An Electron Microscope Study of the Formation of the Yolk Globule in the Oocyte of Zebrafish, *Brachydanio rerio**

Kiichiro YAMAMOTO** and Isao OOTA**

Yolk formation is one of the most important events during oogenesis. Thus, for a long time the vitellogenesis of fish oocytes has been studied and many papers have been published on the subject. However, there are many conflicting opinions about the origin of yolk and various processes of formation have been offerred even for the proteid yolk (Raven, 1961). Because of the very small size at the time of formation, the proteid yolk, as well as related organelles, are certainly beyond the resolving power of the light microscope and thus these controversies appear to be largely due to the limitations of the microscope.

Recently, Droller and Roth (1966) studied the yolk formation of *Lebistes* oocytes with an electron microscope and they concluded that the proteid yolk is formed by action of the egg's own organelles and by the occurrence of highly specific and selective micropinocytotic processes. Golgi complexes and an endoplasmic reticulum were considered to be the organelles which take part in these processes.

As will be described, however, we obtained in the present study results somewhat different than those of Droller and Roth; namely, that the proteid yolk of zebrafish oocytes originate in modified mitochondria, become, through action of pinocytotic vesicles, the primary yolk globule filled homogeneously with dense, minute particles, and finally grow into mature yolk globules with a crystalloid body.

Before going further, the authors wish to express their hearty thanks to Dr. Stanley H. Bennett, Professor of Chicago University. This study was started during the stay of the senior writer in his laboratory.

Materials and Methods

The ovaries of *Brachydanio rerio* were removed and after being cut in small pieces under immersion in fixatives, they were fixed with cold Millonig's or with Weber's solution for about two hours. Dehydration was performed by the routine ethanol method with two changes of propylene oxide. The tissues were embedded

* This study was supported by a grant from the Scientific Research Fund of the Ministry of Education.

** *Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido.*

in an Epon Epoxy resin mixture. Sections were cut on a Porter-Blum microtome with a diamond or glass knife at a thickness of about 400 to 700 Å, and stained by Karnovsky's lead method or by a double staining method using both a saturated uranyl acetate solution (1 to 2 hours) and cacodylate lead of Karnovsky solution (5 to 10 minutes). Photographs were taken with a Hitachi HS-7 electron microscope.

Observations

As already noted by Malone and Hisaoka (1963), yolk vesicles are fairly well formed in an oocyte of zebrafish before the yolk globules begin to appear in the cytoplasm. The oocyte, just before the formation of yolk globules, attains a diameter of about 300 μ (Fig. 1) and is covered completely with a layer of follicular epithelial cells and of theca cells. Between the oocyte and the follicle, a zona radiata is found, 0.8 to 1.0 μ thick and of high electron density. Yolk vesicle, which have grown to about 20 μ in the largest dimension, fill the greater part of the cytoplasm except for a narrow perinuclear zone. The vesicles are almost spherical in shape and are enclosed by a limiting membrane. The interior of the vesicle is sparsely filled with fine granules and has a very low electron density. Occasionally, a moderately dense, crescent-shaped object may be seen in the periphery of the vesicle. Numerous mitochondria are also found distributed throughout the cytoplasm, round to rod-like in form or sometimes filamentous. The round mitochondria measured 0.25 to 0.7 μ in diameter while the large filamentous ones were about 0.4 μ wide by 4.0 μ long. The structure of these mitochondria presents no special features except for cristae which are usually small in number and often appear in an irregular arrangement rather than a straight, parallel one. The matrix of the mitochondria is moderate in density and one to several intramitochondrial granules are found embedded within the matrix.

Golgi complexes are also recognizable in the cytoplasm, but are less in number than in the younger oocytes (Fig. 1). Generally they are located near the yolk vesicles and are composed of three to five flattened sacs and many vesicles with or without Golgi vacuoles. The endoplasmic reticulum of a granular form develops moderately. They are round or tubular in shape and are distributed throughout the cytoplasm. Free ribosomes are abundant throughout the cytoplasm. Pinocytotic vesicles, moderate in density and smaller than 150 mμ in diameter, are found mostly in the periphery of the cytoplasm.

At a somewhat later stage, marked changes in the inner structure of the mitochondria are recognizable. Fig. 2 shows mitochondria in this stage of develop-

ment. They are generally round or rod-like in shape but with a conspicuously uneven contour. Their cristae become obscure and in some mitochondria it is difficult to detect a definite arrangement of cristae. Their matrix increases in electron density. Intramitochondrial granules, about 50 mμ in size, are seen commonly within the mitochondria. Besides these granules, a few small dense particles of approximately 160 Å are found within the matrix frequently forming a regular row or small groups with an indefinite pattern. These changes in the inner structure of the mitochondria are commonly seen in oocytes at this stage of development.

When the oocytes become about 300–350 μ in diameter, bodies of a new type appear in the cytoplasm of the oocyte (Fig. 3), almost spherical in shape, 0.5 to 1.2 μ in diameter and usually enclosed within a limiting membrane. The contents of the body are apparently homogeneous, but vary widely in electron density. These bodies appear to originate in the mitochondria and numerous intermediate stages between the mitochondria and this new type of body can be found.

Another characteristic feature in the oocytes in this stage of development is the abundant occurrence of pinocytotic vesicles. The same vesicles as seen in the previous stage, moderate in density and smaller than 150 mμ in diameter, are numerous, but especially dense in the periphery of the oocyte. In addition to these vesicles, there is still a second type of vesicle found in the oocytes, round to ellipsoid in shape, about 400 mμ along the long axis, dense in content and with a limiting membrane. These vesicles may also be an extra-oocyte product taken in by the oocytes due to pinocytosis. As known in *Lebistes* (Droller and Roth, 1966), many pits filled with a dense substance appear under the edge of the zona radiata which eventually pinch off from the plasma membrane to lie free within the substance of the egg. Thus, the pinocytotic vesicle of the second type also occur widely throughout the cytoplasm.

Fig. 4 shows the mitochondria in an early stage of modification. They are almost spherical in form, smooth in contour and measure 0.4 to 0.5 μ in dimension. The cristae almost disappear from sight. The greater part of the mitochondria is enclosed within double limiting membranes, but the duality of the limiting membrane is lost in some places. The matrix of the mitochondria is homogeneously granular and increases in electron density. Within the matrix intramitochondrial granules and clusters of small particles may also be seen as in the previous stage.

Fig. 5 shows one of the mitochondria in a little later stage of modification. The mitochondrion is nearly round in shape and about 0.9 μ in size. The greater part of the organelle is enclosed by a single membrane, but a part still retains

the duality of the limiting membrane. The contents of the body are granular in texture and moderate in density. Cristae and intramitochondrial granules are not recognized in the body, while in some places dense, slender particles approximate 100 to 200 Å long and 25 to 50 Å wide are found arranged in several parallel bands about 0.1 μ long. Careful observations reveal that small vesicles, round to oval, about 150 mμ in size and moderate in density, are found attached to the organelle or seemingly in the process of being engulfed by the mitochondrion. From size, shape and contents there remains little doubt but that these vesicles are related to the pinocytotic vesicles that are so numerous in the periphery of the oocyte during this stage of development.

In the organelles at a little later stage, it is difficult to detect the characteristics of the mitochondria. As seen in Fig. 6, they have completely lost the duality of the limiting membrane and are now enclosed by a single membrane. However, there is no doubt but that these bodies were derived from the modifying mitochondria. Except for the limiting membrane, the body is quite similar to the modifying mitochondria mentioned above. The little larger size and the greater number of clusters of dense particles are the only differences seen. This body may now be interpreted as a precursor of the yolk globules.

It is significant to note that in addition to the pinocytotic vesicles seen in the previous stage, vesicles of a new type frequently are concentrated in the area around the body. The granules are oval to round in shape, about 300 mμ in the largest dimension, of very high density and enclosed within a limiting membrane. They must be a pinocytotic vesicle of the second type as seen in Fig. 3. Many figures can be found suggestive of vesicles being taken into the body. Some vesicles are found attached to the precursor of yolk globules and at the point of contact the limiting membrane of both the precursor body and the vesicle disappears from sight. Thus, the vesicular contents become directly connected with the matrix of the body. After being taken into the body, the vesicles seem to gradually dissolve and the dense granules cannot be found in the inner region of the body.

