# YOLK PROTEIN UPTAKE IN THE OOCYTE OF THE MOSQUITO *AEDES AEGYPTI*. L.

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ABSTRACT

Yolk proteins are thought to enter certain eggs by a process akin to micropinocytosis but the detailed mechanism has not been previously depicted. In this study the formation of protein yolk was investigated in the mosquito *Aedes aegypti* L. Ovaries were fixed in phosphate-buffered osmium tetroxide, for electron microscopy, before and at intervals after a meal of blood. The deposition of protein yolk in the oocyte was correlated with a 15-fold increase in 140 mμ pit-like depressions on the oocyte surface. These pits form by invagination of the oocyte cell membrane. They have a 20 mμ bristle coat on their convex cytoplasmic side. They also show a layer of protein on their concave extracellular side which we propose accumulates by selective adsorption from the extraoocyte space. The pits, by pinching off from the cell membrane become bristle-coated vesicles which carry the adsorbed protein into the oocyte. These vesicles lose the coat and then fuse to form small crystalline yolk droplets, which subsequently coalesce to form the large proteid yolk bodies of the mature oocyte. Preliminary radioautographs, and certain morphological features of the fat body, ovary, and midgut, suggest that the midgut is the principal site of yolk protein synthesis in the mosquito.

In 1932 Jukes and Kay (21) suggested that yolk protein of the egg of the domestic fowl is chemically related to the serum proteins of the laying hen. Since then, several investigators (7, 19, 20, 24, 25, 38, 42, 43, 45) have confirmed this finding, and the concept has grown that chicken yolk protein is made in the liver and transported *via* the circulation to the egg for storage. Similar studies with the frog (14–18) and rat (27) have also shown egg protein to be nearly identical with protein species found in the maternal serum. Of further significance in this regard is the finding that foreign proteins introduced into the circulation appear in the yolk protein substantially unchanged (4, 24).

These observations on oogenesis in the vertebrates as well as Telfer's work with an invertebrate, a saturniid moth (48–50) demonstrate conclusively that, in many instances of egg protein production and deposition, the protein species is made outside of the ovary, probably in the liver or an analogous organ, is secreted into the extracellular spaces, and is then removed from the circulating blood by the developing egg. There are exceptions to this general scheme, one of which is found in the crayfish, which appears to have a highly developed synthetic mechanism in the oocyte cytoplasm for the formation of yolk protein (3).

In the case of the mosquito, which is the object of this study, there are reasons to suppose that yolk deposition in the developing oocyte is accomplished also by removal of the protein from the blood of the insect. In the first place, yolk develops rapidly. In fact, synthesis and storage are essentially completed in as little as 25 hours after a blood meal. Concomitantly, none of the usual structural mechanisms associated with

rapid synthesis of protein or lipoprotein, especially for segregation and storage in granules, appear in the fine structure of the follicle cells or the oocyte cytoplasm. Indeed, only one unusual structural feature does appear, which seemingly might be involved in yolk deposition, and that is an elaborate development of pits or wells in the surface of the oocyte. It is the major purpose of this report to describe these pits and to present reasons for interpreting them as surface differentiations designed specifically for protein uptake and transport into the oocyte.

## MATERIALS AND METHODS

The ovary of the adult mosquito *Aedes aegypti* L. was studied. The insects were raised by routine methods (51) at 27°C, and the adult form maintained on raisins until needed. The full development and maturation of oocytes was initiated by feeding 1-week-old adults on the shaved back of a rabbit. Thereafter the mosquitoes were sacrificed at intervals as indicated in the next section. To facilitate handling them, the mosquitoes were chilled to 4°C just prior to fixation.

In preparation for light microscopy, entire mosquitoes sacrificed at various intervals after a blood meal were fixed for 6 hours at 4°C in 10 per cent aqueous acrolein (according to the methods of Feder (12, 13) as modified below). The mosquitoes were then dehydrated in a graded series of 1:1 methyl alcohol/1-methoxyethanol, and the abdomens were removed and infiltrated under vacuum for 3 days in six changes of a mixture of 95 per cent glycol methacrylate, 5 per cent Carbowax, and 0.3 per cent α-azodiisobutyronitrile as a catalyst. The final change of the infiltration mixture was allowed to polymerize for 3 days at 60°C before 1.5 μ sections were cut from the blocks. The sections were stained with aqueous 0.5 per cent toluidine blue and 1 per cent acid fuchsin.

For electron microscopic examination, the mosquitoes were fixed with 2 per cent osmium tetroxide, buffered at pH 6.8 and 7.3 according to Millonig (29). Abdomens were removed at predetermined intervals and the cuticles slit to allow rapid penetration of the fixative; fixation was continued for a period of 3 to 4 hours at 4°C. The tissues were thereafter rapidly dehydrated in increasing concentrations of alcohol, with interruption only in 50 per cent alcohol to permit dissection of the ovaries from the abdomen. After dehydration and exposure to two 5-minute changes of propylene oxide, the tissues were infiltrated overnight in a 1:1 mixture of propylene oxide and the embedding monomer of Epon. After a fresh change of monomer, the tissues were flat embedded and polymerized at 60°C for 2 days. The embedding monomer was a 1:1 mixture of methyl nadic anhydride (MNA)/Epon 812 accelerated with 0.5 per cent 2,4,6-tri(dimethylaminomethyl)phenol (26), which gives very hard blocks.

Sections were cut for the most part on a Cambridge (Huxley) microtome. The "silver" sections were mounted on either carbon stabilized celloidin or uncoated grids. Lead staining according to Millonig (30), or Karnovsky (22) at a dilution of 20:1, gave excellent contrast relatively free of contamination. The sectioned material was examined in an EMU 3 (RCA) or a Philips EM 200.

The mosquitoes that were used for the experiment with labeled leucine were fed on the legs and tail of a 14 gm rat that had been injected intraperitoneally 30 hours previously with 6 mc of L-leucine-$H^3$ (specific activity 5.0 c/mM) in 0.5 cc water. At intervals of 0.5, 1, 2, 4.5, 16, and 30 hours thereafter, the mosquitoes were sacrificed and embedded for light microscopy. Sections 1.5 μ thick were cut on a Porter-Blum Servall microtome and prepared for radioautography, following the methods of Caro and van Tubergen (6). Twelve weeks later, the sections were developed, and the number of silver grains compared with the aid of a phase microscope.

## OBSERVATIONS

The structure of the mosquito ovary has been described in detail in several light microscope studies (8, 31–33, 36, 41) and is perhaps best summarized in Christophers' monograph (9). It is important to review first this work and current observations on the structure of the ovary at the time when its development in the adult has reached a steady state. This plateau occurs by 3 days after emergence and persists until the adult feeds on blood. This act of feeding initiates a second stage in ovarian development which culminates in the formation of the mature egg.

### *The Resting Stage*

The ovaries of the mosquito lie in the abdomen between the fourth and sixth segments. Each ovary (Fig. 1), consisting of approximately sixty ovarioles, is contained by a muscular mesothelium beneath which lie many tracheae (Fig. 2). Each ovariole in turn is enwrapped by a thin squamous mesothelium of cells showing a kind of striated myofibril (Fig. 3). The ovarioles contain a maturing follicle, a presumptive follicle, and a germarium, and each of the meroistic-polytrophic follicles is comprised of seven nurse cells and one oocyte within a follicular epithelium.

