THE FORMATION OF THE PRIMARY ENVELOPE DURING OOCYTE DIFFERENTIATION IN TELEOSTS

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ABSTRACT

The differentiation of the primary envelope of oocytes of the seahorse (*Hippocampus erectus*) and the pipefish (*Syngnathus fuscus*) has been investigated by techniques of light- and electron microscopy. The developing oocytes have been divided into four stages according to size. Oogonia are designated as stage I; stages II and III are oocytes; stage IV represents mature eggs. The primary envelope which is produced by the oocyte is initially a tripartite structure; for convenience of description, the portions are referred to as zones 1, 2, and 3, respectively. Zone 1 first appears as a homogeneous substance at approximately the middle of the long axis of each microvillus. Zone 2 is immediately beneath zone 1 and consists of an extremely electron-opaque granular component. Zone 3 is subjacent to zone 2; it is the largest and most complex of the three. Zone 3 consists of an amorphous material organized in a reticular-like network. Staining procedures indicate that the envelope is composed of a glycoprotein. Just before the oocyte matures there is a structural alteration in zones 2 and 3. Zone 2 becomes a compact, dense layer and zone 3 becomes multilaminate. Subsequent to these changes, zone 1 degenerates. The classification of egg envelopes is discussed, and comparisons are made between the primary envelope of the teleosts investigated and the primary envelopes of other species.

INTRODUCTION

As oocytes of certain organisms differentiate they acquire, among other things, external coverings that are important prior to and during the interval between fertilization and emergence of a viable organism. For example, subsequent to the formation of microvilli a homogeneous, and sometimes highly ordered, substance appears and coats the specialized surface of the oocyte (1–4, 6, 18, 53, 56, 57, 59). This layer of material is often referred to as the vitelline envelope and/or zona pellucida. In some echinoderms (5) it is produced in scant amounts, whereas in mammals (6, 16) it is produced in rather copious quantities. Moreover, in some organisms the developing oocytes also obtain a second stratum that overlies the first. This layer has been thought of as also being a part of the so-called vitelline envelope or zona pellucida According to Ludwig (32) if the covering is produced by the oocyte, it is classified as a primary envelope; if it is produced by the encompassing follicle cells, it is designated as a secondary one (see Discussion). The data obtained in the present report substantiate Ludwig's classification, and his terminology will be used.

Despite the numerous papers on the protective coverings of eggs from different groups of organisms, information on the orgin of these coverings is still somewhat confused and incomplete. The

limited amount of information on egg envelopes of teleosts prompted this study of the development of the primary envelope in the seahorse and pipefish, the only two species of the family Syngnathidae. In these forms, the female deposits the eggs in the brood pouch of the male who in turn "incubates" them for a minimum of 8–10 days (11, 50, 51). In these fish we also wanted to determine whether the primary envelope possesses any special morphological features which are not present in the envelope of other teleosts. Therefore, this paper explores (*a*) the origin and ultrastructure of the primary envelope, and (*b*) the alteration of the primary envelope before the egg is deposited in the brood pouch of the male.

MATERIALS AND METHODS

Oocytes obtained from mature, gravid female seahorses (*Hippocampus erectus*) and pipefish (*Syngnathus fuscus*) were used in this study. The seahorses were obtained from Roberts Fish Farm, Inc. (Miami, Fla.) during the months from October to February. The pipefish were obtained from the Marine Biological Laboratory (Woods Hole, Mass.) during the month of June.

After immobilization of the fish with tricane methanesulfonate (31) either the ovaries were fixed *in situ*, or they were excised and processed for both light- and electron microscopy. For light microscopy, pieces of ovary were fixed in the following fixatives: Ammerman's, 10% aqueous acrolein (8), Bouin's, Carnoy's, or Helly's (27). Tissue fixed in Ammerman's, Bouin's or Helley's fixative was washed, dehydrated, infiltrated, and embedded in paraplast; sections were stained with either Heidenhain's iron hematoxylin or Mallory's triple stain. Tissue fixed in Carnoy's fixative was stained by the periodic acid–Schiff technique with and without prior α-amylase digestion. Other sections of the Carnoy's-fixed material were stained accordingly: (*a*) Alcian Blue and Hale's collodial iron technique for the demonstration of acid mucopolysaccharide (40) and (*b*) the mercury bromphenol blue method of Bonhag for basic proteins (10).

Tissue fixed in 10% aqueous acrolein was washed, dehydrated, infiltrated, and embedded in glycol methacrylate according to the procedure of Ashley and Feder (8). 1 μ sections of this material were made and stained with a modified technique of Heidenhain's iron hematoxylin (49).

For electron microscopy, tissue was fixed for 2 hr in a 2% solution (4°C) of glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate buffer (48). After this initial fixation the tissue was washed in buffer and postfixed in a 1% solution (4°C) of phosphate-buffered (pH 7.4) osmium tetroxide. The tissue was then rapidly dehydrated, infiltrated, and embedded in Epon (33). 1 μ sections were made and stained according to the recommendation of Ito and Winchester (29). Thin sections were stained with uranyl acetate followed by the lead citrate stain of Venable and Coggeshall (52) and were examined with RCA EMU-3H and Philips 200 electron microscopes.

OBSERVATIONS

Anatomy and Microstructure

The ovary of the seahorse (Fig. 1) and pipefish (Fig. 2) is a bilobed organ situated in the posterio-dorsal part of the body cavity where it lies against the ventral wall of the swim bladder. The two lobes of the ovary are fused together posteriorly and join a short oviduct which opens by way of the genital orifice. Each ovarian lobe is covered with a layer of the peritoneum (Fig. 3, *P*) beneath which is the thin tunica albuginea ovarii. Although the oocytes of the seahorse are larger than those of the pipefish, this investigation has revealed that the primary envelope of oocytes from both species is produced in the same manner, possesses the same structural configuration, and undergoes equivalent changes before being deposited in the brood pouch. Therefore, the observations presented below are applicable to either.

Fig. 3 is a section of a portion of the ovary and illustrates oogonia and oocytes in different stages of cytomorphosis. These differentiating cells are arranged in a spiral pattern. The innermost portion of the spiral contains oogonia (*OG*) whereas the outer part consists of young oocytes (*OC*) and mature eggs. For convenience of description, the oocytes will be staged according to size. The measurements given below are those of oocytes of the seahorse. These stages are designated as follows.

Stage I

Stage I represents oogonia (Fig. 3, *OG*). They are approximately 20 μ in diameter, have the capacity to divide, and are incompletely encompassed by a layer of follicle cells.

Stage II

The oocytes of stage II, like oocytes of all later stages, have a relatively large nucleus (Fig. 5, *N*), are completely encircled by a layer of follicle cells (Fig. 3, *FC*), and have lost their capacity to divide. The oocytes of stage II range from 30 to 300 μ in diameter. A distinguishing feature is the appear-

ance of a zone located between the oocyte (oocytes about 120 μ in diameter) and the surrounding follicle cells (Fig. 5, *PE*). We shall see presently that this area is the beginning of the highly organized primary envelope. When stained with Heidenhain's iron hematoxylin, the primary envelope of younger stage II oocytes appears as a densely staining line; in older oocytes it is composed of three zones. These zones will be abbreviated hereinafter as Z-1, Z-2, and Z-3 (Fig. 4). When all three zones are discernible, Z-2 is extremely dense whereas Z-1 and Z-3 are refractile to staining with hematoxylin (49) or the toluidine-blue component of the stain recommended by Richardson et al. (46). The primary envelope is PAS positive; the reaction is not abolished by α-amylase digestion. It gives a positive reaction for acid mucopolysaccharide when Alcian Blue and Hale's colloidal iron stains are applied. It is also positive for protein when stained with bromphenol blue.

Stage III

Some oocytes in this group are spherical; some are pear-shaped. The spherical ones range from 300 to 450 μ in diameter whereas the pear-shaped ones are about 450 μ–1.5 mm long and range from 110 to 200 μ in breadth. On the outer edge of the primary envelope of oocytes that are about 450 μ at their widest diameter and 1.5 mm long is a relatively thin layer that stains intensely for basic proteins when bromphenol blue is used.

Stage IV

Stage IV oocytes are mature. They are also pear-shaped and are about 1 mm in diameter and approximately 2 mm long. The primary envelope of oocytes of late stages III (Fig. 6, *PE*) and IV stains so intensely that it appears as a single homogeneous layer rather than one composed of three zones.

Ultrastructure

OOPLASM: Cortical alveolae formation and vitellogenesis in the oocytes of these organisms will be dealt with in another paper. It will be necessary here only to call attention to some general ooplasmic features and the configuration of certain organelle systems prior to and during the formation of the primary envelope.

MITOCHONDRIA: Fig. 7 is a section through young oocyte and depicts large mitochondria (*M*) in the perinuclear ooplasmic matrix. In later stage II oocytes, the mitochondria appear to increase in number. Each organelle shows the typical internal structure of cristae and a few possess some granular dense bodies (Fig. 12, *DB*). These bodies may be related to calcium deposits as found in mitochondria of other cell types (39). Some of the mitochondria are closely associated with patches of dense particles (Fig. 7, *DG*). The mitochondria do not acquire any unique morphological characteristics during late stages of development.

