

SPHEROIDAL AND RING NUCLEOLI IN AMPHIBIAN OOCYTES

Patterns of Uridine Incorporation and Fine Structural Features

NANCY J. LANE

From the Department of Biology, Yale University, New Haven, Connecticut 06520

ABSTRACT

In maturing oocytes of the newt *Triturus viridescens*, the nucleoli undergo a series of morphological changes that are very similar to those described by Callan for the axolotl, *Ambystoma mexicanum*. The nucleoli first assume the form of spheroids which then become extended into ring or necklace shapes that are DNase-sensitive; in mature oocytes the nucleoli revert to a spheroidal form. Short term in vitro incorporation studies with uridine-³H on both species show that RNA synthesis occurs in a restricted, eccentric portion of the spheroidal nucleoli, thereby producing an asymmetrical pattern of labeling. In the ring forms, however, the localization of the radioactivity suggests that synthesis takes place symmetrically throughout their entire length. The changes in nucleolar morphology apparently reflect the fact that the component DNA has undergone a redistribution from a localized region in the spheroidal nucleoli to an extended circle in the rings; the patterns of uridine-³H incorporation, therefore, parallel the distribution of DNA in both the spheroidal and the ring nucleoli. Ultrastructurally, the nucleoli contain a fibrillar component that corresponds in position to that of the DNA. The typical spheroidal nucleolus consists of a fibrillar core situated eccentrically and surrounded by a hull of granular, ribonucleoprotein material. The ring nucleoli are composed of a central fibrous region that is ensheathed all around its circumference by a layer of similar granular material. This granular substance is thicker at intervals along the length of the rings, representing the "beads" of the necklaces.

INTRODUCTION

It is a well established fact that the maturing oocytes of amphibians contain multiple nucleoli. These nucleoli are most frequently characterized by a spheroidal outline; sometimes, however, they assume other shapes. Recent investigators (Miller, 1964; Kezer, 1965; Callan, 1966) report the existence of amphibian oocyte nucleoli in the form of rings or beaded necklaces like those described in a number of organisms by such cytologists as Carnoy and LeBrun (1897); Maréchal (1907),

Jörgensen (1913), and Guyénot and Danon (1953). Miller (1964) has shown that, when the nuclei of the newt *Triturus* are dispersed in dilute saline, the originally spheroidal nucleoli may become transformed into ring-shaped ones, while Kezer (1965) finds that the nucleoli in the salamander *Plethodon* are often normally in the form of sizeable rings. Callan's study (1966), however, indicates that, at least in the case of the axolotl, the ring nucleoli represent a normal but transitory stage

in nucleolar development during the process of oocyte maturation; the spheroidal nucleoli of immature oocytes become extended into rings and then contract back to spheroids again in the mature oocyte (see Fig. 1).

Miller (1964) and Kezer (1965) independently observed that ring nucleoli could be disrupted by treatment with deoxyribonuclease (DNase). This suggested that each necklace contained a continuous DNA component connecting the beads composed of RNA and protein. A few cytologists had earlier described Feulgen-positive granules associated with the spheroidal nucleoli in amphibian oocytes (Brachet, 1940; Painter and Taylor, 1942; Guyénot and Danon, 1953); Gall (1967, personal communication) has recently confirmed the presence of these granules in the nuclei of *Bufo* and *Triturus* oocytes. The changes in nucleolar shape are, therefore, associated with a concomitant change in the distribution of the DNA; the DNA which is restricted to a localized region in the spheroidal nucleoli becomes in some fashion uncoiled and extended into a circle in the ring forms. Accordingly, certain questions arise. Are both types of nucleoli sites of RNA synthesis? Will the patterns of labeling following uridine-³H incorporation parallel the distribution of the DNA? Might the ring forms display sequential RNA synthesis as do some giant loops of amphibian lampbrush chromosomes (Gall and Gallan, 1962)? How do the ring-shaped nucleoli differ in ultrastructural features from the spheroidal ones? To consider these points, I treated nuclei of mature and immature oocytes from both the axolotl and the newt *T. viridescens* with uridine-³H to determine the sites of synthesis of nuclear RNA; they were also studied by electron microscopy.

