and the outline of the nucleus becomes more distinct (Fig. 5 *a*). As shown in Fig. 4, the rate of biosynthesis of fibroin is slowed down gradually after full maturation. Since fibroin synthesis is the most important function of the posterior silk gland, it is quite natural that marked ultrastructural change appears first in the ER from the beginning of the prepupal stage.

The most characteristic change is the appearance or the increase in number of the autophagosomes, as reported by de Duve and Wattiaux (6), by a process in which small portions of the cytoplasm become enclosed by a smooth membrane (7, 8). The cell organelle that is most frequently enclosed is the ER, and the structures thus formed correspond exactly to the ER bodies described by Locke and Collins (9). Since the formation of these autophagosomes is triggered at the beginning of the prepupal stage, it is possible to trace the sequential processes of the formation of these bodies. Probably first a flat saccule of smooth membrane appears in cytoplasm and then flattens more and more until finally most parts of the apposing membranes are fused together to form a sheet of double membrane, approximately 120 A thick (Figs. 7, 8, 11). Although Locke and Collins (9) have suggested that the isolation membranes are derived from Golgi vesicles, we have failed, so far, to obtain convincing evidence for the direct transformation of the Golgi vesicles into the isolation membrane. Usually rough ER is arranged along both sides of the isolation membrane as

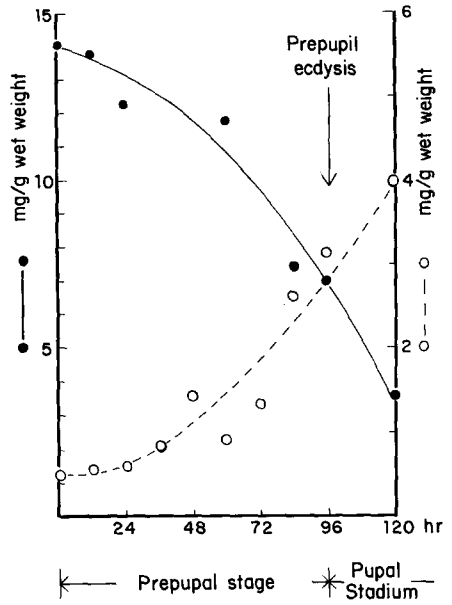


FIGURE 3 Change in RNA (solid circles) and DNA (open circles) content (milligrams per gram fresh tissue) in the posterior silk gland in the prepupal and early pupal stages.

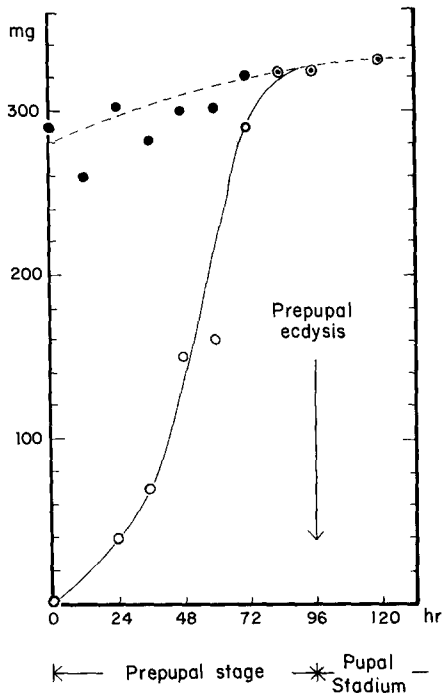


FIGURE 4 Total amount of fibroin synthesized in the posterior silk gland (solid circles) and the time course of spinning of fibroin (open circles) in the prepupal stage.

shown in the figures mentioned. Then the isolation membrane starts to enclose the ER elements on either side; first it forms an incomplete envelope (Figs. 9, 10) and then forms a closed envelope (Fig. 11). Thus the ER body, or autophagosome, is completed. Since these autophagosomes appear in large numbers early in the prepupal stage, they will be called "early autophagosomes." ER and ribosomes enclosed within the cavities of these bodies are subsequently digested (Fig. 12) probably by fusion with lysosomes, as a result autophagosomes are transformed into autolysosomes (6). Occasionally, but rarely, a whorled structure composed of rough ER appears (Fig. 15), the formation of which may be another type of degradation of ER.

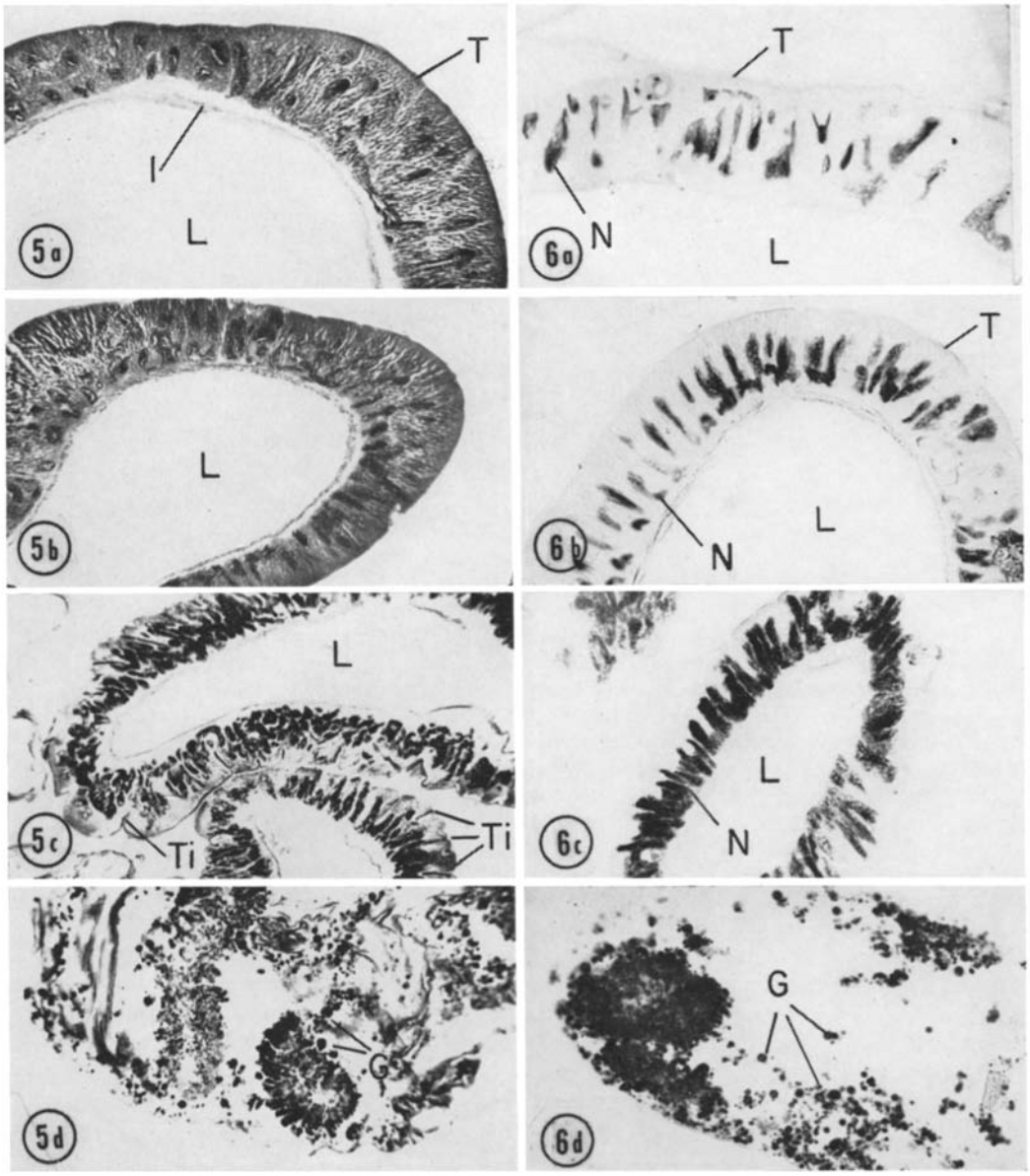
Simultaneously with the appearance of these autolysosomes, the usual lysosomes also appear or increase in number in the cytoplasm in the early prepupal stage. They are dense granules bounded by a single membrane and frequently contain myelin figures (Fig. 13).

