OOCYTE DIFFERENTIATION AND VITELLOGENESIS IN THE ROACH *PERIPLANETA AMERICANA*

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ABSTRACT

The ovary of the roach *Periplaneta americana* has been studied by techniques of light and electron microscopy. Each ovariole (panoistic type) contains a linear array of oocytes in varying stages of development. Newly formed oocytes become encased by a layer of follicle cells and begin pinocytosis. All subsequent growth stages of the oocytes are dependent, in part, on this phenomenon. All of the pinocytotic caveolae show an unique surface modification; *i.e.*, on their internal surface they have an amorphous or filamentous substance and their external surface is studded with many fine radially oriented spike-like projections. The pinosomes of early oocytes do not contain a demonstrable internal structure; they are thought to contain nutritive substances for the developing oocytes rather than yolk precursors. When the oocyte enters its last stage of growth, characterized by yolk deposition, the caveolae become filled with a dense material which is thought to be the precursors of yolk. Hence the conclusion is drawn that yolk formation is independent of any cytoplasmic organelle system of the oocyte and that the precursors of this deutoplasmic substance are manufactured outside the ovary and are internalized by the process of pinocytosis. Under the phase-contrast microscope the nucleoli of early oocytes are large irregular masses and show the phenomenon of nucleolar emission (fragmentation). These "emissions" become randomly dispersed in the nucleoplasm and some of them come to be intimately associated with the fenestrated nuclear envelope. After this process ceases, the main nucleolar mass becomes vacuolated. Electron micrographs suggest that the constituent particles of the nucleolar emissions migrate from the nucleus through patent pores of the nuclear envelope.

Within the past several years investigators have presented pictorial evidence which makes it clear that oocytes of invertebrates and vertebrates possess the same basic substructural organization (Anderson and Beams, 1960; Anderson, 1962; Beams and Kessel, 1962; Kessel and Beams, 1963; Kemp, 1956; King and Devine, 1958; Okada and Waddington, 1959; Sotelo and Porter, 1959). Notwithstanding the agreement which has been reached concerning the basic microanatomy of mature oocytes, no such consensus is evident with respect to the mechanisms of vitellogenesis. Although unanimity may be reached concerning particular features of this phenomenon within the class Insecta, apparently no generalization can be drawn for the entire animal kingdom. In order to inquire into the complicated dynamic processes involved in the development of oocytes (including those in which the cytoplasm is devoid of yolk and those in which it becomes filled with copious amounts of yolk at certain stages), it is evident that a detailed knowledge of the ultrastructural changes which presumably take place during the various stages of their maturation is needed. Such information

might supply invaluable clues as to how particular aspects of growth and/or vitellogenesis are accomplished. Therefore, the present investigation was undertaken in an effort to study (*a*) differentiation of oocytes, paying special attention to yolk deposition, (*b*) the cytoarchitecture of follicular epithelial cells, and (*c*) bacteroides, the symbiotic microorganisms found at the follicular cell-oocyte interface.

MATERIALS AND METHODS

The observations described in this communication are based on ovaries obtained from 150 gravid roaches (*Periplaneta americana*). The ovaries were removed from animals which had been either decapitated or previously anesthetized with carbon dioxide according to the method of Williams (1946). For the study of living material, some of the ovaries were placed in *Drosophila* Ringer's solution (Ephrussi and Beadle, 1936) and the ovarioles examined with phase-contrast optics. For light microscopic analysis, ovaries were fixed in the following: (*a*) Helly's fluid; (*b*) Bouin's; (*c*) Carnoy's; (*d*) Carnoy-Lebrum; (*e*) 10 per cent formalin; or (*f*) 10 per cent acrolein (Luft, 1959). Paraffin sections were cut according to the method of Slifer and King (1933) and stained with either Heidenhain's iron hematoxylin, Giemsa, thionin or Mallory's triple stain. Sections of Carnoy's-fixed material were treated by the following procedures: (*a*) Korson's trichrome stain (1951) for differential staining of nucleic acids with and without prior RNase or DNase digestion,[1] (*b*) Feulgen technique according to the method of Bonhag (1955) with and without prior DNase digestion, and (*c*) the periodic acid–Schiff (PAS) technique for the demonstration of polysaccharides according to the method of Hotchkiss (*cf.* Glick, 1949) with and without prior digestion with salivary amylase. In order to detect protein, ovaries which had been fixed in formalin were stained by Bonhag's modification of the Mazia-Alfert brom-phenol blue technique (*cf.* Pearse, 1961).

For electron microscope studies, ovaries were fixed in either a cold 2 per cent solution of osmium tetroxide which had been buffered (pH 7.5) with Veronal-acetate (Palade, 1952) or a 1 per cent solution of osmium tetroxide containing phosphate buffer (pH 7.5) (Millonig, 1962). In the study of oviposited eggs, oothecae were removed from the roaches, carefully opened, and submerged in the osmium fixatives. Each egg was removed, the chorion being punctured with a fine needle, and placed in fresh fixative for 2 to 3 hours. All of the osmium tetroxide–fixed material was rapidly dehydrated and embedded in Epon (Luft, 1961). Thin sections were stained with either uranyl acetate or according to the lead hydroxide method of Karnovsky (1961) and examined in an electron microscope (RCA-3E).

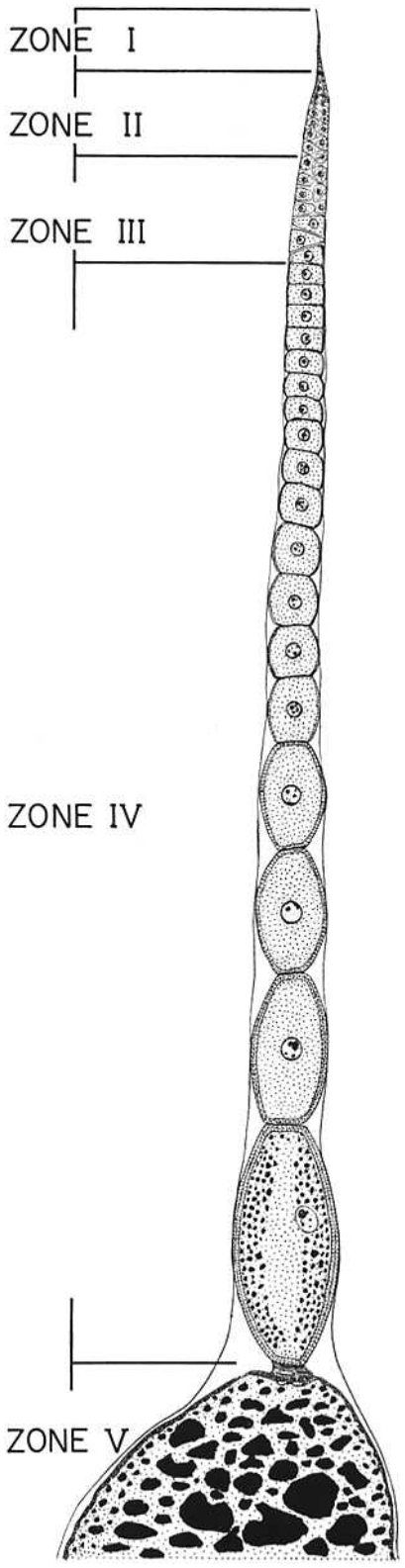
[1] Ribonuclease and deoxyribonuclease were obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

OBSERVATIONS

Histology of Ovariole

An ovary of *Periplaneta americana* consists of eight slender tapering ovarioles. Each ovariole is composed of a variable number of oogonia and oocytes in different stages of cytomorphosis. As each oogonium differentiates into an oocyte it becomes completely surrounded by a layer of follicle cells; each oocyte is separated from adjacent ones by layers of interfollicular tissue. There are no nurse cells associated with these oocytes, hence they are classified as a panoistic type (*cf.* Wigglesworth, 1950). According to Bonhag (1959), each ovariole can be divided, on the basis of its histological features into six zones. Fig. 1 is a diagrammatic representation of an ovariole as seen in longitudinal section illustrating the first five zones. The phase-contrast photomicrographs depicted in Figs. 2 to 8 illustrate some of the salient features of oocytes comprising these zones. The detailed histological picture has been well documented by Bonhag (1959). Certain aspects of these zones, however, merit attention to clarify the interpretation of electron micrographs. Zone I consists of the terminal filament (Fig. 2, *TF*). The terminal filaments, one from each ovariole of an ovary, unite to form a suspensory ligament which attaches the ovary to the wall of the thorax. The germarium constitutes zone II (Fig. 1). It consists of oogonia, young oocytes, and prefollicular cells (Fig. 2, *G*). Zone III (Fig. 1) is composed of oocytes which are not oriented in a linear fashion and in many instances are incompletely encompassed by follicle cells (Fig. 3). The largest portion of the ovariole is zone IV (Fig. 1). It is composed of oocytes linearly arranged; they vary in size and each is completely surrounded by a layer of follicle cells. This large zone may be further divided into (*a*) anterior (Fig. 4), (*b*) middle (Fig. 5), and (*c*) posterior portions (Figs. 6, 7, and 8). Zone V (Fig. 1) contains oocytes filled with numerous yolk bodies. Zone VI is the pedicle and will not be dealt with in this study.

As oocytes differentiate they pass through approximately three auxocyte or growth stages. In



zone II (germarium), the oogonia differentiate into young primary oocytes (Bonhag, 1959). Here relatively little growth is accomplished. If one examines a light microscope preparation of this area previously stained with a basic dye or Korson's trichrome, the cytoplasm appears slightly basophilic. The second growth phase occurs within the third and fourth zones. In these zones there occur (*a*) an increase in cell size, (*b*) a variability in cell shape, (*c*) an increase in nucleolar activity, and (*d*) an increase in cytoplasmic basophilia. There appears to be a decrease in cytoplasmic basophilia in oocytes of the last portion of zone IV and in those which occupy zone V. The third growth phase (posterior part of zone IV and anterior portion of zone V) begins and ends with the deposition of yolk.

Ultrastructure

OOGONIA AND OOCYTES: The cytoplasm of oogonia is similar in its ultrastructural organization to that encountered in young oocytes from zone III. It contains mitochondria evenly distributed in the cytoplasm. The elongate and ovoid profiles of these organelles show their cristae to be transversely oriented. There seems to be an increase in the number of mitochondria in oocytes of the anterior and middle portions of zone IV. In these regions, the organelles become located in the peripheral cytoplasm (Fig. 15, *M*). Golgi complexes are few in number, but, like the mitochondria, they are allocated to the peripheral cytoplasm (Fig. 15, *GC*). A striking feature of the cytoplasm of all oocytes, regardless of their position within the ovariole, is the absence of an organized endoplasmic reticulum. Instead, the cytoplasmic matrix is filled with a host of dense RNP particles. Randomly dispersed within the cytoplasm are relatively large, dense structures whose interiors are composed of two and sometimes three laminated areas enclosed by a thin membrane (Fig. 15, *LB*).

The oolemma of oogonia and young oocytes from the middle of zone II is a smooth membrane with no evidence of morphological specialization

FIGURE 1 Diagrammatic representation of an ovariole (panoistic-type) of *Periplaneta*, as seen in longitudinal section, illustrating the major zones. The division of the ovariole into zones, on the basis of its histological and cytological features, is that employed by Bonhag (1959).

