

ELECTRON MICROSCOPE STUDIES ON THE ORIGIN OF ANNULATE LAMELLAE IN OOCYTES OF *NECTURUS*

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ABSTRACT

Developing oocytes, ranging from approximately 0.1 to 1.0 mm in diameter, in *Necturus* were studied with the electron microscope. The outer layer of the nuclear envelope is actively engaged in the formation of vesicular elements along most of its surface, especially in smaller oocytes. Groups of vesicles appear to be released into the ooplasm at about the same time, resulting in long chains of individual vesicles immediately adjacent to the nuclear membrane. This process is repeated so that chains of vesicles grouped in rather ordered ranks extend progressively into the surrounding cytoplasm. Eventually, the cytoplasm becomes more concentrated with chains of vesicles and the distance between the individual rows becomes less. Very soon after a chain of vesicles has been budded off from the nuclear membrane, fine intervesicular connections appear between certain of the vesicles comprising the rows. Several of the vesicles in a row may then fuse, forming short, flattened cisternae. Fusion of vesicles continues, individual rows of vesicles become more closely packed and, finally, regions appear in the cytoplasm which have the appearance of annulate lamellae. Further growth of the lamellae appears to occur by the progressive fusion of vesicles at the ends of those lamellae already present, as well as by the addition of other fusing rows of vesicles.

INTRODUCTION

In an electron microscope study on the eggs of several genera of sea urchins, Afzelius (1955) noted the presence of cytoplasmic membrane systems which appeared to be morphologically identical with the nuclear membrane. Similar structures were noted by Palade (1955) who used the term "fenestrated cisternae" to describe them. The term "annulate lamellae" was introduced by Swift (1956) to describe similar structures occurring in the ootestes of snails, the ovary of the clam, and the larval pancreas of *Ambystoma*. Rebhun (1956) described the presence of "periodic lamellae" in the oocytes of *Spisula* and in ootestes of *Otala*. These structures are similar to the an-

annulate lamellae of Swift (1956). Annulate lamellae have been described since in the cytoplasm of eggs of the sand dollar, *Dendraster* (Merriam, 1959). In the oocytes of the tunicate, *Boltenia* (Hsu, 1963), annulate lamellae are present in the nucleus as well as in the cytoplasm. In addition, the lamellae occur in rat spermatids (Palade, 1955; Swift, 1956), in spermatocytes of crayfish (Ruthmann, 1958; Kaye *et al.*, 1961), and in a variety of other cells (Okada and Waddington, 1959; Gross, Philpott, and Nass, 1960; Barer, Joseph, and Meek, 1960; Gay, 1955; Wischnitzer, 1960; Mahowald, 1962). Fenestrated membranes of annulate lamellae have been seen sporadically

in various cancer cells (Wessel and Bernhard, 1957; Schulz, 1957; Binggeli, 1959).

No direct evidence is available regarding the method by which annulate lamellae are formed. Because of similarities in structure between annulate lamellae and the nuclear membrane, it has been suggested that the former arise in some manner from the latter. However, the mechanisms postulated for their formation have varied (Afzelius, 1955; Swift, 1956; Rebhun, 1956; Merriam, 1959). Nor is the functional significance of these structures understood. They are seen in some instances to be continuous with rough-surfaced cisternae of the endoplasmic reticulum (Rebhun, 1961), and most, if not all, annulate lamellae are basophilic (Rebhun, 1956; Swift, 1956; Ruthmann, 1958). However, in some instances, small particles resembling ribosomes are associated with annulate lamellae (Afzelius, 1957; Merriam, 1959), while in other instances ribonucleoprotein particles have not been observed to be associated with them (Rebhun, 1956, 1961; Ruthmann, 1958).

The present study implicates the nuclear membrane of developing oocytes of *Necturus* in the role of actively forming numerous vesicles which subsequently take part in the differentiation and growth of annulate lamellae.

MATERIALS AND METHODS

The *Necturus maculosus* used in this study were obtained from the General Biological Supply Company, Chicago, during the months of January, February, March, June, and November. For electron microscope study, individual oocytes or small pieces of the

ovary were transferred directly to the fixative which consisted of a 1 per cent solution of osmium tetroxide buffered with acetate-Veronal (Palade, 1952) to a pH of 7.4 to 7.8. In some cases sucrose was added to the fixative, according to the method of Caulfield (1957). The tissue remained in the ice cold fixative for from 1 to 2 hours. After rapid dehydration in a series of cold ethanols and treatment with propylene oxide, the oocytes were embedded in Epon 812 (Luft, 1961). Thin sections were obtained on a Porter-Blum microtome using glass knives. Sections displaying silver or gold interference colors were mounted on Formvar-coated grids which had been lightly stabilized with carbon. The sections were then stained with a saturated aqueous or alcoholic solution or uranyl acetate or with lead hydroxide (Watson, 1958) and studied with an RCA EMU-3D electron microscope. Some of the sections were mounted on 400-mesh copper grids with no supporting film. Thick sections of the epoxy-embedded oocytes were stained with methylene blue and azure II (Richardson, Jarett, and Finke, 1960).

For light microscope study and cytochemical tests, portions of the ovary were fixed in Bouin's, Carnoy's, and Champy's solutions and 10 per cent neutral buffered formalin. The stains used included Heidenhain's iron hematoxylin, hematoxylin and eosin, and Mallory's. Tests were performed for protein (Mazia, Brewer, and Alfert, 1953) and nucleic acids (Korson, 1951). Tests for alkaline phosphatase (Gomori, 1941) were performed on frozen sections.

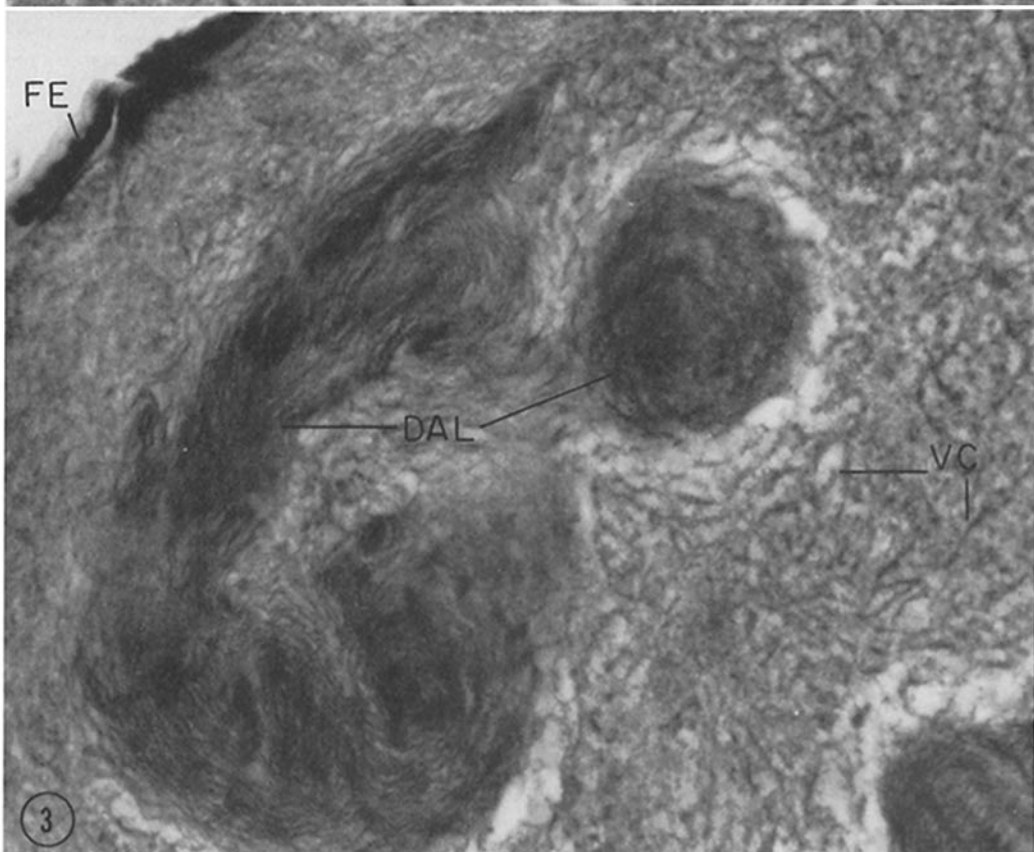
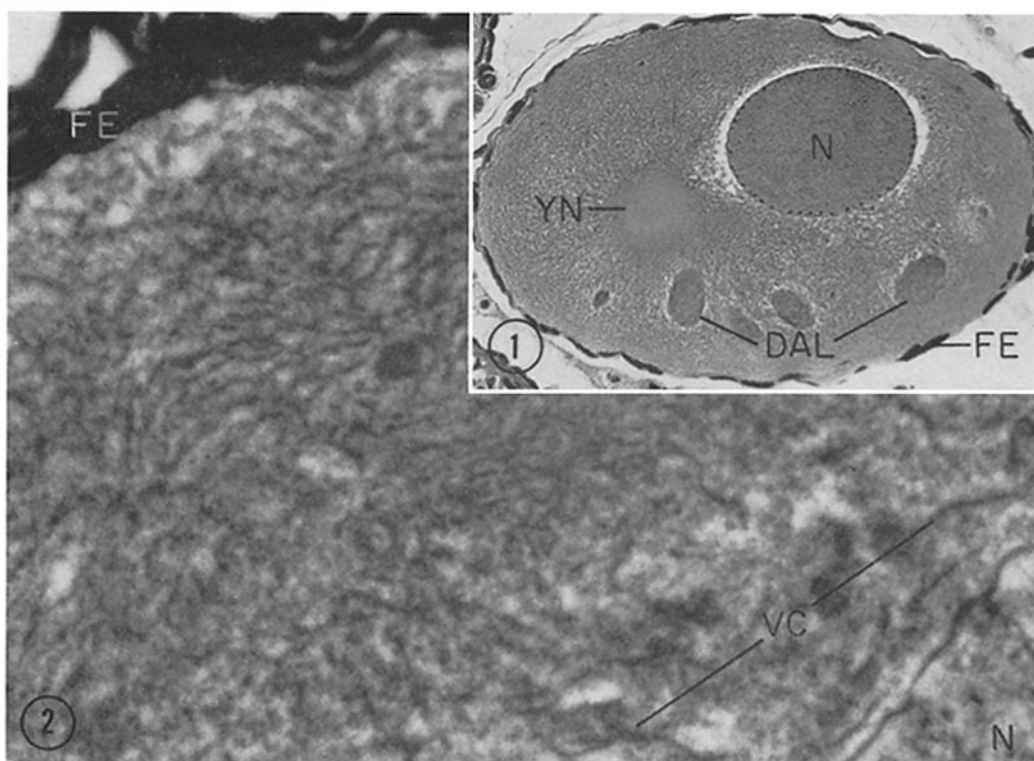
OBSERVATIONS

All observations reported herein were made on young, developing oocytes of *Necturus* ranging from approximately 0.1 to 1.0 mm in diameter. Thus, the observations were made prior to the appearance of the proteinaceous yolk bodies, but

FIGURE 1 Photomicrograph of a developing oocyte showing nucleus (*N*), yolk nucleus (*YN*), follicular envelope (*FE*), and several specialized cytoplasmic regions (*DAL*). Bouin's, H&E. $\times 125$.

