ELECTRON MICROSCOPE STUDIES ON

DEVELOPING CRAYFISH OOCYTES WITH

SPECIAL REFERENCE TO THE ORIGIN OF YOLK

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ABSTRACT

The endoplasmic reticulum is composed, in places, of stacks of parallel cisternae which are limited by membranes having great numbers of ribosomes attached to their outer surface. These are connected with other cisternae of similar structure but with fewer ribosomes and without preferred orientation. The latter extend in all directions from the stacked cisternae, branching and anastomosing freely so that the entire system of membrane-limited cisternae appears interconnected; a morphological condition suitable to serve as the basis for an active transport system. Within the stacked cisternae appear granules about 40 to 60 mμ in diameter. These are thought to represent the precursors of proteinaceous yolk, and the hypothesis is advanced that most of the intracisternal granules are synthesized here, possibly under the influence of the ribosomes. They then "flow" into and along the unoriented cisternae to regions where they collect, expand the cisternae, and undergo transformation into finely granular, relatively large proteinaceous yolk bodies. The mitochondria are somewhat pleomorphic, often show atypical cristae, and frequently contain a few dense granules. Lipid is abundant. Other cytoplasmic components are illustrated.

INTRODUCTION

For over fifty years the origin of yolk in the growing oocyte has been a subject for active research by many investigators. From these studies has come information which suggests that in different species of animals considerable variation exists in the morphological relation between the developing yolk masses and the various cellular organelles. That is to say, the nucleus, mitochondria, Golgi material, and ergastoplasm have all, at one time or another, been implicated in the synthesis of yolk. Yolk has also been described as arising *de novo* from the ground cytoplasm in a manner unrelated to any one of the cellular organelles. Good accounts of the early literature relative to yolk formation may be found in the works of Wilson (1925), MacBride and Hewer (1931), and Raven (1961).

With the advent of the electron microscope, studies were undertaken in an effort to determine whether or not at this level of resolution evidence could be revealed of a close spatial relation between the developing yolk masses and certain specific organelles; a condition which might also imply a close functional relationship as well. Favard and Carasso (1958) have observed a close relationship between the yolk masses and mitochondria in electron micrographs of the snail oocyte. Ward (1962) has been able to show in the frog's oocyte that the first appearance of both protein and lipid yolk droplets is within the mitochondria. Further, in a preliminary report Roth and Porter (1962) have described yolk formation in the mosquito as occurring by an active micropinocytosis of protein substance at the surface of

the oocyte. Moreover, Beams and Kessel (1962) in a short note have observed a close morphological relation between the rough surfaced endoplasmic reticulum and the formation of yolk in the crayfish oocyte. They advanced the hypothesis that the intracisternal granules, which are thought to be the precursors of proteinaceous yolk, are formed largely within the rough surfaced, stacked cisternae and become distributed throughout the ooplasm by moving along the largely smooth surfaced and branched cisternae, where they eventually aggregate and undergo chemical change resulting in definitive yolk bodies. Further work on this subject has amply confirmed and extended this view, as will be evident from the results herein reported.

MATERIALS AND METHODS

Two different genera of crayfish were used in this study. The northern crayfish, *Cambarus* and *Orconectes* sp., was obtained either from the Iowa City area or from the Lemberger Company, Oshkosh, Wisconsin. The southern crayfish, *Procambarus* sp., was obtained from Carolina Biological Supply, Elon College, North Carolina. The animals were obtained during the months of March, May, October, and November.

For electron microscope study, individual eggs or small pieces of the ovary were transferred directly to the fixative, which consisted of a 1 per cent solution of osmium tetroxide buffered with acetate-veronal (Palade, 1952) to a pH of 7.2, 7.6, or 7.8. The tissue remained in the cold fixative (4°C) for 1 to 2 hours. Following rapid dehydration in a series of cold ethanols and treatment with propylene oxide, the tissue was embedded in Epon 812 or Epoxy Araldite (Ciba 502) according to the method of Luft (1961). In addition, some of the eggs were dehydrated in acetone and embedded in Vestopal W according to the method of Ryter and Kellenberger (1958); some were embedded in a 4:1 mixture of *n*-butyl and methyl methacrylate and polymerized at 50°C. Thin

sections of the epoxy-embedded material were obtained with a Porter-Blum microtome using glass knives. Sections displaying silver or gold interference colors were mounted on Formvar-coated grids which had been lightly stabilized with carbon. The sections were stained with a saturated aqueous solution of uranyl acetate or with lead hydroxide (Watson, 1958), and studied with an RCA EMU-3D electron microscope. Some of the sections were mounted on 400-mesh copper grids with no supporting film. Half-micron sections of the epoxy-embedded eggs were stained with methylene blue and azure II according to the method of Richardson et al. (1960) for light microscope study.

For light microscope study and cytochemical tests, portions of the ovary were fixed in Bouin's, Carnoy's, Zenker's, and Champy's solutions and 10 per cent formalin and embedded in paraffin. Stains used included Heidenhain's iron hematoxylin, and tests were performed for protein (Mazia et al., 1953) and for nucleic acids (Korson, 1951).

OBSERVATIONS

The genera of crayfish used in this study lay their eggs in early spring, after which a new group of oocytes are formed. Armitage and Topping (1962) observed that the ovaries of the crayfish *Orconectes nais* reared in the laboratory increased from 75 mg in July, 1961, to 1050 mg in November of the same year. This represents a large portion of the active period of growth, involving an increase in both ooplasm and yolk.

Light Microscope Studies

The ovary of the crayfish is surrounded by a membrane composed of connective tissue and epithelium which is continuous with the stroma of the ovary (Fig. 1). Most of the oocytes of a given ovary are of approximately the same size, but there are always younger stages present even in the nearly mature ovary. Each oocyte is

FIGURES 1 TO 3 Photomicrographs of crayfish oocytes. Fig. 1 shows a portion of the ovary containing oocytes in different stages of development. *N*, nucleus; *FC*, follicle cell layer. In Fig. 2 the nucleus (*N*) is present and contains several nucleoli (*NL*) adjacent to the nuclear membrane. Note the granular perinuclear zone of cytoplasm (*PNZ*). Small, dense masses of material are scattered throughout the central or intermediate region of the cytoplasm (*IZ*). Very small yolk masses are beginning to appear in the peripheral region of the cytoplasm (*PZ*). Follicle cells are shown at *FC*. In Fig. 3, a portion of the nucleus and the granular, perinuclear zone of cytoplasm (*PNZ*) are shown at higher magnification. In addition, the small, elongate, basophilic staining masses (ergastoplasm) (*SBB*) in the intermediate zone of cytoplasm (*IZ*) appear to be striated. A network of "filaments" (*ICC*) may be faintly seen between the striated basophilic bodies (*SBB*). Champy's solution, Heidenhain's hematoxylin. Fig. 1, × 200; Fig. 2, × 500; Fig. 3, × 1200.
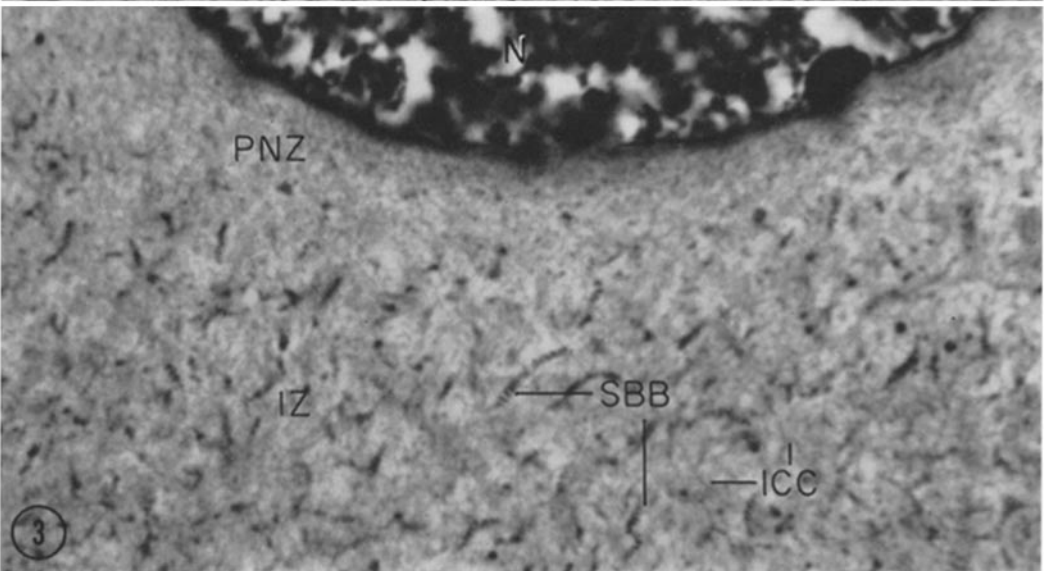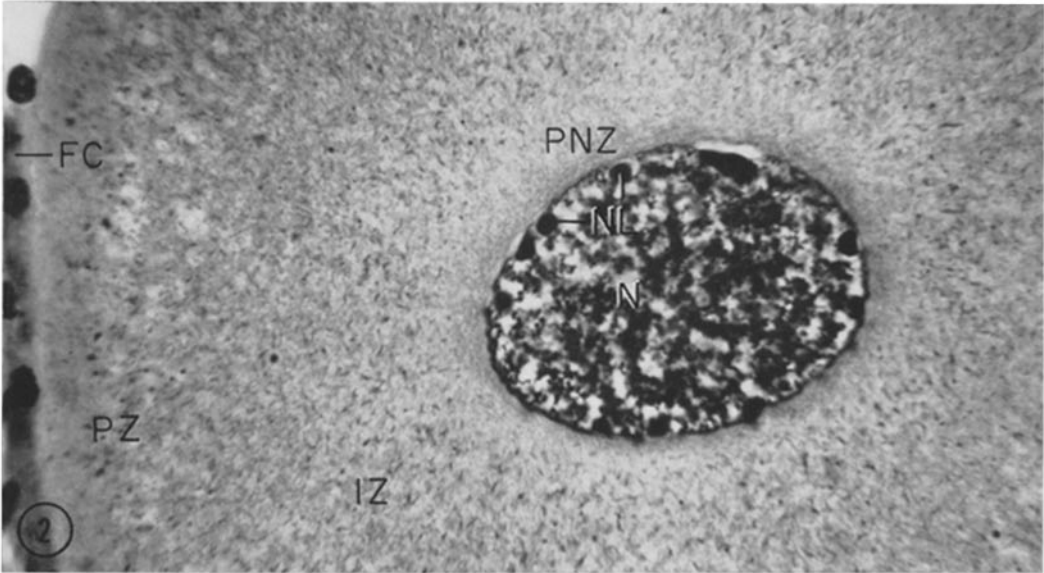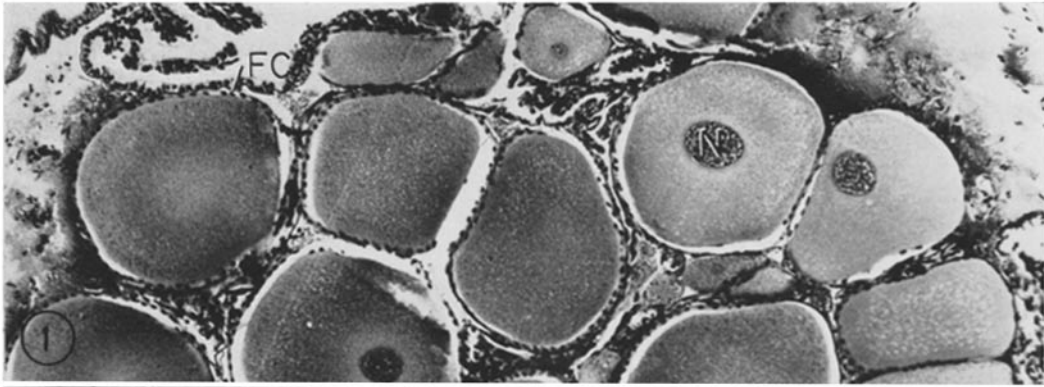
surrounded by a layer of follicle cells which are cuboidal in shape (Fig. 2, *FC*), and, as will be revealed in the electron micrographs to be described below, possess numerous microvilli which penetrate the chorion of the oocyte. The chorion can sometimes be seen in stained preparations as a narrow membrane, but no evidence has been revealed in either the light or the electron microscope to show that a direct morphological connection exists between the follicle cells and the cytoplasm of the oocyte. In young oocytes the germinal vesicle is relatively large and contains an abundance of densely staining chromatin arranged in a loose netlike pattern (Fig. 2, *N*). The nucleus is limited by a sharply staining membrane adjacent to which are located several prominent basophilic staining nucleoli (Fig. 2, *NL*). Evidence that these nucleoli migrate intact from the nucleus to the cytoplasm, as suggested by Kater (1928), was not observed. The young oocytes contain many basophilic staining masses (ergastoplasm) which in their distribution within the ooplasm are reminiscent of the Nissl bodies seen in certain nerve cells (Fig. 3, *SBB*). This resemblance is due to the fact that both the Nissl bodies (Palay and Palade, 1955) and the basophilic bodies of the crayfish oocytes are largely made up of rough surfaced membranes of the endoplasmic reticulum which are arranged in a compact and parallel fashion. Larger oocytes reveal a perinuclear zone containing numerous small basophilic staining granules (Figs. 2 and 3, *PNZ*). These, as will be seen in the electron micrographs later to be discussed, appear to represent small groups of ribosomes. Numerous basophilic bodies which often show a banded structure (Fig. 3, *SBB*) are located in the intermediate zone of cytoplasm (Fig. 2, *IZ*) beyond the perinuclear region. These, as will be noted below, constitute the stacked

cisternae of the endoplasmic reticulum. Parts of the complicated network constituting the branched cisternae of endoplasmic reticulum may be seen, less clearly, in the ooplasm between the stacked cisternae (Fig. 3, *ICC*). Proteinaceous and lipid yolk bodies in various stages of growth usually are first observed in the peripheral region of the cytoplasm (Fig. 2, *PZ*). The proteinaceous yolk bodies, the striated basophilic bodies, and in many instances the interconnecting network stain for protein with mercuric bromphenol blue (Mazia *et al.*, 1953).