The cells of the follicular epithelium are at this

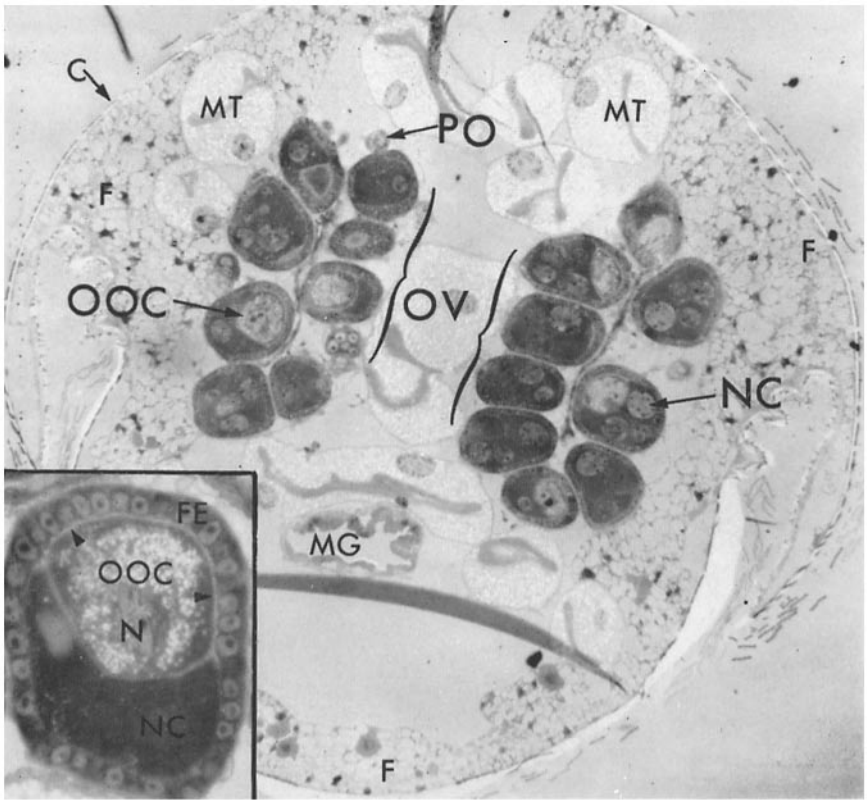



FIGURE 1 A light micrograph of a cross-section through a mosquito abdomen at a time just prior to yolk deposition. The section was stained with toluidine blue and acid fuchsin. The ovaries consist of two clusters of ovarioles (*OV*) situated centrally with respect to the spongy fat body (*F*) which encircles the abdomen beneath the cuticle (*C*). A presumptive follicle (*PO*) is joined to each maturing follicle in the region of the nurse cells (*NC*). The oocyte (*OOC*) is easily identified by the clear, non-staining lipid inclusions in its cytoplasm. In the Malphigian tubules (*MT*) the refractile inclusions, polytene chromosomes, and the lumen are especially evident. An undistended, scalloped midgut (*MG*) is situated in the central portion of the hemocoel just above a fold in the section. × 150.

The inset depicts a single follicle at greater magnification. Two nurse cells (*NC*), at the lower half of the follicle, exhibit intense basophilia in the cytoplasm. Numerous refractile lipid droplets surround the nucleus (*N*) of the oocyte (*OOC*). A light region (arrow heads) at the periphery of the oocyte, just within the follicular epithelium (*FE*), is the zone of yolk protein uptake. × 570.

stage cuboidal with large nuclei and little cytoplasm. They are closely applied to each other and joined, neighbor to neighbor, at their apical margins by desmosomes. Their basal surfaces rest on a basement membrane (Fig. 3). At higher magnifications provided by electron microscopy, the nuclei of these cells are seen to have a prominent polymorphic nucleolus enveloped by distinctly granular chromosomal elements superimposed on a lighter nucleoplasm (Fig. 2). The nuclear envelope possesses numerous pores. The cytoplasm, which is relatively sparse in amount, is rich in ribosomes but poor in elements of the endoplasmic reticulum (ER). These flattened cisternae are rough surfaced and occur most especially in the basal halves of the follicle cells. Mitochondria are numerous and possess well ordered, parallel arrays of cristae and several dense granules which in other types of cells are binding sites for divalent cations (37). Occasional Golgi elements and infrequent lysosomes are the other major inclusions.

The single oocyte and seven nurse cells reside beneath the follicular epithelium. The nurse cells, to be described more fully in a subsequent paper, are relatively large (27 $\mu$), the nuclei alone having the width of three follicular epithelial cells. The granular nucleoplasm, with several discrete nucleoli, is limited by a nuclear envelope perforated by a large number of pores. Immediately outside the nuclear envelope is an accumulation of dense granular material which probably corresponds to the localized thickening of the nuclear envelope reported earlier in *Drosophila* (23). This material at some places extends about 0.5 $\mu$ into the "sea" of ribosomes which represents, in part, the matrix of the cytoplasm. The nurse cells also have many rounded mitochondria, occasional lipid droplets, and a few elements of a rough-surfaced endoplasmic reticulum scattered throughout the cytoplasm. Although the nurse cells are closely applied to each other and to the oocyte and to the follicular epithelium, no evidence exists of cytoplasmic bridges.

The oocyte, as noted above, is readily identified by its uncommon nucleus in which two distinct bodies stand out against the relatively clear nucleoplasm (Fig. 2). One of these, the nucleolus, is a dense, meandering, polymorphic structure which sends long branches out into the nucleoplasm. The other, the chromosomal mass, consists of a unique configuration of synaptic chromosomes which have migrated from their previous peripheral position in the nucleus. This structure will be described in detail elsewhere. The enveloping nuclear membrane, regularly perforated with pores, provides a distinct separation from the rest of the oocyte.

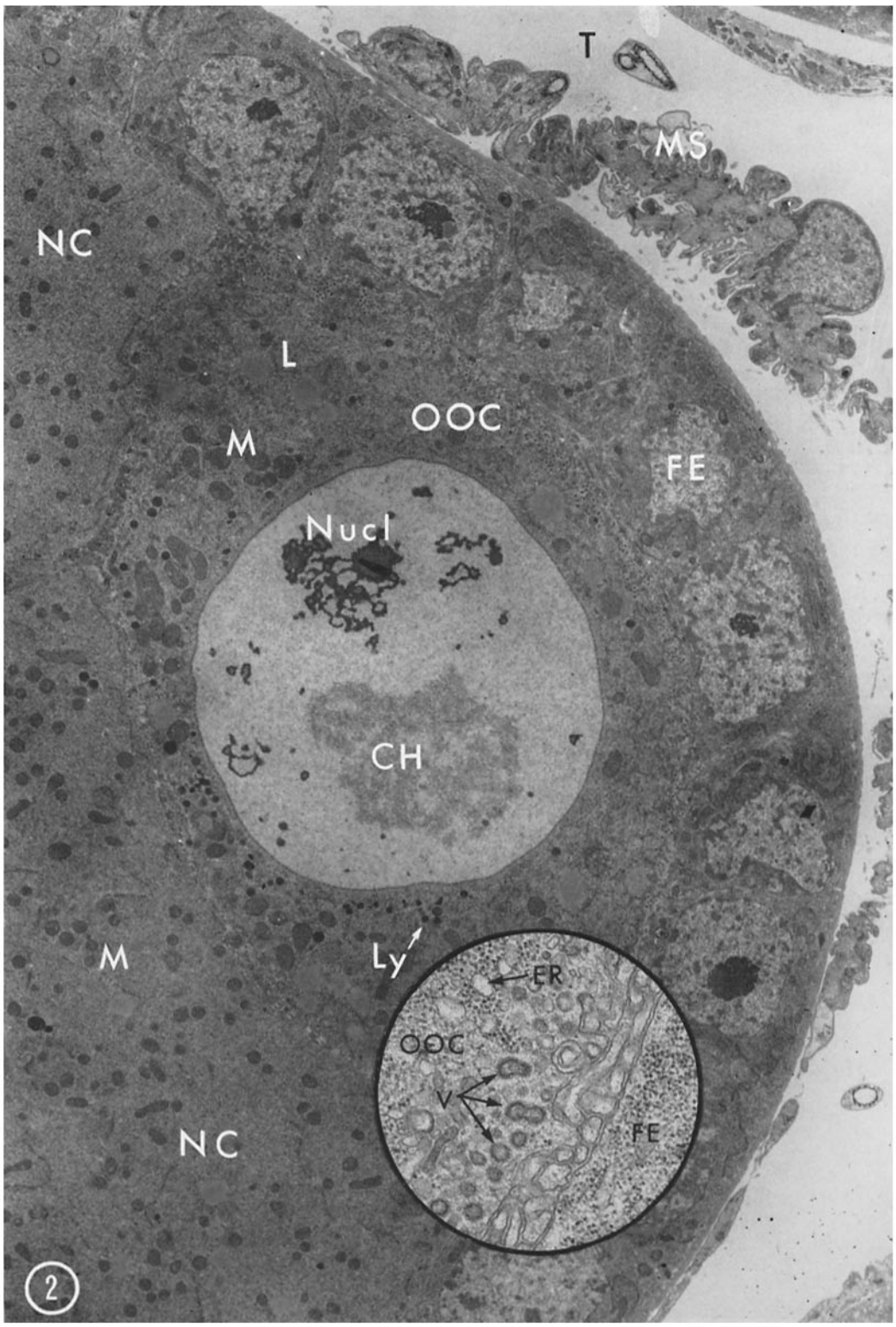
The cytoplasm of the oocyte is characteristically different from that of the surrounding cells (Fig. 2). Mitochondria here are larger with well ordered cristae; dense lysosome-like bodies are more numerous and many multilobed lipid droplets are common among the prominent background of free ribosomes. No yolk granules or precursors are evident.