(Fig. 11, *OL*). The plasma membrane of follicle cells (Fig. 11, *FP*) is closely applied to the oocyte, being separated from the oolemma by a space approximately 200 to 300 A wide. Specialization of the oolemma, in the form of microvilli, is first noted in oocytes from the lower portion of zone III. Fig. 12 illustrates a small area at the surface of one such oocyte and shows long and circular profiles of a few microvilli (*MV*). On oocytes of the remaining zones the formation of microvilli becomes complete, that is, the entire surface of each oocyte is covered with a multitude of these protoplasmic projections (Figs. 15, 20, 22, and 24, *MV*). At light microscopic levels the oolemma of all oocytes is PAS-positive, a reaction that is not abolished by prior salivary amylase digestion.

When the follicle cells come to completely encompass the oocyte (zone III) the latter enters the second part of the growth phase which commences with, *inter alia*, pinocytosis. The insert in Fig. 11 shows the beginning of this process. The magnitude of the process increases and in oocytes of zone IV the peripheral cytoplasm teems with the resulting pinosomes; they are devoid of any internal structure (Fig. 20, *P*). Attention is called to the structure of the membrane of the caveolae and pinosomes. In Fig. 11 (see arrows on insert) are profiles of caveolae which were fixed in the process of being brought into the general cytoplasm. Here, as well as in other oocytes (zone III: Fig. 15, arrows), one can discern, on the surface of the caveolae adjacent to the ooplasm, fine radially oriented filaments or spike-like structures. The inner membrane portion of the caveolae appears to contain a small quantity of a homogeneous, electron-transparent amorphous substance which may be responsible for the PAS-positive reaction at the surface of all oocytes. When the caveolae become pinosomes they initially retain their surface modifications (Fig. 20, *P*).

Oocytes from the posterior portion of zone IV enter the third and final phase of growth; *i.e.*, the deposition of yolk. A light microscope section of such an oocyte stained with Heidenhain's hematoxylin is illustrated in Fig. 21. There are many basophilic bodies of varying diameters in the peripheral cytoplasm of these oocytes. These structures give a positive reaction for protein when Bonhag's modification of the Mazia-Alfert bromphenol blue technique is used. They are PAS-positive and salivary amylase-resistant. Such reactions suggest that these bodies are composed of at least two components: protein and polysaccharide. These observations confirm those made by Bonhag (1956, 1959). From electron micrographs of an area corresponding to that shown in Fig. 21, it is evident that this period of the growth phase is likewise dependent, in some way, on the mechanism of pinocytosis (Figs. 22 and 24). Close examination of caveolae reveal that they possess a surface structure like that seen on caveolae of the oolemma of oocytes from zone III, and anterior and middle portions of zone IV (Fig. 23, C_4). These caveolae, however, do have a relatively large quantity of a dense, filamentous, or amorphous substance (PAS-positive) on their outer membranes (Fig. 24, C_2, C_3). Not only is the filamentous material found associated with caveolae but it is also found along the edges of microvilli. When the microvilli are viewed in cross-section their surfaces are studded with fine filaments (Fig. 20, *MW*). This material is similar to the "antennulae microvillares" described by Yamada (1955*a*) on the microvilli of cells of the epithelium of the gall bladder and the filamentous material seen by Ito and Winchester (1963) on the microvilli of mucous cells from the stomachs of bats. (see also Peachey and Rasmussen, 1961; Sjöstrand and Elfvin, 1962).

If one could follow the complete process whereby the caveolae are eventually internalized as discrete units, one might designate caveolae C_2 (Fig. 24) as the beginning of this process which would continue to C_3 (Fig. 24) and to C_4 (Fig. 23), when they are eventually pinched off and become constituents of the ooplasm (Fig. 24, YB_1). Since these bodies are thought to contain the precursors of yolk, they will be referred to hereafter as yolk bodies. A profile of a yolk body presents the following organization. It is bounded by a double-membrane envelope. The surface of the inner membrane is laden with an amorphous material and the outer one is studded with numerous radially arranged spike-like projections. In the interior of these structures is an electron-opaque material. The growth of the yolk bodies is accomplished either by synthesis of the internal material or by a coalescence of smaller yolk bodies. The latter method appears more plausible (Fig. 22, *YBC*). The yolk bodies do not show any preferred orientation in the cytoplasm, nor do they have any unique anatomical association with a particular organelle system, such as mitochondria or Golgi complexes. In the cytoplasm are also found remnants of oil droplets (Figs. 22 and 24, *OD*).

NUCLEUS: Another aspect of the growth phase of oocytes is seen in the activity of nuclear contents. In living material the nuclei of all oocytes are large and spherical. The nucleoli of oogonia and oocytes from zone II (Fig. 2) to the anterior portion of zone IV (Fig. 4) are massive and irregular in shape, while those of the lower portions of zone IV are usually spherical and display many vacuolated areas (Fig. 6).

The nucleolus emits small masses of material and reaches its highest point of activity in oocytes of the upper portion of zone IV. These masses have been referred to as nucleolar emissions (*cf.* Montgomery, 1898; Bonhag, 1958). One can record some aspects of this process by phase-contrast microscopy 2 to 3 minutes after the removal of the ovary from the animal. A perfect example of this process is demonstrated in Fig. 9. This picture of an oocyte was obtained from the anterior region of zone IV. Here, initially, the nucleolar emissions appear like "droplets of dew" (Witschi, 1956) on the nucleolus. If one observes such an oocyte for 10 minutes, the nucleolar emissions move out into the nucleoplasm where they become randomly distributed as illustrated in Fig. 10, *NE*. Many of the phase-contrast pictures, as well as fixed and stained preparations of ovarioles, show that the nucleolar emissions can become intimately associated with the inner surface of the nuclear envelope. After the cessation of the nucleolar emission phenomenon, it appears that the main nucleolus becomes vacuolated (Fig. 10, *VN*). The author has not been able to demonstrate nucleolar emissions in the cytoplasm adjacent the nuclear envelope, nor has he observed similar structures in the cytoplasm in light microscope preparations. What has been observed, however, is a small rim of basophilic material at the periphery of the nuclear envelope (Fig. 13, *NB*). The basophilia of the nucleolus, the nuclear emissions, the basophilia at the surface of the nuclear envelope and within the general cytoplasm are all abolished by prior digestion with RNase. Such a reaction indicates that these areas contain a high content of ribonucleoprotein.

Fig. 16 is an electron micrograph of a nucleolus from an oocyte in the anterior portion of zone IV. Most of the central area is occupied by a compact mass of dense particles and a few light areas. Extending out from this area are 100 to 150 A particles arranged in reticular fashion (nucleolonema of Estable and Sotelo, 1955). A nucleolar emission is observed in the upper left portion of the figure as well as in Figs. 11 and 18 (*E*). In Fig. 11 are two areas, each consisting of small clusters of dense particles similar in size to those composing the nucleolonema. Some of these clusters appear to be arising from the nucleolar emission (Fig. 11, *NB*).

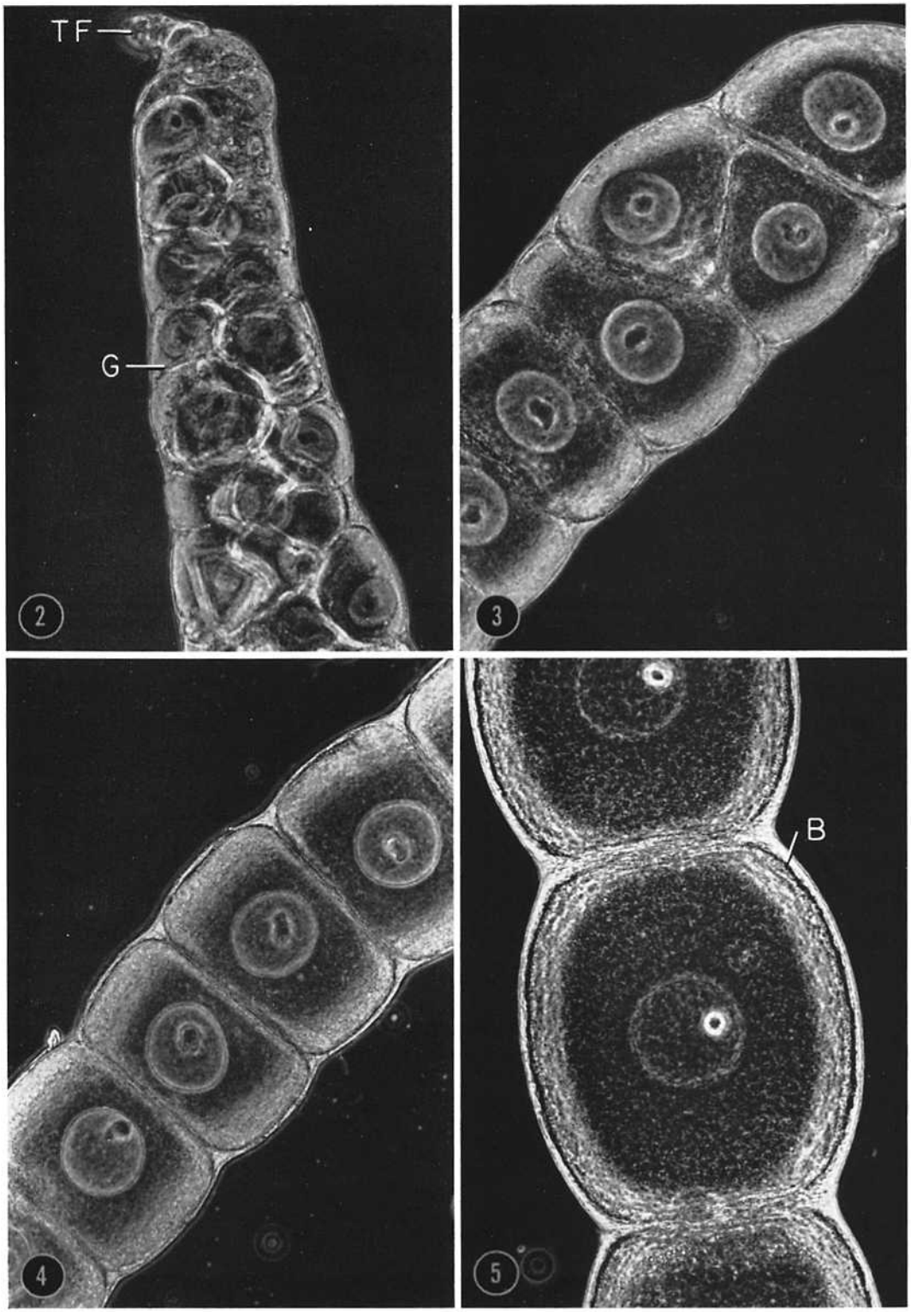
The nucleus is limited by a fenestrated double-membrane envelope (Figs. 11 and 18, *NE*). In sections made tangential to the surface of the nuclear envelope the pores are clearly visible (Figs. 17 and 18, *NP*). Attention is called to the small clusters of dense particles closely associated with the nuclear envelope as well as with the pores (Figs. 17 and 18, arrows). These clusters of dense particles may be responsible for the rim of basophilia seen at the surface of the nuclear envelope in light microscope preparations.