Electron Microscope Studies

NUCLEUS

The nucleus of the oocyte is limited by a well defined, porous double membrane envelope (Fig. 5, *NP*). Adjacent to the nuclear membrane are found nucleoli which are free of a surface membrane and composed of a finely granular substance (Figs. 5 and 8, *NL*). The granules are about 150 A in diameter, closely aggregated, and often arranged in an irregular pattern of relatively wide anastomosing strands. The nucleoli show a highly irregular surface with small islands of granular material near by in the nucleoplasm; a condition which suggests that the latter were probably formed by a process of shedding or delamination from the former (Fig. 5, *NL*, arrows). This condition is especially striking on the side of the nucleoli adjacent to the nuclear membrane. That some of the particles composing the nucleolus may then pass through the nuclear pores into the perinuclear zone of ooplasm is suggested in certain regions (Fig. 8, arrows). If nuclear-cytoplasmic transfer does occur in this way, the granules from the nucleolus upon entering the ooplasm may reaggregate into small masses which are often

FIGURES 4 AND 5 Electron micrographs of young oocytes showing the nucleocytoplasmic junction. *N*, nucleus. In Fig. 5, a large nucleolus (*NL*) is seen to be situated in close contact with the porous, double nuclear membrane (*NP*). The nucleolus is composed of closely packed granules except in the region adjacent to the nuclear membrane, where it is more loosely organized (arrows). Clusters of dense masses of granules (*RA*) are scattered in the cytoplasm immediately outside the nucleus. These appear similar in size and density to masses associated with the nucleolus. Large numbers of parallel membranes traverse the ooplasm of the perinuclear region and do not appear to have any preferred orientation at this time (*AER*). Most of these lamellae have no ribosomes attached to their surface. The differentiation of cisternal stacks of rough surfaced endoplasmic reticulum is beginning (*ERS*), but they are not so large as in later stages. A few intracisternal granules are present at this time. *GB*, Golgi bodies; *M*, mitochondrion; *L*, lipid. Epon, uranyl acetate. Fig. 4, × 13,000; Fig. 5, × 14,000.
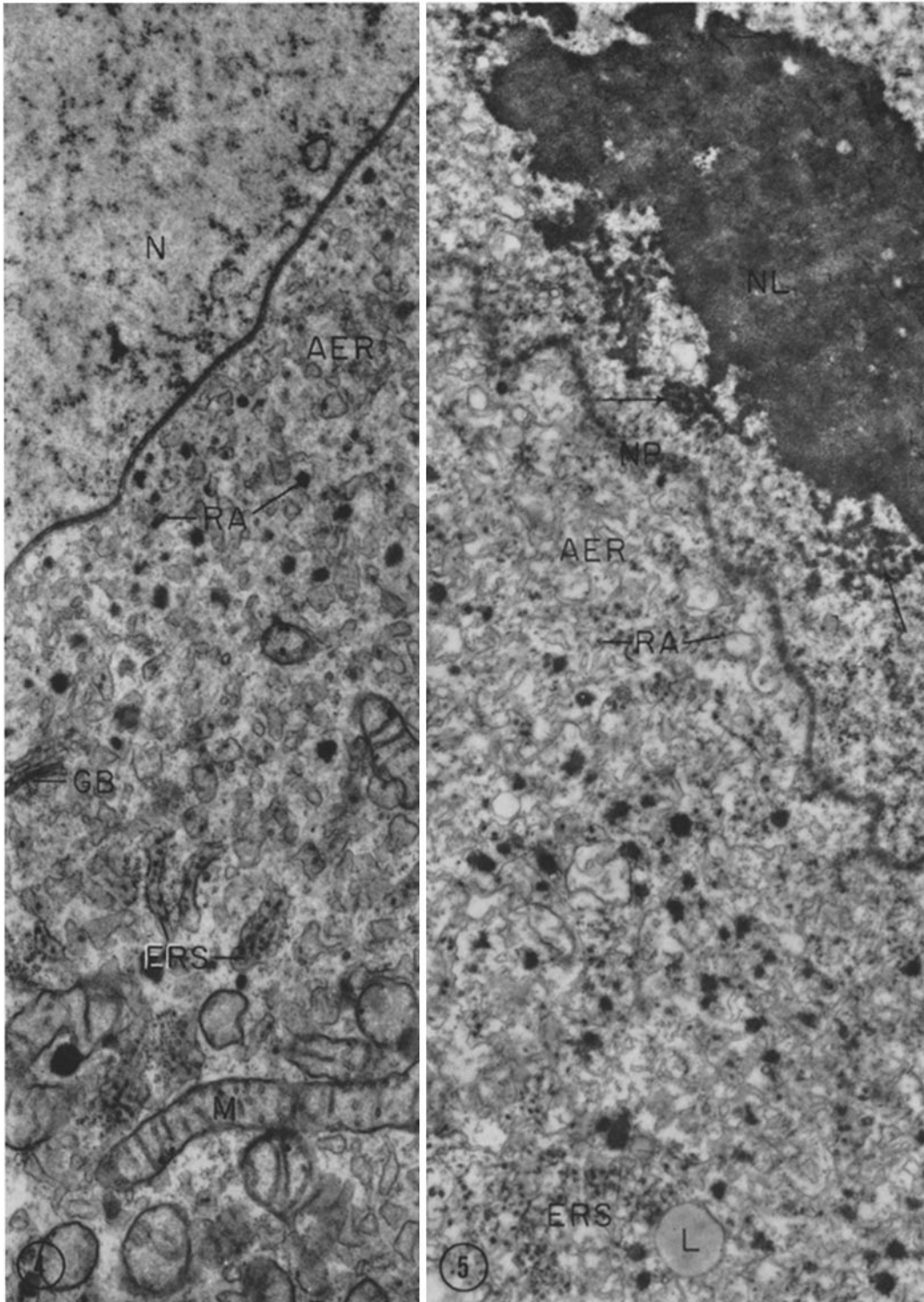
observed in the perinuclear zone of young oocytes (Figs. 4 and 5, *RA*; *cf.* Anderson and Beams, 1956).

ENDOPLASMIC RETICULUM

Scattered vesicular and cisternal elements of agranular endoplasmic reticulum are present in the perinuclear ooplasm in addition to the clusters of ribosomes (Figs. 4, 5, 7, and 8, *AER*). Some degree of anastomosis seems to exist between the cisternae in this region, and there is some evidence of a close morphological relationship between the outer layer of the nuclear envelope and the smooth surfaced membranes of the endoplasmic reticulum (Fig. 7, arrows). The possibility exists that the agranular membranes in the perinuclear zone of ooplasm may have their origin from the outer layer of the nuclear envelope. Such a process is demonstrable in oocytes of *Necturus* (Kessel, 1963). Few ribosomes are associated with the membranes of the reticulum in the perinuclear zone of ooplasm (Figs. 7 and 8, *AER*).

In the ooplasm located more centrifugally to the nucleus the agranular cisternae grow, become more branched, and eventually aggregate to form stacks of rough surfaced cisternae (Figs. 4 and 6, *ERS*). At this time, other important changes relative to the endoplasmic reticulum are noted; namely, the appearance of numerous ribosomes on the surface of the membranes in the stacked cisternae and in the ooplasm between the cisternae (Figs. 4 to 6, *ERS*). As the stacks of rough surfaced endoplasmic reticulum are thus differentiated, one can note the appearance of discrete particles within the cisternae (Figs. 4 to 6, *ERS*). Although the number of cisternae comprising a stack is variable, as many as 12 to 16 have been observed in a single stack of a rapidly growing oocyte.

Some of the cisternae composing a stack may interconnect (Fig. 11, arrows). In addition, the various cisternae composing the stack may be connected at points on their surface with unoriented, smooth surfaced, intercommunicating and branched portions of the endoplasmic reticulum (Figs. 10 and 11, *ICC*). The transition from the rough surfaced membranes of the stacks to the smooth surfaced ones between and connecting the stacks is usually abrupt. This system of single, branching, and agranular cisternae permeates throughout the ooplasm, branching and anastomosing freely so that the entire system of membrane-lined cisternae appears interconnected (Fig. 9); a morphological condition suitable to serve as the basis for an active transport system.

In the intermediate region of the ooplasm of growing oocytes, the cisternal stacks become filled with intracisternal granules which have been undergoing synthesis presumably under the influence of the ribosomes located on the surface membranes (Figs. 10 to 12, *R*). At higher magnification, the intracisternal granule appears as a disc-shaped structure displaying a relatively dense cortex and a less dense central region (Figs. 12 and 13, *ICG*; Fig. 13, arrows). They are of fairly uniform size, measuring approximately 60 to 100 mμ in diameter and 20 to 40 mμ in thickness. As the granules reach a certain density within the cisternae of the stacks they seem to migrate or "flow" out from the stacks into and along the highly branched and largely smooth surfaced cisternae (Fig. 13, *ICC*). When this occurs, the system of intercommunicating cisternae becomes more prominent as they are filled with granules. As the granules are transported along the cisternae to different regions of the ooplasm, some tend to aggregate to form large granular masses, particu-

FIGURE 6   A later stage in the differentiation of the endoplasmic reticulum than that shown in Fig. 4. The lamellae comprising the stacks of rough surfaced endoplasmic reticulum are more numerous (*ERS*). All the cisternae of the stacks contain intracisternal granules (*ICG*). Two small forming yolk bodies (*FYB*) are shown and are attached to elements of the endoplasmic reticulum. The perinuclear zone (*PNZ*) of ooplasm consists of numerous membranous elements which are largely smooth surfaced. Small dense masses are located in the perinuclear zone. Numerous mitochondria (*M*) and several Golgi elements (*GB*) are present. *N*, nucleus; *L*, lipid. Epon, uranyl acetate. × 9500.