The cortex of the "resting" oocyte is also remarkable in a number of respects. The surface adjacent to the follicular epithelial cells is covered by many unoriented villi. Then, just within this border of microvilli, there are many pit-like invaginations (140 m$\mu$ in diameter) of the oocyte membrane and many vesicles with diameters similar to those of the pits (Fig. 2, insert). These structures represent the first manifestation of a cortical differentiation which, as will be described below, appears to prepare the oocyte for the ingestion of large quantities of yolk protein. Although quite numerous even in this early resting stage, before the insect obtains a blood meal, the vesicles and pits are uniformly small and relatively undeveloped as compared with those present later when yolk deposition begins. This uniformity of size and structure is an important point in interpreting subsequent changes in this region. By contrast, that part of the oocyte facing the nurse cells shows relatively few microvilli, pits, and pit-vesicles.

The structure of the "resting" follicle constitutes the base-line on which the substantial alterations that occur in the oocyte of the blood-fed mosquito may be superimposed. A meal of blood or a controlled diet (44) sets in motion a series of changes in cell structure that quickly result in the formation of the mature egg. By 4 hours after the meal, these cytological changes are already in evidence, and after 7 hours they are striking.

FIGURE 2 This micrograph shows part of a follicle fixed before feeding, with a portion of two adjacent nurse cells (*NC*) and an oocyte (*OOC*) enclosed by a follicular epithelial layer (*FE*). A granular, ribosome-filled cytoplasm and scattered mitochondria characterize the nurse cells, while lipid droplets (*L*), and some denser droplets (*LY*), clumped chromatin (*CH*), and complex nucleolus (*Nucl*) are typical of the oocyte (*OOC*) at this stage. Great numbers of small, dense vesicles are present in the cortex of the oocyte (see insert). Peripheral to the ovariole is the discontinuous muscular sheath (*MS*). Tracheoles and trachea (*T*) lie exterior to this sheath tissue, but within that which surrounds the entire ovary. × 3,700.

The insert depicts at higher magnification a small segment of the vesiculated interface between the oocyte (OOC) and the follicular epithelium (FE). Bristle-coated vesicles (*V*) and elements of the endoplasmic reticulum (*ER*) are common in the oocyte cortex immediately beneath the interface. × 20,000.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## *Structural Changes Associated With Yolk Formation*

### A. The Follicular Epithelium

With the onset of yolk deposition after the insect has a blood meal, some striking changes appear in the follicular epithelium. Most obvious are the development of large intercellular spaces (Fig. 3) and a concomitant decrease in desmosomal connections which may be associated with this separation. In some instances, the spaces encountered were large and extended from the basement membrane of the follicle to the oocyte surface. Continuity of the epithelium is, however, not lost and there is a suggestion in the micrograph in Fig. 3 that contact with the basement membrane is retained by a fine reticulation of thin cell extensions. Obviously this behavior of the follicle cells produces open channels between the extrafollicular space and the surface of the oocyte—open, that is, except for the basement membrane.

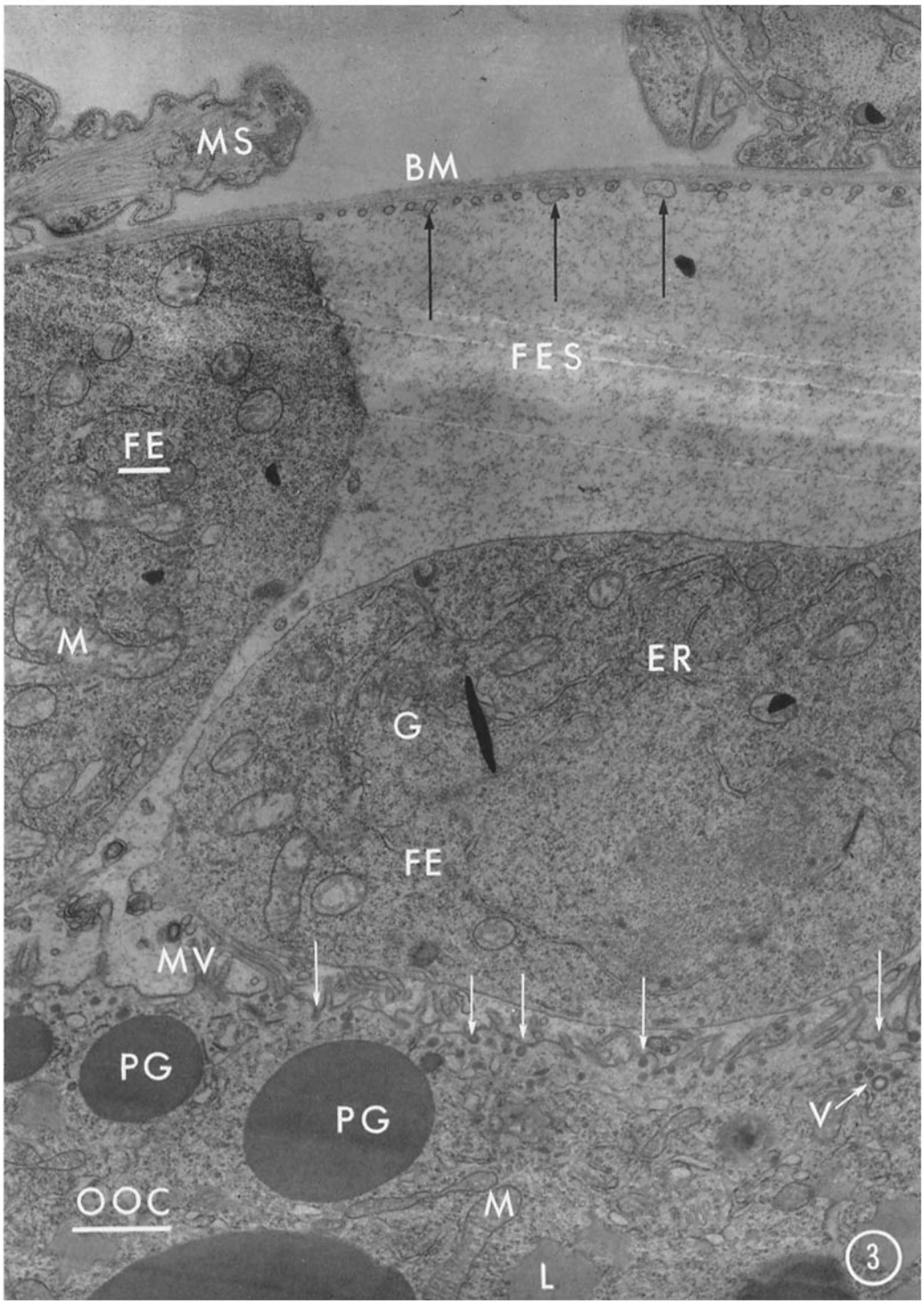
Some note should be taken of the material that fills these extracellular spaces. It is finely particulate and somewhat flocculent in appearance. Clearly the same material is not present in the interstitial spaces beyond the basement membrane of the ovariole. Throughout the intercellular spaces the distribution of this material is essentially uniform. At the oocyte surface it is continuous with a more condensed layer of similar texture, representing apparently an adsorbed layer of the same material. This tendency to concentrate on this surface is shown only in relation to the oocyte; adjoining surfaces of follicle cells and nurse cells are not so covered (Fig. 4). Even the extremities of the microvilli from the oocyte surface are relatively free.

### B. The Oocyte Surface

Thus the surface of the oocyte in the maturing ovariole faces on a greatly expanded space under and between the cells of the follicular epithelium (Figs. 3 and 4). It is especially in this cortical region of the maturing oocyte that a series of important alterations occurs, which are the particular interest of this report. The area of the oocyte facing the follicular epithelium is covered with great numbers of microvilli (also somewhat evident in the resting oocyte) which, by 7 hours after the blood meal, push into the extracellular spaces created by the separation of the follicular epithelial cells (Fig. 3). These microvilli vary somewhat in length and are not in any sense regularly disposed like those, for example, of the typical intestinal epithelium. They seem, however, to achieve a uniform diameter (60 m$\mu$) especially in their greatest extension from the surface. One gets the impression from the variation in length and orientation that the population may in life be actively changing, some retracting and others forming.

In between the microvilli, and also disposed irregularly, are numerous dense vesicles which are morphologically similar to the dense vesicles present in the resting oocyte (Figs. 2, 3, and 4). These connect in some instances with the oocyte surface and appear in profiles as small pits or wells (140 m$\mu$ in diameter). In other cases they are free and embedded in the cortex up to 0.5 $\mu$ from the surface (Fig. 4).