FIGURE 2 Photomicrograph of an oocyte smaller than that shown in Fig. 1 and at high magnification. Note that the cytoplasm appears less differentiated and consists of numerous, long filamentous structures (*VC*) extending from the nucleus (*N*) to the plasma membrane and follicular envelope (*FE*). Electron micrographs show these structures to be composed of long rows of vesicles. Champy's, hematoxylin. $\times 2,000$.

FIGURE 3 Photomicrograph of a developing oocyte showing at higher magnification the differentiated regions of the cytoplasm (*DAL*) similar to those shown at lower magnification in Fig. 1. Note the lamellar nature of these areas which represent the forming annulate lamellae. Long filaments (*VC*) consisting of rows of vesicles are present in the cytoplasm adjacent to the lamellae and in some instances appear to enter them. Follicular envelope, (*FE*). Bouin's, hematoxylin. $\times 2000$.



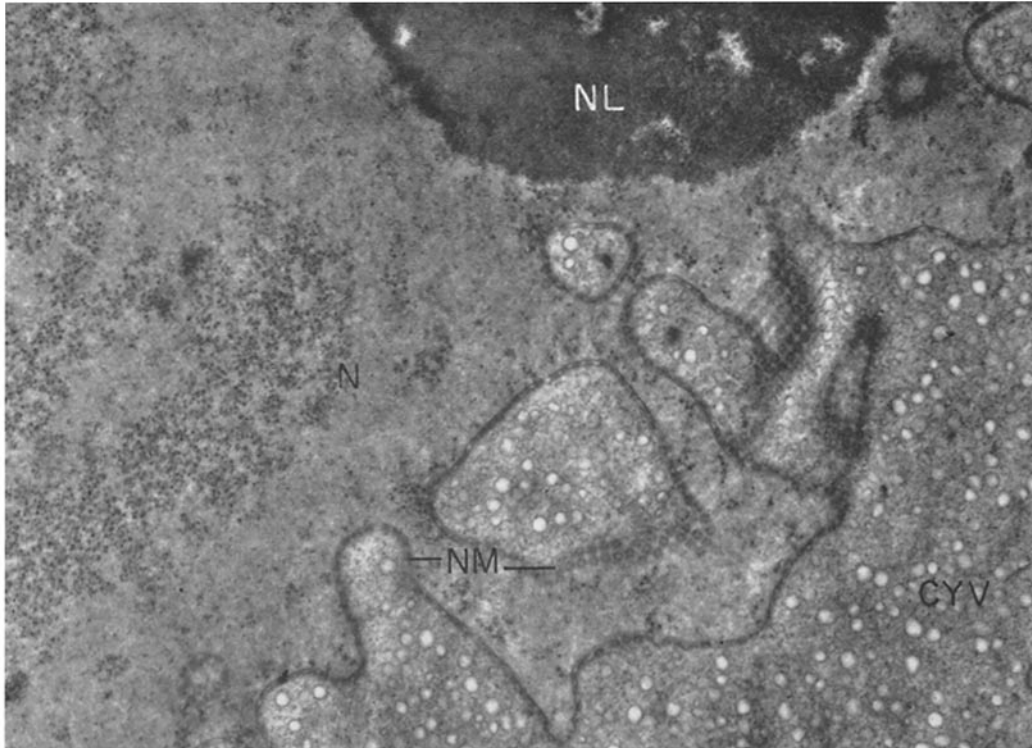


FIGURE 4 Electron micrograph of the nuclear-cytoplasmic region of a developing oocyte showing the nature of the nuclear membrane (NM) in tangential and transverse sections. Nucleolus (NL) in the nucleus (N). Note the numerous cytoplasmic vesicles (CYV) of rather uniform size located close to the outer nuclear membrane. $\times 14,000$.

not before considerable amounts of lipid had been formed in the cytoplasm of the oocytes.

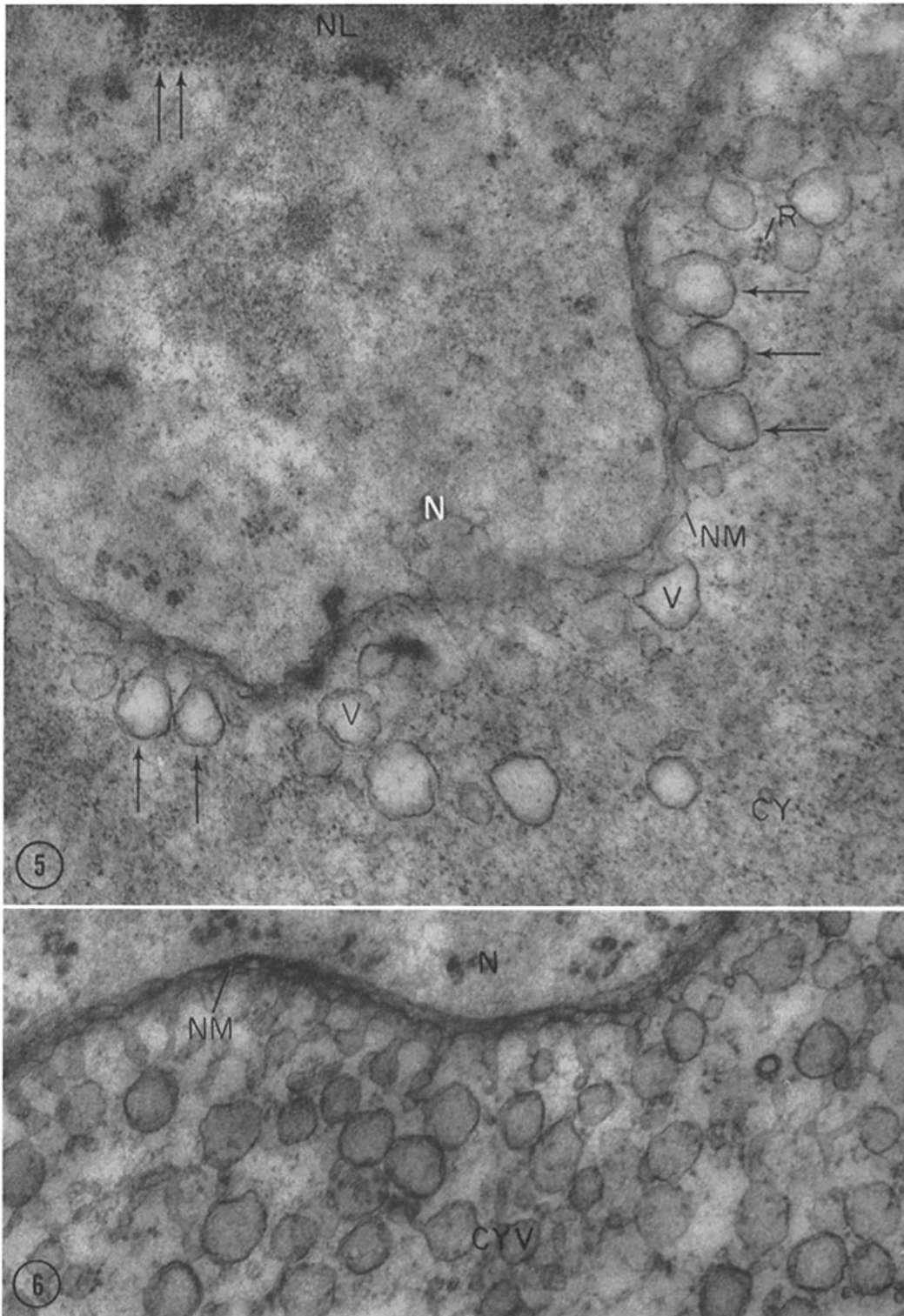
I. Light Microscope

Photomicrographs of portions of developing oocytes are represented in Figs. 1 to 3. In Fig. 1,

the nucleus (N) is present and multiple nucleoli are closely associated with the nuclear membrane. A portion of the yolk nucleus (YN) is demonstrated also as well as several differentiated cytoplasmic regions (DAL) which will be shown later to represent regions of differentiating annulate

FIGURE 5 Electron micrograph showing the extensive blebbing activity of the outer nuclear membrane observed in oocytes at stages of development studied. Nucleus (N). Spherical vesicles which are still attached to the outer nuclear membrane (NM) by a narrow stalk are apparent in the areas marked by the single arrows. Vesicles (V) which do not appear attached to the nuclear membrane at the section level are observed immediately adjacent to the nuclear membrane. The content of the vesicles is of low density. Individual particles (double arrows) comprising the nucleolus (NL) are similar in size to occasional groups of particles (R) in the cytoplasm (CY). The remainder of the cytoplasm (CY) is finely granular and in most cases these granules are smaller than those seen at R. $\times 53,000$.

FIGURE 6 Shows the extent to which blebbing of the outer nuclear membrane (NM) may occur. The blebs seem to form from the interannular regions all along the outer membrane. Nucleus (N), cytoplasmic vesicles (CYV). $\times 53,000$.



lamellae. Fig. 2 is a section of a smaller oocyte in which the ooplasm appears to contain numerous, long filamentous structures (*VC*) which are present in all regions of the cytoplasm. These structures can be seen more clearly in the oocyte shown in Fig. 3 (*VC*). Subsequent electron micrographs will reveal that these structures are long rows or chains of vesicles. In Fig. 3, it is possible also to note the lamellar nature of the regions differentiating into annulate lamellae (*DAL*). The follicular envelope is shown also (Figs. 1 to 3, *FE*). Cytochemical tests (Gomori, 1941) on frozen sections of oocytes of different sizes indicate a strong reaction for alkaline phosphatase in the follicular envelope, but none within the oocyte itself. The cytoplasm of the oocytes stains with mercuric bromphenol blue (Mazia *et al.*, 1953), indicating considerable amounts of protein. Cytochemical tests for ribonucleic acid (Korson, 1951) have proved somewhat variable, but generally the regions of differentiating annulate lamellae do not stain intensely. Further cytochemical studies on annulate lamellae are needed.