GOLGI COMPLEX: The Golgi complexes in oogonia (stage I) and young oocytes have a perinuclear position (Fig. 7, *GC*). This organelle often consists of flattened cisternae with which is associated a population of vesicles of varied sizes. As oogonia develop into oocytes (stage II), the Golgi complexes migrate from a perinuclear position toward the periphery of the ooplasm. Concomitant with this movement, certain of the Golgi complexes show an increase in the number of cisternae and associated vesicles. Indeed, some of the cisternae appear to vesiculate into numerous, elongate structures and become scattered within the ooplasmic matrix (Fig. 7). From the organization of these Golgi complex-derived cisternae one gains the impression that they belong to the smooth endoplasmic reticulum, i.e., their surfaces are not adorned with ribosomal particles. The interior of the components of the Golgi complex and their derived vesicles in these oocytes (stage II) contain a fine, wispy substance (Fig. 7, *V*). This substance is found within these vesicles for the duration of formation of Z-1 (see below) of the primary envelope.

During the formation of each zone of the primary envelope the vesicular units derived from the Golgi complex are usually found within the peripheral ooplasm In later stages, the vesicles contain a rather homogeneous substance. Many of these vesicles are often confluent with the oolemma. Hence, it is thought that the contents of the elongate and vesicular units contain the precursor(s) of the primary envelope (Fig. 14,V_1; Fig. 15, V_2; Fig. 16,V_3). The vesicles containing the precursor(s) of the vitelline envelope remind one of the pits and invaginations that are formed on the oolemma during the initial stages of micropinocytosis (Fig. 16, V_3). These vesicles, however, are not interpreted as micropinocytotic,for, when this phenomenon is initiated on the oolemma, the ooplasmic side of the invagination is fuzzy, like that shown in Fig. 17 (*PV*).

ENDOPLASMIC RETICULUM: Within the ooplasm of oogonia and very young oocytes (stage II) there is no organelle system that one could definitely identify as rough-surfaced endoplasmic reticulum. However, when the developing oocytes of stage II reach about 80 μ in diameter there appear scattered within the ooplasm slender cisternae of the rough endoplasmic reticulum. Prior to the appearance and during the production of Z-2 and Z-3 of the primary envelope, the cisternae of the endoplasmic reticulum become filled with what appears as a finely stippled component (Figs. 11–13, *ER*). Another feature of these cisternae is the formation of evaginations (Fig. 13, *ER**) on their surfaces as well as bulbous enlargements at their tips. Both the evaginations and bulbous tips are usually devoid of ribosomal particles. Those vesicles located near the cisternae of the endoplasmic reticulum contain a material similar in density to that found in the endoplasmic reticulum. The evaginations and bulbous tips of the cisternae of the endoplasmic reticulum are interpreted to be early stages in the formation of the vesicles found in the vicinity of this organelle. When all zones of the primary envelope are formed the endoplasmic reticulum is not dilated. One finds only a few cisternae within the ooplasm of a mature egg.

Primary Envelope

The primary envelope appears on the surface of oocytes subsequent to the formation of microvilli. Thus, it is evident that the envelope cannot be described apart from the specialized surface of the oolemma.

OOLEMMA: The oolemma (Fig. 8, *OL*) of young oocytes is closely associated with the plasmalemma of follicle cells (Fig. 8, *FC*), being separated from it by a space of about 300–400 A. The first sign of a morphological specialization of the oolemma (stage II oocytes) is the formation of microvilli (Fig. 9, *MV*). By stage III, the surface of the oocyte is completely covered with microvilli. These microvilli are long and project into the space which is formed between the oocyte and follicle cells. This space is produced when the follicle cells move away from the surface of the oocyte during the early growth period (stage II). Some of the microvilli are so long that they project into the intercellular space of the follicle cells; some indent the face of the follicle cell adjacent to the oocyte. Microvilli of the mature egg (stage IV) are thin, slender units and possess a more dense interior than those of oocytes of stage II and III. Microvilli during all stages of differentiation possess a core of filaments, 60–70 A in diameter. These filaments proiect into the microvilli from the ooplasm (Fig. 11, *FO*). Although microtubules are found within the ooplasm they were never observed projecting into the microvilli.

Microvilli that appear during early stages of oocyte maturation are devoid of an intermicrovillous substance (Fig. 9, *MV*). It is not until the oocyte is about 90 μ in diameter (stage II) that one sees a homogeneous intermicrovillous material (Fig. 10, *Z-1*). This substance is the be-

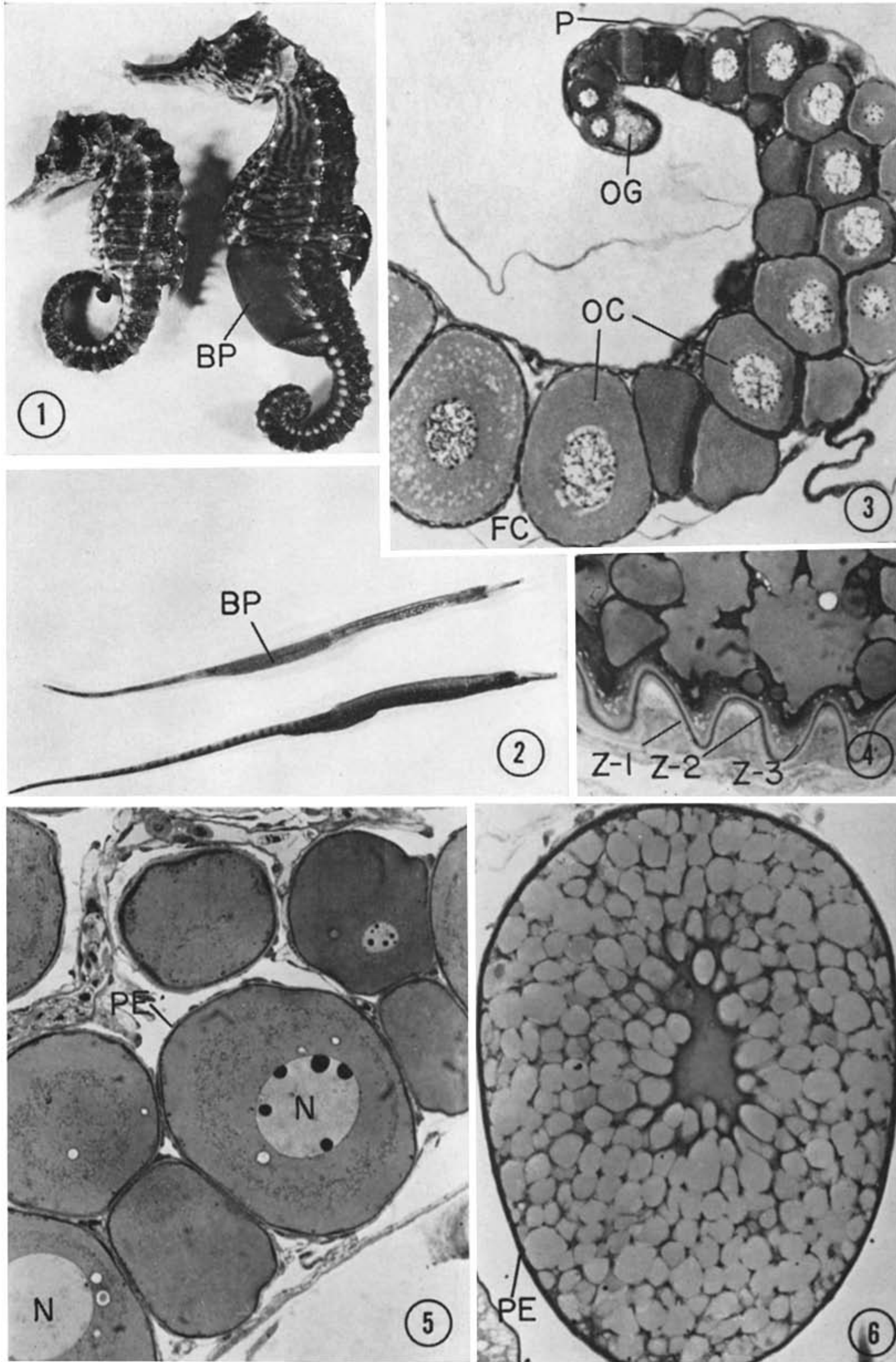
FIGURES 1 and 2 Male and female of the seahorse (Fig. 1) and pipefish (Fig. 2). The brood pouch of the males is labeled *BP*. Figs. 1 and 2, × .5.

FIGURE 3 A section of a portion of the ovary of the seahorse showing oogonia (*OG*) and oocytes (*OC*). The oocytes are encircled by a layer of follicle cells (*FC*) and the limiting boundary of the ovary by the peritoneum (*P*). Paraplast embedded; Heidenhain's iron hematoxylin stained. × 100.

FIGURE 4 Section of a stage II oocyte illustrating the three zones of the primary envelope (*Z-1*, *Z-2*, and *Z-3*), Epon embedded; toulidine-blue stained (30). × 700.

FIGURE 5 Section of various sized oocytes illustrating nuclei (*N*) and the densely stained primary envelope (*PE*). Epon embedded; toulidine-blue stained (30). × 200.

FIGURE 6 A stage III oocyte filled with yolk bodies and enringed by the densely stained primary envelope (*PE*). Glycol methacrylate embedded; hematoxylin stained (52). × 500.