Mitochondria have a normal appearance at the beginning of the prepupal stage. They are usually large and elongated. Occasionally, but rarely, however, the mitochondria contain tubular cristae longitudinally oriented (Fig. 7). Karnovsky has described a similar transformation of cristae in mitochondria in the proximal tubular cells of a fasted summer frog (10). Occasionally mitochondria are enclosed within the early autophagosomes, and thus autolysosomes containing ER as well as mitochondria are formed.

Most of the Golgi apparatus apparently remain unchanged (Figs. 8, 9, 13). Three or four Golgi vacuoles surrounded by a number of minute Golgi vesicles are observed.

The nuclei contain a large number of chromatin blocks more condensed than in the fifth instar larvae. In most of the nucleoli, however, a pars amorpha appears from the very beginning of the prepupal stage; this indicates depressed activity in the nucleoli.

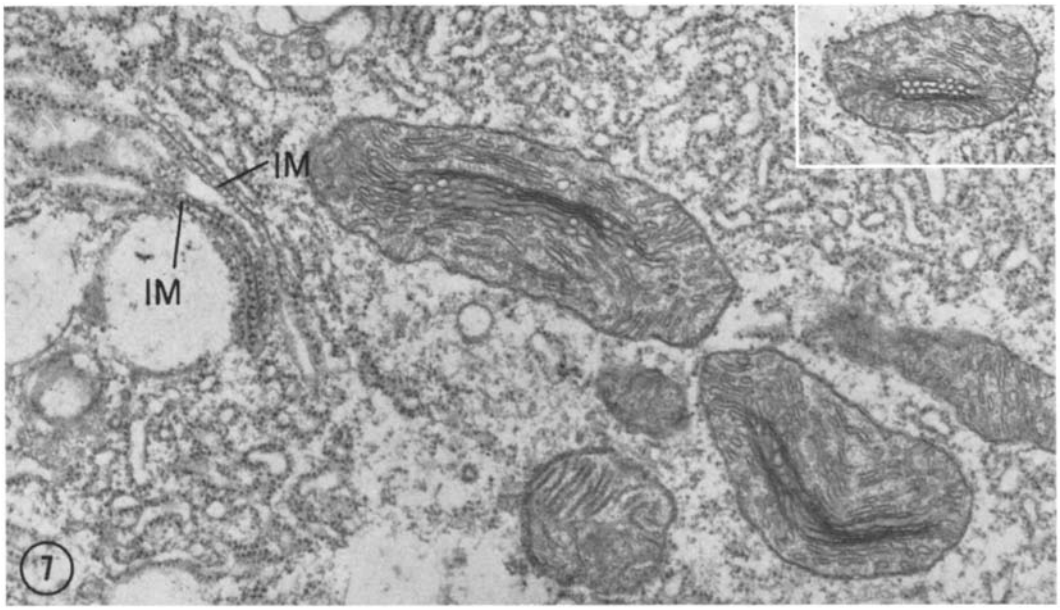
Histochemical studies show that the acid phosphatase reaction is positive in the usual lysosomes (Fig. 14). We have failed so far to demonstrate a positive reaction in the early autophagosomes.



FIGURES 5 and 6 Light micrographs of the posterior silk gland in the prepupal and early pupal stages. Fig. 5 series were stained with Mallory's method, while Fig. 6 series were stained with Feulgen's method. 5 *a* and 6 *a*, prepupa 0 hr; 5 *b* and 6 *b*, prepupa 72 hr; 5 *c* and 6 *c*, prepupa 84 hr; 5 *d* and 6 *d*, pupa 24 hr. *T*, tunica propria; *Ti*, invaginated tunica propria; *L*, glandular lumen; *N*, nucleus (more exactly, branch of nucleus); *G*, chromatin globule; *I*, intima. × 160.

MIDDLE PREPUPAL STAGE (PREPUPA 48–72 HR OLD)

During this period the silkworms are still vigorously spinning, but at the end of this stage (~72 hr) they stop spinning, as previously explained. Light microscopical changes observed in the early prepupal stage (0–36 hr) become more pronounced; the radial diameter of the cells decreases further and the nucleus becomes more



FIGURES 7–11 Electron micrographs of posterior silk gland cell in the prepupal stage 24 hr. Figs. 7 and 8 show in the cytoplasm isolation membrane (*IM*) which is sandwiched between elements of the rough ER. Most parts of the isolation membrane show fusion of paired apposing membranes. Figs. 9 and 10 show incomplete autophagosomes (*Ap*) containing rough ER and ribosomes, while Fig. 11 shows closed autophagosomes. Branching of isolation membrane is also observed in Fig. 11 (arrow). Mitochondria in Fig. 7 contain tubular cristae longitudinally oriented (see insert). *G*, Golgi region. OsO_4 fixation. Fig. 7, × 24,000; insert × 20,000; Fig. 8, × 45,000; Fig. 9, × 60,000; Fig. 10, × 24,000; Fig. 11, ×45,000.

condensed and rounder in shape than in the previous stage.

Electron microscopical observation also shows that the various ultrastructural changes which are observed in the previous stage (0–36 hr) become much more prominent in the following stage (48–72 hr).