II. Electron Microscope

Fig. 4 shows a portion of the nuclear envelope in the oocyte of *Necturus*, in both tangential and transverse sections. The nuclear membrane is a double membrane interrupted at frequent and regular intervals by pores or annuli (Fig. 4, *NM*). The regularity in the arrangement of the annuli is evident. The structure of the nuclear envelope

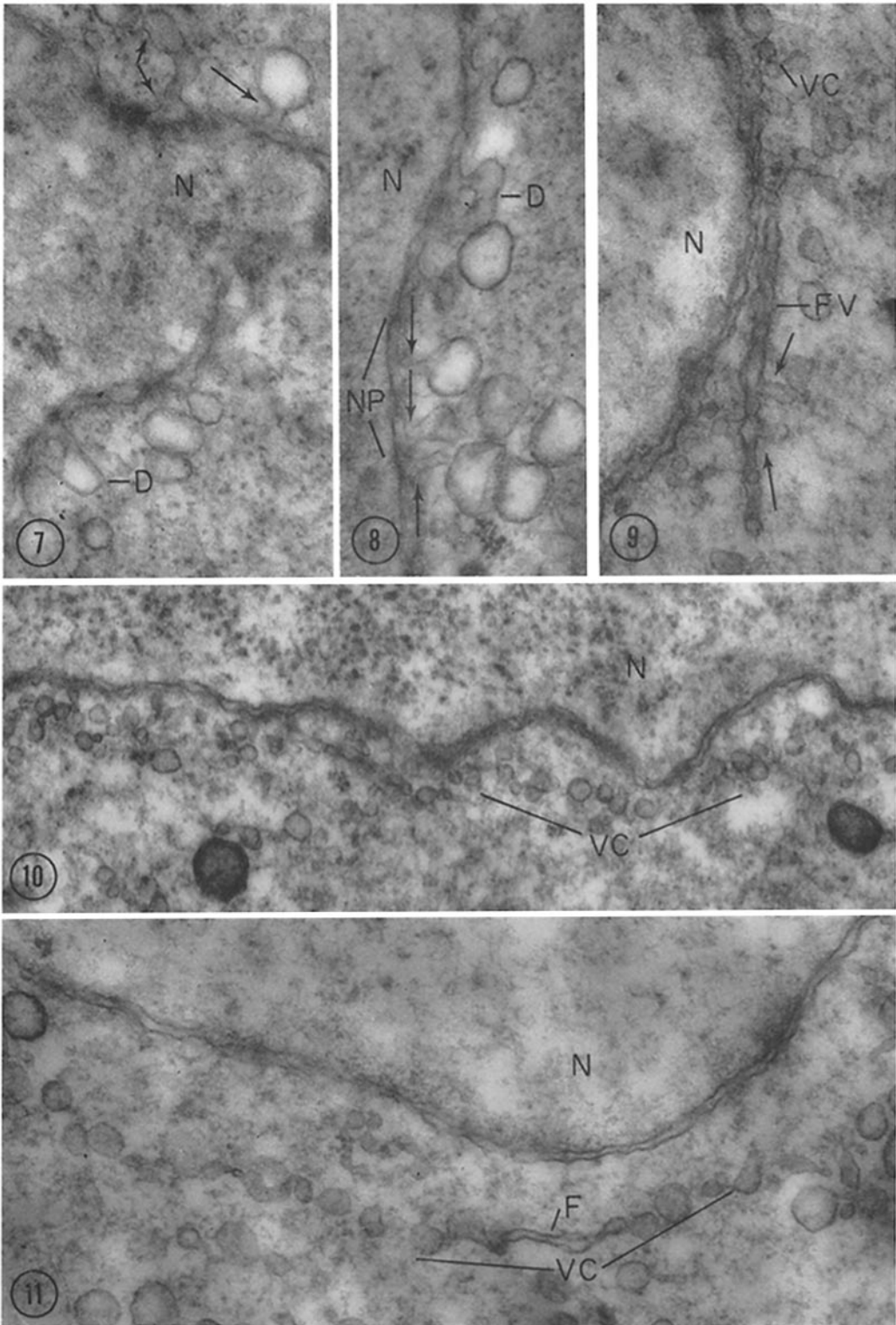
appears to be essentially similar to that described earlier for other amphibian oocytes (Wischitzer, 1958). The nucleolus is composed of many densely packed particles 100 to 200 Å in diameter. The nucleoli are numerous at this stage and are located close to the nuclear membrane. In some cases the nucleoli are extremely dense (Fig. 4, *NL*), while in others a nucleolonema arrangement is apparent (Figs. 23 and 24, *NL*). The particles comprising the nucleolus were observed to be more loosely packed on the periphery and especially in the region adjacent to the nuclear membrane. Groups of small particles present in the cytoplasm close to the nuclear membrane are similar in appearance to fragmented clusters of nucleolar particles in the nucleus (Fig. 5, *R*). This suggests that components of the nucleolus may pass into the cytoplasm through the nuclear membrane. However, in the stages examined in this study, few particles comparable in size to ribosomes, as described in other cells, were observed in the ooplasm.

In addition to abundant mitochondria, Golgi elements, and lipid particles, the cytoplasm of the young oocyte contains numerous membrane-limited vesicles of fairly uniform size (Fig. 4, *CYV*). The largest vesicles range from 150 to 200 m μ in diameter. The interior of the vesicles is homogeneous and of low density. No discrete structural elements ever were observed within the vesicles. In some cases, the content of the vesicles is slightly denser than the surrounding cytoplasm, which

FIGURES 7 AND 8 Show additional examples of blebbing of the outer nuclear membrane (arrows). Occasionally two adjacent blebs may fuse at their distal ends, forming a single, short cisterna connected by two stalks with the nuclear membrane (*D*). Annuli are visible in certain regions of the nuclear membrane (*NP*). Nucleus (*N*). $\times 53,000$.

FIGURES 9 AND 10 Groups of adjacent blebs appear to be released from the nuclear membrane at approximately the same time, resulting in the formation of a long row of vesicles (*VC*) located in linear array in the ooplasm immediately adjacent to the nucleus (*N*). The vesicles are quite close together and somewhat smaller than when they were still a part of the nuclear membrane. Figure 9 shows a situation in which several vesicles have fused very soon after being formed, producing a lamella (*FV*) similar to the adjacent nuclear membrane. In addition, this lamella has begun to form blebs of its own (arrows), away from the nuclear membrane. This condition is encountered infrequently. Fig. 9, $\times 53,000$; Fig. 10, $\times 32,000$.

FIGURE 11 Soon after a chain of vesicles is released into the cytoplasm some flattening of the vesicles occurs and fine intervesicular connections may be observed. Several adjacent vesicles in a chain (*VC*) may fuse (*F*) to form a cisterna of variable length. Nucleus (*N*). $\times 53,000$.



may be due to the plane of section through the vesicles or to some unknown factor (Fig. 6, *CYV*). That the cytoplasmic vesicles have their origin from the nuclear membrane is clearly shown in the micrographs. Spherical vesicles which are attached to the outer nuclear membrane by a narrow stalk are apparent in many of the micrographs (Figs. 5 to 8, arrows). Only the layer of the nuclear membrane adjacent to the cytoplasm ever was observed to take part in the blebbing activity. The formation of the vesicle begins as a small swelling or expansion of the outer nuclear membrane. The process continues, and eventually a narrow, finger-like projection of the membrane is formed. Subsequently, the most distal portion of the projection expands and the resulting structure is a tubular extension or stalk with a distal bleb (Fig. 5). Active blebbing apparently can occur along the entire surface of the interannular regions of the outer nuclear membrane. Furthermore, blebbing of the nuclear membrane was observed in oocytes of all stages examined but with greater frequency in smaller oocytes. The vesicles may be formed so close together that the distal portions of adjacent blebs often touch. In fact, a fusion of two adjacent blebs may occur at their distal ends, resulting in the formation of a single, short cisterna which is connected by two stalks with the nuclear membrane (Figs. 7 and 8, *D*). As the vesicles pinch off or become detached from the nuclear membrane, small stubs or expansions of the outer membrane remain.

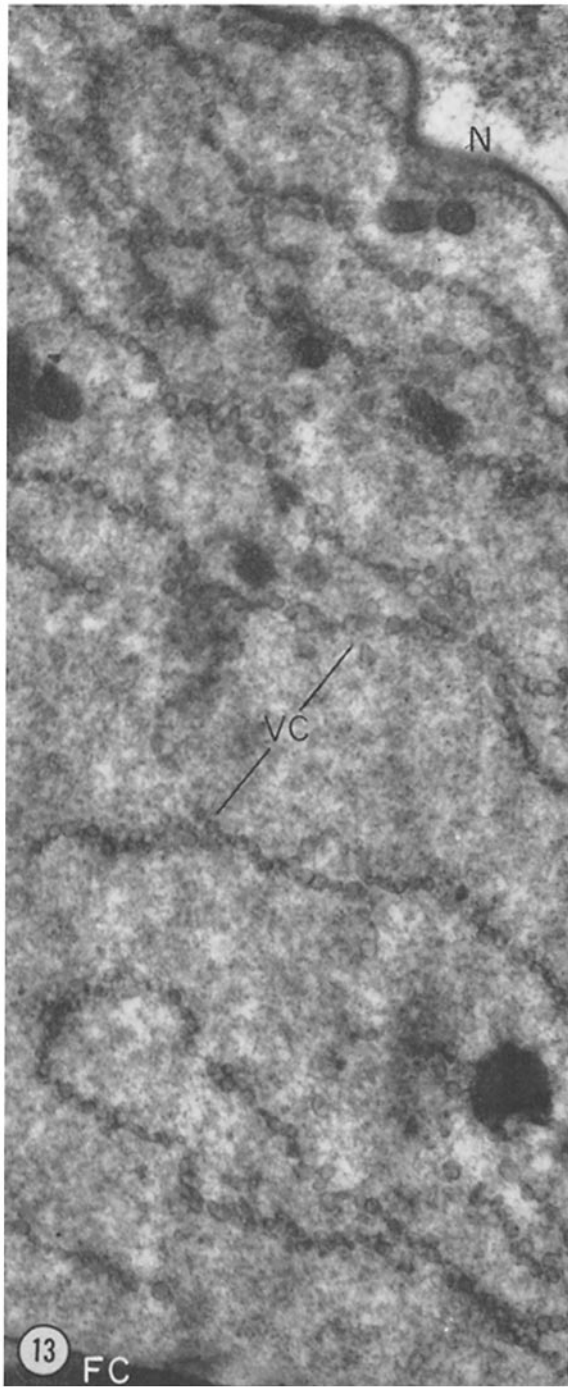
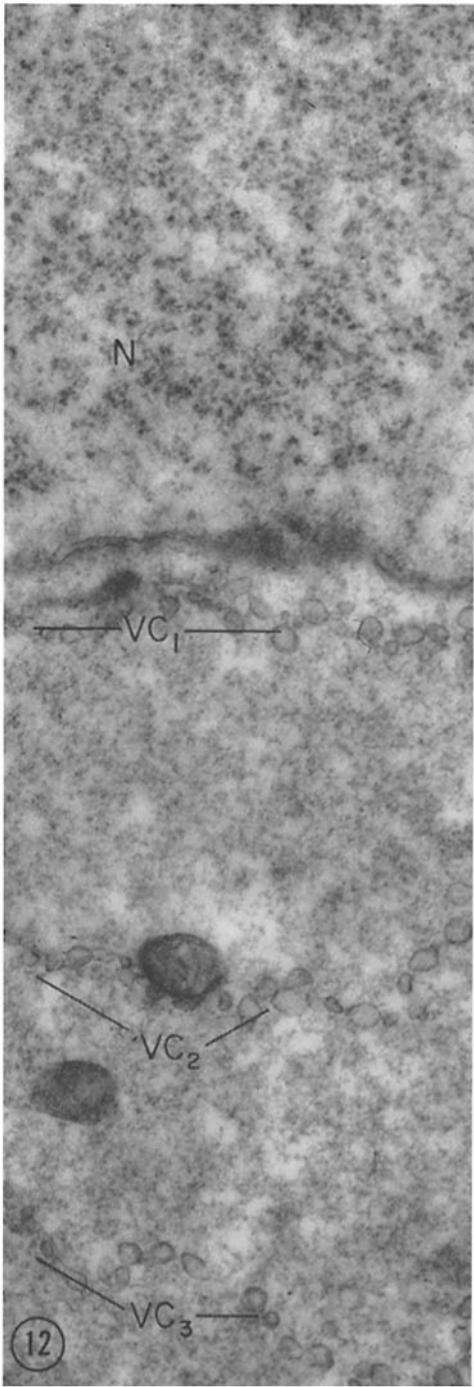
Groups of adjacent blebs appear to be released from the nuclear membrane at approximately the same time. This results in the formation of a long row of vesicles, no longer connected with the nuclear membrane, located in linear array in the ooplasm immediately adjacent to the nucleus (Fig. 10, *VC*). The vesicles comprising the vesicular chains are usually close together and appear some-