BP
1
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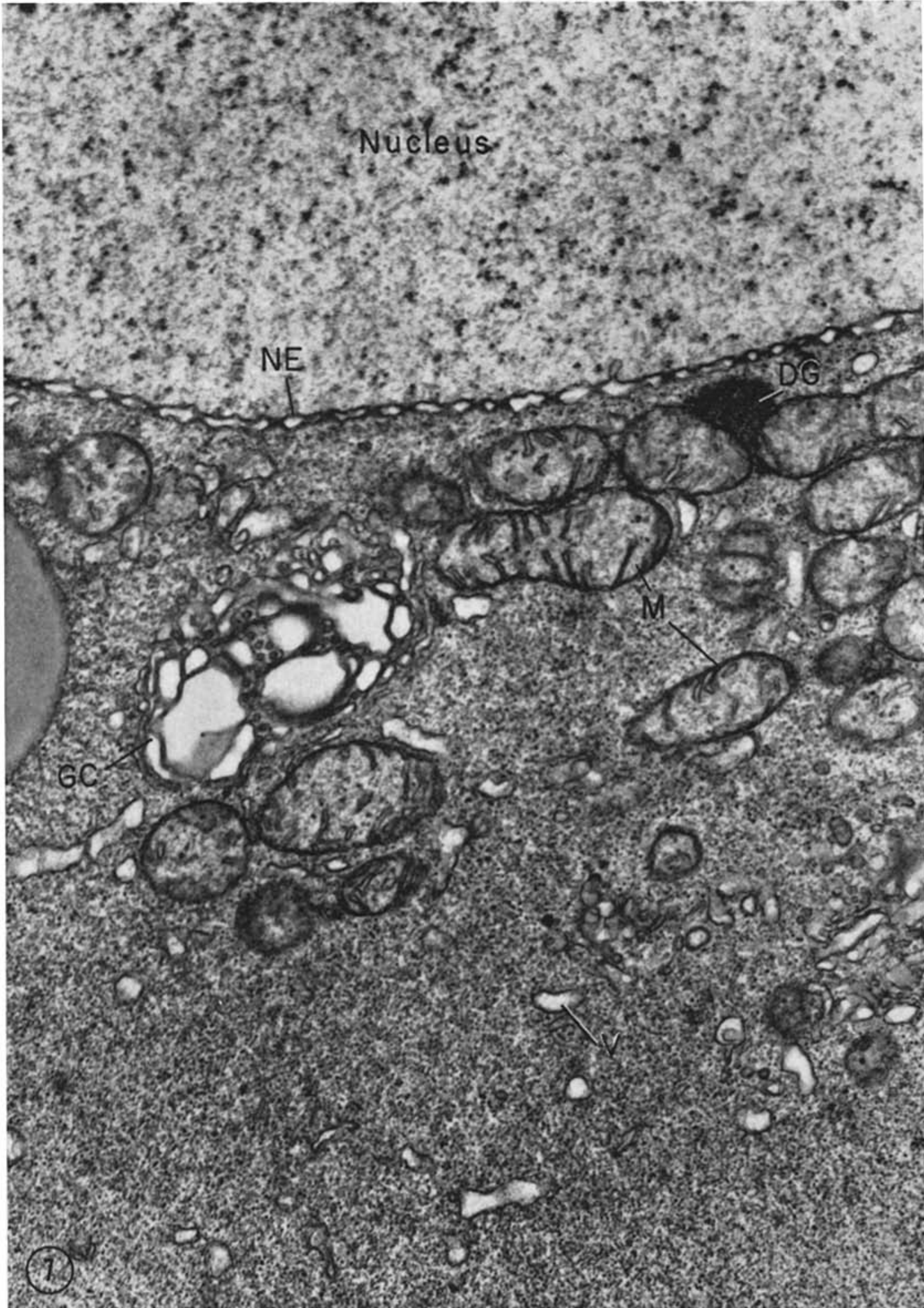


FIGURE 7 A section through a young oocyte illustrating the nucleus, nuclear envelope (*NE*), Golgi complex (*GC*), smooth-surfaced vesicles (*V*), dense granules (*DG*), and mitochondria (*M*). × 20,000.

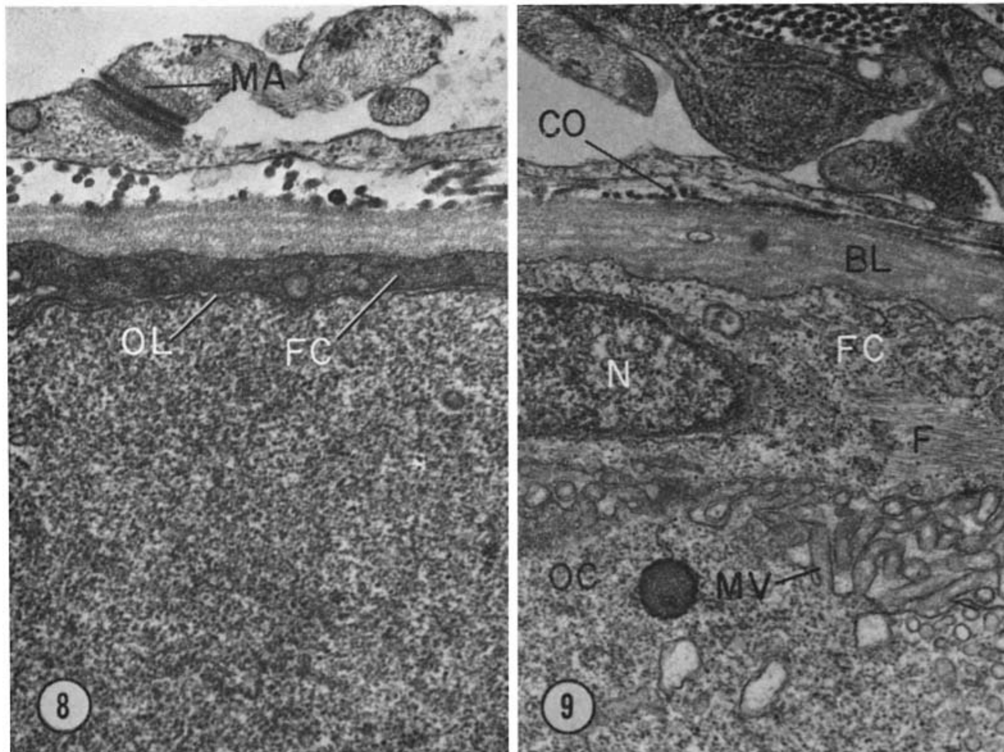


FIGURE 8 A section through the surface of a young oocyte illustrating the morphologically nonspecialized oolemma (*OL*) closely associated with the plasmalemma of a follicle cell (*FC*). The stromal cells are associated with each other by maculae adhaerentes (*MA*). × 15,000

FIGURE 9 A small portion of a young oocyte (*OC*) depicting microvilli (*MV*). The encompassing follicle cell (*FC*) shows its nucleus (*N*) and some fine filaments (*F*). The outer surface of the follicle cells rest on a basement lamina (*BL*) that is in contact with some collagenous fibers (*CO*). × 15,000

ginning of the formation of the first zone of the primary envelope. The constituents of each zone are not deposited simultaneously over the entire surface of the oocyte. In the case of Z-1 (the outermost zone), the component is initially deposited in several places on the oocyte and continues until it is almost as thick as the microvilli are long (Fig. 11, *Z-1*). A section transverse to the long axis of the microvilli reveals that a space is always present between the components of the envelope (Z-1, Z-2, and Z-3) and each microvillus (Figs. 18 and 19,*).

When oocytes are about 150 μ in diameter (stage II), one sees the first appearance of Z-2 (Fig. 15). It develops on the inner aspect of Z-1 in the form of dense vesicular units. To these units others are added until Z-2 appears as a rather dense granular region in which are located some slitlike areas. Finally, Z-2 (Fig. 20) becomes one-fourth the height of Z-1 and is now a dense, homogeneous layer when compared with the less dense component of Z-1.

Z-3 of the primary envelope first appears on oocytes that are approximately 250 μ in diameter. It is first seen on the inner portion of Z-2 as open circular units (Fig. 16, *Z-3*). As Z-3 continues its development, it appears as a filigree of an amorphous material. When Z-3 is complete (stage III), it is the largest zone of the primary envelope (Fig. 20).