The results of this investigation indicate that, when very short term incubation times are employed, the patterns of incorporation of uridine-³H in the nucleoli parallel the distribution of the nucleolar DNA. Electron microscopic studies show that the fibrillar nucleolar component, part of which may represent the DNA moiety, is asymmetrically displaced to one side in the spheroidal nucleoli. In the ring nucleoli, however, the fibrillar area is circumferentially distributed like a string running through and connecting the beads in a necklace.

A preliminary report of this work has been published earlier in abstract form (Lane, 1966).

MATERIAL AND METHODS

The amphibians used in this study were mature females of the Mexican axolotl, *Ambystoma mexicanum*, and the newt, *Triturus viridescens*. The animals were maintained at a constant temperature of 20°C in aged water which was changed twice weekly. For each experiment, some 20–30 oocytes were removed from an animal anesthetized with MS 222 (1 mg/ml) according to the technique described by Gall (1965). Clumps of oocytes were incubated “in vitro” in 1 ml amphibian Ringer’s solution containing 100 μ C uridine-³H (specific activity 21.0 or 25.0 c/mmole; obtained from Schwarz BioResearch, Orangeburg, New York). The oocytes were incubated for 15, 30, and 45 min and at hourly intervals up to 8 hr. The short-term incubation experiments were repeated a total of 18 times, each experiment involving 10 or more oocytes. After each period of incorporation, a clump of oocytes was removed and washed in a number of changes of fresh Ringer’s solution. Several oocytes were separated out, the diameter of each measured, and the nucleus isolated; spread preparations were made of each nucleus in a depression slide as specified by Gall (1965). Oocytes of varying diameter from a single specimen of *T. viridescens* were studied to determine whether, during maturation, the nucleoli undergo a cycle of morphological changes as in the axolotl; this experiment was repeated a number of times with different animals. The solution used for isolating the newt nuclei was unbuffered 5:1 salt solution (0.1 M KCl: 0.1 M NaCl) (Callan and Lloyd, 1960). For isolating axolotl nuclei, the modified isolation medium proposed by Callan (1966) was employed. The nuclear preparations were exposed to neutralized formalin vapor for 2–3 min, allowed to disperse, and were subsequently treated for 30 min with the vapor from a solution of 50% ethanol. They were then dehydrated through an ascending series of alcohols to acetone, and air dried. Control preparations were treated with ribonuclease (RNase) (1 mg/ml) and DNase (50 μ g/ml) in 0.1 M phosphate buffer for 2–4 hr at 37°C.

For radioautographic preparations, the slides on which the nuclei had been spread were coated with a “subbing” solution of 0.01% gelatin and 0.01% chrome alum. This presented a smooth surface for adherence of the Kodak NTB-2 liquid emulsion into which they were subsequently dipped. The slides were exposed at 4°C from 1 wk to 7 months; they were then developed in D-19 for 3 min at room temperature, fixed, washed, and dried.

Unfixed spread preparations were treated with DNase and RNase (60 μ g/ml and 1 mg/ml, respectively) in 0.1 M 5:1 salt solution in 0.1 M phosphate buffer at pH 6.8; magnesium chloride (0.003 M) was