Intermingling with these bodies is another kind of body with a much higher electron density than the yolk precursors mentioned above. These bodies are enclosed within a limiting membrane and are filled with dense slender particles which are almost similar to small clusters of particles seen in the precursor of the yolk globules. Frequently there is a narrow region with low electron density in the periphery of the body (Fig. 7). Thus, there is no doubt but that these bodies are yolk globules in an early stage of development and therefore are designated as the primary yolk globules.

When the oocyte reaches the size of about 400 μ in diameter, yolk globules of a mature type are found composed of three components: a main body, a superficial layer, and a limiting membrane (Fig. 8). The main body has a high electron density and one to several can be recognized within a single yolk globule. Generally, the small yolk globules have a single main body rod-like in form, while the large yolk globules contain two or more main bodies of very complicated form. As already reported by the writers (Yamamoto and Oota, 1967), the main body has a crystalline pattern. The profile of the main body is either in a rectangular net pattern or a parallel band pattern. The rectangular net pattern consists of highly ordered light meshes of ca. 50 Å high and 150 Å wide and dense threads about 25 to 50 Å wide (Fig. 9). The parallel band pattern is composed of parallel, straight, alternately dense and less dense bands whose center-distance is ca. 175 Å in some cases and ca. 125 Å in others (Figs. 10 and 11). From their findings, Yamamoto and Oota (1967) believe that the main body may be composed of cuboid units closely arranged side by side with an orthorhombic crystalline form.

When the yolk globule contains several main bodies, the crystalline patterns are not always orientated the same, but usually differ for each main body (Fig. 12). This suggests that the yolk globule becomes larger by the growth of each globule and by the fusion of several globules. In figure 13 and 14 are shown yolk globules apparently just after the fusion of two smaller globules.

A superficial layer surrounds the main bodies of each globule and is medium in density because of a moderate distribution of fine granules. The granules are of high density, round in form and about 50 Å in diameter.

Near the main body the particles display a gradual change from a disordered pattern to the regular periodic pattern of the main body (Fig. 15). This suggests that the superficial layer is where synthesis of the elements in the main body takes place.

The limiting membrane, about 60 Å thick, is usually found surrounding both the superficial layer and main body. Sometimes the membrane shows the triple-layered profiles characteristic of the unit membrane (Fig. 16), but frequently the membrane is only a series of small vesicles. At time it is difficult even to detect the presence of a membrane.

With the growth of oocytes the yolk globules become more numerous and gradually enlarge until they occupy most of the cytoplasm.

Discussion

One of the reasons why many workers have arrived at conflicting opinions

about the origin of yolk may be attributed to the obscure distinction of yolk components studied. As summarized by Raven (1961), yolk may be separated by its chemical nature into three main components: carbohydrate yolk, fatty yolk and proteid yolk. The yolk globule discussed in this paper corresponds to the proteid yolk. In general, this component of the yolk appears after the carbohydrate yolk and fatty yolk have been formed; its formation takes place most actively during the late phase of vitellogenesis. In teleosts a large number of yolk globules accumulates in the eggs and serves as the most important nutrient for the development of embryos.

Yolk globules begin to appear in the zebrafish oocyte after the yolk vesicle (carbohydrate yolk) has occupied a large part of the cytoplasm and they actively accumulate during the late phase of vitellogenesis until the globules fill most of the oocyte. Yolk globules are rich in lipid and protein (Malone and Hisaoka, 1963). Further, they are characterized by the presence of a main body with a crystalline pattern (Yamamoto and Oota, 1967).

Many studies have been done with the light microscope on the origin of the proteid yolk in teleosts. However, since the precursors of yolk globules are very small in size and are beyond the resolving power of the light microscope, the results obtained have raised many doubtful points. Recently, Droller and Roth (1966) studied the vitellogenesis of *Lebistes* oocytes with an electron microscope. They concluded that proteid yolk is formed as a result of action of the egg's own organelles (Endoplasmic reticulum and Golgi complex) as well as the occurrence of highly specific and selective micropinocytotic processes. Evidence from the present study, however, does not confirm their conclusion. As mentioned above, in zebrafish oocyte mitochondria are modified at first into the precursor of yolk globules, then into the primary yolk globule and finally into yolk globules with crystalloid bodies. Thus in the present species, the organelles related to the formation of the proteid yolk are believed not to be the Golgi complexes and the endoplasmic reticulum, but mitochondria. This agrees with Malone and Hisaoka's opinion (1963) based on studies with a light microscope.

In the zebrafish oocyte Golgi complexes appear to take an important role in the formation of the carbohydrate yolk (yolk vesicle) similar to the role of the organelles in the goldfish oocyte (Yamamoto and Onozato, 1965). This aspect will be dealt with in detail in another paper.

As already pointed out by Malone and Hisaoka (1963) for zebrafish and by Yamamoto (1958) for several teleosts, it is unlikely that yolk globules originate in the yolk vesicles. These two components of yolk differ markedly in their chemical nature even in the early stages of development. Moreover, it is evident

from the present findings that the yolk globules accumulate a crystalloid main body until they attain a size of about one micra in diameter while the yolk vesicles containing a light, granular substance were observed even in a large one of more than 20 micra in size.

The mitochondrial origin of the proteid yolk has already been reported in amphibian oocytes by Lanzavecchia (1960, 1966), Ward (1962), Balinsky and Davis (1963) and Tokins (1964), and in molluscan oocytes by Carraso and Favard (1958). However, the way in which mitochondria are transformed into the proteid yolk in the oocyte of zebrafish seems to be quite different than those observed by the above mentioned authors. The proteid yolk in those species have also main bodies with a crystalline structure. According to Lanzavecchia (1960, 1966), Ward (1962), Tokins (1964) and Carraso and Favard (1958), the crystalline bodies in these species appear early in the mitochondria with double limiting membranes and a few intact cristae. While, the crystalline bodies of the zebrafish can be demonstrated only in the mature yolk globules through the primary yolk globules which are homogeneously packed with fine particles. In *Xenopus*, the proteid yolk with a crystalline body is also formed through the primary yolk platelets composed of a non-crystallized substance and an enclosing membrane (Balinsky and Davis, 1963). Between these two species, however, the processes of mitochondrial transformation into the primary yolk are somewhat different. In *Xenopus*, the cristae of mitochondria are changed into stacks of concentric membranes and then transformed into a more densely packed body similar to the component of the primary yolk platelets, while in zebrafish mitochondrial cristae become obscure and disappear from sight, along with the formation of minute dense particles which are a main component of the primary yolk globules.

A characteristic feature seen in the present species is the clear participation of pinocytotic substance in the formation of yolk globules. At the time of yolk formation, the important role of extra-oocyte substance taken into the oocytes by pinocytosis has been emphasized by many workers such as Knight and Schechtman (1954), Glass (1959), Telfer (1960, 1961), Kessel and Beams (1963), Anderson (1964), Ramamurty (1964), Roth and Porter (1964) and Stay (1965). These authors, however, have not referred to the role of the organelles in the formation of proteid yolk. In this respect, their opinions differ markedly from the findings obtained in the present study. As mentioned above, in zebrafish oocytes the small and light pinocytotic vesicles are involved early in the modifying mitochondia, and the large and dense pinocytotic vesicles late in the precursor of yolk globules. The two kinds of vesicles correspond to the pinocytotic substance revealed by Droller and Roth (1966) at the time of yolk formation of

Guppy oocytes. Although Droller and Roth (1966) observed that the pinocytotic substance appears to be deposited within the precursors of yolk globules as it is, the pinocytotic vesicles taken into the precursors of zebrafish oocytes in contrast seem to be dissolved and then resynthesized into crystalloid main bodies. Since Ringle and Gross (1962) have demonstrated nucleic acid present in the yolk platelets of Amphibia and more recently Nass and Afzelius (1965) demonstrated the presence of DNA in mitochondria, the assumption that a crystalline proteid yolk may be synthesized from the vesicular material involved is not unreasonable.