Once the primary envelope reaches its final form, i.e. when all three zones are formed, there is an alteration of its over-all configuration. The first change begins in Z-3 with the thickening and increase in density of the amorphous component (Fig. 21). This increase in thickness of the amor-

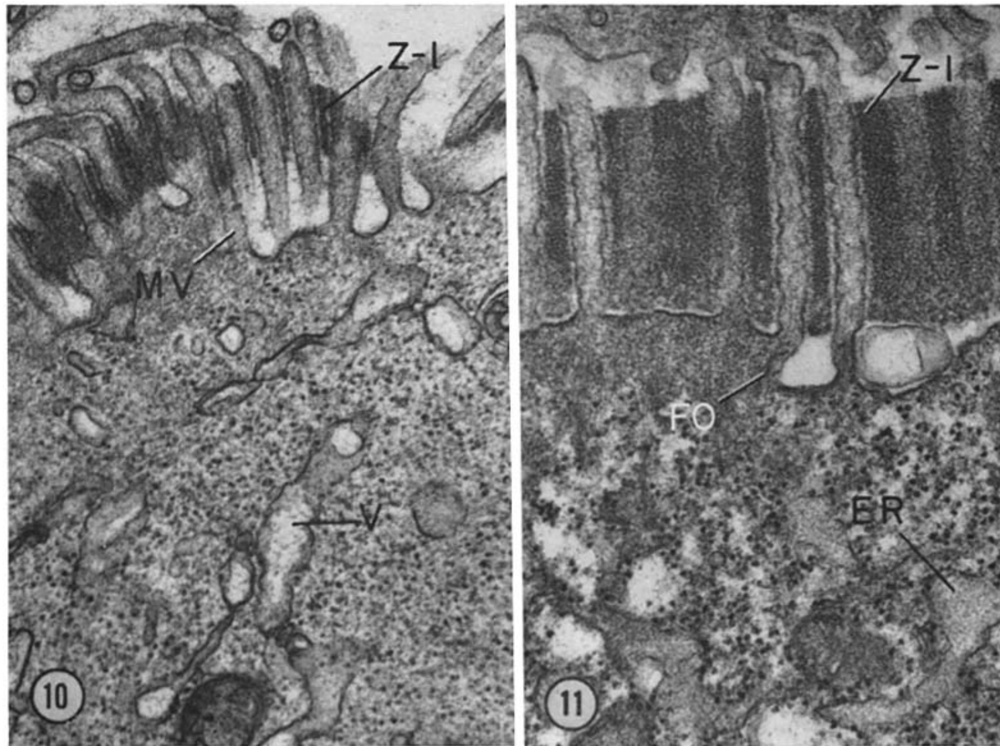
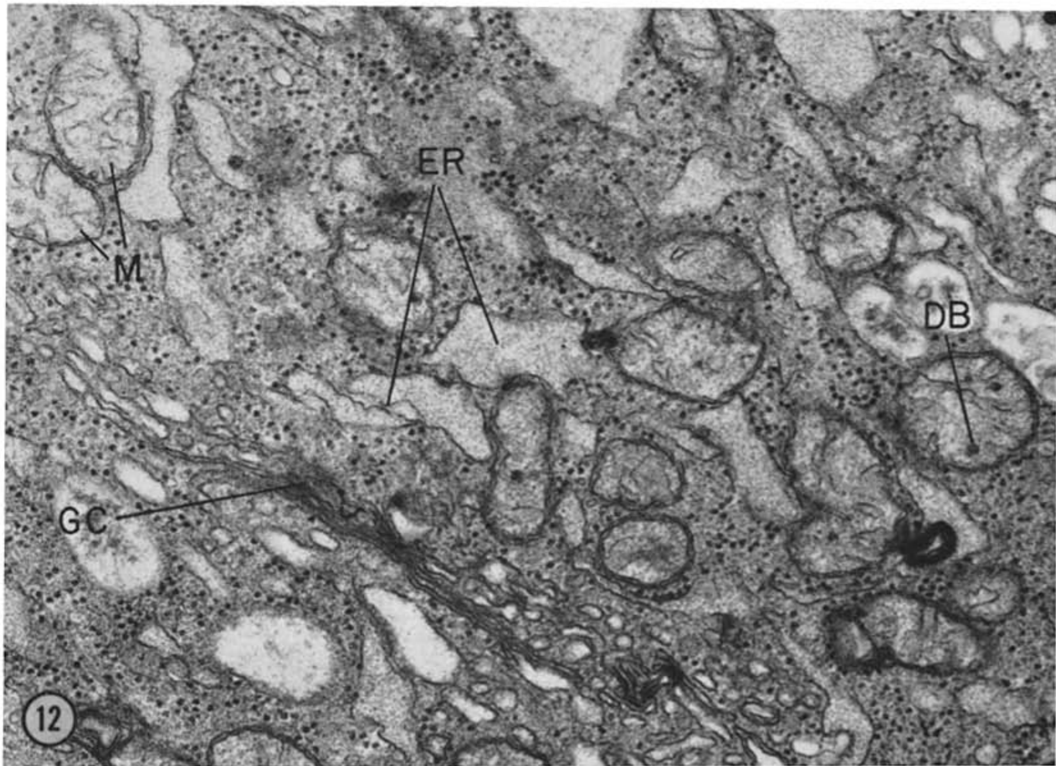


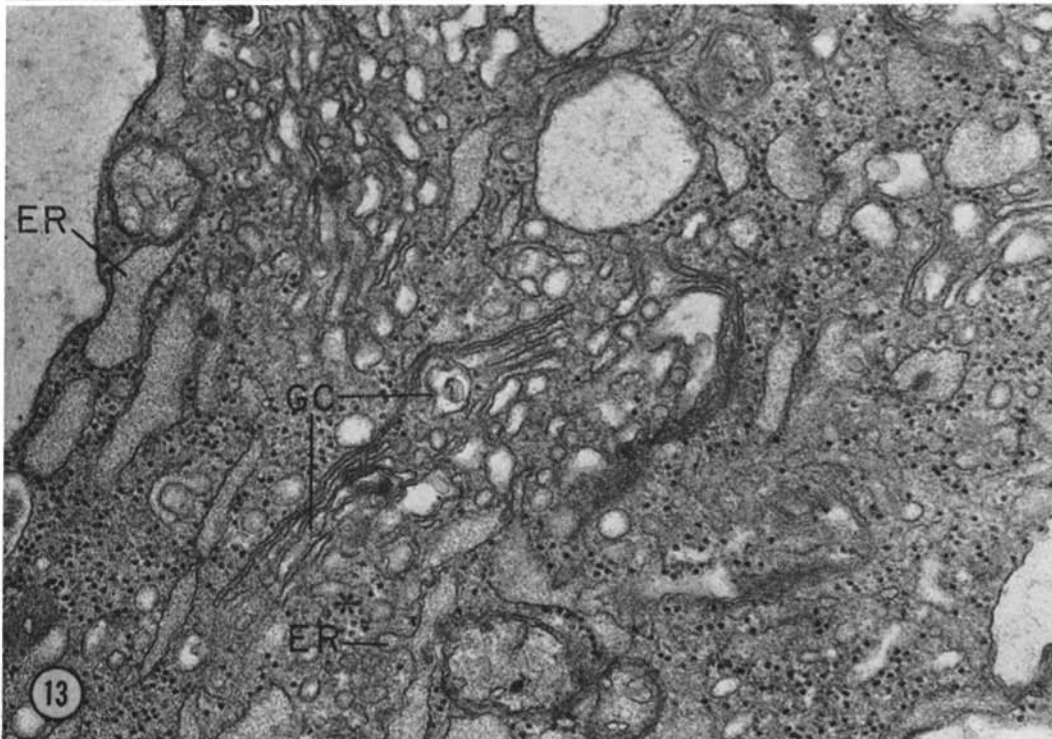
FIGURE 10 A section through a young oocyte showing microvilli (*MV*), smooth-surfaced vesicles (*V*), and the initial formation of zone one (*Z-1*) of the primary envelope. × 30,000

FIGURE 11 A portion of an oocyte showing the first zone (*Z-1*) of the primary envelope, fine filaments (*FO*) projecting from the ooplasm into a microvillus, and cisternae of the endoplasmic reticulum (*ER*). × 30,000