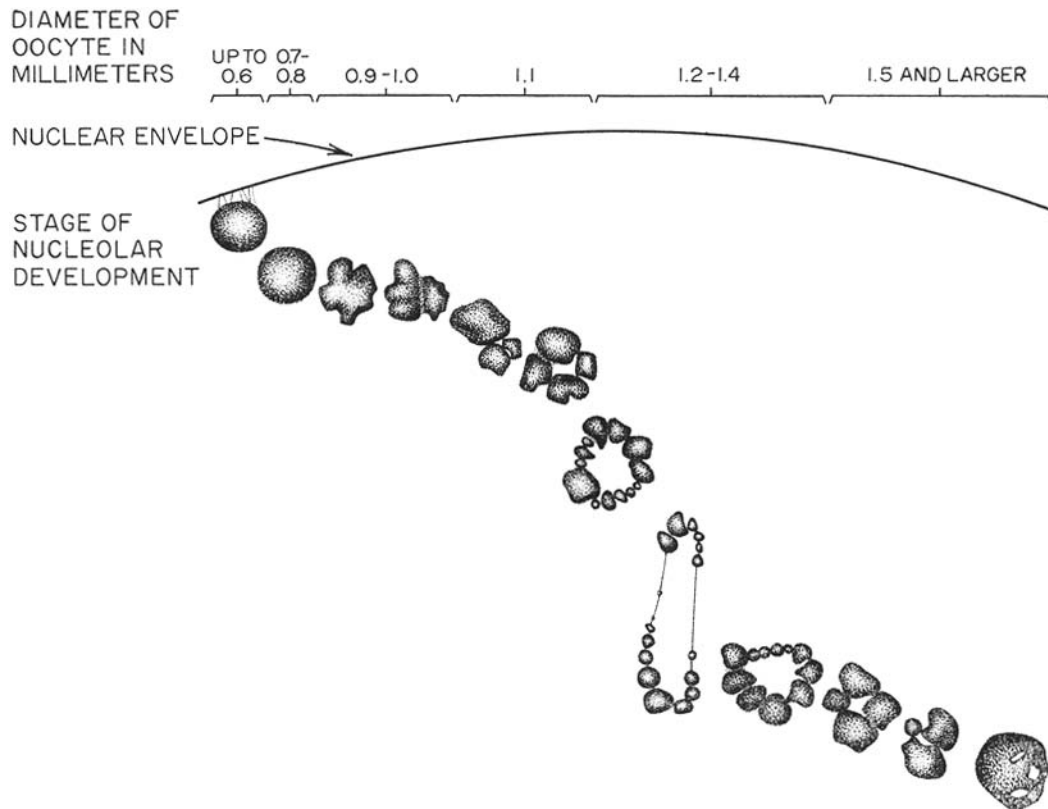


FIGURE 1 Diagram illustrating the transformations in nucleolar form that occur during oocyte maturation in the newt *Triturus viridescens*.

added to the former. A drop of one of these enzyme solutions was added to a depression slide in which a nucleus had been spread in 5:1 salt solution; the final concentration of the enzyme was thereby reduced by at least one-half. Particular attention was paid to the effects of the enzymes on nucleoli in the ring conformation.

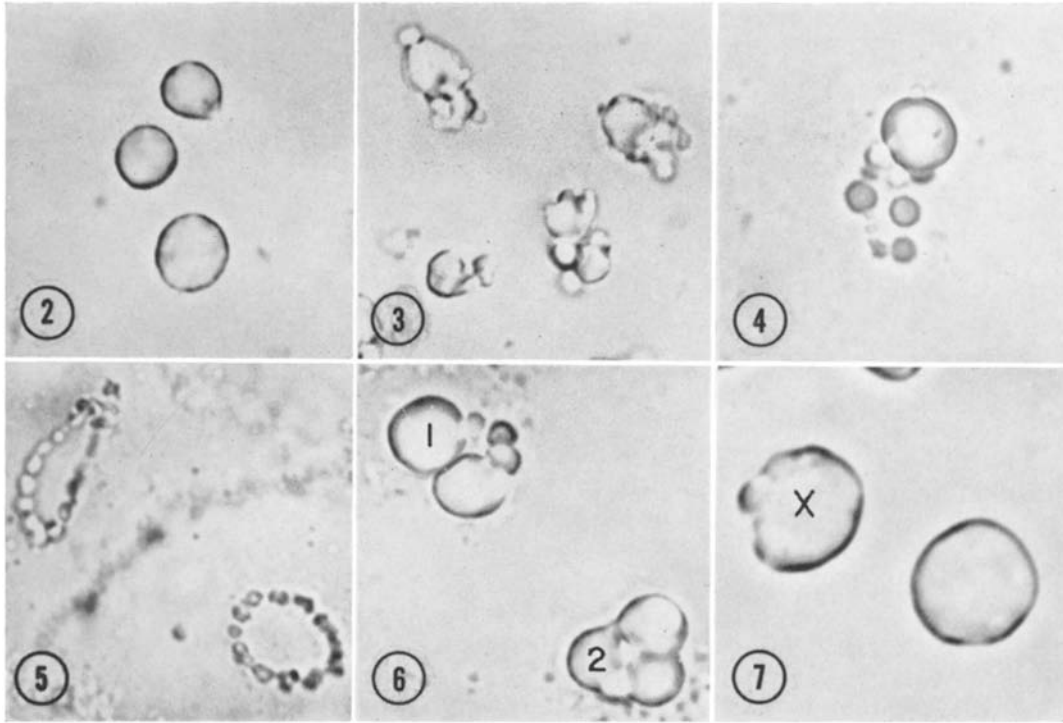
For electron microscopic studies, both isolated nuclei and whole oocytes were fixed for 1-3 hr in 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 (Sabatini, Bensch, and Barnett, 1963) and then washed for 3 hr or overnight in phosphate buffer with added 0.2 M sucrose. The nuclei were isolated either directly in buffered glutaraldehyde or in 0.1 M 5:1 salt solution in 0.1 M phosphate buffer at pH 6.8. In the latter case, the nuclei were transferred to glutaraldehyde immediately after being separated from the yolky cytoplasm so that the interval between isolation and fixation was only a matter of seconds. Isolated nuclei are not structurally different from nuclei in intact oocytes, but the preservation of nuclear pore structure is better in isolated than in intact nuclei when large oocytes are being studied,