In addition to the above opinions on the origin of the proteid yolk, Beams and Kessel (1962, 1963) insisted that in the crayfish oocyte, the endoplasmic reticulum is the first site where the proteid yolk is synthesized. Further, Watenberg (1962) and Hope *et al.* (1964) ascribes the origin of proteid yolk in amphibia for certain vesicles of unknown origin, Takamoto (1964) for vesicles of nuclear origin and Lanzavecchia (1966) for the multivesicular body. However, no evidence to support the above opinions could be found in the present study. Golgi complexes are also an area where yolk may be synthesized (Afzelius, 1958; Yasuzumi and Tanaka, 1957; Hsu, 1961; Kessel, 1964). As already mentioned above, however, Golgi complexes in zebrafish oocytes appear to assist in the formation of carbohydrate yolk but in the formation of proteid yolk.

Summary

The formation of proteid yolk in the zebrafish oocytes has been studied with an electron microscope and the following results were obtained.

1. When the oocyte attain about 300 μ in diameter, a characteristic modification of mitochondria appears. The gradual disappearance of cristae and intramitochondrial granules, the appearance of dense round particles about 160 Å in diameter and the loss of duality in the limiting membrane occur in succession along with changes in form.

2. The precursors of the yolk globule appear to be formed by modification of mitochondria as well as by small and light pinocytotic vesicles about 150 mμ. The precursors with a single limiting membrane are oval or round in form, 0.7 to 1.2 μ in diameter, granular and of moderate density; they contain small clusters of dense, slender particles about 100 to 200 Å long.

3. The precursors appear to develop into the primary yolk globules with the participation of large and dense pinocytotic vesicles about 300 mμ in size. The primary yolk globules are usually round in form and somewhat larger than the precursors. They are homogeneously filled with minute particles of high density and enclosed within a limiting membrane.

4. When the oocyte grow to about 400 μ in diameter, yolk globules of a mature type begin to appear in the cytoplasm. They consist of three components: one to several main bodies with a crystalline pattern, a superficial layer embedded with fine, dense granules and an enclosing membrane. The yolk globules seem to enlarge by both the growth of each globule and the fusion of several small globules.

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Explanation of Plates

Abbreviation

ER-Endoplasmic reticulum; FC-Follicular epithelial cell; G-Golgi complex; IG-Intramitochondrial granule; IP-Intramitochondrial particle; M-Mitochondrion; MB-Main body: PPV-Primary pinocytotic vesicle; PeYG-Precursor of yolk globule; PiYG-Primary yolk globule; R-Ribosome; SL-Superficial layer; SPV-Secondary pinocytotic vesicle; YV-Yolk vesicle; ZR-Zona radiata.

PLATE I

Fig. 1. Low-power micrograph of the oocyte just before the beginning of yolk globule formation. One of the yolk vesicles (YV) which has grown to about 20 μ in maximum size and filled the great part of the cytoplasm is seen in the lower-left corner. $\times$24,800

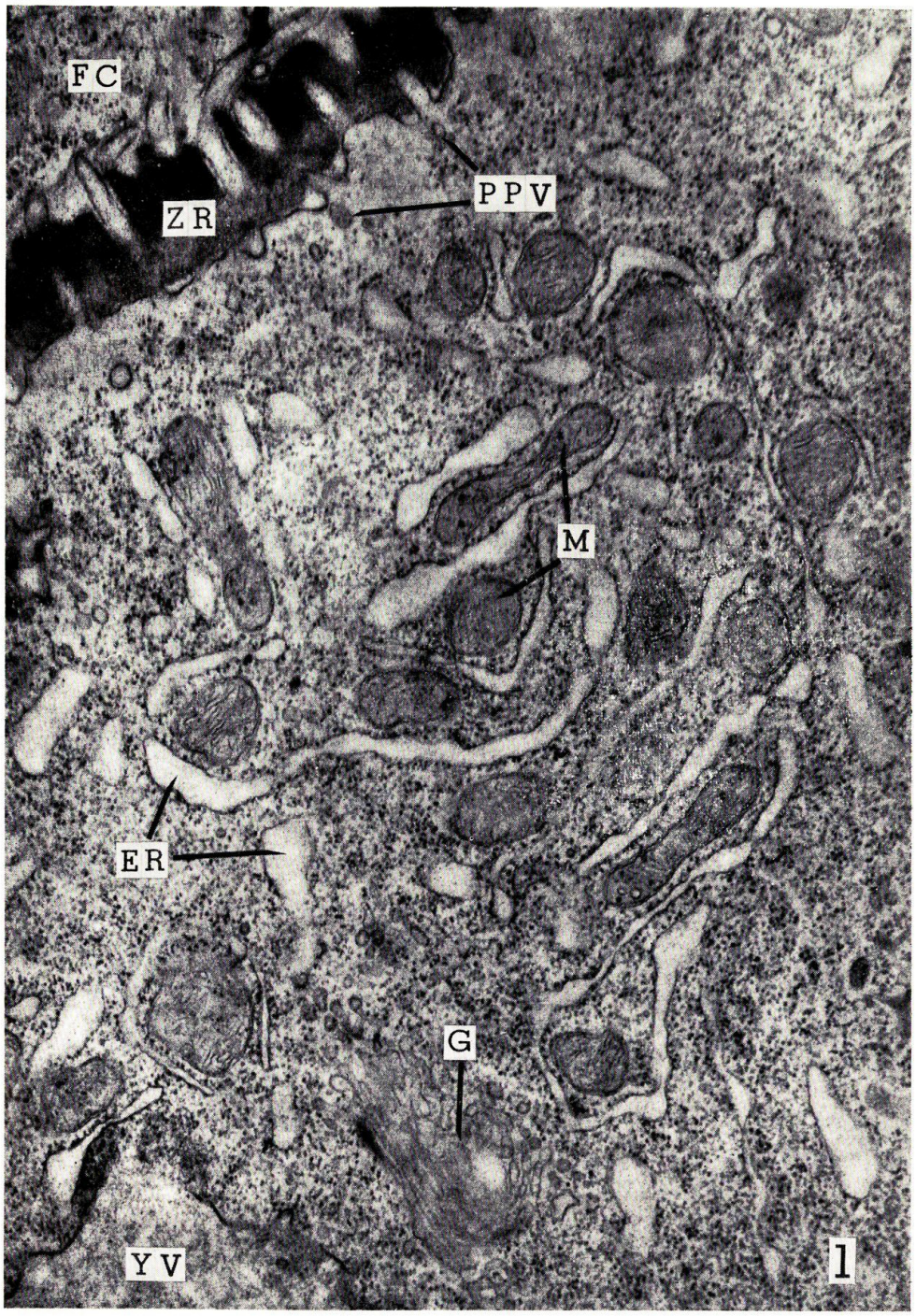

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PLATE II

Fig. 2. Modifying mitochondria of the earliest stage. Small dense particles approximate 160 Å (IP) are found within the matrix of some mitochondria. ×75,000

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PLATE III

Fig. 3. Low-power micrograph of the oocyte at the beginning of yolk globule formation. Two kinds of pinocytotic vesicles, small light vesicles and large dense ones, are seen thoroughly distributed in the cytoplasm, but especially numerous in the periphery. ×13,200

