# ORGANIZATION AND ACTIVITY IN THE PRE- AND POSTOVULATORY FOLLICLE OF *NECTURUS MACULOSUS*

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ABSTRACT

The established follicle envelope of *Necturus maculosus* consists of a layer of follicle cells (granulosa) surrounding the developing oocyte, a layer of theca comprised of connective tissue cells, fibers, and matrix, and a layer of serosal cells. The changes in shape and fine structure of these layers during differentiation accompanying oogenesis are described. The cells and capillaries of the follicle envelope are engaged in an extensive pinocytotic activity, the details of which are described. We used cytochemical techniques to analyze the activity of the follicle envelope with respect to lipid accumulation and alkaline phosphatase activity. Radioautographic results indicate that cells of the follicle envelope are capable of incorporating tritium-labeled uridine and amino acids at certain times during oocyte growth. A comparative analysis was made of the soluble proteins in follicle envelopes isolated from immature oocytes and of those in follicle envelopes isolated from nearly mature oocytes and in postovulatory follicles. After the oocyte is ovulated, the cells of the follicle envelope are converted into a postovulatory follicle. The cells of the postovulatory follicle undergo further differentiation resulting in their becoming actively engaged in the formation of a secretion, the details of which are described at the electron microscope level. Analysis of the postovulatory follicle by thin-layer chromatography and cytochemistry demonstrated the presence of a wide variety of lipid substances and the possible presence of steroid. That the postovulatory follicle may be engaged in steroid biosynthesis is also suggested by studies involving the demonstration of 3 $\beta$-hydroxysteroid dehydrogenase activity with cytochemical techniques applied to frozen sections and to soluble proteins separated by gel electrophoresis.

## INTRODUCTION

In almost all organisms, the developing oocyte is invested by cells and formed elements collectively designated the follicle envelope. This structure thus represents a morphological and physiological boundary between the oocyte and its environment. Because of the intimate relationship existing between the follicular envelope and developing oocyte, it has long been held that the follicle cells, in particular, play an important role in the nourishment of the oocyte and in mediating the transport of materials into the growing egg. Several different functions have been proposed for the vertebrate follicle envelope. These include: (*a*) the synthesis of nucleic acids and proteins which may then be utilized by the oocyte (see 29, 59); (*b*) the formation of secondary egg membranes (see 69); (*c*) the mediation of the transport of materials from the capillaries in the connective tissue of the follicle envelope to the oocyte (see 89); and (*d*) the biosynthesis of cholesterol and steroid (see 8, 60). Recently, Masui (57) has obtained results with *Rana pipiens* which . . .

"indicate that pituitary gonadotropin acts on the follicle cells to stimulate them to release a hormone that directly acts on the oocyte to induce maturation." Much of the information suggesting that the follicle cells play a role in the synthesis of materials which are then supplied to the oocyte is based on studies of insect ovaries (3, 4, 6, 7, 40, 80, 94). Notwithstanding the fact that amphibian oocytes have been extensively studied at the electron microscope level, few studies have been concerned with variations in structure and activity occurring in the follicle envelope throughout the entire period of oogenesis (see 9, 24, 38, 85, 86, 91). That this is the case probably reflects the technical difficulties of dealing with the follicle envelope separated from its oocyte, and of observing the activity in the follicle envelope over a long period of development.

The present report summarizes information collected by a variety of means about the activity of the follicle envelope during the course of oogenesis in the neotonous salamander, *Necturus maculosus*.

## MATERIALS AND METHODS

### *Electron Microscopy*

*Necturus* oocytes of different sizes, mechanically isolated follicle envelopes (i.e., stripped from oocytes with watchmaker's forceps under a dissecting microscope) from oocytes ranging from 2.5 to 4.5 mm in diameter, and postovulatory follicles were fixed for 2 hr in an ice-cold 1% solution of glutaraldehyde (76) in either 0.1 or 0.05 M Sörensen's phosphate buffer (pH 7.4). In some cases, 0.5 mg of calcium chloride or 0.05 M sucrose was added to the fixative. Similar tissues were also fixed for 2 hr in ice-cold, 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). The fixative, therefore, ranged from approximately 255 to 270 milliosmols. Glutaraldehyde-fixed tissues were subsequently washed in several changes of 0.1 M phosphate buffer containing 0.05 M sucrose for a period of 6–12 hr and postfixed for 2 hr in cold, 1% osmium tetroxide (63) in 0.1 M phosphate (pH 7.4). After rapid dehydration in a series of cold ethanols and treatment with propylene oxide, the tissues were embedded in Epon 812 (55). Sections obtained with a Sorval MT-2 ultramicrotome were stained with uranyl acetate (87) and lead citrate (71) and studied in an RCA EMU-3G electron microscope.

### *Histochemistry*

Schultz's modification of the Liebermann-Burchardt reaction as described by Thompson (84) was used in detecting cholesterol. Whole mounts of the follicle envelope and squashed preparations of the postovulatory follicle were treated and photographed within a 10- to 30-min period. An aqueous Nile blue staining method as described by Thompson (84) was also used in demonstrating the presence of acidic and nonacidic lipids. Both whole mounts and fresh-frozen sections of follicle envelopes and postovulatory follicles were stained at 60°C with Nile blue, rinsed with distilled water at room temperature, and immediately examined. Gomori's (33) method for the detection of alkaline phosphatase was used for the demonstration of this enzyme in fresh-frozen sections of the follicle envelope. Portions of the *Necturus* ovary were fixed in Bouin's or Champy's solution, and paraffin sections were stained with mercuric bromphenol blue (58) or Heidenhain's iron hematoxylin, respectively.

### *Lipid Extraction–Thin Layer Chromatography*

Lipids were extracted from the postovulatory follicles by the method of Folch et al. (31) under an atmosphere of argon. The preparations were examined cytologically prior to extraction, for elimination of possible contamination of the postovulatory follicles with egg debris, atretic follicles, or small oocytes. 5-$\mu$l samples of the lipid extract were spotted on activated (110°C for 30 min) Silica Gel G plates (82) and placed into one of six different solvent systems for the specific lipid or steroid separation. Solvent A consisted of 90 parts *n*-pentane, 10 parts diethyl ether, and 1 part acetic acid; solvent B consisted of 65 parts chloroform, 34 parts methanol, and 4 parts water; solvent C, 3 parts benzene, 2 parts ethyl acetate; solvent D, 4 parts benzene, 1 part ethyl acetate; solvent E, 4 parts chloroform, 1 part ethyl acetate; solvent F, 9 parts chloroform, 1 part acetone. The separated lipids and steroid were visually demonstrated by charring the plates with 50% sulfuric acid. The classes of lipids and steroids were identified by their rf values according to Stahl (82) and Randerath (68). In addition, specific color reagents Nos. 123 and 60 were used in detecting the presence of steroids (82).

### *Soluble Proteins-3 β-Hydroxysteroid Dehydrogenase Activity*

A modification of Wattenberg's (88) method for 3 β-hydroxysteroid dehydrogenase (3 β-HSD) localization was used for the demonstration of possible sites of steroid biosynthesis. The technique was applied to frozen sections of postovulatory follicles as well as to soluble proteins separated in polyacrylamide gels. The purpose of using polyacrylamide gel electrophoresis in this portion of the study was to provide a more concentrated enzyme that could be histochemically analyzed for 3 β-HSD activity. In this technique,

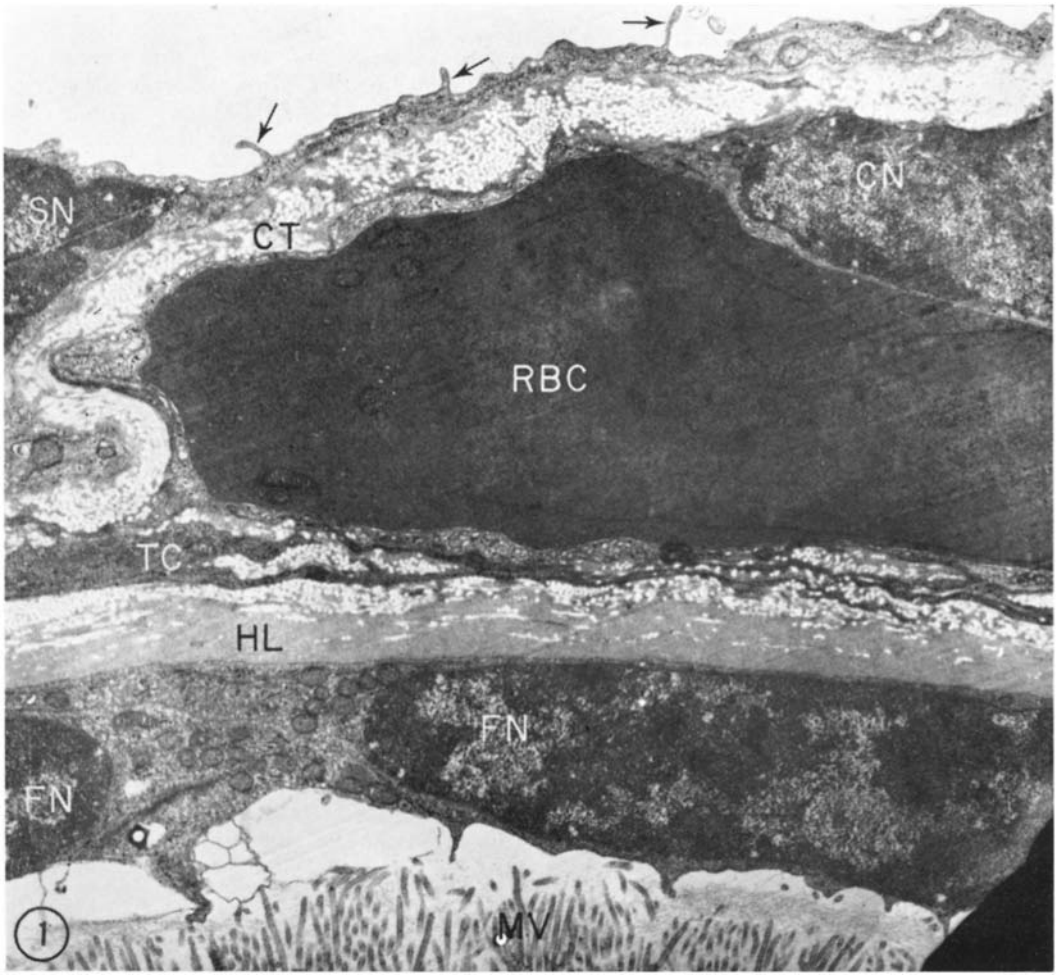



FIGURE 1 Oocyte-follicle complex ~0.75 mm in diameter. A portion of two overlapping follicle cells and their nuclei (*FN*) is illustrated adjacent to the microvilli (*MV*) of the oocyte. The theca contains a capillary with an enclosed erythrocyte (*RBC*). The nucleus (*CN*) of the capillary endothelial cell and a process from a fibroblast cell (*TC*) are also illustrated in the theca. The connective tissue fibers and matrix of the theca are identified (*CT*, *HL*). The thin serosal cell layer includes a portion of the nucleus (*SN*), and microvilli project from the outer surface of the serosal cells (arrows). Glycogen granules and pinocytotic vesicles are present in the serosal, endothelial, and thecal cells. × 10,200.

approximately 30 postovulatory follicles in each experiment were homogenized at 4°C in 0.58 M sucrose in 0.2 M Tris-HCl buffer, pH 7.54, and the cellular debris was sedimented in an International B-20 refrigerated centrifuge at 20,000 *g* for 1 hr at 4°C. 100-$\mu$l samples of the supernatant fraction were loaded onto 7.5% polyacrylamide gels (21) and separated at 3.3 milliamps/tube for 90 min in a Tris-glycine buffer at pH 8.6. The gels were removed and incubated for 20–30 min in the following medium at 37°C: 3 mg of nicotinamide adenine dinucleotide; 0.2 mg of dehydroepiandrosterone (dissolved in dimethyl formamide); 1 mg of nitro-blue tetrazolium salt; 0.03 mg of phenzaine methosulfate; 7 ml of 0.2 M Tris-HCl buffer at pH 7.54; and 3 mg of potassium cyanide. The incubation mixture was then removed, and the gels were washed in tap water and stored in a 7.5% acetic acid solution. For demonstration of unspecific diaphorase reaction, control gels were incubated in the substrate which lacked the dehydroepiandrosterone.

Soluble proteins of the pre- and postovulatory

follicles were demonstrated by treating the electrophoresed gels with a 1% naphthol blue-black stain in a 7.5% acetic acid solution for 30 min. The proteins appeared as blue bands after leaching or electrophoretic destaining.

### *Radioautography*

Small numbers of oocytes with associated follicle envelopes were cultured in 2, 10, or 20 ml of sterile Holtfreter's solution containing uridine-$^3$H (1.71 c/mmole, Schwarz BioResearch, Orangeburg, N.Y.), uridine-5-$^3$H (25 c/mmole, New England Nuclear Corp., Boston), lysine-$^3$H (1.0 c/mmole, Schwarz BioResearch), or L-leucine-4,5-$^3$H (5 c/mmole, New England Nuclear Corp.) in amounts ranging from 10 to 100 $\mu$c for durations ranging from 30 min to 22 hr. Adult females were also injected with 250–500 $\mu$c of uridine-$^3$H or 150–250 $\mu$c of leucine-$^3$H or lysine-$^3$H and maintained for 1–14 days thereafter. In all cases, the oocytes were rinsed briefly in several changes of cold, sterile Holtfreter's solution. They were then fixed in ice-cold, 6% formalin containing 0.5 or 3% trichloracetic acid (TCA) and in some cases the corresponding amino acid in the amount of 0.1 mg/ml of total fixative for 24 hr, or they were fixed in Bouin's solution for 24 hr. The oocytes were embedded in paraffin, sectioned at 5 $\mu$, and processed for radioautography according to the techniques described by Kopriwa and Leblond (50). Deparaffinized and hydrated sections treated with 5% TCA were coated with Kodak NTB-2 emulsion and exposed for periods of 4–8 wk.

## RESULTS

### *Preovulatory Follicle*

#### General

The ovary of *Necturus* is a saccular structure with peritoneum covering the outside and an inner ovarian epithelium lining the inside. Numerous oogonia are dispersed throughout the connective tissue stroma which is interposed between the inner ovarian wall and the peritoneum or serosa. As the oocytes grow, they bulge into the interior of the ovary. During the early prophase of oogenesis, the fibroblasts of the connective tissue differentiate into a layer of cells, the follicle epithelium or granulosa layer, immediately surrounding the oocyte surface. An early stage in the formation of the follicle envelope is illustrated in Fig. 2. In this figure a fibroblast nucleus is closely apposed to the gonial cell, and its contour reflects the curvature of the oogonium. The zona pellucida, which includes the zona radiata (processes from the follicle cells and plasma membrane of the oocyte) and (in urodeles) an acellular or homogeneous layer, is located between the follicle epithelium and the oocyte surface. The follicle epithelium is surrounded by the theca, composed of connective tissue fibers, matrix, fibroblasts, and capillaries. The theca, in turn, is surrounded by the surface epithelium or serosa, constituting the outermost layer of the follicle. These components of the *Necturus* follicle envelope are illustrated in Figs. 1 and 3. In part, the serosa results from the enlargement of the oocyte during its period of tremendous growth so that the oocyte protrudes against the inner ovarian epithelium. Eventually, the inner ovarian epithelium completely surrounds the oocyte, but remains continuous with the peritoneum in a small region which marks the point through which the mature egg will rupture at the time of ovulation (see 91, 92). During the course of oogenesis in *Necturus*, the oocyte and its associated follicle envelope increase in size to approximately 5 mm in diameter at maturity. Previous studies have demonstrated that protein yolk deposition begins in those oocytes 1.1–1.2 mm in diameter (49). Studies dealing with various aspects of the amphibian oocyte and follicle envelope include those by Kemp (45–48), Dollander (24), Wartenberg and Gusek (86), Wartenberg (85), Wischnitzer (91), and Hope et al. (38).

#### Fine Structure

GRANULOSA: The typical organization of the follicle envelope surrounding oocytes 0.5–1.0 mm in diameter is illustrated in Figs. 1 and 3. The fibrous network associated with the tips of the oocyte microvilli in Fig. 1 represents an early stage in the formation of the vitelline membrane. At this stage of development, the follicle cells are squamous in shape and contain a large, ellipsoidal nucleus. Slender projections of the follicle cell extend between the oocyte microvilli and end in close proximity to the oolemma (Fig. 2). In the area of close contact, the plasma membranes of the two cells appear more dense than in any other region (Fig. 2). The cytoplasm of the follicle cells at this period of development consists of mitochondria, rough-surfaced endoplasmic reticulum, free ribosomes, and one or more Golgi complexes (Fig. 4). In this early period of oogenesis, it is common for adjacent follicle cells to overlap each other for considerable distances as is

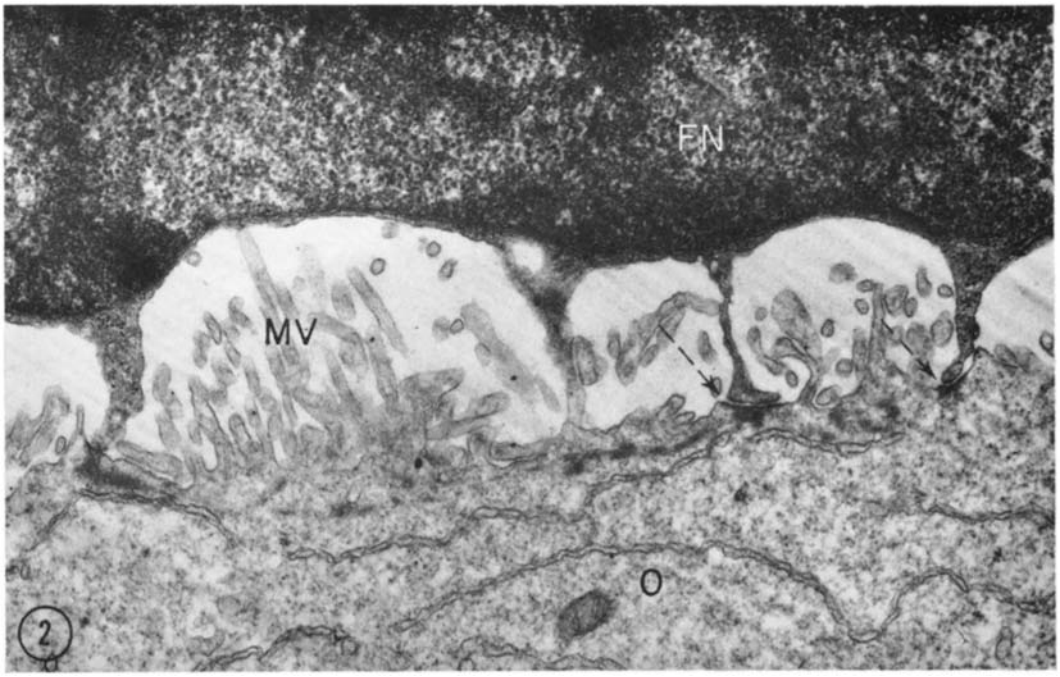



FIGURE 2 Oocyte-follicle complex ~0.5 mm in diameter. The figure illustrates a portion of the oocyte (*O*) and the large, elliptical-shaped nucleus of the follicle cell (*FN*). Processes from the follicle cell extend between the oocyte microvilli (*MV*) and terminate in close proximity to the oolemma (arrows). × 22,000.

apparent in Figs. 1 and 3, and no intercellular spaces are evident between the follicle cells.