FIGURES 7 AND 8   Region of the nuclear membrane in young oocytes. The nucleus is shown at *N* with a portion of a nucleolus (*NL*, Fig. 8). A close association is often seen to exist between the outer nuclear membrane and agranular membranous elements (*AER*) of the perinuclear zone of cytoplasm (Fig. 7, arrows). Several annuli are observed in Fig. 8 (arrows) and ribosomelike structures appear to be in the process of passing through the pores (three center arrows). Epon, uranul acetate. × 32,000.
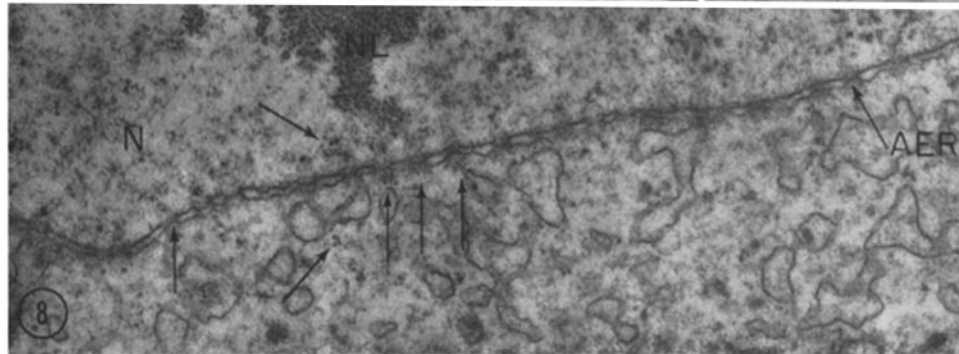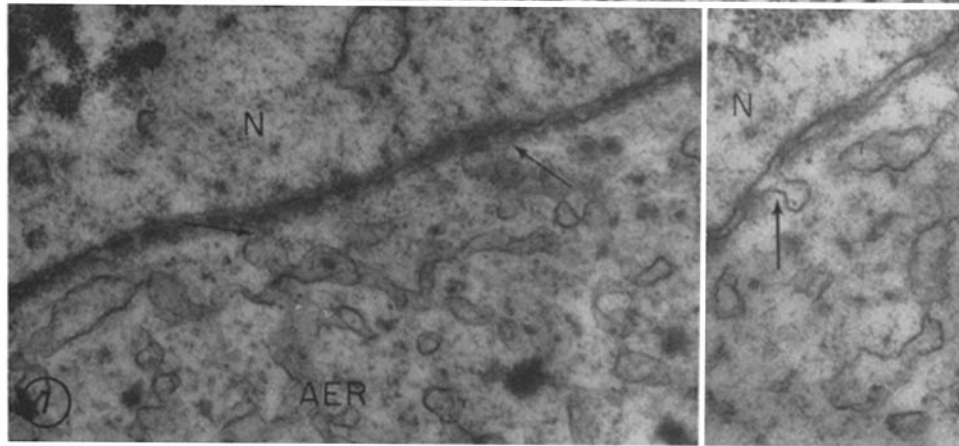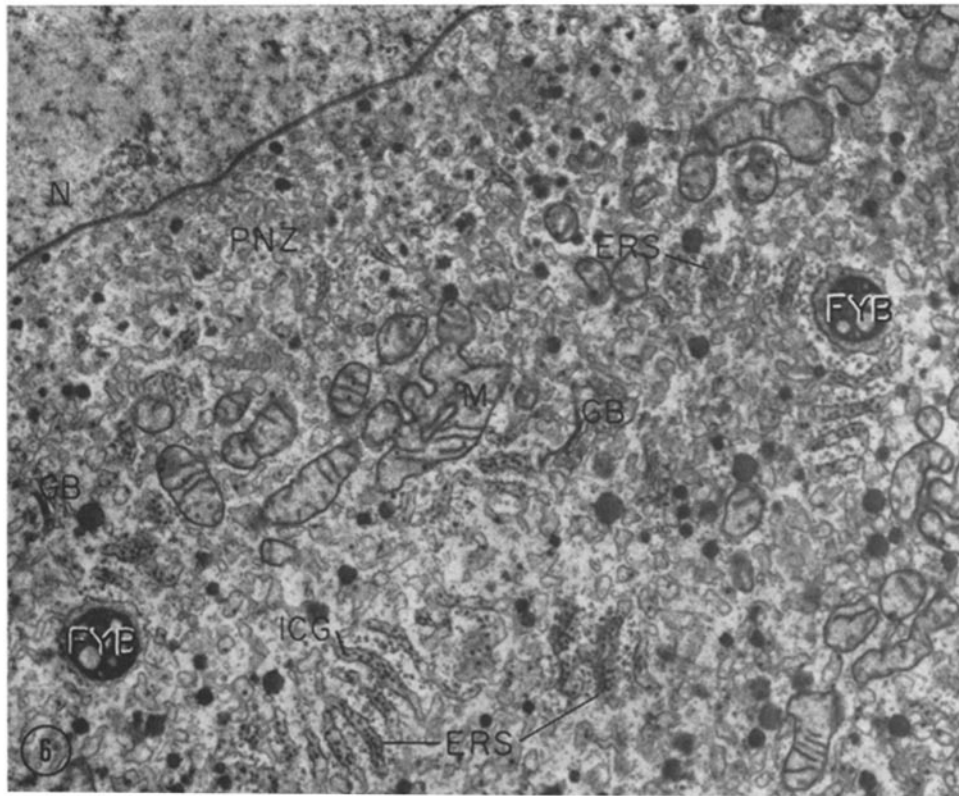
larly in the region where anastomosis of several cisternae has occurred (Figs. 13 to 15, *ACG*). The granules within the aggregates increase in number and expand the cisternae, but retain their form and appearance for an unknown period of time. Eventually they undergo physiochemical changes resulting in a finely granular protein-aceous yolk body (Figs. 16 and 17, *PYB*). By this stage, several cisternae have joined in the region of the developing yolk body and no doubt they have all contributed granules to its growth. The membranes enveloping the yolk bodies are usually agranular, but not always, as is evident in Fig. 17 (*R*). Here, ribosomes are present on the membrane surrounding the lower right side of the yolk body. Present also in this figure are numerous intracisternal granules which are arranged around the periphery of the yolk mass. Some of these may have formed here under the influence of ribosomes, since presumably synthesis can occur wherever ribosomes are present. Fig. 17 also illustrates at the top of the yolk body several intracisternal granules that show evidence of breaking down to form definitive yolk.

Small bodies which appear to represent incompletely formed yolk masses are infrequently observed in areas of the ooplasm adjacent to the nucleus in young oocytes (Fig. 6, *FYB*). Their presence in this particular region of the oocyte cytoplasm at this time is unusual, and no definite explanation for this condition is available.

In large oocytes the cortical region becomes loaded with yolk masses of both the proteinaceous and the lipid type. As this occurs the stacks become disorganized and the interconnecting cisternae restricted in their distribution. The intracisternal granules remain relatively numerous in the older oocytes (Fig. 19, *ICC*). Small groups of ribosomes may still be observed on the outer surface of the membranes in certain localized regions of the intercommunicating cisternae. It seems probable that many of the intracisternal granules present at this stage have been synthesized at an earlier time in the maturation of

the oocyte. However, it seems possible that any portion of the endoplasmic reticulum bearing ribosomes is capable of synthesizing intracisternal granules regardless of whether or not the ribosomes are associated with stacks. The mature proteinaceous yolk bodies in the older oocytes are usually large, dense, and more or less homogeneous (Fig. 19, *PY*).

Fatty yolk globules are seen in most of the figures (*L*, *LY*). The lipid makes its appearance in the ooplasm earlier than does the proteinaceous yolk. The lipid particles usually vary considerably in size and are irregular in outline. It has not been possible to discover morphological evidence of a nature that would permit speculation on how this material is formed in the cell. It can only be stated that it appears to arise *de novo* from the ooplasm without close association with any one of the cytoplasmic organelles.

MITOCHONDRIA

The mitochondria in the developing oocyte of the crayfish are relatively numerous and largely filamentous and show considerable pleomorphism (Figs. 14, 16, 22, and 23, *M*). They are much better preserved in oocytes embedded in epoxy resin than in oocytes embedded in methacrylate. In sections of the latter, only faintly outlined, clear bodies with poorly preserved cristae are observed (Fig. 9, *M*). However, in sections of material embedded in epoxy resin, the mitochondria typically show an inner and outer membrane with a varying number of cristae formed by the infolding of the inner membrane (Fig. 6, *M*). The cristae sometimes branch and anastomose (Fig. 23, *M*). In some mitochondria regions are observed where the cristae aggregate into a tightly packed group with their long axes arranged parallel to one another, resembling in some respects a honeycomb (Figs. 22 and 24, *M*). Small dense granules are often present in the mitochondrial matrix (Fig. 24, arrows) and they appear similar to those described in mitochondria from other types of cells (*cf.* Haguenau, 1958). Their signifi-

FIGURES 9 AND 10 Both figures show portions of the cytoplasm of young oocytes containing as yet few protein yolk bodies. Several stacks of rough surfaced endoplasmic reticulum (*R*) are present in each figure and contain numerous intracisternal granules (*ICG*). Interconnections between the cisternal stacks are effected by means of a system of single, branching cisternae (*ICC*) which, for the most part, are smooth surfaced. Only a few intracisternal granules are located within the interconnecting cisterna at this time. *L*, lipid; *M*, mitochondrion. Fig. 9, methacrylate, lead hydroxide, × 14,000; Fig. 10, Epon, uranyl acetate, × 14,000.
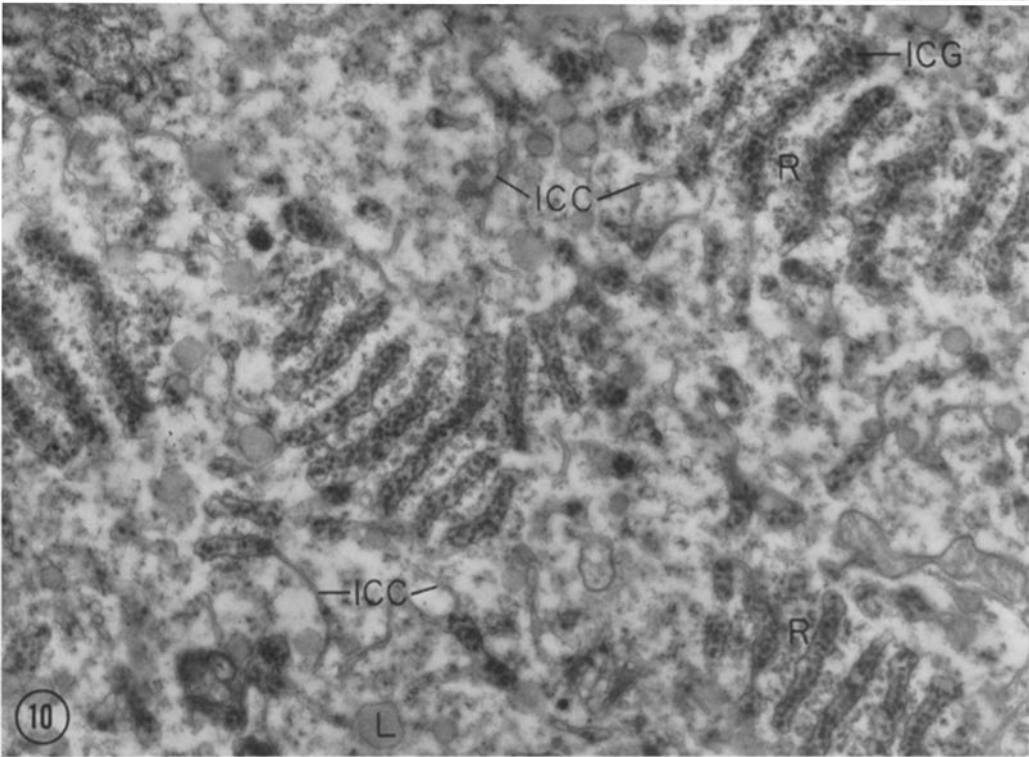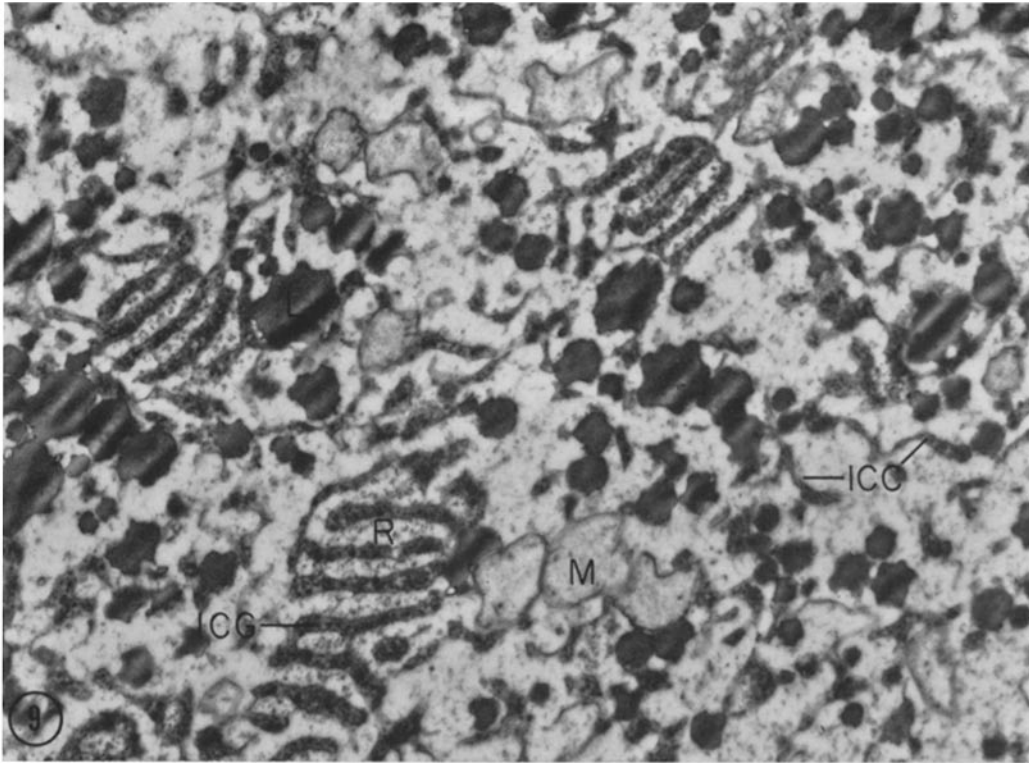
cance is unknown, but they do not appear to be involved in the synthesis of yolk. Furthermore, no evidence was found that the mitochondria play a direct role in the synthesis of yolk in the crayfish oocyte as they appear to do both in the oocyte of the snail (Favard and Carasso, 1958) and in that of the frog (Ward, 1962).

Golgi Material

The Golgi material is not well preserved in methacrylate sections of crayfish oocytes. However, in sections of Epon-embedded material it is present in the form of dictyosomes which display the usual parallel agranular membranes and associated vesicles (Figs. 4 and 6, *GB*; Fig. 28, *GA, V*). They are less numerous than the mitochondria and do not appear to be involved directly in yolk formation. However, granules similar in size and density to the intracisternal granules are sometimes observed within their cisternae (Fig. 28, arrows). A few cases have been observed where a close relationship seems to exist between the cisternae of the Golgi complex and those of the endoplasmic reticulum (Fig. 28, *ICC*). In fact, some preparations appear to show them as continuous, and if this is true, it is then understandable how the granules could move from one type of agranular cisternae to another.

Other Ooplasmic Bodies

It is not uncommon to find scattered in the ooplasm of epoxy-embedded oocytes large bodies of the type illustrated in Figs. 6 and 18 (*PY, FYB*). It is not certain how these bodies are formed, but part of their appearance may be due to the technique used, especially the epoxy resin, for they are rarely observed in sections of methacrylate-embedded material. In any case, their outer border often appears composed of proteinaceous yolk and they are often attached to cisternae in the same way as are developing yolk bodies (Fig. 18, arrows). Thus, it appears that they are probably yolk bodies part of whose structure has for some unknown reason been modified either in their formation or by the techniques used to demonstrate them.