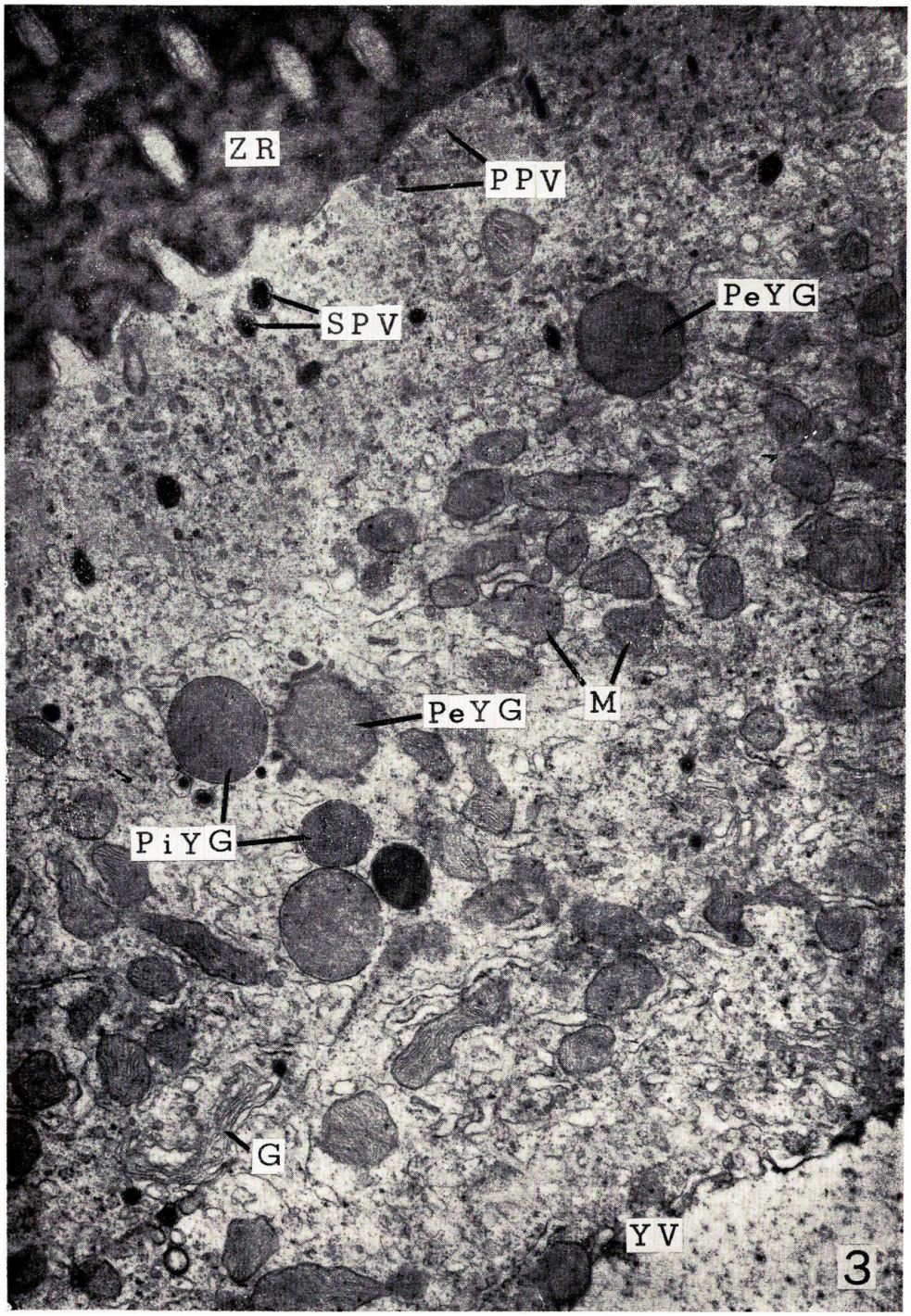

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PLATE IV

Fig. 4. Modifying mitochondria whose cristae have already disappeared from sight. ×100,000

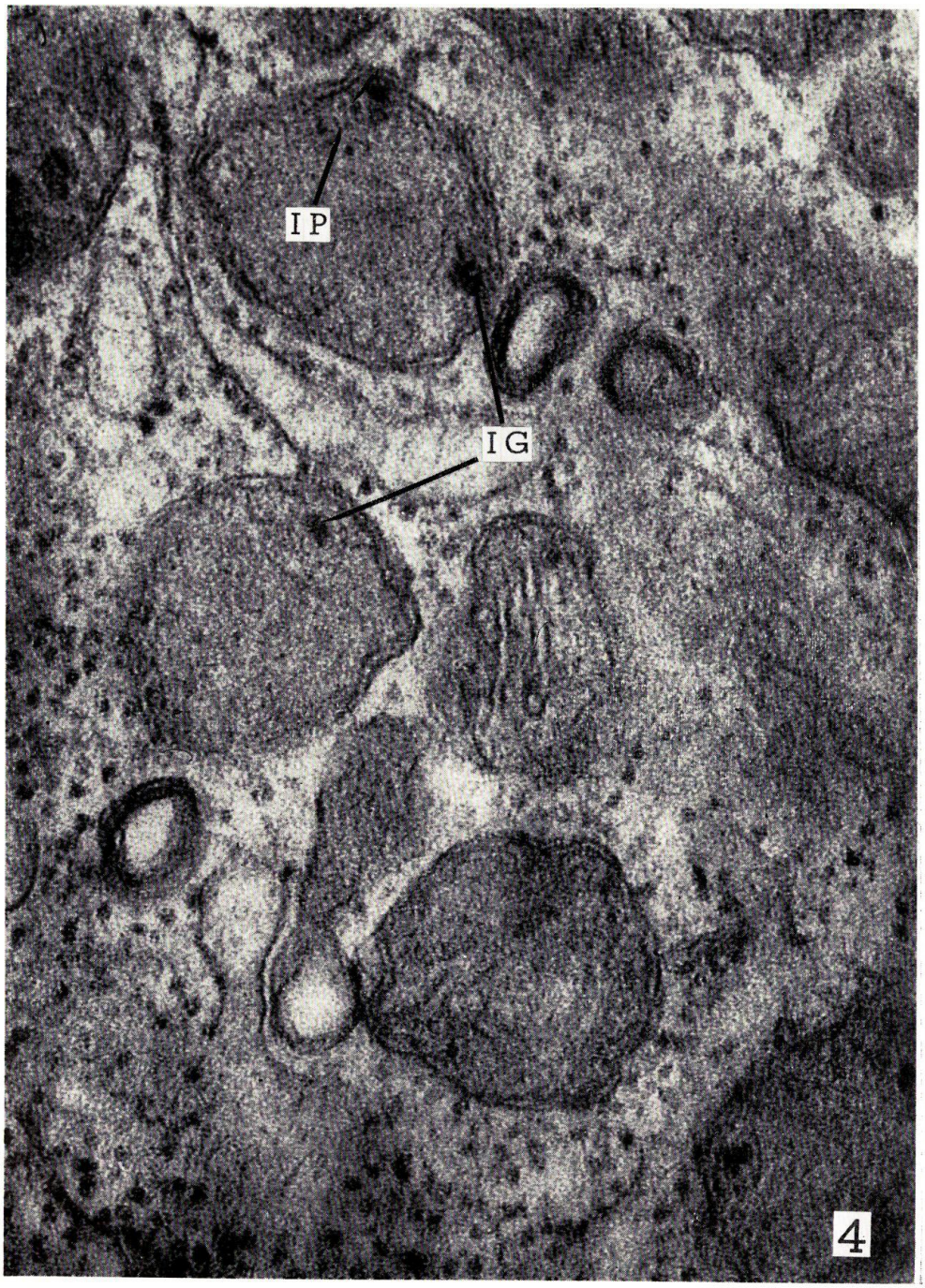

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PLATE V

Fig. 5. Modifying mitochodrion at the latest stage. It has almost lost the duality of the limiting membrane. The dense particles (IP) become numerous and are found arranged in the shape of several parallel stripes. ×100,000