As the oocyte-follicle complex increases in size from 1.3 to 2.0 mm in diameter, several changes occur in the follicle cells. For one, the shape of the follicle cell changes from squamous to cuboidal and the nuclei change from ellipsoidal to ovoid in shape (Figs. 5, 6). Further, by the time the oocytes have grown to ~1.3 mm in diameter, overlapping of adjacent follicular cells is uncommon, but large intercellular spaces are apparent which become extensive channels between the follicle cells of larger oocytes (Figs. 7, 8). Moreover, the distance between the follicle cell layer and the oocyte increases as the length of the oocyte microvilli increases and the deposition of the vitelline membrane is completed. At this time, long, slender extensions of the follicle cell extend between the oocyte microvilli and occupy deep but narrow pits of the oocyte surface. Cytoplasmic changes in the follicle cells also occur at this time. An extensive system of branching and anastomosing smooth-surfaced tubules becomes evident, this system representing an elaboration of smooth endoplasmic reticulum (Fig. 10). While free ribosomes are still numerous, the rough endoplasmic reticulum does not appear so extensively developed (Figs. 7–9). Dense, irregularly shaped secretion granules are present in the follicle cells early in development and persist throughout much of oogenesis (Figs. 4, 8, 10). They first appear in the Golgi region (Fig. 4) and later are more concentrated in this area than in any other region of the follicle cell cytoplasm. These secretion granules in the follicle cells appear similar in size, shape, and density to secretion granules in the serosa and theca cells. Although a tubular, smooth endoplasmic reticulum is encountered in those follicle cells fixed in glutaraldehyde as the primary fixative, numerous vesicles of rather uniform size are present in those follicle cells fixed in osmium tetroxide as the primary fixative. Lipid droplets begin to accumulate in the follicle cell cytoplasm during this period of development (Fig. 6). Lipid deposition begins when the oocyte-follicle complex is ~1.3 mm in diameter and increases during the growth to 2.0 mm. Histological sections of the oocyte-follicle cell complex which are suitable for

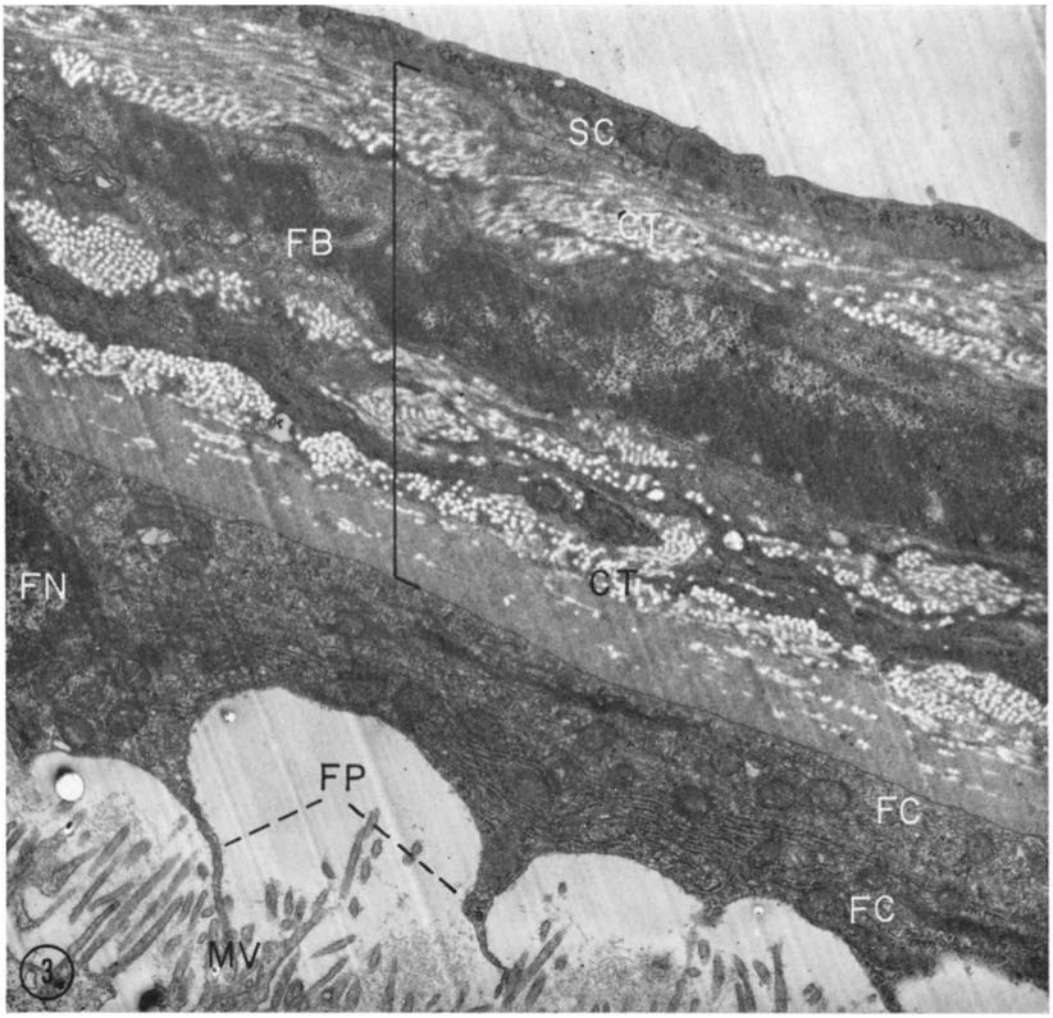



FIGURE 3 Oocyte-follicle complex ~0.75 mm in diameter. The thin serosal cell layer is identified at *SC*. The extensive theca indicated by the dark vertical line contains fibroblast cells (*FB*) and connective tissue fibers and matrix (*CT*). A portion of two overlapping follicle cells (*FC*) and a follicle cell nucleus (*FN*) are identified. Processes of the follicle cell (*FP*) extend between oocyte microvilli (*MV*). A fibrillar material is associated with the tips of the oocyte microvilli at this stage. Glycogen granules and pinocytotic vesicles are apparent in the theca and serosa cells. × 14,500.

the preservation of lipid indicate that in some of the follicle cells lipid appears packed at 2 mm whereas in other cells lipid accumulation has not progressed to such an extent (Figs. 5, 6). The accumulation of lipid in the follicle cells continues as oogenesis proceeds. A portion of a follicle cell surrounding an oocyte ~4 mm in diameter is illustrated in Fig. 11. The cytoplasm of this cell is nearly filled with lipid. Thus, one major function of the follicle cell during oogenesis in *Necturus* appears to be the synthesis and accumulation of large amounts of lipid.

During the terminal stages of oogenesis (oocytes 4-5 mm in diameter), proteinaceous yolk platelets appear in some, but not all, of the follicle cells (Figs. 12–14). These yolk platelets as visualized in light microscope preparations are similar in size to those in the ooplasm and stain intensely with

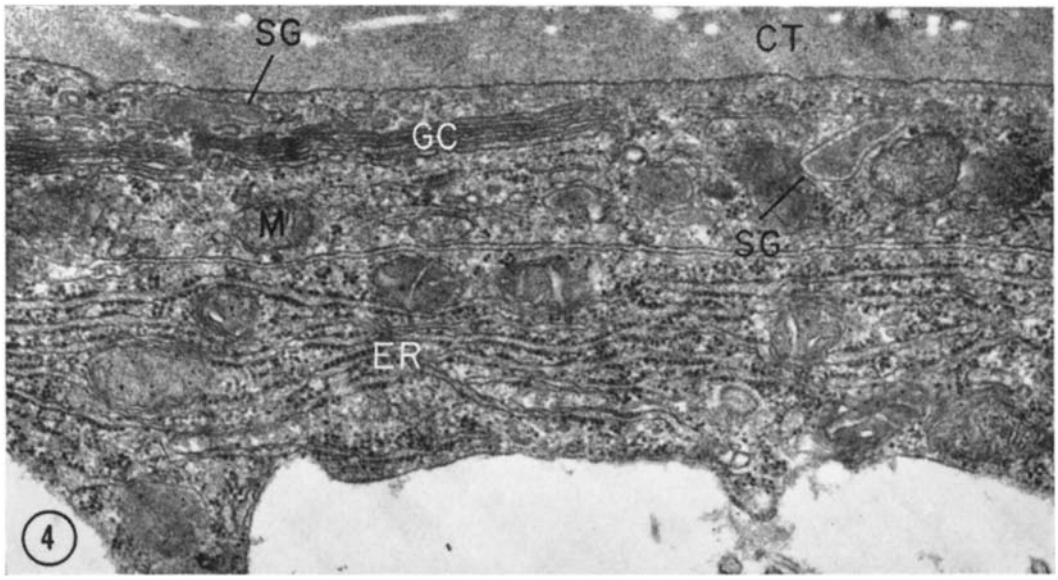
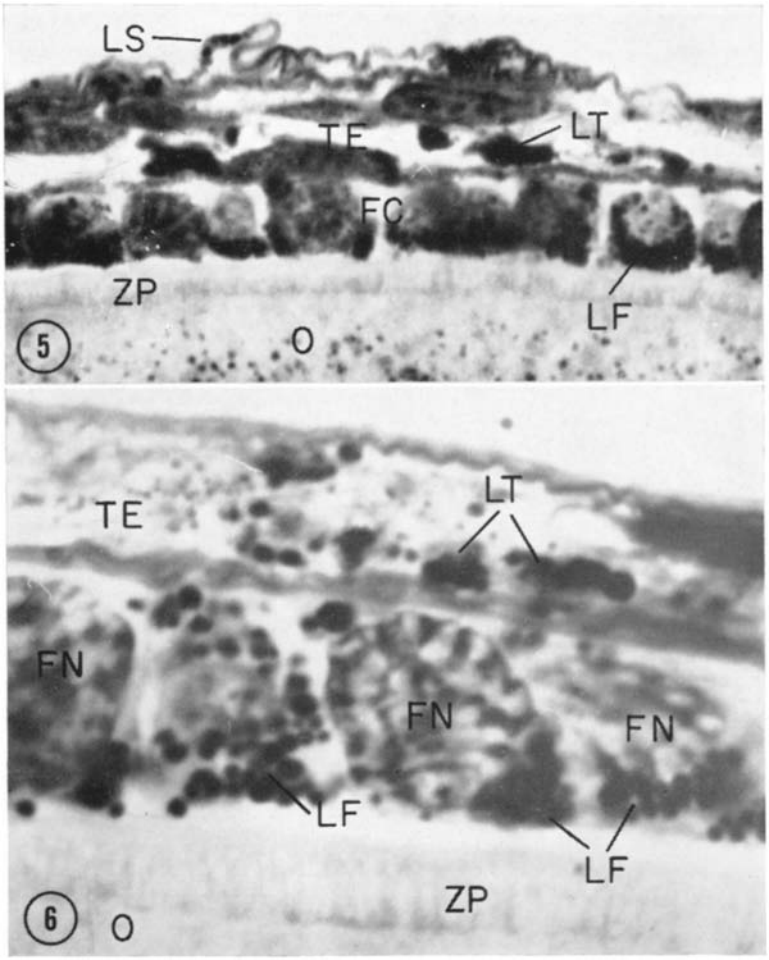



FIGURE 4 Oocyte-follicle complex ~0.75 mm. The figure illustrates a portion of the cytoplasm of two follicle cells. Mitochondria (*M*), rough-surfaced endoplasmic reticulum (*ER*), Golgi saccules (*GC*), and forming secretory granules (*SG*) are identified. A portion of the connective tissue of the theca is present at *CT*. × 30,000.

mercuric bromphenol blue (Fig. 13). Electron micrographs of the follicle cells around an oocyte nearing maturity confirm the presence of yolk platelets in some of the follicle cells. Further, such yolk platelets are enclosed by numerous membranous whorls (Figs. 12–14) similar to those described as being associated with the process of yolk demolition in embryonic cells of other species (43, 44). Some regions of the follicle cell cytoplasm contain extensive membranous whorls which probably have resulted from the complete destruction of the yolk platelets (Fig. 12). However, there is no evidence that any of the yolk platelets within the oocyte are undergoing demolition at this stage. Thick Epon sections of the entire oocyte-follicle complex were stained and examined for possible evidence of atresia. As far as could be determined, no evidence of atresia in the egg was evident when yolk platelets were encountered in some of the follicle cells late in oogenesis.

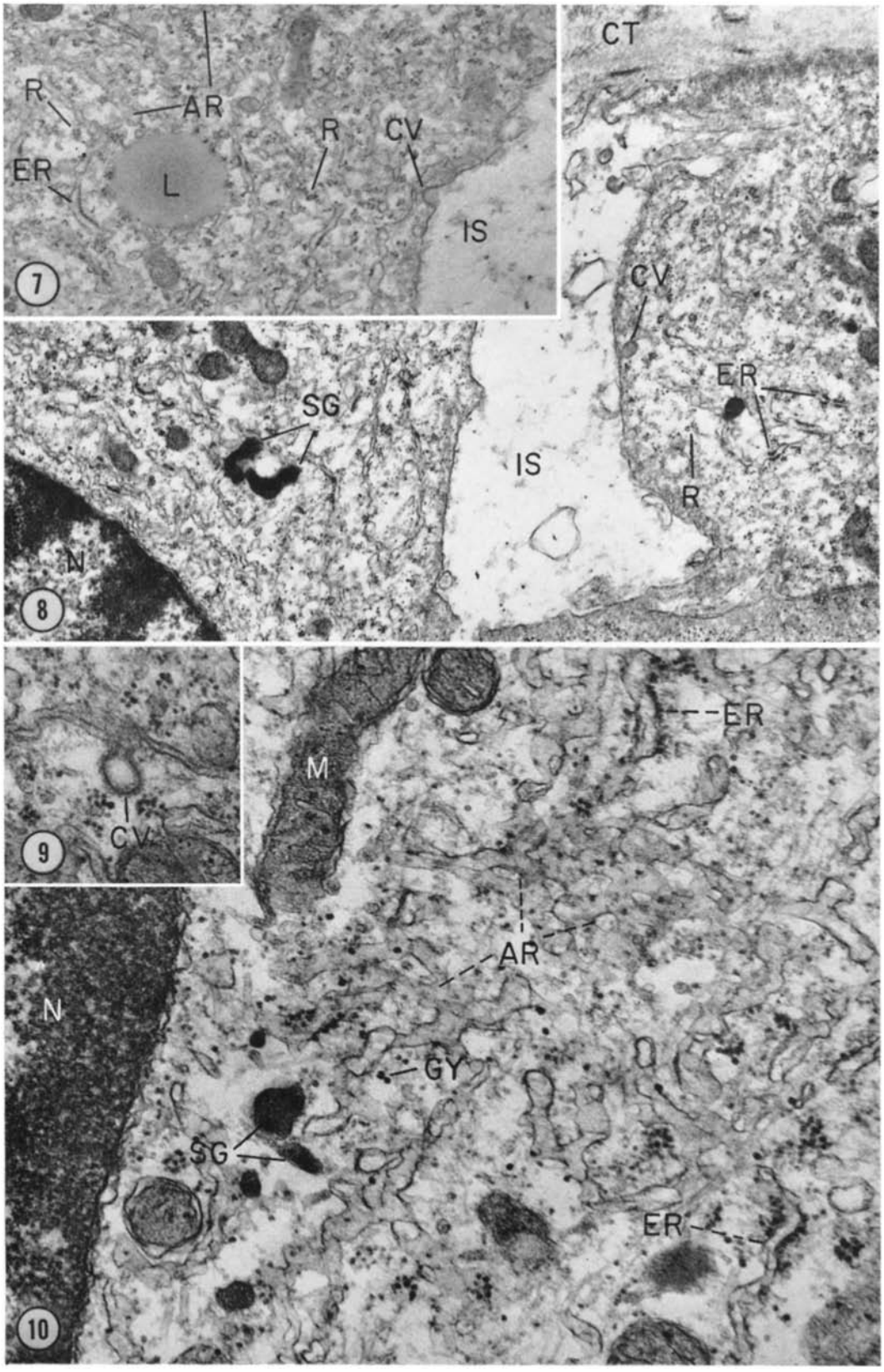
THECA: The outer surface of the follicle cells is generally quite smooth and surrounded by a structureless-appearing matrix in which connective tissue fibers are embedded (Figs. 1–3). This region begins the theca, a layer consisting of numerous fibroblasts which are stellate in shape with numerous, long processes interdigitating with adjacent cells (Fig. 3). The cytoplasm of the theca cells contains mitochondria, free ribosomes, both smooth and rough forms of endoplasmic reticulum, and numerous granules similar in appearance to particulate glycogen (70). Lipid droplets are frequently observed in these cells with the electron microscope and, in suitable histological preparations, some of the cells appear to be filled with lipid droplets (Figs. 5, 6). The space between the cells in the theca is occupied by collagenic fibers, matrix, and capillaries (Figs. 1–3).