A number of early autophagosomes, usually lysosomes and telolysosomes (6) are observed in accordance with the degenerative changes of the cytoplasm. Fig. 16 shows a typical electron micrograph of the cytoplasm of the cell in this prepupal stage.

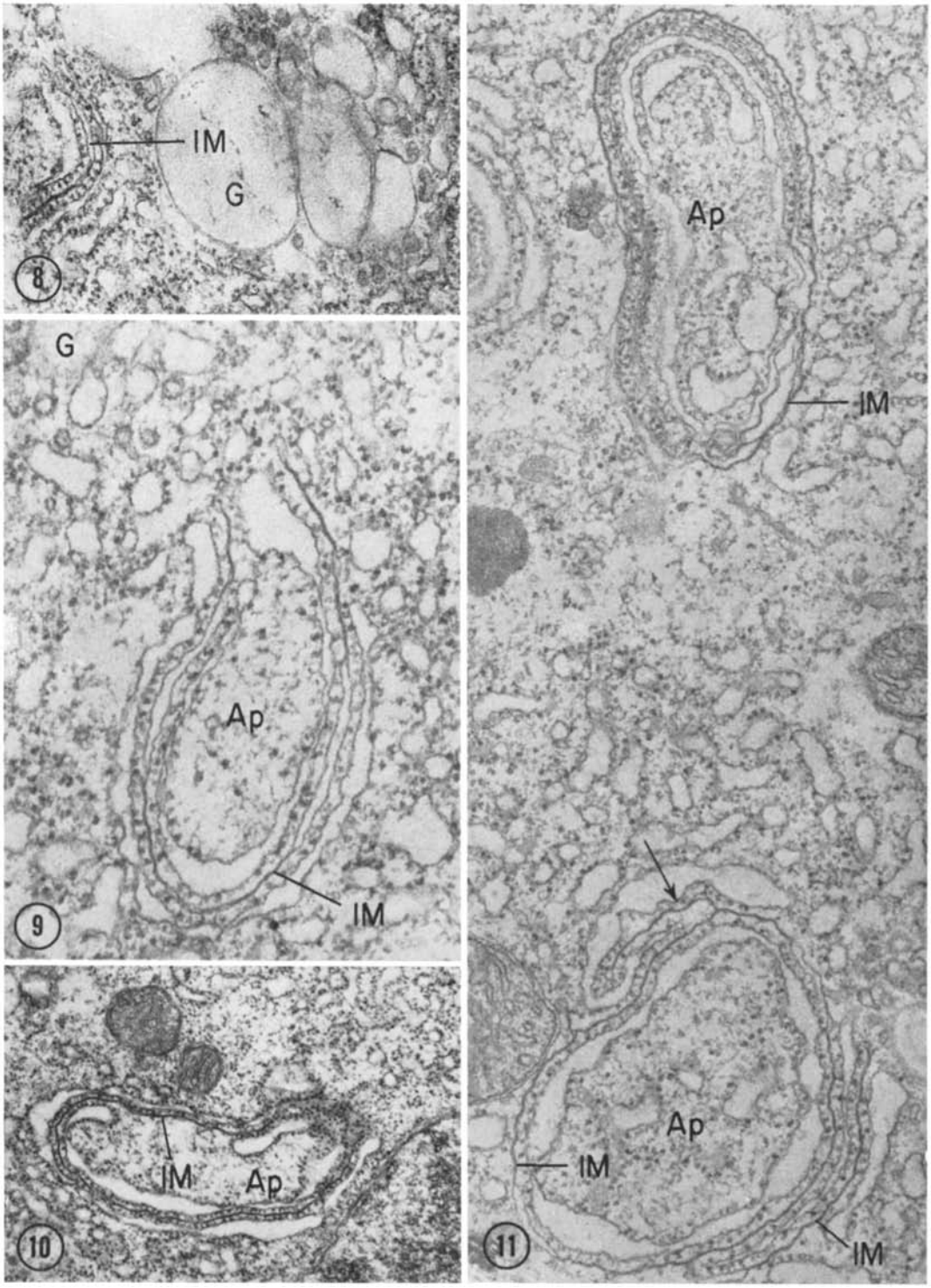
Another interesting finding in the cytoplasm is the appearance of a number of smooth membrane–bounded vacuoles; the cytoplasm thus becomes extensively vacuolated (Fig. 16). These vacuoles are usually elongated and irregular in shape and sometimes are arranged in linear arrays. Some of them may be formed by the deep invagination of the basal plasma membranes or by the marked distension of the intracisternal space of the rough ER. It is also possible that some of these vacuoles are formed by complete autodigestion of the contents of the autolysosomes.

In the nucleus, the chromatin blocks are fused together and greatly condensed. Some of them are attached to the inner nuclear membrane. This state probably corresponds to the so-called pyknosis. The pars amorpha of the nucleoli enlarges further and the nucleoli at this stage are less granular and surrounded by dense nucleolus-associated chromatin as shown in Fig. 17.

LATER PREPUPAL TO EARLY PUPAL STAGE (PREPUPA 84 HR OLD TO PUPA 24 HR

As previously described, the silkworm larva stops spinning at about 72 hr after full maturation and, after another 24 hr, carries out ecdysis in the cocoon to become pupa (prepupal ecdysis). The degradation of the posterior silk gland seems to be further accelerated after the spinning, especially after prepupal ecdysis.

Light microscopical observations clearly show remarkable changes. First, the tunica propria invaginates deeply into the cytoplasm as shown in



FIGURES 8–11 See legends under Fig. 7.

