

# FINE STRUCTURAL AND RADIOAUTOGRAPHIC OBSERVATIONS ON DENSE PERINUCLEAR CYTOPLASMIC MATERIAL IN TADPOLE OOCYTES

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## ABSTRACT

Dense fibrous material is first seen in association with mitochondria in tadpole oogonia but is most prominent in oocytes during the extended first meiotic prophase when it aggregates into dense bodies in the perinuclear cytoplasm. The origin of this material has been attributed to 350-A nuclear granules which form cytoplasmic streamers of fibrous material upon passage through nuclear pores. This has commonly been interpreted as the transfer of ribonucleoprotein to the cytoplasm for storage. However, cytochemical reactions for nucleic acids have indicated an absence of detectable RNA in this dense material, and the results of radioautographic studies with labeled uridine, thymidine, or actinomycin D argue against the presence of nucleic acids. When sites of incorporation of tritiated amino acids were radioautographically localized, an appreciable number of silver grains were present over the dense bodies. Uptake of certain amino acids occurs fairly promptly but the degree of labeling levels off after about 6 hr, suggesting a rapid turnover of the material in the dense bodies. Attention is drawn to the similarity of the dense bodies to structures present in germ cells of a number of other species, and possible functions of the dense bodies in germ cell differentiation are considered.

## INTRODUCTION

Quite a number of reports have appeared on the fine structure of oocyte development in the frog. An understanding of some aspects of this complex process appears to be emerging from the varied pieces of information that have resulted. This is particularly true for the aggregates of dense material present in the perinuclear zone of amphibian oocytes. These have usually been interpreted as a product of nucleocytoplasmic transfer (Pollister et al., 1954; Ornstein, 1956; Wischnitzer, 1958; Lanzavecchia, 1962; Miller, 1962; Wartenberg, 1962; Balinsky and Devis, 1963; Swift, 1965; Takamoto, 1966; Clérot, 1968; Hay, 1968; Massover, 1968; Kessel, 1969). Linear arrays of 300-

350-A granules just inside the nuclear envelope of amphibian oocytes associated with the pore-annular complex presumably move through the annuli of the nuclear envelope (Swift, 1965) to form small perinuclear "nuages" (André and Rouiller, 1956) which subsequently aggregate into larger masses of dense material (Ornstein, 1956). The larger aggregates of dense material present in amphibian oocytes frequently have mitochondria associated with their surfaces or embedded within their substance (Ornstein, 1956; Miller, 1962; Massover, 1968; Hay, 1968; Clérot, 1968; Kessel, 1969).

This feature is not peculiar to frog oocytes, for

similar dense bodies have been observed in the perinuclear zone of echinoderm oocytes (Kessel, 1966), spider oocytes (André and Rouiller, 1956), insect (Anderson and Beams, 1956; King, 1960; Lima-de-Faria and Moses, 1966; Kessel and Beams, 1968 *b*), crayfish (Beams and Kessel, 1963; Kessel and Beams, 1968 *a*), tunicate (Hsu, 1963; Kessel, 1966), and fish oocytes (Anderson, 1968; Scharrer and Wurzelman, 1969). Also, mitochondrial clusters, containing small amounts of dense material, have been reported in tunicate (Hsu, 1963; Kessel, 1966), fish (Anderson, 1967), and mammalian oocytes (Odor, 1960, 1965; Adams and Hertig, 1964; Hope, 1965; Weakley 1967, 1969; Szollosi, 1969); as well as in fish (Follenius, 1965), amphibian (Clérot, 1968), and mammalian spermatocytes (André, 1962; Fawcett and Phillips, 1967; Fawcett et al., 1970).

The fine structural similarity of 300–350-Å nuclear granules to ribosomes and components of amphibian oocyte nucleoli led to the suggestion that the granules may be ribonucleoprotein particles en route to the cytoplasm via nuclear pores (Swift, 1965; Lane, 1967; Franke and Scheer, 1970). Also, Miller (1962) noted a resemblance of the dense material to the fibrous component of the nucleolus, and other authors have been tempted to implicate the nucleolus in the formation of the dense bodies of amphibian oocytes (De Robertis et al., 1965; Hay, 1968; Scharrer and Wurzelman, 1969; Kessel, 1969).

In the study reported here we have systematically examined the ultrastructural morphology of the dense bodies and their relationship to other cell components in the earlier stages of oogenesis. This was done to determine if the prior observations, sometimes based on rather small samples or without regard for staging, do indeed represent various aspects of one continuous process. We have also employed special techniques to demonstrate nucleic acids at the electron microscope level (Watson and Aldridge, 1961; Swift et al., 1964), to test the hypothesis that the dense material is a ribonucleoprotein. The dense bodies were observed in association with isolated nuclei and in intact cells to determine their form and distribution under these conditions. In addition, living oocytes were incubated in medium containing isotopically labeled metabolic precursors in order to explore aspects of formation and composition of the dense material.

The findings of the present study on the means

and sequence of formation of the dense bodies are consistent with the presumed process as outlined above. However, it will be shown that nucleic acids are not detected in the dense material and that incorporation of amino acids indicates a major protein component.

## MATERIAL AND METHODS

The dense bodies are found in the smaller oocytes of adult frogs. However, the ovaries of *Rana pipiens*, *Rana clamitans*, and *Rana catesbeiana* tadpoles (Lemberger Co., Oshkosh, Wis.) were found to be a more suitable source of tissue for the present study because of the relative abundance of oocytes containing the dense bodies.

Ovaries were fixed at room temperature by immersion in 2–6% glutaraldehyde buffered to pH 7.4 with 0.2 M *s*-collidine, or in a cacodylate-buffered formaldehyde-glutaraldehyde-cresol (FGC) combination fixative (Ito and Karnovsky, 1968), with or without 0.33% dichromate before postfixation with buffered osmium tetroxide. To demonstrate nucleic acids, some small pieces of ovaries fixed in glutaraldehyde for 15 min or 1 hr were subjected to ribonuclease digestion (Sigma type II-A, 5 × recrystallized, Sigma Chemical Company, St. Louis, Mo.) (Swift et al., 1964) or the appropriate control conditions, and then postfixated in osmium tetroxide. Nucleic acid staining with the indium trichloride method of Watson and Aldridge (1961), with or without prior ribonuclease digestion, was used to confirm the sites of RNA- and DNA-containing structures.

For the radioautographic study, 0.5 mm cubes of minced tadpole ovaries were incubated at room temperatures (22–24°C) in vitro in tissue culture medium 199 diluted to 2/3 of its usual concentration with distilled water, 1000 units of penicillin, and 10 µg of streptomycin per ml of medium. One of the tritium-labeled compounds was added to this culture medium, which was then incubated for 15 min–72 hr. Some samples were transferred to medium lacking isotope for further incubation after 2–6 hr. Compounds used were the amino acids L-arginine (SA 1.1 Ci/mM), L-lysine (SA 4.38 Ci/mM), L-leucine (SA 58.2 Ci/mM), L-phenylalanine (SA 8.3 Ci/mM), and DL-tryptophan (SA 179 Ci/mM); the nucleotides uridine (SA 9.38 Ci/mM) and thymidine (SA 6.7 Ci/mM); and the antibiotic actinomycin D (SA 3.38 Ci/mM). Also used were D-galactose (SA 6.9 Ci/mM), L-fucose (SA 4.3 Ci/mM), and *n*-acetyl galactosamine (SA 0.4 Ci/mM). The incubations were carried out in a 4 ml Pyrex tube containing 0.25 ml of culture medium with 50 µCi of the radioactive compound. The tubes containing the media were gassed with oxygen,

initially and at later intervals, to maintain the appropriate pH. After incubation, the pieces of ovary were routinely fixed in FGC but some samples were fixed in phosphate-buffered 10% formaldehyde, as well as directly in osmium tetroxide, before embedding in Epon or Epon-Araldite. Sections were prepared for radioautography by a method similar to that described by Caro and van Tubergen (1962). For light microscope radioautography 0.5 and 1  $\mu$  sections were dried on gelatinized slides and dipped in melted Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) diluted with three parts water, allowed to dry, and exposed for 1 wk to several weeks before development in Kodak Dektol developer (Eastman Kodak Co., Rochester, N. Y.) for 5 min. The slides were then cleared in Kodak Hypo for 2-3 min, washed, and stained with 1% toluidine blue "O" buffered to pH 7.2 for 5-10 min at 60°C. The slides were then washed several times in neutral distilled water, air-dried, and cover slips were applied over heavy immersion oil (Cargille type B, Cargille Laboratories, Inc., Cedar Grove, N. J.).

For electron microscope radioautography, thin sections were collected on bare copper grids, coated with a thin film of emulsion, and dried and boxed for appropriate exposure. The film was formed over a wire loop with Ilford L4 emulsion diluted with an equal volume of water, and was used to affix the grid on a glass slide as described by Caro (1962). The grids were developed in Kodak D-19 or by the physical developer technique of Bachmann and Salpeter (1965) after exposure for 2 wk to several months. After the specimens were cleared in Kodak Acid Fix Hypo for 1 min, they were stained for 5 min in lead citrate stain made according to Venable and Coggeshall (1965). The radioautographs were subsequently examined in the electron microscope.