SEROSA: The cells comprising the serosa are thin, flattened, and are characterized by numerous and extensive desmosomes (Figs. 1, 3, 17, 18). Large numbers of tonofilaments extend from the desmosomal plaques into the serosal cells and occur throughout much of the cytoplasm (Figs. 18–20). Small, dense granules probably representing particulate glycogen are present early in the development of the serosa cells, and they increase in number during subsequent periods of development (Figs. 18–20). Some overlapping of adjacent serosal cells occurs, and intercellular spaces, sometimes containing a flocculent material, are commonly encountered (Figs. 15–17). In addition to




FIGURES 5 and 6 Oocyte-follicle complex ~2 mm in diameter. The photomicrographs illustrate the follicle cell layer (*FC*) and their nuclei (*FN*), the theca (*TE*), serosa (*LS*), zona pellucida (*ZP*), and a portion of the oocyte (*O*). In this preparation, lipid droplets are present in many of the follicle cells (*LF*), theca cells (*LT*), and serosa cells (*LS*). Champy's fixation, Heidenhain's iron hematoxylin. Fig. 5, ×1500; Fig. 6, × 2400.

FIGURES 7–10 Oocyte-follicle complex ~2 mm in diameter. All figures illustrate a portion of the follicle cell cytoplasm which contains extensive arrays of smooth-surfaced endoplasmic reticulum (*AR*), rough-surfaced endoplasmic reticulum (*ER*), and numerous polysomes (*R*). A portion of an extensive intercellular space (*IS*) is included. Pinocytotic vesicles of the coated variety (*CV*) in the process of forming from the plasma membrane are illustrated in Figs. 7–9. Dense, irregularly shaped secretory granules (*SG*) are identified in Figs. 8 and 10. Particulate glycogen indicated at *GY* in Fig. 10. Follicle cell nucleus (*N*). Mitochondria (*M*). Connective tissue fibers of theca (*CT*). Lipid (*L*). Fig. 7, × 23,700; Fig. 8, × 19,000; Figs. 9 and 10, × 50,000.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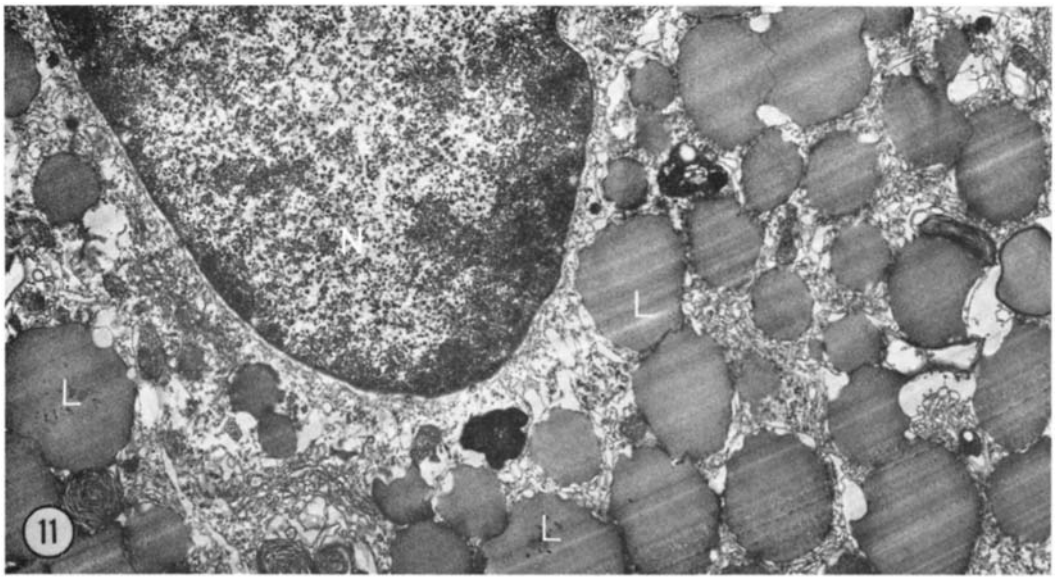



FIGURE 11 Oocyte-follicle complex 3.5–4.0 mm in diameter. The figure illustrates a portion of the nucleus (*N*) and cytoplasm of a follicle cell. Note the large number of lipid droplets (*L*) now present at this stage of oogenesis × 10,200.

the extensive array of tonofilaments, mitochondria, particulate glycogen, and pinocytotic vesicles, other membranous structures appear in the cytoplasm of the serosal cells shortly after the formation of this layer. A small amount of rough-surfaced endoplasmic reticulum is present in the cells, as well as a more extensive branching and tubular form of smooth-surfaced endoplasmic reticulum (Figs. 18–20). One or more Golgi complexes are present in each of the serosal cells (Figs. 19, 20). As was the case for the follicle and theca cells, the serosa cells also accumulate lipid droplets during the course of oogenesis (Figs. 5, 16). The number of such lipid droplets, however, varies among serosal cells in the same follicle envelope. In addition to lipid, another type of inclusion product is observed in some of the serosa cells as illustrated in Fig. 19. These inclusions are dense, irregularly shaped granules which seem to have their origin in association with the Golgi complex (Fig. 20) and which in appearance resemble granules also present in the granulosa and theca cells. Microtubules are randomly distributed in all cells of the follicle envelope.

### MICROPINOCYTOSIS

SEROSA: Micropinocytosis appears to occur in all cells of the *Necturus* follicle envelope. However, not all cell types in the envelope are equally active, nor does micropinocytosis in these cells occur with constant frequency during the course of oogenesis. The earliest pinocytotic activity is encountered in the serosal cells. From the time the serosa becomes associated with the young oocyte until the oocytes have grown to ~1 mm in diameter, the serosal cells contain numerous vesicles of the nonalveolate or noncoated type which measure 100–130 mμ in diameter (Fig. 15). These vesicles are frequently observed to be continuous with the plasma membrane of the inner, outer, and lateral margins of the cells (Figs. 15, 17, 18). The number of caveolae and isolated micropinocytotic vesicles remains high in the serosal cells as the oocyte grows to a diameter of ~2.5 mm. During subsequent periods of oocyte growth, however, micropinocytotic activity continues but at an apparently reduced rate. The extensive pinocytotic activity in the serosa as evidenced in the electron micrographs suggests that this portion of the follicle envelope is important in the uptake of materials from outside of the follicle and their transport to deeper regions of the follicle envelope.

THECA: Vesicles of the nonalveolate variety similar in size to those seen in the serosa cells are also observed in the theca cells. The vesicles may be isolated in the cytoplasm of these cells or

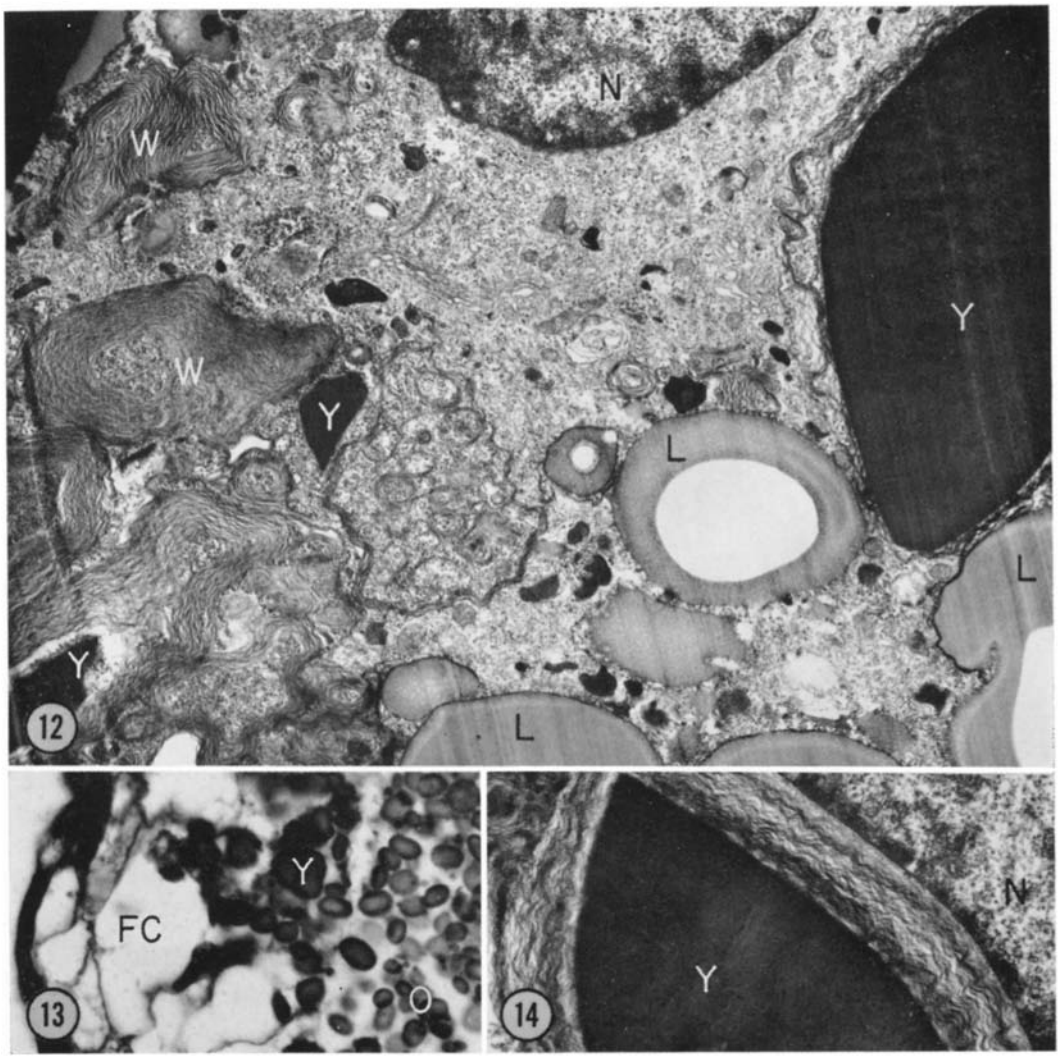



FIGURE 12 Oocyte-follicle complex ~4.5 mm in diameter. The figure illustrates a portion of a follicle cell with nucleus (*N*), lipid droplets (*L*), and yolk platelets (*Y*) surrounded by concentrically arranged membranes. Other regions of the follicle cell cytoplasm contain concentric membranous whorls (*W*) which do not enclose yolk platelets. × 10,200.

FIGURE 13 Oocyte-follicle complex ~5 mm in diameter. The photomicrograph illustrates that some of the follicle cell cytoplasm appears vacuolated (*FC*), while in other cells intensely stained yolk platelets (*Y*) are present and are similar in size and appearance to those in the oocyte (*O*). Bouin's fixation. Mercuric bromphenol blue stain. × 2500.

FIGURE 14 Oocyte-follicle complex ~5 mm in diameter. The figure illustrates a portion of a follicle cell with nucleus (*N*) and a yolk platelet (*Y*) surrounded by concentric membranes. × 19,000.

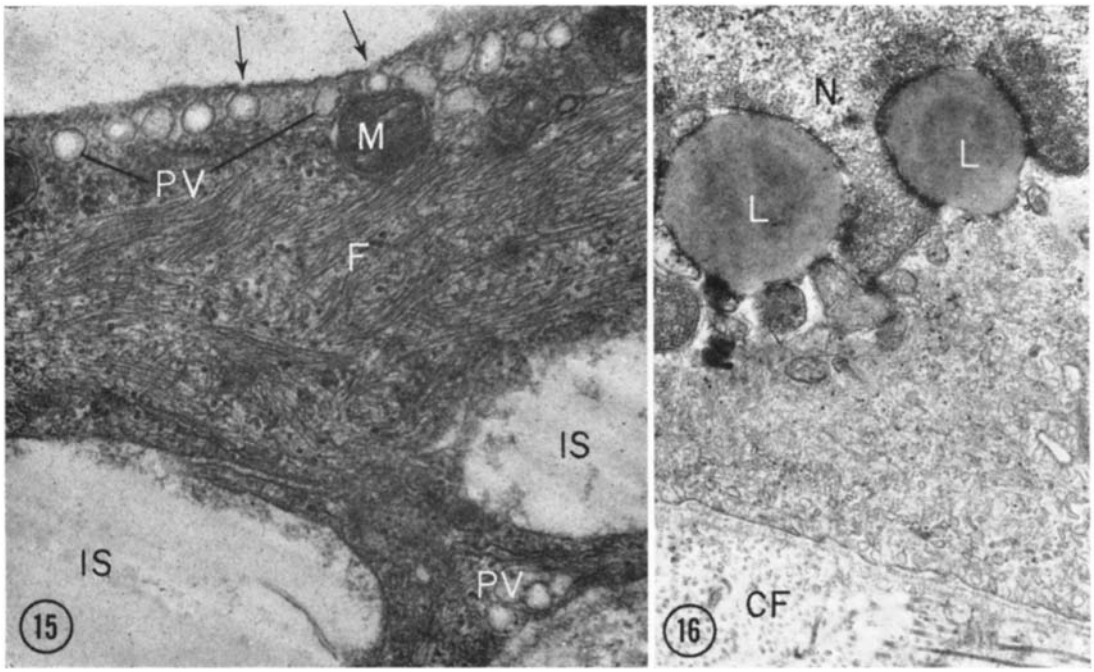



FIGURE 15 Oocyte-follicle complex ~0.5 mm in diameter. Portions of two overlapping serosa cells are illustrated. Intercellular spaces (*IS*) between adjacent cells contain a flocculent material. Note alignment of pinocytotic vesicles along outer and inner surface of the cell (*PV*). Some of the vesicles are continuous with the plasma membrane (arrows). Mitochondria (*M*) are aligned close to the pinocytotic vesicles, and numerous intracytoplasmic filaments are apparent (*F*). × 37,500.

FIGURE 16 Oocyte-follicle complex ~2 mm in diameter. Lipid (*L*) droplets are present in the serosa cell cytoplasm. The nucleus (*N*) of the serosal cell and collagenic fibers of the theca (*CF*) are identified. × 18,900.

continuous at any point with the plasma membrane of the cells. However, the number of such vesicles is not so great in the theca cells as in the serosa. The micropinocytotic vesicles in the theca cells are observed very early in oogenesis and persist throughout all stages studied with the electron microscope.

FOLLICLE CELLS: During the early stages of oogenesis, the follicle cells appear to be the least active of all cells in the follicle envelope. The follicle cells associated with oocytes less than 1 mm in diameter show little or no evidence of pinocytotic activity (Fig. 3). During subsequent stages of oocyte growth, however, occasional micropinocytotic vesicles are found in the follicle cells. These vesicles are similar in size to those described in other cells of the follicle envelope, but those associated with the plasma membrane of the follicle cells are usually of the alveolate (coated) variety (Figs. 7–9).

CAPILLARIES OF THECA: Numerous capillaries traverse the region of the theca. A traversing capillary with an enclosed red blood cell is illustrated in Fig. 1. Perhaps the most extensive micropinycytotic activity occurs in the capillary endothelial cells. The cytoplasm of these cells often appears filled with smooth-surfaced vesicles exhibiting a variety of configurations (Figs. 21–23). Numerous flask-shaped invaginations of the plasma membrane are observed at both the inner and outer surfaces of the endothelial cells, and they are similar in size and shape to intracytoplasmic microvesicles. In addition to the microvesicles, microvesicular rosettes are present in the capillary endothelium (Figs. 21–23). The rosettes consist of a number of microvesicles which sur-

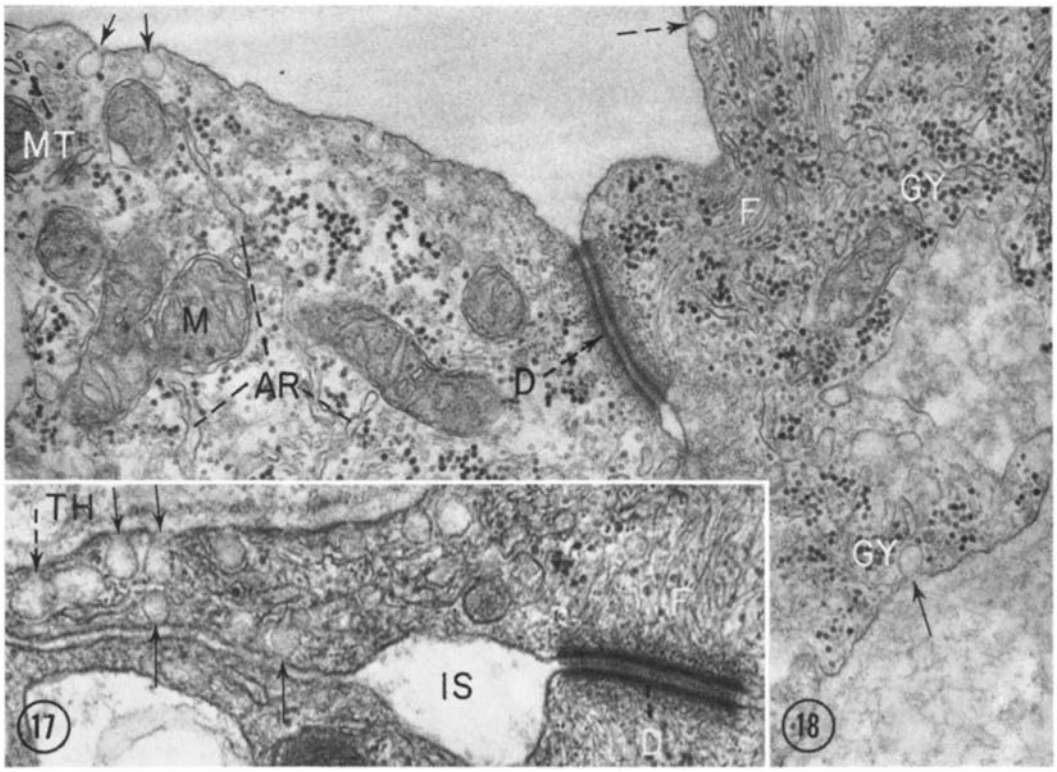



FIGURE 17 Oocyte-follicle complex ~0.5 mm in diameter. Junction of two serosal cells. Desmosome (*D*), intercellular space (*IS*), and tonofilaments (*F*) are indicated. Note caveolae associated with the lateral margins of the serosa cells (upper arrows). Pinocytotic vesicles are also continuous with the plasma membrane of the inner surface of the cell (lower arrows). Basement membrane and connective tissue fibers of the theca (*TH*) are present in upper left of figure. × 50,000.