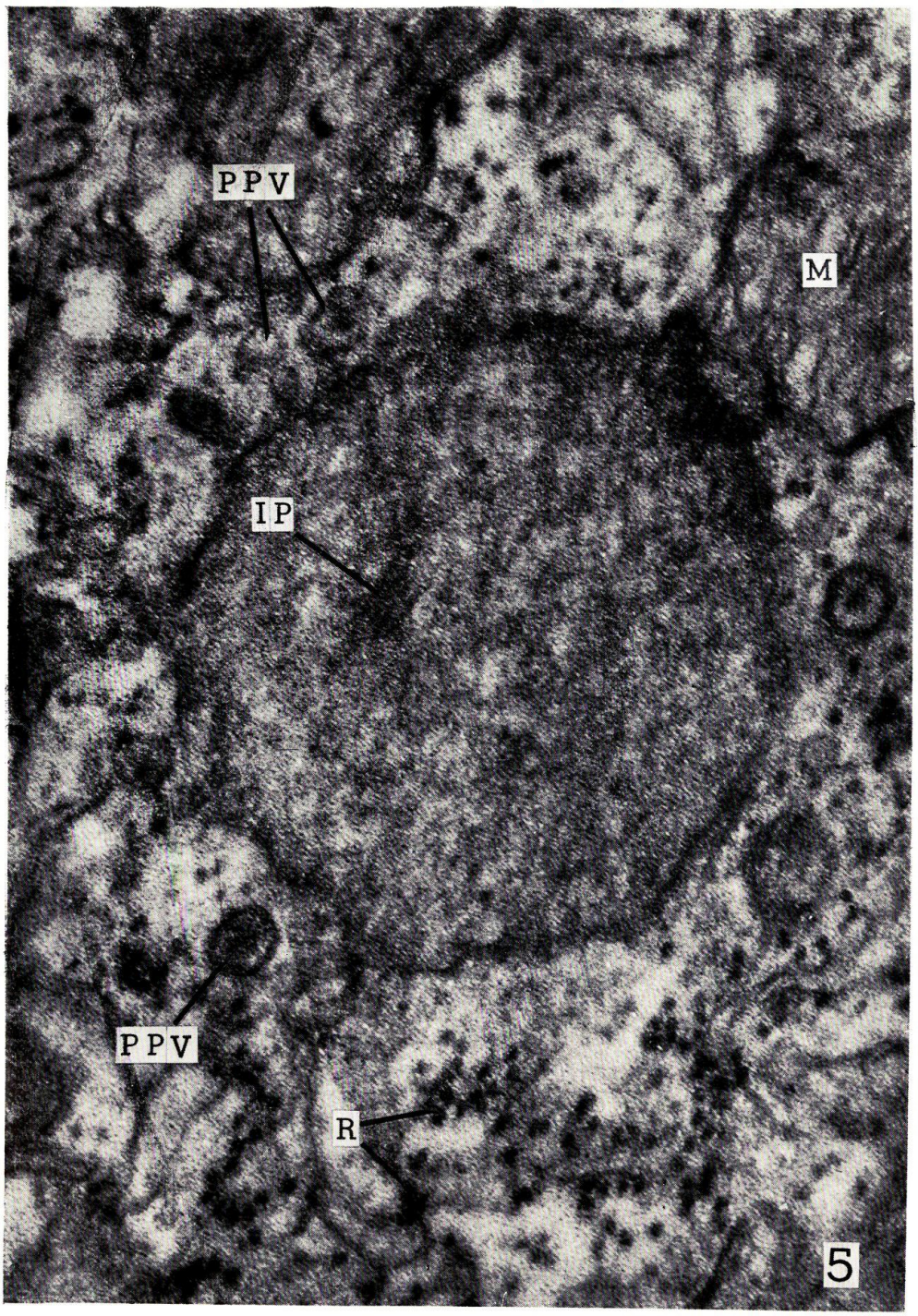

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PLATE VI

Fig. 6. Precursors of yolk globules. The precursors are enclosed totally within a single limiting membrane. Note that many pinocytotic vesicles are attached to the precursors. ×35,000

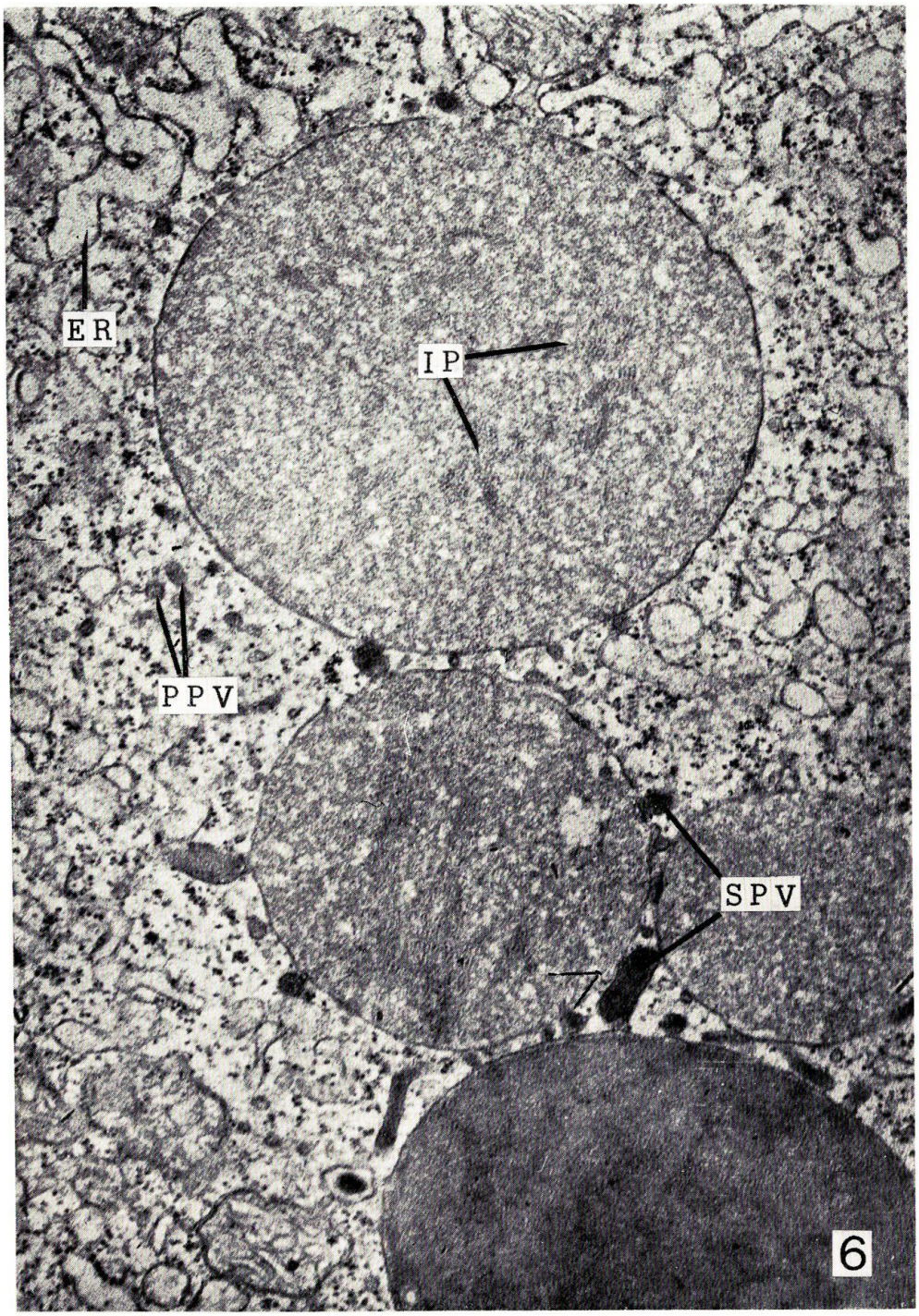

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PLATE VII

Fig. 7. Primary yolk globules. The globule is densely and homogeneously filled with small particles. The arrows indicate the parts of the limiting membrane which show the triple layers characteristic of the unit membrane. ×100,000