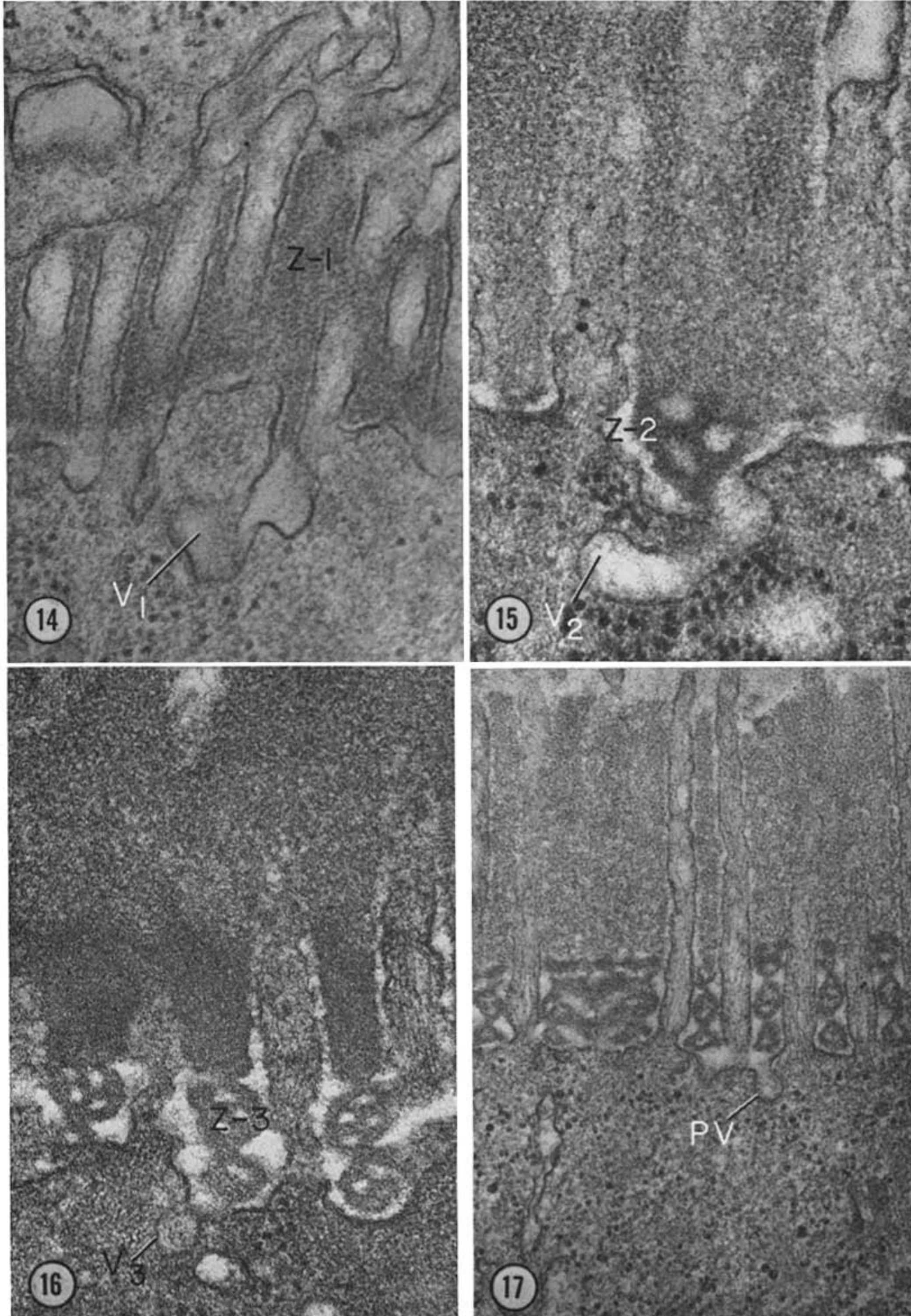
phous component progresses outwardly until the pattern that distinguishes Z-3 is completely obliterated. Z-3 eventually appears (oocytes approximately 400 μ in diameter) as a rather compact structure with alternating light and dense laminae (Figs. 22 and 23, *Z-3*). In the second change Z-2 appears to become thinner when compared with its equivalent on late stage II oocytes. It is now a rather compressed, dense layer (Figs. 21–23, *Z-2*) and is thought to be the same stratum that stains so intensely with bromphenol blue. The third change is the loss of Z-1. Just before the alteration in Z-2 and Z-3 in late stage III oocytes, one observes a disorganization of the component of Z-1, i.e. from an area that was originally homogeneous and organized about microvilli to one that appears flocculent (Fig. 22, *Z-1*). When the oocyte becomes a mature egg, only scant amounts of the remnants of Z-1 (Fig. 23, *Z-1**) are found associated with the now compressed constituent of Z-2.

FOLLICLE CELLS: Follicle cells that partially surround oogonia and completely encircle young oocytes are squamous. Each cell contains a rather spindle-shaped nucleus (Fig. 9, *N*) and the normally occurring organelle systems such as centrioles (Fig. 24, *C*), mitochondria, (Fig. 24, *M*, inset), Golgi complexes (Fig. 24, *GC*), and rough endoplasmic reticulum (Fig. 24, *ER**), fine filaments (60–70 A in diameter), (Fig. 9, *F*), microtubules, some dense bodies, and a host of ribosomes. With the exception of centrioles, the numbers of these organelles increase along with the cytoplasmic volume. This is particularly true of the rough endoplasmic reticulum and the Golgi complex (stage III). The saccules of the Golgi complex acquire a rather dense substance. Associated with these saccules are numerous smooth-surfaced vesicles.





FIGURES 12 and 13 Portions of the ooplasm of stage II oocytes showing cisternae of the endoplasmic reticulum (*ER*), mitochondria (*M*) that contain dense bodies (*DB*), and Golgi complexes (*GC*). Note the evagination from the surface of a cisterna of the endoplasmic reticulum (*ER**). Figs. 12 and 13, × 40,000.



Figures 14–16 The formation of all three zones (*Z-1*, Fig. 14; *Z-2*, Fig. 15; *Z-3*, Fig. 16) of the primary envelope by the fusing of vesicles with the oolemma. (V_1, Fig. 14; V_2, Fig. 15; V_3, Fig. 16). Fig. 14, × 78,000; Fig. 15, × 90,000; Fig. 16, × 78,000.

Figure 17 A fuzzy coated pit (*PV*) which is an early stage in the formation of pinocytotic vesicle. × 43,000.

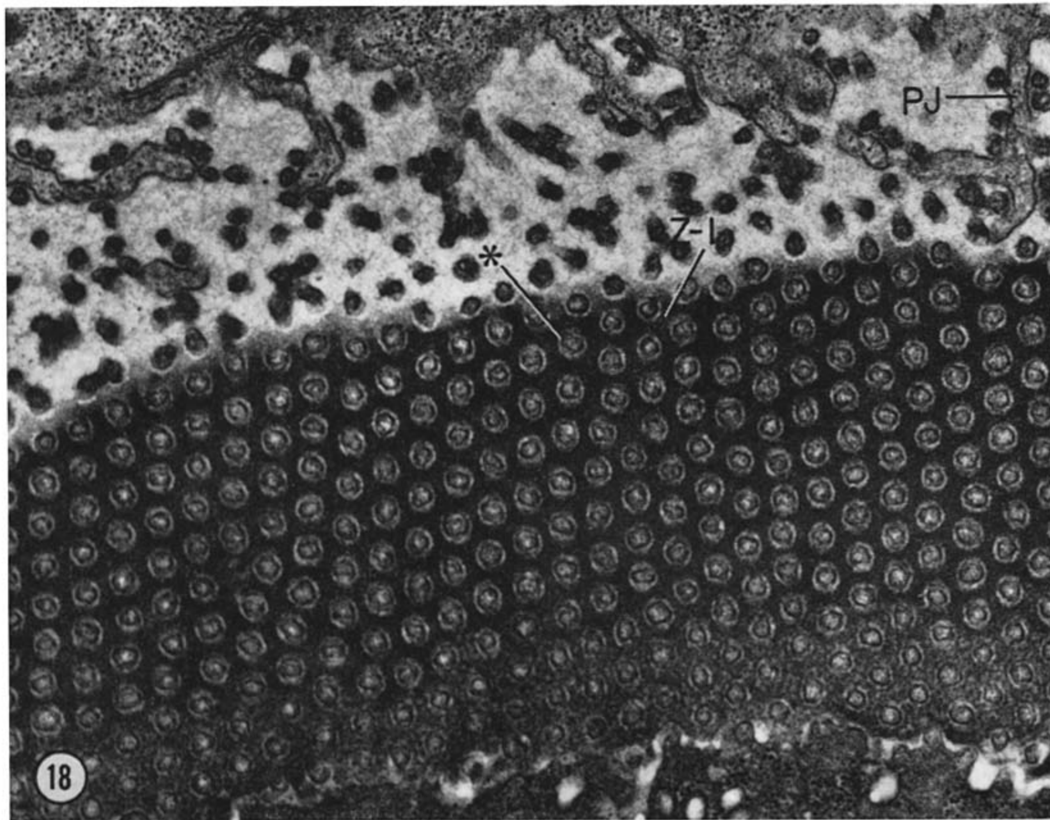


FIGURE 18 Tangential section through the first zone (*Z-1*) of the primary envelope of a stage II oocyte. A space is always present between the components of the envelope and the microvilli (*). Note the protoplasmic projection of a follicle cell at *PJ*. × 30,000.

In stage III oocytes, just before and during the changes which take place in the zones of the primary envelope, the cisternae of the rough endoplasmic reticulum of the follicle cells become dilated and filled with a rather homogeneous substance. Some of these cisternae show evaginations at various points along their surfaces (Fig. 24, *ER**, inset).

Concomitant with the increase in cell volume, the previously morphologically nonpolarized follicle cells become polarized, i.e. the plasmalemma of the follicle cells facing the oocyte sends out cytoplasmic projections (Fig. 18, *PJ*) that infrequently interdigitate with the microvilli of the oocyte (Fig. 23, *PJ*). Sometimes a cytoplasmic projection from a follicle cell is closely applied to the oolemma, being separated from it by a space of about 200 A. The follicle cells are connected to each other by maculae adhaerentes.

By the time the oocyte reaches late stage III, the cytoplasm of the follicle cells contain many dense bodies. These may be lysosomes. Another interesting feature of the cytoplasm is an increase in the over-all electron density of the cytoplasmic matrix. This may reflect cytonecrosis. It should be pointed out that the follicle cells do not encompass the egg when it is deposited in the brood pouch of the male.

The oocyte and the follicle cells are enclosed by a homogeneous substance which occasionally appears laminated (Figs. 8 and 9, *BL*). This material may be the basement lamina of the follicle cells. The basement lamina is sometimes separated from other elements of the ovarial stroma by a layer of collagenous fibers (Fig. 9, *CO*). The stromal cells are joined by maculae adhaerentes (Fig. 8, *MA*).