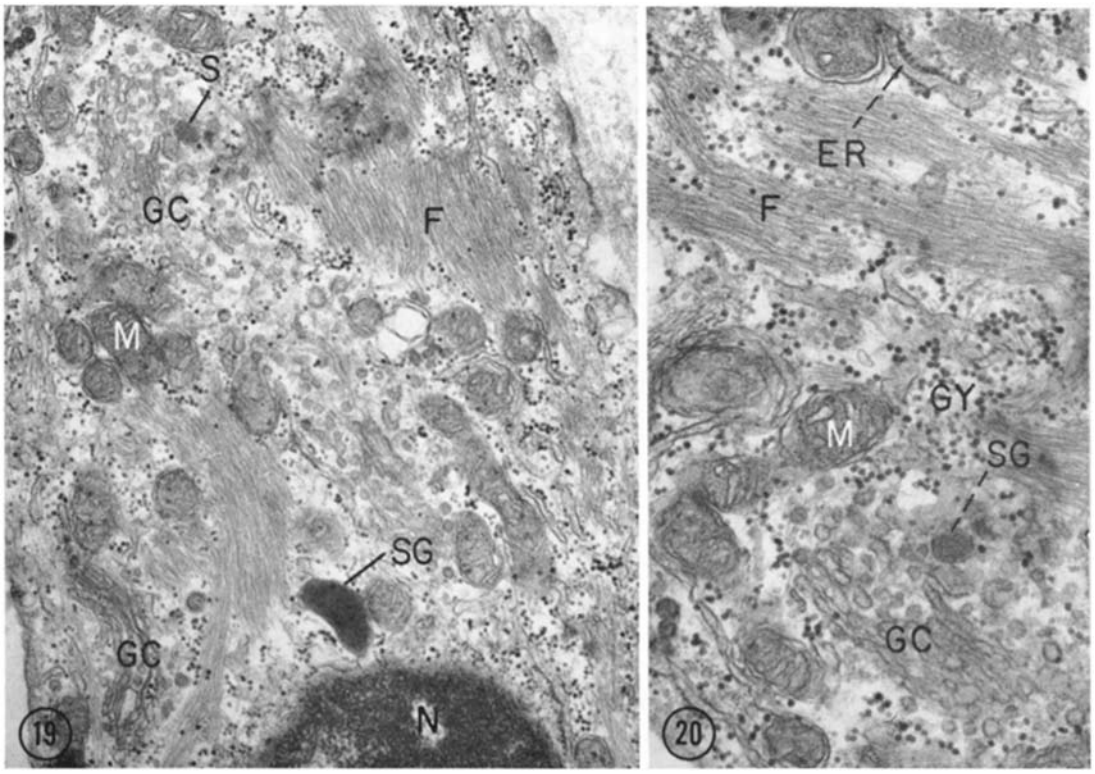
FIGURE 18 Oocyte-follicle complex ~1.5 mm in diameter. The figure illustrates the junction of two serosa cells. Extensive desmosomes (*D*) are formed at the junction of the two cells, and numerous tonofilaments (*F*) extend from the desmosomal plaques into the cytoplasm. The cytoplasm contains elements of the smooth endoplasmic reticulum (*AR*), mitochondria (*M*), microtubules (*MT*), and glycogen (*GY*). Pinocytotic vesicles are continuous with the plasma membrane at arrows. × 37,500.

round and are continuous with a larger, central vesicle: from one to five small vesicles communicate with the larger, central vesicle (Fig. 23). On occasion, the limiting membrane of the rosette appears to be continuous with the plasma membrane, thus establishing a continuity between the microvesicular rosettes and the extracellular space of the theca (Fig. 21). The capillaries of the theca thus appear to be involved in the transport of large quantities of material from the blood into the follicle envelope. The rate at which such activity occurs does not appear to be constant. If the number of micropinocytotic vesicles present in the capillary endothelium can be used as an index of the amount of material incorporated into the developing follicle, then the activity appears to be greatest in those oocyte-follicle complexes ranging from 1 to 3 mm in diameter.

## *Postovulatory Follicle*

### STRUCTURE

Female *Necturus* commonly lay their eggs during the early spring months after an extensive behavioral ritual involving the male. When an egg is ovulated, the follicle envelope becomes converted into a rounded body with a reddish-orange coloration (Fig. 40). Histological preparations of the

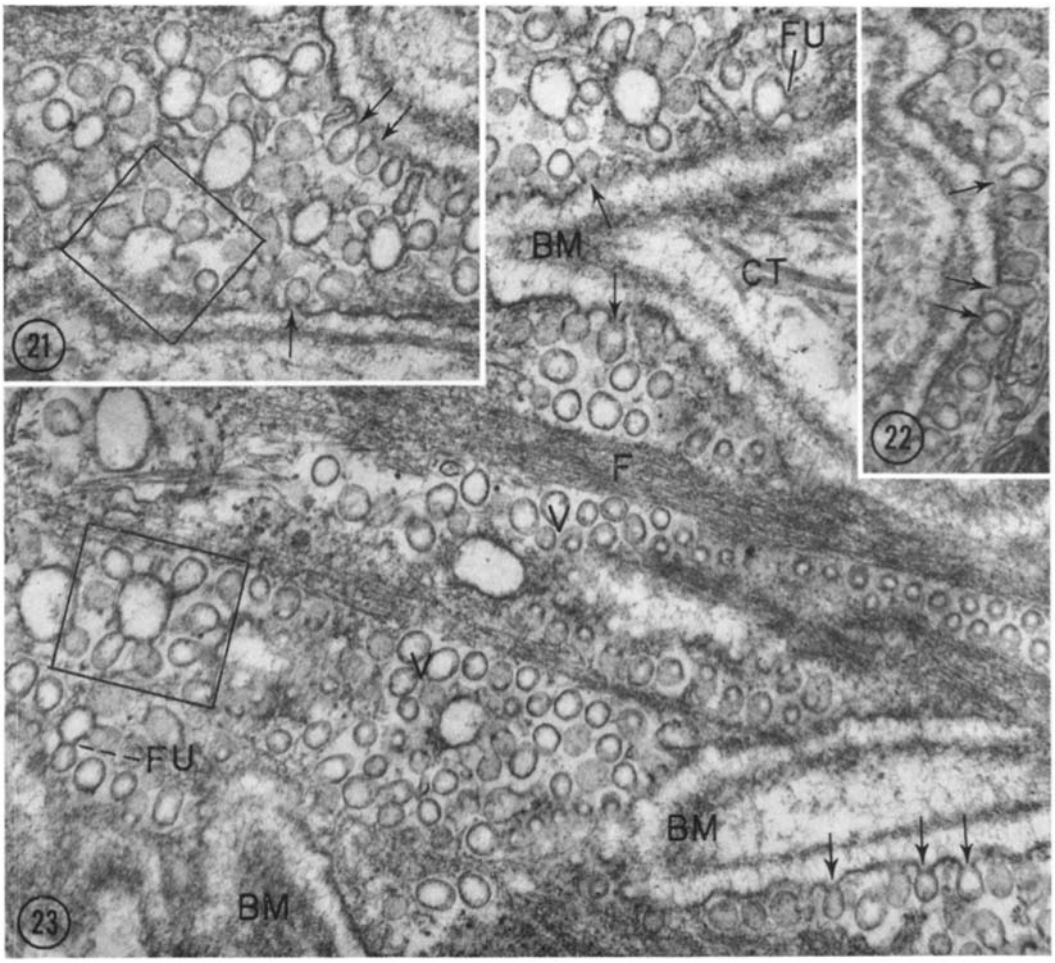



FIGURES 19 and 20 Oocyte-follicle complex ~2 mm in diameter. The Golgi complexes (*GC*) in the serosa cells are illustrated, as are mitochondria (*M*), tonofilaments (*F*), and glycogen (*GY*). The dense, irregularly shaped secretion granule (*SG*) in Fig. 19 appears to have its origin from the Golgi components (Fig. 20, *SG*). The secretory granules are similar in appearance to those present in the follicle cells. Granular endoplasmic reticulum (*ER*). Serosa cell nucleus (*N*). Fig. 19, × 37,500; Fig. 20, × 50,000.

postovulatory follicles show that these follicles are loosely organized, contain connective tissue fibers (probably those of the preovulatory follicle), and are richly vascularized. The maximum diameter of the postovulatory follicles ranges from 1.25 to 1.50 mm. After the egg-laying period in the spring, some of the mature eggs not ovulated appear to undergo degeneration. This activity is accomplished by stellate-shaped cells which invade the oocyte in large numbers (Fig. 24). The postovulatory follicles can be distinguished from atretic follicles on the basis of their coloration and consistency. The postovulatory follicles examined with the electron microscope ranged in size from 1.25 to 1.4 mm. As will become evident, the postovulatory follicle is composed of a number of cell types, each of which is active over a considerable period of time. The description of the fine structure of the postovulatory follicle included here is primarily for the purpose of determining whether this follicle contains cell types similar to those occurring in known steroid-producing organs.

Before the ultrastructure of the cell types encountered in the postovulatory follicle is described, several comments can be made which apply generally to all the cells comprising this follicle. Thus, all the cells have a well developed endoplasmic reticulum (rough and/or smooth) and a large number of lipid droplets and mitochondria. Many of the cells are filled with small, dense granules approximately 250 A in diameter, which appear similar to particulate glycogen, as well as intracytoplasmic filaments.

The most frequently encountered cell type in the postovulatory follicle is characterized by an entensive, centrally located Golgi complex consisting of flattened saccules and both bristle-coated and uncoated vesicles (Fig. 25). Dense granules in




FIGURES 21–23 Portion of capillary endothelial cells in theca. Oocyte-follicle complex ~3 mm in diameter. Note the presence of large numbers of intracytoplasmic microvesicles (*V*). Fusion of microvesicles is indicated at *FU*. Many of the microvesicles appear continuous with the plasma membrane of the endothelial cell (arrows). Microvesicular rosettes (inside squares) are commonly present in the endothelial cells and occasionally are continuous with the plasma membrane (Fig. 21). Intracytoplasmic filaments (*F*), basement membrane (*BM*), and connective tissue fibers of the theca (*CT*) are identified. × 37,500.

a variety of shapes and sizes are closely associated with the Golgi components and, in fact, some of the Golgi cisternae contain a product similar in appearance to the isolated granules (Fig. 25). Elements of the rough-surfaced endoplasmic reticulum and lipid droplets surround the Golgi elements in this cell and occupy most of the remainder of the cell (Fig. 26). In some instances, the cisternae of the rough endoplasmic reticulum are dilated by a finely granular product of medium electron opacity.

Another cell type in the postovulatory follicle contains, in addition to the components already mentioned, a predominantly smooth form of the endoplasmic reticulum (Figs. 27–29). In some regions of this cell, these membranes are arranged into concentric whorls (Fig. 27). In these regions, as well as elsewhere in the cytoplasm, the short cisternae of agranular reticulum are expanded and contain a dense secretory product (Figs. 27, 28). Only a small amount of granular endoplasmic reticulum is present in this cell. What appears to

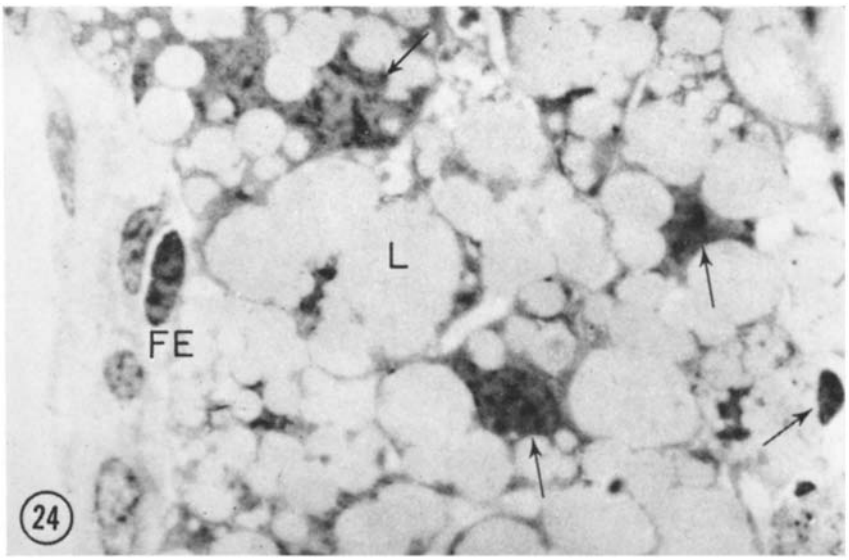



FIGURE 24 Atretic egg. Follicle envelope (*FE*) is present at left in figure. Note that numerous, stellate-shaped cells (arrows) have invade the ooplasm which contains large numbers of lipid droplets (*L*). Epon section. Azure II, methylene blue stain. × 1500.

represent the mature formed product of this cell is illustrated in Fig. 29.

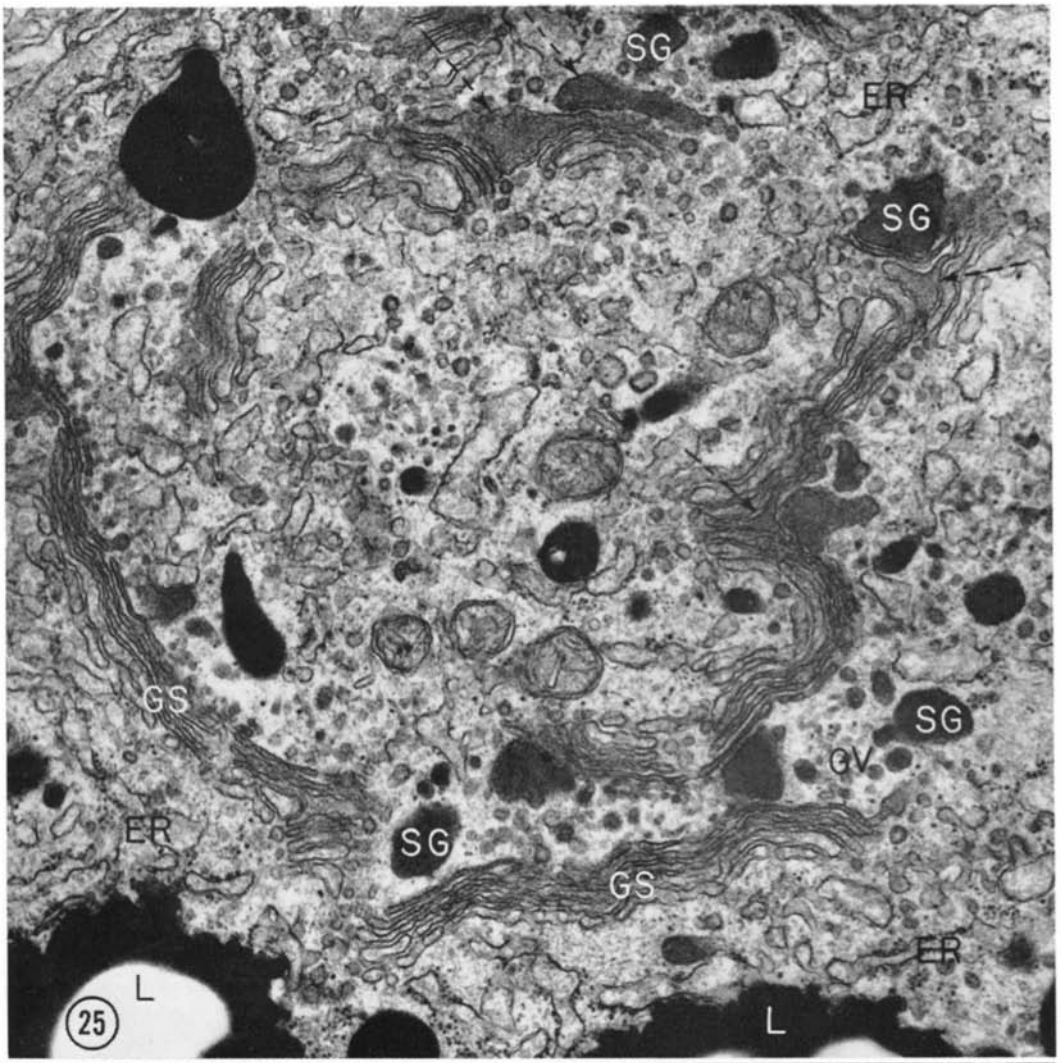
A third cell type in the postovulatory follicle is illustrated in Figs. 30 and 31. These cells contain an extensive Golgi complex and agranular reticulum. They are also characterized by numerous inclusion bodies which become extremely large and dense. The large inclusion bodies appear to result from the fusion of smaller granules (Fig. 31). Lipid and particulate glycogen are also present.

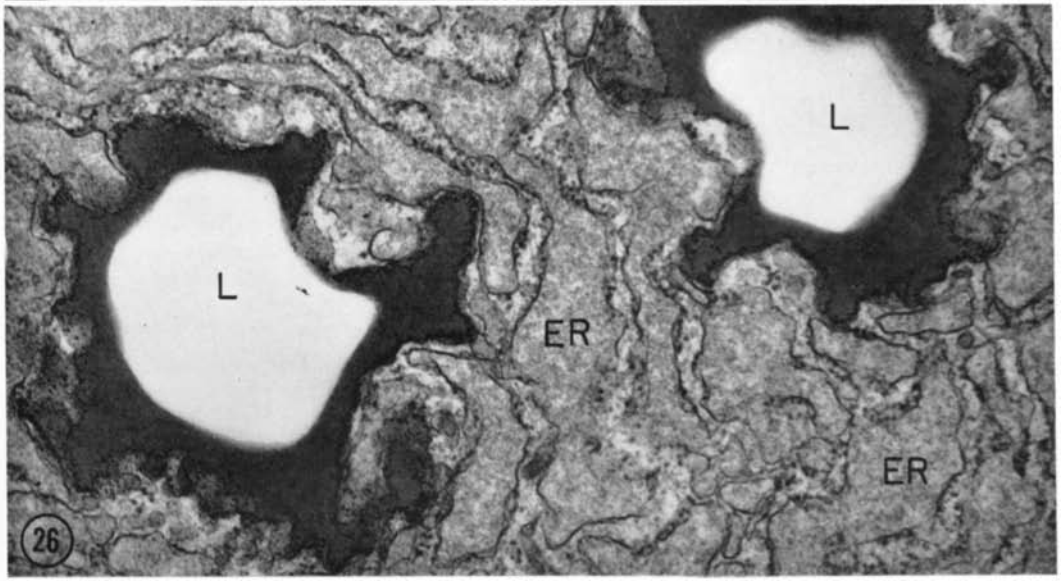
Occasionally, cells are encountered in the postovulatory follicle as illustrated in Fig. 32 which contain predominantly granular endoplasmic reticulum. Dense, homogeneous granules are present within the cisternae of this reticulum. These dense intracisternal granules vary in size and number, but typically are surrounded by a more loosely organized granular material.