possibly because the yolk granules interfere with the penetration of the fixative in whole oocytes. In both cases, the oocytes and nuclei were subsequently postosmicated for 1 hr, dehydrated through propylene oxide, and embedded in Araldite. Sections for light microscopy were made from these blocks by cutting at 1-2 μ on a Porter-Blum MT 2 microtome. Such sections were stained with 1% toluidine blue in 1% borax for observations on the form and distribution of the nucleoli. Thin sections cut from these same blocks were used for study under a Philips EM 200, operated at 60 kv.

OBSERVATIONS

Morphological Transformations of Nucleoli

Spread preparations of nuclei of *T. viridescens* taken from oocytes covering a wide range of diameter showed that the multiple nucleoli undergo a distinct cycle of morphological changes (Fig. 1). The majority of these nucleoli, which number in



FIGURES 2-7 Light micrographs of the changes in nucleolar morphology during oocyte maturation in *T. viridescens*. Spread preparations made by isolating nuclei in 5:1 salt solution.

FIGURE 2 Spheroidal nucleoli detached from nuclear envelope, found in oocytes measuring from 0.7 to 0.8 mm in diameter. The point of former attachment to the nuclear envelope can still be seen in the nucleolus at the top of the micrograph. $\times 1,600$.

FIGURE 3 Angular nucleoli from oocytes around 0.9-1.0 mm in diameter. $\times 1,600$.

FIGURE 4 Ring nucleolus from oocyte of about 1.1 mm in diameter. Note that it is composed of "beads" of different sizes. $\times 1,600$.

FIGURE 5 Extended ring or necklace nucleoli present in oocytes ranging from 1.2 to 1.4 mm in diameter. $\times 870$.

FIGURE 6 Coalescence of nucleolar material from extended ring forms into smaller rings composed of a few, larger beads. Typical of oocytes around 1.5 mm in diameter. $\times 2,000$. Compare 1 and 2 with those shown in Figs. 15 and 16.