what smaller than when they were still a part of the nuclear membrane. Some flattening of the vesicles occurs, and thin intervesicular connections may be observed in some cases (Fig. 11, *VC*). Occasionally, two or more vesicles may fuse completely to form a short cisterna (Fig. 11, *F*). The time and extent of the fusion of vesicles are generally variable and may differ from region to region of the cytoplasm. In Fig. 9 (*VC*), a considerable amount of fusion has already occurred in a portion of the vesicular chain (*FV*) located close to the nucleus. In addition, this portion appears to be in the process of forming its own vesicles by a blebbing process (Fig. 9, arrows). This condition, however, appears to be extremely rare since it has been observed only once. The groups of vesicles arranged in rows migrate through the ooplasm to more peripheral regions (Fig. 12, *VC*₁, *VC*₂, *VC*₃). Much of the ground cytoplasm between the chains of vesicles appears finely granular (Fig. 14, *CY*). The individual vesicles comprising a chain appear to move through the ooplasm at rather uniform speeds, judging by the regularity of the rows (Fig. 12, *VC*). The ordered migration of chains of vesicles throughout the cytoplasm may be due to individual affinities between the vesicles. In addition, the regularity of movements may be due in part to the fact that some vesicles in a chain may be interconnected. However, this condition is a variable one and in some chains few intervesicular connections or cisternae are visible.

The formation and migration of chains of vesicles in the ooplasm continues so that eventually the rows extend to the plasma membrane. Such a condition is shown in Fig. 13 (*VC*) which displays an oocyte approximately 0.25 mm in diameter. As the oocyte increases in size, the distance between chains of vesicles becomes variable in different regions of the ooplasm (Fig. 14). For

FIGURE 12 Electron micrograph of young oocyte showing portions of three linear arrays of vesicles (*VC*₁, *VC*₂, *VC*₃). One chain of vesicles is located close to the nuclear membrane and has formed most recently. Note the distance between the rows of vesicles and the nature of the finely granular ooplasm between the chains. Nucleus (*N*). All vesicles within a row appear to move at approximately similar speeds through the ooplasm to more peripheral regions. $\times 15,000$.

FIGURE 13 Electron micrograph of a young oocyte approximately 0.25 mm in diameter. At this stage the cytoplasm is filled with long rows of vesicles (*VC*) extending from the nucleus (*N*) to the plasma membrane and follicular epithelium (*FC*). $\times 32,000$.



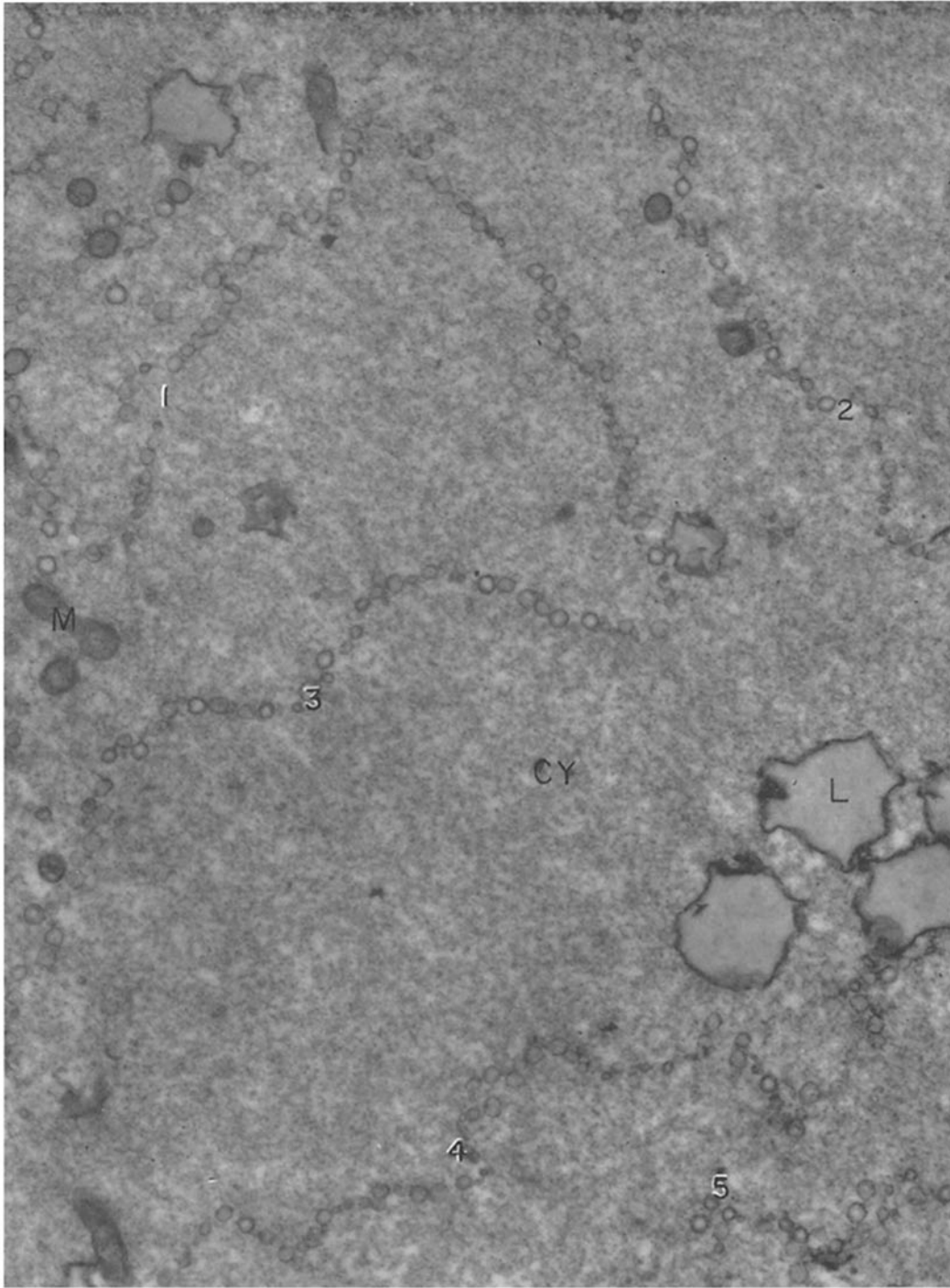


FIGURE 14 A section of the same oocyte as that shown in Fig. 12, but more peripherally located. Portions of five (1 to 5) distinct vesicular chains are present. Granular cytoplasm, (CY); mitochondria, (M); lipid (L). $\times 15,000$.

example, in certain regions, the rows of vesicles may be separated by short distances (Fig. 15, *CVC*). The number of vesicles constituting a given chain is variable, and it is impossible to determine their number with accuracy because of the thickness of the sections. However, over a hundred have been counted in certain of the individual rows.

After a considerable number of vesicular chains have been formed and as the chains move closer together, they tend to become organized into a circular or oval configuration (Fig. 15, *CVC*) with the vesicles arranged in concentric layers. Several such areas may be present in a single oocyte (Fig. 1, *DAL*), and it is possible to observe several areas showing fusion of adjacent vesicles (Fig. 15, arrows). The differentiation of annulate lamellae occurs within the oval packet of concentric rows of vesicles. This transformation does not occur instantaneously but is a progressive activity. Further, there appears to be no regional preference with regard to the first activities of differentiation. Initial stages in the formation of annulate lamellae by the progressive fusion of rows of vesicles are shown in Fig. 16 (*AL*).

Although some difficulty was encountered in accurately measuring the diameter of oocytes, the beginning of differentiation of annulate lamellae was first observed in oocytes approximately 0.5 mm in diameter. These events can be observed also in oocytes ranging up to 1 mm in size which represent approximately the largest oocytes examined. The first step in the differentiation of the annulate lamellae involves the progressive fusion of vesicles in a row. Examples of this are shown in Fig. 17 (*FVC*), 18 (*FV*), and 22 (*FV*). As continuity is established between adjacent vesicles, they become smaller and the point of fusion is typically narrower than the other portions of the vesicle. A row of vesicles with only a few intervesicular connections is outlined in Fig. 17 (line). This unit will subsequently transform into annulate lamellae similar to those shown in the same figure. Additional examples are present in other micrographs (Fig. 16, *VC*). As the vesicles of a row begin to fuse, short cisternae are formed which become progressively longer as other vesicles participate in the fusion process. Very shortly thereafter, annuli are observed in the lamellae and a matrix of moderate density becomes associated with the annuli (Fig. 18, *A*). Because of the rapidity with which events occur at this point, it has been impossible to determine either the precise sequence

of this activity or exactly what processes are responsible for the formation of annuli as the vesicles fuse. Possibly, the point at which individual vesicles fuse might play some role in determining the position of an annulus as well as the formation of the diaphragm associated with it. Evidence for this from the micrographs is by no means conclusive, however.

An individual annulate lamella consists of two parallel membranes which contain a series of pores or annuli. It is in this respect that their structure is identical with that of the nuclear envelope. It is also in this respect that the lamellae differ structurally from other elements of the endoplasmic reticulum. The pores in both the nuclear membrane and the lamellae are conspicuous in both transverse sections and sections cut parallel to the membranes (Figs. 8, 22, and 23). In transverse sections, it can be observed that the paired membranes of each lamella join with each other at intervals to form the pores (Fig. 22, *A*). In terms of three-dimensional structure, the merger of the paired membranes represents the inner walls of the pore. Traversing the pore in many instances is a thin membrane or diaphragm exactly like that associated with the annuli of the nuclear envelope (Figs. 8, *NP*; 18, 22, *A*). The diaphragm is not evident in every annulus and its presence or absence may depend on the thickness of the section as well as the portion of the annulus present in the section. Whether or not the diaphragm is universally present and traverses the entire pore, however, is questionable. The possibility also exists that the structure referred to as the annular diaphragm might only represent a special view of the pore due to the plane of section and, therefore, not a discrete structural component of the annulus. An amorphous material of moderate density is associated with both the annulate lamellae and the nuclear pores. The homogeneous material is observed with equal density both within the annular space and outside the annular walls (Figs. 22 and 23, *A*).

In surface view, each of the pores in the annulate lamellae is seen to be surrounded by an annulus (Fig. 23, *A*). The inside diameter of the annulus is approximately 600 Å. This diameter is essentially the same as that of annuli in the nuclear membrane. In instances where several annuli are shown in surface view, the outer wall of an annulus is separated from its adjacent annulus by a distance of approximately 300 m μ . Occasionally, a small

dense granule approximately 100 to 150 A in diameter is located in the central region of an annulus as has been observed, for example, in the annuli of the egg of *Dendraster* (Merriam, 1959). The function of the structure is not known, but it may represent some configuration associated with the annular diaphragm.