Finally, a cell type such as that illustrated in Fig. 33 is occasionally observed. These cells contain large lipid droplets and numerous, small dense bodies some of which are comparable to lysosomes in structure. In this connection, it should be indicated that structures which could be identified as lysosomes were rarely found in the previous cell types during the period of activity described.

It is apparent from the description of the fine structure of the postovulatory follicle that considerable differentiation occurs in the cells of the follicle envelope as they transform into cells of the postovulatory follicle. Furthermore, the cells of the postovulatory follicle are much more active than those of the preovulatory follicle in terms of synthetic activities, as judged from the increase in the extent of their organelle systems and the increase

FIGURES 25 and 26 Postovulatory follicle. The peripheral portion of this cell type contains lipid droplets (*L*) and an extensive development of granular endoplasmic reticulum (*ER*), the cisternae of which are dilated by a homogeneous product. The central portion of the cell contains an extensively developed Golgi complex consisting of Golgi saccules (*GS*) and vesicles (*GV*). The origin of the secretory granules (*SG*) appears to be associated with the Golgi complex since some of the Golgi cisternae are filled and expanded by a dense, homogeneous product (arrows) similar in appearance to the isolated secretory granules. Fig. 25, × 29,700; Fig. 26, × 37,500.


SG
ER
SG
GS
SG
GV
ER
SG
GS
ER
L
25
L


L
L
ER
ER
26

in the amount and kind of formed product observed in the cytoplasm of these cells.

### Cytochemistry

That all cell types found in the *Necturus* follicle envelope accumulate lipid droplets during the course of oogenesis was illustrated both in photomicrographs of histological preparations and in electron micrographs. The accumulation of lipid in the follicle envelope is also demonstrated with Nile blue staining. Thus, when follicle envelopes are mechanically isolated from oocytes less than 1 mm in diameter and either the fresh preparations or frozen sections are stained with Nile blue, no red-staining droplets characteristic of neutral lipids are observed in the cells (Fig. 34). However, all follicle envelopes removed from oocytes ranging from 2.5 to 5 mm in diameter showed large numbers of reddish-stained droplets in their cells, these results indicating the accumulation of large quantities of neutral lipid in the follicle (Figs. 35, 36). Follicle envelopes prepared by methods which remove lipid show follicle cells with a highly vacuolated cytoplasm (Fig. 13). Large numbers of red- to pink-staining globules are also present in some of the cells of the postovulatory follicle (Figs. 37, 38). The purple coloration noted in some cells of the postovulatory follicle suggests that acidic lipids are also present in addition to neutral lipids.

Fresh-frozen sections of postovulatory follicles, stained with the Schultz method for the identification and demonstration of cholesterol and cholesteryl esters, contain numerous blue-green colored granules characteristic of a positive reaction (Fig. 39). On the other hand, fresh-frozen sections of follicle envelopes removed from oocytes less than 1.5 mm in diameter did not give a positive reaction for cholesterol. However, similar preparations of follicle envelopes removed from oocytes nearing maturity did contain some blue-green granules characteristic of a positive reaction.

Application of the Gomori method for the demonstration of alkaline phosphatase was made on the follicle envelope at different periods of oogenesis. The results of this technique indicate that large amounts of alkaline phosphatase are localized in the follicle envelope (Fig. 41). While all regions of the envelope appear to contain the enzyme, the reaction is particularly intense in the follicle cells (Fig. 42). The intensity of the reaction appears greater in follicle cells surrounding oocytes <3 mm in diameter than in those greater than 3 mm in diameter.

### Soluble Proteins

With the technique of polyacrylamide-gel electrophoresis, an analysis was made of the soluble proteins occurring in the follicle envelope during oogenesis. This analysis was performed on follicle envelopes mechanically isolated from oocytes less than 1 mm in diameter, from oocytes 4–5 mm in diameter, as well as on the postovulatory follicle. Photomicrographs of these three gels are illustrated in Figs. 43–45. With the techniques employed, 22 distinct soluble-protein bands were detected in the original gels of follicle envelopes removed from immature oocytes. In contrast, 14 distinct soluble-protein bands were detected in the original gels of follicle envelopes removed from nearly mature oocytes. In the postovulatory follicle, 18 distinct soluble-protein bands were detected in the original gels. Changes in the migration of many of the soluble proteins in the follicle envelope during oogenesis is evident in Figs. 43–45.

## *Radioautography*

The incorporation of tritium-labeled uridine and amino acids was used as a measure of RNA and protein biosynthesis in the follicle envelope.

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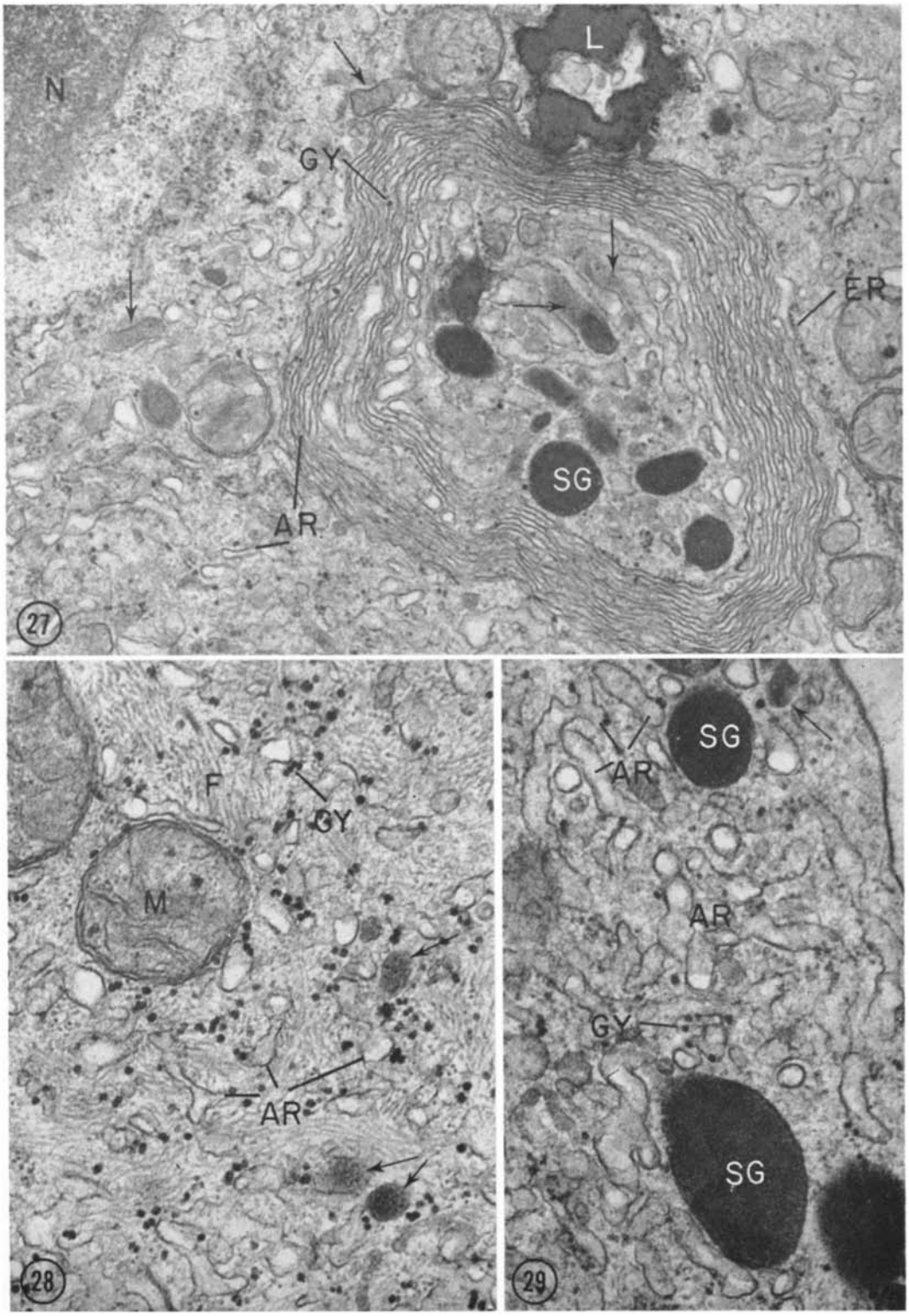
Figures 27–29 Postovulatory follicle. Another cell type encountered in the postovulatory follicle contains extensive amounts of a branching and tubular form of smooth-surfaced endoplasmic reticulum (*AR*). A concentric array of smooth membranes is illustrated in Fig. 27. Spherical or oval dense granules representing the formed product of this cell are illustrated in Figs. 27 and 29 (*SG*). The unlabeled arrows direct attention to those regions in which the forming secretory product appears to be located within the cisternae of the smooth reticulum. Small, dense granules (probably glycogen) are numerous (*GY*), as are the mitochondria (*M*). Only a small amount of the rough-surfaced endoplasmic reticulum is present (*ER*). Lipid (*L*). Nucleus (*N*). Intracytoplasmic filaments (*F*). Fig. 27, × 34,000; Figs. 28 and 29, × 50,000.


N
L
GY
ER
SG
AR
27
F
GY
M
AR
28
SG
AR
AR
GY
SG
29

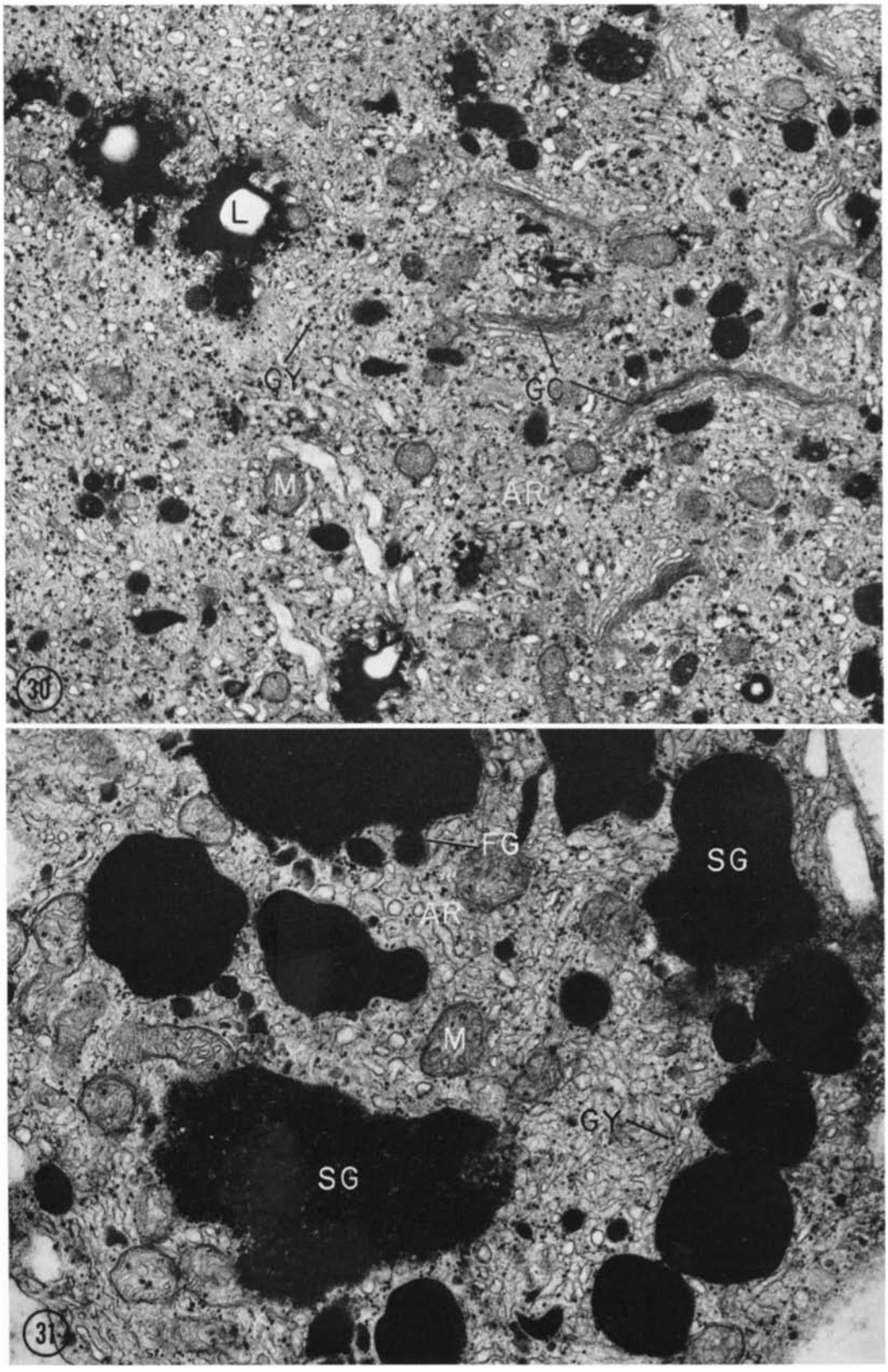
Figs. 46–49 illustrate an intense labeling of the follicular envelope with uridine-$^3$H. Fig. 46 illustrates an oogonium as well as two fibroblast nuclei during a very early stage in the formation of the follicle envelope. After 2 hr of in vitro incorporation of uridine-$^3$H, an intense labeling is apparent over the oogonial nucleus and perinuclear cytoplasm. Furthermore, numerous developed silver grains are apparent over the nuclei and cytoplasm of the fibroblast cells. Labeling of the cells of the follicle envelope, especially the follicle cells, with uridine-$^3$H under in vitro conditions remains high as oogenesis proceeds. The oocyte-follicle complexes illustrated in Figs. 47–49 range up to 1 mm in diameter; whereas lipid yolk is already present in such oocytes, protein yolk deposition has not yet begun (49). In Fig. 49, it is apparent that the intensity of the labeling with uridine-$^3$H per unit area over the follicle cell and fibroblast nuclei approaches that apparent over the oocyte nucleus. The cells of the follicle envelope are also labeled with uridine-$^3$H under in vivo conditions. In fact, the cells of the follicle envelope are labeled with uridine-$^3$H under both in vivo and in vitro conditions for a considerable period of oogenesis. In the oocyte-follicle complexes some variation in the intensity of labeling is apparent among oocytes as well as among follicle cells in a single follicle. In some complexes little labeling of the follicle envelope occurs with uridine-$^3$H, whereas in others some of the cells comprising the follicle envelope are more intensely labeled than others. The variation in labeling patterns noted among some of the oocytes may reflect, in part, the fact that not all of the oocytes are in the same state of activity. In general, the labeling of the follicle envelope with uridine-$^3$H is not so significant in those oocyte-follicle complexes nearing the end of their growth period (i.e., 3.5–5 mm in diameter) as was demonstrated during the earlier period of oogenesis. Thus, in oocyte-follicle complexes 4–5 mm in diameter, an occasional follicle cell, theca cell, or serosa cell may have a considerable amount of label associated with it, under both in vitro and in vivo conditions, but the remainder of the cells in the envelope are not heavily labeled.

The cells of the follicle envelope also appear capable of incorporating leucine-$^3$H and lysine-$^3$H under both in vivo and in vitro conditions, but the pattern of labeling differs somewhat from that of uridine-$^3$H. The variations in the pattern of labeling which have been observed with uridine-$^3$H are also apparent with the tritium-labeled amino acids. In general, during the first half of oogenesis (i.e., oocyte-follicle complexes $<3$ mm), labeling of the follicle envelope, especially the follicle cells, is at a low level as judged from the small number of grains usually located over the envelope. However, as the oocyte-follicle complex increases in size from $\sim 3.5$ to 5.0 mm in diameter, the number of developed silver grains located over many of the cells in the follicle envelope increases considerably in number under both in vitro and in vivo conditions, as illustrated in Fig. 50. In this radioautograph, a fairly intense labeling of the follicle cells, theca cells, and serosa cells with leucine-$^3$H is apparent under in vitro conditions.

### *Thin-Layer Chromatography*

Tracings of thin-layer chromatograms of lipids extracted from postovulatory follicles are illustrated in Fig. 51. Six different solvent systems were used in an attempt to identify the lipids and to determine whether a steroid material was present in the postovulatory follicle. The possible identification of the materials separated with the different solvents is indicated in Table I. The identification of the compounds is based on the Rf values of the spots as compared to Rf values listed for various materials by Randerath (68) and Stahl (82). It can be noted that a number of the spots obtained with the steroid solvent systems do have Rf values comparable to those of several different steroids. Because of the limitations inherent in the identification of steroids by using Rf values and color reagents applied to thin-layer chromatographic

FIGURES 30 and 31 Postovulatory follicle. Third cell type is characterized by an extensive development of smooth endoplasmic reticulum (*AR*). Golgi complexes are numerous (*GC*). Specific secretory granules (*SG*) vary widely in size probably through fusion of smaller secretory granules (*FG*). Small dense granules (arrows) are concentrated around lipid droplets (*L*), but they are also widely dispersed in the cytoplasm (*GY*). Mitochondria (*M*). Fig. 30, × 19,000; Fig. 31, × 25,200.