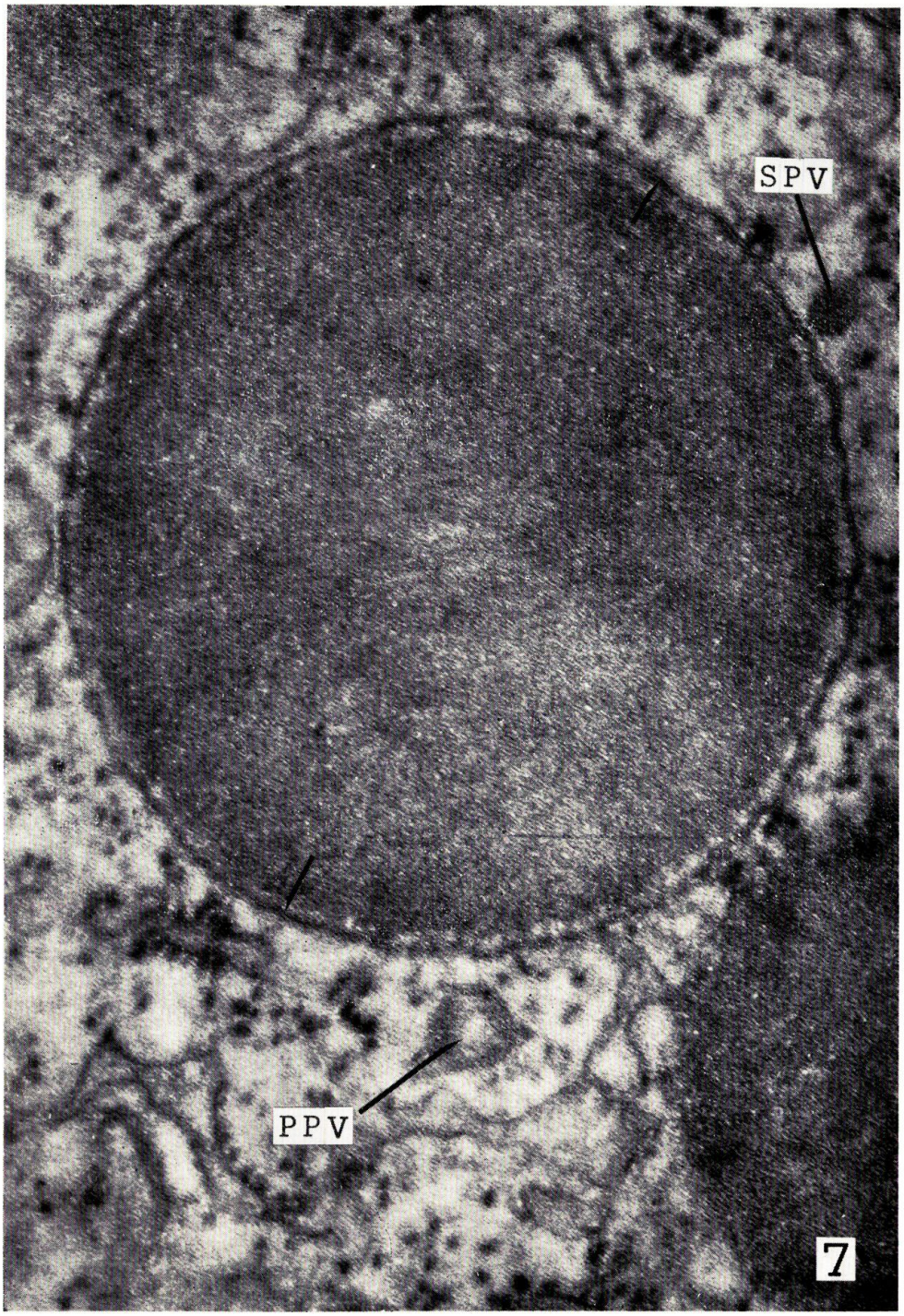

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PLATE VIII

Fig. 8. Low-power micrograph of mature yolk globules. The yolk globule consists of three components: a main body (MB), a superficial layer (SL) and a limiting membrane. ×6,800

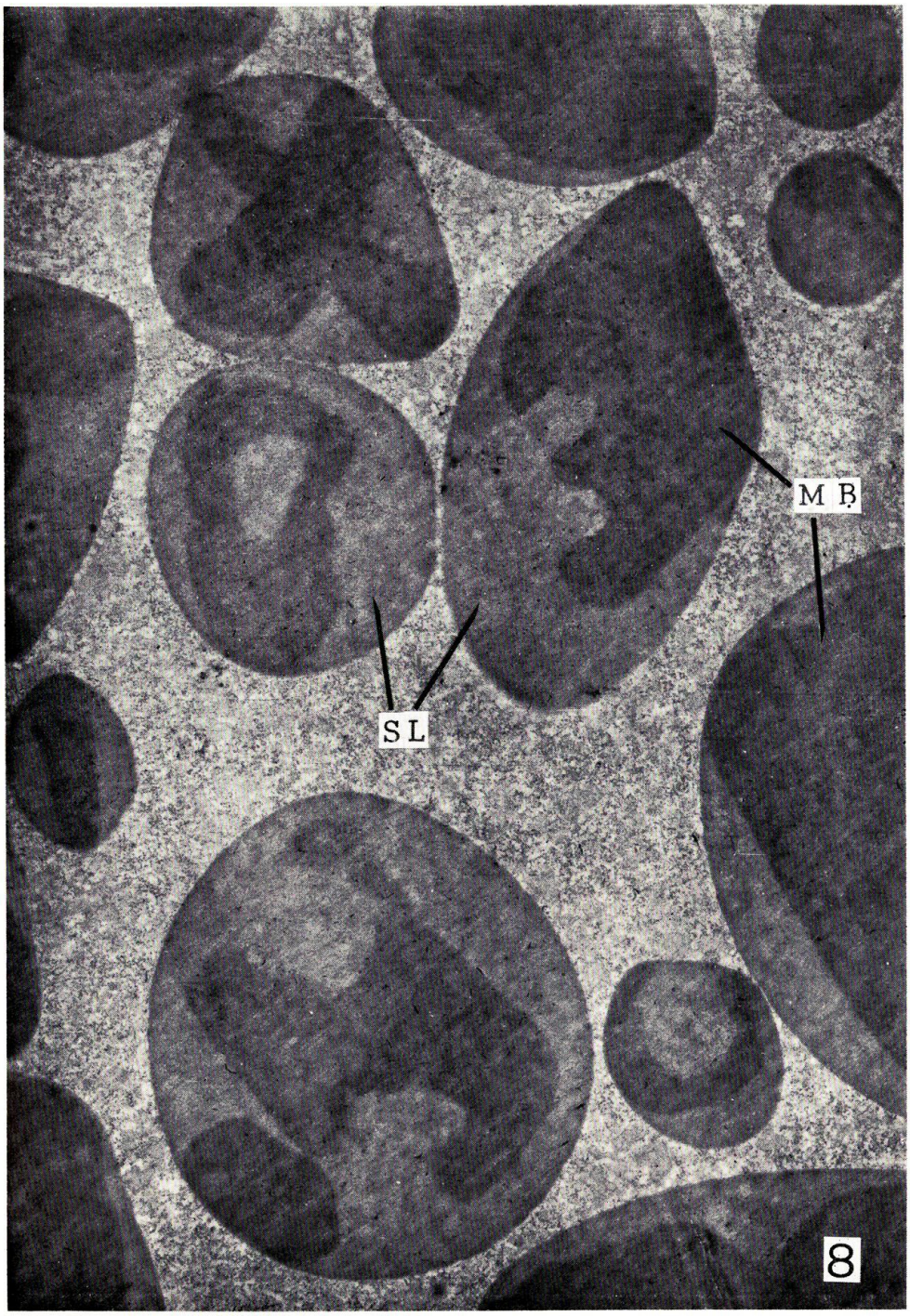

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PLATE IX

Figs. 9 and 10. Crystalline patterns in the main body. (9) Rectangular net pattern. (10) Parallel band pattern with spacings ca. 175 Å. ×200,000