## OBSERVATIONS

Various types of germinal cells are identifiable in a photomicrograph of a 0.5  $\mu$  thick Epon section of tadpole ovary (Fig. 1). The smallest of these are *oogonia* (15-20  $\mu$  in diameter), which represent the earliest stage of oogenesis and are cells encountered only infrequently, usually near the periphery of the ovary. They have an irregularly shaped nucleus and contain darkly stained structures in the otherwise lightly staining cytoplasm. More numerous are *small primary oocytes* (20-50  $\mu$  in diameter) which are also found towards the periphery of the ovary. They have rounded nuclei containing several prominent nucleoli and have darker staining cytoplasm than do oogonia. The most prominent germinal cells are *intermediate-sized primary oocytes* (50-80  $\mu$  in diameter). They

are located centrally in the ovary and have larger nuclei and more intensely stained cytoplasm than the earlier stages. The primary oocytes frequently have densely stained cytoplasmic bodies on, or adjacent to, the membrane of the germinal vesicle.

The structures of primary interest in this study are the darkly staining bodies in the cytoplasm of oogonia and the prominent perinuclear cytoplasmic dense bodies of oocytes. These are larger and more frequently observed in the small primary oocytes, but are also present in intermediate-sized primary oocytes (Fig. 1). By the time yolk accumulation begins in large oocytes, the dense material is no longer observable.

A low magnification electron micrograph of an oocyte (Fig. 2) illustrates the irregular contours of the dense bodies which extend short projections toward the nucleus. The bodies tend to lie in shallow indentations of the nucleus and their density is similar to that of the nucleoli. Some of the larger bodies are aggregates of several smaller units.

At slightly higher magnification, the dense bodies are seen to be bounded on the cytoplasmic side by mitochondria, some of which may be embedded in the dense bodies (Fig. 3). The dense material is separated from the nuclear envelope by a narrow space of uniform width. The nucleus does not appear to be locally specialized.

When small primary oocytes are examined in the living state with Nomarski interference contrast optics (Fig. 4), the germinal vesicles containing multiple nucleoli are prominent. The juxtannuclear dense bodies are also apparent in unfixed cells, just as they are in material prepared for electron microscopy. Nuclei of tadpole oocytes can be isolated and washed essentially free of cytoplasm without dislodging the dense bodies (Fig. 5). This is interpreted to indicate that the dense bodies are stable morphological structures having a rather strong physical attachment to the nucleus.

The origin of the large dense bodies was traced by systematically studying the earlier stages of oogenesis in tadpole ovaries. Particular attention was paid to stages not frequently described in earlier reports. When oogonia are examined in the electron microscope, the cells lack juxtannuclear dense bodies, but there are mitochondrial clusters with small amounts of dense material in their interstices (Fig. 6). In small oocytes approximately 20  $\mu$  in diameter in the zygotene stage of meiotic

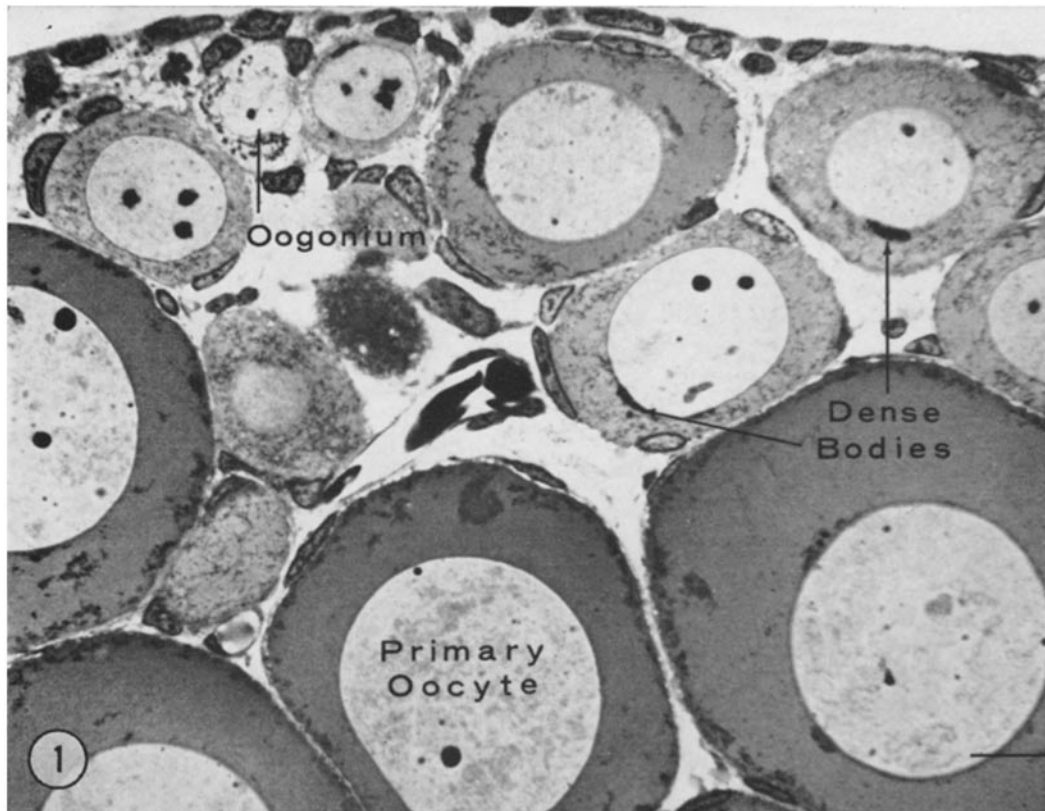


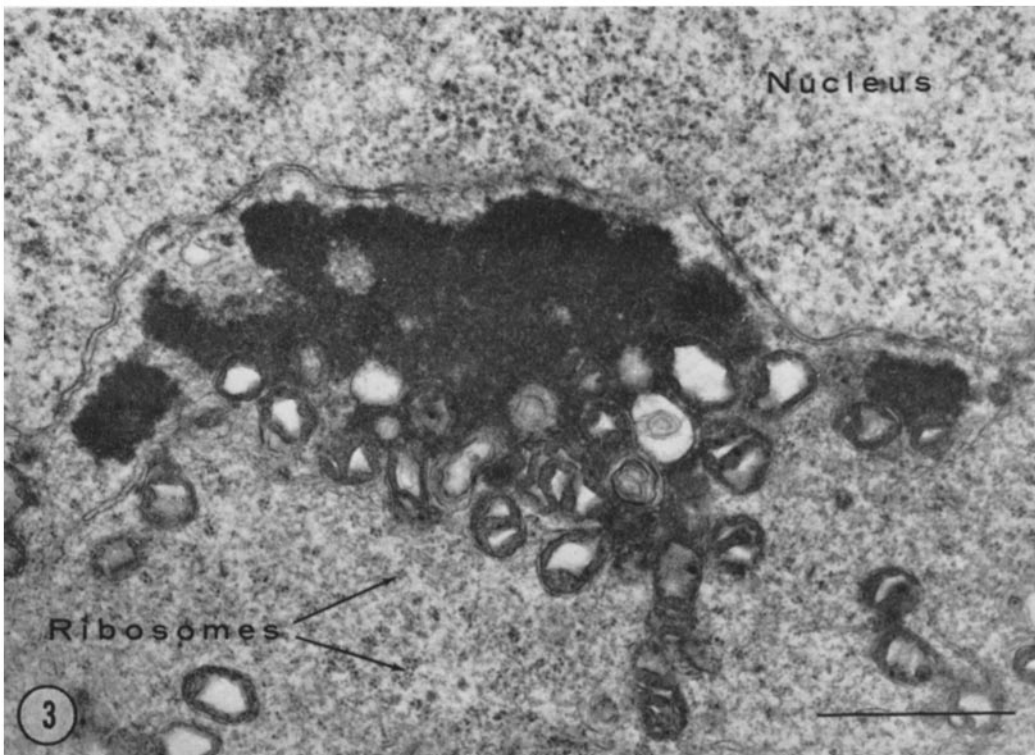
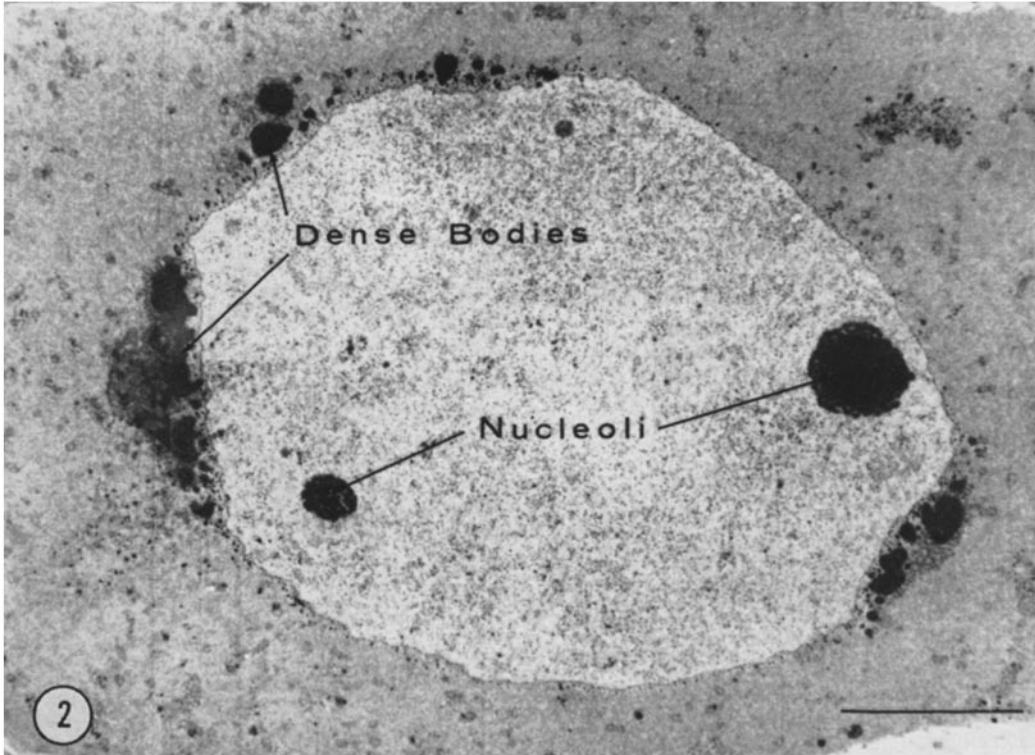
FIGURE 1 Ovary of *Rana clamitans* tadpole as seen in a light micrograph of an Epon section,  $0.5\ \mu$  in thickness, stained with toluidine blue. The periphery of the organ is at the top of the figure. An oogonium and several small primary oocytes are situated near this margin while more densely stained intermediate-sized primary oocytes are present deeper in the tissue. Dense bodies are commonly seen as intensely stained structures adjacent to the nuclear membrane in small primary oocytes. Bar,  $10\ \mu$ .  $\times 960$ .

prophase (identifiable by the synaptonemal complexes in the nucleus), some mitochondrial clusters with dense material are present (Fig. 7). In addition, there are sometimes large accumulations of material of similar density and texture free in the cytoplasmic matrix but not intimately associated with either the nucleus or mitochondria.