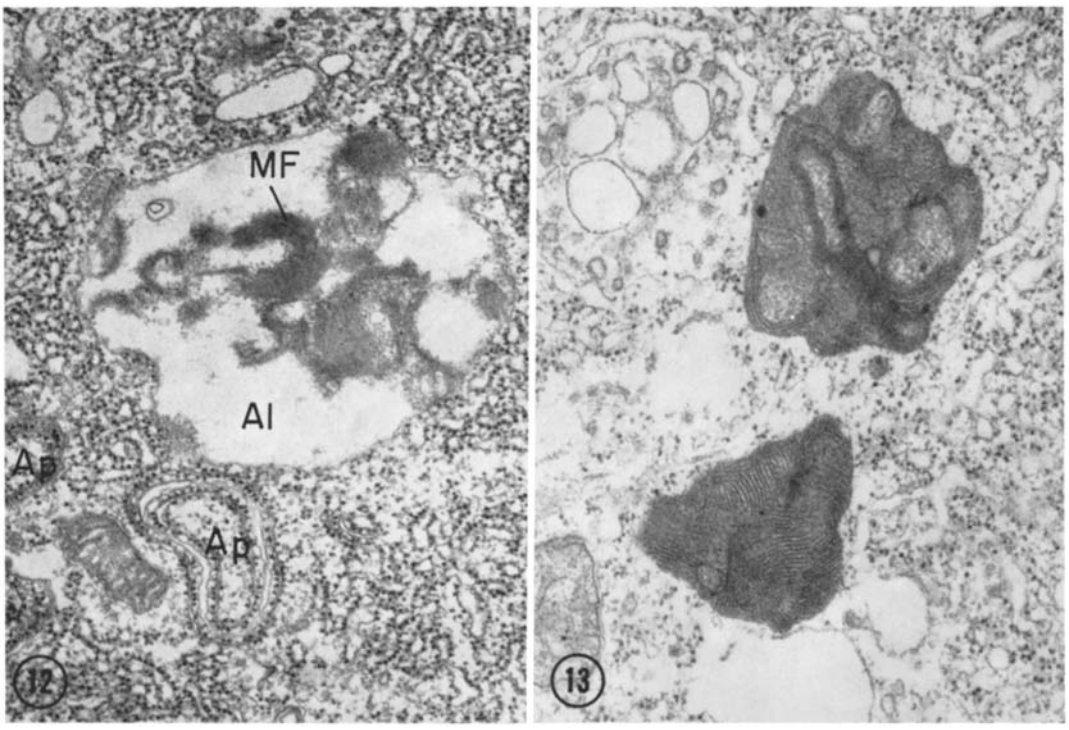


FIGURE 12 An autolysosome (*Al*) in which ribosomes, ER and mitochondria are already extensively digested, and in which residual myelin figures (*MF*) are observed. Some autophagosomes (*Ap*) lie near the autolysosome. Glutaraldehyde-OsO_4 fixation. × 25,000.

FIGURE 13 Lysosomes in the early prepupal stage. They are dense and contain myelin figures. The membrane around each of these lysosomes is not clearly observed in this figure. OsO_4 fixation. × 30,000.

Fig. 5 *c*. The nucleus becomes more and more condensed and the ratio of nuclear volume to cytoplasmic volume increases markedly, probably by the selective degradation of the cytoplasm. This result is consistent with the rapid increase in the amount of DNA per gram wet weight of posterior silk gland as shown in Fig. 3. Sometimes, vacuolization of the cytoplasm is apparent even at the light microscopical level. After prepupal ecdysis, the nucleus disintegrates into a large number of dense globules as shown in Fig. 5 *d*. Since these globules are Feulgen positive (Fig. 6 *d*), it is certain that they contain DNA, and hence they will be called chromatin globules. The cytoplasm is degraded further and disappears almost completely in the early pupal stage.

The most interesting ultrastructural change which appears in the cytoplasm in this stage is the segregation of subcellular components into distinct masses, i.e., partition of the cytoplasm. Sometimes, the surviving cytoplasmic organelles, especially the ER, seem to be preferentially gathered together, and thus a region is formed in which mainly or exclusively the ER exists (Fig. 18). Subsequently, a smooth membrane starts to enclose these regions, and thus large globules containing ER and/or other cytoplasmic organelles are formed. A globule containing exclusively vesicular ER is shown in Fig. 19. These globules probably are a kind of autophagosome, because it is apparent that by this process a part of the cytoplasm is segregated from the rest of the cytoplasm by a smooth membrane. The origin of the smooth membrane is not clear at the present moment. One possibility is that it is derived from the membranes of a number of vacuoles in cytoplasm.

Lipoidal bodies are occasionally observed in this stage as shown in Fig. 20. They are circular or oval in profile, contain a homogeneous matrix of moderate density, and are bounded by a single membrane. These bodies are, therefore, quite similar in appearance to the usual lipid droplets.

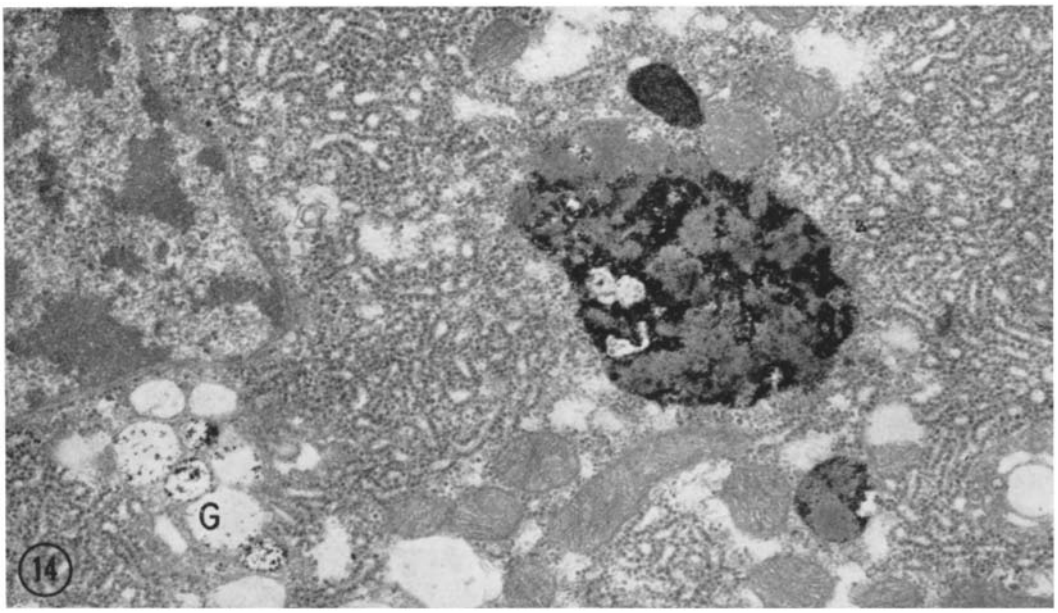


FIGURE 14 Acid phosphatase reaction of the posterior silk gland cell in the prepupal stage 24 hr. The usual lysosomes show a positive reaction. A slight precipitation of lead is also observed in some of the Golgi vacuoles (*G*). × 30,000.