FOLLICLE CELLS: The follicle cells which incompletely surround young oocytes and completely encompass those which appear in zone III are of the squamous type. As the oocytes enlarge, the follicle cells rapidly increase in number, becoming cuboidal in the middle portion of zone IV. Their cuboidal nature is maintained until the oocytes reach the posterior portion of zone IV where they become columnar, only to change

FIGURES 2 through 10 Phase-contrast photomicrographs illustrating various zones of an ovariole. Fig. 2 shows the first zone, composed of the terminal filament (*TF*), and zone II, the germarium (*G*), which consists of oogonia in its upper portion and young oocytes in the lower portion. Fig. 3 is a segment of the ovariole showing a small region of zone III distinguished by oocytes arranged in a non-linear fashion. Zone IV is the largest zone of the ovariole and may be divided into three regions: Fig. 4 shows oocytes from the anterior portion; Fig. 5, from the middle portion and Figs. 6, 7, and 8, from the posterior portion. Note the large, irregularly shaped nucleoli in the oocytes illustrated in Figs. 3 and 4.

Fig. 9 is an oocyte from the anterior region of zone IV showing a stage during the formation of nucleolar emissions. Ten minutes later (Fig. 10) the nucleolar emissions appear as dense spherical bodies in the nucleoplasm (*NE*), and the main nucleolus becomes vacuolated (*VN*). Figs. 2 through 8, × 200; Figs. 9 and 10, × 975.

(*For figures, see following pages.*)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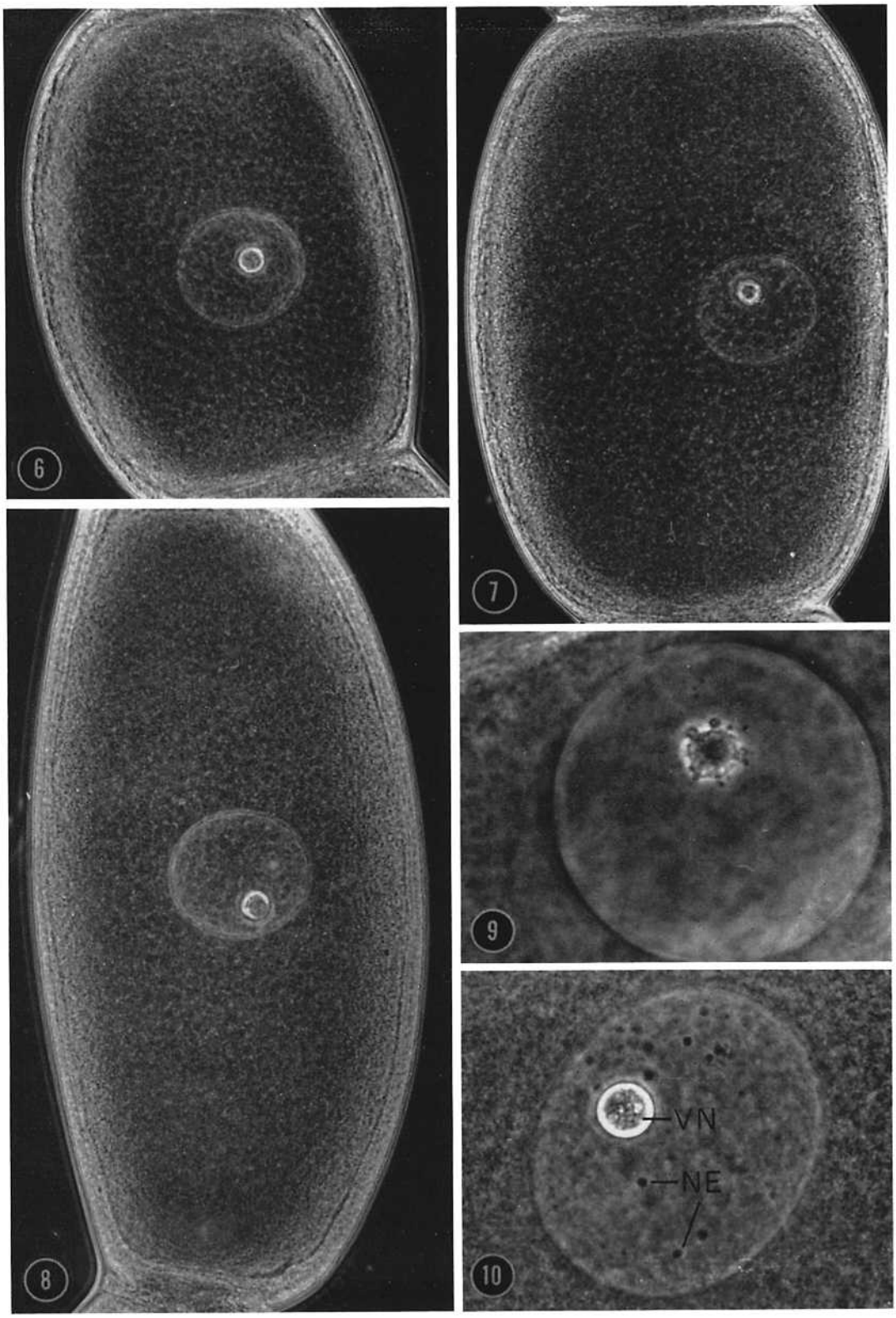
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shape again in zone V where they become somewhat spherical.

For convenience, that portion of the follicle cell adjacent to the oocyte will be referred to as apical, and the opposite end will be designated basal. In zone III the apical ends of these cells are closely applied to the oolemma (Fig. 12, *FP*). When the oolemma produces microvilli, blunt and slender processes extend out from the apical ends of the follicle cells; some of the processes interdigitate with the microvilli of the oocytes. The plasma membrane of follicle cells is closely applied to the microvilli of the oocytes. This association is so close that the plasma membrane of the follicle cells shows a crenated pattern (Fig. 12). This is particularly true for those oocytes which occupy the posterior part of zone III and the anterior and middle portions of zone IV.

Just prior to the deposition of yolk bodies in oocytes in the posterior part of zone IV, the follicle cells undergo an interesting change. They begin to retract from the specialized surface of the oolemma. The resulting space is intensely PAS-positive and salivary amylase–resistant. An electron micrograph of this area shows it to be filled with a rather fine, wispy material (Fig. 20, *W*).

The plasma membrane on the lateral aspects of the follicle cells is folded and shows the commonly described desmosomes; *i.e.*, local thickenings of the inner surface of plasma membrane. Occasionally "septate desmosomes," similar to those described in certain coelenterates (Wood, 1959; Overton, 1963), are found. They are more prevalent, however, between cells comprising the interfollicular tissue (inset, Fig. 19, *SD*). Also found in this area are many adhesion plates like those depicted by Smith (1963) in the photocytes of the firefly, *Photuris pennsylvanica*.

The basal portions of the follicle cells are somewhat flattened and show no unique specialization. They do, however, send out pseudopodial extensions much like that of an epitheliomuscular cell of hydra. Profiles of these are found in Fig. 19 (*PE*).

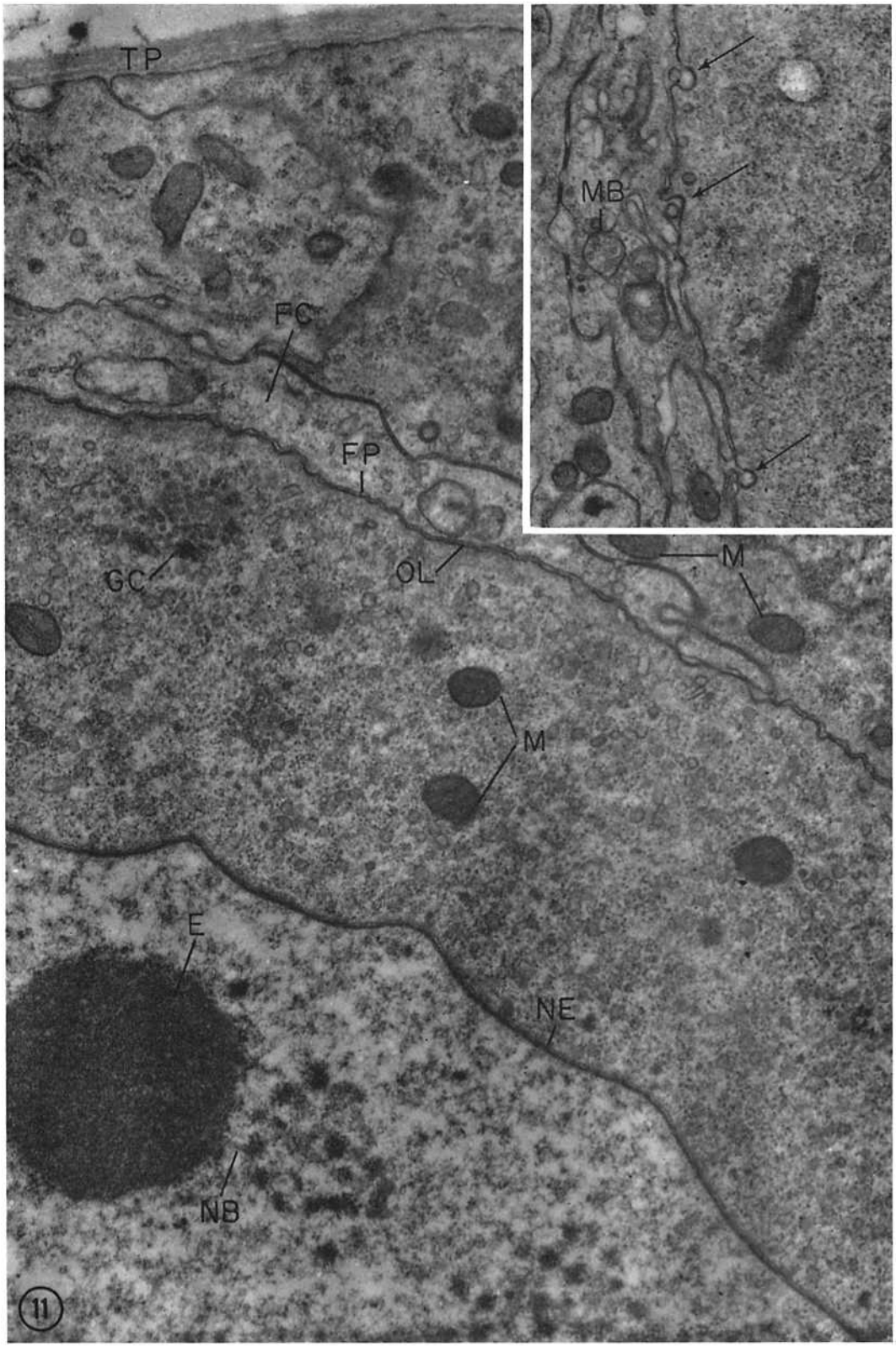
The follicle cells, as well as the cells of the interfollicular tissue, are endowed with an abundant cytoplasm containing many mitochondria (Fig. 19, *M*), multivesicular bodies (Fig. 11, insert, *MB*), Golgi complexes (Fig. 12, *GC*), endoplasmic reticulum of the rough variety (Fig. 19, *ER*), and a vast assemblage of RNP particles. One can also find lysosome-like structures (Fig. 12, *L*), a system of circularly arranged membranes and diplosomes occupying a juxtanuclear position.

Surrounding the entire ovariole is a material which has been referred to as a sheath or tunica propia (Fig. 14, *TP*). (A second sheath which is cellular is also present and will not be dealt with in this study). It is thinnest around oocytes of zone II and becomes thickest around those of zone IV. It is PAS-positive, stains pale with Heidenhain's hematoxylin, and blue with Giemsa and Mallory's triple connective tissue stains. This covering consists of a finely granular, relatively non-compact material and at times appears laminated (Figs. 11 and 19, *TP*). It is conceivable that this coating, which in content and position resembles the basement membrane of other tissue types, may be a product of the follicle cells and/or interfollicular tissue (Bonhag and Arnold, 1961).