L
GY
GC
M
AR
30
FG
SG
AR
M
GY
SG
31

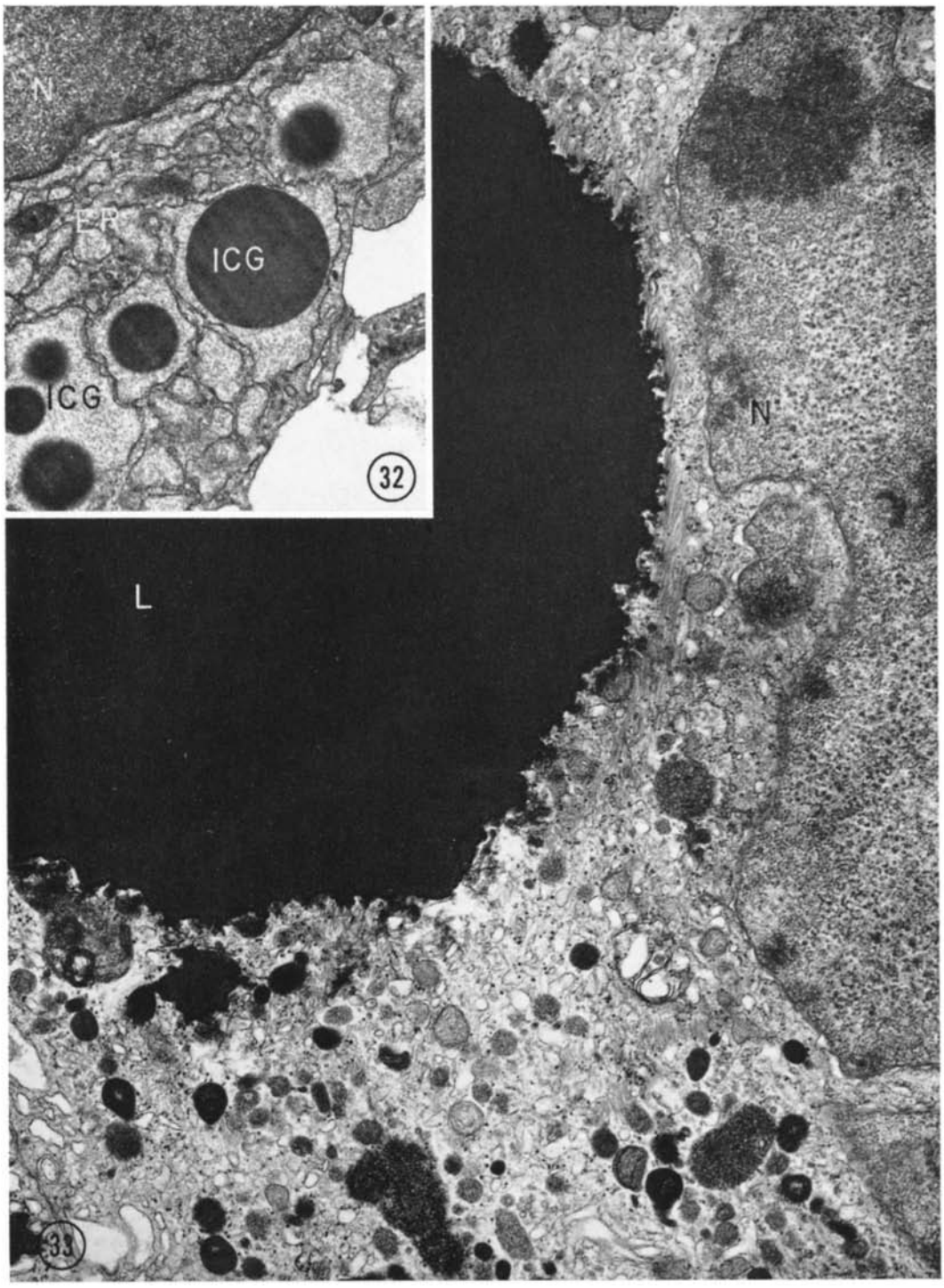



FIGURE 32 Postovulatory follicle. The cell type illustrated here is only occasionally encountered in the postovulatory follicle and contains granular endoplasmic reticulum (*ER*), the cisternae of which are expanded with intracisternal granules (*ICG*). Nucleus (*N*). × 15,750.

FIGURE 33 Postovulatory follicle. This cell type is characterized by extremely large lipid droplets (*L*) and by granules, some of which have the appearance of lysosomes. Nucleus (*N*). × 19,000.

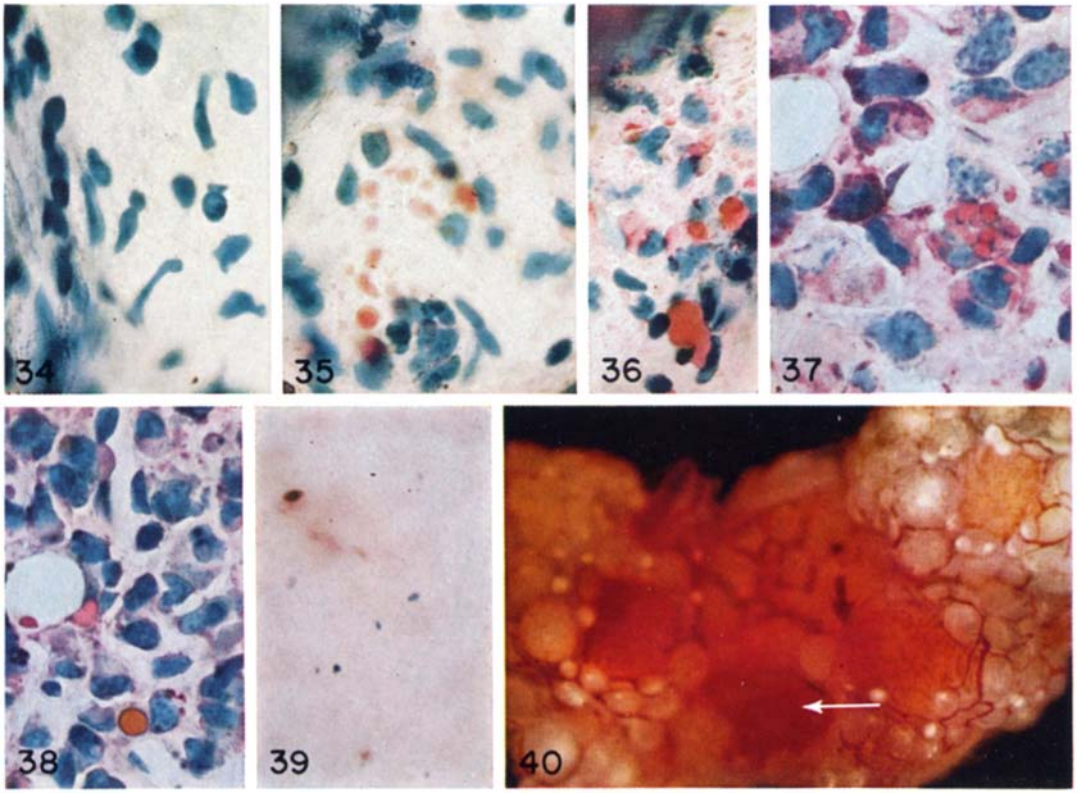



FIGURE 34 Whole mount preparation of follicle envelope removed from oocyte 1 mm in diameter and stained with Nile blue. Note absence of reddish-staining globules and hence of neutral lipid. × 400.

FIGURES 35–36 Whole mount preparations of follicle envelopes removed from oocyte 3–5 mm in diameter and stained with Nile blue. Large number of reddish-staining globules are evident in the cells of the follicle envelope, indicating presence of neutral lipid. × 400.

FIGURES 37–38 Frozen sections of postovulatory follicle stained with Nile blue. × 600.

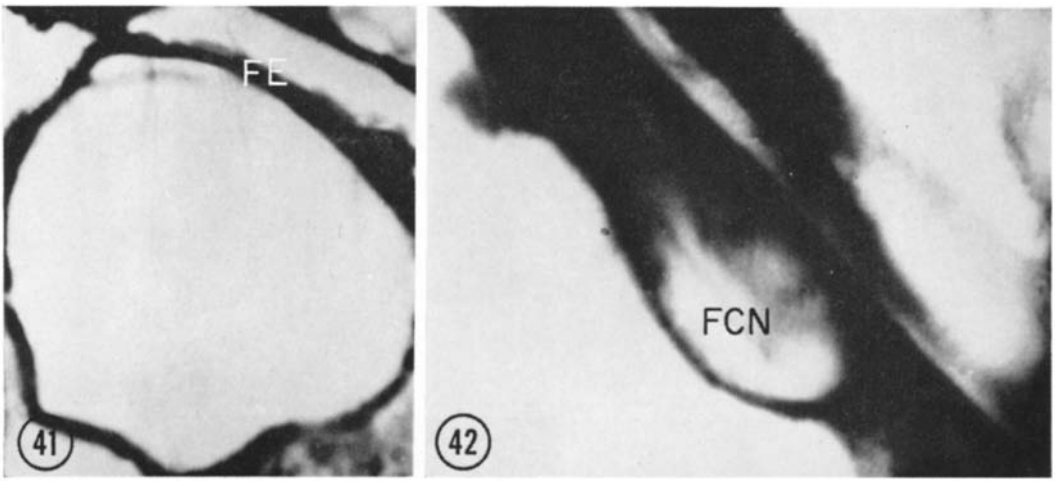
FIGURE 39 Postovulatory follicle, frozen section, stained for demonstration of cholesterol. Cholesterol is indicated in the bluish-green stained granules. × 400.

FIGURE 40 Gross appearance (unstained) of a *Necturus* ovary. Postovulatory follicles are reddish-orange in color (arrow). × 8.

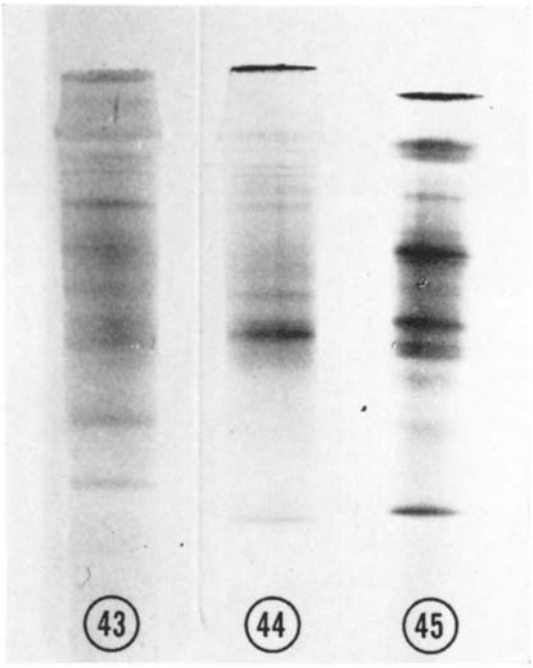
separations (see 68, 82), the results can be considered as only suggestive evidence for the presence of steroids in the postovulatory follicle.

### *Steroid Dehydrogenase Activity*

The results of the cytochemical localization of 3 β-hydroxysteroid dehydrogenase (3 β-HSD) activity in the postovulatory follicle are illustrated in Figs. 52 and 53. In general, the reaction product appeared finely granular (Fig. 53), but on occasion large purple-staining granules were evident. According to Wattenberg (88), this coloration indicates the most intense activity. In addition, large lipid droplets were commonly encountered which had a number of small, purple-staining granules attached to their surface (Fig. 52). Treatment of frozen sections of postovulatory follicles with acetone prior to incubation in the substrate resulted in the removal of lipids and enzyme activity. In fact, much of the enzyme activity appears to be




FIGURES 41–42 Frozen sections of follicle envelope from oocyte ~0.75 mm in diameter which have been incubated for alkaline phosphatase reaction. Note intense reaction in entire follicle envelope (*FE*) in Fig. 41. The reaction is particularly intense in follicle cells (Fig. 42). Follicle cell nucleus (*FCN*).




FIGURES 43–45 Polyacrylamide gel electrophoretograms of soluble proteins extracted from follicle envelopes of immature oocytes (<1 mm in diameter) in Fig. 43, from follicle envelopes of nearly mature oocytes in Fig. 44, and in the postovulatory follicle in Fig. 45.

associated with the lipid droplets in the postovulatory follicle, since treatment of the frozen sections with acetone following incubation in the steroid substrate resulted in the disappearance of most of the lipid droplets as well as most of the 3 β-HSD activity. It has previously been shown that a nonspecific deposition of formazan occurs on lipid droplets (52). Although true 3 β-HSD activity is usually stable to acetone, Levy et al. (52) found that 3 β-HSD activity in the theca interna of the rat ovary was acetone labile. Frozen sections of the postovulatory follicle treated for diaphorase activity gave positive results similar to those obtained with 3 β-HSD; however, the reaction for diaphorase was less intense than that obtained for 3 β-HSD.

Attempts were also made to localize more specifically the 3 β-HSD activity in the soluble-protein bands evident after disc electrophoresis on polyacrylamide gels. In the process, the electrophoresed gels were incubated in the steroid substrate (dehydroepiandrosterone). In these preparations, eight narrow bands were apparent in the original gels (Fig. 54). In contrast, only five thin bands were evident in the original electrophoresed gels incubated for diaphorase activity (Fig. 55). Furthermore, in those instances in which the diaphorase- and steroid substrate–reactive soluble-protein bands were identical in position, the intensity of staining of the diaphorase bands was much less (Figs. 54, 55).

In this connection, it should be pointed out that we have recently been concerned with a study of the multiple forms of lactic acid and malic acid dehydrogenases in a variety of vertebrate and

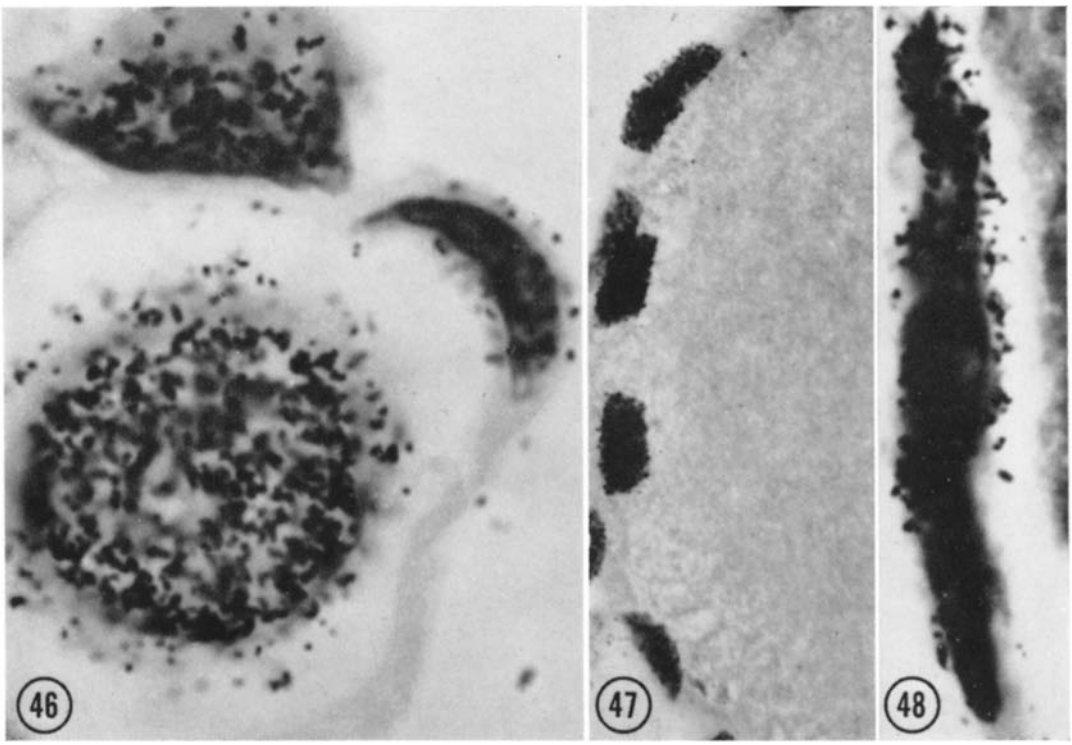



FIGURE 46 Radioautograph of oogonium surrounded by two fibroblast cells during early stage in the formation of follicle envelope. Note intense incorporation of labeled precursor (uridine-$^3$H) into oogonial nucleus and perinuclear cytoplasm as well as into fibroblast nuclei and cytoplasm. In vitro exposure, 11 $\mu$c uridine-$^3$H for 5 hr.

FIGURES 47–48 Follicle cells surrounding oocytes <1 mm in diameter. Follicle cell nuclei are heavily labeled after 22-hr in vitro exposure to 11 $\mu$c uridine-$^3$H. Label also appears associated with narrow rim of follicle cell cytoplasm in Fig. 48.

invertebrate tissues. Our preliminary results have suggested that some of the dehydrogenase enzymes may exhibit a lack of substrate specificity and/or that a substrate is not needed for a positive histochemical reaction. For example, treatment of frozen sections of mouse ovaries for LDH and 3 $\beta$-HSD activity gave similar results for the localization of both enzymes in the tissue. In addition, electrophoretically separated soluble proteins of *Necturus* and mouse ovaries demonstrate an identical localization for both LDH with a sodium lactate substrate and 3 $\beta$-HSD with a dehydroepiandrosterone substrate (cf. 52). Furthermore, treatment of the separated soluble proteins for a diaphorase reaction gave results identical with those obtained in LDH and 3 $\beta$-HSD separations, although the intensity of staining of the bands was greatly reduced. The results suggest that, under the conditions employed, the pyridine nucleotide–linked dehydrogenase either is capable of accepting different substrates or is the result of endogenous NAD reacting with the tetrazolium salt to produce a "nothing" dehydrogenase reaction (cf. 95).