FIGURE 3 This micrograph shows parts of two adjacent follicular epithelial cells (*FE*) and an associated oocyte (*OOC*) fixed 7 hours after a blood meal. The follicle cells, separated by large spaces (*FES*), rest upon a typical basement membrane (*BM*). At some points along the basement membrane extremely attenuated extensions of the epithelial cells appear as a row of circular profiles (black arrows). The openings between these provide easy access to the intercellular space for any materials that traverse the basement membrane. The ovariole is covered by a thin reticular sheath of mesothelial cells (*MS*) which contain muscle fibrils. The oocyte at this stage in its development contains large proteid granules (*PG*), lipid droplets (*L*), mitochondria (*M*), and elements of the endoplasmic reticulum in a cytoplasmic matrix rich in ribosomes. The cortical zone, depicted here, possesses other features of special interest. Numerous microvilli (*MV*) extend from the oocyte surface into the intercellular space. Between the microvilli are several small dense pits (white arrows) still continuous with the cell surface. Small pit-derived vesicles (*V*) of the same size, density, and structure as the pits reside in the cortex of the oocyte. A part of a follicle cell in the center shows some profiles of the endoplasmic reticulum (*ER*) and a prominent Golgi component (*G*). × 15,500.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During this period of active yolk deposition there are roughly 300,000 pits on the surface of the oocyte facing the follicle cells. This figure is nearly 15 times the number of pits on the resting oocyte fixed only 7 hours earlier.

The pits and vesicles have structural features in common which help to relate them. It is, for example, characteristic for a cortical vesicle near the surface to be about 140 mμ in over-all diameter and to show, morphologically, 3 distinct layers. Of these, the middle one is most dense and the thinnest (*ca.* 75 A). This layer is obviously equivalent in all its characteristics to the plasma membrane of the oocyte (Fig. 4, insert, and Fig. 5). Internal to this line there is a layer of material 250 to 400 A thick, which by its density and texture can be identified with the layer of material on the external surface of the oocyte cell membrane. External to the middle layer, and continuous with the cytoplasmic matrix, there is a layer or coat about 200 A thick which seems to be made up of small bristles or hairs radiating outward from the dense line. We shall refer to this as the "bristle coat" and to the vesicles as "coated."

It is not difficult to see a close structural relationship between the coated vesicles and the pits. Both show the same 3-layered structure, and it is evident in the pits in Fig. 5 that the middle one of the layers has the thickness and molecular structure of a unit membrane. In the same image, the structure of the inner, adsorbed layer and the outer bristle coat are shown to better advantage than elsewhere.

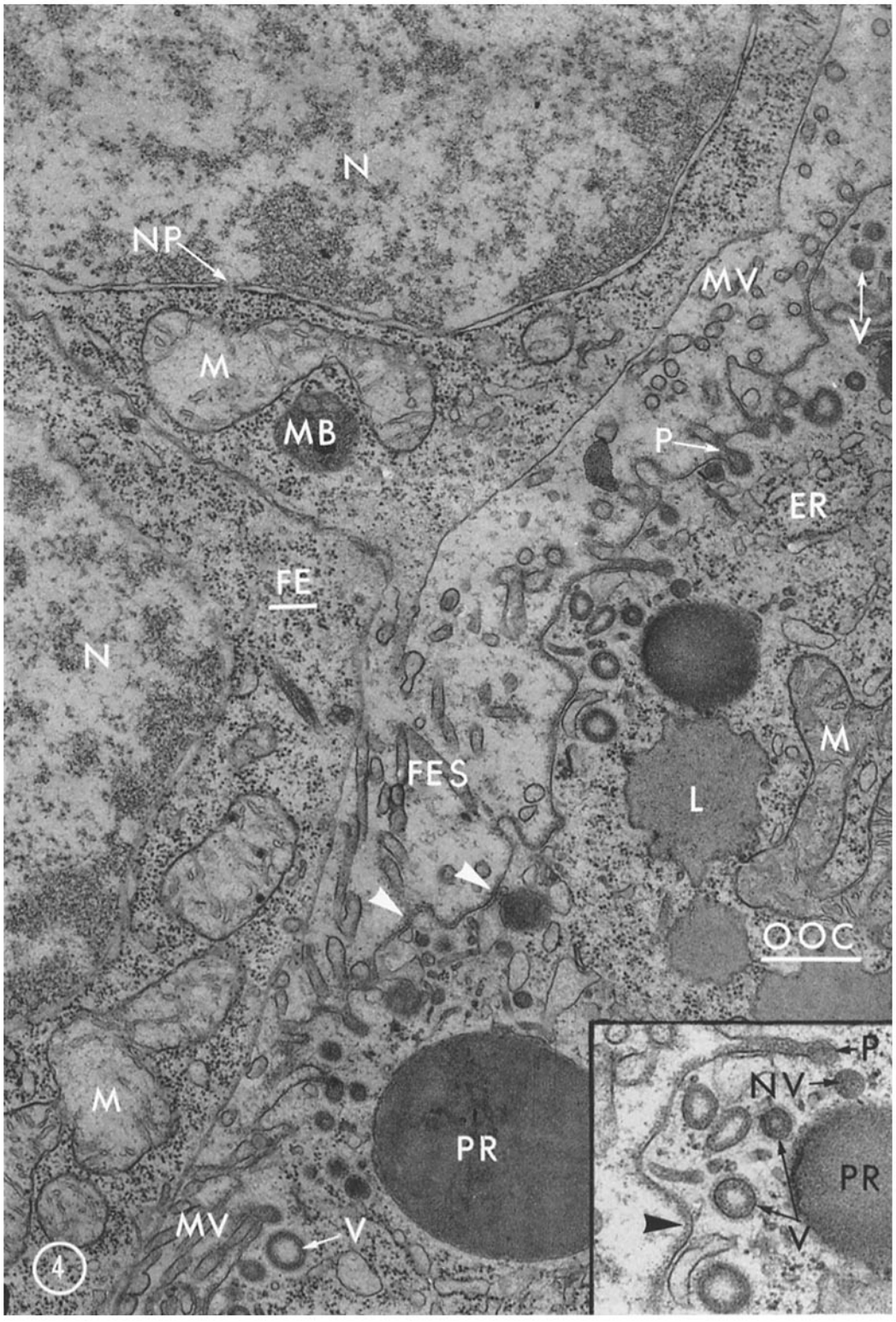
In other respects the pits seem to represent simply a coated vesicle connected with the surface of the oocyte by a neck. The neck, which is usually about 60 mμ in diameter, varies greatly in length. It may be as long as 150 mμ or essentially nonexistent. In the latter case, the pit is a shallow depression in the oocyte surface, identified, however, as a pit of this type by the characteristic coat on the cytoplasmic side. Apparently these variants represent stages in the development of deep pits from shallow ones and the eventual pinching off of the deepest part of the pit to form a coated vesicle. Ordinarily the bristle coating is not present on the neck of the pit but only over its deepest part, the diameter of which exceeds that of the neck.

The individual cortical vesicles in the cortical zone of a maturing oocyte are no longer of uniform size as they were prior to the blood meal. They display, in fact, a broad spectrum of sizes crowded into the region immediately beneath the membrane and slightly deeper in the cytoplasm (Figs. 3, 4, and 6). Close to the surface the majority are 140 to 150 mμ in diameter and still have the bristle coating. Farther in, there are larger vesicular units with a content of the same density as that of the coated vesicles, but without the characteristic coating. And deeper still, there are vesicles whose content is similar to that of both pits and yolk granules (Fig. 6). Apparently through a fusion of these various units the larger proteid bodies of the mature oocyte gradually develop (Figs. 6, 7, and 8). The yolk material, which first appears as a layer adsorbed onto the oocyte surface, is therefore thought to be taken into the cell *via* the coated pit and vesicle, possibly changed in character as the vesicles coalesce, and finally incorporated into yolk granules.

It should be noted that profiles of flattened

FIGURE 4 Micrograph of the interface (*FES*) between the oocyte (*OOC*) and the follicular epithelium (*FE*) 7 hours after a blood meal. A few mitochondria (*M*) and a multivesicular body or lysosome (*MB*) are among the organelles in this region of the follicular epithelial cytoplasm. The nuclei (*N*) show the usual nuclear envelope with pores (*NP*). Between the microvilli (*MV*), which arise from the oocyte surface, are frequent pits (*P*) filled with a dense content. Pit-derived vesicles (*V*), lipid (*L*), mitochondria (*M*), elements of the endoplasmic reticulum (*ER*), and graded stages in proteid droplet (*PR*) formation crowd the oocyte cortex. A uniform layer of dense material (arrows) adheres to the oocyte plasma membrane and is similar in density and texture to that found in the pits, vesicles, and proteid droplets. × 34,500.