BACTEROIDES: Between the follicle cells and the developing oocytes of *Periplaneta* and other insects there are microorganisms which live as symbiotes. These organisms, since their precise identification is unknown, are referred to as bacteroides. The presence of such microorganisms in insect tissues was first recorded in 1887 by Blochman and has since been studied assiduously by Buchner (1953) in Germany and by Richards and Brooks (1958) in the United States.

The bacteroides are Feulgen-positive, a reaction which is abolished by prior digestion with DNase. They are rod-shaped, 2.5 to 3.5 microns long, and about 0.5 to 1.5 microns wide. Usually they appear as single organisms, but some chains are encountered. In phase-contrast photomicrographs

FIGURE 11 Small portion of an oocyte from the middle portion of zone II and associated follicle cells (*FC*) with mitochondria (*M*) and a multivesicular body (*MB*, insert). Note the tunica propia (*TP*) in the upper part of the micrograph. The plasma membrane of follicle cells (*FP*) is closely applied to the oolemma (*OL*). At the surface of these oocytes pinocytosis begins, and the caveolae show fine, radiating spike-like structures (see arrows on insert). Within the ooplasm are mitochondria (*M*) and elements of the Golgi complex (*GC*). The nuclear envelope (*NE*) and a nucleolar emission (*E*) are shown. To the right of the nucleolar emission are small clusters of similar particles, some of which appear to be attached (*NB*) to the nucleolar emission. Fig. 11 and insert, × 23,000.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they are seen *en masse* as a dark line between the follicle cells and the oocyte (Fig. 5, *B*).

If a thin section is made of a young oocyte (anterior part of zone III), the precise relationship of the microorganisms to the surrounding cells can be observed (Fig. 12, B). Here the bacteroides are few in number. They subsequently increase their population by fission (Fig. 20, B_1) and eventually come to completely surround the oocyte (Fig. 13, *B*). Fig. 14 is a photomicrograph of a transverse section through an oocyte equivalent to that illustrated in Fig. 6. The microorganisms of this oocyte are arranged in palisade fashion and are so numerous as to cause indentations in the oolemma.

The bacteroides are bounded by a double-membrane envelope and in thin sections present many different profiles (Fig. 20). Their internal structure varies; dense masses of material may appear terminally, laterally, centrally or eccentrically placed. In their plasm are found areas of dark and light densities; occasionally a concentrically arranged membrane system (mesosome) (Fig. 22, *MS*) similar to that which occurs in *Bacillus megatherium* and other bacteria is found (*cf.* Fitz-James, 1960).

It is understandable that a controversy has arisen among light microscopists with respect to the location of these microorganisms, that is, whether they are intracellular or extracellular. Contrary to the finding in a recent electron microscope investigation of similar microorganisms associated with oocytes of the roach *Blatta* (Gresson and Threadgold, 1960), the bacteroides of *Periplaneta* are extracellular (*cf.* Bush and Chapman, 1961). The close spatial relation of the bacteroides, along with the fact that they are demonstrable at the surface of all oocytes during varying stages of the animal's development, excited investigators to ponder the question: How are these microorganisms transmitted? Some workers have envisioned that this is accomplished *via* the egg. In turkeys, the etiological agent of "blackheads," the protozoan, *Histomonas meleagridis*, for example, is also thought to be transmitted transovarially. This is thought to be achieved through the eggs of the nematode, *Heterakis gallinae* (*cf.* Manwell, 1961). A discussion of how the bacterioides of *Periplaneta* are transmitted is beyond the scope of this paper. However, in 1936 Gier stated that, "Before the egg is oviposited the original oocyte membrane breaks down and permits the bacteroids to enter the cytoplasm." Difficult technical problems are encountered when one attempts to preserve the fine structure of the cytoplasm of eggs contained within oothecae. In such poorly preserved material the author was not able to substantiate the claim made by Gier (1936).

For a discussion of the functional significance of this microorganism as well as other interesting aspects of their biology, the reader is referred to the papers of Brooks and Richards (1955, 1956), Brooks (1960), and Glaser (1930).

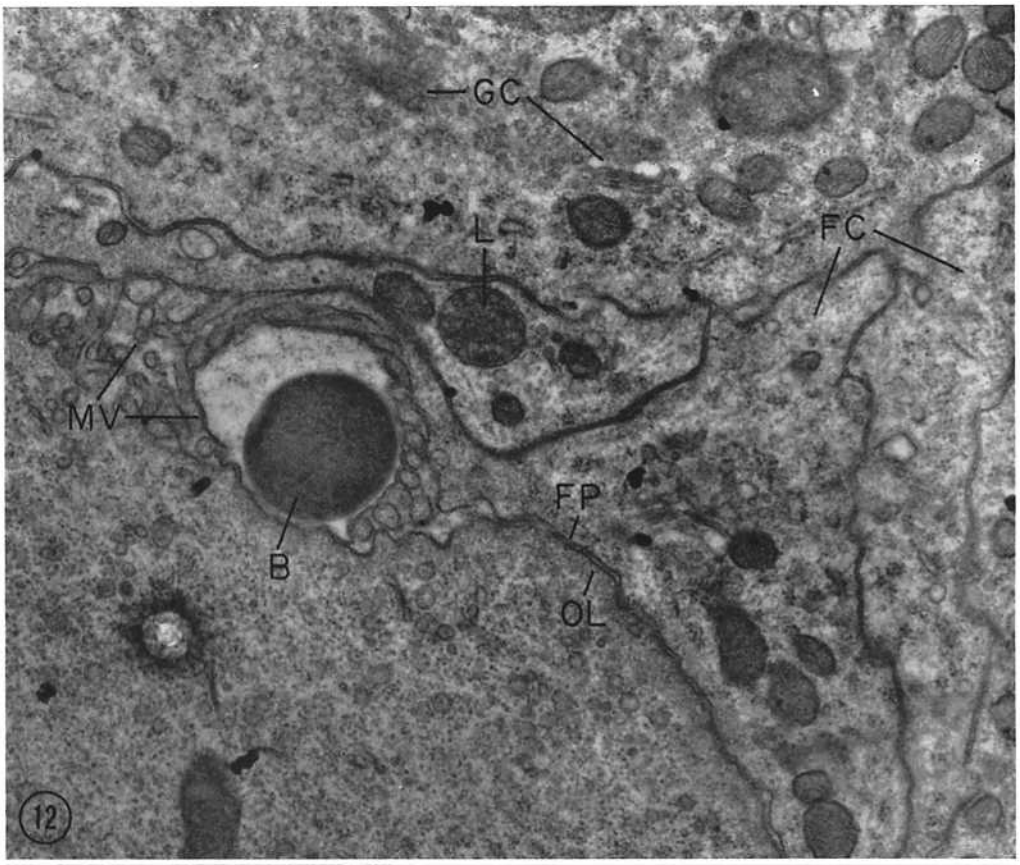
DISCUSSION

The origin of yolk in the class Insecta has long been a subject of controversy. This controversy has primarily centered around three speculative suggestions: (*a*) that yolk bodies are transformed organelle systems; (*b*) that they are the secretory products of the Golgi complex; and (*c*) that they are the maturation products of nucleolar emissions. Evidence derived from classical light microscopy, cytochemistry, and immunological techniques supports the view that in certain insects yolk bodies

FIGURE 12 Small area of a young oocyte from zone III showing the appearance of microvilli (*MV*). The surrounding follicle cells (*FC*) are closely applied to the oolemma (*OL*) and its microvilli. Between the two cell types is a bacteroid (*B*). In the cytoplasm of the follicle cells are Golgi complexes (*GC*) and a lysosome (*L*). × 23,000.

FIGURE 13 Photomicrograph of a longitudinal section through 6 oocytes from zone III showing their incomplete encompassment by follicle cells (*FC*). At this stage of development, the bacteroides (*B*) form a single layer. In many of these oocytes a rim of basophilic material (*NB*) is closely associated with the outer aspect of the nuclear envelope. Bouin's fixative, Heidenhain's hematoxylin stain. × 375.

FIGURE 14 Transverse section of an oocyte from the posterior region of zone IV. Note the large number of bacteroides (*B*), arranged in a palisade fashion, and the densely stained tunica propria (*TP*) at the surface of follicular epithelial cells. Two mitotic figures are also evident. Helly's fixative, Giemsa stain. × 375.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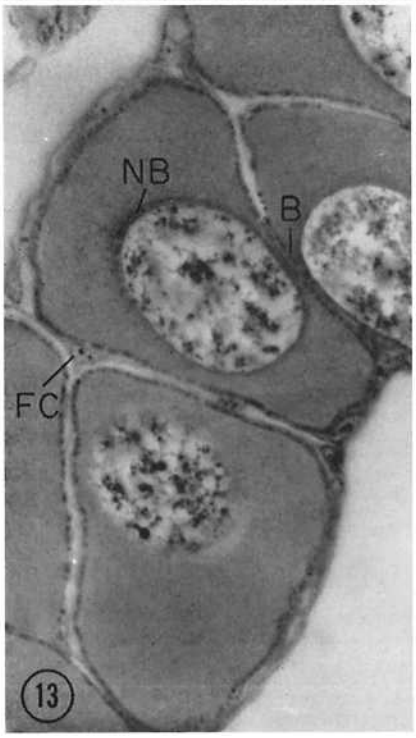
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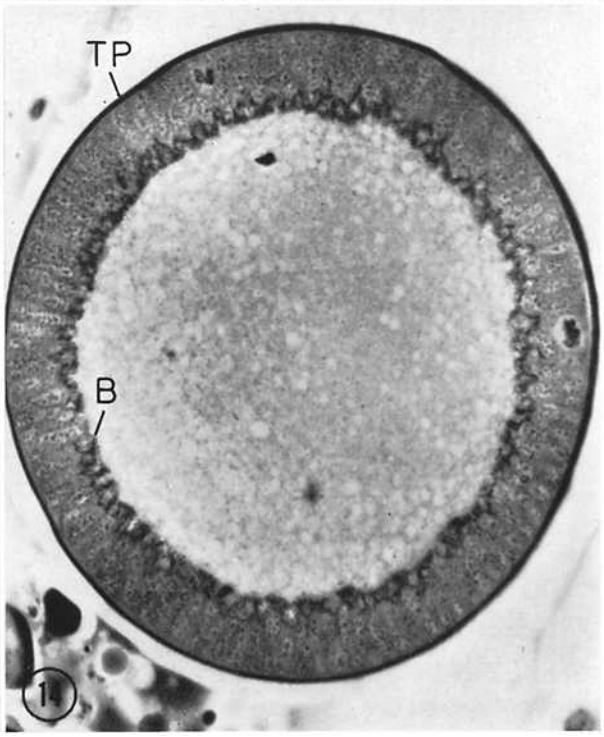
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arise independently of oocyte cytoplasmic organelles, an opinion which has been confirmed, in part, by electron microscopy. The literature concerning vitellogenesis in insects is voluminous and will not be reviewed here. The reader is referred to the review by Bonhag (1958) and the book by Raven (1961).