In patches of differentiating annulate lamellae, it is quite common to observe interconnections between adjacent annulate lamellae (Figs. 17 and 19, *C*). In addition, lateral projections of an annulate lamella are encountered frequently (Fig. 17, arrows). These projections involve the interannular portion of the lamellae. Quite often the projection consists of a stalk with a distal bleb or vesicle (Fig. 19, arrow). This could suggest that once the annulate lamellae are differentiated, they then acquire the capacity to form additional vesicles by a blebbing process, in order to produce additional raw material for the production of annulate lamellae. However, when this condition is observed, a chain of vesicles is usually located adjacent to an annulate lamella which is forming the projections. Thus, individual vesicles may be connected with adjacent annulate lamellae by a narrow stalk (Fig. 18, arrows). This condition suggests that a vesicle in another row may fuse with an adjoining annulate lamella as well as in an opposite plane with adjacent vesicles comprising a portion of its own row. This activity then results in the formation of cross-chains connecting individual lamellae. Such activity may also play a role in the formation of branched annulate lamellae which are occasionally observed (Fig. 24, *BAL*).

In sections cut parallel to the membranes of the annulate lamellae, structures in addition to the annuli are often observed. These are in the form of smaller rings about half the size of the annuli (Fig. 20, arrows). They are arranged with considerable regularity and lie adjacent to the inter-

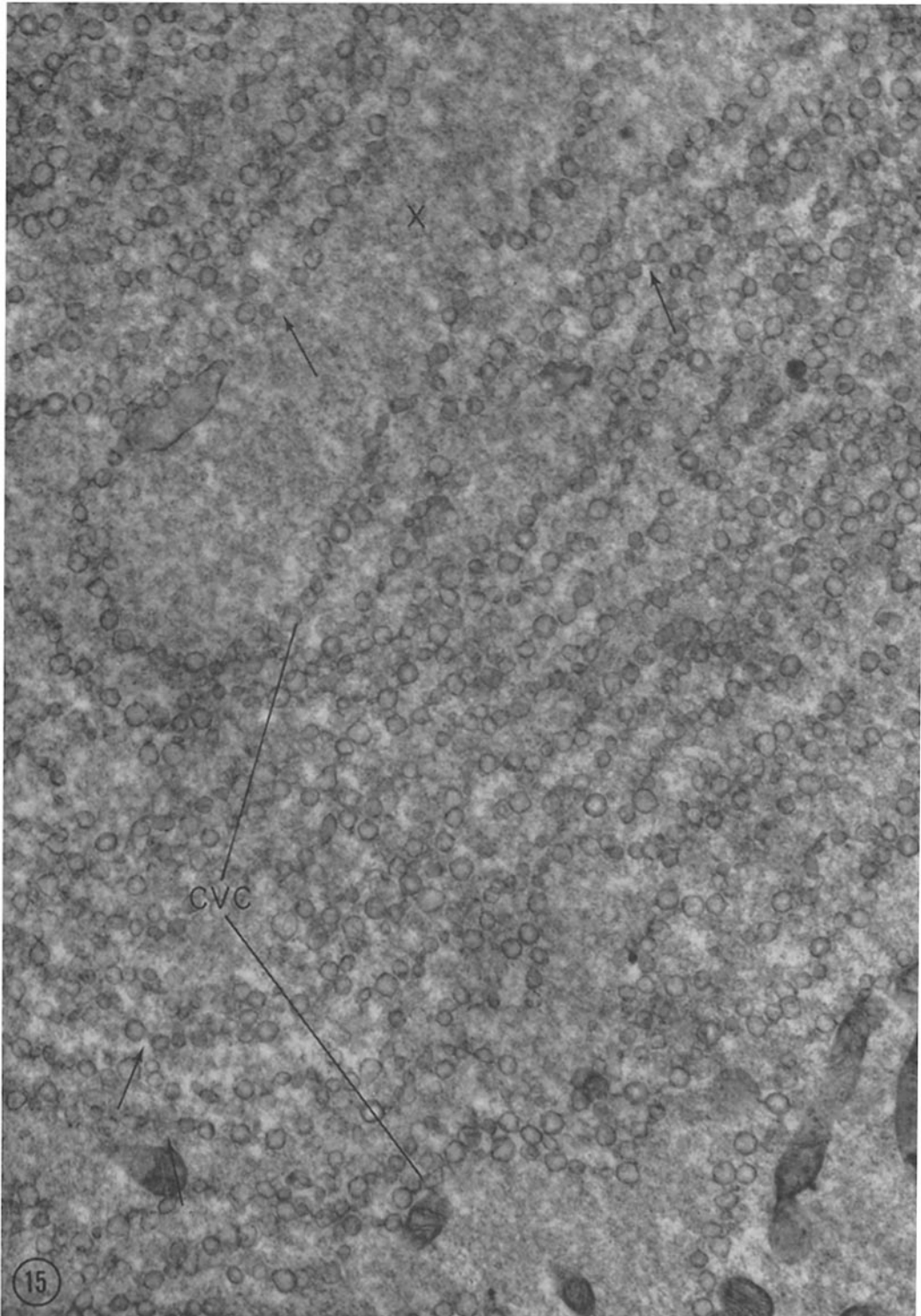
annular region of a lamella. It is believed that these structures represent transverse sections through the lateral stalks, which connect the lamellae with vesicles of adjacent rows, or through connections between individual lamellae.

While it is quite common for the annulate lamellae to differentiate from chains of vesicles which have become arranged into concentric rows of vesicles, this condition does not appear to be a necessary preliminary. If annulate lamellae do differentiate in this way, there are usually several major patches of lamellae produced in a single oocyte and these are usually located in the intermediate or even peripheral regions of the oocyte cytoplasm (Fig. 1, *DAL*). However, it is also possible for annulate lamellae to differentiate from long, straight chains of vesicles which have become closely applied to each other. Such an example is shown in Figs. 23 and 24 (*A*, *AL*); and, in this instance, the annulate lamellae are usually located in the perinuclear region of the ooplasm. The annulate lamellae continue to increase in length and number with time. This occurs by (a) the progressive fusion of vesicles at the ends of differentiated annulate lamellae (Fig. 21, arrows) and (b) the transformation of additional rows of vesicles into annulate lamellae by fusion (Figs. 17, *FVC*; 18, *FV*).

Because, in part, of the density characteristics of the annulus, little internal structure can be discerned. There is some suggestion that small units, often appearing as vesicles approximately 100 to 200 A in diameter, are embedded in the dense matrix of the annular wall (Fig. 20, *A*), similar to those described by Merriam (1959) for annulate lamellae in the eggs of *Dendraster*.

Little doubt can be held about the sequence of events postulated here for the formation of annulate lamellae in *Necturus*. It is unlikely for several reasons, that the process could take place just as well in a reverse sequence. The blebbing of the

FIGURE 15 A later stage in the differentiation of vesicular chains in the oocyte. After a considerable number of rows of vesicles have been formed through continued activity of the outer nuclear membrane, they move closer together and in many instances become organized into an oval configuration consisting of concentric layers of vesicles (*CVC*). A portion of one of these is shown with the approximate center of the structure located at (*X*). Several such regions may be present in a single oocyte. Intervesicular connections are present in the chains, and some fusion has occurred between adjacent vesicles (arrows). The degree of fusion of vesicles at this stage is quite variable, and in other cases more fusion has occurred than that shown in this figure. $\times 20,500$.



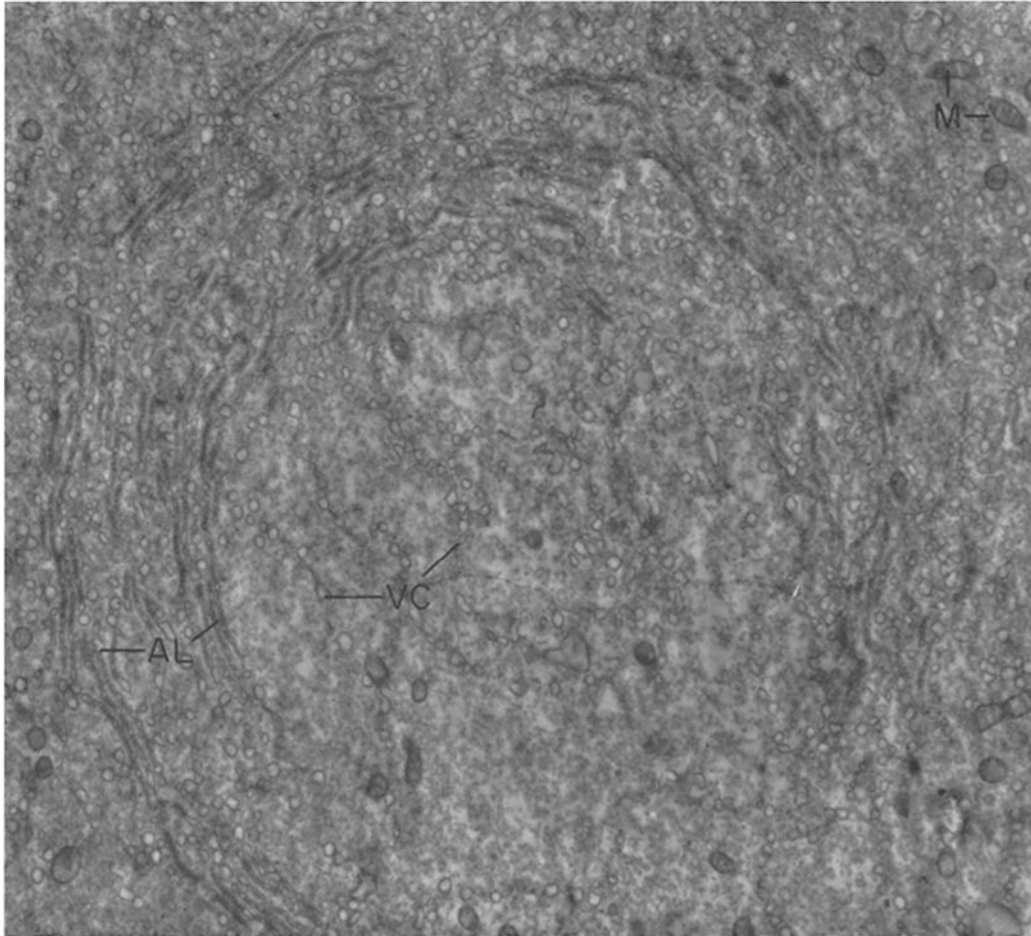


FIGURE 16 Stage showing the beginning differentiation of annulate lamellae. After the numerous rows of vesicles have become organized into an oval packet, differentiation of the annulate lamellae from these begins. Areas already exhibiting the structure of annulate lamellae (*AL*) are observed, while numerous chains of vesicles (*VC*) are also present which will eventually become transferred into lamellae. As the process continues, the entire region will become a mass of annulate lamellae similar to the area shown in the photomicrographs (see Figs. 1 and 3, *DAL*). Mitochondrion (*M*). $\times 10,000$.

outer nuclear membrane occurs for a considerable time before any annulate lamellae are present in the oocyte. Likewise, numerous rows of vesicles are also present in the oocyte cytoplasm before any annulate lamellae are observed. It has been possible to obtain information as to the presence or absence of annulate lamellae by examining serial sections of several oocytes in the electron microscope as well as in the light microscope.