The inset at the lower right shows at greater enlargement a portion of the oocyte cortex. The materials contained in the pit (*P*), bristle-coated vesicles (*V*), naked vesicle (*NV*), and proteid droplet (*PR*) all have the same density and granularity as that on the oocyte surface (arrow). Where a vesicle membrane is cut in vertical section, it has the same dimensions as the plasma membrane. × 50,000.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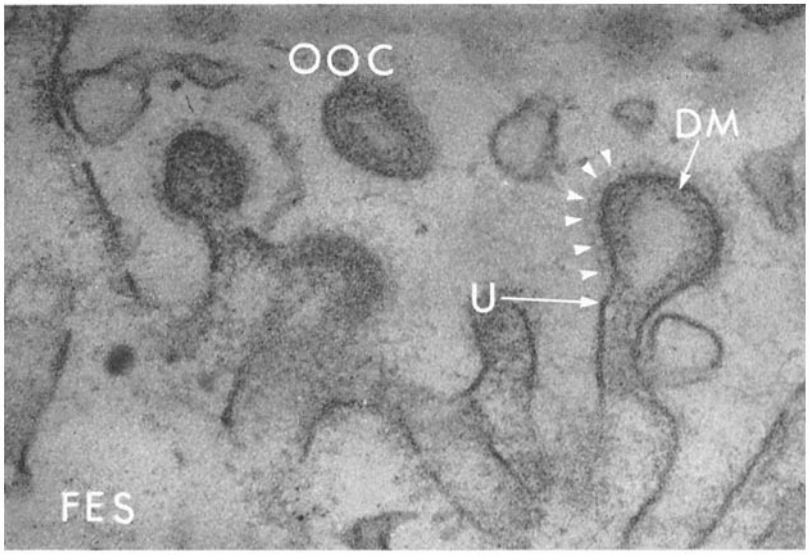



FIGURE 5 Several pits are shown in greater detail. A uniform layer of dense material (*DM*) adheres to the 75 A unit membrane (*U*) on the free surface of the pit, while on the cytoplasmic side of the membrane the 200 A bristles (arrows) radiate into the oocyte cortex. The unit membrane has the same thickness and 3-layered structure as that limiting the oocyte. The bristles, in some images, appear joined near their outer ends. *OOC*, oocyte; *FES*, interface between oocyte and follicular epithelium. × 106,000.

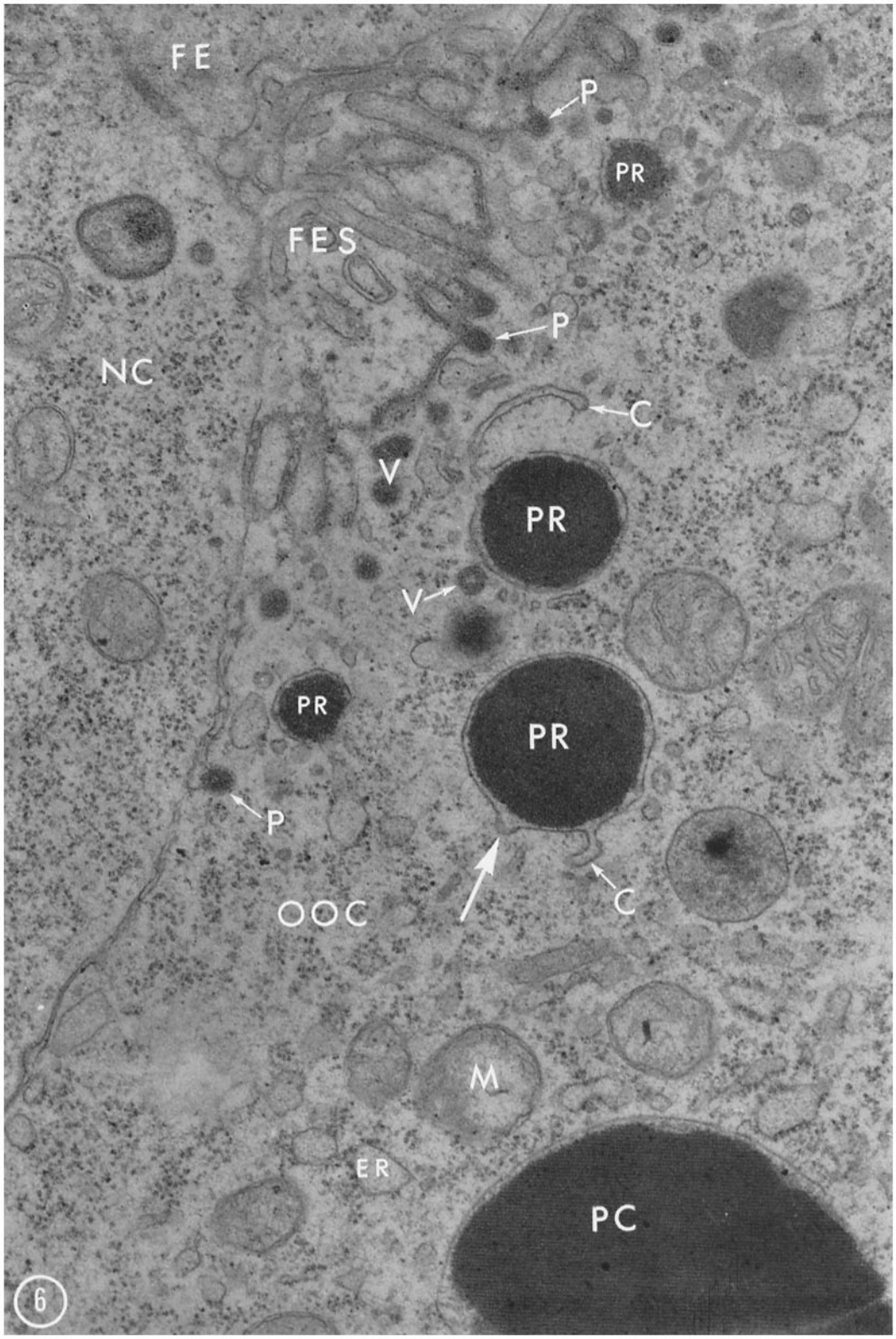
vesicles often appear attached to the limiting membrane of the larger proteid droplets and show open continuity with the content of the droplet (Fig. 6). According to one interpretation, these may represent the remnant of a recently emptied "excoated" vesicle that has fused with a droplet. They might equally well represent small elements of the endoplasmic reticulum or Golgi system possibly contributing some material, maybe an enzyme, to the droplet.

The larger, dense bodies present in the egg cytoplasm, the yolk granules, display a crystalline pattern in their content, and this may exhibit various orientations in a single plane of section (Figs. 6 and 8). This shift in orientation presumably arises from the fusion of several smaller units which had adopted crystalline order before fusion. These large, highly ordered, dense bodies give positive protein and PAS histochemical tests, and thus are considered to be identical with the protein yolk granules observed by the light microscopists (32, 33).

### *Site of Yolk Synthesis*

The implication from the above observations and the literature references on yolk synthesis is that these cortical pits take up material from the extracellular space of the follicle and contribute it to yolk granule formation. The question of immediate interest is where in the insect are the

FIGURE 6 At the juncture shown here between the follicular epithelium (*FE*), nurse cells (*NC*) and the oocyte (*OOC*), microvilli extend into the widening follicular epithelial space (*FES*) of the maturing ovary. Pits (*P*) along the oocyte cell membrane are filled with a dense material similar to that in the developing proteid droplets (*PR*). In some cases out pocketings appear on the membrane of the droplet (arrow) which are interpreted as vesicles (*V*) that have just emptied their contents into the membrane-limited space (also see Fig. 7). Small cisternae (*C*) are also continuous with the membrane of the droplet. These may represent the membrane remnant of large vesicles that have recently coalesced with the droplet. The content of the yolk protein body assumes a crystalline-like configuration when it matures (*PC*). *ER*, endoplasmic reticulum; *M*, mitochondria. × 41,500.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yolk proteins etc., synthesized. To obtain some clues to this, we made a survey of the cells in the mosquito's abdomen, paying special attention to the occurrence of systems at the fine structure level usually associated with protein synthesis for export.

The cells of the Malpighian tubules, for example, do not possess the ergastoplasmic form of the ER, and show little evidence of an enlarged Golgi component or of secretory granules. Cells of the fat body do show rough ER in substantial amounts, but no secretory granules. From the survey, only the cells of the midgut epithelium emerged as likely candidates for the role of synthesizing yolk protein.