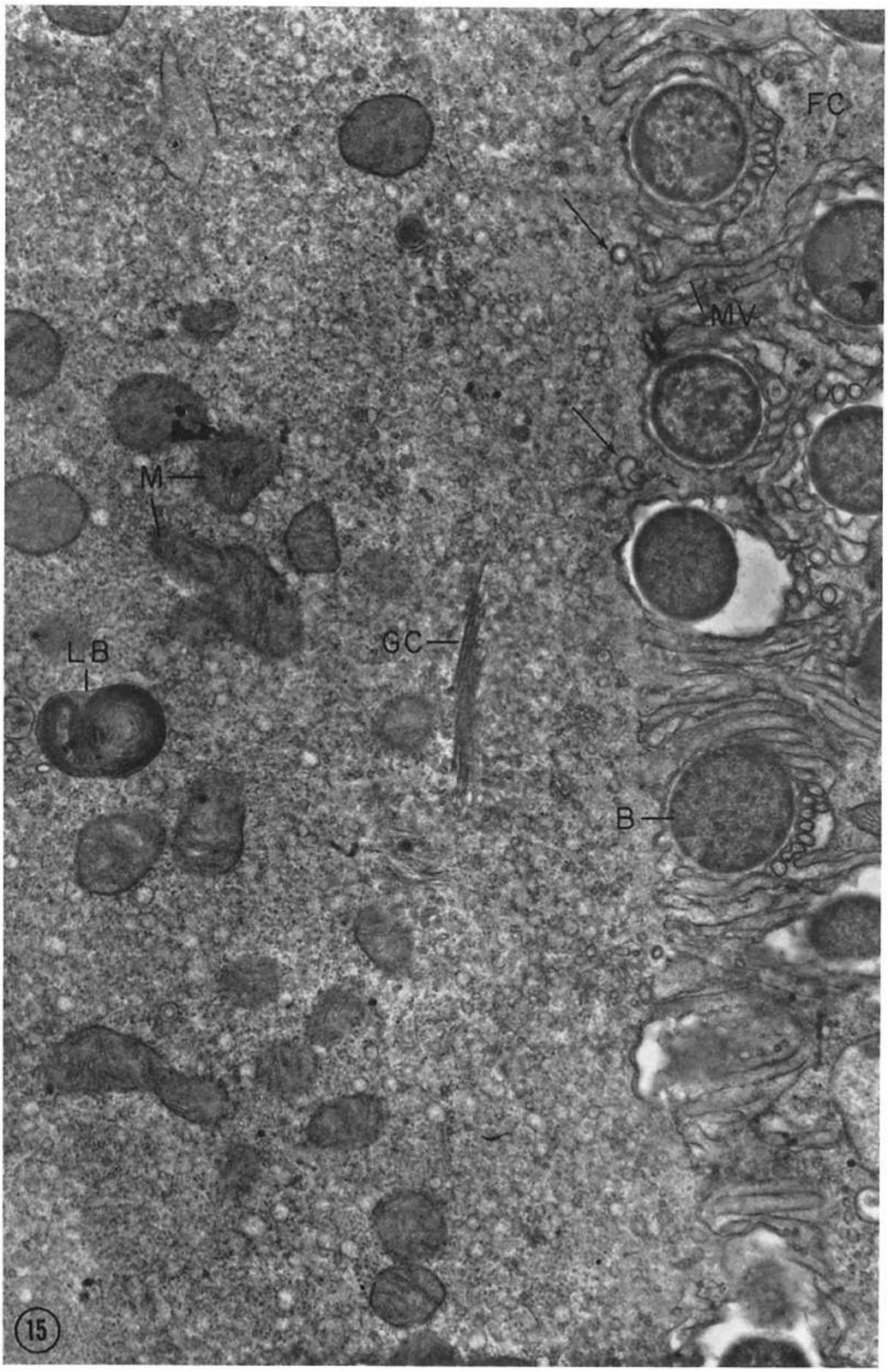
In electron micrographs illustrating this paper, it has been shown that at the time yolk is deposited in the cytoplasm of oocytes in zone IV many caveolae appear on the oolemma. Unlike the caveolae which appear on the oolemma of oocytes from other zones, these are filled with a dense amorphous material and when internalized become yolk bodies. During further differentiation the yolk bodies become larger primarily by coalescence of smaller ones. These yolk bodies do not assume a preferred orientation within the cytoplasm, nor are they uniquely associated in any way with a particular organelle system. Thus, such observations permit one to conclude that the precursors of yolk are formed outside of the oocyte. In order for the precursors to become internalized they are dependent upon the ability of the oolemma (*a*) to "realize" their attachment and (*b*) to pinocytose.

Evidence in the literature indicates that in the *Cecropia* moth the protein precursors of yolk are found in the blood. The first critical study of the metamorphosis of insect blood proteins was an immunological investigation by Telfer and Williams (1953). These authors demonstrated that the blood of the *Cecropia* moth contains at least nine substances, all of which have properties of proteins and are antigenic to rabbits. Of the six antigens intensively studied, one (antigen 3) was identified as a carotenoid. Another, antigen 7, was among the most interesting, in that it was found in the blood of pupal females and only in minute quantities in the pupae of males. In 1954, Telfer meticulously studied antigen 7. From his detailed analysis he showed that antigen 7 makes its appearance after the larva has spun its cocoon and is demonstrable in all of the succeeding stages of metamorphosis. Antigen 7 is concentrated in female blood approximately one thousand times higher than the similar antigen of males. At the time when eggs are actively being produced in the *Cecropia* moth (pupal-adult transformation) the antigen decreases significantly. In females which had been ovariectomized, there is an increase rather than the normal decrease of antigen 7. Ovaries which had been transplanted to the haemocoel of males produced eggs, but there was no incorporation of antigen 7. Furthermore, Telfer (1954) transfused the ovaries of *Anthereae polyphemus* females with cecropia blood and their eggs incorporated antigen 7. From these elegant experiments that author concluded "that antigen 7 is secreted into the blood by some tissue other than the ovaries and that it is subsequently drawn from the blood and deposited in the yolk." A similar conclusion has been reached by Telfer and his associate in other communications (Telfer and Rutberg, 1960; Telfer, 1960).

Bonhag (1955, 1959) has shown, with the techniques of classical light microscopy as well as those of cytochemistry, that yolk bodies of *Periplaneta* as in other insects (the milkweed bug, *Oncopeltus fasciatus*), first make their appearance in the peripheral cytoplasm. Telfer (1961) has recently confirmed his physiological findings, and the morphological observations made by Bonhag, with fluorescence microscopy. Telfer found, as a result of his studies with fluorescein-labeled blood protein antibodies in the *Cecropia* moth, that the tunica propia, which is presumably permeable to protein (see also Bonhag and Arnold, 1961), all extracellular spaces, and the myriad yolk bodies, were intensely fluorescent.

The significance of the perpendicularly oriented spikes associated with the caveolae of the oolemma of *Periplaneta* is not known. This structural modification of caveolae, first demonstrated by Ashford and Porter (*cf.* Roth and Porter, 1962) at the surface of liver cells facing the space of Disse, has also been demonstrated on the oolemma of the polytrophic ovary of mosquitoes (Roth and Porter, 1962) and on the oolemma of the telotrophic ovary of *Rhodnius prolixus* (Anderson, unpublished

FIGURE 15 Section made between two oocytes from the anterior region of zone IV. A small part of a follicle cell (*FC*) with a process extending between microvilli (*MV*) of the oolemma is shown. Between the follicle cells and the oocytes are a number of bacteroides (*B*). In the cytoplasm of the oocyte are mitochondria (*M*), Golgi complex (*GC*), and a dense laminated body (*LB*). Note caveolae (at arrows) with their modified surfaces. × 23,000.

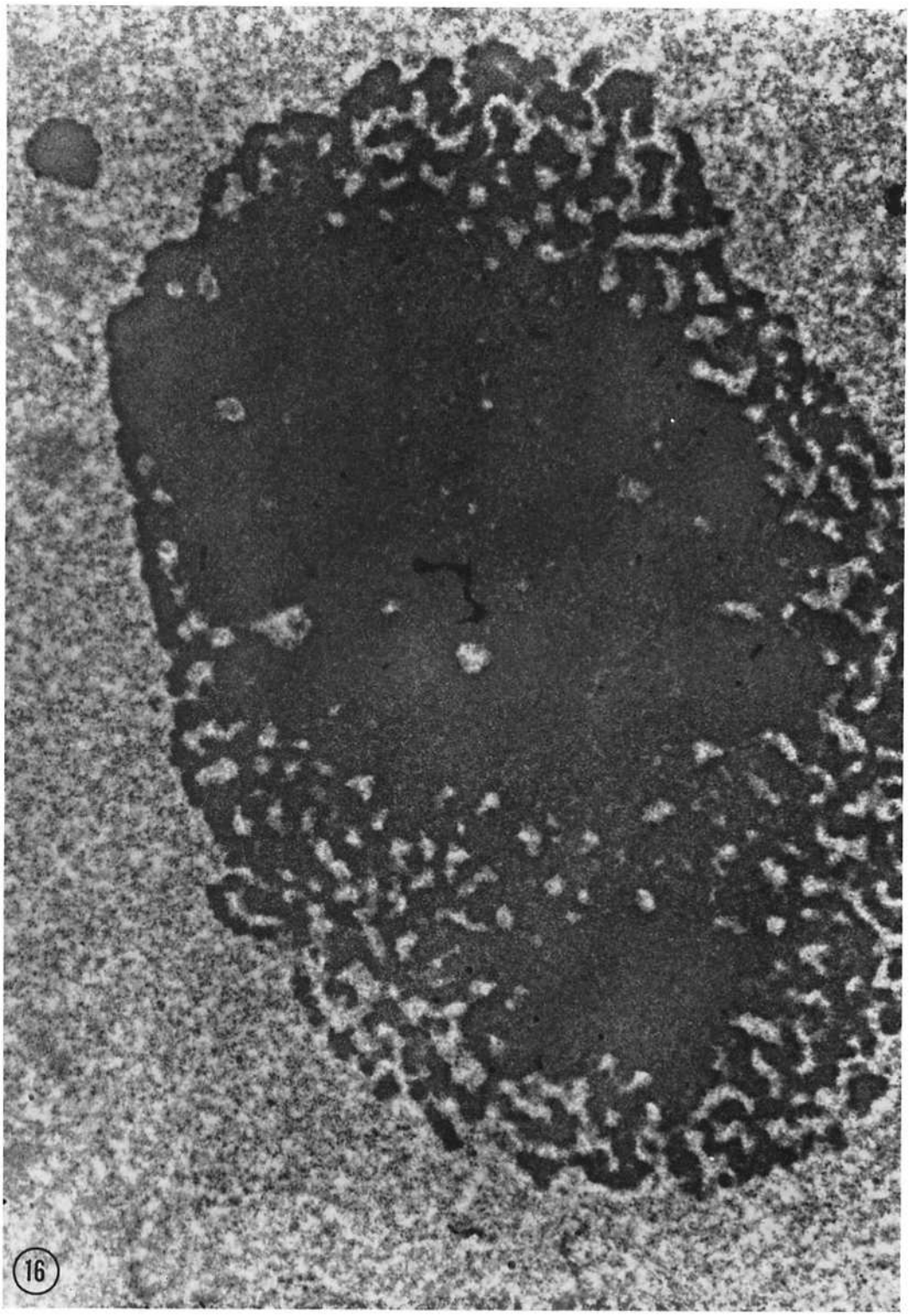

FC
MV
M
LB
GC
B
15

observations). These spike-like structures are apparently well preserved with the newer methods of fixation and embedding, for they have been found on some, but not all, caveolae of the plasma membrane of smooth muscle cells from the crop of pigeons (Dumont, unpublished observations). Roth and Porter (1962) offered the suggestion that the fine, radially oriented spikes might be associated with the mechanism of infolding and pinching off of the pinosome. They further suggested that they may be sites for protein binding. In this connection, one is reminded of Bennett's (1956) concept of membrane flow and vesiculation and his statement as follows: "The extracellular fluid may contain particles (such as ions or protein molecules) capable of engaging binding sites on the cell surface. As a result of appropriate collisions and engagement of specific complementary groups, the particles would become bound to the external surface of the membrane. If the cell responded by causing the membrane to invaginate in the region of the bound particles, the portion of the membrane bearing the particles would soon be located in a recess or pocket or *caveola intracellularis* of Yamada (1955*b*)." Experimental evidence (Brandt, 1958; Brandt and Pappas, 1960, 1962; Schumaker, 1958) supports Bennett's hypothesis which now forms an important part of our knowledge concerning the entrance of material into the cell.