Particles comparable in size and density to ribosomes never were observed directly associated with the annulate lamellae in the stages covered

by this study. However, similar particles were seen either singly or in clusters in the general region of the differentiating annulate lamellae (Fig. 17). The formation of intranuclear blebs or annulate lamellae within the nucleus, as has been shown in other oocytes (Hsu, 1963; Merriam, 1959), was not observed in *Necturus*. The presence of "heavy bodies" such as those described in other oocytes (Afzelius, 1957; Merriam, 1959; Gross, Philpott, and Nass, 1960) was not found in stages of oocytes of *Necturus* examined in this study.

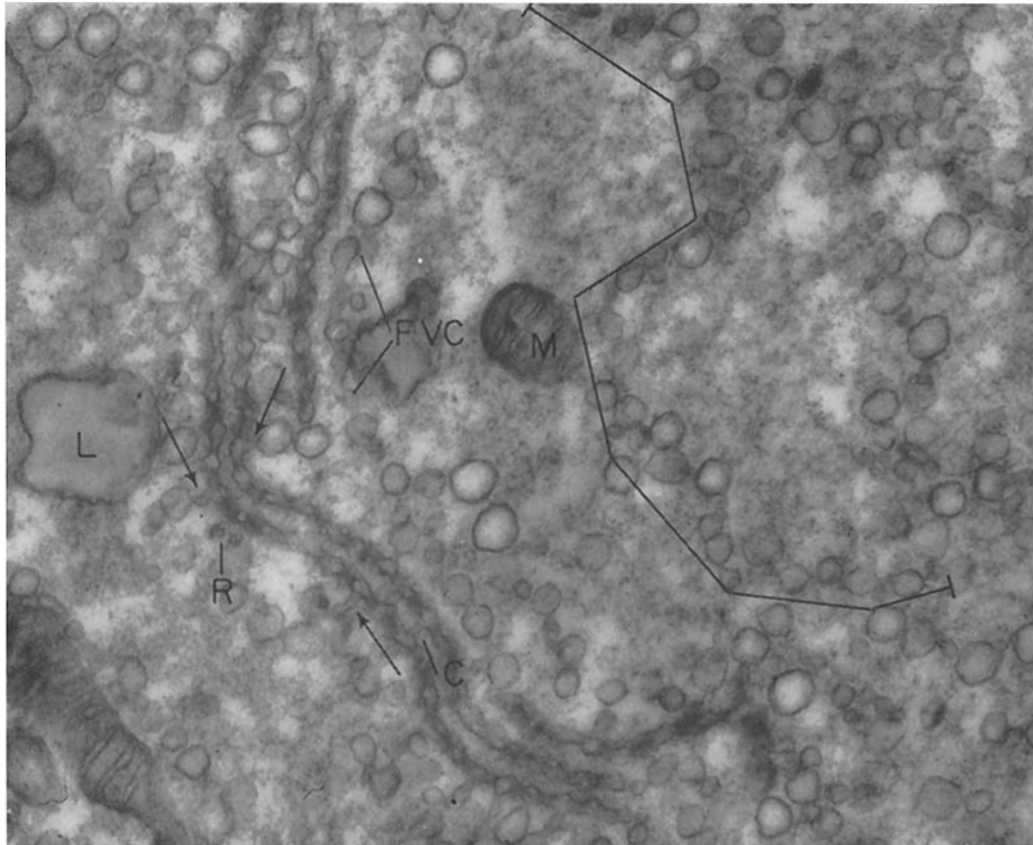


FIGURE 17 Portions of three differentiated annulate lamellae. Intercommunications between lamellae are shown (*C*). Connections are also observed between annulate lamellae and individual vesicles of adjacent rows (unlabeled rows). A row of vesicles in the process of fusing to form a lamella is indicated at (*FVC*). A chain of individual vesicles which is destined to undergo transformation into annulate lamellae is outlined. Lipid (*L*). Mitochondria (*M*). Ribosomes (*R*). $\times 32,000$.

DISCUSSION

Subsequent to the electron microscopic descriptions of the nuclear membrane and associated annuli by Callan and Tomlin (1950), Gall (1954), Afzelius (1955), and Watson (1955), numerous observations have implicated the nuclear membrane in a variety of dynamic cell processes. The pores or annuli in the nuclear membrane appear in some cases to be sites of nuclear-cytoplasmic exchange (Anderson and Beams, 1956; André and Rouiller, 1957; Pollister *et al.*, 1954; Wischnitzer, 1958; and others). That a continuity may exist between the nuclear membrane and membranes of the endoplasmic reticulum was early shown by Watson (1955), and has been observed since by

others in a variety of cell types. Evidence has also been presented that the endoplasmic reticulum may form the nuclear membrane at the end of mitosis (Amano and Tanake, 1957; Barer, Joseph, and Meek, 1959). Formation of mitochondria at the nuclear membrane has also been suggested in the work of Hoffman and Grigg (1958) and Brandt and Pappas (1959). Ribosomes have been observed attached to all regions of the nuclear membrane, and the possibility that "membrane flow" may carry material from the nucleus to the cytoplasm has also been suggested (Bennett, 1956). The possibility that annulate lamellae also may form by a delamination of the nuclear membrane or some other process has already been mentioned (Swift, 1956; Rebhun, 1956; Merriam, 1959).

In addition, a variety of blebs or outpocketings of the nuclear envelope have been observed, and their role in the transfer of nuclear material to the cytoplasm has been stressed (Hadek and Swift, 1962). Sometimes only the outer layer of the nuclear membrane is involved (Watson, 1955; Swift, Rebhun, Rasch, and Woodward, 1956; Swift, 1958; Clark, 1960), or both layers as in *Drosophila* (Gay, 1955, 1956).

While blebbing phenomena involving the outer nuclear membrane have been observed previously in other cells, none of the cells have demonstrated the frequency of blebbing that occurs in oocytes of *Necturus*. Hsu (1962) found several blebs of the outer nuclear membrane in oocytes of the tunicate (*Boltenia*) and believed that, as the bleb was set free in the cytoplasm, the membrane of the bleb seemed to "disintegrate into strings of ribosomes." The evidence for this seems somewhat inconclusive, however. Wischnitzer (1963) also noted a blebbing of the outer nuclear membrane in oocytes of *Rana*, but, since vesicle formation was only occasionally observed, it was felt that it occurred at irregular intervals in response to some transient physiological condition of unknown significance. Thus, the significance of structural variations of the nuclear membrane, in terms of actual cell function, is still largely obscure.

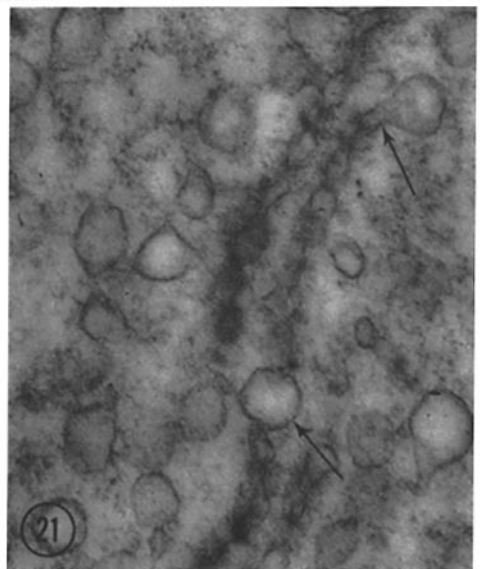
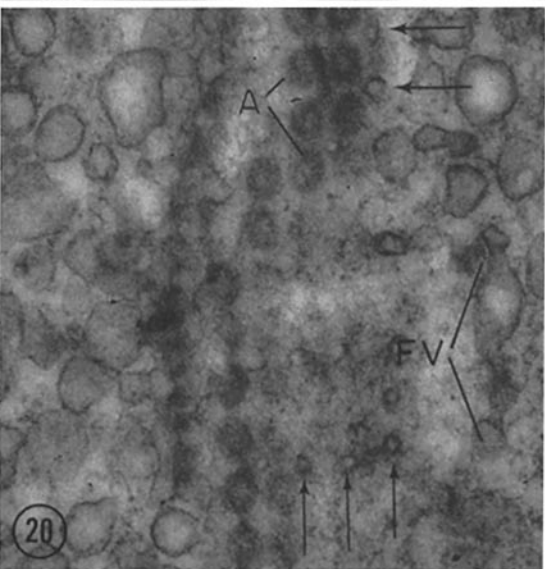
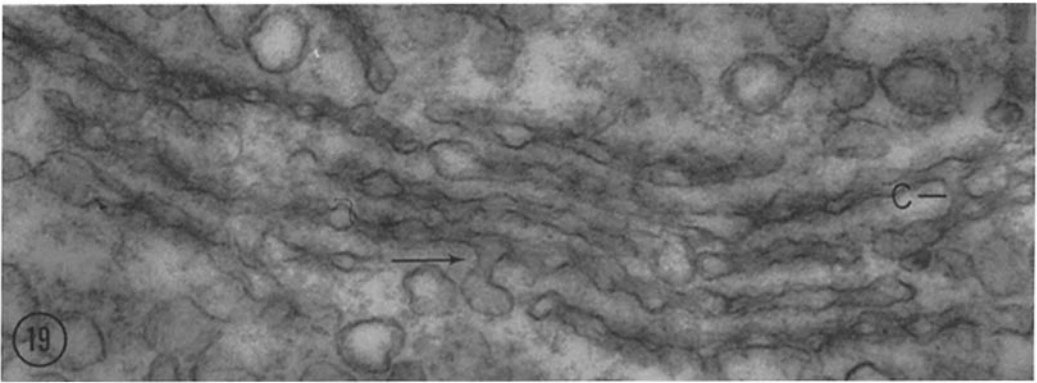
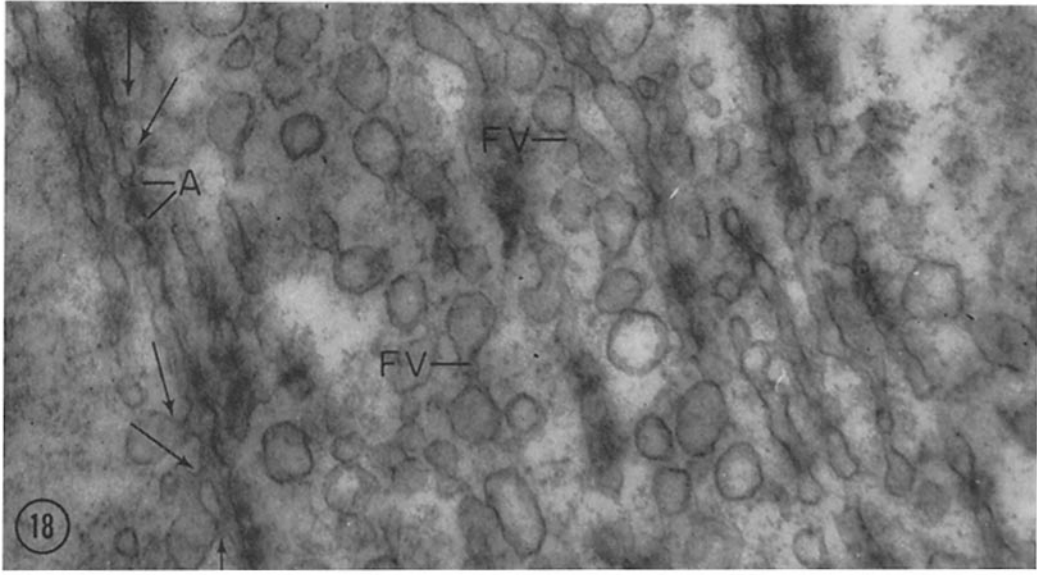
Since annulate lamellae are morphologically similar to the nuclear envelope, and since the lamellae frequently have been observed close to the nucleus, it would seem reasonable that all explanations regarding the origin of these structures involve the nuclear envelope in some way. Thus, Afzelius (1955, 1957) considered the annulate lamellae to be fragments of the nuclear membrane remaining after nuclear breakdown in