DISCUSSION

The study of the envelopes that are formed on the surface of oocytes during their maturation has

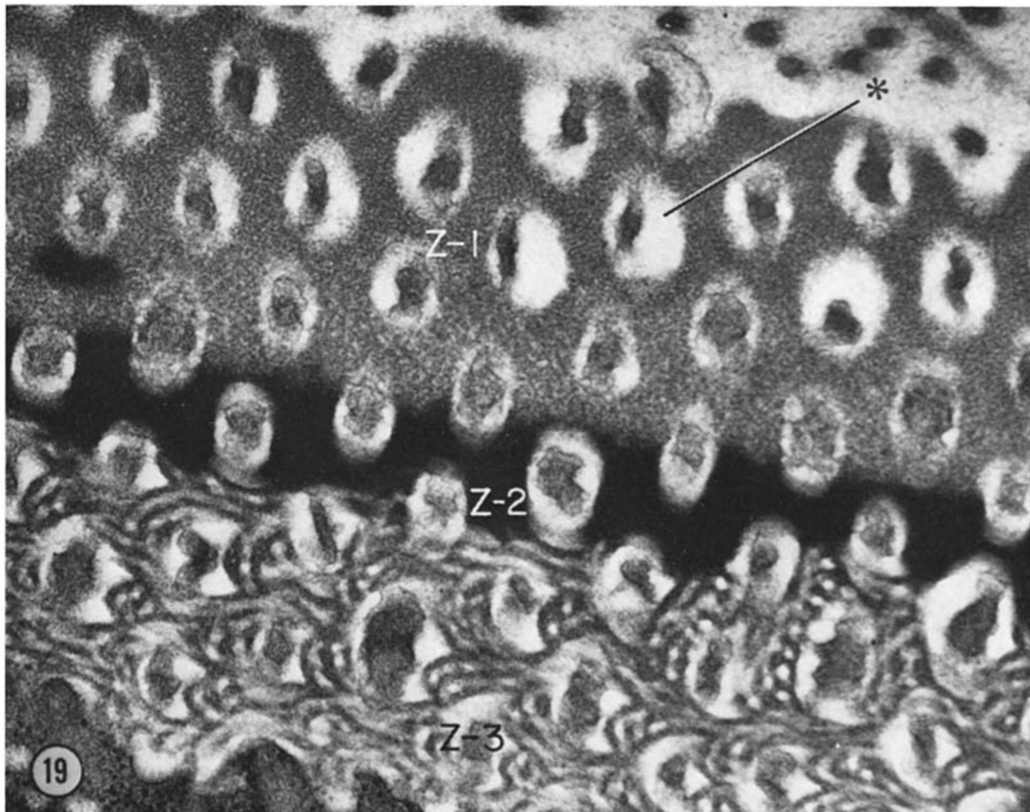


FIGURE 19 Section, similar to that in Fig. 18, through all three zones (*Z-1*, *Z-2*, and *Z-3*) of the envelope of a stage III oocyte. × 30,000.

attracted the attention of a host of able investigators (9, 15, 20, 22, 28, 34, 35, 43, 44). While unanimity exists concerning certain phenomena associated with the differentiation of the oocyte, there is no general agreement with respect to nomenclature of the noncellular material that is assembled on its surface during development. Evidence presented in this study and evidence obtained by others on a variety of organisms make it abundantly clear that, as an oocyte matures, there appears on its morphologically and physiologically specialized surface a substance that may be either homogeneous or architecturally complex. Since, in many instances, this substance is refractile to staining with dyes commonly used in histology and cytology, some investigators have referred to the refractile area as the zona pellucida while others have called it the vitelline membrane. With the resolution afforded by the light microscope, early cytologists were unable to discern that the oolemma became specialized during differentiation by the formation of microvilli. These researchers did, however, recognize the fact that fine striations appeared within the so-called zona pellucida. Now it is known that, at least in some oocytes, the structures responsible for the fine striations may have a dual origin since, when oocytes are enringed by follicle cells, cytoplasmic projections from these follicle cells often, but not always, interdigitate with microvilli of the oocyte. The arrangement of the microvilli on the oocyte and the presence of cytoplasmic projections of the follicle cell within the substance comprising the so-called zona pellucida and/or vitelline membrane during its development prompted early cytologists to call this area the zona radiata. Such a term, while used for many years in the literature, says little, complicates an already confused picture, and should be discontinued.

As indicated above, some authors have used the

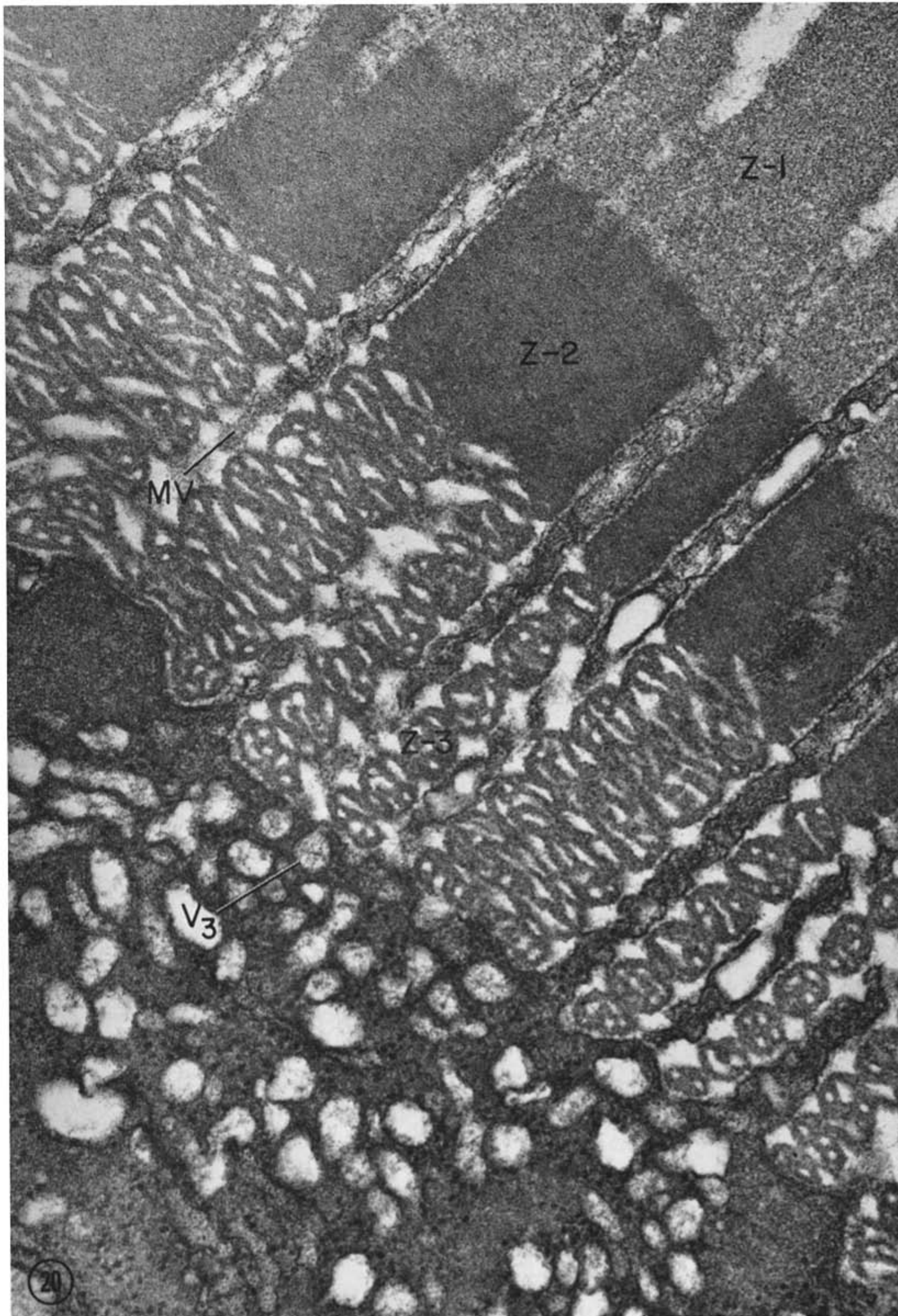
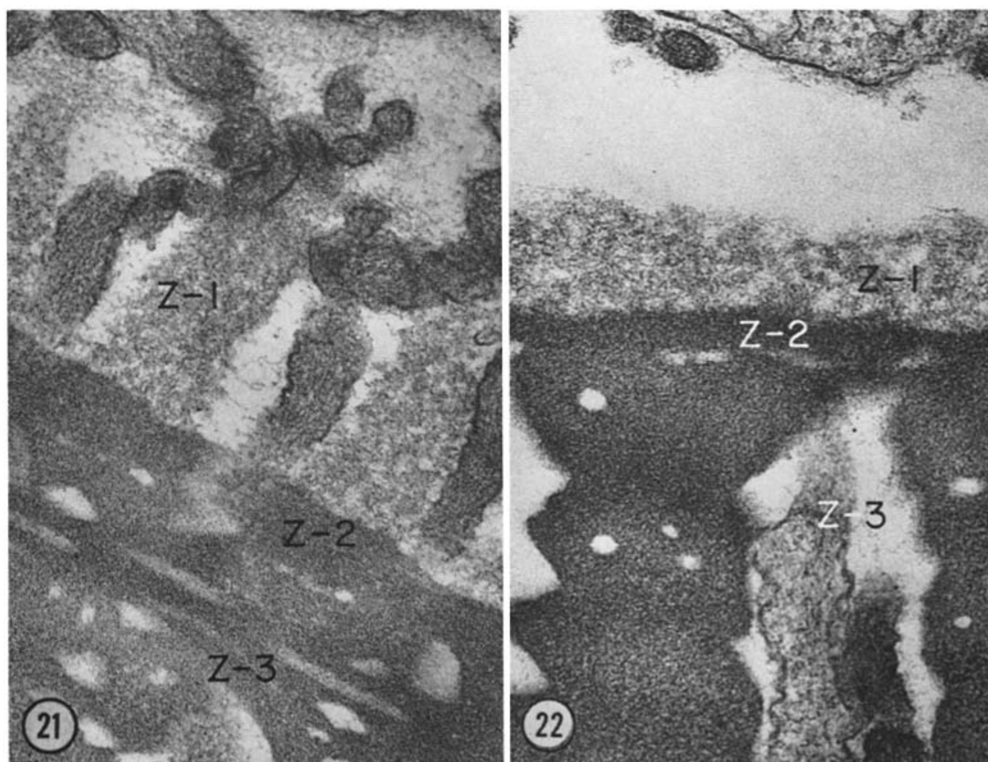


FIGURE 20 A tangential section through a stage III oocyte showing zone 1 (*Z-1*), zone 2 (*Z-2*), and zone 3 (*Z-3*) of the primary envelope, a microvillus (*MV*) of the oocyte, and a vesicle (V_4) fixed at the time its content was being added to zone 3. × 60,000.