To elaborate further on vesiculation of the plasma membrane, Brandt (1962) has called attention to the well known structure, usually of an amorphous or filamentous nature, which is found external to the plasma membrane. He refers to this material as the extraneous coat of the plasma membrane. According to Bennett (1963), this extraneous coat is equivalent to such structures as the basement membrane, zona pellucida, mucous coatings, and the like. Since most of these are rich in polysaccharides, Bennett has assigned to them the general inclusive term "glycocalyx." Brandt envisions that the superficial coating ". . . may be a barrier or an operative of substance in and out of cells." He cites many illustrative examples to show the importance of this area, recalling in particular the well known experiments on the plasmalemma of amoeba by Shumaker (1958) and Brandt and Pappas (1960), and the experiments dealing with the uptake of ferritin by endothelial cells of capillaries (Palade, 1960; Wissig, 1958). From the above experiments, particularly those concerning amoeba, the concept has been derived that pinocytosis is a two stage event: (*a*) binding to the plasmalemma to concentrate the solute and (*b*) the formation of pinocytotic vesicles by the invagination of the plasma membrane (Brandt, 1962). Brandt (1962) believes that "The extraneous coat of the cell can bind and concentrate protein which is dissolved in the environmental water phase." He further suggests that pinocytosis ". . . beginning with the binding of substance to the extraneous coat, is most applicable to molecules, particularly large ones, but not to ionized atoms or their molecular analogues." In this connection also, regarding the specificity of pinocytotic caveolae, Marchesi and Barrnett (1963) found enzymatic activity to nucleotide substrates localized in the pinocytotic vesicles of blood capillaries. Bennett (1963) suggests that the glycocalyx may also bind selectively and would include such ions as calcium and potassium.

As has already been pointed out, pinocytosis commences in young oocytes in zone III but the resulting pinosomes are devoid of any particulate internal structure. This is also true for pinosomes which develop in oocytes from the middle portion of zone IV. The external limiting membrane of these pinosomes, however, does show the structural modifications just mentioned. The fate and function of pinosomes in oocytes from the aforementioned zones is not known. Such activity of the oolemma brings up the long standing question of how oocytes obtain their nutrient: Is it primarily a process of diffusion or do the pinosomes contain nutritive materials? It will be recalled that classical light microscopists, influenced by what they called "canals" or "ducts" in the zona radiata of oocytes of higher vertebrates, were prompted to suggest that these structures established direct protoplasmic connection between the oocyte and surrounding follicle cells. They envisioned that follicle cells are a form of nurse cells. This kind of morphological continuity has been demonstrated to be incorrect (Anderson and Beams, 1960;

FIGURE 16 Nucleolus from an oocyte in the upper region of zone IV. Extending from the compact mass of particles are similar particles arranged in a reticular fashion (nucleolonema). At the upper left is a nucleolar emission. × 24,500.


16

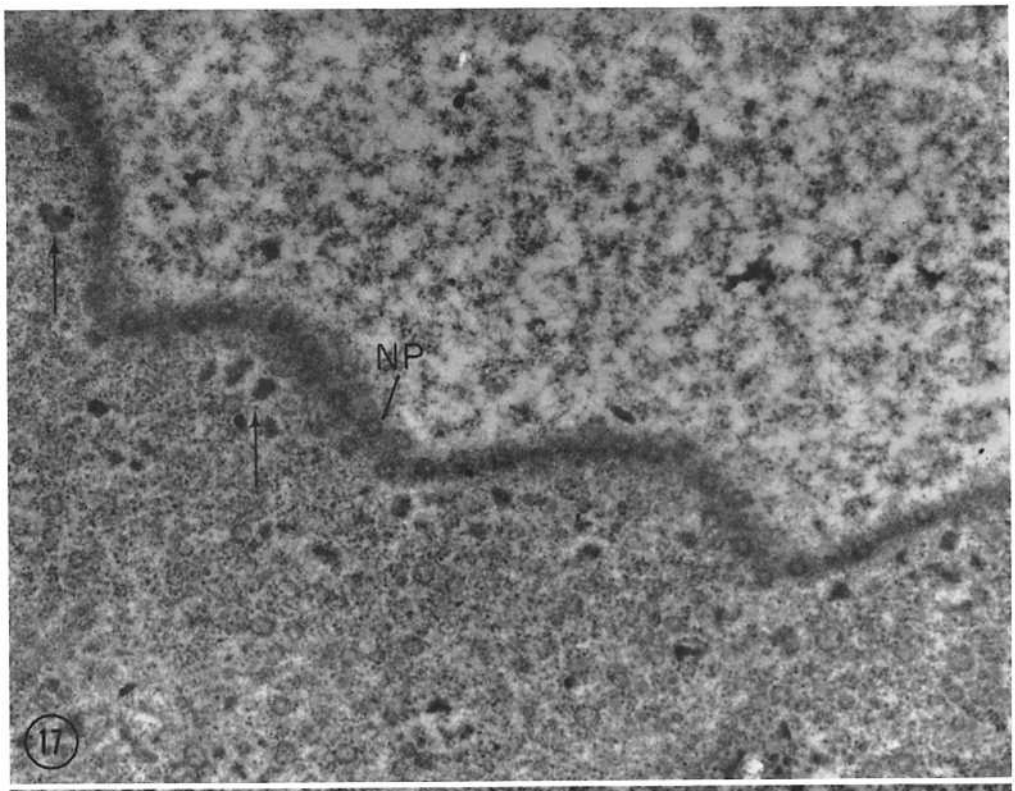
Kemp, 1956; Sotelo and Porter, 1959). The suggestion has been made, however, that the observable pinocytosis which takes place at the surface of certain mammalian oocytes could carry nutritive substances into the ooplasm (Anderson and Beams, 1960). Since the pinosomes of oocytes of zone III and the middle portion of zone IV are devoid of any definite internal particulate material, and since the oocytes in this stage of development are not depositing yolk in their cytoplasm, pinocytosis may be, in these zones, a rapid method of internalizing droplets of nutritive material which are subsequently utilized for maintaining the machinery of developing oocytes, rather than for the deposition of yolk during these stages.

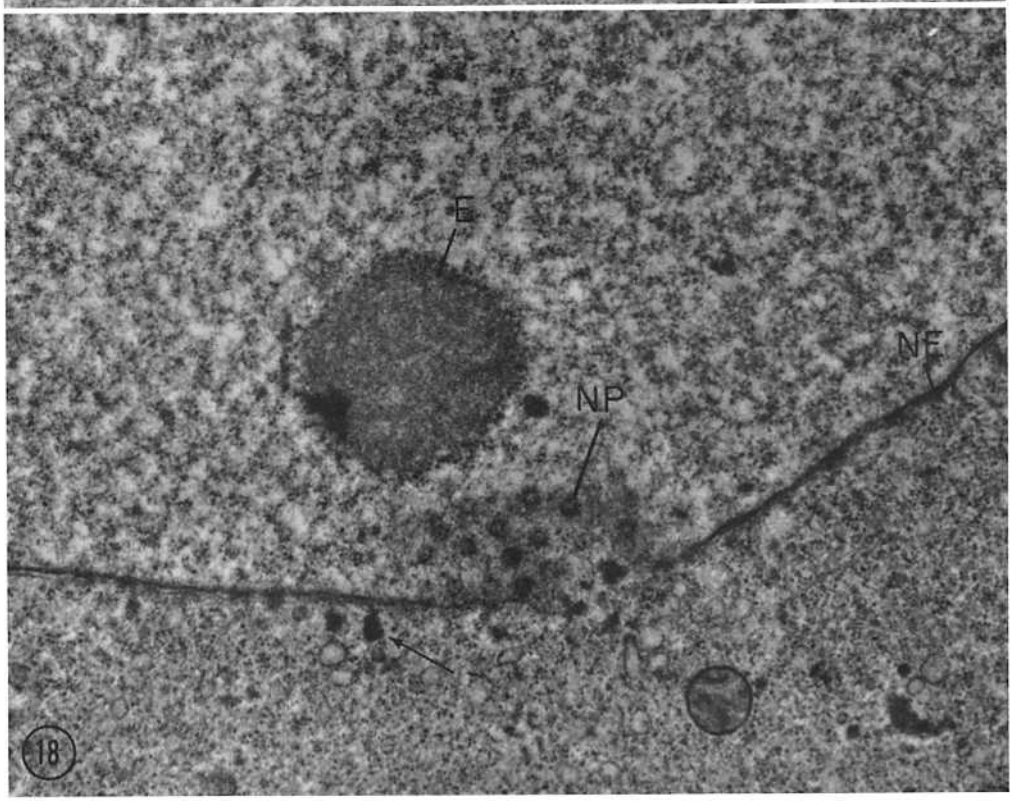
In the large oocytes (zone IV) which are actively depositing yolk in their cytoplasm, the membranes of pinosomes also show a similar surface architecture. However, unlike the pinosomes which are produced on the oolemma of oocytes of zone III, they have a definite internal electron-opaque substance. If it is assumed that yolk precursor protein(s) is available in the blood to all oocytes of all zones, *i.e.* that there is no anterior-posterior or transverse concentration gradient, and further assumed that the protein becomes enmeshed within the amorphous PAS-positive portion of caveolae, it is then possible to suggest that the caveolae of oocytes of zone IV, which are actively depositing yolk material, become selective for precursor protein(s), since only these show an accumulation of an electron-opaque material. From this suggestion, and recalling the physiological data of Telfer (1954, 1960) and Brandt (1962), and the speculation of Bennett (1963), one gains the impression that during the development of the oocyte the oolemma changes physiologically in some way which would allow for the accumulation of more of the amorphous material at its surface, and hence an increase in binding capacity.

Alternatively, one may suggest that a physiological change of the oolemma may not be of primary significance but that the retraction of the follicle cells from the surface of the oocytes located in the lower portion of zone IV leaves a relatively large fluid-filled area containing large amounts of the precursor yolk protein, hence making it more readily available to the developing cell. If the yolk precursor protein then becomes bound in the caveolae in large quantities, it may account for the internal electron-opaque material in these caveolae. Polysaccharide material, which presumably constitutes the amorphous substance at the inner surface of the caveolae, may also be made more available in this manner. Such an increase in the availability of the proteins would make it easy for them to engage the binding sites of the caveolae in large quantities and finally become internalized in the oocyte as yolk bodies. Just what role, if any, the follicle cells play in the production of protein is not known. It will be recalled that in his studies dealing with fluorescent-labeled protein, Telfer was not able to demonstrate fluorescent material within the cytoplasm of the surrounding follicle cells. It may be that these cells secrete substances which are incorporated into the oocyte by pinocytosis (Telfer, 1961). The follicle cells may also form the complex chorion of the egg (King and Koch, 1963).