If one examines somewhat larger oocytes ( $20\text{--}30\ \mu$  in diameter) in the early pachytene stage of meiotic prophase, many small dense bodies are present (Fig. 8). When viewed at higher magnification, the frequent association of dense bodies with mitochondria is evident (Fig. 9). Three other fine structural features which seem to be related to the dense bodies are prominent at this level of magnification: (a) Small tufts of dense material are present around their periphery, (b) streamers of filamentous material are found on the cytoplasmic

side of nuclear pores, and (c) nuclear granules approximately  $350\text{-}\text{\AA}$  in diameter are present in the nucleoplasm near the nuclear envelope. In still larger oocytes ( $30\text{--}50\ \mu$  in diameter) the large aggregates of dense material are present. This is the stage commonly described by earlier workers. It is noted that annulate lamellae are not commonly seen until this period of oogenesis and, although they occasionally lie in proximity to dense bodies, they become prominent well after the formation of dense material begins.

Careful examination of the perinuclear streamers of fibrous material reveals their frequent close association with the nuclear pores. It is also common to find arrays of  $350\text{-}\text{\AA}$  nuclear granules aligned opposite the nuclear pores (Figs. 10–13). Swift (1965) was the first to suggest that the granules pass into the cytoplasm through nuclear



pores and that during this process they are altered to form small tufts of fibrous material.

The prevailing interpretation of these images has been that the dense material is a ribonucleoprotein produced in the nucleus and transferred to the cytoplasm. Methods for demonstration of nucleic acids at the electron microscope level were employed to test this hypothesis. When tissues were treated with indium trichloride according to Watson and Aldridge (1961), a distinctive pattern of staining was observed in oocytes. The homogeneous, centrally located pars fibrosa of the nucleoli was rimmed with clusters of the densely staining pars granulosa (Fig. 14). These nucleolar complexes are often embedded in a loose meshwork of filaments which also stain with indium. In the cytoplasm, granules which are identified as ribosomes were stained as well (Fig. 14). When tissues were digested with ribonuclease and then subjected to the indium treatment, the fibrous and granular portions of nucleoli and the ribosomes were unstained (Fig. 15). However, the fine filaments in the nucleoplasm near the nucleoli continued to stain and are interpreted to represent the positive staining of chromosomal DNA (Fig. 15).

When pieces of ovaries fixed in glutaraldehyde were digested with ribonuclease and then postfixated in osmium tetroxide, it was found that the nuclear granules, streamers, and dense bodies are quite resistant to the enzyme, whereas the nucleic acid-containing components of the nucleoli and the ribosomes are considerably altered or entirely removed from the cells by this treatment (Fig. 16). These findings are in good agreement with the results of application of indium which failed to stain the 350-A nuclear granules, the cytoplasmic streamers, or the dense bodies. In such preparations the mitochondrial clusters are recognizable

as negative images in a field of distinctly stained ribosomes (Fig. 17).

To explore further the composition of the dense bodies and to study their origin, pieces of tadpole ovaries were incubated in culture medium containing radioactive compounds which might be incorporated as precursors. Fine structural examination following the *in vitro* incubations indicated that the cells tolerate this treatment without any marked changes in their ultrastructure. Radioautography after extended incubation with tritiated uridine, thymidine, or actinomycin D did not reveal an incorporation of these compounds into dense bodies as would be expected if they contained nucleic acids. However, there was significant incorporation of the aliphatic amino acid leucine, the basic amino acids arginine and lysine, and the aromatic amino acids tryptophan and phenylalanine, thus establishing that the dense bodies are, at least in part, composed of proteins. Although there is widespread incorporation of all the amino acids used into both the nucleus and cytoplasm (Fig. 18), examination of radioautographs at the light microscope level reveals a definite aggregation of silver grains over the dense bodies (Figs. 19 and 20), as well as over other cellular components. Incubation of ovarian tissue with tritiated phenylalanine resulted in radioautograms indicating a greater uptake of this amino acid by dense bodies as compared to other amino acids. By varying the length of time of exposure of the cells to medium containing the tritiated phenylalanine and the subsequent period of incubation in medium lacking isotope, it was found that the intensity of labeling of dense bodies could be varied. Although there was no attempt to quantitate this in the present study, the greatest labeling was apparent after 6 hr of incubation with isotope followed by 6 hr in medium

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FIGURE 2 A low magnification electron micrograph of a small primary oocyte of a *Rana clamitans* tadpole with several dense bodies in the perinuclear zone. As seen in this section, relatively large amounts of the material may be present in cells at this stage. The dense bodies are irregularly shaped and frequently consist of aggregations of smaller masses. They are situated in slight indentations of the nuclear envelope but the intervention of a narrow zone of cytoplasm prevents their direct application to the nucleus. Bar, 10  $\mu$ .  $\times$  2400.

FIGURE 3 A dense body present in the cytoplasm of a small primary oocyte of a *Rana clamitans* tadpole. Mitochondria are frequently embedded in the dense material at the periphery of larger bodies. The nuclear envelope does not appear to be locally specialized adjacent to the dense body but there is a constant separation. Bar, 1  $\mu$ .  $\times$  25,700.

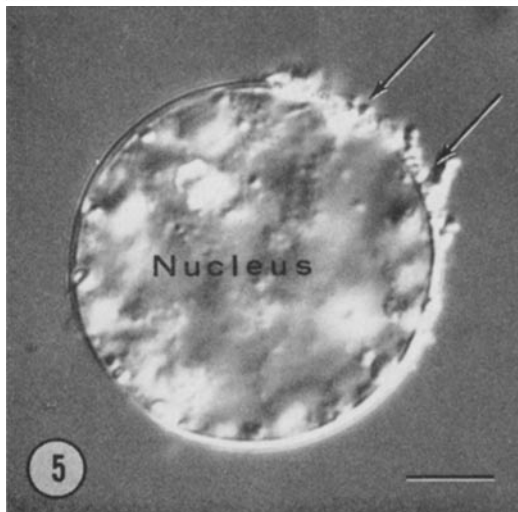
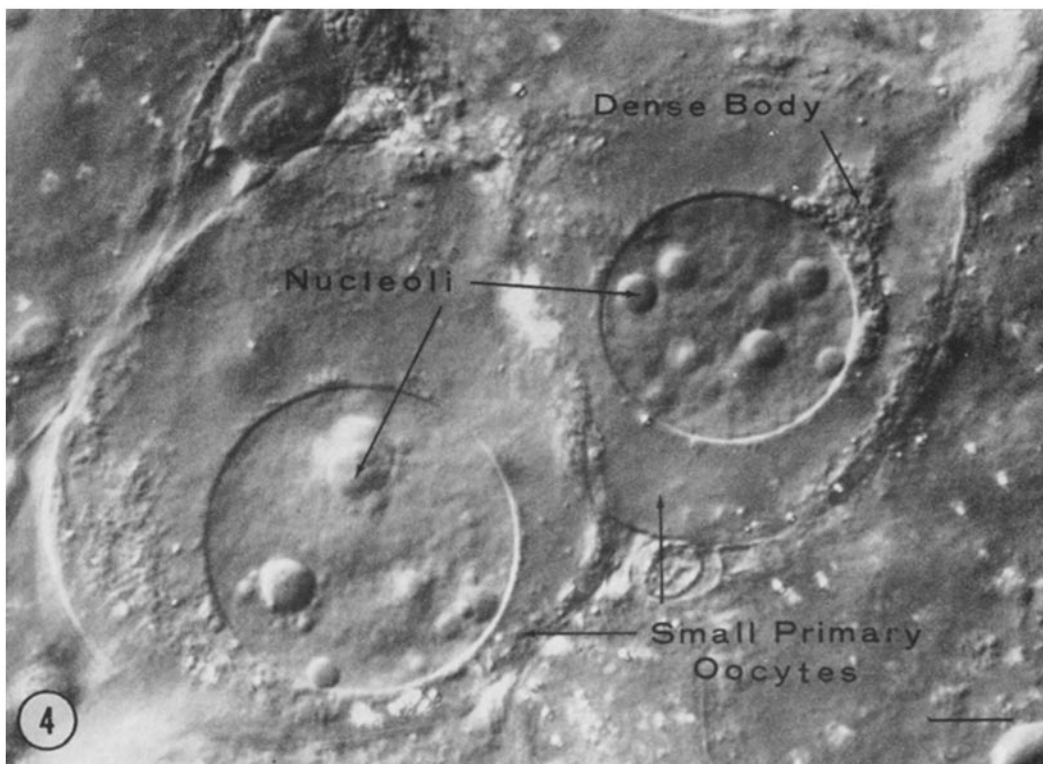


FIGURE 4 A light micrograph of living small primary oocytes of a *Rana clamitans* tadpole as seen with Nomarski interference contrast optics. Several nucleoli are visible in each nucleus at this level of focus. A large dense body is present in the juxtannuclear region of one oocyte. There is no apparent relationship between distribution of nucleoli and location of dense bodies. Bar,  $10 \mu$ .  $\times 1160$ .