Two types of cells are found in the midgut of the mosquito, one a regenerative and the other an absorptive type (Fig. 9). The apical pole of the latter is covered with closely packed microvilli, each about 140 mμ in diameter and of undetermined length. These have, in turn, upon them a number of tiny microvilli (micromicrovilli) about 200 A in diameter, designed presumably to increase the extent of the cell surface. In the subcortical zone, under the free surface of these cells, numerous mitochondria are crowded into a layer 2 to 4 μ deep which blends into a cytoplasm that is characterized by arrays of rough ER, free ribosomes, and extensive infoldings from the basal and lateral surfaces of the cell. The nucleus, at this postfeeding phase, contains a prominent nucleolus. The prominent development of rough-surfaced ER, and the numerous Golgi profiles with some evidence of secretory granules, encourage the thought that these cells, beside being absorptive, are active in synthesis and secretion. In their fine structure they remind one of vertebrate liver cells.

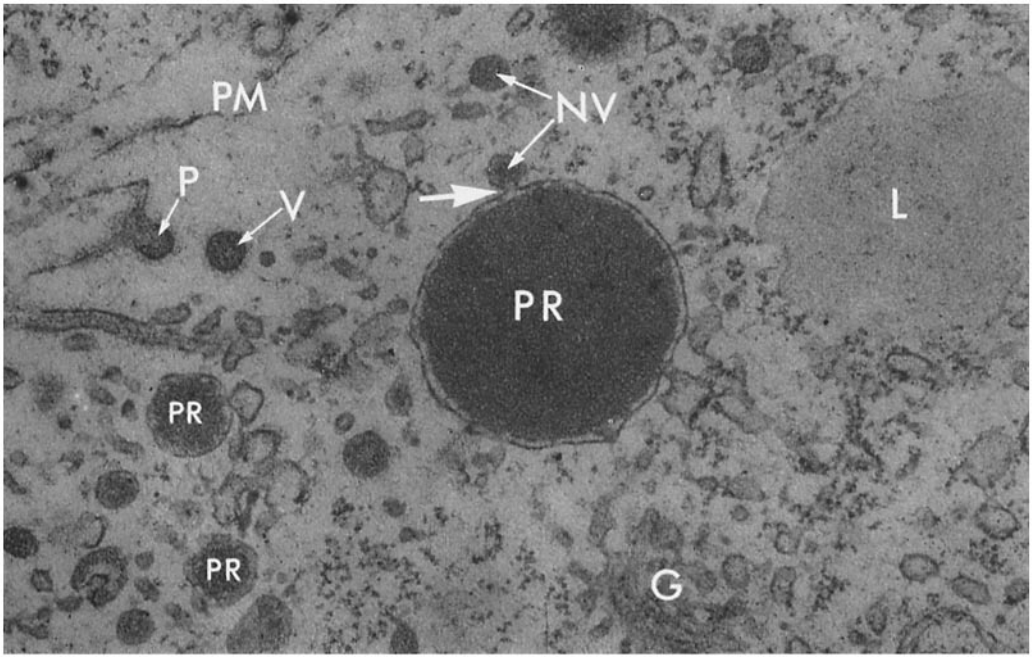



FIGURE 7 This micrograph of the cortex of the oocyte shows what we interpret as a naked vesicle (*NV*) in the act of fusing (arrow) with the membrane of the yolk droplet (*PR*). Smaller irregularly shaped droplets (*PR*) and coated vesicles (*V*) are abundant in the area immediately beneath the pit-studded (P) plasma membrane (*PM*). Lipid bodies (*L*) and Golgi components (*G*) are other common inclusions of the egg cytoplasm at this period of yolk formation. × 56,500.

## AUTORADIOGRAPHIC EXPERIMENTS

In an attempt to determine the pathway by which the proteins, formed after a blood meal, enter the different tissues of the abdomen, the mosquitoes were fed on an anesthesized rat made

radioactive by an intraperitoneal injection of DL-leucine-$H^3$. Although the results are quite preliminary, and are now being repeated at the resolution offered by the electron microscope, they give some information of possible significance to this study.

In all samples, silver grains were located in high concentration over the blood meal in the midgut. Above the midgut epithelium, however, the number of silver grains increased from just above background at 0.5 hours after the blood meal to a plateau of about triple background at 4.5 hours, and then were maintained near that level in all samples taken later in yolk formation. In the ovary a similar rise in radioactivity occurred in the nurse cells and oocyte during the early 0.5 to 4.5 hours after a blood meal. Later, in the 16- and 30-hour samples, the number of silver grains increased notably above the protein yolk bodies.

The abdominal fat body does not appear to show a significant increase above background labeling until the 16- and 30-hour samples. The Malphighian tubules exhibit a pattern of labeling similar to that observed above the midgut cells.

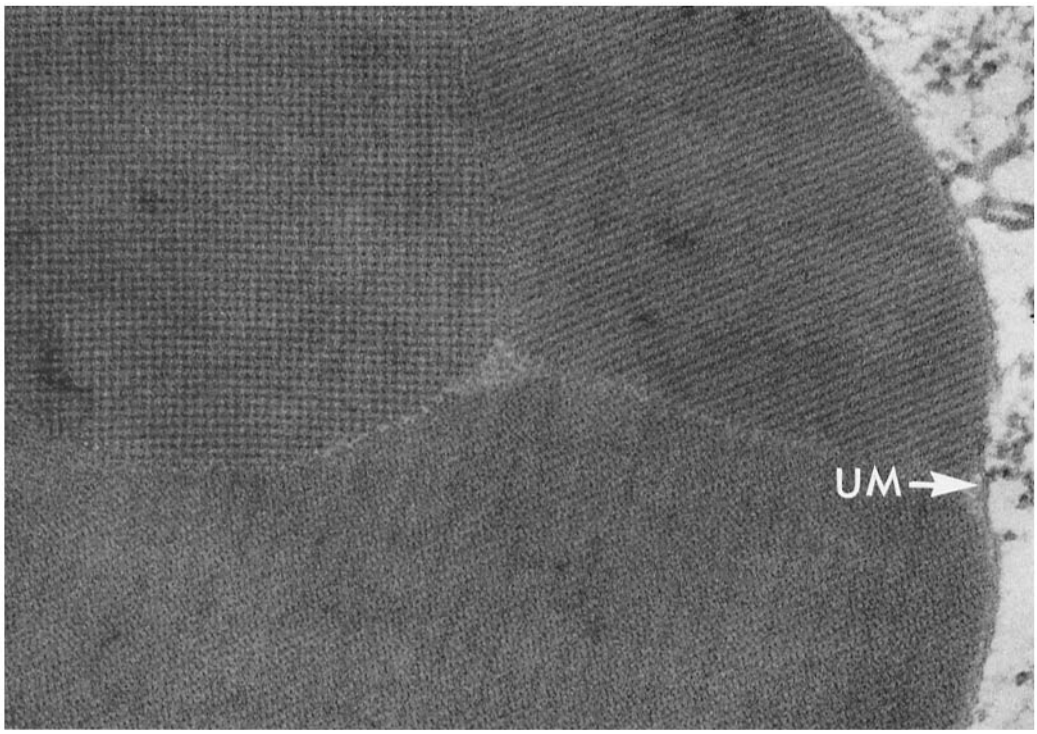



FIGURE 8 The different orientation of the crystal lattice planes in the small granules that fuse to form the yolk proteid body is shown in this micrograph. A unit membrane (*UM*) limits the droplets. × 108,000.

## DISCUSSION

The proteid droplets in *Aedes aegypti* constitute the major form of yolk protein in the egg (31, 32). By studying the formation of these bodies, we have sought to shed some light on the site of synthesis, transport, and method of uptake of yolk protein by the oocyte.

### *Sites of Yolk Synthesis*

The possible sites of synthesis of yolk protein in the mosquito ovary can be considered, on morphological grounds, to be restricted to three. One could be the follicular epithelial cells; their proximity to the oocyte, for example, suggests a possible involvement in the synthesis of material for oocyte growth and differentiation (Fig. 3). However, these cells contain almost none of the machinery usually associated with intracellular

synthesis of protein for secretion (35). They have little of the rough form of the endoplasmic reticulum, infrequent Golgi bodies, and, at this stage, no evidence of secretory granules or other secretory activity at the cell surface. Only after yolk deposition is essentially completed do the cells of this epithelium show any proliferation of the ER and Golgi complex. When this occurs, these cells are engaged in producing the chorion of the egg (31, 33) and most yolk deposition has already been completed.

The nurse cells and the oocyte itself are the other two possible sites of synthesis, but here, as in the follicular epithelium, the machinery (cytoplasmic inclusions and secretory granules) usually present for such purposes is absent during the short period of yolk deposition. The presence of great numbers of free ribosomes in the cytoplasm of both cells suggests active protein synthesis. This is, however, the pattern found in rapidly growing undifferentiated cells in which the components of the cytoplasmic matrix are being formed, and there is no associated mechanism for the transport and assembling of the protein into yolk droplets.