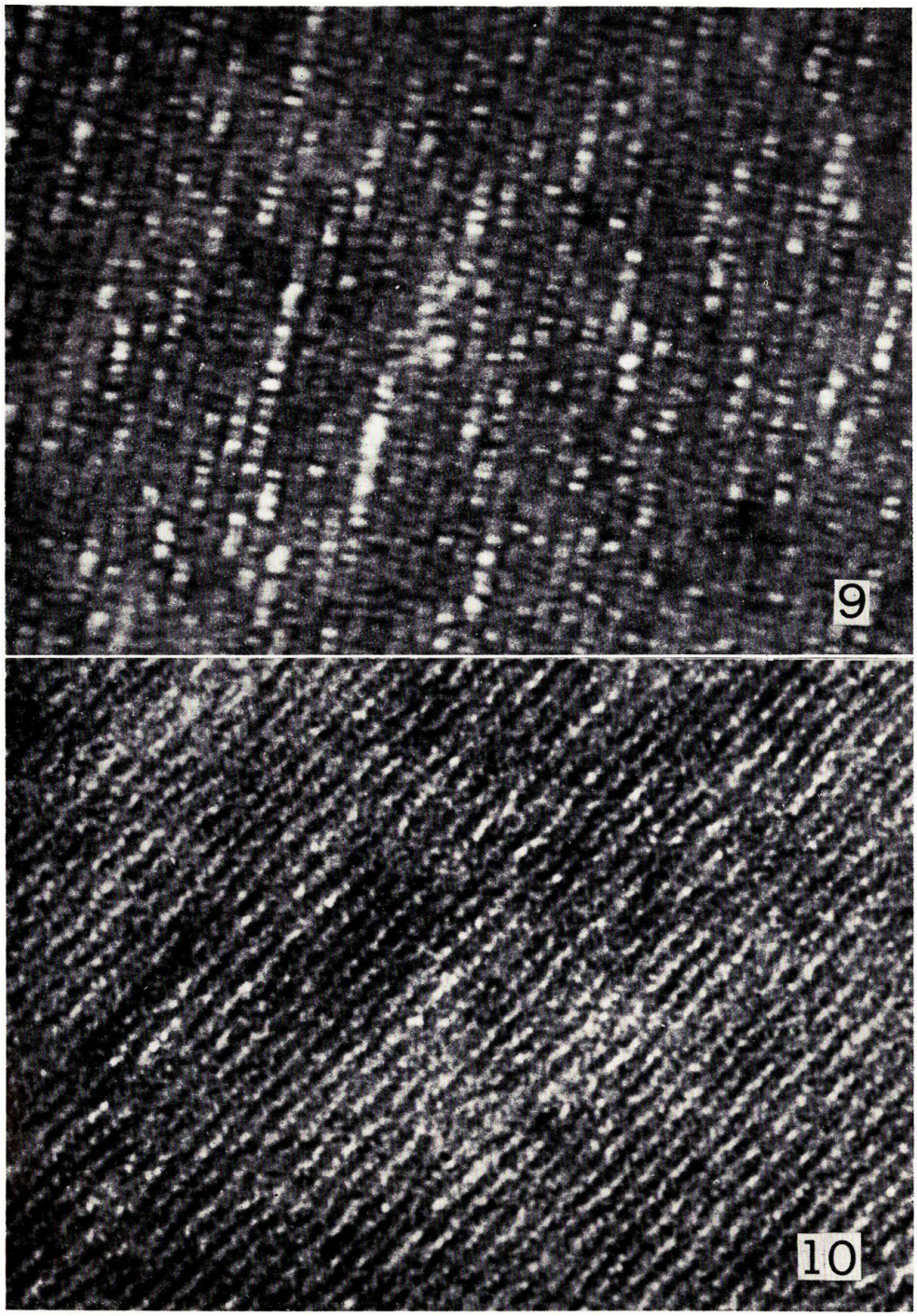

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PLATE X

Figs. 11 and 12. Crystalline patterns in the main body. (11) Parallel band pattern with spacing ca. 125 Å. ×200,000 (12) Main bodies showing parallel band pattern in a different orientation. ×120,000

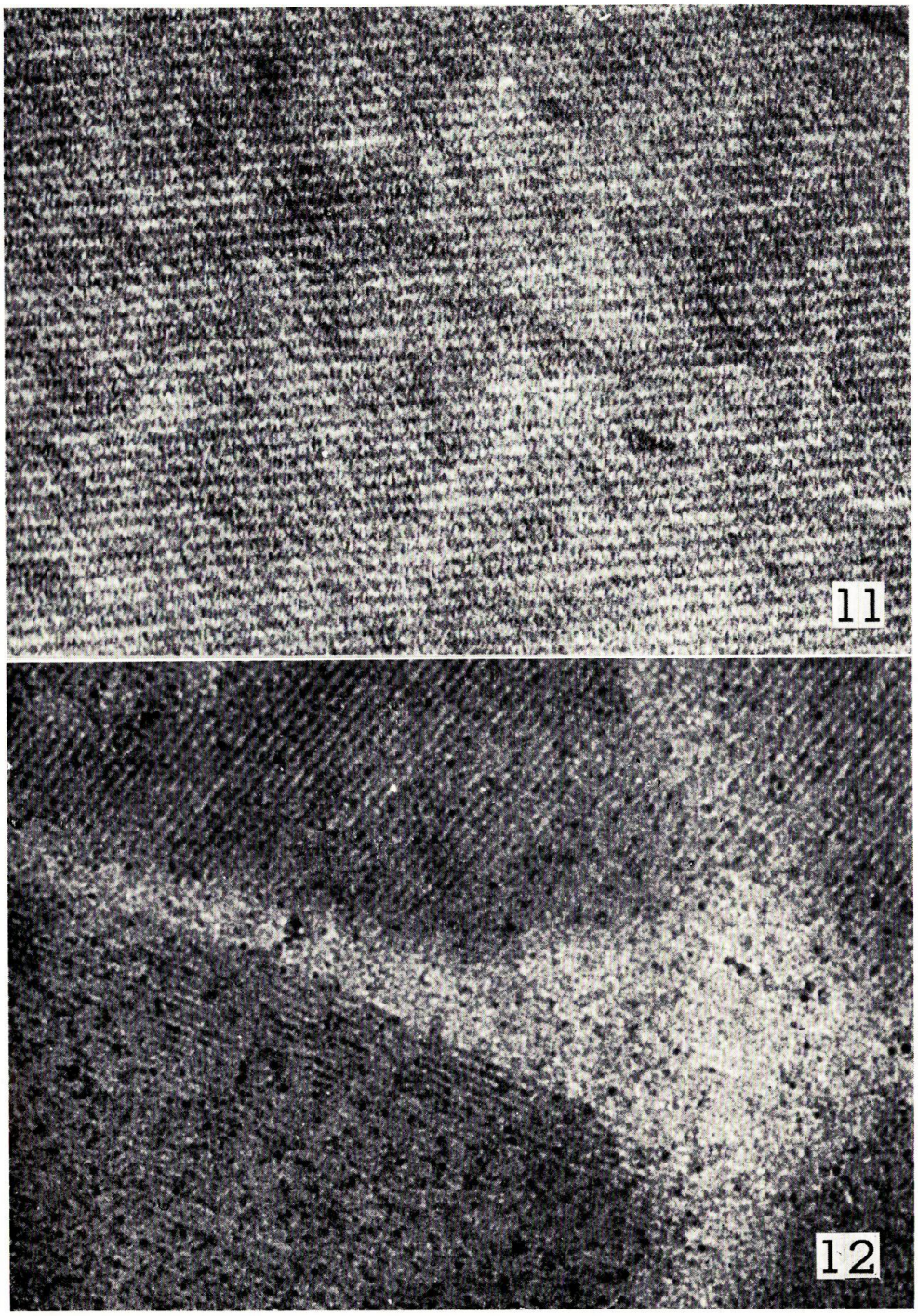

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PLATE XI

Figs. 13 and 14. Yolk globules suggesting the fusion of small globules. (13) ×19,800, (14) ×11,400