FIGURE 5 Nucleus isolated from a small primary oocyte of a *Rana clamitans* tadpole and washed free of its cytoplasm, photographed with Nomarski interference contrast optics. The dense body (arrows) continues to be related to the nuclear membrane, suggesting that the dense body is a rather stable morphological feature of these cells and does have some physical attachment to the nucleus. Bar,  $10 \mu$ .  $\times 1160$ .

without the tritiated compound. There were obviously fewer grains after shorter periods of incubation (3 hr) but no evident increase if these periods were extended to 24 hr. This suggests that the material may be synthesized elsewhere in the cell and requires about 6 hr to be concentrated in the dense bodies.

On sections processed for electron microscope radioautography, the silver grains are infrequent over mitochondria but are commonly found over the periphery of the dense bodies (Figs. 21 and 22). An occasional grain lies over or near the nuclear pores or between them and the dense bodies. Also, small dense aggregates not associated with mito-

chondrial clusters were labeled in these preparations. Silver grains were associated with the granular portion of the nucleolus, frequently at its periphery (Fig. 21), as well as being scattered over the nucleoplasm. The distribution of silver grains after fixation of the tissue with 10% formaldehyde or directly in osmium tetroxide was not qualitatively different than after glutaraldehyde or FGC fixation.

## DISCUSSION

Although a number of authors have reported the presence of dense material in the perinuclear cytoplasm of frog oocytes and have speculated on its origin, these observations have not correlated the occurrence of dense material with specific stages of egg formation. The present study indicates that the size and relative abundance of dense bodies in germinal cells of amphibian ovaries are related to particular stages of oogenesis. Dense material is first seen in the interstices of mitochondrial clusters present in oogonia. To our knowledge this is the first time that dense cytoplasmic material has been described at this early stage of germ cell differentiation. However, it is more apparent in small primary oocytes, here being situated in small dense bodies lying near the nucleus. Streamers of fine filamentous material extending to the dense bodies from nuclear pores are quite numerous in primary oocytes and would appear to represent addition of new material to the dense bodies. These streamers probably correspond to the ill-defined densities around oocyte nuclei described as nuages by earlier workers (André and Rouiller, 1956).

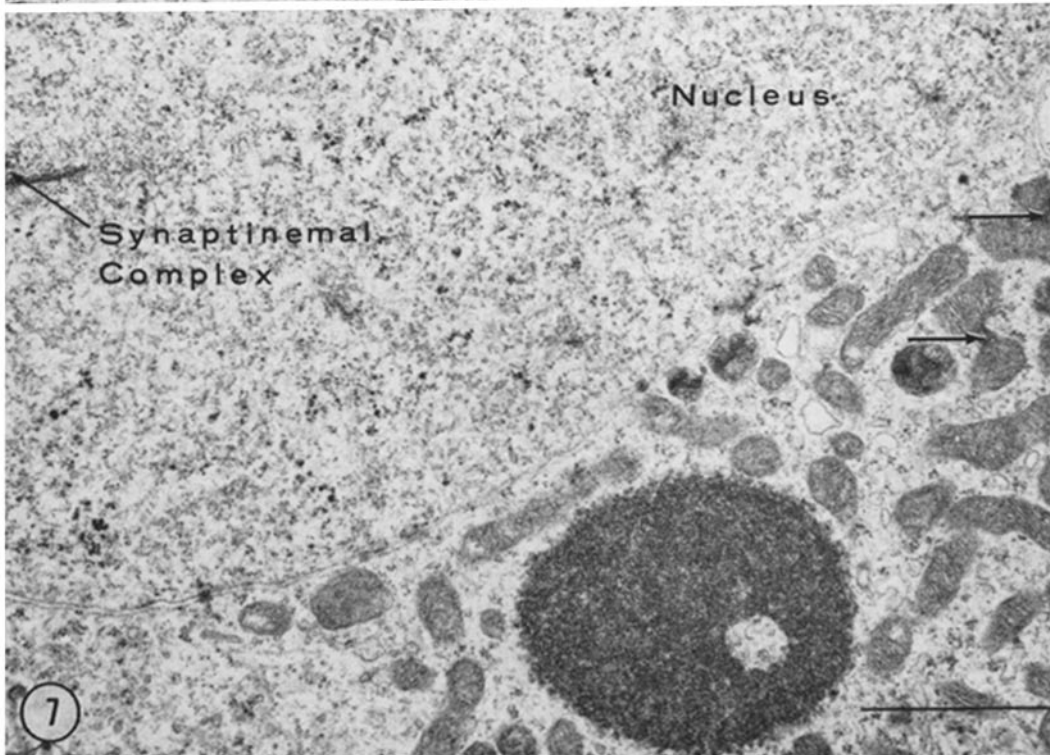
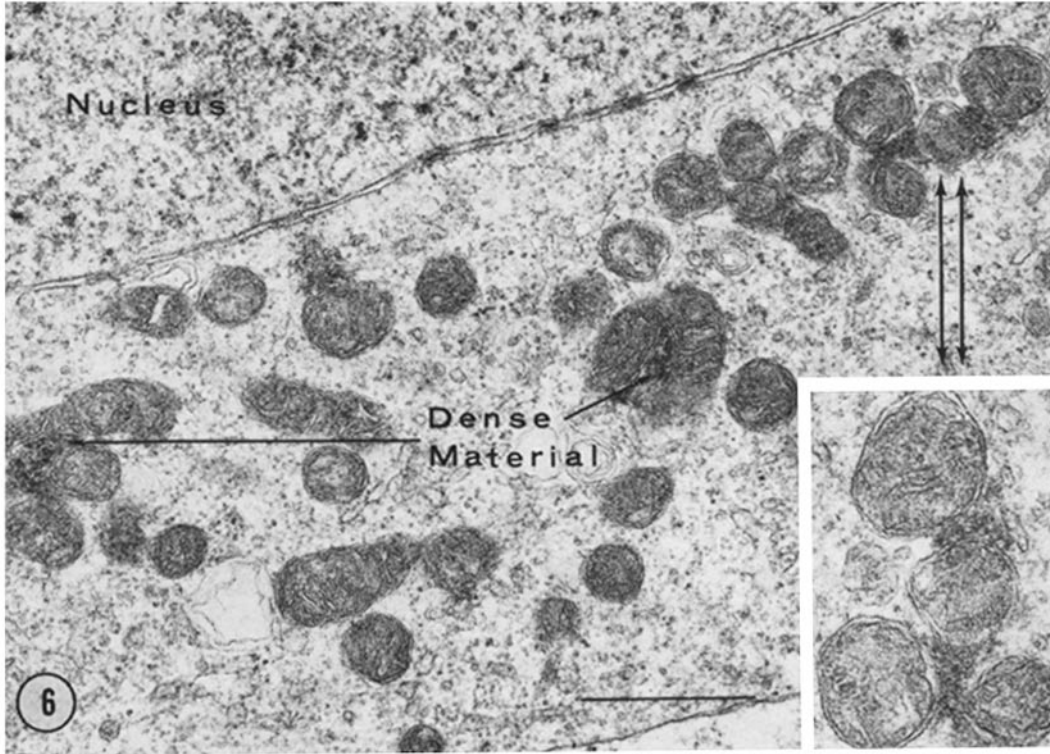
In the latter part of small primary oocyte development, the dense bodies aggregate to form large accumulations of the material. These are situated near the nuclear envelope and have many mitochondria around their periphery or embedded within their substance. As the cells continue to grow and reach the stage of intermediate-sized oocytes, the dense bodies become smaller and less abundant. In large, nearly mature oocytes (up to 1.5 mm in diameter) which have already accumulated stores of yolk, the dense bodies are no longer recognizable (Ward, 1962; Kessel, 1969).

It has been suggested repeatedly that the nuclear granules in amphibian oocytes (Swift, 1965; Takamoto, 1966; Lane, 1967) and the dense material in the cytoplasm of amphibian oocytes

(De Robertis et al., 1965; Hay, 1968; Kessel, 1969) and fish oocytes (Scharrer and Wurzelman, 1969) contain ribonucleoprotein. However, this is an assumption based on morphology rather than on the results of experimental determination. An effort has been made in the present study to obtain evidence as to composition of the material, by using essentially morphological techniques. Neither reactions for the demonstration of nucleic acids at the electron microscope level combined with the ribonuclease digestion method of Swift et al., (1964), nor the indium technique of Watson and Aldridge (1961), supported the contention of others that the dense bodies are ribonucleoprotein. Although ribosomes and nucleoli, which are known to contain nucleic acids, stained positively with indium, the nuclear granules, cytoplasmic streamers, and dense bodies did not, and their morphology was not visibly altered by ribonuclease digestion before embedding. Similar results were reported for the dense material of the chromatoid body in rat spermatids (Eddy, 1970). Furthermore, radioautography after incubation of oocytes in culture medium containing tritium-labeled uridine, thymidine, or actinomycin D revealed that the dense bodies have no detectable incorporation or binding of radioactivity. Clérot (1968) reported that the dense material was not removed from thin sections by RNase digestion. The present observations confirm and extend his tentative conclusions that significant amounts of nucleic acids are not present in the dense cytoplasmic bodies of amphibian oocytes.