## DISCUSSION

The presence and distribution of intercellular spaces in the *Necturus* follicle envelope are of considerable interest in connection with the problem of the incorporation of materials into the oocyte from sources external to the oocyte and ovary. Intercellular spaces become evident in both the theca and serosa layers very soon after the completed follicle envelope has been established. Intercellular spaces between follicle cells develop by the time the oocyte-follicle complex has

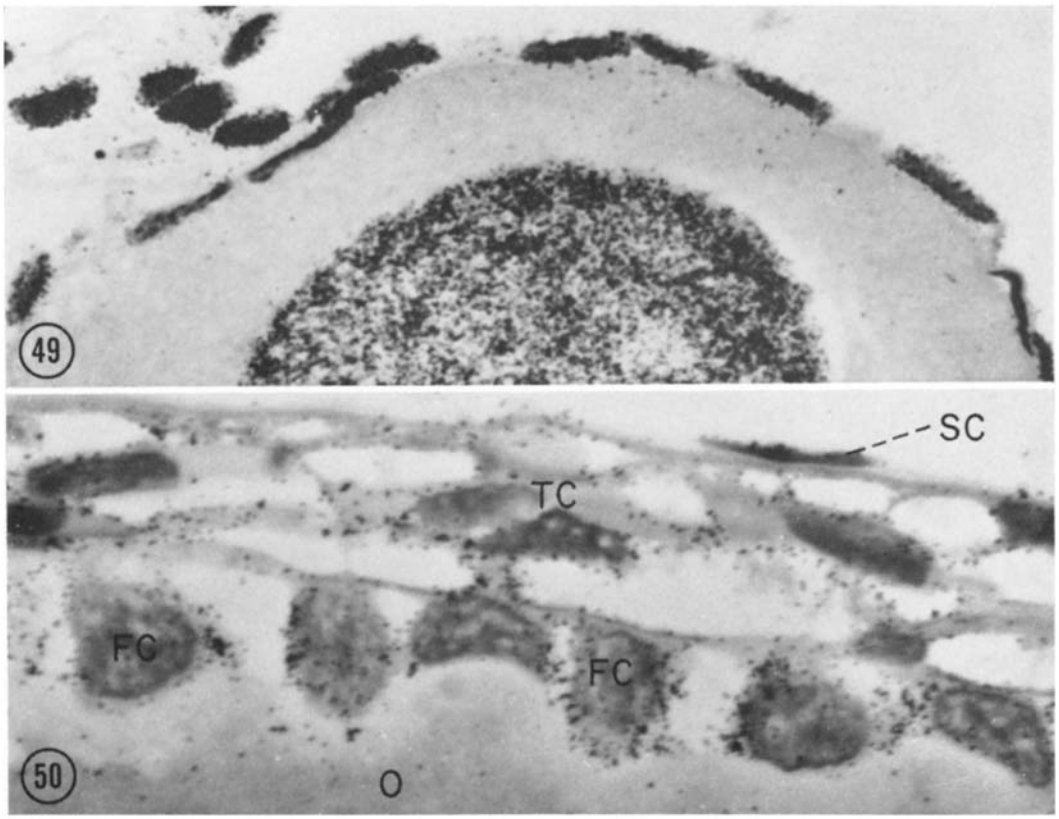



FIGURE 49 Oocyte-follicle complex ~0.75 mm in diameter. Oocyte nucleus, follicle cell nuclei, and ovarian fibroblast nuclei are intensely labeled after 2-hr in vitro exposure to 11 $\mu$c uridine-$^3$H. Little labeling is associated with the oocyte cytoplasm.

FIGURE 50 Oocyte-follicle complex ~4 mm in diameter. Note the label associated with the follicle cells (*FC*) after one-hour exposure in vitro to leucine-4,5-$^3$H. Some of the cells in the theca (*TC*) and serosa (*SC*) are also labeled. Oocyte periphery (*O*). × 2400.

grown to ~1.3 mm in diameter, and they persist throughout most of oogenesis. Since such channels exist, materials destined for passage into the growing oocyte need not necessarily pass through the follicle cells. Attention has been called to the presence of intercellular spaces between the follicle cells in *Triturus* oocytes as well (39). The ubiquity of follicle cell processes, oocyte microvilli, and pinocytotic vesicles associated with the oocyte surface in amphibia has inspired the concept that nutritive materials from the blood capillaries in the theca reach the oocyte by diffusion through the follicle cells and their processes (see 1, 16). While such a pathway may be operable in the *Necturus* follicle envelope, it is most probable that the intercellular spaces in the follicle envelope represent a major pathway for the migration of materials from the capillaries in the theca and from those outside of the follicle envelope to the oocyte surface where these materials are incorporated into the oocyte via micropinocytotic vesicles. The flocculent material present in many of the intercellular spaces of the follicle envelope may represent material coagulated by the fixative while in transit through these spaces. There is evidence to suggest that intact blood proteins are capable of passing through the barrier formed by the follicle cell layer and reaching the oocyte (see 30, 59, 77). In this regard, the results of a soluble-protein analysis on the oocyte, follicle envelope, blood, and liver of *Necturus* are of interest (64). For oocytes ranging from 1.0 to 1.5 mm in diameter, the soluble pro-

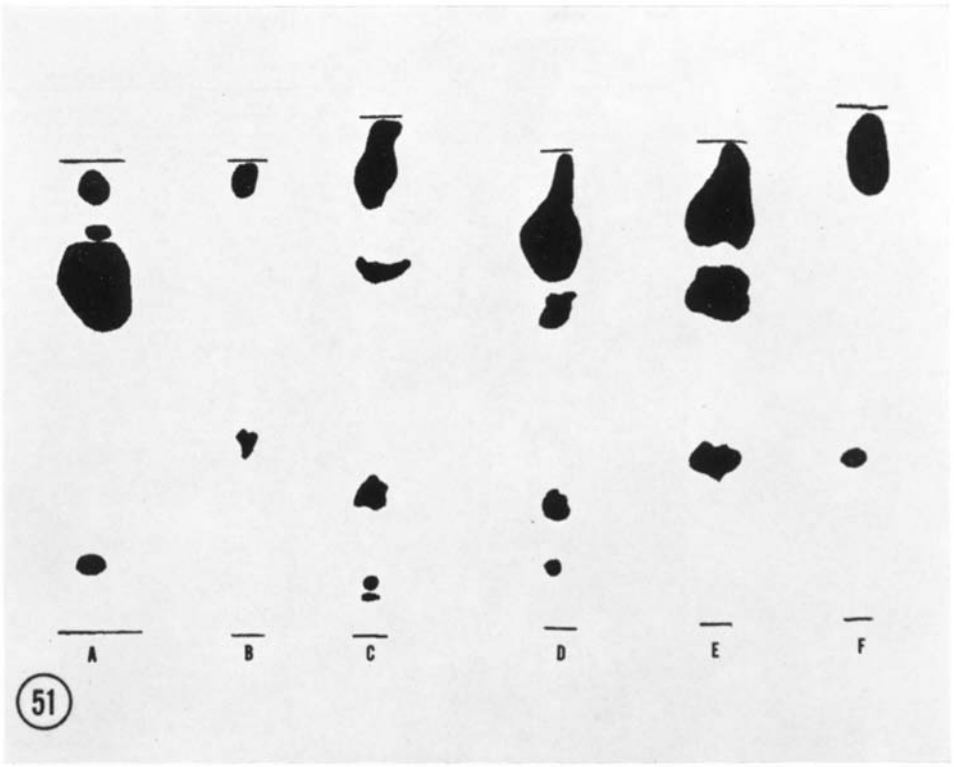



FIGURE 51 Tracings of thin-layer chromatograms of lipids extracted from postovulatory follicles. Solvent for *A*, 90 parts *n*-pentane, 10 parts diethyl ether, and 1 part acetic acid; solvent for *B*, 65 parts chloroform, 34 parts methanol, and 4 parts water; solvent for *C*, 3 parts benzene, 2 parts ethyl acetate; solvent for *D*, 4 parts benzene, 1 part ethyl acetate; solvent for *E*, 4 parts chloroform, 1 part ethyl acetate; solvent for *F*, 9 parts chloroform, 1 part acetone.

teins extracted from either the follicle envelopes or oocytes are present in greatest numbers (64). Furthermore, those soluble proteins isolated from either the follicle envelopes or oocytes during this period of oogenesis are strikingly similar in number and migration to those isolated from the liver or blood (64). Studies on the mechanisms of yolk deposition in the oocyte indicate that this deposition is initiated in oocytes ~1.1 mm in diameter (49). In oocytes ranging from 1.1 to 2.0 mm, the yolk precursor bodies increase considerably in size and number.[1] The results thus suggest that soluble proteins may pass in large amounts from the blood capillaries of the follicle envelope to the developing oocyte at a period of oogenesis in which the initial stages of protein yolk deposition occur. The formation of intercellular spaces in the follicle cell layer at a time closely correlated with the aforementioned activity would appear to be significant in this activity.

That the cells of the follicle envelope are directly and actively engaged in the processes of transport within the envelope is indicated by the large numbers of micropinocytotic vesicles occurring in these cells. Microvesicles described as associated with a transport function have been observed in a number of cells (see, 27, 42, 72, 90). Although some controversy exists regarding the origin, content, and direction of transport by microvesicles, it is likely that these activities vary with the metabolic state of the cell (see 90). Pinocytotic vesicles are most numerous in the serosa cells, theca cells, and capillary endothelial cells of the *Necturus* follicle envelope, and these vesicles are generally of the noncoated or nonalveolate variety. The pinocytotic vesicles associated with the follicle cells, however, are typically of the coated or alveolate variety (see 74), and they are much less numerous.

[1] R. G. Kessel. Unpublished observations.

TABLE I

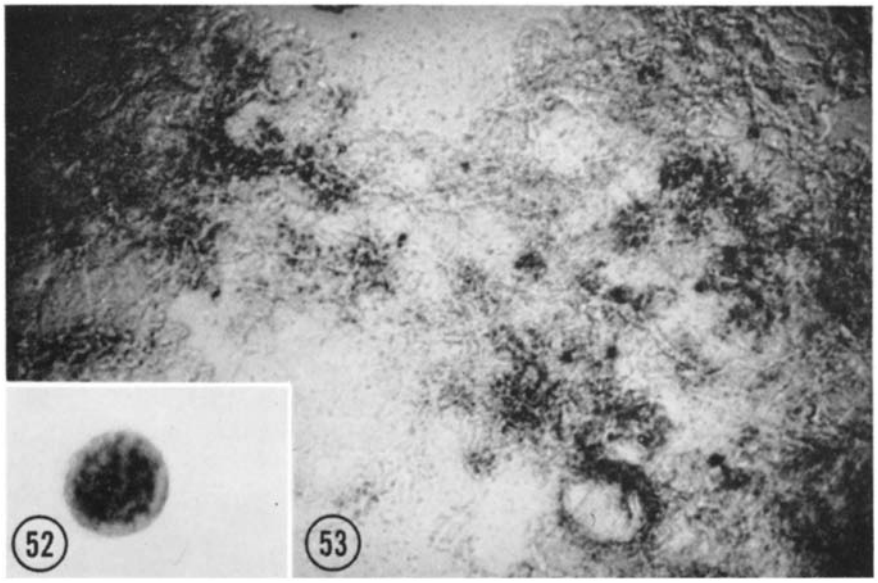
*Chromatography of Postovulatory Follicle*

| | Solvent | Rf × 100 | Possible identification* |
|---|---|---|---|
| A | *n*-Pentane: 90<br>Diethyl ether: 10<br>Acetic acid: 1 | 92<br>83<br>71<br>14 | Sterol esters<br>Triglycerides of nonoxygenated fatty acids<br>Triglycerides or free fatty acids<br>Steroids, cholesterol, or triglycerides containing hydroxy acids |
| B | Chloroform: 65<br>Methanol: 34<br>Water: 4 | 96<br>40 | Neutral lipids<br>Phosphatidyl choline or cephalin |
| C | Benzene: 3<br>Ethyl acetate: 2 | 88<br>69<br>27<br>10<br>8 | Neutral lipids |
| D | Benzene: 4<br>Ethyl acetate: 1 | 83<br>65<br>25<br>13 | Neutral lipids and progesterone<br>Estrone, and/or androstenedione and/or cholesterol<br>5-Androstenediol and/or adrenosterone<br>Pregnanediol |
| E | Chloroform: 4<br>Ethyl acetate: 1 | 83<br>67<br>33 | Neutral lipids<br>Testosterone propionate and/or androstane-3,17-dione and/or progesterone<br>17 $\alpha$-hydroxyprogesterone, and/or androsterone, and/or estradiol |
| F | Chloroform: 9<br>Acetone: 1 | 88<br>31 | Neutral lipids<br>Pregnane-3$\alpha$,20 $\alpha$-diol, and/or estradiol |

* After Stahl (82), for solvents A, B, E, F; after Randerath (68), for solvent D.

Evidence that microvesicles and microvesicular rosettes in fat cells and endothelium may constitute a mechanism for the transport of free fatty acids has been provided by Williamson (90). In their structure and number, the microvesicles and microvesicular rosettes in the capillaries of the *Necturus* follicle envelope are so similar to those described in fat cells that it is tempting to speculate that they may be involved to some extent in the transport of free fatty acids from the blood into the follicle envelope. That such activity appears correlated with the progressive accumulation of lipid in cells of the follicle envelope lends further support to such a viewpoint. However, it is apparent that a number of different molecular species in addition to fatty acids are transported in the capillaries and into the cells of the *Necturus* follicle envelope.

Most of the cytoplasmic differentiation in cells constituting the follicle envelope in *Necturus* takes place as the oocyte-follicle complex grows from ~1.3 to 2.0 mm in diameter. These changes involve, among others, the elaboration of a smooth form of the endoplasmic reticulum. The rough-surfaced endoplasmic reticulum is also present in varying amount in all cells of the follicle envelope throughout oogenesis. The development and presence of both rough and smooth-surfaced forms of endoplasmic reticulum may be related to the biosynthesis of lipid in these cells. All three cell types in the follicle possess Golgi material that appears to be associated with the origin of dense secretory granules which are similar in structure in all cell types comprising the three layers of the follicle envelope. The nature and significance of these secretory granules are unknown. All cells of

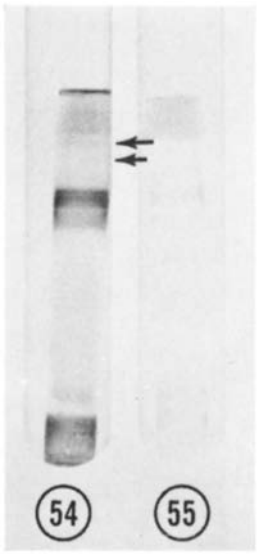



FIGURES 52 and 53 Photomicrographs of frozen sections of postovulatory follicle illustrating reaction product (dark granular material) when assayed for 3 β-HSD activity (Fig. 53). Reaction product is associated with surface of lipid droplet in Fig. 52.

the follicle envelope contain considerable amounts of glycogen, and the accumulation of lipid in all three cell types is particularly active in oocyte-follicle complexes ranging from 1.3 to 2.0 mm in diameter. Thus, the major activities occurring in the preovulatory follicle appear to be the accumulation of lipid and glycogen. These activities proceed during the course of oogenesis so that at its completion most of the follicle cells and many of the serosa and theca cells are filled with lipid droplets. Lipid droplets in generally small amounts have been pointed out in other amphibian follicle cells (see 39). In this connection, it is of interest that in the case of birds the cholesterol content of the follicle epithelium and of the theca interna increases strongly in the last phase of vitellogenesis (56). More recently, it has been demonstrated by Popjak and Tietz (67) that the follicle cells of birds are capable of synthesizing fatty acids and cholesterol from added acetate. Only a small amount of cholesterol was detected in the *Necturus* follicle envelopes at the end of oogenesis. The accumulation of lipid and glycogen in cells of the follicle envelope during oogenesis in *Necturus* appears to be preparation of the cells for their subsequent functional activity as a postovulatory follicle, since cells constituting the postovulatory follicle also contain large amounts of lipid, glycogen, and cholesterol.

Analysis of the number of soluble proteins present in the follicle envelope during oogenesis and in the postovulatory follicle demonstrated a change in the number of soluble proteins in the follicle envelope during development, or a possible change in the solubilities of certain of the proteins. Changes in the patterns of migration of the soluble proteins were also evident. Since soluble proteins have been shown to consist, in part, of enzymes, it is probable that the enzymatic activity of the cells of the follicle envelope may also be changing during the cells' period of development and activity. That this differentiation may, in part, involve loss of the ability to synthesize a pre-existing protein and/or changes in the solubilities of pre-existing proteins has been suggested and discussed by Spiegel (81).

The significance of the intense reaction for alkaline phosphatase obtained in the *Necturus* follicle envelope in general and in the follicle cells in particular may be related to the transport of materials through the follicle envelope. Osawa (62) has reported a strong alkaline phosphatase reaction in the follicle cells of *Triturus*. In a study demonstrating the presence of alkaline phosphatase




FIGURES 54 and 55 Polyacrylamide gel electrophoretograms of soluble proteins extracted from postovulatory follicle. Gel in Fig. 54 was incubated for 3 β-HSD activity. Gel in Fig. 55 was incubated in same medium but without substrate. Note faint bands (arrows) in Fig. 54.

in the follicle cells of *Chirocephalopsis*, Linder (53) commented that the presence of the enzyme in the follicle is probably related to the follicle's function as a selective membrane controlling the passage of food substances across cell boundaries from the blood to the oocyte.

Based on either cytochemical or radioautographic techniques, it has been suggested that follicle cells in general are rich in RNA (see 69). The radioautographic studies of Ficq (29) indicate the occurrence of DNA synthesis and protein synthesis in follicle cells in other amphibian species. The results of the incorporation of uridine-$^3$H into the *Necturus* follicle envelope suggest that the follicle envelope is capable of the biosynthesis of RNA. That synthesis of RNA rather than accumulation of RNA occurs in the follicle envelope is suggested by the results of the in vitro studies and the treatment of the radioautographs with trichloroacetic acid. Since the radioautographic procedures utilized probably removed the soluble RNA, the incorporation of uridine-$^3$H would appear to occur in ribosomal and perhaps some messenger RNA. The results also indicate that the incorporation of amino acids into proteins occurs in the follicle envelope especially during the later part of oogenesis. The labeling of the follicle cells late in oogenesis may be related to the appearance of yolk platelets in some, but not all, of the follicle cells as demonstrated in the electron micrographs. The radioautographic results thus provide evidence that in *Necturus* the cells of the follicle envelope actively take up uridine, probably into RNA, and later in development are more active in the uptake of protein precursors. Quantitative information regarding the amount of RNA and proteins in isolated amphibian follicle envelopes has only recently become available. Panje and Kessel (64) have recently reported the results of a quantitative analysis of RNA and protein in isolated follicle envelopes of *Necturus* during selected stages of oogenesis. This study showed that in follicle envelopes removed from oocytes 0.7–2.5 mm in diameter there is a rapid rise in the amount of RNA but only a small increase in total protein during this period of oogenesis. In follicle envelopes removed from oocytes ranging from 2.5 to 4.0 mm in diameter, the total RNA continues to increase slowly; but there is, in contrast, a sharp increase in the total protein in the isolated follicle envelopes at these same stages. These quantitative results (64) correlate with the demonstration that the follicle cells intensely incorporate uridine-$^3$H especially during the first half of oogenesis (based on oocyte size) whereas incorporation of leucine-$^3$H and lysine-$^3$H in the follicle cells is particularly high during the latter half of oogenesis.