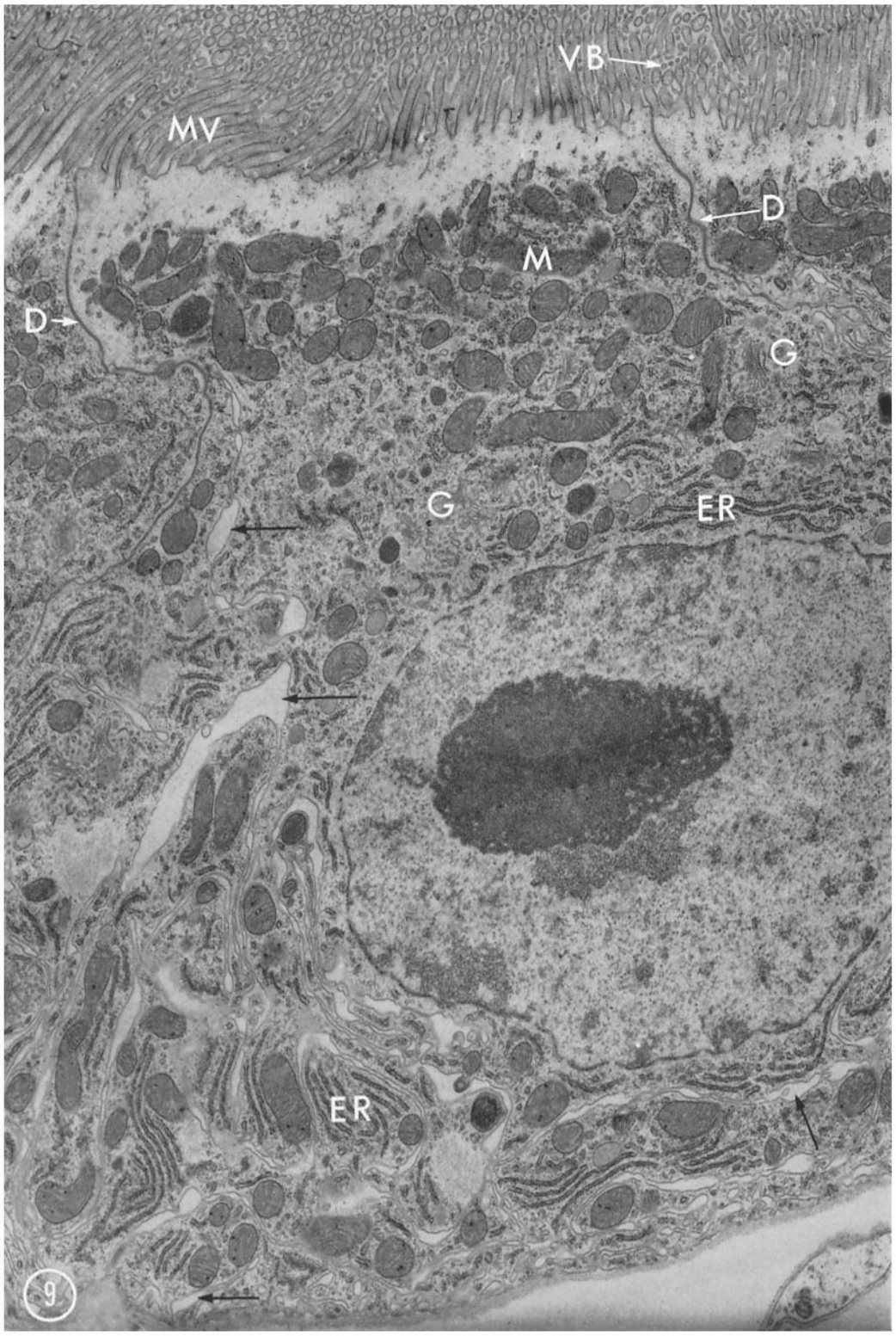
Therefore, unlike the crayfish oocyte in which the ER is clearly involved in the synthesis of yolk protein (3), or the frog oocyte (52) where the mitochondria are associated with yolk deposition, or the snail oocyte where Favard and Carasso (11) implicate both mitochondria and ER in yolk formation, the mosquito oocyte shows no obvious involvement in the manufacture of its own yolk.

This conclusion is in no way surprising. The uptake and deposition in the oocyte of yolk proteins from extraovarian sources appears to be the common pattern among the vertebrates. And even more pertinent here are the findings of Telfer that the same pattern is true for the insects he has studied. Particularly significant for the interpretation of our observations is Telfer's demonstration, by immunohistochemical studies on the saturniid moth, that certain foreign proteins are taken into the oocyte. He also demonstrated that naturally occurring proteins in the hemolymph are preferentially concentrated in the ovary, and that no yolk protein could be detected in the follicular epithelial cells, the nurse cells, or in the cytoplasm of the oocyte apart from the yolk droplets. He concludes justifiably that yolk proteins are synthesized at some extraovarian point in the insect and transported to the ovary for deposition. Though no similar studies have been made on the mosquito, it seems reasonable to expect that the same mechanisms would be operative in this form.

If the cells of the ovary are not involved, where does yolk synthesis take place? Thus far, as reported here, our studies of the tissues of the mosquito abdomen have identified only certain cells of the midgut as having the structural equipment needed for this function. This consists of ribosome-studded cisternae in parallel arrays and other elements of fine structure usually associated with secretion. These cells at their apical pole are in contact with the contents of the intestine following a blood meal, and on their other side are in a favorable position to secrete into the hemocoel. Furthermore, the autoradiographs in the experiments which included tritiated leucine in the blood, while preliminary, showed definite accumulation and concentration of label over these cells. It seems highly probable, therefore, that these units of the midgut are at least one site of yolk synthesis.

The only other candidates for this role are the

FIGURE 9 This micrograph shows the greater part of an epithelial cell from the midgut of the mosquito. The apical pole is covered with microvilli (*MV*) about 140 mμ in diameter, which appear here in both longitudinal and cross-section. Smaller microvilli (*VB*), *ca.* 20 mμ, bud off from the larger ones. In the subapical region, immediately beneath a relatively clear cortex, is a zone densely populated with mitochondria (*M*). At other levels in the cytoplasm, profiles of the endoplasmic reticulum (*ER*) dominate the picture. These profiles describe the system as being constructed in part of large lamellar cisternae, their surfaces heavily encrusted with ribosomes, typical for cells active in protein synthesis. Sections through the Golgi complex (*G*) are scattered about in the supranuclear zone. Prominent also are extensive infoldings of the plasma membrane along the lateral surfaces of the cell, and most especially the basal surface. These infoldings reach in some instances almost to the apical pole of the cell and show irregular dilations (arrows). *D* marks the separation between adjacent cells. × approximately 21,600.