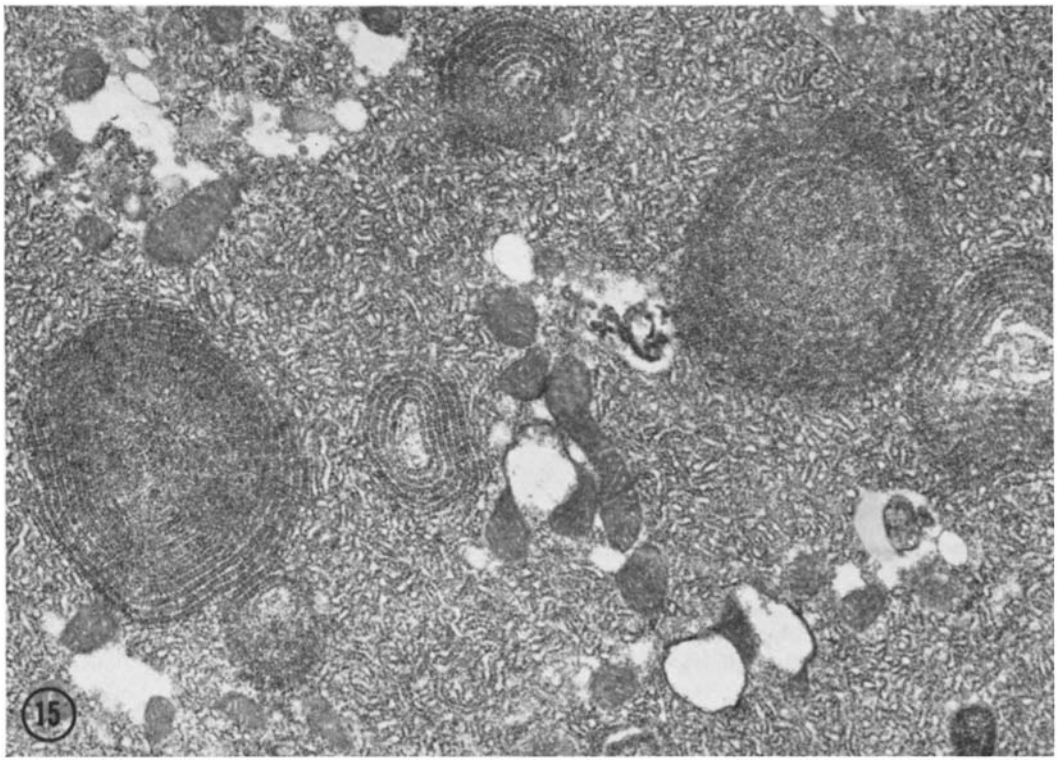


FIGURE 15 Concentric lamellar ER structures observed in the prepupal stage 36 hr. Glutaraldehyde-OsO_4 fixation. × 20,000.

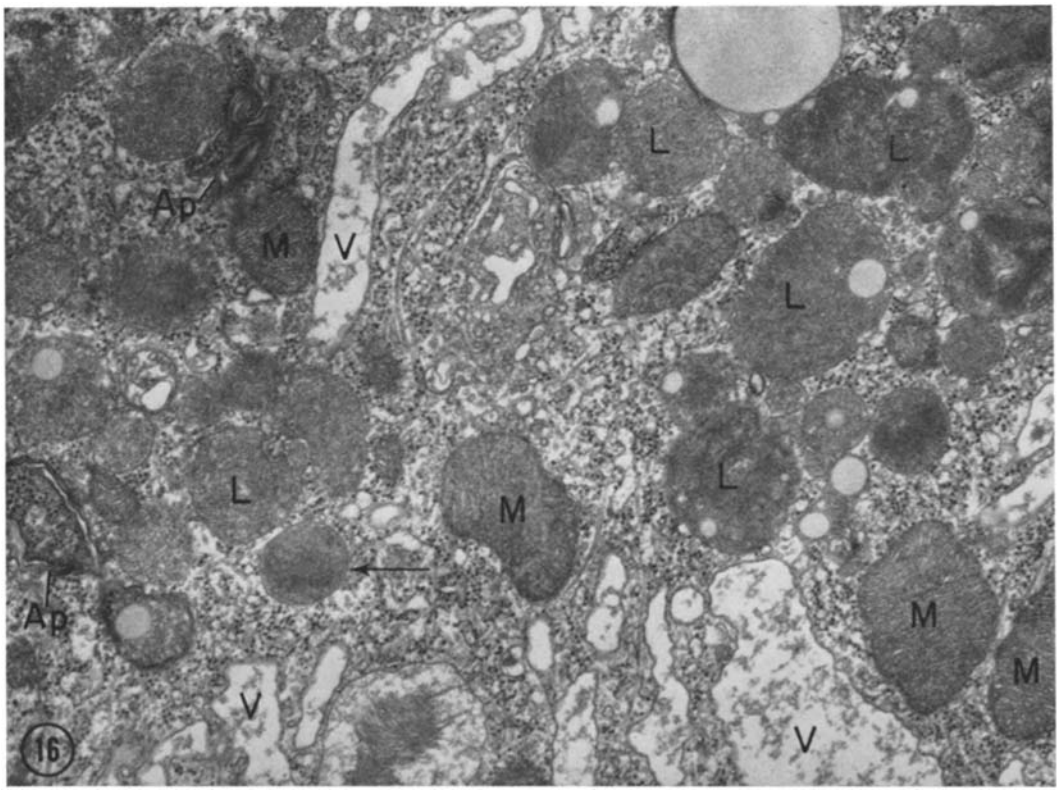


FIGURE 16 Cytoplasm of posterior silk gland cell in the prepupal stage 72 hr. A number of usual lysosomes (*L*) each bounded by a single membrane appear in cytoplasm. Some of them contain myelin figures (arrows) and vacuoles. Several autophagosomes (*Ap*) and mitochondria (*M*) are also observed. Vacuolization (*V*) of cytoplasm is apparent. Glutaraldehyde-OsO_4 fixation. × 26,000.

Some of them, however, contain, in addition to the usual homogeneous matrix, a denser granular matrix at the periphery, and occasionally myelin figures are found in this granular matrix (Fig. 20). The proportions of these two matrices differ from one body to another, and in the extreme case bodies filled exclusively with the denser granular matrix are found. Such bodies are difficult to distinguish from typical lysosomes (compare Figs. 16 and 20).

The intranuclear condensation of chromatin blocks becomes much more pronounced, and at the same time a partition of the nucleus, similar to the partition of cytoplasm, starts to appear as shown in Fig. 21. That is, the nucleus is divided into smaller parts which are then enclosed by a smooth membrane which invaginates deeply into the partitioned nucleus. Thus autophagosomes containing chromatin blocks are formed which most probably correspond to the dense globules observed by light microscopy (Fig. 5 *d*). Frequently, a part of the cytoplasm is segregated together with a part of the nucleus and thus autophagosomes containing a part of nucleus as well as a part of cytoplasm are also found.

These autophagosomes formed in the later prepupal or in the early pupal stage by the partition of both the cytoplasm and the nucleus of the cells are different in some respects from the early autophagosomes and therefore will be called "late autophagosomes." They are probably released into the hemolymph. The extensive formation of these late autophagosomes leads to the complete disintegration of the cellular organization of the posterior silk gland in the pupal stage.