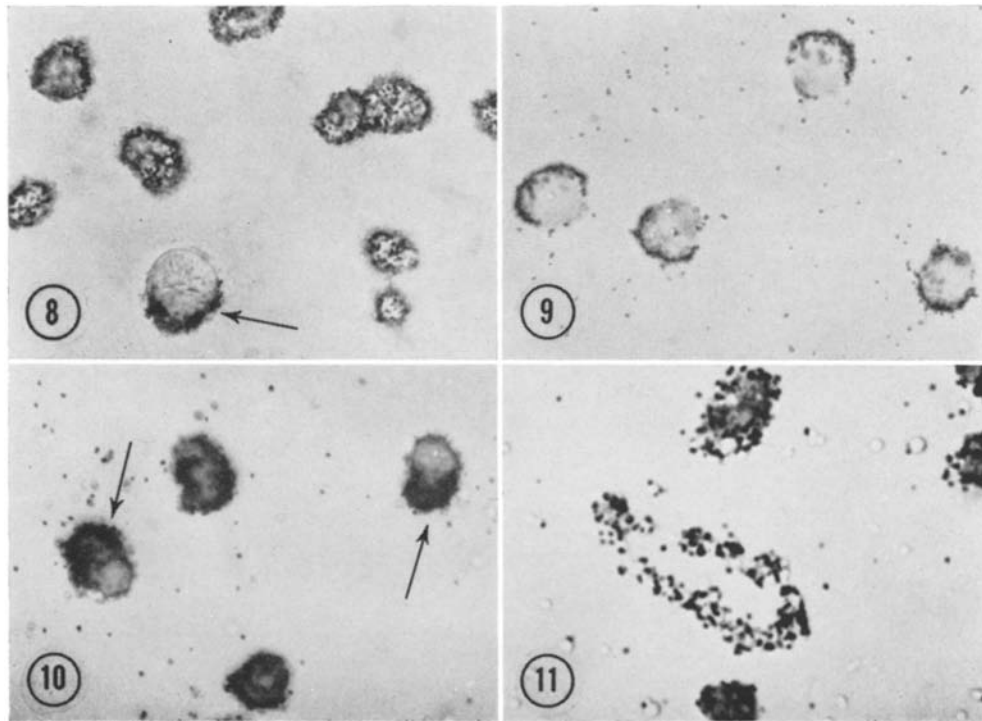
FIGURE 7 Nucleoli from mature oocytes; these nucleoli have completely regained their spheroidal form, with the exception of X which is still somewhat lumpy in outline. $\times 2,700$.

the hundreds, are unattached to the meiotic chromosomes. In small, immature oocytes, up to 0.5 and 0.6 mm in diameter, the nucleoli are spheroidal and attached to the nuclear envelope. They remain adherent to it even after the nucleus is broken open. At 0.7-0.8 mm, the nucleoli are detached from the nuclear envelope but still have the form of spheroids with a peripheral distribu-

tion (Fig. 2). When the oocytes attain a diameter of 0.9-1.0 mm, the nucleoli become angular in outline (Fig. 3). These nucleoli then become pulled out into the form of rings in oocytes of 1.1 mm diameter. These rings are composed of beads of RNA and protein (Miller, 1966) strung out along a connecting strand; often they consist of one or two large beads and a number of small ones (Fig.

4). In medium-sized oocytes, 1.2–1.4 mm diameter, the nucleoli attain extended ring or “necklace” shapes where many of the beads are of a fairly similar size (Fig. 5). With maturation, when the oocytes are 1.5–1.6 or more mm in diameter, the nucleoli reaggregate into spheroidal bodies first by reclumping of the necklaces into smaller rings composed again of fewer, larger beads (Fig.

6) and then finally merging into a single spheroidal mass (Fig. 7). These spheroidal forms are larger than those in immature oocytes, presumably owing to accumulation of newly synthesized RNA and protein. Frequently they contain vacuoles of variable size and number (Figs. 17 and 19). In oocytes of medium size, the majority of the nucleoli in a single nucleus are at approximately the same



FIGURES 8–11 All the micrographs in this plate are radioautographic preparations made by coating spread nuclear specimens with Kodak NTB 2 liquid emulsion. The nuclei were isolated from oocytes that had been incubated for varying periods of time in medium containing uridine- ^3H .

FIGURE 8 Preparation from an immature oocyte, 1.1 mm in diameter, from *A. mexicanum*. Incubated in uridine- ^3H for 30 min. Note that the radioautographic label is restricted to one eccentric region of the spheroidal nucleolus (arrow), but is evenly dispersed over the angular and doughnut-shaped nucleoli. $\times 1,000$.