Large numbers of 350-A nuclear granules are not commonly observed in all germinal cells. They have been reported in frog primary spermatocytes, (Clérot, 1968) and amphibian oocytes (Swift, 1965; Lane, 1967; Massover, 1968; Kessel, 1969) and fish oocytes (Scharrer and Wurzelman, 1969). Perhaps closer examination will reveal the presence of numerous 350-A nuclear granules in germinal cells of some other species, but they were not obvious in spermatocytes of mammals studied earlier in this laboratory (Fawcett et al., 1970). The germinal cells of most animals are appreciably smaller than amphibian oocytes, and the mitochondrial clusters usually have only small amounts of dense material in their interstices. It was observed in the present study that smaller germinal cells of the tadpole, i.e. the oogonia and young oocytes, apparently lack nuclear granules and cytoplasmic streamers even though mitochondrial





clusters are present. It may be that only in certain stages in amphibia and a few other groups is the material produced in amounts sufficient to aggregate into discrete, easily visible nuclear granules.

If the origin of the nuclear granules was known, one could begin to understand whether they represent material destined for a specific cytoplasmic role or whether they have already served their function by the time they cross the nuclear pores. Swift (1965) suggested that they are formed on the lampbrush loops of the oocyte chromosomes and that they move to the periphery of the nucleoplasm for their eventual release through the nuclear pores. Although Swift saw no apparent relation to nucleoli, Lane (1967) reported "streams" of nuclear granules lying between the nucleoli and the nuclear pores, both in intact cells and in isolated nuclei, and suggested a nucleolar origin for them.

Nuclear granules bearing some similarity to those present in amphibian oocyte nuclei have been observed in other cell types of other species. Watson (1962) reported that "perichromatin granules" about 300 Å in diameter are present in mouse and rat hepatic nuclei and are stainable with indium trichloride or uranyl acetate. However, he was unable to detect similar granules in *Xenopus* liver nuclei. Stevens and Swift (1966) reported that Balbiani ring granules 400 Å in diameter, present in association with chromosomal puffs in *Chironomus* salivary glands, contain RNA. This was based on the stainability of the granules with uranyl acetate or sodium tungstate on thin sections of aldehyde-fixed tissue, and the loss of staining following extraction with ribonuclease. It would seem, however, that the morphological similarity between these granules and the nuclear

granules in germinal cells of amphibia is an insufficient reason to equate them in composition or function.

As a result of the present study, several more comments of a general nature can be made on the formation and composition of the dense bodies. For example, techniques for the demonstration of nucleic acids were consistently negative, but the pattern of uptake of several amino acids indicates that the dense bodies have an appreciable protein content. The fact that incorporation of the aromatic amino acid phenylalanine is especially active is of interest in relation to recent observations by Clérot (1968). He reported that the dense material in frog oocytes can be digested from thin glycol-methacrylate sections with pepsin, an endopeptidase known to cleave preferentially the peptide bond involving aromatic amino acids. Additionally, the finding that silver grains are usually located over the periphery of the dense bodies indicates that newly formed material is being added to the surface of already existing dense bodies. This is consistent with Swift's (1965) hypothesis that the net movement of material is towards the dense bodies, rather than from the dense bodies into the nucleus. It also indicates that the dense material is being actively synthesized at this time and is not, therefore, the end product of catabolic events. Finally, labeling of the dense bodies levels off after about 6 hr of incubation. It would seem from this that the dense bodies actively turn over the material and are not a final storage site.

Aggregates of mitochondria have been reported in germinal cells in a number of other species. Although there is no experimental evidence as to the significance of this relationship, it has generated some interesting suggestions involving mito-

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FIGURE 6 Although oogonia lack juxtannuclear dense bodies, they do contain clusters of mitochondria with dense material in their interstices, as seen in this oogonium from a *Rana pipiens* tadpole. A higher magnification view of one of these clusters (double arrows) is shown in the *inset*. In this electron micrograph the nucleus is at the top of the figure and a small portion of the plasmalemma of the cell is visible at the lower edge. Unlike oocytes, oogonia are not completely surrounded by a layer of follicle cells. Bar, 1  $\mu$ .  $\times$  22,800. *Inset*,  $\times$  37,600.

FIGURE 7 An oocyte in the zygotene stage of meiotic prophase, as indicated by the presence of a synaptonemal complex. A few mitochondrial clusters with dense material are present (arrows) in this cell from a *Rana pipiens* tadpole, but they are fewer than in oogonia (Fig. 6). An occasional large accumulation of dense material may be seen which does not have a close association with mitochondria or the nuclear envelopes. Bar, 1  $\mu$ .  $\times$  25,600.

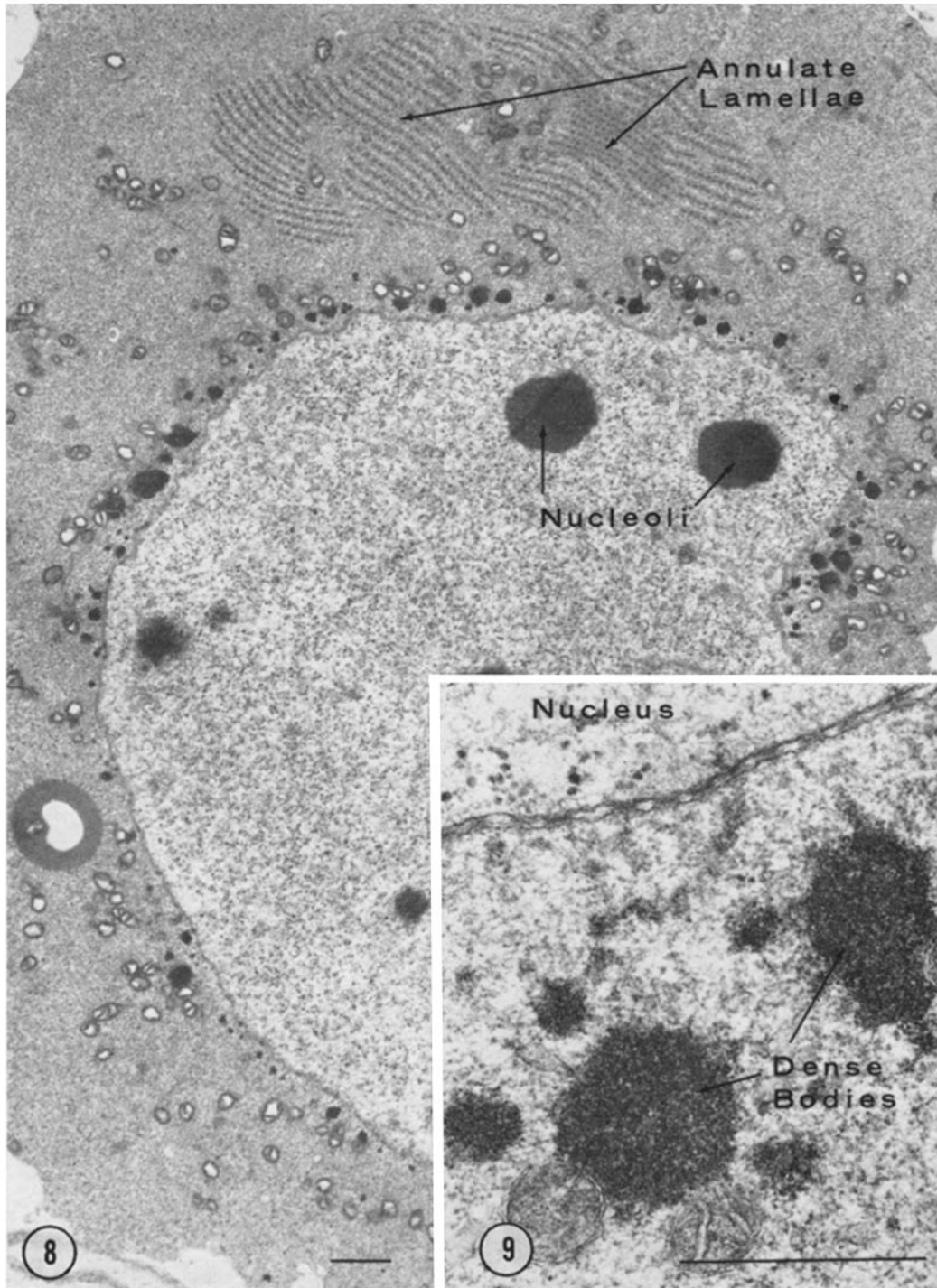
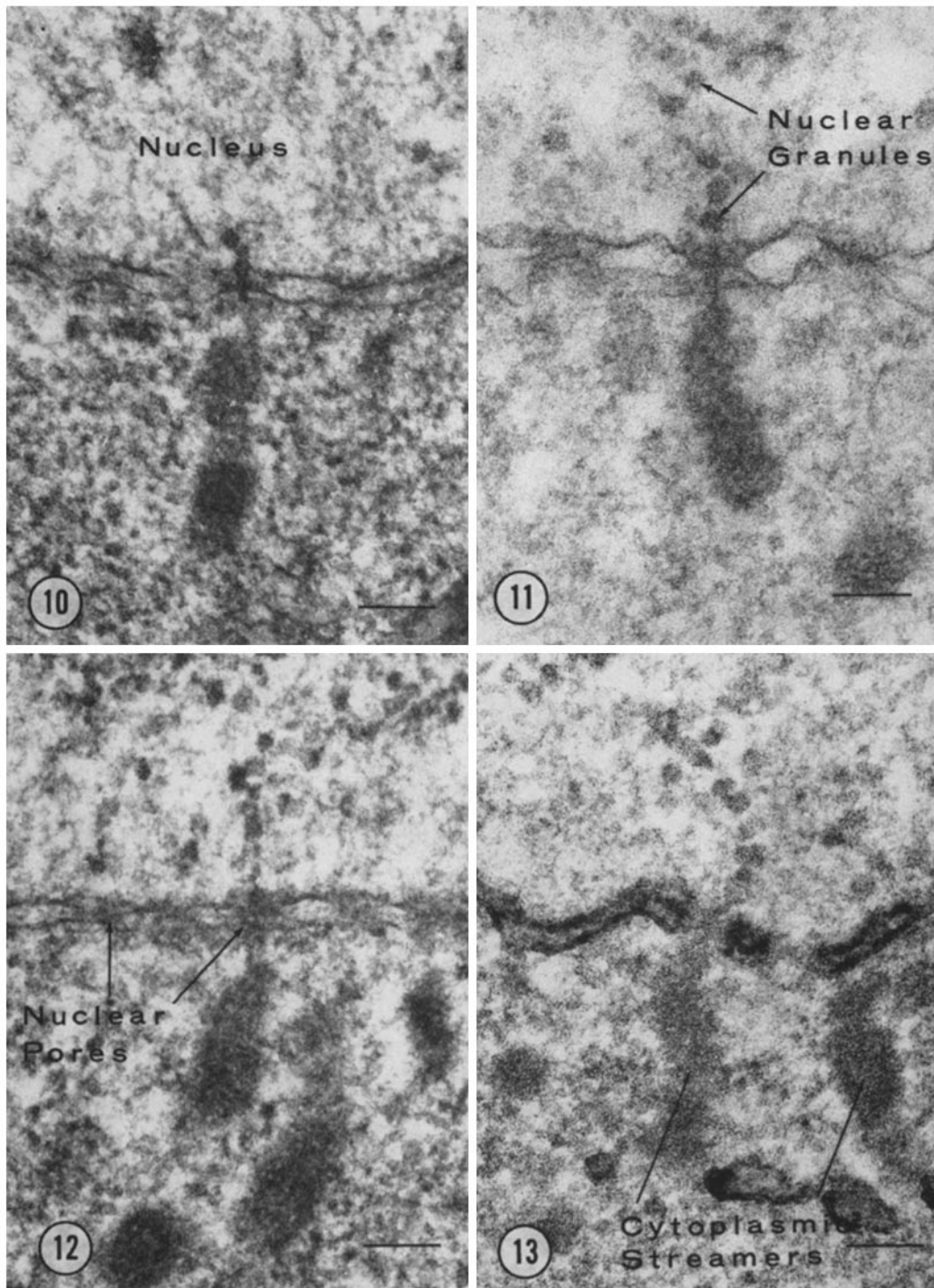