metaphase, but this does not appear to be the case in other oocytes (Swift, 1956). Rebhun (1956) envisioned a delamination of the nuclear membrane as the process whereby the annulate lamellae are formed. Merriam (1959) has advanced the hypothesis that the nuclear envelope forms inside the nucleus with subsequent delamination into the cytoplasm. Swift (1956) also suggested that annulate lamellae owe their structure in some unknown manner to the nuclear membrane, possibly by the fragmentation of the preexisting membrane or by being synthesized upon the nuclear membrane, using the latter as a mold. A third possibility also was considered, namely, that annulate lamellae are not directly associated with the nuclear membrane but that similar forces shape the formation of both. Heretofore, direct evidence for the origin of annulate lamellae has been lacking. Formation of annulate lamellae by delamination of portions of the nuclear envelope does not appear to occur in oocytes of *Necturus*. The nuclear membrane is directly involved, however, in producing the raw material (*i.e.*, vesicles by a blebbing process involving only the outer nuclear membrane) for the subsequent construction of annulate lamellae. The observations in *Necturus* also demonstrate that annulate lamellae may differentiate in regions of the cytoplasm far removed from the nuclear membrane. The possibility still exists, however, that more than one method may be available by which the cell can construct these lamellae, and only future observations at crucial periods in its differentiation will provide additional information. It is interesting to note, in this regard, that other investigators (Swift, 1956; Merriam, 1959) have suggested that once the annulate lamellae are formed they move out

FIGURES 18 AND 19 Differentiating annulate lamellae showing that (at *A*) the paired membranes of each lamella join at intervals to form the pores. Traversing these pores is a thin membrane (*A*). Vesicles which are in the process of fusing to form lamellae are indicated (*FV*). Connections between an annulate lamella and adjacent vesicles are indicated at arrows. Interconnections between differentiated annulate lamellae are shown at *C*. Figs. 18 and 19, $\times 53,000$.

FIGURE 20 Transverse view of lamellae showing spacing and nature of the annuli (*A*). Smaller vesicles or rings (arrows) are located adjacent to the interannular regions and probably represent transverse sections of lateral projections of the lamellae. A fusing row of vesicles is shown at *FV*. $\times 53,000$.

FIGURE 21 Shows that an annulate lamella may increase in length by fusing with additional individual vesicles (arrows). $\times 53,000$.



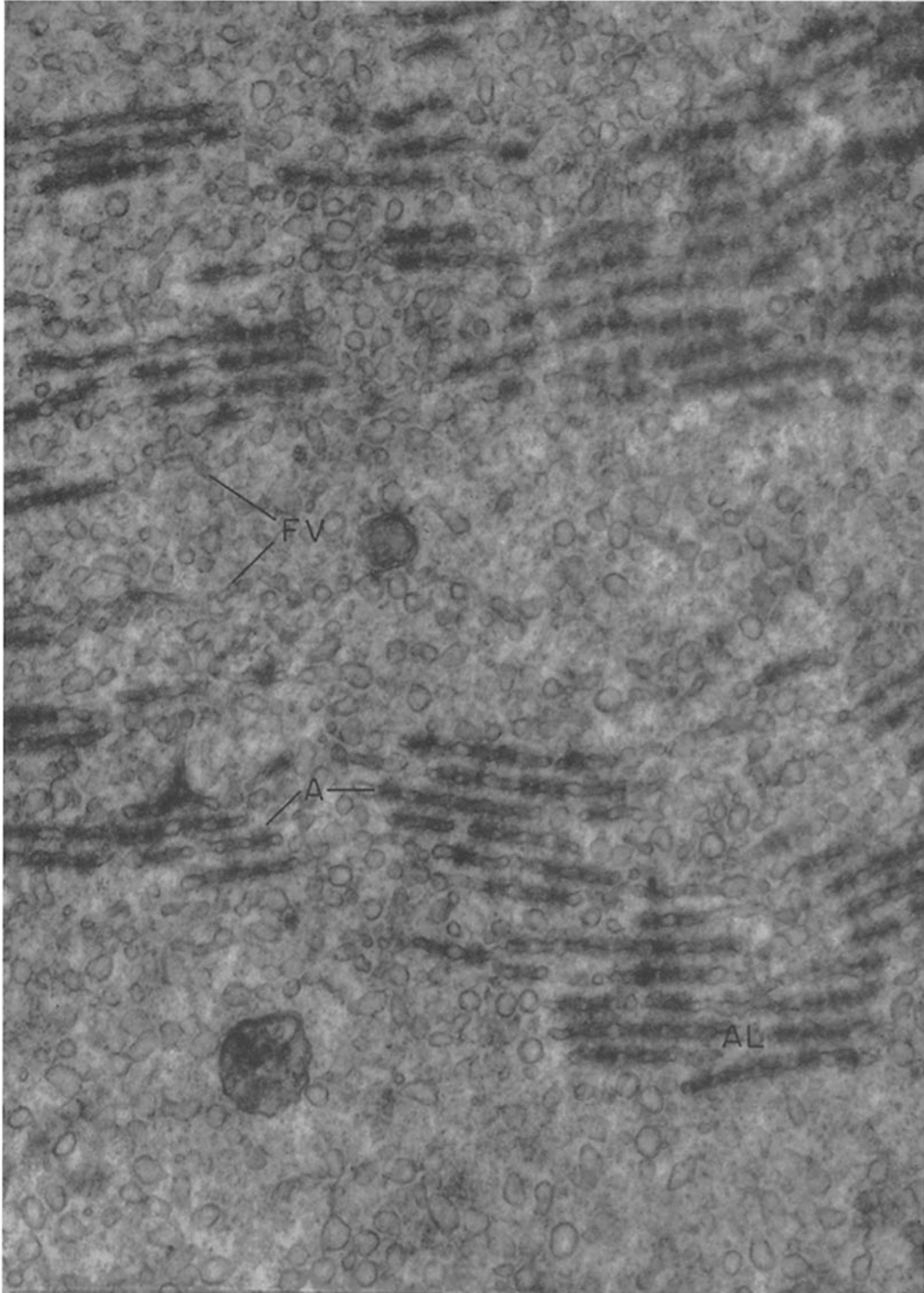


FIGURE 22. Several patches of annulate lamellae (AL). The annular region is shown to good advantage at A. The annular diaphragm and the dense material associated with it are obvious. Fusing rows of vesicles are indicated at FV. $\times 32,000$.

into the cytoplasm and possibly undergo a breakdown or degenerate into vesicles. Essentially this represents a reversal of the process described for the origin of annulate lamellae in *Necturus*.

The formation of cytoplasmic vesicles from the nuclear membrane in *Necturus* occurs over a considerable period of time in the life history of the oocyte. As judged from the many thousands of vesicles which are needed as raw material for the annulate lamellae, the frequency with which the vesicles are formed must be very rapid. The nuclear membrane then must be able to replace this material and at a sufficient rate so as to maintain a harmonious interaction of rapid membrane synthesis and membrane loss. No information is available, of course, to suggest what factors initiate this activity or stop the process at the appropriate time. Of considerable interest is the fact that individual vesicles, after being formed from the nuclear membrane and moving considerable distances in rather ordered ranks through the cytoplasm, have the capacity to reform a structure similar to that which produced them. It appears that the vesicles somehow possess, after their formation, the information that governs their subsequent activities. The information appears useful also in producing a high degree of order in a system which involves the movement of numerous chains of vesicles to a certain region of the ooplasm in which differentiation is to occur. While the vesicles contain a material of some kind, and which in part helps to maintain the shape and integrity of the vesicle, this material does not possess any degree of density in the electron microscope. If the vesicles do have within them a material derived from the nucleus (there is no direct evidence for this assumption), then the material apparently would have to traverse the inner nuclear membrane to reach the cisterna of the nuclear membrane and remain, since the cisterna is for a time continuous with the forming vesicle. If true, this would suggest differential permeability properties between the inner and outer nuclear membrane.

The functional significance of the annulate lamellae is, unfortunately, completely unknown. They have been observed in both vertebrate and invertebrate cells, but more are prevalent thus far in the invertebrates. As Porter (1961) has pointed out, tissues in which annulate lamellae have been described share one characteristic, namely, for the most part they are embryonic or fetal tissues

which are rapidly developing or differentiating. This has been emphasized in a recent study by Ross (1962) who found small patches of annulate lamellae in the adrenal cortex of fetal rats. These lamellae were encountered most frequently in 16-day fetuses and presumably disappeared after the 19th day of gestation. This study as well as others (Swift, 1956) suggest that annulate lamellae are short lived structures. It is hoped that additional studies on older oocytes of *Necturus* will reveal the fate of the annulate lamellae in this cell. Because of the close relationship of the annulate lamellae with the nuclear envelope and because of the high concentration of RNA that they contain, Swift (1956) has suggested that these lamellae may function in the transfer of genetic specificities from the nucleus to the cytoplasm, a view discussed also by Ruthmann (1958). This may well prove to be the case, but, if so, cells which do not possess annulate lamellae must achieve transfer of specificities by other means. An unusual feature of annulate lamellae is their close association, in certain cells, with rough-surfaced endoplasmic reticulum (Rebhun, 1961; Ruthmann, 1958; Ross, 1962). Continuity of the two systems is well illustrated in the oocytes of *Spisula* (Rebhun, 1961) but the functional significance of this association is not known. The suggestion has been put forth that annulate lamellae in the egg of *Dendraster* may synthesize 150 A particles (Merriam, 1959). A continuity between annulate lamellae and agranular vesicular reticulum has been described in spermatocytes of the crayfish (Kaye, Pappas, Yasuzumi and Yamamoto, 1961). Rebhun (1960) has noted that annulate lamellae on occasion have been seen to follow the general orientation of the ergastoplasm relative to the asters.

As yet, no information has been obtained to determine the source or nature of the dense matrix that becomes associated with the annulate lamellae. It appears to form just subsequent to the fusion of a chain of vesicles to form lamellae since they have been observed with little or no dense material present. Since the matrix becomes evident shortly after fusion of the vesicles, the contents of these vesicles may play some unknown role in its formation. Another point of interest is in determining how pores form in the lamellae. Conceivably, they may arise at the point of fusion of adjacent vesicles, but no direct evidence has been obtained for this possibility. The pores of the annuli appear to be embedded in a

moderately dense matrix when viewed in tangential section, but the functional relationship between the pores and the surrounding dense matrix is unknown as yet. Merriam (1959) has noted that the matrix of the annuli does not disappear when sections are bleached with hydrogen peroxide and hence reacts to the treatment in the same way as the 150 A particles of the heavy bodies, suggesting that the annular matrix may contain nucleic acid in non-particulate form.