It has been reported in the literature, particularly in the papers of Laskowski (1938), Cooper (1950), and Flickenger and Rounds (1956), that yolk proteins of frog eggs are synthesized in some tissue other than the ovary. As indicated by these authors, this synthesis takes place in the liver. Recent cytological studies on the oocytes of *Rana pipiens*, however, suggest that mitochondria participate in the formation of fatty and proteinaceous yolk (Ward, 1962; see also Malone and Hisaoka, 1963). Similar conclusions were reached by Favard and Carasso (1958) for *Planorbis* and Lanzavecchia (1961) for *Rana esculenta*. Beams and Kessel (1962, 1963), studying yolk formation in the oocytes of the crayfish, offer good morphological evidence that the endoplasmic reticulum is responsible for the formation of proteinaceous yolk in this form. In the oocytes of crayfish examined by Beams and Kessel (1962), the endoplasmic reticulum is well developed. It abounds in the cytoplasm as stacks of cisternae, each being interconnected with the others by smooth tubular ele-

FIGURES 17 and 18 Small areas of nuclei from oocytes in the upper region of zone IV. The nuclear envelope (*NE*) when cut tangentially shows numerous pores (*NP*). A nucleolar emission is seen at *E* (Fig. 18), and small clusters of RNP particles (at arrows) are closely associated with the pores. Fig. 17, × 24,500; Fig. 18, × 20,000.


NP
17


E
NE
NP
18

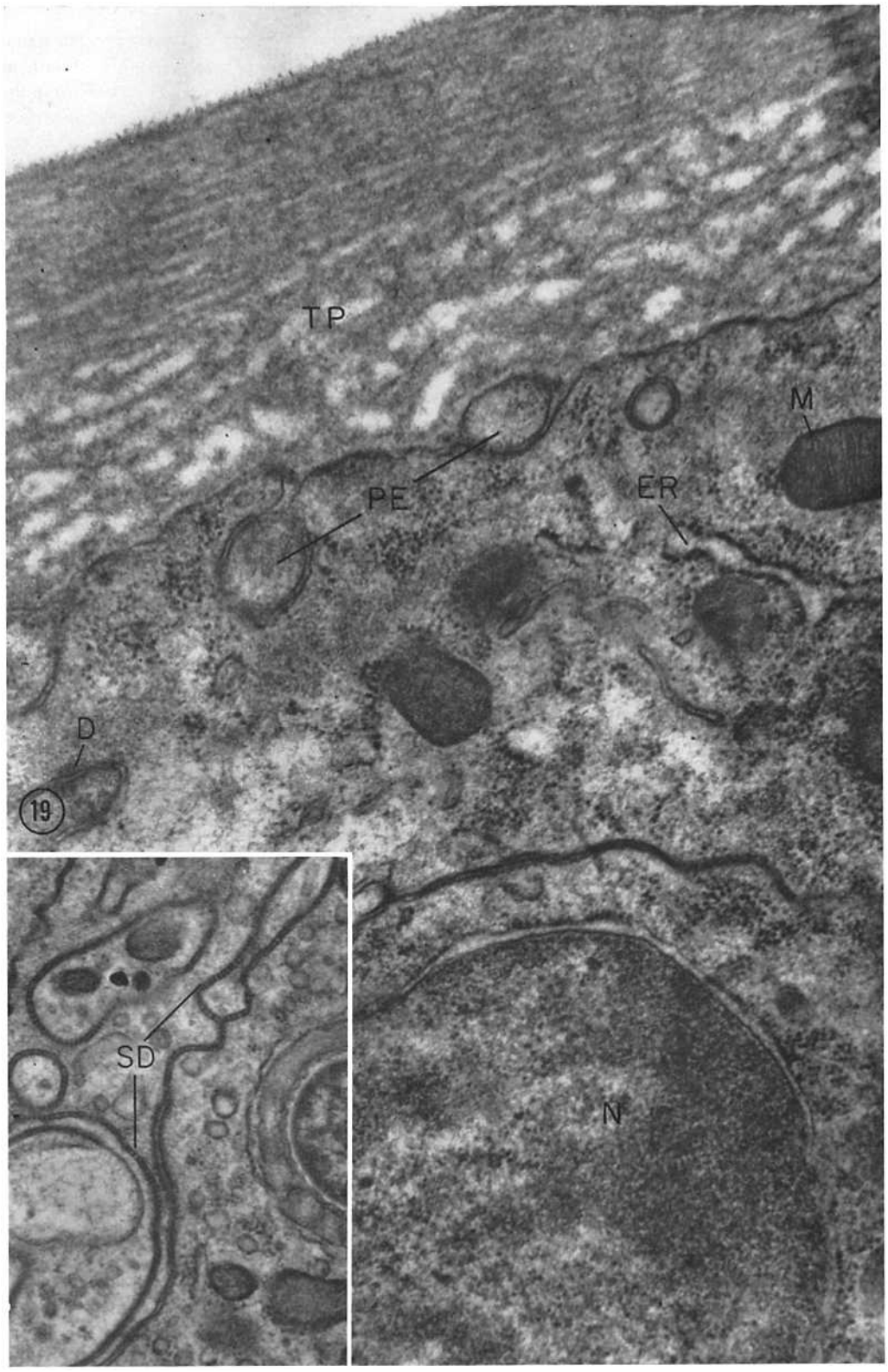
ments of the cisternal system. Within the interior of the cisternae are definite formed structures. From these observations the authors suggested "... that the intracisternal granules, believed to represent the precursors of the proteinaceous yolk, arise chiefly in the region of oriented cisternae possibly under the influence of ribosomes. They then "flow" into and along the unoriented cisternae to regions or pockets where they collect, expand the cisternae, and undergo transformation into a finely granular, relatively large yolk body which may or may not remain associated with the cisternae."

It is well known that during the growth processes of many kinds of cells a concomitant increase in nucleolar size usually occurs. As illustrated in this study, a rim of basophilic material is present at the surface of the nuclear envelope. Such a picture obtained from a large array of cells has prompted investigators to formulate the hypothesis that the nucleolus is a site of protein synthesis and that the synthesized protein, in the form of ribonucleoprotein, traverses the nuclear envelope and becomes localized within the cytoplasm where it engages in particular cytoplasmic protein synthesis (Brachet, 1957; Caspersson, 1950, 1962). Other investigators, however, do not agree with this concept (*cf.* Swift, 1962). It is apparent that the fragmentation (nucleolar emissions) of the nucleolus is not peculiar to oocytes of *Periplaneta*, since a similar phenomenon has been observed during the development of frog oocytes (*cf.* Witschi, 1956). Those investigators who have studied vitellogenesis in *Periplaneta* have offered many suggestions concerning the fate of the nucleolar emissions. Gressen and Threadgold (1962) make the following statement concerning *Blatta*: "The nucleolar extrusions move away from the nucleus and towards the periphery of the cell. As each extrusion migrates through the cytoplasm it becomes closely applied to its surface. When the extrusions reach the peripheral region of the cell, large clear spaces make their appearance in each extrusion; the extrusion's membrane becomes less clearly defined and finally disappears." Others have suggested that the nucleolar emissions are individually extruded from the nucleus and actually become transformed into yolk bodies (Nath and Mohan, 1929). It is conceivable that what these investigators observed, in their light microscope preparations, and interpreted as nucleolar emissions are structures similar to the laminated bodies shown in Fig. 15 (*LB*).

Bonhag (1958) has stated in his review that "If nucleolar emissions give rise to yolk bodies, one should be able to follow the structural transformation of the former into the latter with the electron microscope." Evidence gathered from the present study shows that the nucleus is limited by a double-membrane envelope which is perforated at intervals by pores. This study also presents evidence that the main nucleolar mass, nucleolar emissions, and certain cytoplasmic areas are basophilic, and that they are digestible with RNase, a property which is due to the high content of ribonucleoprotein of these constituents. There is no evidence that the nucleolar emissions are extruded into the cytoplasm in such large masses. However, since the nuclear envelope has a fenestrated structure and is in close association with clumps of RNP particles on both its inner and outer aspects, one can think of the outer particles as being derived from the inner ones and moving to the cytoplasmic side through the pores of the nuclear envelope. This interpretation is substantially in agreement with morphological and experimental data obtained for other cell types (Anderson and Beams, 1956; Goldstein and Plaut, 1955).

As has already been emphasized, the protein of the yolk bodies is not synthesized in the ooplasm. Furthermore, in all oocytes examined there was no evidence of an organized endoplasmic reticulum, an observation that would further indicate that these cells are not actively engaged in the synthesis of yolk protein. The cytoplasmic RNA may be needed for the initial and subsequent growth of the oocyte or it may be utilized for internal maintenance synthesis. In this connection, unpublished observations on the ovarioles of *Rhodnius prolixus* (telotrophic-type) indicate that the mechanism of yolk deposition is similar

FIGURE 19 Portions of two follicular epithelial cells, depicting the nucleus (*N*), desmosomes (*D*) and (*SD*) (see inset), endoplasmic reticulum (*ER*), a mitochondrion (*M*), profiles of pseudopodial extensions of other follicle cells (*PE*), and the thick, finely granular, tunica propria (*TP*). × 32,500; inset × 14,500.


TP
M
PE
ER
D
19
SD
N

to that of *Periplaneta*. In neither case is there any correlation between nuclear or nucleolar phenomena and yolk formation. Presumably the ribonucleoprotein produced during oocyte development in both cases is used for maintenance rather than yolk synthesis.

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FIGURE 20 Small area of an oocyte from zone IV just prior to yolk deposition. The follicle cells (*FC*) are not intimately associated with the microvilli (*MV*) of the oocyte. The intervening space is filled with a fine wispy material (*W*), some of which (*MW*) appears to adhere to the surface of microvilli. Also found in this space are bacteroids (*B*), one of which was fixed while in the process of fission (B_1). The peripheral ooplasm (*OP*) swarms with many pinosomes (*P*). × 20,000.