FIGURE 8 In small primary oocytes in the early pachytene stage of meiotic prophase, many small dense bodies are present in the perinuclear zone of the cytoplasm. This appears to be a period of particularly active formation of the dense material. The annulate lamellae that are present in cells during this phase of differentiation may be seen at the top of this electron micrograph of a *Rana clamitans* oocyte. Bar, 1  $\mu$ .  $\times$  7500.

FIGURE 9 A higher magnification view of a portion of a *Rana pipiens* oocyte in about the same stage as that in Fig. 8. The nucleus contains small dense granules approximately 350  $\text{\AA}$  in diameter. The perinuclear cytoplasm contains small tufts and streamers of dense material, as well as dense bodies which may have mitochondria associated with their surface. Bar, 1  $\mu$ .  $\times$  35,400.



FIGURES 10-13 The nucleus is situated above the nuclear envelope in each of these electron micrographs which show portions of small primary oocytes from *Rana clamitans* tadpoles. Swift (1965) was the first to suggest that dense material present in the perinuclear zone may arise from nuclear granules crossing the nuclear pores. Although the nucleoplasmic zone adjacent to the nuclear envelope usually is free of granules, sometimes granules are arrayed opposite to cytoplasmic streamers at the nuclear pores. In some instances there appears to be continuity between the material in the cytoplasmic streamers and that in nuclear granules (Figs. 10 and 13). Bar,  $0.1 \mu$ .  $\times 112,000$ .

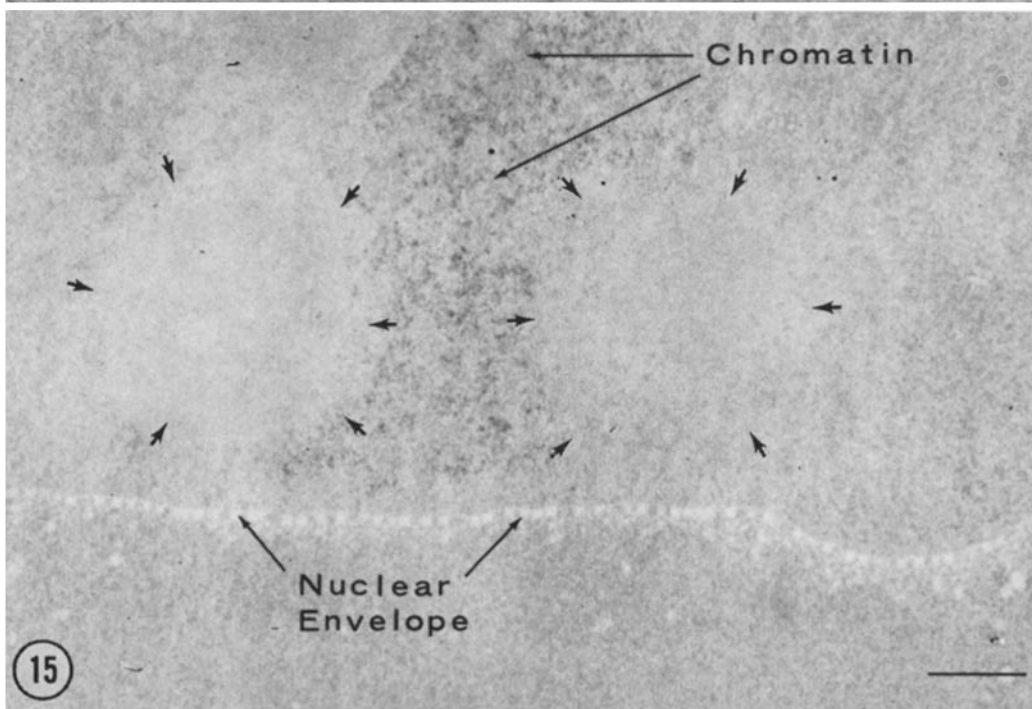
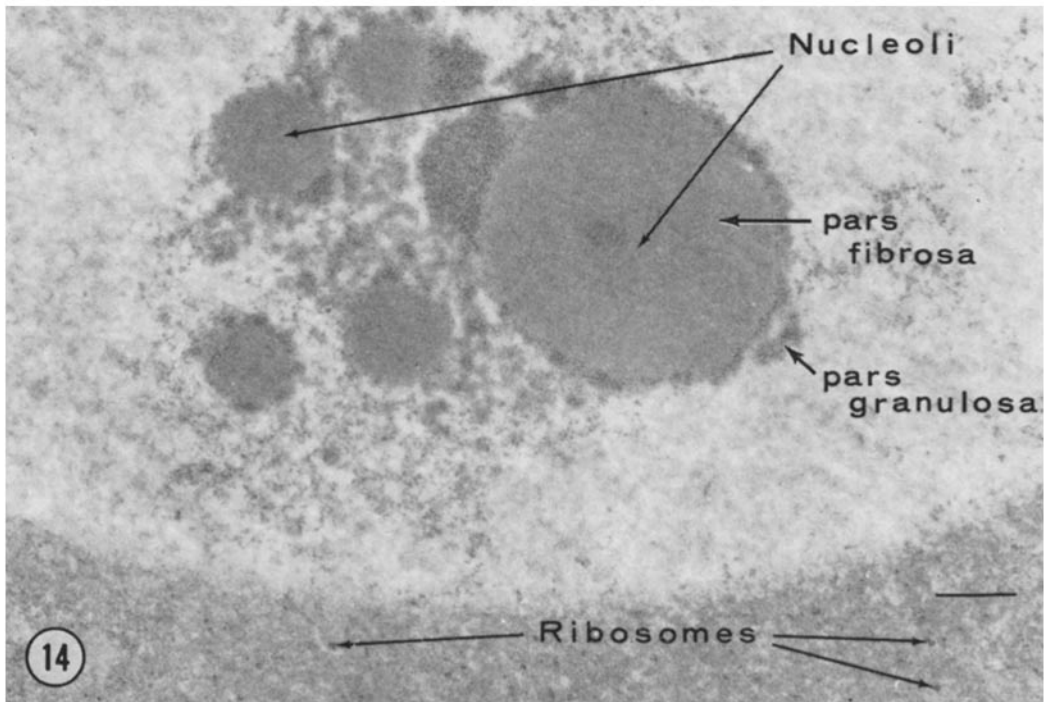


FIGURE 14 Oocyte of *Rana pipiens* tadpole stained for nucleic acids by the indium trichloride technique. The cytoplasm stains with relative density, and clusters of ribosomes are just visible at this level of magnification. Nucleic acids are less diffuse in the nucleoplasm, however, with staining confined to nucleolar components and scattered areas of fibrous material. The pars fibrosa of nucleoli stains less intensely than the surrounding pars granulosa. Nuclear granules cannot be detected. Bar, 1  $\mu$ .  $\times 10,400$ .