FIGURE 9 Preparation from a mature oocyte of about 1.8 mm diameter from *A. mexicanum*. Incubated in uridine- ^3H for 45 min. Note that each spheroidal nucleolus is asymmetrically labeled with silver grains on one edge only. $\times 1,000$.

FIGURE 10 Preparation from a mature oocyte of approximately 1.5 mm diameter from *T. viridescens*. Incubated in uridine- ^3H for 60 min. After this period of incorporation, the radioautographic label is localized over half or more of the surface of the spheroidal nucleoli. The arrows point to nucleoli in which the asymmetrical pattern of labeling is still evident $\times 1,000$.

FIGURE 11 Preparation from a medium-sized oocyte of about 1.3 mm in diameter from *T. viridescens*. Incubated in uridine- ^3H for 45 min. The radioautographic label is distributed fairly evenly over the entire extent of the ring nucleolus. $\times 1,200$.

stage morphologically; however, some nucleoli in the same nucleus may be at different stages of ring extension and, sometimes, while the rest are in ring forms, a few nucleoli may still be spheroidal (Fig. 8).

Correlated with these changes in morphology, the nucleoli in the newt become altered in their intranuclear disposition. In very young oocytes, they are, as described above, attached to the nuclear envelope. From this peripheral disposition they become detached from the nuclear membrane and gradually move in toward the center of the nucleus. This occurs when most of the nucleoli are in the ring forms in medium-sized oocytes. In mature oocytes, when the nucleoli are again in the shape of spheroids, they are aggregated in the center of the nucleus around the clumped chromosomes. The process of oocyte maturation is a relatively lengthy one, so that the progression of the oocyte through the various size classes and the concomitant changes in nucleolar morphology (as shown in Fig. 1) occur over a period of some months.

The spheroidal nucleoli of *Triturus* measure up to 10 μ in diameter, in comparison with those of *Ambystoma* which may be as much as 20 μ in di-

ameter. The ring nucleoli of both species show different degrees of extension, but attain maximum circumferences ranging from 200–300 μ .

Deoxyribonuclease Treatment

Nucleoli in the ring or necklace form are disrupted when unfixed, spread preparations are treated with DNase. The breaks occur at intervals along the length of the ring so that ultimately all that is left are the separate beads that were originally linked up to comprise the necklace. RNase does not have this effect. This phenomenon is more readily demonstrated in the newt than in the axolotl since in the latter it is often difficult to separate the necklace forms from the rigid nuclear sap surrounding them.

Radioautography

Examination of radioautographs of spread nuclear preparations from oocytes incubated for 15, 30, or 45 min in uridine- ^3H solution shows that each spheroidal nucleolus incorporates the RNA precursor in a restricted eccentric area (Figs. 8 and 9). This asymmetrical pattern of labeling is present in spheroidal nucleoli from both mature