FIGURES 21 and 22 Tangential sections through the primary envelope of late stage III oocytes illustrating the compactness of *Z-2* and loss of the pattern that distinguishes *Z-3*. In Fig. 21 zone 1 (*Z-1*) shows a disorganization of its constituent and in Fig. 22 zone 1 (*Z-1*) appears as a flocculent material. Fig. 21, × 40,000; Fig. 22, × 60,000.

terms zona pellucida and vitelline membrane in reference to the same structure. In its organization this stratum obviously does not follow the conventional, structural configuration of membranes, although it might possess physiological properties that are similar and/or equivalent to those of the plasma membrane. Therefore, the terms vitelline membrane and zona pellucida should be abandoned.

In 1874, Ludwig (32) suggested a suitable classification for the noncellular layers that encompass oocytes of a wide variety of organisms. Wilson (55) reemphasized Ludwig's classification and wrote the following: "...it is convenient to class the egg envelopes as primary, formed by the egg itself, secondary, formed by the follicle cells immediately surrounding the egg, and tertiary, formed by the oviduct or other maternal structures not immediately connected with the egg." In reference to the classification of Ludwig (32), Wischnitzer (58) has recently written that "...it is conceivable that the zona pellucida in urodeles at least, may be a product of both the ovum and follicle cells. Because of this such a classification system may not have any validity." For the formulation of a working classification that might be applied to external coverings of oocytes of organisms throughout the animal kingdom, great flexibility is desired. It seems to this author that the classification as proposed by Ludwig (32) possesses such plasticity. Within the animal kingdom, particularly among the invertebrates, there are many organisms whose oocytes are not surrounded by follicle cells, for example, the cnidarian, *Metridium* (Anderson, unpublished data), the polychaetous annelid, *Diopatra* (7), and the arthropod, *Limulus* (19; Dumont and Anderson, unpublished data). In the forementioned organisms it is clearly evident that the oocyte produces the primary envelope. In *Fundulus* and in the amphineuran mollusc, *Mopalia* (Ander-

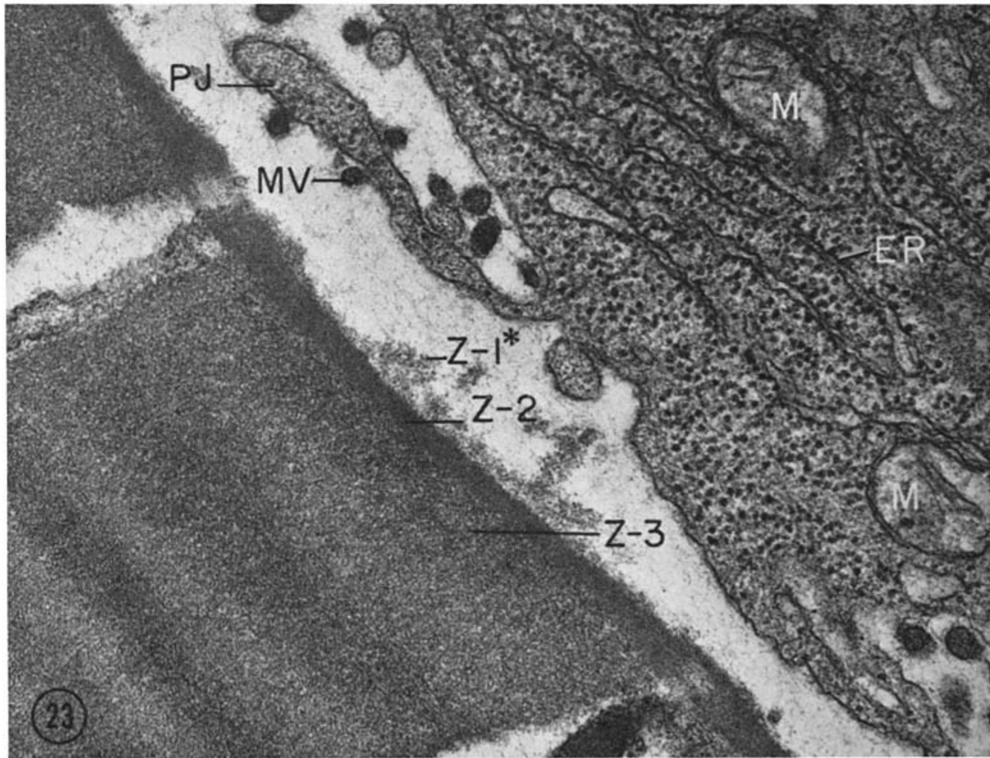


FIGURE 23 A tangential section through the surface of a mature egg showing remnants of zone 1 (*Z-1**), the dense compact material comprising zone 2 (*Z-2*), and the laminated appearance of zone 3 (*Z-3*). Note the loosely associated follicle cell containing mitochondria (*M*) and endoplasmic reticulum (*ER*). A cytoplasmic projection of the follicle cell is labeled *PJ* and a microvillus from the oocyte *MV*. × 50,000.

son, unpublished data), the stratum that overlies the primary envelope is produced by the follicle cells and, according to Ludwig's classification, would then be a secondary envelope. There appears to be some evidence that in other forms, such as amphibians (54), a secondary envelope is presumably formed; however, while displaying no morphological differences this secondary envelope gives a positive reaction for acid mucopolysaccharide. It should be emphasized again that the term zona pellucida was applied to the material encompassing oocytes and was so named only on the basis that it did not stain with dyes commonly used in cytology.

Whereas the tertiary coat is not the issue in this paper, it is nevertheless not formed by *ovarian oocytes* but, as indicated by Ludwig, by the oviduct or other maternal structures. These coverings are well known, and one might cite such examples as the gelatinous coverings found on the eggs of the frog (47) and the chemically complex egg case of certain elasmobranchs (17). Attention should be directed to the fact that the classification of envelopes of oocytes as proposed by Ludwig was developed by him only for ovarian oocytes and not for fertilized eggs. It is well known in Ascaris for example that, when the egg is activated, certain metabolic processes are initiated that are directed toward the synthesis of new structures and the release of certain other structures formed during oogenesis that are utilized in the formation of three noncellular coats (21, 24). Perhaps in those organisms in which envelopes are formed by the fertilized egg and their composition is unknown one should initially refer to the envelopes by number only to indicate the order in which each appears. When the chemical composition of each envelope is known, the terminology for any given species should reflect its chemical organization. For example, Foor (25) terms the second envelope that is