Complex membranous bodies such as are shown in Fig. 27 (*HMB*) may be numerous in the oocyte cytoplasm even in young stages. These heterogeneous structures usually consist of dense granules and many concentrically layered membranes. The entire structure may be completely surrounded by a limiting membrane or the membrane may be incomplete. The possibility exists that these regions may represent localized areas of atrophy or degeneration.

An even more puzzling structure which may be found rather uniformly distributed in both intermediate and peripheral zones of ooplasm of growing oocytes is shown in Fig. 25 (*MT*). This structure is composed of groups of elongate filaments or tubules usually varying from 12 to 24 in number (Fig. 20, *MT*). In transverse section the tubules consist of a dense periphery with a

FIGURE 11 Portion of the cytoplasm of a young oocyte showing the arrangement of the endoplasmic reticulum. In the center of the field is shown a series of stacked, parallel membranes bearing small particles or ribosomes. These stacked cisternae may interconnect (arrows). Ribosomes are also located between the parallel membranes, in which case they are usually arranged in the form of rosettes (*R*). Numerous dense particles of rather uniform size are located within the cisternae of the endoplasmic reticulum (*ICG*). The cisternal stacks, which are very numerous in the young oocyte, are all interconnected with a system of single, branching cisternae usually devoid of ribosomes (*ICC*). Only a few granules are located within the single branching cisternae at this stage, but they are similar to those within the cisternae of the stacked endoplasmic reticulum. Two forms of endoplasmic reticulum are thus observed in this egg; they are interconnected and appear to have different functions, as subsequent micrographs will show. *M*, mitochondrion; *L*, lipid. Epon, uranyl acetate. × 20,000.

FIGURE 12 High magnification of a portion of a cisternal stack of endoplasmic reticulum showing the morphology of the intracisternal granules (*ICG*). The granule is a disc-shaped structure, as can be seen in surface and side views (arrows). The central region of the granule is less dense and gives the granule a "doughnut" appearance, in some instances. The ribosomes (*R*) associated with the lamellae of the cisternal stack are both attached to the membranous elements and located in rosettes between them. *M*, mitochondrion. × 53,000.
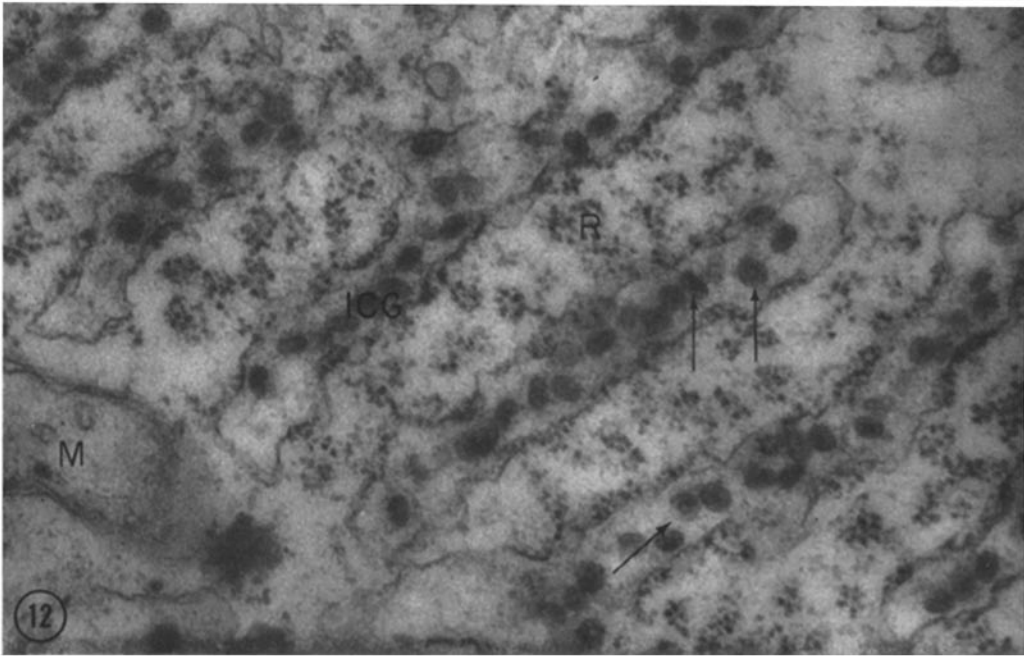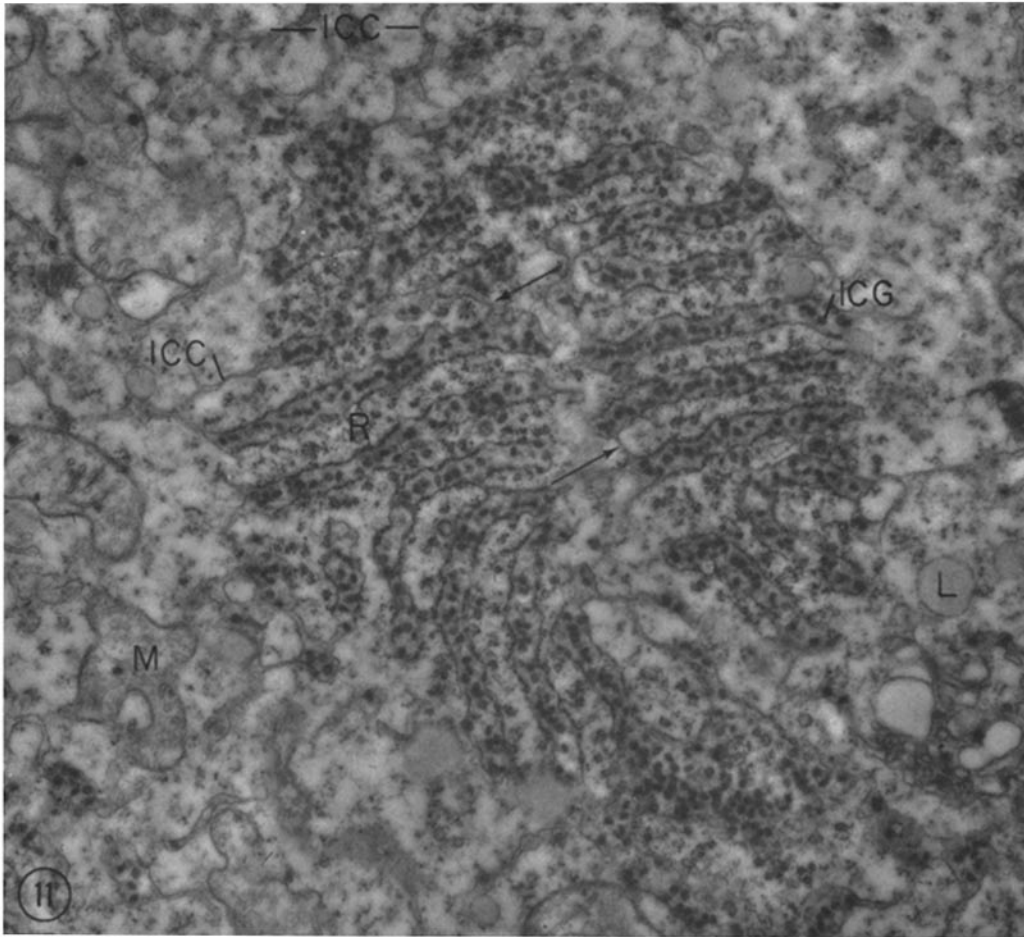
central region which may either be "empty" or contain a small, dense body (Fig. 26, *MT*, arrow). In longitudinal section the tubules appear to have a regular axial periodicity of approximately 150 to 200 A (Fig. 25, *MT*), and in certain regions small granules can be seen distributed within the tubules. Of some interest also is the fact that in some instances the microtubules appear to be continuous with granule-filled cisternae of the endoplasmic reticulum (Fig. 25, *ICC* arrow).

Oocyte Surface and Follicle Cells

A chorion is located between the follicle cells and the oocyte. The latter is beset by numerous microvilli which penetrate the chorion and in many places make contact with the follicle cell membrane. At and just below the surface of the oocyte in the space between the microvilli are seen numerous small vesicles (Fig. 21, *PV*). Certain of these appear attached to the surface membrane (Fig. 21, arrow) and probably reflect an activity of incorporation of material into the egg by a process of micropinocytosis.

The follicle cells have relatively large nuclei, numerous mitochondria, elements of endoplasmic reticulum, Golgi material, and isolated vesicles of different sizes especially on the side adjacent to the oocyte. Multivesicular bodies may be of common occurrence in the follicle cell cytoplasm at certain stages. The plasma membrane on its basal side is highly infolded. In certain regions, the plasma membrane of the follicle cells may be specialized in the form of desmosomes. A basement membrane surrounds the outside of the follicle cell layer. Fig. 29 diagrammatically summarizes the events of yolk formation in the crayfish oocyte.

DISCUSSION

During the growth of the oocyte several special and important cytological changes occur within it; *i.e.*, meiosis, the synthesis and deposition of nutritive material collectively referred to as yolk, and an active synthesis of cytoplasm. In certain forms at least, it is in the oocyte that the basic structural organization is established for the determination of such conditions as polarity, bilaterality, cortical and ooplasmic differentiation, and other little-understood conditions (coding) involved in the subsequent development of the egg and embryo. It is the analysis of the way in which these conditions arise that constitutes the ultimate goal of biologists. However, for the present, the ultrastructural organization associated with these conditions seems beyond the reach of present day electron microscopes. It appears that some of the various microscopically visible materials in the oocyte or in the egg are not necessarily essential for the development of certain organisms. This is evidenced by the fact that they may be completely displaced, stratified, and in some instances removed from the egg without marked effect upon its subsequent development (Harvey, 1932; Harvey, 1939). Furthermore, it has been demonstrated that *Ascaris* eggs can withstand a centrifugal force of 900,000 times gravity for 10 hours or 350,000 times gravity for 6 days without being killed; a condition which argues that the protoplasm of this egg must possess a basic ultrastructural organization of a substantial physical nature (Beams and King, 1940).

The one aspect of the cytology of the growing oocyte in addition to nuclear studies which has been profitably investigated by both the light and electron microscopes is that of the origin and deposition of yolk. As evidenced by the literature cited in the Introduction, the problem concerning the origin of yolk is one of long standing and current difference of opinion; a condition which probably indicates that this process may differ in different species of animals. In the case of the Crustacea alone several different views have been

Figure 13 Stage in the initial development of the proteinaceous yolk in crayfish oocytes. At this time the interconnecting system of single, branching cisternae of endoplasmic reticulum is filled with intracisternal granules so that the network now appears more prominent (*ICC*). It is believed that most of these granules (*ICG*) were derived from the cisternal stacks (especially in northern crayfish, where the cisternal stacks are more prominent) and subsequently migrated into the single, interbranching cisternae. The granules have begun to aggregate and expand certain of the cisternae at this time (*ACG*). In most cases the single lamellae do not possess ribosomes; this condition seems to be especially true in the northern crayfish, whereas in the southern crayfish more of the lamellae do have ribosomes. *L*, lipid; *M*, mitochondrion. Epon, uranyl acetate. × 13,000.
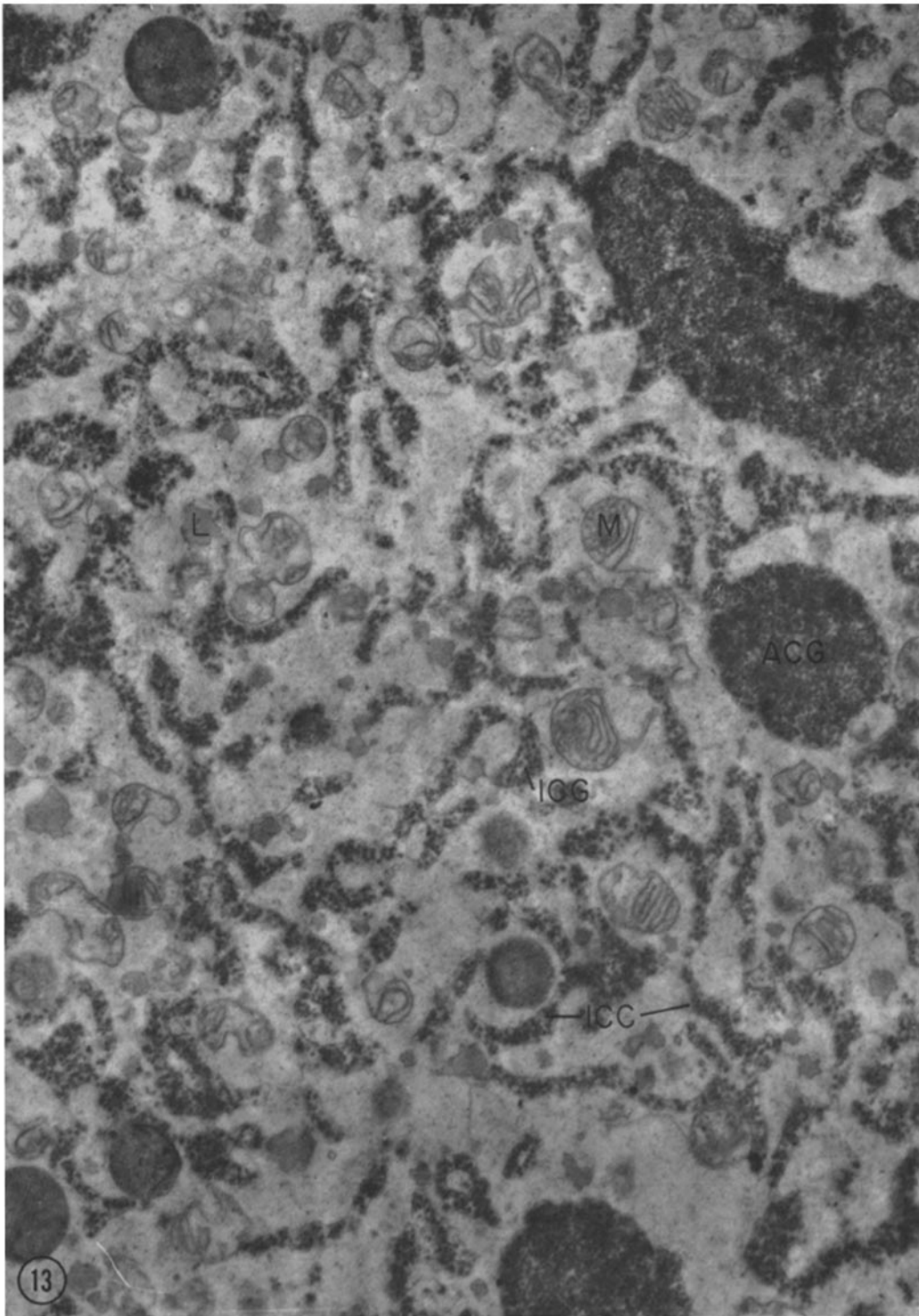
expressed concerning yolk formation. In *Oniscus*, according to King (1926), there are two kinds of yolk: albuminous yolk which is formed in close morphological relationship with mitochondria, and fatty yolk which is formed from the Golgi material. In the crab *Carcinus*, Harvey (1929) found the albuminous yolk to arise in close relationship with the Golgi bodies and with nucleolar extrusions. Bhatia and Nath (1931) found that in the prawn *Palaemon lamarrei* the yolk is formed by a swelling and transformation of the mitochondria, whereas in the crab *Paratalphusa spinigera* it arises directly from nucleolar extrusions.