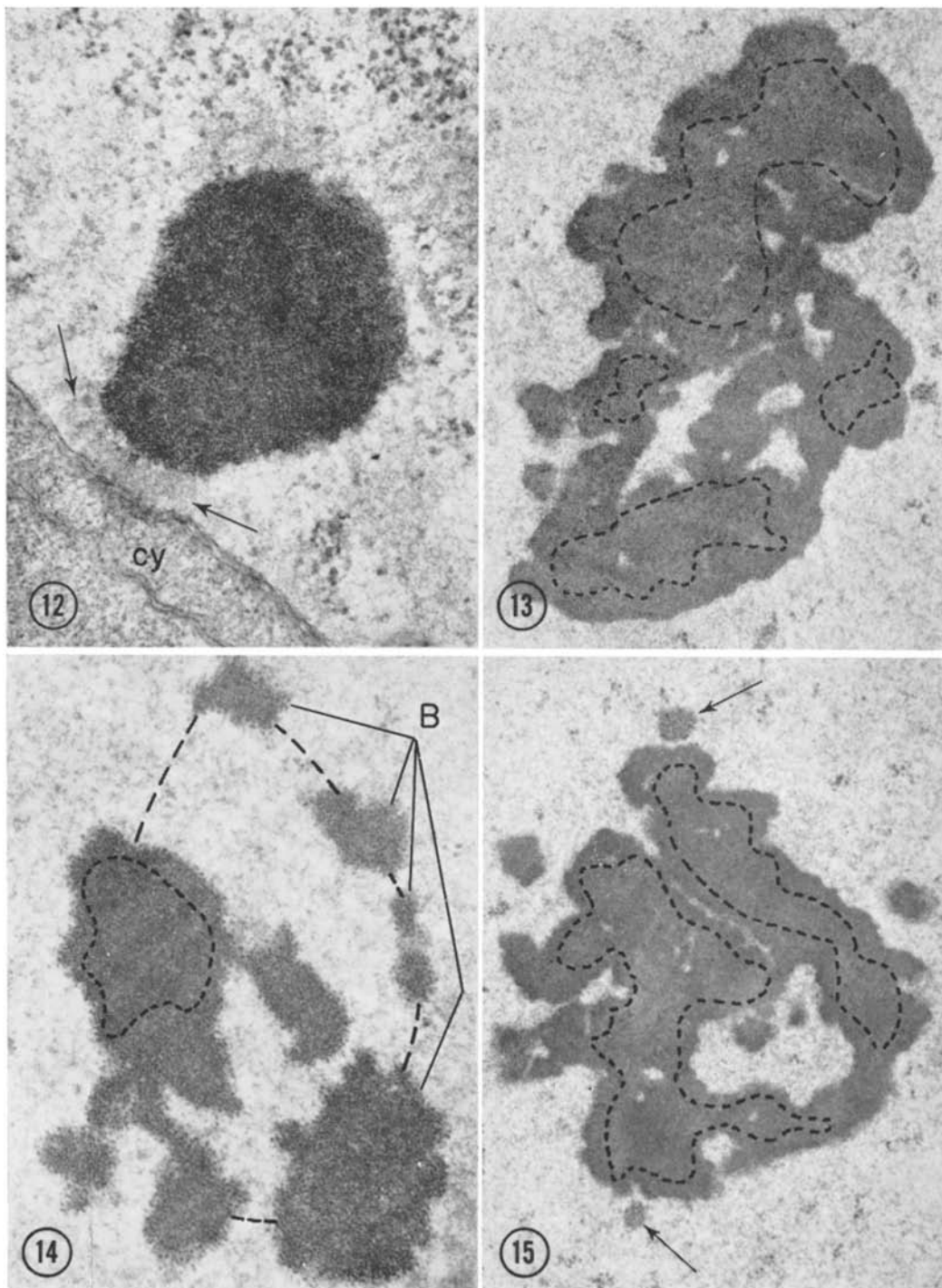
FIGURES 12–15 All the electron micrographs in this plate are nucleoli from sections of amphibian nuclei fixed in 4% glutaraldehyde, postosmicated, and embedded in Araldite. The thin sections were stained in uranyl acetate. At these magnifications, the distinction between fibrous internum and granular externum is not clear. Hence, the fibrous core material of each nucleolus is encircled by dotted lines; the granular hull substance lies outside. Fig. 12 is from the axolotl, *A. mexicanum*, and Figs. 13–15 are from *T. viridescens*.