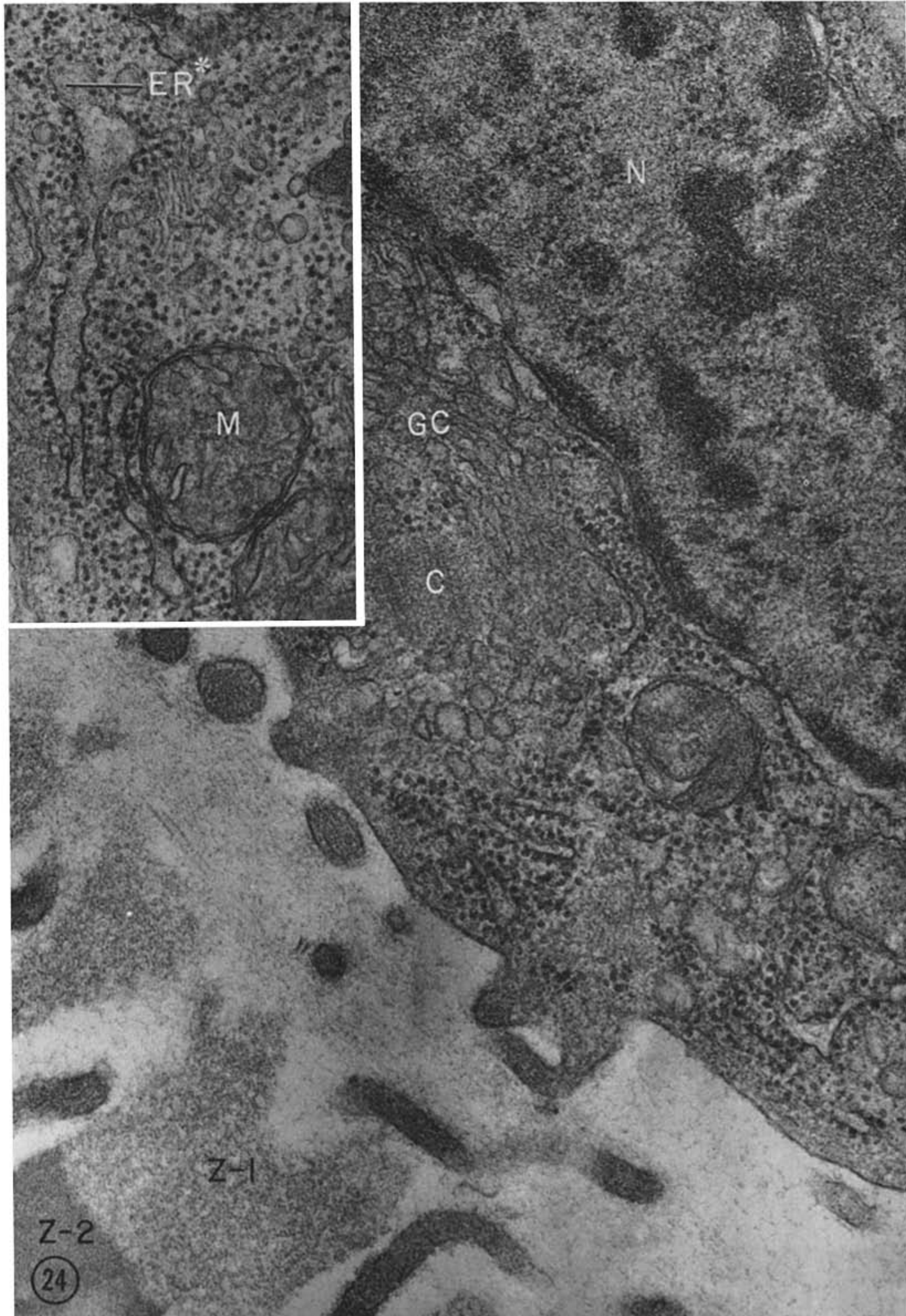


FIGURE 24 Section through a portion of a follicle cell surrounding a stage III oocyte showing nucleus (*N*), centriole (*C*), Golgi complex (*GC*), mitochondria (*M*, inset), and a cisterna of the endoplasmic reticulum with a bulbous tip (*ER**, inset). Note zone 1 (*Z-1*) and a portion of zone 2 (*Z-2*) of the primary envelope. Fig. 24, × 50,000; inset, × 50,000.

produced by the fertilized *Ascaris* egg as the chitinous covering and the third one as the ascaroside coat. In those cases in which the tertiary envelope is formed it would make no difference when it is produced, for in many organisms the tertiary envelope is produced before fertilization whereas in others it is produced after this complex phenomenon has been completed.

The term chorion according to Jaeger (30) is "...the membrane that encloses the fetus." It is not applicable to the coats of ovarian oocytes. If the term is to be retained, it should only be applied to the *protective covering surrounding an embryo*. Therefore, the chorion, regardless of its origin, could be (*a*) an augmented primary envelope like that found in the seahorse, pipefish, and *Diopatra*, (*b*) an augmented primary and secondary envelope as in *Fundulus*, (*c*) those coverings that are produced by the oocyte prior to and after fertilization, for example in *Ascaris*, or (*d*) the materials of the tertiary envelope.

Origin of Primary Envelope

Staining procedures utilized for light microscopy indicated that the primary envelope is composed primarily of a protein–acid mucopolysaccharide complex or a glycoprotein. The crucial question now is what is the origin of the material that constitutes the tripartite primary envelope? It has already been shown that the primary envelope commences with the production of the constituents of Z-1. As differentiation proceeds, there appear a second (Z-2) and a third (Z-3) zone, each of which is added in succession to the inner surface of the preceding one facing the oolemma. During the formation of each zone, vesicular and elongate structures are found in the peripheral ooplasm. The lumens of these structures are found to be confluent with the material comprising the egg coat during its formation. These temporally morphological observations favor the interpretation that the primary envelope is a product of the metabolic machinery of the oocyte.

It has been noted previously that during the early growth period of stage II oocytes there are no cisternae of the rough endoplasmic reticulum. As the oocyte continues to grow, the Golgi complexes move toward the periphery. Just before the appearance of Z-1 of the primary envelope, the saccules and the associated vesicles of the Golgi complex increase in number and contain a wispy material. The vesicles move into the peripheral ooplasm and fuse with the oolemma, thereby spilling their contents around the microvilli. The formation of Z-1 of the primary envelope ensues.

Before the formation of Z-2, many cisternae of the rough endoplasmic reticulum, filled with a finely stippled substance, appear within the ooplasm. These cisternae produce vesicles that are devoid of ribosomal particles and contain a material whose density is similar to that within the cisternae from which they are derived. When the endoplasmic reticulum becomes filled with the homogeneous material, the Golgi complex is also noted to produce vesicles from the margins of its saccules. These vesicles also contain a relatively dense substance. At the time Z-2 and Z-3 are produced there is a population of vesicles within the peripheral ooplasm. Interestingly enough, the internal material of these vesicles is not wispy but is somewhat dense and flocculent. These vesicles fuse with the oolemma and their contents are utilized in the formation of Z-2 and Z-3, respectively. The phenomenon of vesicle fusion ceases when Z-3 shows the first signs of alteration, i.e., the thickening of its highly ordered component.

Since no rough endoplasmic reticulum is found within the ooplasm of oocytes during the formation of Z-1 of the primary envelope, it is thought that the vesicles derived from the Golgi complex contain the precursor(s) for this first zone. In other words, certain of the Golgi complexes are involved in the initial synthesis of the constituent(s) utilized in the fabrication of Z-1 of the primary envelope. After Z-1 is formed, rough endoplasmic reticulum is found scattered within the ooplasm and the cisternae of this organelle contains a rather homogeneous substance. It is possible that the homogeneous substance within the cisternae of the endoplasmic reticulum is protein. Here it should be recalled that the primary coat is composed of a protein–acid mucopolysaccharide complex. If one assumes that the material within the cisternae of the endoplasmic reticulum is indeed protein and further assumes that this protein is one of the precursors of Z-2 and Z-3, the next question is where would the protein be made available to become complexed with the polysaccharide component? This might be accomplished in the following manner. The protein component of the endoplasmic reticulum could leave the cisternae of this organelle in the form of vesicles that bud off either from their surfaces or tips. These vesicles may subsequently fuse with the saccules of the Golgi complex where

the polysaccharide moiety may be added. When the complex is produced within the saccules of the Golgi complex, such a substance may leave the Golgi complex sequestered within vesicles. The vesicles containing the protein–acid mucopolysaccharide complex leave the region of Golgi complex and migrate to the periphery and fuse with the oolemma. Just what the different texture and density of the substance comprising each zone means is at present obscure. However, it could reflect a difference in the amounts of polysaccharide and/or protein contained in each zone. While no experiments have been made to substantiate the involvement of the Golgi complex and the endoplasmic reticulum in the synthesis of the components of the primary envelope, there is strong evidence in the literature that the rough endoplasmic reticulum does indeed transfer its contents to the Golgi complex. For example, since the radioautographic study of Caro and Palade (14), which indicated that the Golgi complex is a site for the concentration of protein, many investigators have shown that this organelle is not only a site for the concentration of protein but is also a site for the synthesis and/or concentration of polysaccharide (12, 13, 37, 38, 41, 42, 45).

Comparative Aspects of Teleost Egg Envelopes

From the accounts dealing with the envelopes of oocytes from other kinds of fish (22, 23) and the unpublished results of the present author on the sunfish (*Lepomis microcuris*), the brown trout (*Salmo trutta*), and the killifish (*Fundulus heteroclitus*), it is clear that the primary envelope of oocytes of teleosts studied to date does not possess a zone that would be equivalent to Z-1 of the primary envelope of oocytes of the seahorse and pipefish. The organization of Z-2 and Z-3 in the primary envelope of the seahorse and pipefish is, nevertheless, similar to that seen in the primary envelope of the teleosts *Cynolebias belotti* (36) and *Agonus cataphactus* (26). During oocyte differentiation in the latter two teleosts the zones also change in a manner similar to that indicated for the highly ordered Z-3 in the seahorse and pipefish. In medaka, *Oryzias latipes*, the inner portion of the envelope is organized like Z-3 in the Syngnathidae and also undergoes similar structural changes (60). However, in *Oryzias*, the highly ordered pattern is overlaid by a dense layer and has attached to it some adhesive fibers like those found in *Fundulus* (20). It may be found that in *Oryzias* the outer, dense layer and fibers are produced by the follicle cells as they are in *Fundulus* (Anderson, unpublished data) and will, therefore, constitute a secondary envelope.

This study has also shown that, as the oocyte reaches maturity, Z-1 of the primary envelope becomes disorganized and eventually degenerates. Why this zone is discarded and the others are retained is unknown. It is also obscure what functional significance this zone might serve during oocyte differentiation.

The function of follicle cells in the seahorse and pipefish remains unexplained. However, they might be involved in the production of certain enzymes necessary for the destruction of Z-1 and the alteration of Z-2 and Z-3. It seems unlikely that the apparent differentiation exemplified by follicle cells is of little importance.

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