Kater (1928) has given the best account of the morphological aspects of protoplasmic and deutoplasmic synthesis in the oogenesis of the crayfish. He found that during protoplasmic synthesis the cytoplasm is highly basophilic. Part of the basophilia is due, he thought, to the diffusion of chromatin through the nuclear membrane and to the extrusion of nucleoli into the cytoplasm. The elaboration of yolk is marked by a change from a basophilic to an acidophilic condition of the cytoplasm and to an increase in number of mitochondria and Golgi bodies. In regions of active yolk synthesis the mitochondria become granular and increase in size. After the completion of yolk synthesis they shrink but do not disappear; a condition which suggests that their relationship to yolk formation must be that of a catalyst or synthetic agent. The Golgi bodies were observed in close association with developing fatty yolk globules; a relationship which is thought to indicate their active participation in the synthesis of this material.

Much literature has accumulated since the early studies which showed that ergastoplasm actively participates in the synthesis of protein (see Caspersson, 1950, and Brachet, 1960, for review of literature). Later studies demonstrated that the incorporation of amino acids into proteins is associated with ribosomes (see Siekevitz, 1959, and Porter, 1961, for review of literature). Few cells show a closer fine structural relationship between the rough surfaced endoplasmic reticulum and

their secretion product (yolk) than does the oocyte of the crayfish. That is, the endoplasmic reticulum of this cell can be visualized as a highly organized system for the synthesis, transport, and deposition of proteinaceous yolk. In fact, the mechanism involved in the synthesis of yolk seems closely to parallel that described by Palade (1956) and Siekevitz and Palade (1958a, 1958b, 1958c) for the origin of secretion in the guinea pig pancreas. They present evidence which suggests that the ribosomes synthesize the enzymes which traverse the cisternal membranes and give rise to relatively large intracisternal (prozymogen) granules; the latter seem to have chemical properties similar to those of the definitive zymogen granules. How the intracisternal granules are delivered to other regions of the cytoplasm and to the surface of the cell is not clear; they may exit in some way through the Golgi complex. Other gland cells have been noted to possess dilated cisternae, but their contents, rather than consisting of discrete intracisternal granules, are more homogeneous and probably represent the presence of a colloidal material (Hendler *et al.*, 1957; Dempsey and Peterson, 1955; Wissig, 1960; Brandes and Portela, 1960; Porter and Pappas, 1959; Godman and Porter, 1960; Kessel, 1960). Opaque bodies of unknown function appear within the cisternae of the spider oocyte (André and Rouiller, 1957), and some synthesis of yolk in the snail *Planorbis* may be related to the ergastoplasmic membrane system (Favard and Carasso, 1958).

The endoplasmic reticulum in the crayfish oocyte, unlike that observed in many other oocytes, must be regarded as a highly differentiated system. Both the granular and agranular forms are present in the same cell and the two forms of endoplasmic reticulum are interconnected; a condition which is of limited occurrence thus far. Different functions can be ascribed to the two forms of the endoplasmic reticulum in the crayfish oocyte. Thus, the granular portion seems to be the synthetic region where the yolk precursors first make their appearance. The agranular membranes then appear (1) to function in activities of conduction of

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FIGURES 14 AND 15   Portions of developing oocytes selected to show the accumulation and walling-off of masses of intracisternal granules (*ACG*) from single, branched cisternae (*ICC*, Fig. 14). In many cases it can be observed that several cisternae (*ICC*) contribute granules to the presumptive proteinaceous yolk body. In certain regions the cisternal granules appear to be fused (Fig. 15, arrows). *M*, mitochondrion; *R*, ribosomes. Epon, uranyl acetate. Both figures × 32,000.
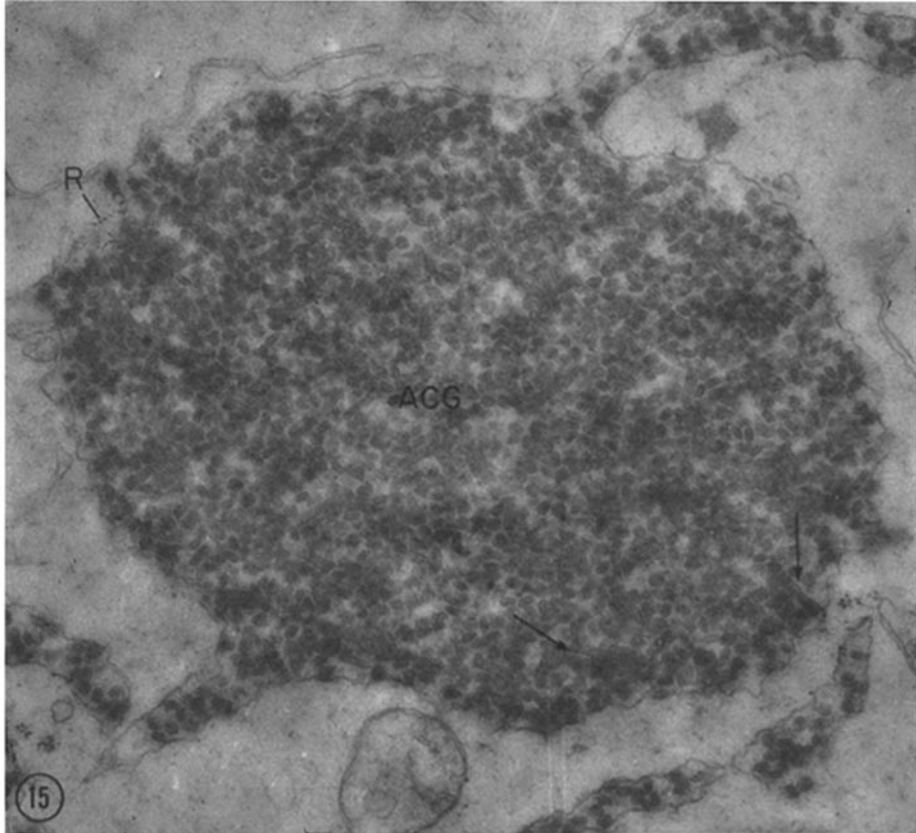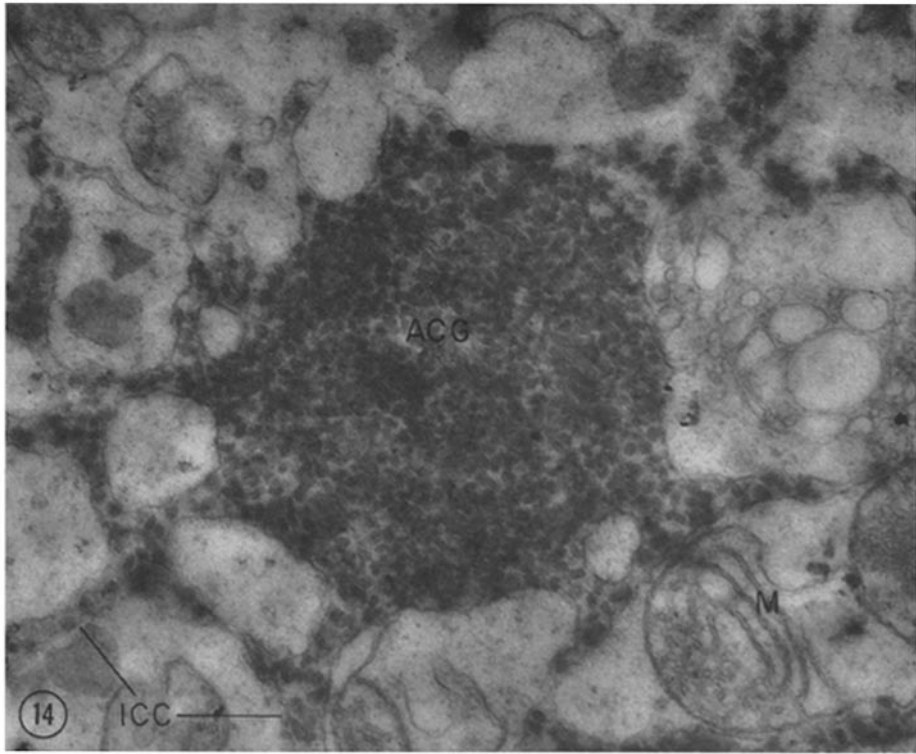
intracisternal granules throughout the oocyte, and (2) to serve as a formative region for the proteinaceous yolk body. There are only a few other instances in which the agranular membranes of the endoplasmic reticulum have been conceived as playing a role in the function of intracellular conduction. These include the sarcoplasmic reticulum of muscles (Ruska et al., 1958; Peachey and Porter, 1959) and the "chloride cells" on the gill filaments of Fundulus (Kessel and Beams, 1962). The endoplasmic reticulum in intestinal epithelial cells has also been implicated in the transport of lipid particles, on the basis of experimental work by Palay (1960).

All genera of crayfish used in this study were found to form their proteinaceous yolk in the same manner. The only difference noted was in the extent to which the cisternal stacks were developed. The latter were more prominent in the northern genera, more diffuse in the southern genus. In addition, a larger part of the branching cisternae in the southern genus was provided with ribosomes.

The results of our study support those derived from biochemical and autoradiographical methods (see review by Prescott, 1960); namely, that the RNA is synthesized in the nucleus, especially the nucleolus, from which it is transferred through the nuclear pores to the cytoplasm. Here as ribosomal RNA it becomes associated with the membranes of the endoplasmic reticulum, which seem to be derived, at least in part, from the outer membrane of the nuclear envelope. As the synthesis of yolk begins it is deposited in the form of precursor granules in the cisternae of the endoplasmic reticulum.

In the oocyte of the mosquito Roth and Porter (1962) have observed the accumulation of protein (yolk) within the oocyte by a process of micropinocytosis. Vesicles are at first pinched off from the surface of the egg and later fuse to produce the definitive yolk bodies. This condition has also been observed by Anderson in the cockroach oocyte (personal communication) and in the milkweed bug oocyte (Kessel and Beams, 1963). Press (1959) has reported that portions of the follicle cells are engulfed into the cytoplasm in the case of the avian egg. Micropinocytotic vesicles are also seen in the crayfish oocyte, but whether or not they contribute to the origin of yolk in this organism is not clear. However, it would appear that if they do play a direct part it is a minor one, and perhaps the material taken into the oocyte in this way is more involved in the over-all metabolism of the cell than it is in the direct synthesis and storage of yolk.