There is no information regarding the kinds or relative amounts of RNA in the *Necturus* envelope. Thus, it would be of interest to determine whether or not sRNA, mRNA, and rRNA are all present in the follicle cells, what their relative amounts are, and whether or not they vary in amount during the growth period of the oocyte. Nace and Lavin (59) have suggested the possibility that the synthetic machinery (e.g., messenger RNA) may be formed in the amphibian follicle cell nuclei and transported into the oocyte to take part in autosynthetic processes associated with yolk deposition. This activity, they postulate, is consistent with evidence of intense uptake of nucleic acid and protein precursors in the follicle cells (29) which do not appear to accumulate the products of the synthesis implied by such uptake. Although several studies have failed to detect the presence of mRNA in amphibian oocyte nuclei or cytoplasm (11, 12, 19, 32), a more recent study by Davidson et al. (20) reports the presence of mRNA in *Xenopus*

oocytes from which the follicle envelopes had been mechanically removed.

During the process of yolk utilization or demolition in various embryological stages of the frog, Karasaki (44) described a condition in which the yolk platelet became progressively surrounded by membranous whorls. As a result of this process, the main body of the yolk platelet was eventually transformed into large concentric membranous structures having the appearance of myelin figures. Karasaki (44) has proposed that these membranes, which are 70 A thick and show a unit membrane structure, are utilized during the course of embryogenesis for the formation of various cytomembranes needed during differentiation. In the present study, when the yolk platelets were observed within the follicle cells, they were surrounded by varying numbers of concentric membranes similar to those suggested as resulting from the demolition of the yolk platelet. Furthermore, the demolition process appears to involve all of the yolk platelets since none were observed in the cells of the postovulatory follicle. In view of the increased differentiation which the cells of the postovulatory follicle undergo compared to their condition in the preovulatory follicle, it is tempting to speculate that the accumulation of yolk in some cells of the follicle envelope late in oogenesis may be associated with their preparation to eventually become highly active in secretion, a process that involves a much more extensive development of cytomembrane systems in those cells of the postovulatory follicle than was the case in the preovulatory follicle cells.

A matter of considerable interest concerns the problem of whether or not the *Necturus* postovulatory follicle is capable of synthesizing steroid and playing a role in the regulation of ovarian activity. Several lines of evidence suggest that the postovulatory follicle may act, among other functions, as a steroid-producing organ: (*a*) studies based on thin-layer chromatography of lipids extracted from the postovulatory follicle; (*b*) cytochemical studies for the localization of 3 $\beta$-HSD activity and the application of this reaction to soluble proteins separated by polyacrylamide gel electrophoresis; (*c*) the suggested presence of cholesterol (prerequisite for steroid biosynthesis) in the postovulatory follicle based on techniques of thin-layer chromatography and cytochemistry; (*d*) the fine structure of certain cells comprising the postovulatory follicle; and (*e*) the fact that the postovulatory follicle is formed as a result of ovulation and then engages in pronounced cellular activity for a period of time is reminiscent of the activity associated with the mammalian corpus luteum.

The smooth-surfaced endoplasmic reticulum is characteristically a well developed cytomembrane system in known steroid-producing cells. Thus, a tubular form of smooth-surfaced endoplasmic reticulum is encountered in the corpus luteum (5, 25, 26, 93), in the interstitial cells of the testis (17, 18), and in the adrenal cortex (51, 54, 73). In addition, steroid-producing cells are characterized by the presence in the cytoplasm of mitochondria, Golgi complexes, lipid, and lysosomes (see 54). In general, the rough endoplasmic reticulum is not so well developed as the smooth form. The point to be made here is that the postovulatory follicle of *Necturus* does contain cells which, on the basis of kind, number, and disposition of intracellular organelles and formed product, resemble known steroid-producing cells in other animals. In general, no visible accumulation of secretory product occurs in cells known to produce steroid hormones, and the process whereby steroid hormones are secreted is poorly understood. However, Belt et al. (2) have reported electron-opaque granules bounded by a smooth membrane within the cytoplasm of pelican adrenal cells. Granules of similar dimensions and electron opacity but without the membrane were observed between adjacent parenchymal cells and in the subendothelial space. Belt et al. (2) suggested that the dense granules represent hormone which is synthesized within the smooth membranes, carried to the cell surface, and secreted by a process of reverse pinocytosis.

Much of the cholesterol and cholesterol ester in adrenal cortical cells is located in lipid droplets as indicated by the Schultz reaction (see 22). Although cholesterol in certain steroid-producing cells may be stored in solution in the neutral fat droplets, there is also evidence that the extensive agranular reticulum in steroid-producing cells is involved with the storage and metabolism of cholesterol in relation to the biosynthesis of steroid hormones (see 28). Biochemical studies on a variety of cell types indicate that the enzymes required for the synthesis of triglyceride and phospholipids are associated with the rough- and smooth-surfaced elements of the endoplasmic reticulum (see 83, 10, 78, 23, 41). The enzymes catalyzing fatty acid esterification thus appear to be bound to the

membranes of the endoplasmic reticulum, so that this activity must occur at, or very near, the membrane (83). The subsequent step, in which the triglyceride molecules aggregate into a visible lipid droplet, has been localized to the intracisternal portion of the endoplasmic reticulum, and the growth of lipid droplets has been suggested to occur by intracisternal accumulation and fusion of smaller droplets (83). The biochemical pathways in the formation of steroids and the subcellular localization of the enzymes involved have been studied by Popjak and Cornforth (66), Olson (61), Halkerston et al. (34), Byer and Samuels (13), Hoffmann (37), Ryan and Engel (75), Sharma et al. (79), and Hayano and Dorfman (35).

Little information is available regarding the significance of the presence of a large Golgi complex in steroid-producing cells. Since recent biochemical studies have emphasized the importance of steroid conjugates, particularly sulfates, in biosynthetic reactions (14), Long and Jones (54) have suggested "that conjugation of steroid hormones occurs in the Golgi apparatus of adrenocortical cells and that the product is secreted as the more water-soluble sulfate or glucuronide."

Some evidence is available to indicate that steroid biosynthesis occurs in the postovulatory follicles of other amphibians. In histochemical studies for the demonstration of 3 $\beta$-HSD activity, Botte and Cottino (8) have obtained positive reactions in the ovarian follicle and postovulatory follicles of both *Rana esculenta* and *Triturus cristatus*. On the other hand, such histochemical activity could not be demonstrated in either the ovarian follicle or postovulatory follicles in *Xenopus laevis* and *Bufo bufo* (65). Of particular interest with respect to the capability of steroid biosynthesis by the *Necturus* postovulatory follicle are the results of Callard and Leathem (15) who have recently described studies in which ovarian fragments of *Necturus maculosus* were incubated with labeled pregnenolone, progesterone, or testosterone. Their data suggested that both pregnenolone and progesterone could be used as substrates for in vitro ovarian steroid synthesis in *Necturus*.

The functions of the other cell types constituting the postovulatory follicle are unknown. Some of the cells may function in the continued biosynthesis of lipid and cholesterol. Other cells may be capable of phagocytosis and take part in cleaning up the follicle components after ovulation and after the postovulatory follicle has completed its function, as occurs in other vertebrate species (see 36).

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## REFERENCES

1. ANDERSON, E., and H. W. BEAMS. 1960. *J. Ultrastruct. Res.* **3**:432.
2. BELT, W. D., M. N. SHERIDAN, R. A. KNOUFF, and F. A. HARTMAN. 1965. *Z. Zellforsch.* **68**:864.
3. BIER, K. 1962. *Naturwissenschaften.* **49**:332.
4. BIER, K. 1963. *J. Cell Biol.* **16**:436.
5. BLANCHETTE, E. J. 1966. *J. Cell Biol.* **31**:501, 542.
6. BONHAG, P. F. 1955. *J. Morphol.* **96**:381.
7. BONHAG, P. F. 1956. *J. Morphol.* **99**:433.
8. BOTTE, V., and E. COTTINO. 1964. *Boll. Zool.* **31**: 491.
9. BRACHET, J. 1947. *Experientia.* **3**:329.
10. BRINDLEY, D. N., and G. HÜBSCHER. 1965. *Biochim. Biophys. Acta.* **106**:495.
11. BROWN, D. D., and E. LITTNA. 1964. *J. Mol. Biol.* **8**:669.
12. BROWN, D. D., and E. LITTNA. 1964. *J. Mol. Biol.* **8**:688.
13. BYER, K. F., and L. T. SAMUELS. 1956. *J. Biol. Chem.* **219**:69.
14. CALVIN, H. I., R. L. VANDEWIELE, and S. LIEBERMAN. 1963. *Biochemistry.* **2**:648.
15. CALLARD, I. P., and J. H. LEATHEM. 1966. *Gen. Comp. Endocrinol.* **7**:80.
16. CHIQUOINE, A. D. 1960. *Am. J. Anat.* **106**:149.
17. CHRISTENSEN, A. K., and D. W. FAWCETT. 1961. *J. Biophys. Biochem. Cytol.* **9**:653.
18. CHRISTENSEN, A. K., and D. W. FAWCETT. 1966. *Am. J. Anat.* **118**:551.
19. DAVIDSON, E. H., V. G. ALLFREY, and A. E. MIRSKY. 1964. *Proc. Natl. Acad. Sci. U.S.A.* **52**:501.
20. DAVIDSON, E. H., M. CRIPPA, F. R. CRAMER, and A. E. MIRSKY. 1966. *Proc. Natl. Acad. Sci. U.S.A.* **56**:856.
21. DAVIS, B. J. 1964. *Ann. N. Y. Acad. Sci.* **121**:404.
22. DEANE, H. W. 1962. *In* Handbuch der Experimentellen Pharmakologie. 1962. O. Eichler and A. Farah, editors. pt. 1. **14**:1. Springer-Verlag, Berlin.

23. DEDUVE, C., R. WATTIAUX, and P. BAUDHUIN. 1962. *Advan. Enzymol.* **24**:291.
24. DOLLANDER, A. 1956. *Compt. Rend. Soc. Biol.* **150**: 998.
25. ENDERS, A. C. 1962. *J. Cell Biol.* **12**:101.
26. ENDERS, A. C., and W. R. LYONS. 1964. *J. Cell Biol.* **22**:127.
27. FAWCETT, D. W. 1959. *In* The Microcirculation. S. R. M. Reynolds and B. W. Zweifach, editors. University of Illinois Press, Urbana. 1.
28. FAWCETT, D. W. 1963. *In* Intracellular Membranous Structure. S. Seno and E. V. Cowdry, editors Okayama, Japan. *Symp. Soc. Cellular Chem.*, **14**:15.
29. FICQ, A. 1961. Metabolisme de l'oogenese chez les amphibiens. *In* Symposium on germ cells and development. Instit. Intern. Embryol; 1st. Lombardo, Fondazione A Baselli, Milan. 121.
30. FLICKINGER, R. A., and D. E. ROUNDS. 1956. *Biochim. Biophys. Acta.* **22**:38.
31. FOLCH, J., M. LEES, and G. H. SLOANE STANLEY. 1957. *J. Biol. Chem.* **226**:497.
32. GALL, J. G. 1966. *In Natl. Cancer Inst. Monograph.* **23**:475.
33. GOMORI, G. 1952. Microscopic Histochemistry. The University of Chicago Press, Chicago.
34. HALKERSTON, I. D. K., J. EICHHORN, and O. HECHTER. 1961. *J. Biol. Chem.* **236**:374.
35. HAYANO, M., and R. I. DORFMAN. 1962. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. 503.
36. HISAW, F. L., JR., and F. L. HISAW. 1959. *Anat. Record.* **135**:269.
37. HOFMANN, F. G. 1960. *Biochim. Biophys. Actr.* **37**: 566.
38. HOPE, J., A. A. HUMPHRIES, JR., and G. H. BOURNE. 1964. *J. Ultrastruct. Res.* **10**:547.
39. HOPE, J., A. A. HUMPHRIES, JR., and G. H. BOURNE. 1963. *J. Ultrastruct. Res.* **9**:302.
40. HSU, W. S. 1952. *Quart. J. Microscop. Sci.* **93**:191.
41. ISSELBACHER, K. J. 1965. *Federation Proc.* **24**:16.
42. JENNINGS, M. A., V. T. MARCHESI, and H. FLOREY. 1962. *Proc. Roy. Soc. (London), Ser. B.* **156**:14.
43. KARASAKI, S. 1959. *Embryologia.* **4**:247.
44. KARASAKI, S. 1963. *J. Ultrastruct. Res.* **9**:225.
45. KEMP, N. E. 1956. *J. Biophys. Biochem. Cytol.* **2**: 281.
46. KEMP, N. E. 1956. *J. Biophys. Biochem. Cytol.* **2**(4, suppl.): 281.
47. KEMP, N. E. 1958. *Anat. Record.* **130**:324.
48. KEMP, N. E. 1961. *J. Appl. Phys.* **32**:1643.
49. KESSEL, R. G. 1966. *J. Cell Biol.* **31**:148A. Abstr.
50. KOPRIWA, B. M., and C. P. LEBLOND. 1962. *J. Histochem. Cytochem.* **10**:269.
51. LEVER, J. D. 1955. *Am. J. Anat.* **97**:409.
52. LEVY, H., H. W. DEANE, and B. L. RUBIN. 1959. *Endocrinology* **65**:932.
53. LINDER, H. J. 1959. *J. Morphol.* **104**:1.
54. LONG, J. A., and A. L. JONES. 1967. *Am. J. Anat.* **120**:463.
55. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.
56. MARZA, V. D., and E. V. MARZA. 1935. *Quart. J. Microscop. Sci.* **78**:133.
57. MASUI, Y. 1967. *J. Exptl. Zool.* **166**:365.
58. MAZIA, D., P. A. BREWER, and M. ALFERT. 1953. *Biol. Bull.* **104**:57.
59. NACE, G. W., and L. H. LAVIN. 1963. *Am. Zool.* **3**:193.
60. NANDI, J. 1967. *Am. Zool.* **7**:115.
61. OLSON, J. A. 1965. *Ergb. Physiol.* **56**:173.
62. OSAWA, S. 1951. *Embryologia* **2**:1.
63. PALADE, G. E. 1952. *J. Exptl. Med.* **95**:285.
64. PANJE, W. R., and R. G. KESSEL. 1968. *Exptl. Cell Res.* In press.
65. PESONEN, S., and J. RAPOLA. 1962. *Gen. Comp. Endocrinol.* **2**:425.
66. POPJAK, G., and J. W. CORNFORTH. 1960. *Advan. Enzymol.* Interscience Publishers, Inc., New York. **22**:281.
67. POPJAK, G., and A. TIETZ. 1953. *Biochiem. J.* **54**: 590.
68. RANDERATH, K. 1965. Thin-Layer Chromatography. Academic Press, Inc., New York.
69. RAVEN, C. P. 1961. Oogenesis: The Storage of Developmental Information. Pergamon Press, New York.
70. REVEL, J. P. 1964. *J. Histochem. Cytochem.* **12**:104.
71. REYNOLDS, E. S. 1963. *J. Cell Biol.* **17**:208.
72. RHODIN, J. A. G. 1962. *Physiol. Rev.* **42**(Suppl. 5, pt. II):48.
73. ROSS, M. H., G. D. PAPPAS, J. T. LANMAN, and J. LIND. 1958. *J. Biophys. Biochem. Cytol.* **4**:659.
74. ROTH, T. F., and K. R. PORTER. 1964. *J. Cell Biol.* **20**:313.
75. RYAN, K. J., and L. L. ENGEL. 1957. *J. Biol. Chem.* **225**:103.
76. SABATINI, D., K. BENSCH, and R. J. BARRNETT. 1963. *J. Cell Biol.* **17**:19.
77. SCHECHTMAN, A. M. 1947. *J. Exptl. Zool.* **105**:329.
78. SCHNEIDER, W. C. 1963. *J. Biol. Chem.* **238**:3572.
79. SHARMA, D. E., E. FORCHIELLI, and R. I. DORFMAN. 1962. *J. Biol. Chem.* **237**:1495.
80. SIRLIN, J. L., and J. JACOB. 1960. *Exptl. Cell Res.* **20**:283.
81. SPIEGEL, M. 1960. *Biol. Bull.* **118**:451.
82. STAHL, E. 1965. Thin-Layer Chromatography. Academic Press Inc., New York.
83. STEIN, O., and Y. STEIN, 1967. *J. Cell Biol.* **33**: 319.
84. THOMPSON, S. W. 1966. Selected Histochemical and Histopathological Methods. Charles C Thomas, Springfield, Illinois.

85. WARTENBERG, H. 1962. *Z. Zellforsch.* **58**:427.
86. WARTENBERG, H., and W. GUSEK. 1960. *Exptl. Cell Res.* **19**:199.
87. WATSON, M. L. 1958. *J. Biophys. Biochem. Cytol.* **4**:475.
88. WATTENBERG, L. W. 1958. *J. Histochem. Cytochem.* **6**:225.
89. WILLIAMS, J. 1969. *In* The Biochemistry of Animal Development R. Weber, editor. Academic Press Inc., New York. **1**:1.
90. WILLIAMSON, J. R. 1964. *J. Cell Biol.* **20**:57.
91. WISCHNITZER, S. 1966. *In* Advances in Morphogenesis. M. Abercrombie and J. Brachet, editors Academic Press Inc., New York. **5**:131.
92. WITSCHI, E. 1966. Development of Vertebrates. W. B. Saunders Company, Philadelphia.
93. YAMADA, E., and T. M. ISHIKAWA. 1960. *Kyushu J. Med. Sci.* **11**:235.
94. ZALOKAR, M. 1960. *Exptl. Cell Res.* **19**:183.
95. ZIMMERMANN, H., and A. G. E. PEARSE. 1959. *J. Histochem. Cytochem.* **7**:271.