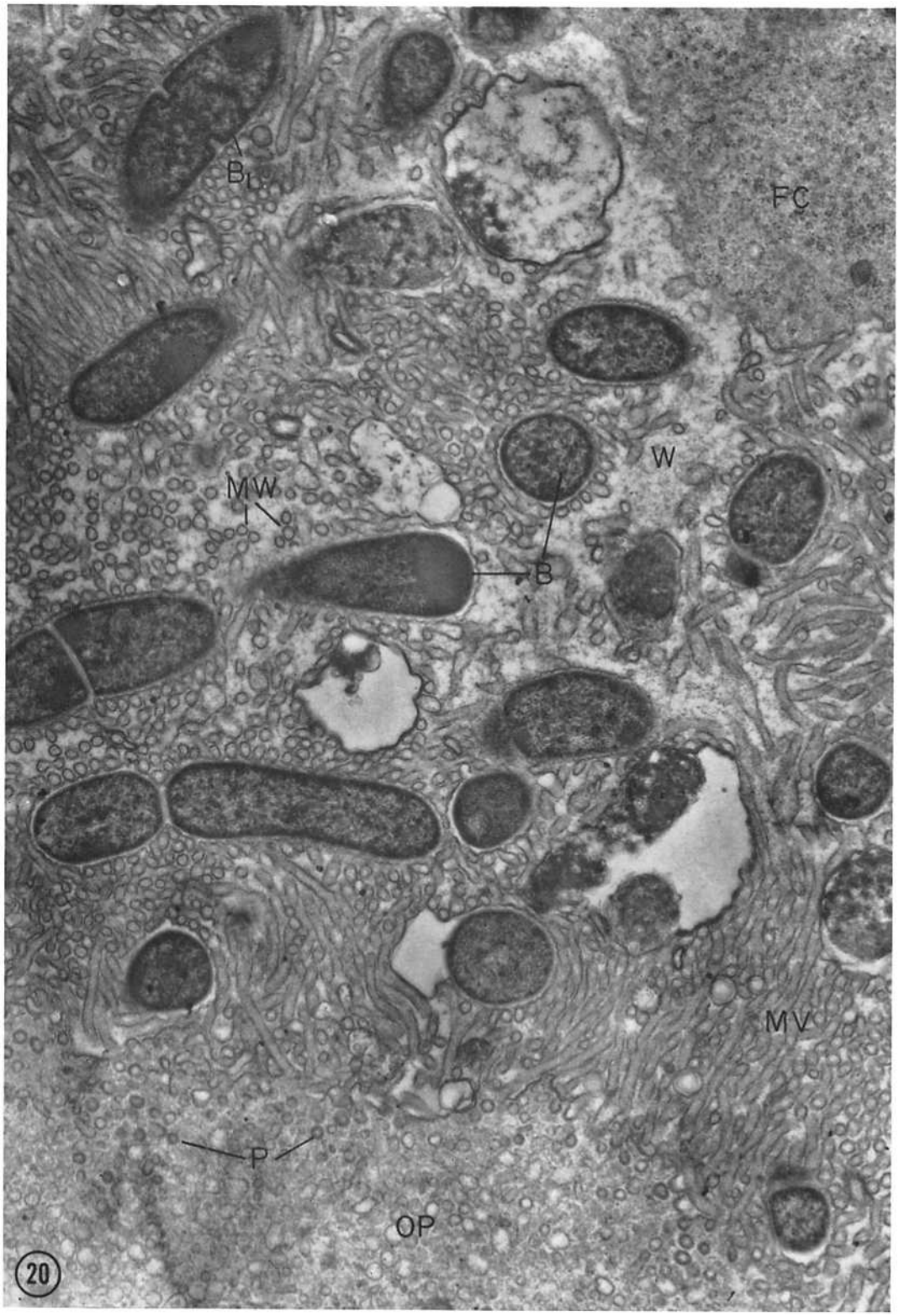
BL
FC
W
MW
B
MV
P
OP
20

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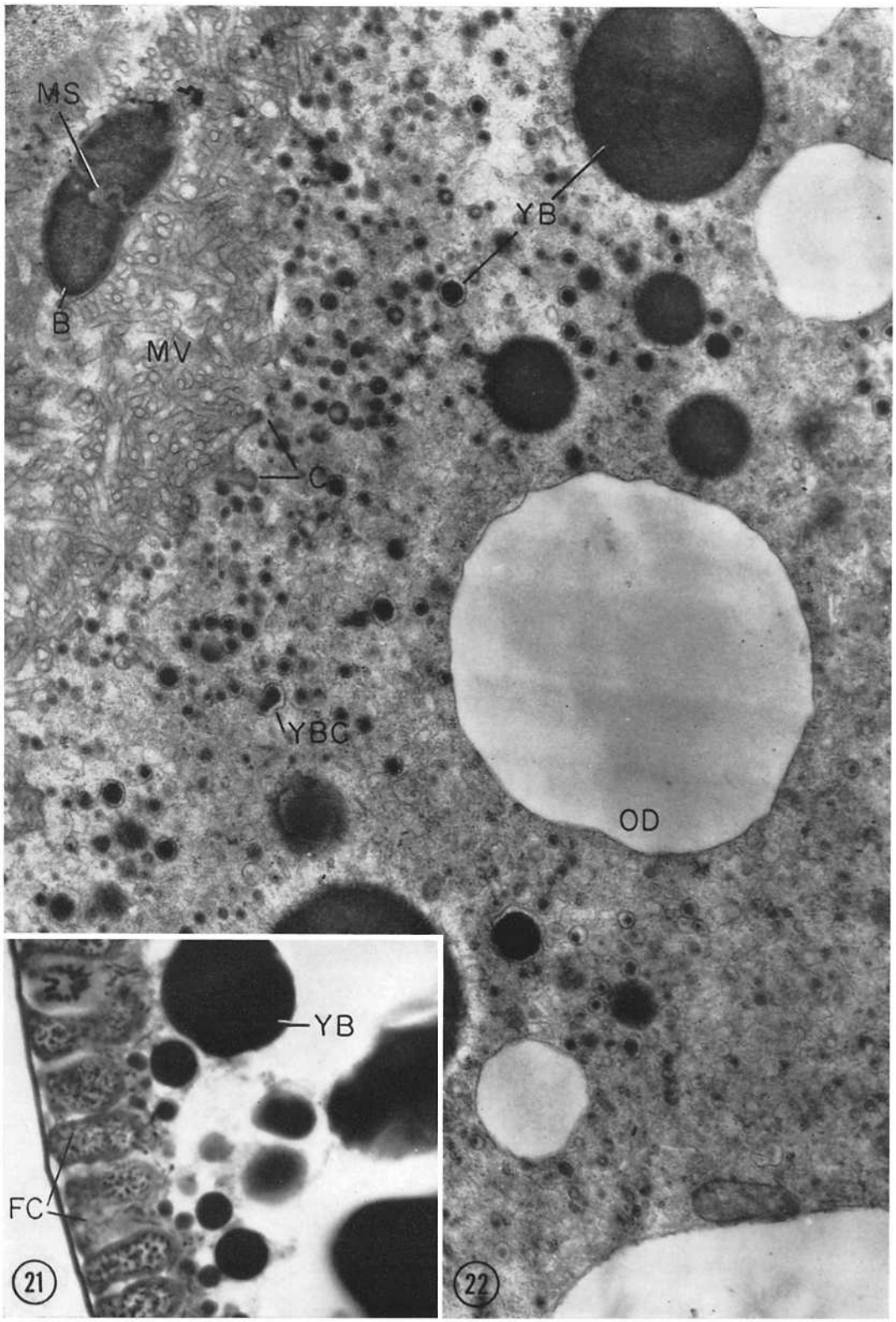
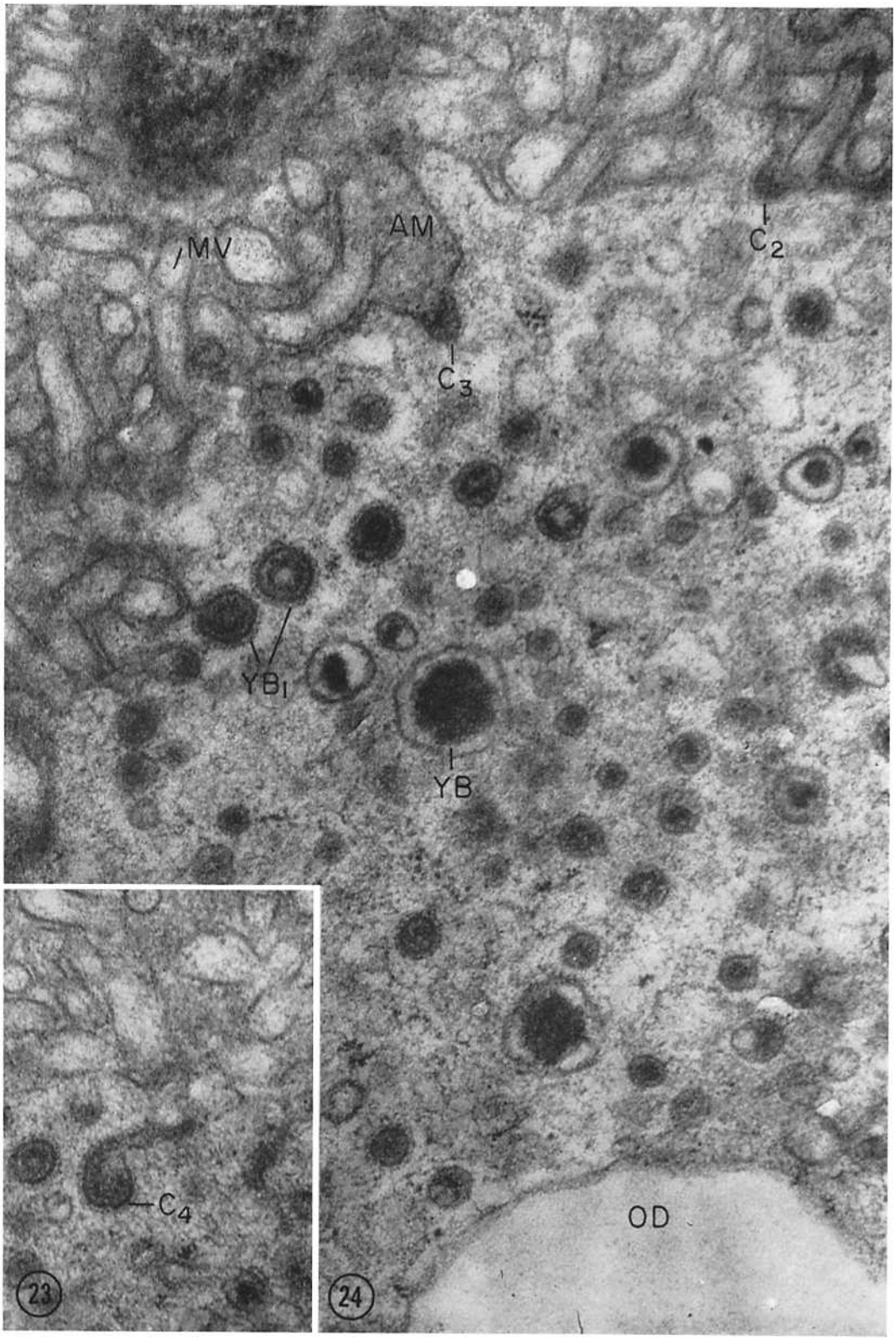
FIGURE 21 Photomicrograph of an oocyte (lower region of zone IV) showing yolk bodies (*YB*) and follicle cells (*FC*). Note the mitotic figure of a follicle cell at the upper left. Helly's fixative; Heidenhain's hematoxylin stain. × 375.

FIGURE 22 Electron micrograph of a region similar to that shown in Fig. 21. A bacteroid (*B*) with a lamellar structure (*MS*) is shown amongst the multitude of microvilli (*MV*). The cytoplasm is primarily filled with yolk bodies of graded sizes (*YB*). Note fusion of small yolk bodies (*YBC*), a mitochondrion (*M*), the caveolae at *C*, and a part of a large vacuole presumably occupied previously by oil (*OD*). × 15,000.


MS
B
MV
YB
C
YBC
OD
YB
FC
21
22

FIGURES 23 and 24 Small areas of the peripheral portion of oocytes (lower region of zone IV) during the time when yolk precursors are internalized by the process of pinocytosis, stages of which are represented at C_2, C_3, and C_4 (Fig. 23). Profiles of initially formed yolk bodies are shown at YB_1 and a later stage at YB. Note microvilli (MV) with some relatively dense amorphous material (AM) between adjacent ones, and a vacuole (OD) which was previously occupied by an oil droplet. × 49,000.


MV
AM
C2
C3
YB1
YB
C4
OD
23
24