In some eggs it appears that the raw materials are taken into the oocyte and transformed into both ooplasm and yolk. As pointed out by Raven (1961) and in the literature cited above, it is clear that the nature of the nutritive materials taken into the egg may vary. That is, they may be chemically uncomplicated materials such as simple amino acids, and in this case synthesis would probably take place within the egg, as for example in the crayfish oocyte. If the substances taken into the oocyte are prefabricated, so to speak, by other cells of the body such as the liver (Flickinger and Rounds, 1956), they need only be transported to the ovary by the blood stream, taken into the oocyte, and stored as yolk (Telfer, 1961; Roth and Porter, 1962). The latter condition would help to explain the appearance of crystalline yolk bodies within the mitochondria, since the yolk may be only segregated and stored here rather than actually synthesized. However, Ward (1962) finds that extraoocyte synthesis of all proteinaceous yolk in Rana pipiens is difficult to accept. It is not within the scope of this paper to discuss the large amount of biochemical information accumulating with regard to the developing amphibian oocyte, but much of this information is contained in the

FIGURES 16 AND 17 Later stages in the formation of the proteinaceous yolk body by the accumulation of intracisternal granules. Dissolution of most of the intracisternal granules has occurred, resulting in a more homogeneous and finely granular body (PYB). Discrete intracisternal granules (ICG) are still apparent in the peripheral region of certain of the forming yolk bodies, suggesting that a transformation of the intracisternal granules is initiated while the structure is still being formed. Numerous connections with granule-filled cisternae of endoplasmic reticulum are still evident. In some cases (mostly southern crayfish) ribosomes (R) may be present on the membranes limiting the forming yolk body, as well as the interconnecting cisternae. L, lipid; M, mitochondrion. Fig. 16, × 25,000; Fig. 17, × 42,000.
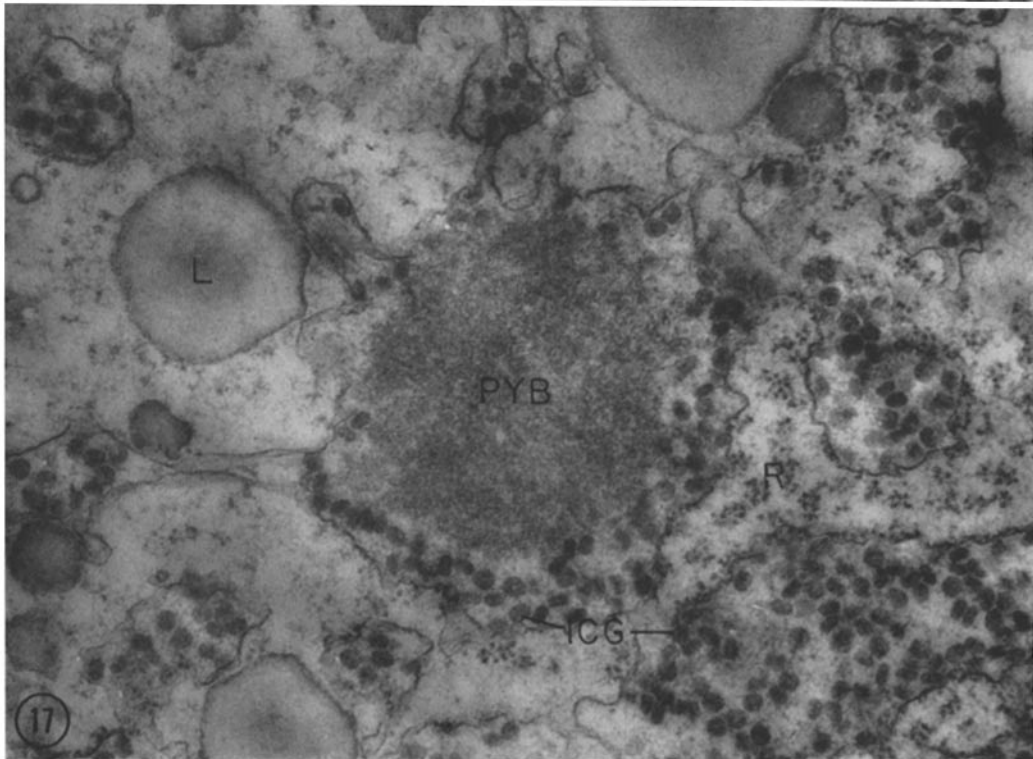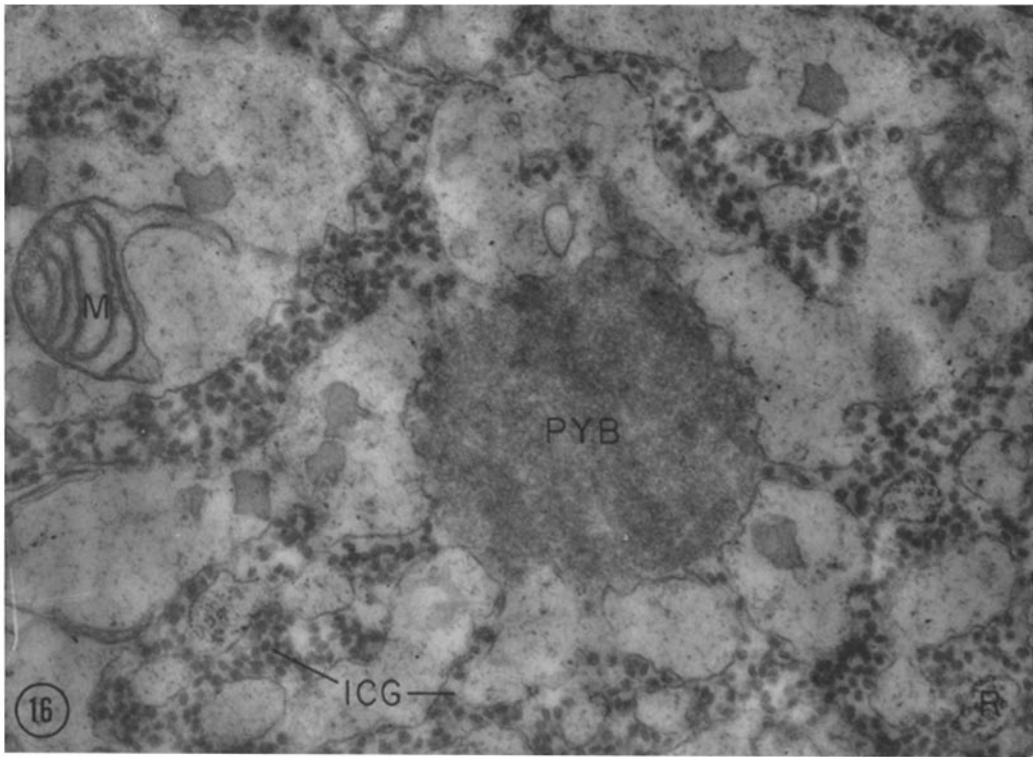
work of Ficq (1955), Kemp (1955), Pantelouris (1958), and Brachet (1960) and is well summarized in the review by Raven (1961).

Little evidence was obtained in the study of the oocyte of the crayfish to reveal the method of the origin of fatty yolk. It seems simply to arise *de novo* from the cytoplasm without close morphological relationship to any specific cytoplasmic organelle. In the frog oocyte, Ward (1962) has described the "fatty yolk" as appearing within the mitochondria, and several light microscope studies have also directly related fatty yolk formation to the activity of the Golgi material and/or mitochondria (see Raven, 1961, for review).

No evidence has been revealed which would tend to link either the Golgi material or mitochondria with the synthesis of yolk in the crayfish. However, some mitochondria show regions which exhibit unusual and strikingly complex morphological patterns in their cristae. These appear to arise from an extensive branching and packing of the cristae into an oriented pattern which resembles to some degree the tubular pattern found in the mitochondria of *Pelomyxa carolinensis* (Pappas and Brandt, 1959), but more especially they are like the stacking of the membranes sometimes observed in chloroplasts (see Granick, 1961, for review of literature). The significance of this condition is obscure; it may be correlated in some way with a special and localized enzymatic activity within the mitochondria. The matrix of the mitochondria is of about the same density as the surrounding ooplasm except for the presence of occasional dense granules. The latter have been reported many times especially in cells in which a significant amount of water and cations flow (see Haguenau, 1958, for review of literature).

Bodies similar in shape and structure to the striated microtubules present in the crayfish oocyte have been observed in certain cancer cells infected with virus (Leduc and Bernhard, 1962). However, no relationship between these bodies and the virus infection has been revealed and their nature remains unknown.

Finally, in those cases where the yolk is presumably formed elsewhere than in the oocyte, it would be interesting to know whether or not the endoplasmic reticulum plays a major role in its synthesis.

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FIGURE 18 Several protein yolk bodies (PY) are shown to have a heterogeneous appearance. This condition may be due to some stage of the embedding procedure. This appearance is not a constant one. Several discrete granules may be observed in the most peripheral region of these yolk bodies, and in several places (arrows) attachments are still evident between the yolk body and the intercommunicating cisternae. Numerous small lipid bodies (L) are also present. Epon, uranyl acetate. × 20,000.

FIGURE 19 Electron micrograph of a large, mature oocyte obtained during November. The cytoplasm of the oocytes at this stage is filled with globules of lipid (LY) and proteinaceous yolk (PY). The proteinaceous yolk globules, though variable in size, may be several microns in diameter. The large proteinaceous yolk globules do not appear to be connected with elements of the endoplasmic reticulum at this time. Between the closely packed lipid and protein yolk globules are located the single branching lamellae of the smooth form of the endoplasmic reticulum (ICC). Intracisternal granules are still present in quantity and were probably synthesized earlier in development. Walling-off of groups of intracisternal granules and subsequent transformation into yolk particles occurs late in the growth of the oocyte, mainly in the peripheral region of the cytoplasm. Vestopal W, uranyl acetate. × 15,000.
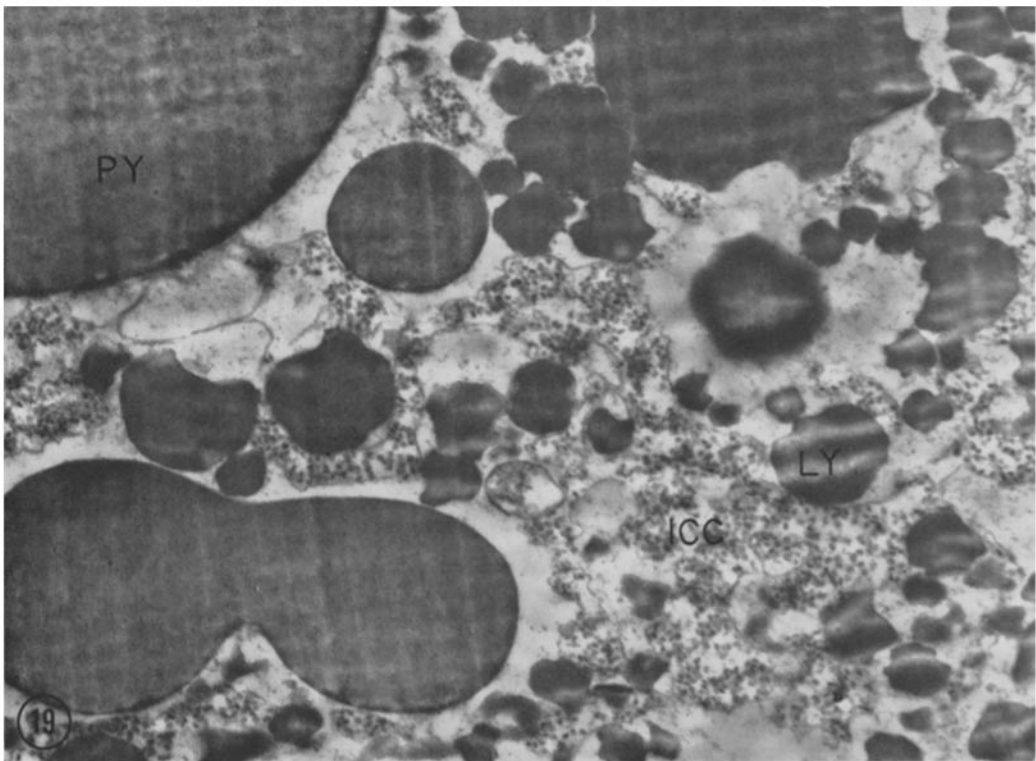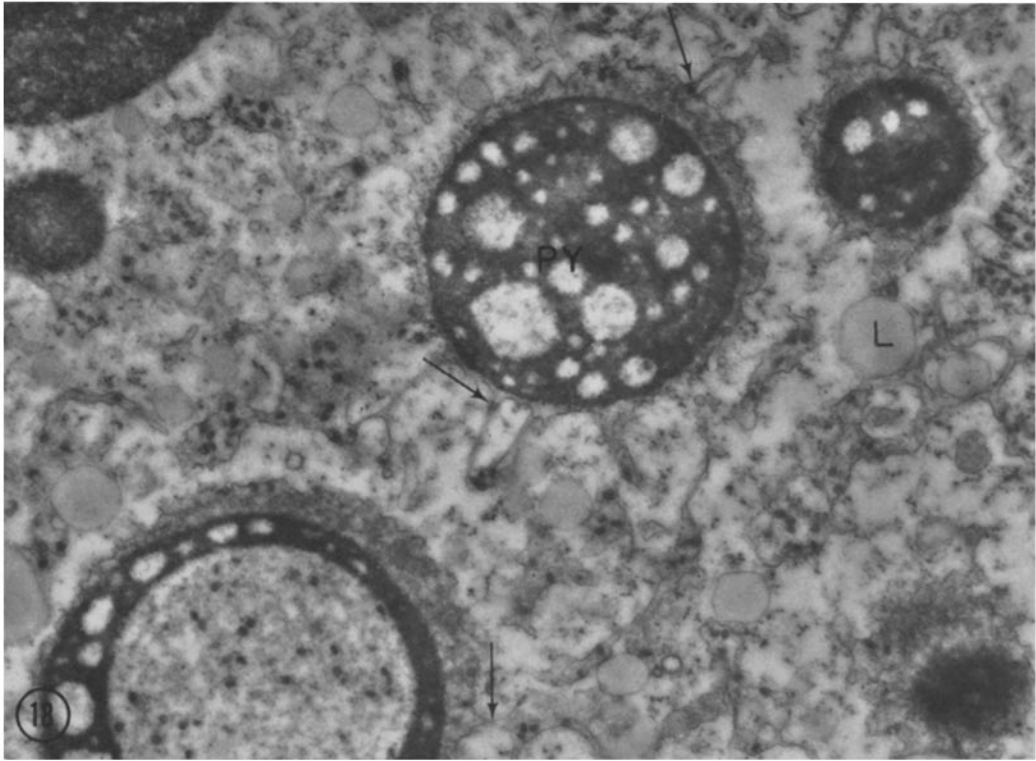
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FIGURE 20 The relation between the periphery of the oocyte and the follicle cell (FC) is shown. Numerous microvilli (MV) are shown. In the oocyte, a portion of a large proteinaceous yolk body (PY) is shown, as well as a stack of rough surfaced endoplasmic reticulum (ERS). ICC, intercommunicating cisternae; M, mitochondrion. Clusters of dense microtubules are seen at MT. Epon, uranyl acetate. × 14,000.