DISCUSSION

During the metamorphosis of the silkworm, *Bombyx mori*, from larva to pupa and from pupa to adult, a

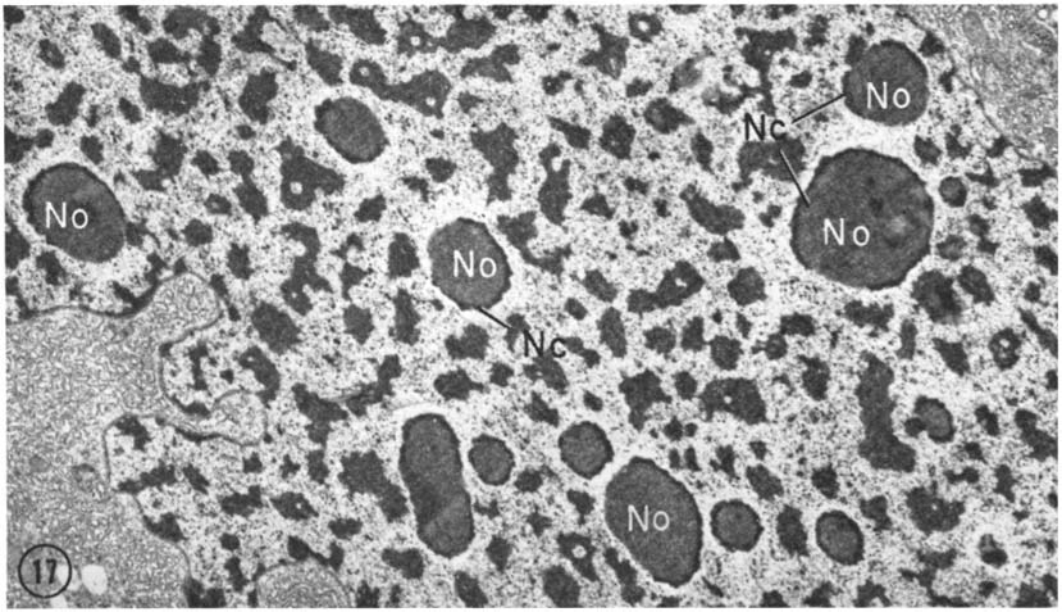


FIGURE 17 Nucleus of a posterior silk gland cell in the prepupal stage 72 hr. The pars amorpha occupies most of each of the nucleoli (*No*). Nucleolus-associated chromatin (*NC*) is observed at their periphery. Glutaraldehyde-OsO_4 fixation. × 5,000

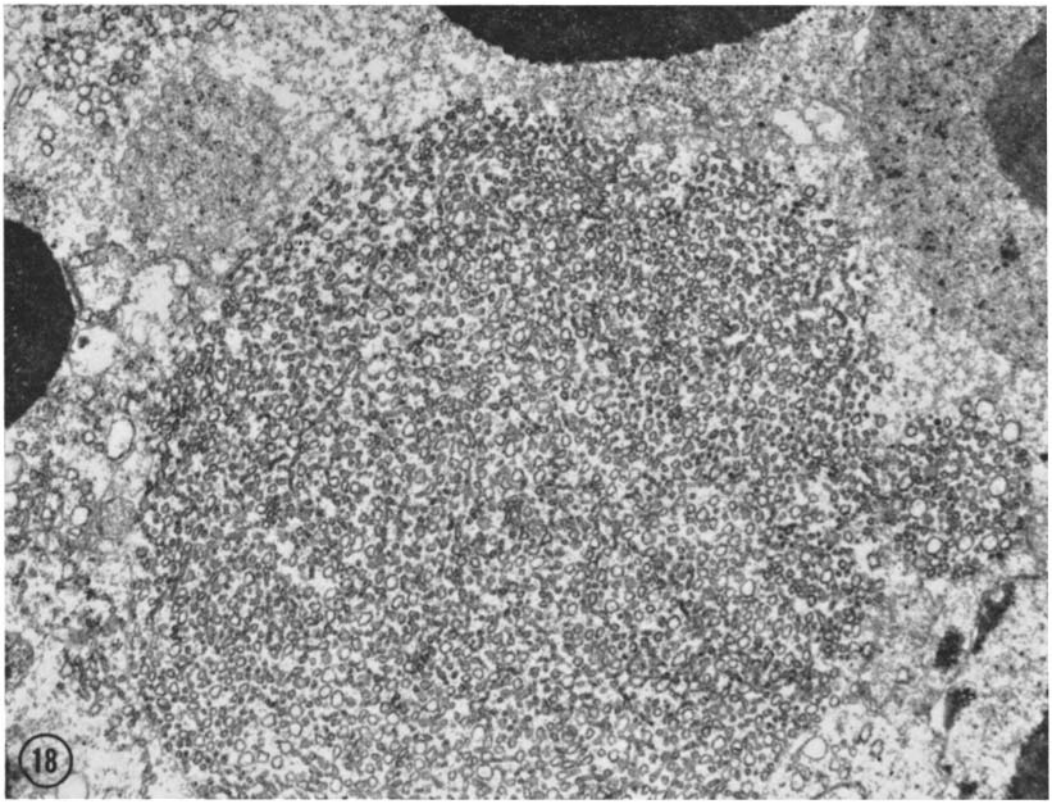


FIGURE 18 Segregation of subcellular components in the cytoplasm of a posterior silk gland cell at 96 hr of the prepupal stage. Vesicular ER elements are aggregated and are partly surrounded by a smooth membrane. Glutaraldehyde-OsO_4 fixation. × 9,300.