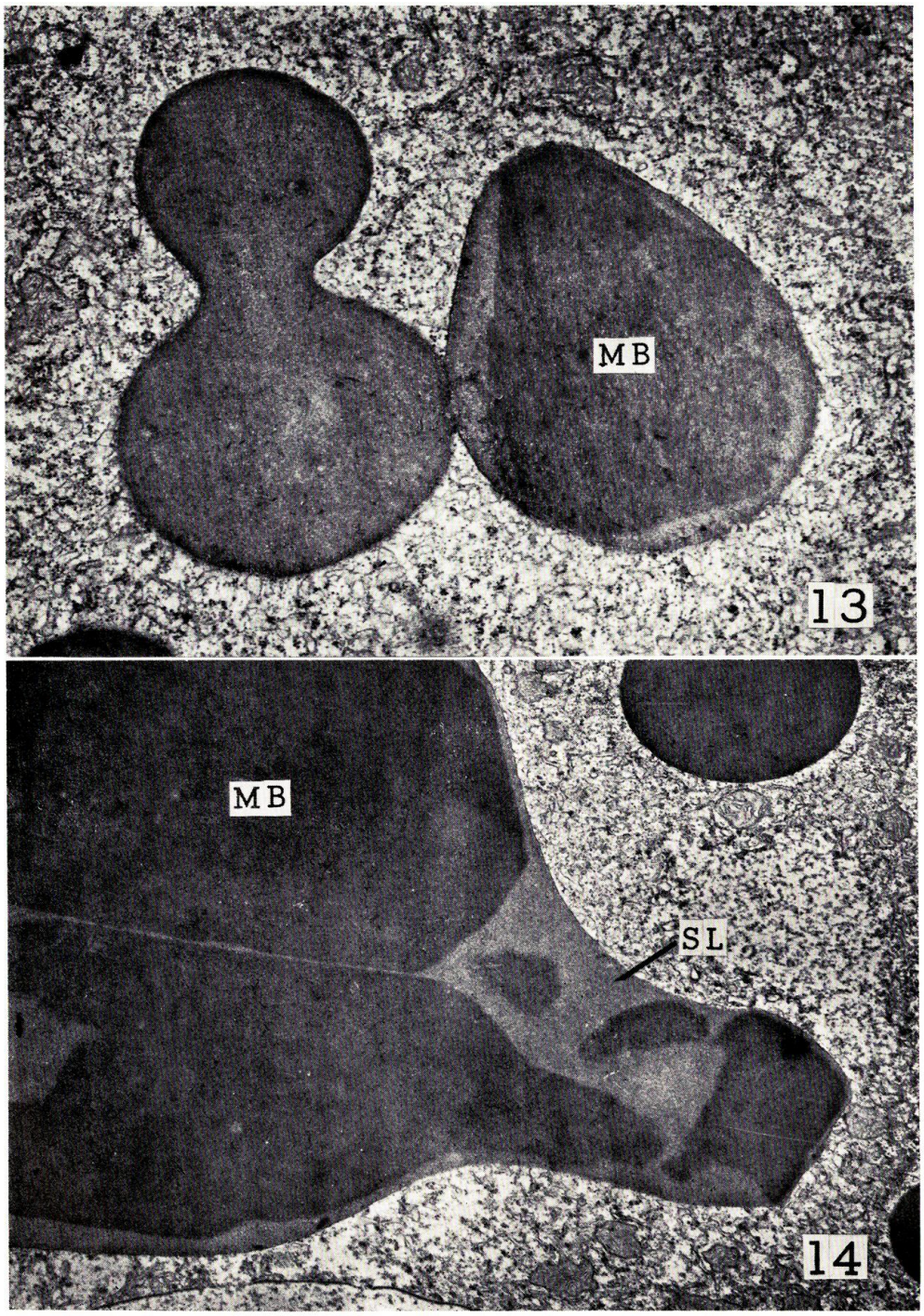

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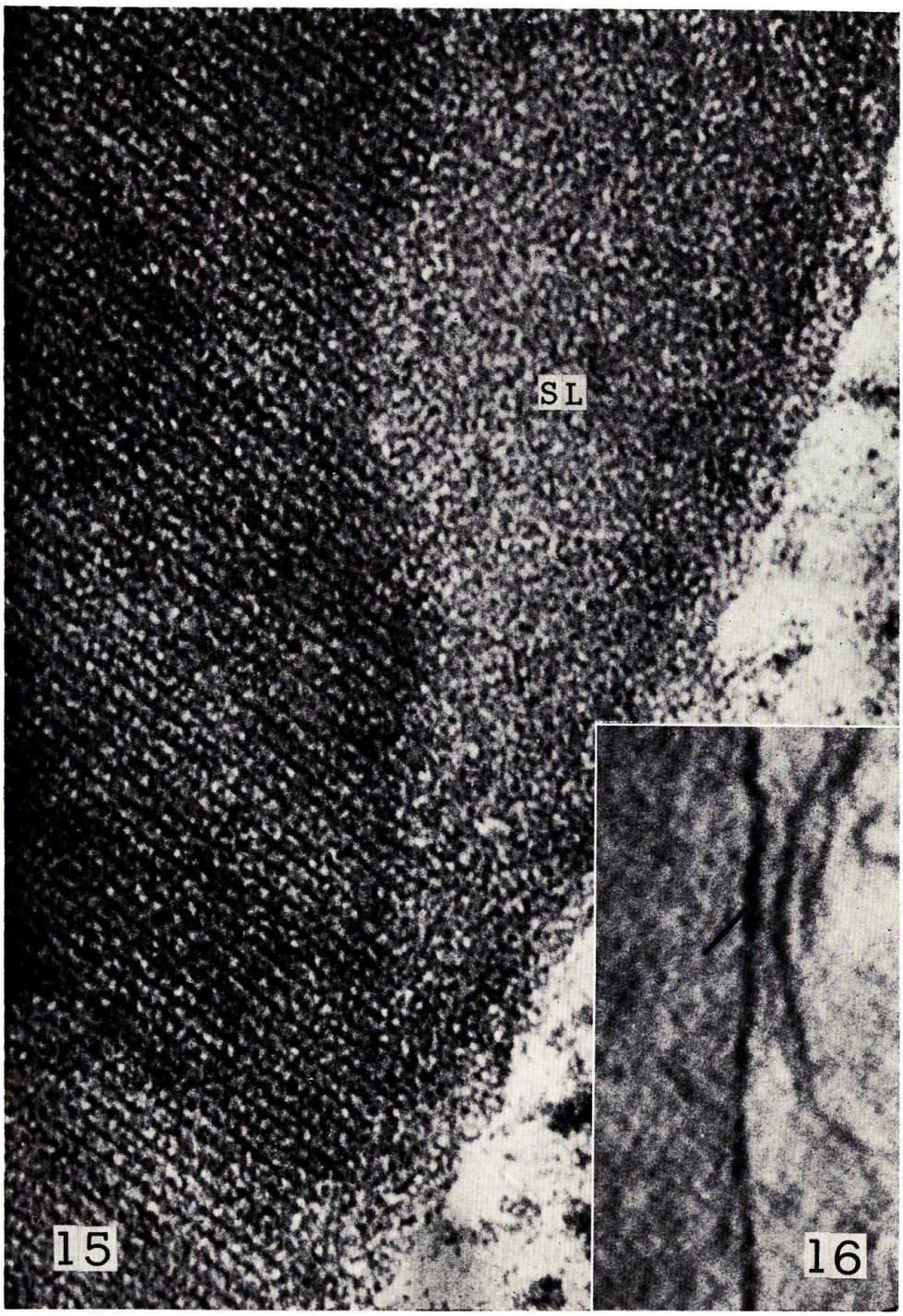
PLATE XII

Figs. 15 and 16. Superficial layer (SL) and limiting membrane (indicated by arrow) from the yolk globules of mature eggs. ×200,000



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