FIGURE 21 Higher magnification of oocyte (O) and follicle cell (FC) junction. Numerous dense vesicles (PV) are observed (arrow). Their significance is not known but they may represent a pinocytotic activity by the oocyte surface or a stage in the formation of a chorion by the oocyte. Epon, uranyl acetate. × 53,000.
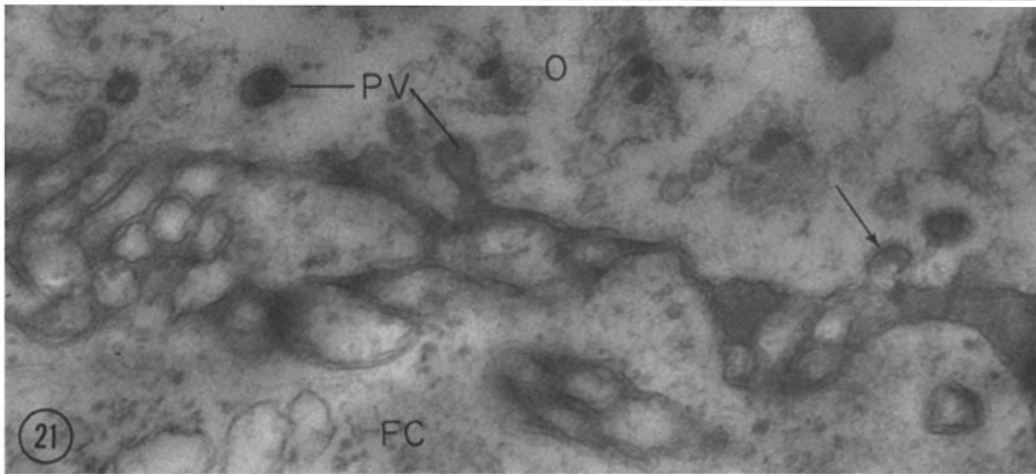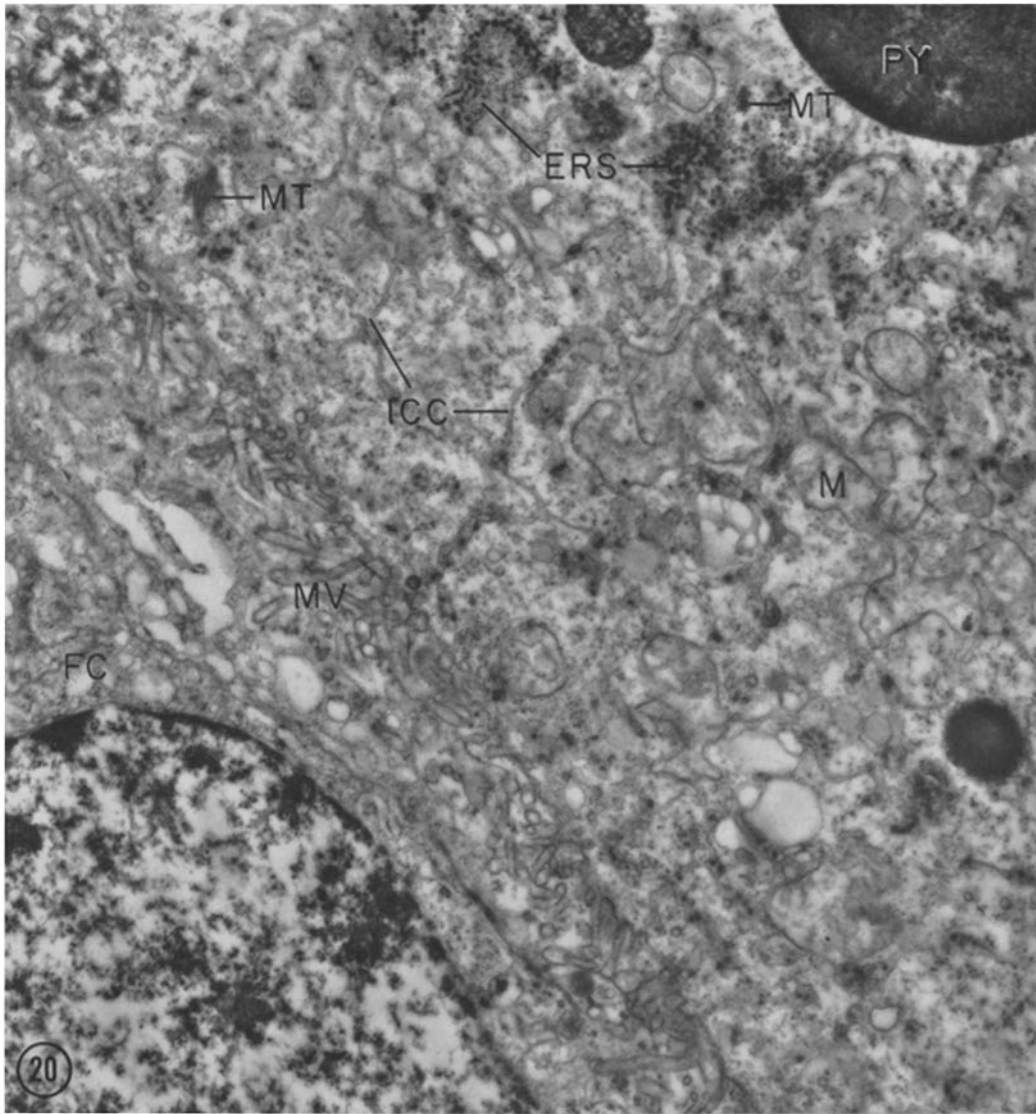
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Figures 22 to 24 Examples of pleomorphism in the mitochondria. In some cases, the cristae are closely packed in certain regions of the mitochondria (Figs. 22 and 24, *M*). This condition is encountered quite frequently. Dense bodies (Fig. 24, arrows) are often found in the mitochondria. The mitochondrial cristae often show considerable branching and anastomosis (Fig. 23, *M*). *ERS* (Fig. 22), cisternal stack of endoplasmic reticulum. *PYB* (Fig. 24), a forming proteinaceous yolk body. Epon, uranyl acetate. Fig. 22, × 32,000; Fig. 23, × 53,000; Fig. 24, × 53,000.
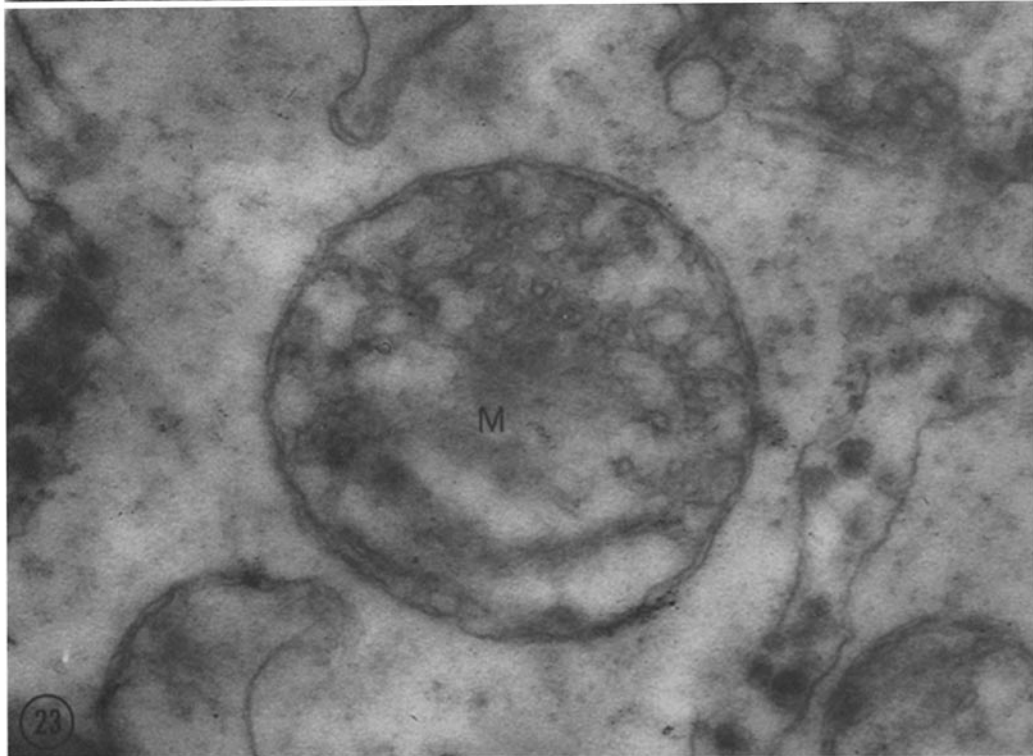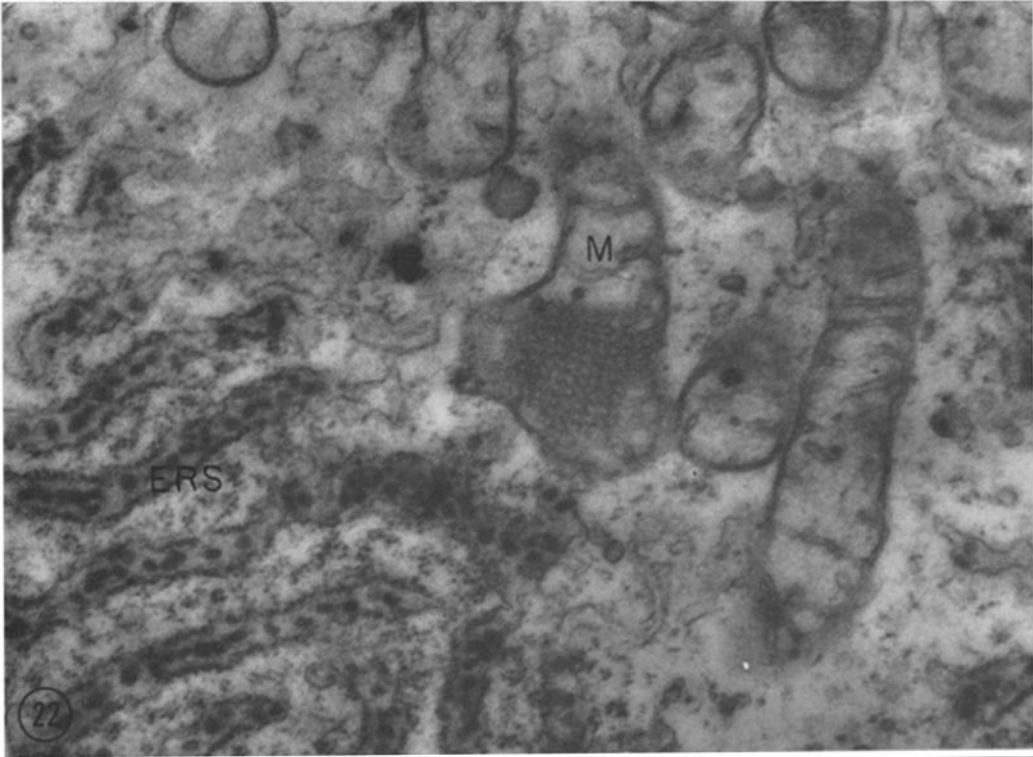
Figures 25 and 26   A group of closely packed filaments or tubules within the oocyte cytoplasm (*MT*). These microtubules appear to be striated or to have an axial periodicity. Small dense granules are observed in association with the structures (Fig. 25, arrows). In some cases the tubules have a close association with intercommunicating cisternae (*ICC*, arrow, Fig. 25), but the significance of this is unknown. The microtubules have been observed with moderate frequency in the oocyte and do not appear to be restricted to any particular region. The structure of the microtubules (*MT*) is somewhat more evident in transverse section. The periphery of the tubules is of considerable density. In some cases a small dense granule appears within the tubule (Fig. 26, arrow). Epon, uranyl acetate. Fig. 25, $\times$ 53,000; Fig. 26, $\times$ 46,000.
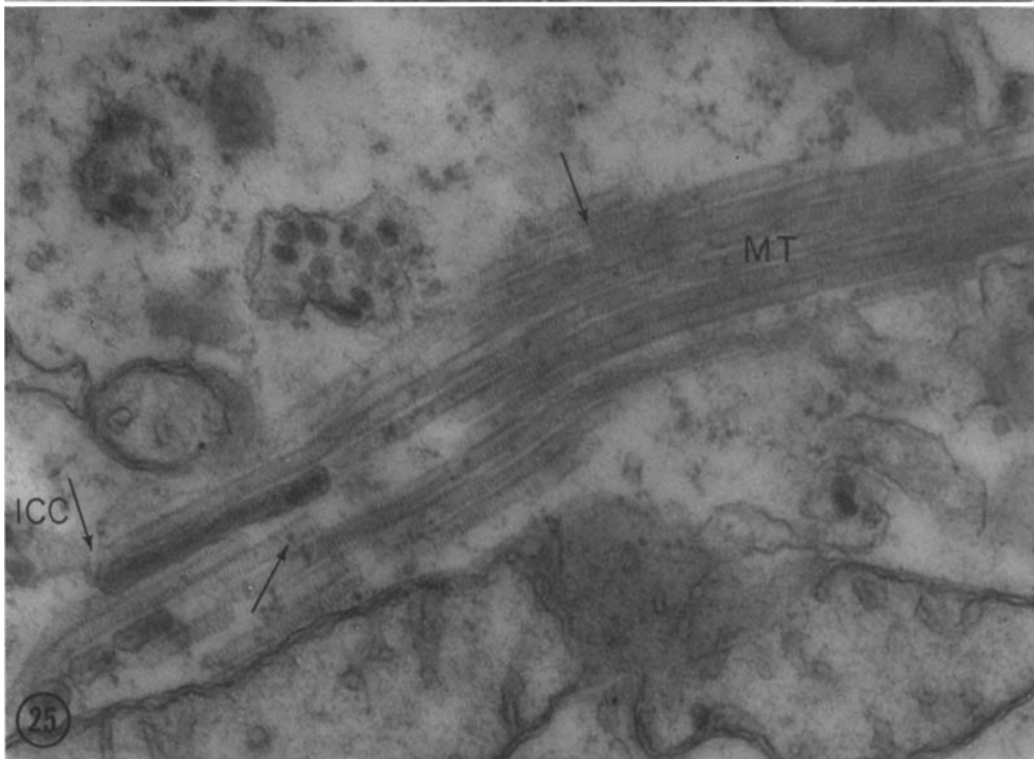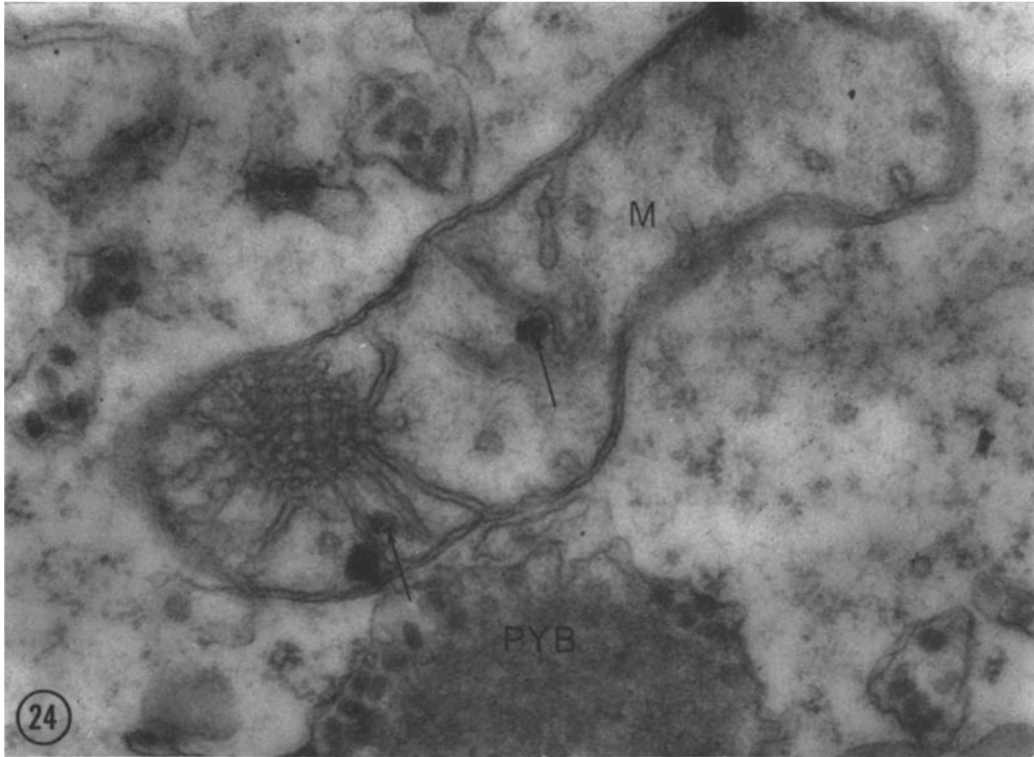
FIGURE 27 Complex membranous structures (*HMB*) are often observed even in young oocytes. They usually contain dense granules and numerous units composed of concentrically arranged membranes. The body may be completely surrounded by a single membrane or only partially enclosed as observed in the figure. *ERS*, a stack of rough surfaced endoplasmic reticulum. Epon, uranyl acetate. × 32,000.