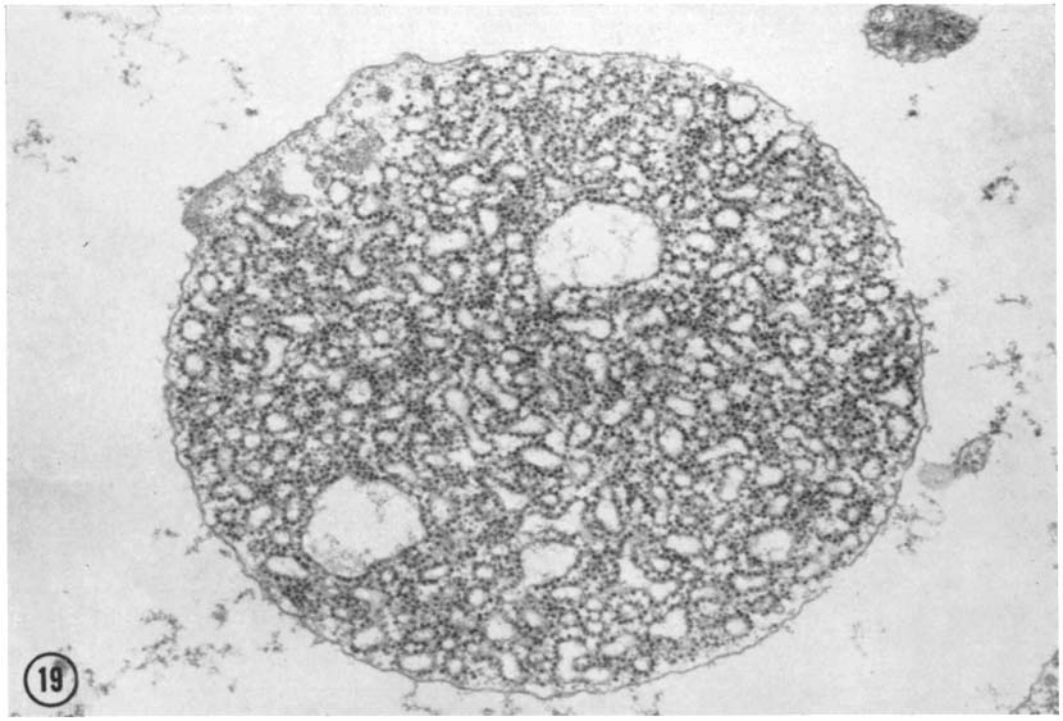


FIGURE 19 An autophagosome observed in the degraded posterior silk gland at 24 hr of the pupal stage. This globule contains exclusively rough ER. Glutaraldehyde-OsO_4. × 25,000.

number of larval organs disappear and a number of new adult organs are formed. In the prepupal and pupal stages, the insect does not eat and yet the new organs for the adult insect must be formed. So that such events can be made possible, macromolecules composing larval organs must be decomposed to smaller molecules before reutilization. The silk gland of the silkworm is one of the most important organs which are degraded during metamorphosis, and it is interesting to study the cellular or subcellular mechanisms by which the posterior silk glands undergo autodegradation.

The first organelles which appear or increase markedly in number in the cytoplasm at the beginning of the prepupal stage are autophagosomes containing ER, or early autophagosomes as we have called them. The formation of autophagosomes, of course, is not a special phenomenon for insect metamorphosis but is commonly found under various conditions, as has recently been reviewed by de Duve and Wattiaux (6). Important questions now arise: why these autophagosomes should appear or increase in the cytoplasm at the beginning of prepupal stage and what the function of these bodies is in the course of programmed death of the cells. In the early half of the prepupal stage, the cells are still functioning and fibroin is synthesized. One possibility is, therefore, that the ER which has suffered from some damage is enclosed within autophagosomes so that the pathological changes can be localized as in the case of usual focal degradation. Another possibility is that the autophagosomes are formed to degrade ER selectively. Since the cell organelles which are enclosed within the early autophagosomes are exclusively elements of the ER, it is likely that some of the ER of the cells can be degraded in this way. The volume of cytoplasm which is enclosed within the early autophagosomes, however, is probably not more than a small percentage of the total volume of cytoplasm, and the process of the enclosing of ER itself does not seem to play an important role in the over-all degradation of ER. Further studies will be necessary to solve this question.

Another important organelle which increases in number in the cytoplasm in the prepupal stage is the usual lysosome. This is to be expected because

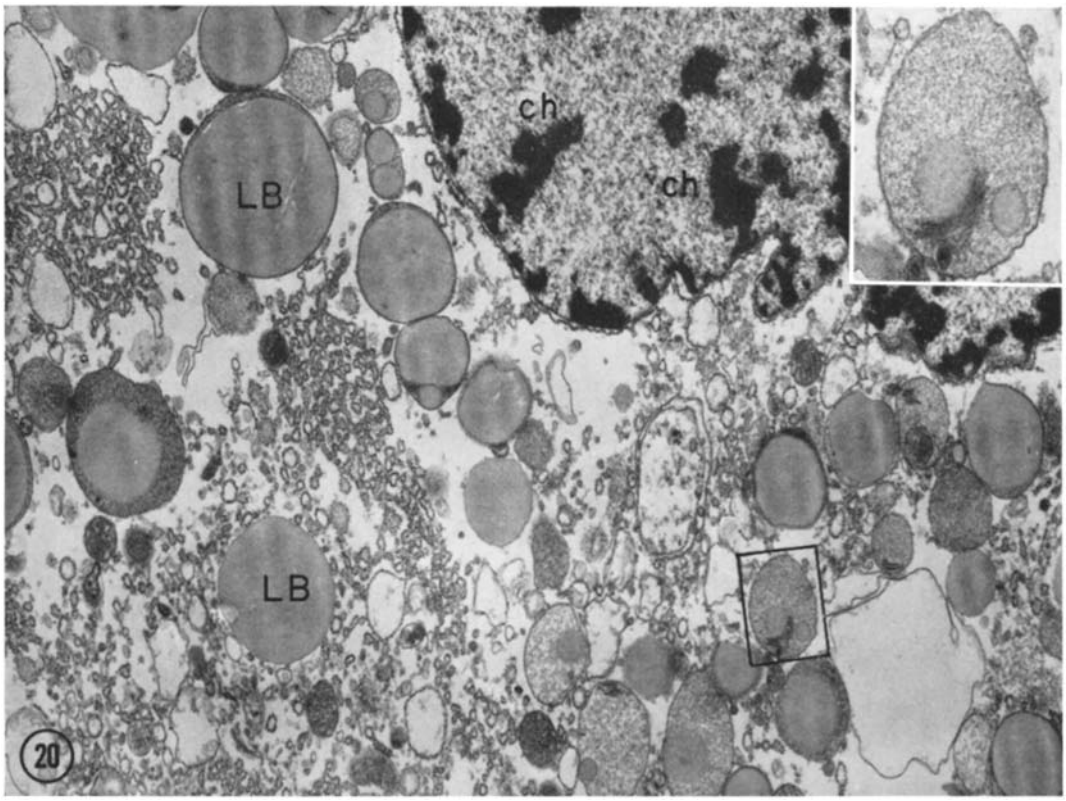


FIG. 20 A number of lipoidal bodies (*LB*) observed at 96 hr of the prepupal stage. Most of them contain a homogeneous matrix of moderate density. Some of them, however, contain, in addition to the homogeneous matrix, a denser granular matrix at the periphery. Occasionally, myelin figures (see insert) are found in this granular matrix. *Ch*, condensed chromatin blocks. Glutaraldehyde-OsO_4 fixation. Fig. 20, × 10,500; insert × 26,000.

the amounts of RNA, protein, and total lipids in the posterior silk gland decrease rapidly in the prepupal stage as shown in Fig. 2. Probably the enzymes which are released from the lysosomes are mainly responsible for the rapid degradation of these compounds.