It has been suggested throughout this discussion that all the raw material for the differentiation of annulate lamellae in *Necturus* is derived from the outer nuclear membrane. Indeed, the prolonged observations of this material suggest this to be the case. However, this does not preclude absolutely the possibility that some other membrane system in the oocyte may be potentially

capable of participating in the formation of vesicles which might take part to some extent in the differentiation of annulate lamellae. Few or no pinocytotic vesicles were observed associated with the plasma membrane of the oocyte in these stages. In addition, while considerable amounts of Golgi material were observed scattered throughout the ooplasm in the stages examined, the vesicles associated with the Golgi material seemed to remain concentrated in a disorderly fashion near the membranous lamellar component of this structure.

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(For References, see page 414.)

FIGURES 23 AND 24 Annulate lamellae may form from closely packed rows of vesicles without first becoming organized into concentric whorls of vesicles. Several such areas are shown in the perinuclear region of the ooplasm (*A*, *AL*). Occasionally a branched annulate lamella is observed (*BAL*). Surface views of annuli in both the nuclear membrane and annulate lamellae are shown at *A*. Numerous vesicles (*CV*) are closely packed in the ooplasm. Nucleus (*N*), nucleolus (*NL*). Fig. 23, $\times 15,000$; Fig. 24, $\times 22,000$.

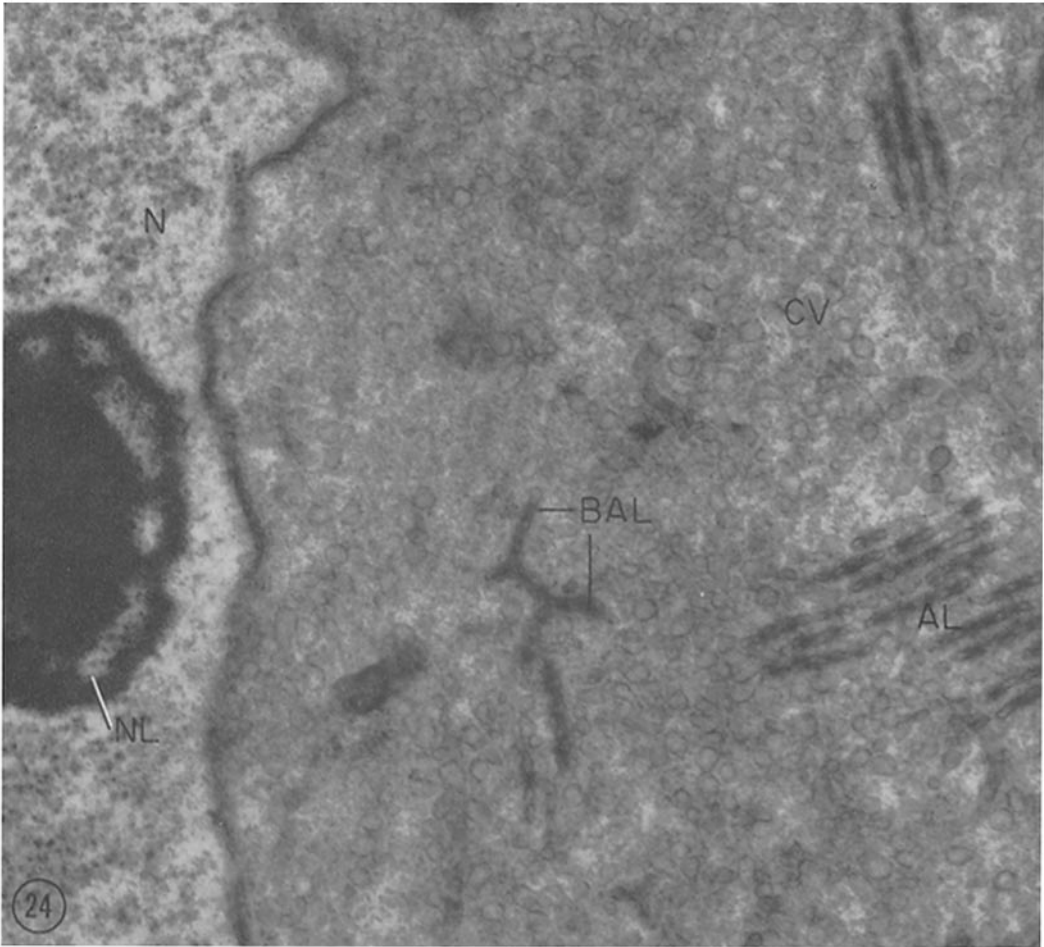
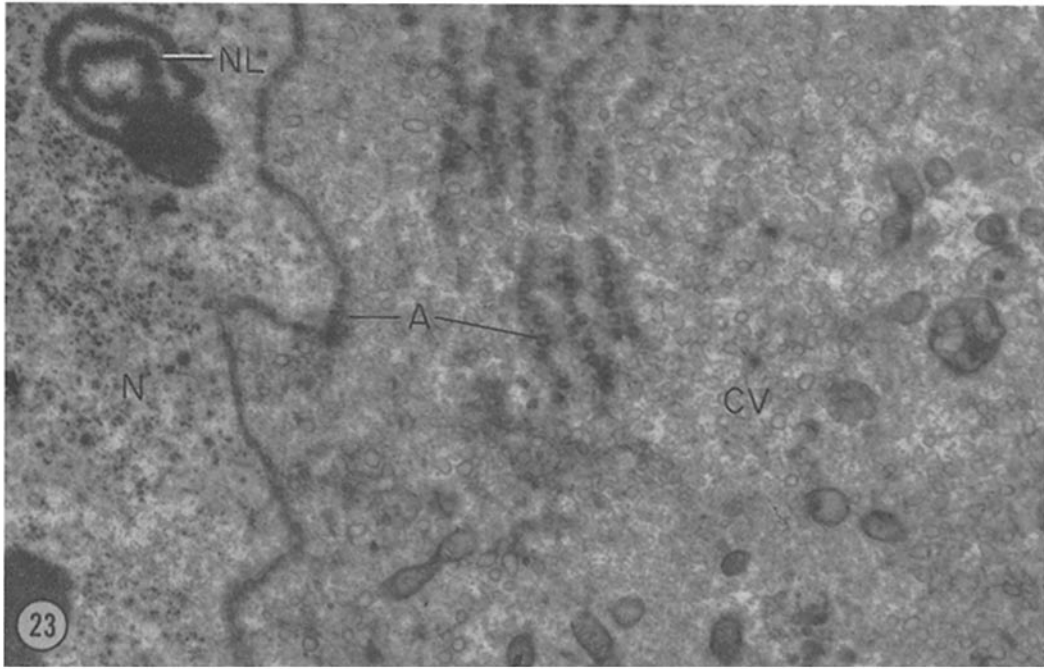
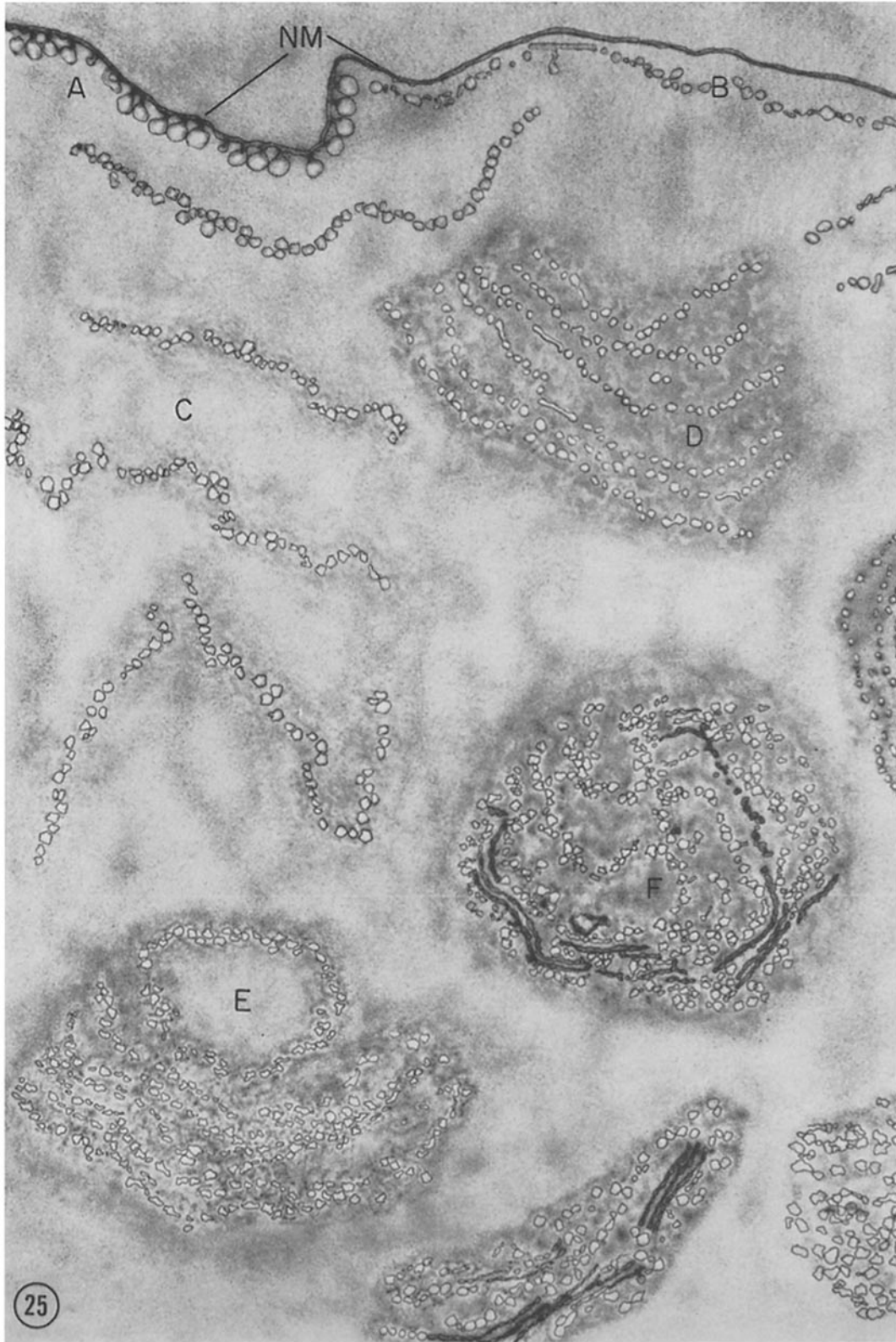


FIGURE 25 A diagrammatic summary of the major events in the construction of annulate lamellae in oocytes of *Necturus*. Extensive blebbing of the outer nuclear membrane is shown (*A*). Groups of blebs detach from the membrane at approximately the same time, resulting in formation of a long row of vesicles in the ooplasm immediately adjacent to the outer nuclear membrane (*B*). The groups of vesicles move in rather ordered rows to more peripheral regions of the cytoplasm (*C*). The rows of vesicles come closer together as additional rows are formed and released from the nuclear membrane. (*D*). Intervesicular connections or short cisternae may be present in the rows or chains of vesicles, indicating the beginning of a fusion activity by the vesicles. The rows of vesicles eventually may become organized into concentric rings of vesicles (*E*) followed by the differentiation of the annulate lamellae by progressive fusion of vesicles (*F*).



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