FIGURE 28 The Golgi material in the oocyte is widely distributed and consists of severa parallel, agranular, flattened membranous cisternae (*GA*) and associated vesicles (*V*). A close association is often observed between elements of the Golgi material and the intercommunicating cisternae (*ICC*) of the endoplasmic reticulum. In some cases a few granules resembling those located within the endoplasmic reticulum are observed within the Golgi elements (arrows). Epon, uranyl acetate. × 53,000.
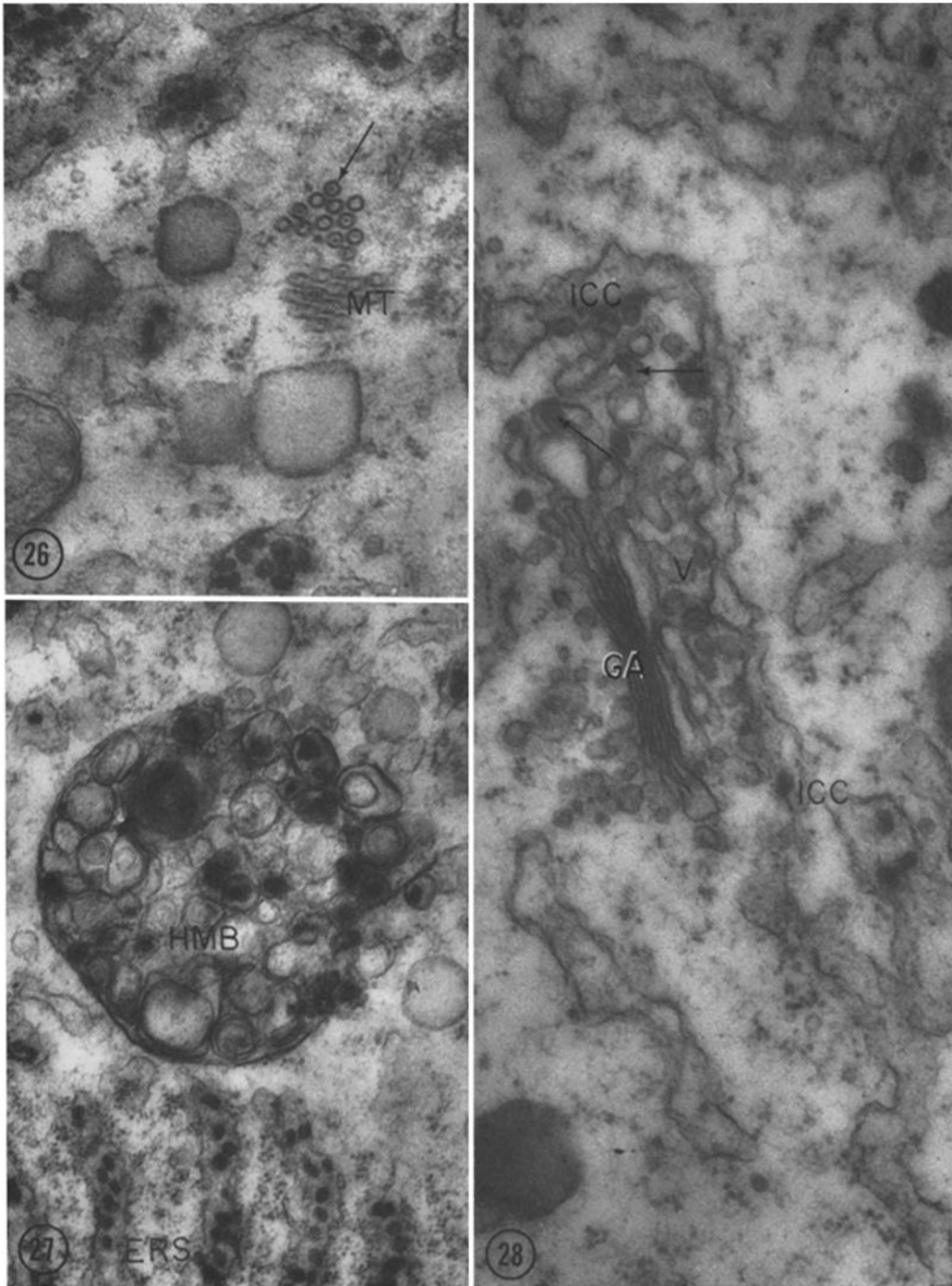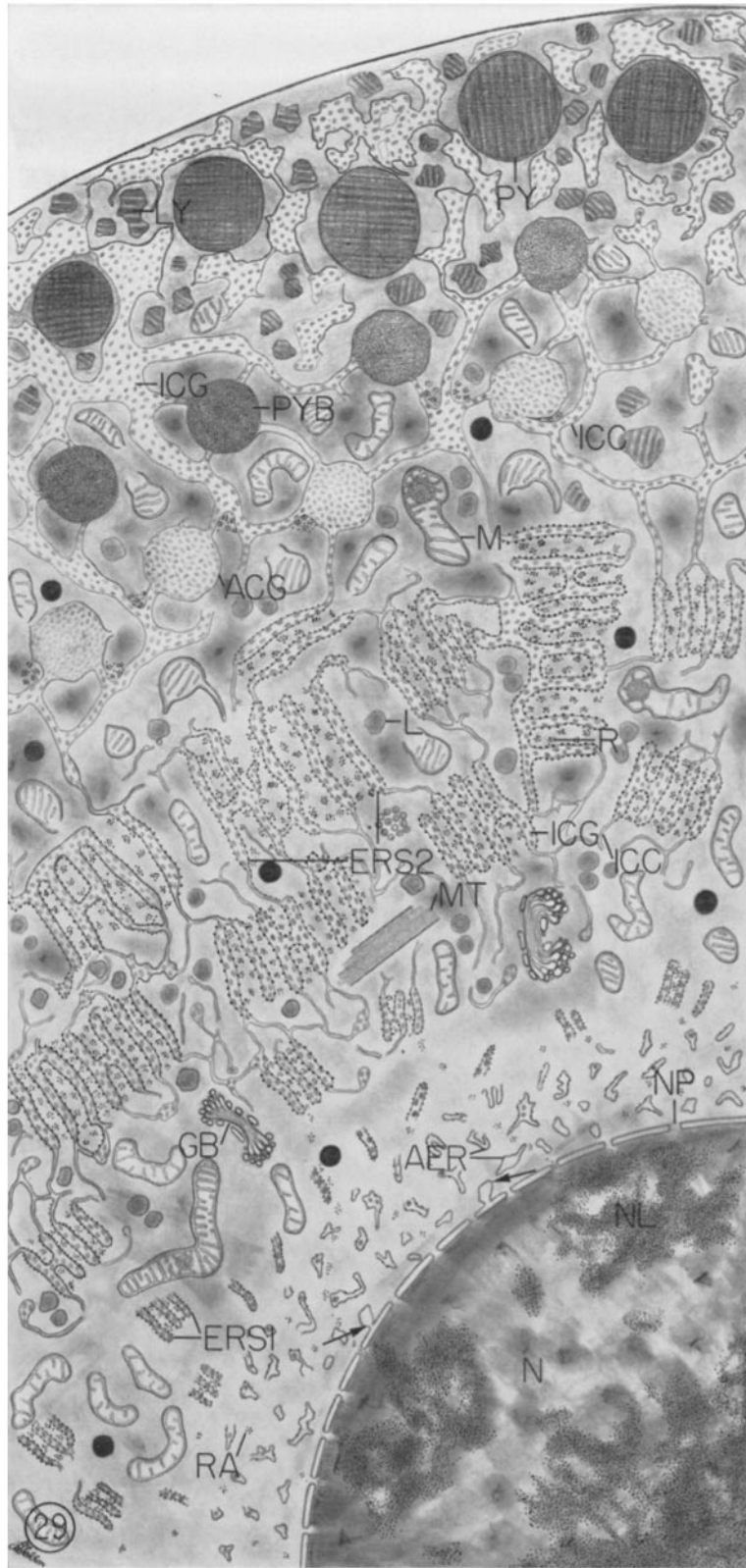
FIGURE 29 Diagram summarizing the events of differentiation and growth in the cray-
fish oocyte. The stages in the differentiation and activity of the endoplasmic reticulum and
its role in the formation of proteinaceous yolk are emphasized. The structures shown are:
nucleus (N), nucleolus (NL), nuclear pore (NP), ribosomal aggregates (RA), agranular
endoplasmic reticulum (AER), forming cisternal stacks of rough surfaced endoplasmic
reticulum (ERS1), differentiated stacks of rough surfaced endoplasmic reticulum (ERS2),
intercommunicating smooth surfaced cisternae (ICC), intracisternal granules (ICG),
ribosomes (R), mitochondria (M), small lipid particles (L), Golgi material (GB), aggregates
of intracisternal granules (ACG), immature yolk bodies (PYB), striated microtubules (MT),
lipid yolk (LY), proteinaceous yolk (PY). Areas showing blebbing of the outer nuclear
membrane are seen at unlabeled arrows.