FIGURE 15 When the oocytes of *Rana pipiens* tadpoles are treated with ribonuclease before exposure to indium trichloride, cytoplasmic components are not stained and nucleoli are not visible. Areas of low density (short arrows) are presumed to represent sites where nonnucleic acid components of nucleoli remain. Fibrous material in the nucleus which is not removed by ribonuclease digestion and which stains with indium is interpreted as chromatin. Bar, 1  $\mu$ .  $\times 12,800$ .

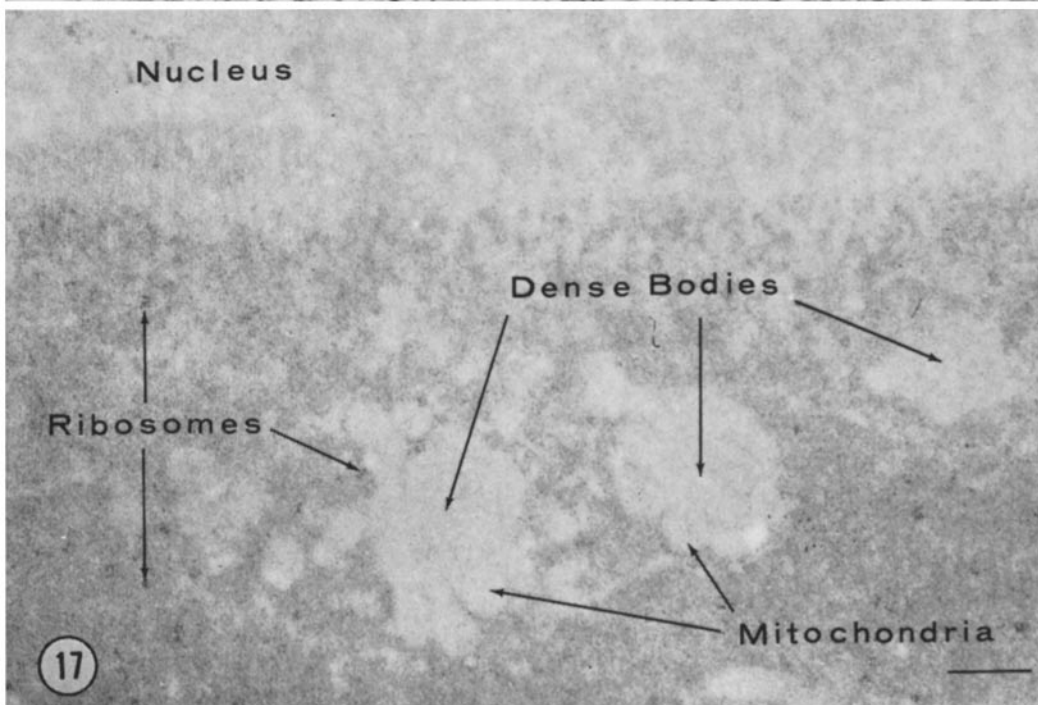
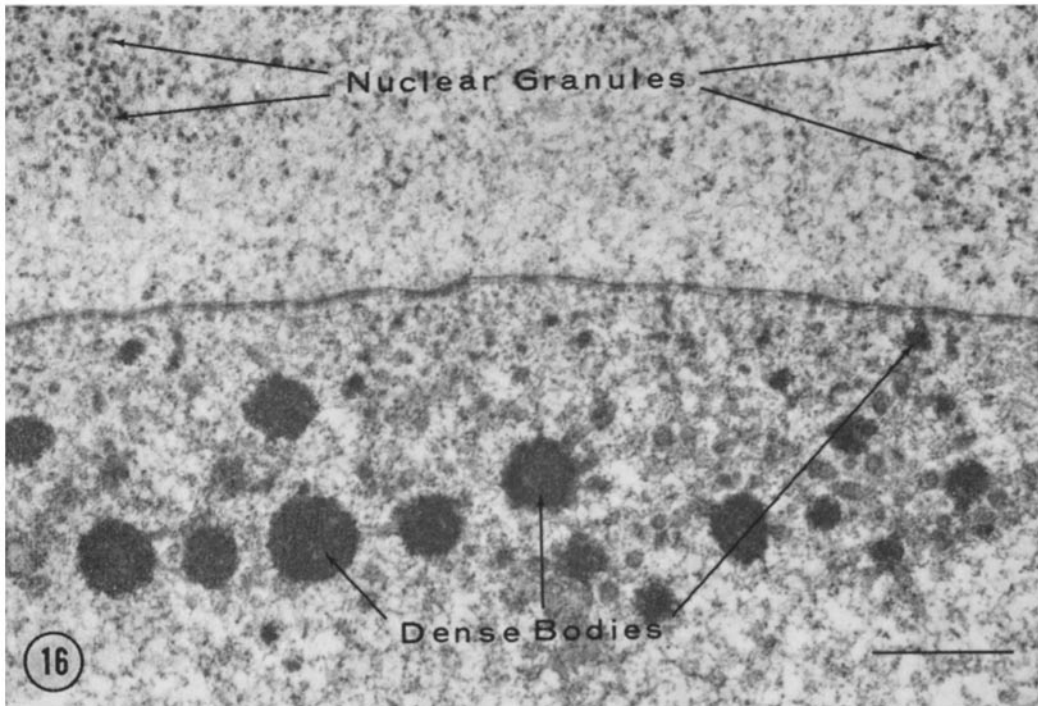


FIGURE 16 This tissue was digested with ribonuclease and then postfixed in osmium tetroxide. The enzymatic treatment was sufficient to eliminate indium staining of structures known to contain ribonucleic acids (Fig. 15) but the nuclear granules and cytoplasmic dense bodies are still present, as shown here. Bar,  $1 \mu$ .  $\times 18,500$ .

FIGURE 17 Oocyte of a *Rana pipiens* tadpole stained with indium trichloride. Dense bodies and structures interpreted as their surrounding mitochondria, although unstained themselves, stand out as negative images in the homogeneously stained, ribosome-rich cytoplasm. Ribosomal clusters are indicated by the arrows. Nuclear granules are unstained. Bar,  $1 \mu$ .  $\times 11,000$ .

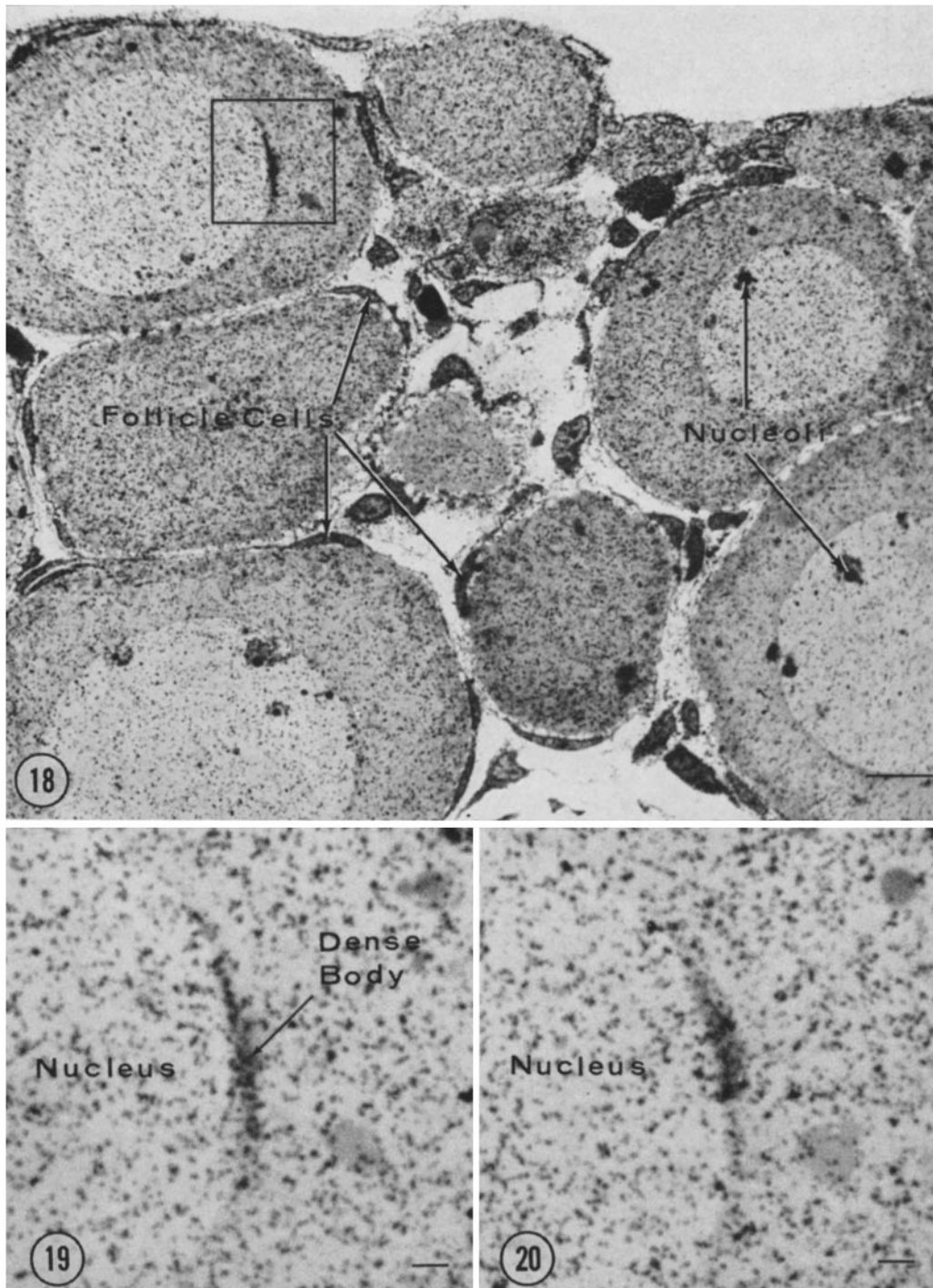


FIGURE 18 Radioautograph of a  $0.5 \mu$  thick Epon section. Pieces of ovary from a *Rana clamitans* tadpole were incubated in tissue culture medium containing tritiated phenylalanine for 6 hr and transferred to medium lacking isotope for an additional 6 hr before fixation. Both nuclear and cytoplasmic structures incorporate the aromatic amino acid. Heavy concentrations of silver grains over dense bodies (in box) indicate that a relatively high concentration of phenylalanine is present in the dense material. Bar,  $10 \mu$ .  $\times 950$ .