VB
MV
D
M
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G
G
ER
ER
9

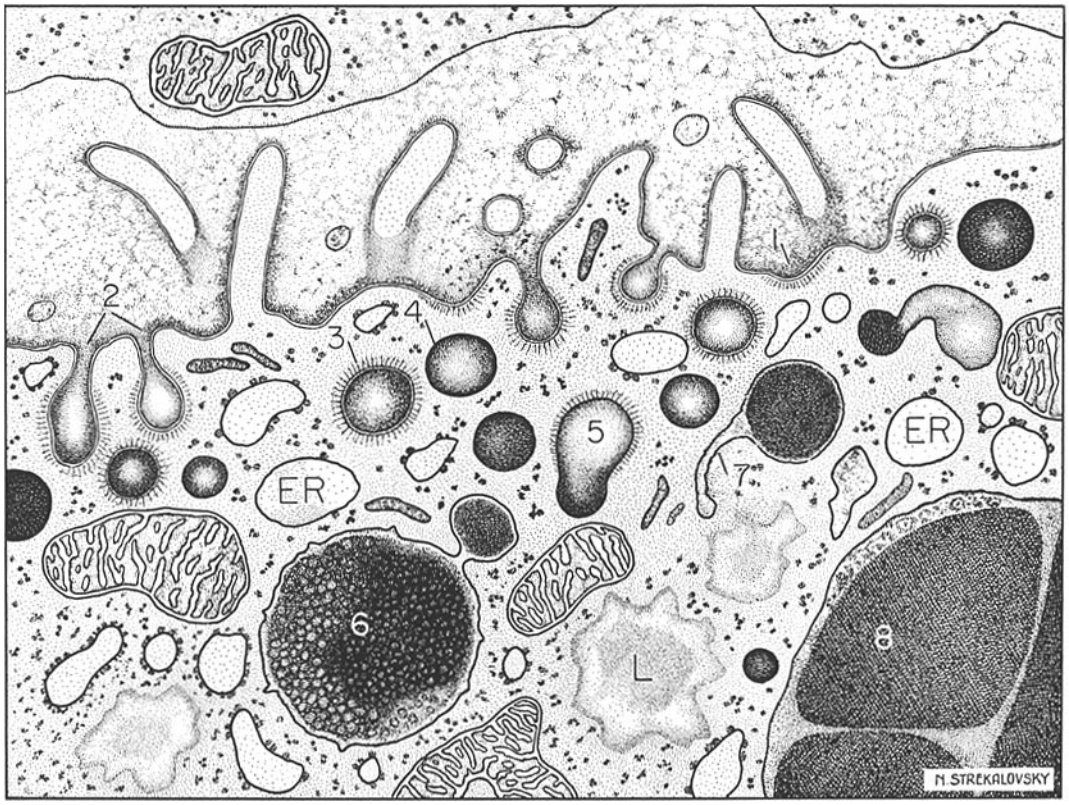


FIGURE 10 This schematic drawing interprets the micrographs examined in this study. It depicts changes and events involving the cortical pits and coated vesicles of the mosquito oocyte. At (*1*) is shown the first stage of invagination into the oocyte of the protein-coated plasma membrane from the intercellular space. The fully developed pit (*2*), by pinching off, forms the coated vesicle (*3*). These vesicles lose their bristles to form dense spheres of similar size (*4*) which then fuse with other dense spheres (*5*). Often a flattened empty sac is attached to the droplet (*7*). This sac may be the membrane remnant of a vesicle or perhaps some element of the Golgi complex that has recently fused with the droplet. The larger droplets (*6*) coalesce to form the large crystalline proteid yolk bodies (*8*) of the oocyte. Other conspicuous and characteristic inclusions and organelles of the oocyte cytoplasm are mitochondria, vesicles of the rough surfaced endoplasmic reticulum (*ER*), lipid (*L*) and ribosomes. At the top of the drawing, microvilli project into the intercellular space fronting on the follicular epithelium. Note the absence of adhering material on the membranes of the follicular epithelial cells (also see Fig. 4). × approximately 60,000.

cells of the fat body which likewise show rich developments of the rough ER. Here, however, the accumulation of label following the feeding of tritiated leucine is delayed until late in yolk deposition.

The suggestion that the midgut is the principal site of protein synthesis for yolk formation is at variance with a long standing bias among insect physiologists toward the fat body for this role. It is well established that most insects contain protein stored in droplets in the fat body, and as proteins or amino acids are needed this source is mobilized. Our autoradiographic results may be due to the absence of protein in the fat bodies of the 1-week-old mosquitoes we used, and the resultant absence of this potential protein source during oogenesis.

The mechanisms of yolk transport to and uptake by the ovariole, and subsequent concentration in the oocyte, have been considered only briefly by previous investigators except Telfer. In a studious examination of the question, he implicates the oocyte surface and the basement membrane in the selective uptake. The surface

of the oocyte, he speculates, might possess a mechanism for selective absorption akin to that demonstrated for the surface of the ameba (5). Uptake and transport might be achieved by a process similar to pinocytosis.

The observations derived from the present study bear mostly on these questions and, in general, support Telfer's speculations.

The spaces between the follicular epithelial cells begin to widen by 4 hours after a blood meal, and by 7 hours have opened sufficiently to allow unobstructed communication from the basement layer to the oocyte surface by this intercellular route (Fig. 3). Before yolk proteins can enter this newly formed space they must pass through the basement layer enclosing the ovariole. Although not all investigators agree that a basement layer is permeable to whole proteins, there is good evidence that certain foreign and maternal proteins present in the blood can penetrate this layer in certain tissues (10, 24, 28, 47, 49).

Again, with respect to the present study, Telfer's findings are especially significant, for they demonstrate that proteins in the hemocoel do traverse the basement layer and are present in the interfollicular space just within. Some device probably exists capable of holding the yolk proteins inside the basement membrane and continuing their inward diffusion. The micrographic evidence bearing on this question describes a coarsely granular material on the inside of the basement membrane, and no resolvable material outside. Furthermore, it suggests that once inside, the protein is complexed into relatively large aggregates which are too large to diffuse out through the membrane. Thus the conditions required for the continuing inward diffusion of the smaller molecular species are maintained with the basement membrane functioning as part of a diffusion pump. What serves to aggregate the proteins into larger complexes is not known, but a factor or factors synthesized in either the follicular epithelial cells or the oocyte could be secreted into the extracellular space to perform the task.

An equally valid possibility is that the serum proteins coprecipitate in the interfollicular space and thus fulfill the same requirement for inward diffusion. In this connection, it is worth noting that Schjeide and Urist (43) have shown that two serum and yolk proteins of the chicken are very similar and that the two components of each, the $X_1$ phosphoprotein and the $X_2$ "glycolipoprotein," coprecipitate at a lowered pH and/or lowered ionic strength with the release of protein-bound calcium. If either or both of these conditions were to be met *in vivo*, coprecipitation would explain the aggregates encountered in the interfollicular space.

The same inward migration of yolk protein is doubtlessly influenced as well by the condensation of material on the oocyte surface and its incorporation into the egg. Just what it is, in or on the oocyte surface, that encourages the development of such a layer is likewise not known. That it represents yolk protein is indicated by the observation that this oocyte-associated material in the *Cecropia* moth, for example, is immunologically similar to the yolk protein within the oocyte and the serum proteins in the hemocoel (50).

### *The Uptake Mechanism*

It has been shown here that, in the region of the oocyte surface adjoining the follicular epithelial cells, numerous small depressions or pits extend into the egg cortex. Similar differentiations have been noted by Wartenberg (53) in the oocyte of several amphibians, but without attention to their characteristic fine structure. He interpreted these as engaged in pinocytosis associated with the uptake of materials for yolk formation. We find that the cortical pits number approximately 300,000 in the stage of oocyte development achieved 7 hours after feeding. This represents a 15-fold increase over the number of pits found in the resting stage preceding feeding. Their structure is uniform. Each pit contains a quantity of dense material having the same texture as that adsorbed on the oocyte surface at other points, which in turn seems related to the flocculent material in the follicular spaces. On their inner convex surfaces facing the cytoplasm, the pits are covered with a border of fine bristles about 200 A long.

Several significant reasons are apparent for associating these pits with yolk protein uptake and the formation of proteid droplets and granules of the maturing egg. The first one is the fact that uptake is in progress when the pit development reaches its most prominent expression. One could argue further that other mechanisms of uptake are not evident and that simple diffusion of yolk through the plasma membrane is highly improbable. Then, it is obvious that vesicles deeper in the cortex are related to the pits and are de-

rivatives of them. These vesicles possess the same content and the same border of bristles.

To this point the animation of this uptake mechanism is not hard to picture and is essentially that which characterizes pinocytosis. When the process of yolk absorption is at its peak, the formation of pits and their derivatives is probably quite rapid. The subsequent fate of the pit-vesicles is somewhat less clear. They apparently lose the bristle border, fuse to form larger vesicles, pick up other satellite vesicles, and progressively grow into "proteid droplets" several times greater in diameter than the pit-vesicles. This interpretation is depicted in Fig. 10.

The content of these larger dense yolk bodies also changes. From a beginning of homogenous density, there gradually emerges evidence of order in the arrangement of the particles, a crystallinity of structure common to yolk granules. This could depend on a process of selective hydrolysis within the vesicle, resulting finally in a high degree of purity in the residual yolk. Therefore, even though the pits and the oocyte surface may be selective, to a degree, in what they adsorb from the perioocyte lymph, subsequent purification is not unlikely.

The role of the bristle border in all of this is hard to imagine. It may have a mechanical function, giving, by virtue of a natural repulsion of the outer ends of the bristles, the spherical form to the base of the pit and the pit-vesicles. It is obvious from other observations, however, that cortical pits of this general character form and apparently separate from the surface without this apparatus (34). Perhaps this proposal has less merit than that which associates the specificity of materials adsorbed with characteristics of the bristle border. It is not possible at the moment to be other than vague in these suggestions.

Pits of similar structure have been reported in many cell types (1, 2, 39, 40, 46). Their occurrence is indeed widespread, as will be reported in a subsequent paper. They are not to be confused with the simpler pits found especially in smooth muscle cells and blood vascular endothelial cells (34, 53). The latter have simple, clean limiting membranes and a smaller diameter. At this time we present the working hypothesis that pits of the type found here in the mosquito are, in general, involved in selective uptake of materials (proteins and perhaps non-proteins) from the cell's environment. Since the mechanism evinces some selectivity (39), it is reasonable to propose that different cell types may manifest different specificities, with some cells, such as those of the reticuloendothelial system, showing a broader spectrum of interests than others.

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