In the later prepupal or early pupal stages, an important change occurs in both the cytoplasm and the nucleus of the cells. This change is the formation of a number of late autophagosomes containing ER and/or mitochondria or condensed chromatin blocks. These autophagosomes are subsequently released into the hemolymph. Thus structural elements of the cells which have survived until the later prepupal or early pupal stage are completely decomposed by such a procedure. This process seems to be especially important for the disintegration of the nucleus because most of the DNA in the cell is preserved until this stage of involution (Fig. 2). It is interesting to point out here that those late autophagosomes which contain exclusively ER are very similar in appearance to the RNA granules described by Locke and Collins (9). The modes of formation of the two granules, however, seem to be quite different; formation of late autophagosomes by the coalescence of early autophagosomes has never been observed in the cells.

It has been pointed out that macrophages play an important role in the metamorphosis of the tadpole tail (11). We have observed that the posterior silk gland is tightly covered by tunica propria, at least until the prepupal ecdysis and, moreover, we could not find any macrophage in the posterior silk gland during the prepupal stage. We can safely conclude, therefore, that at least

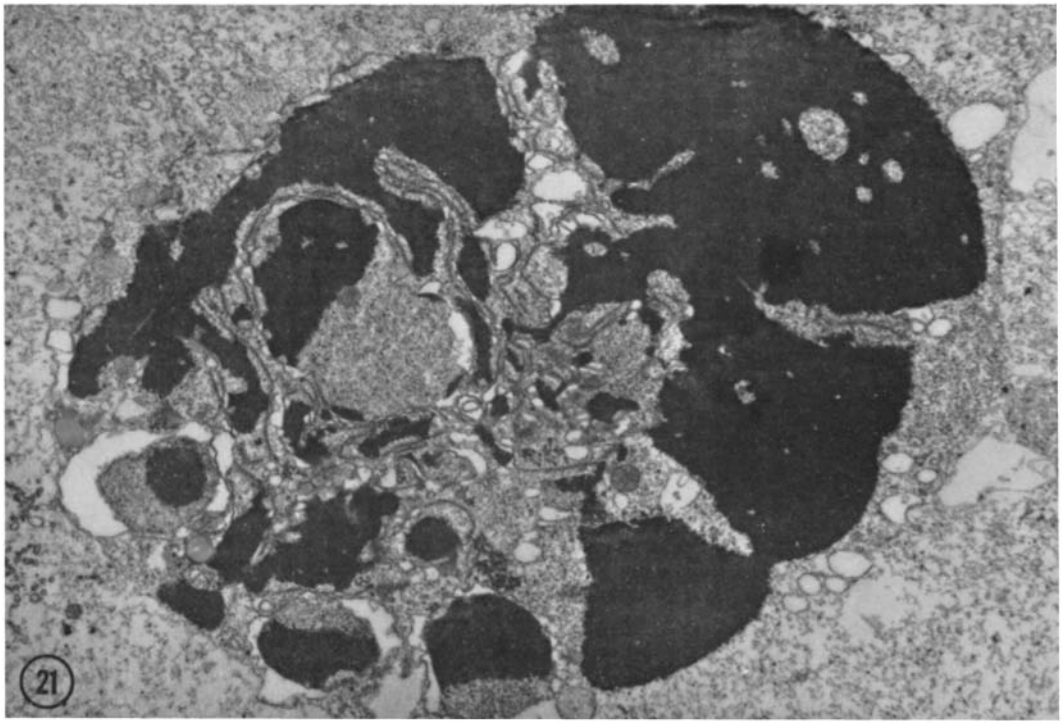


FIGURE 21 Degradation of nucleus in the posterior silk gland at 96 hr of the prepupal stage. Markedly condensed chromatin blocks are partitioned and partially surrounded by smooth membranes. Glutaraldehyde-OsO_4 fixation. × 6,500.

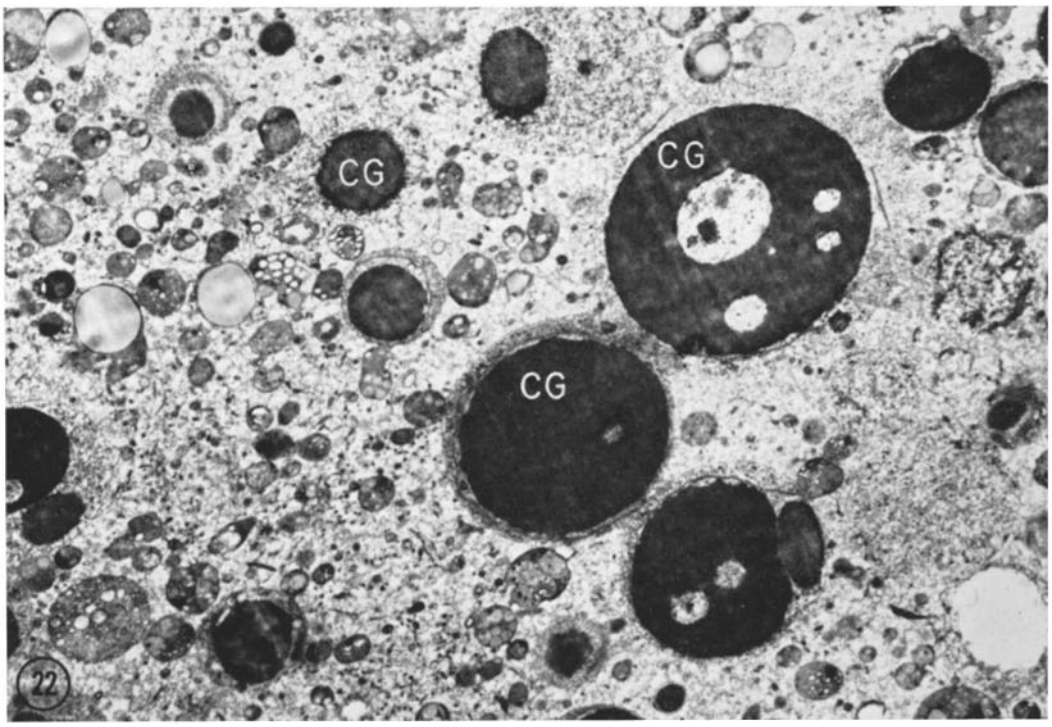


FIGURE 22 Autophagosomes containing chromatin blocks, or chromatin globules (*CG*) as we have called them, are observed in the degraded posterior silk gland at 24 hr of the pupal stage. Glutaraldehyde-OsO_4 fixation. × 3,200.

during this stage phagocytosis does not play any role in the cytolytic processes of the cells.

We should like to thank Professor A. Inouye of Kyoto University for his encouragement and Doctors T. Matsumoto and K. Hayashiya of Kyoto University of Industrial Arts 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.

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