FIGURES 19-20 Light micrographs of the dense body outlined in Fig. 18 as seen in parallel sections photographed with a blue filter. In addition to the dense body, silver grains are present over the nucleoplasm and cytoplasm. Some of this labeling presumably represents incorporation of phenylalanine into structural and functional proteins of the oocyte unrelated to the dense material. Bar,  $1 \mu$ .  $\times 5200$ .

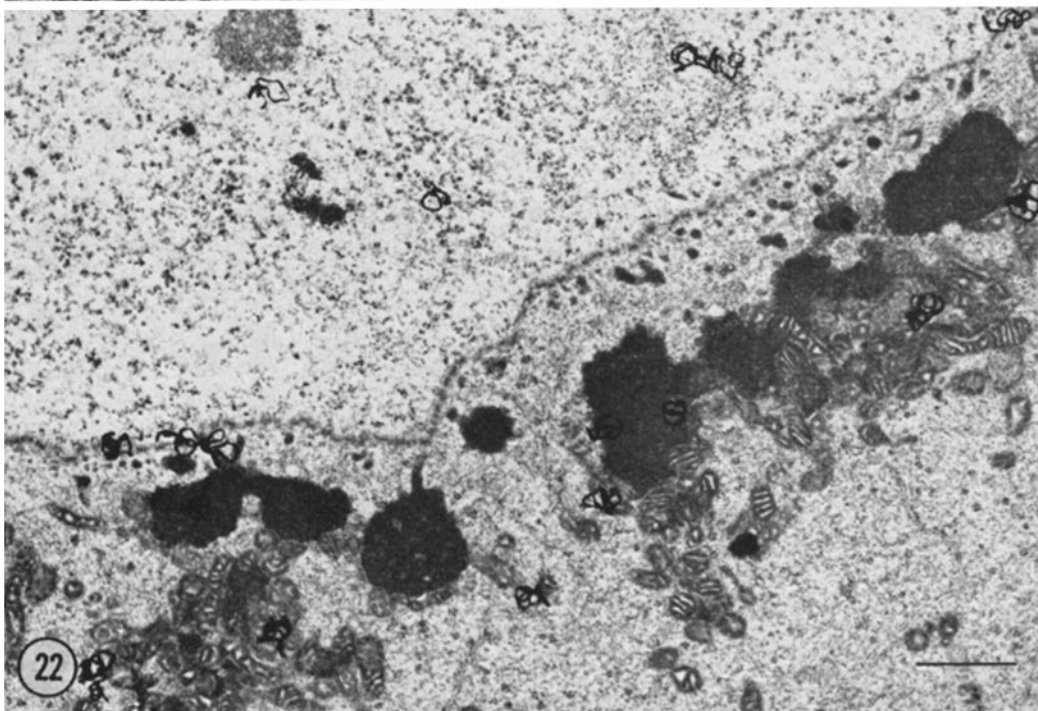
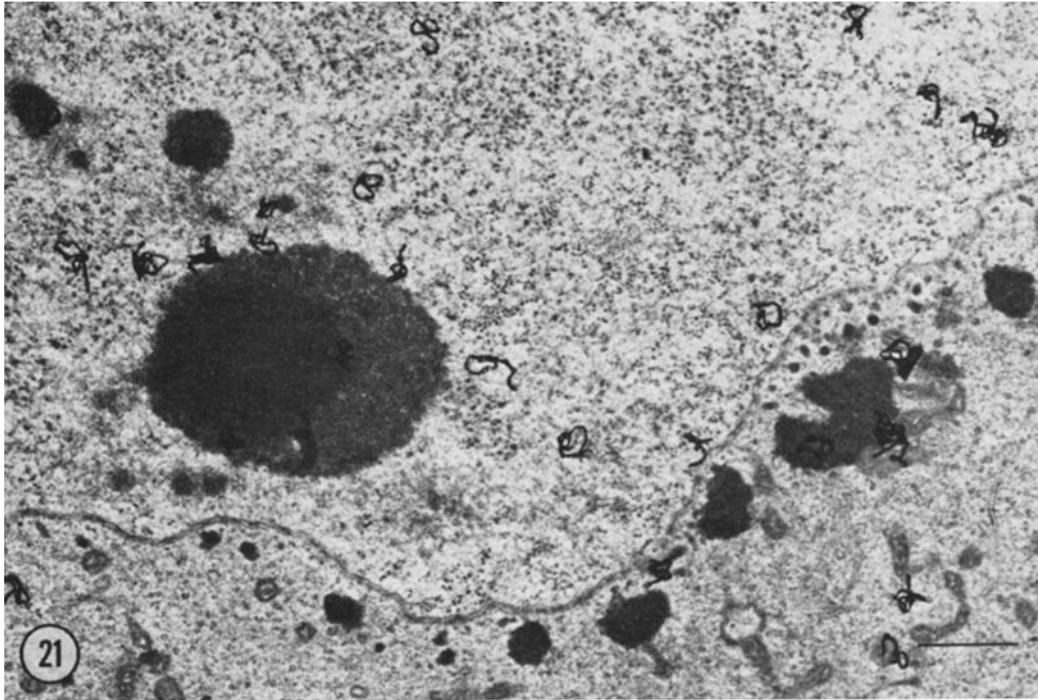


FIGURE 21 An electron microscope radioautograph of an oocyte from a *Rana clamitans* tadpole. The tissue was treated like that shown in Fig. 18. Some of the silver grains over the nucleus frequently are associated with the pars granulosa of nucleoli. Some labeling of a dense body may be seen. Bar,  $1 \mu$ .  $\times 13,000$ .

FIGURE 22 Dense bodies in oocytes incubated with phenylalanine as in Fig. 18. Silver grains may occasionally be seen over the nuclear envelope. After 6 hr of exposure to isotope followed by a 6 hr chase, the label is frequently associated with the periphery of the dense bodies (see also Fig. 21), suggesting accretion of newly formed protein to material already present. The labeling of dense bodies is less pronounced after longer chase periods. Bar,  $1 \mu$ .  $\times 13,000$ .



chondrial function and formation during gametogenesis. André (1962) proposed that the dense material present in rat spermatocytes either forms mitochondria *de novo* or is used in the growth of existing mitochondria which subsequently divide. This hypothesis was extended by Clérot (1968), on the basis of fine structural observations on frog testis and ovary. He suggested that the dense material may participate in mitochondrial multiplication by simple addition to mitochondrial structures already in existence, or may represent mitochondrial enzymes or polypeptide precursors of mitochondrial enzymes which are utilized during mitochondrial growth. Szollosi (1969) further surmised that the close morphological association between mitochondrial clusters and rough endoplasmic reticulum in oocytes and spermatocytes in a number of mammalian species represents a functional relationship important to mitochondrial duplication.

Rather than contributing to mitochondrialogenesis it was suggested by Fawcett et al. (1970) that the dense cytoplasmic material in mammalian spermatocytes may be, at least in part, a product of the mitochondrial protein synthetic system. The apparent ability of frog ovarian mitochondria to form small yolk platelets internally (Ward, 1962; Kessel, 1966), and the accumulation of large proteinaceous crystalloids within spermatid mitochondria of insects (Phillips, 1970), would seem to indicate that germinal cell mitochondria can synthesize appreciable amounts of material beyond that required for their own structural or metabolic needs.

Another possibility that apparently has not been suggested before is that the mitochondria may in some way transform or activate the dense material to permit its utilization in the functional activities of the cell. This type of relationship would be consistent with the apparent sequence of formation of the dense bodies from material arising elsewhere and its brief period of confinement in association with mitochondria.

Each of these suggestions has the common fault of being based on indirect evidence, and they are far from compelling schemes at the present time. All have been derived by reasoning a priori that since the mitochondria and dense material are intimately related morphologically, then they must also be closely linked functionally. Perhaps we will find that they have a much less specific affinity. The dense substance may just as well be a

nucleoprotein manufactured in excess and stored in the cytoplasm. It could then be used during that period of vigorous cell division in early embryogenesis prior to reinitiation of normal synthetic activity.

Germ cells have many morphological features in common with somatic cells and rely upon many of the same functional processes for their integrity. However, as we have seen here, there are some structural components and cytoplasmic associations which are unique to differentiating germ cells. By continued systematic study of morphological features unique to germ cells in several different systems and under a variety of experimental conditions, it should be possible to define more closely the role of these features in germ cell differentiation.

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