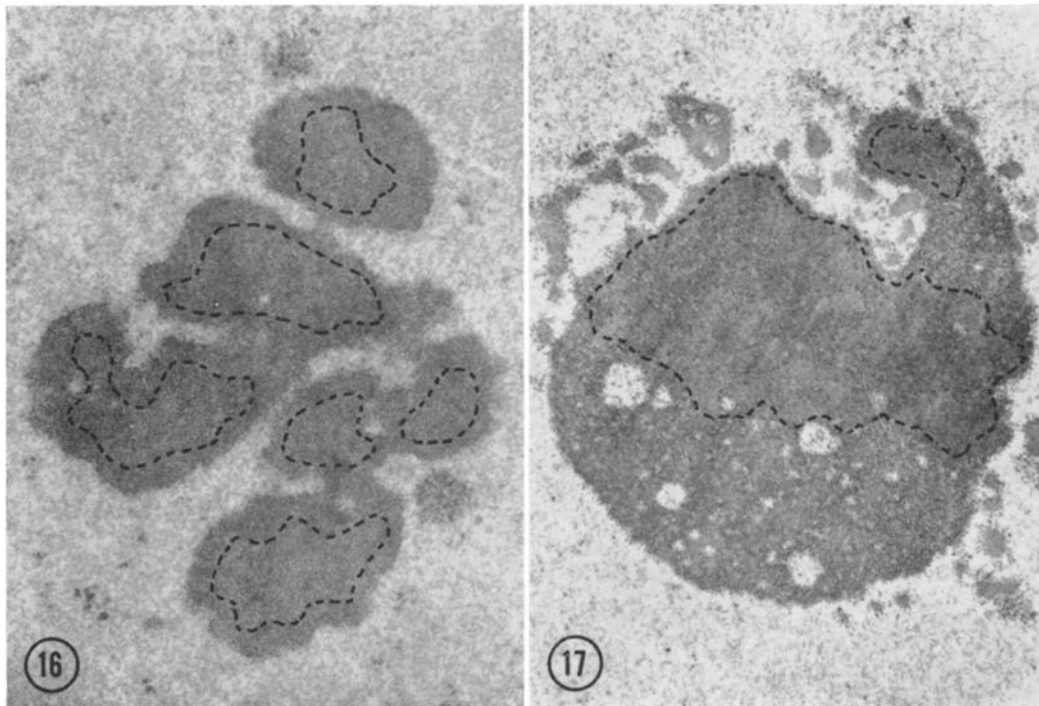
FIGURE 12 Portion of a nucleus from an immature oocyte. The spheroidal nucleolus is composed entirely of fibrous core material and is connected to the nuclear envelope by fibrillar attachments (arrows). Compare its shape with that of nucleoli shown in Fig. 2. Cy, cytoplasm. $\times 34,000$.

FIGURE 13 Nucleolus typical of a nucleus from an oocyte of approximately 1.0 mm in diameter. The nucleolus has an irregular shape corresponding to the angular forms shown in Fig. 3. $\times 19,000$.

FIGURE 14 Nucleolus from the nucleus of a medium-sized oocyte, 1.3 mm in diameter. The nucleolus is in the form of a small ring like that shown in Fig. 4, but the section cuts through only part of its circumference. The dashed line indicates how the beads would probably be joined together if serial sections were cut. Note that the beads (B) of the necklace in this section are composed of granular material. $\times 26,000$.

FIGURE 15 Nucleolus from a nucleus of an oocyte of about 1.3 mm in diameter. The nucleolus is probably reclumping after being in an extended necklace phase, and corresponds roughly to 1 in Fig. 6. Note the granular masses (arrows) located peripherally. $\times 15,500$.





FIGURES 16-17 The electron micrographs in this plate are nucleoli from oocytes prepared in the same way as in Figs. 12-15. Again, the fibrous cores of the nucleoli are circumscribed by dotted lines.

FIGURE 16 Nucleolus from a nucleus of an oocyte, about 1.5 mm, from *T. viridescens*. The nucleolus is in the process of reaggregating to a spheroidal form. Compare its shape with that of 2 in Fig. 6. $\times 26,000$.

FIGURE 17 Nucleolus from a nucleus of a mature oocyte from *A. mexicanum*, about 1.9 mm in diameter. The nucleolus has almost completely regained the form of a spheroid. The fibrous material has clumped in the central area and the granular material remains vacuolated on the periphery. Compare with X in Fig. 7. $\times 12,000$.

and immature oocytes, in other words, from nucleoli both before (Fig. 8) and after (Fig. 9) necklace formation. However, in the case of spheroidal nucleoli from extremely young oocytes, particularly those still attached to the nuclear envelope, the labeling is symmetrical. Radioautographs from both *T. viridescens* and *A. mexicanum* are essentially the same except that the localized labeling is more marked in the axolotl than in the newt. Owing to the short incubation times employed, this label is most clearly demonstrated in preparations that have had a lengthy exposure to the emulsion, for example up to 5-7 months. In the case of oocytes incubated for 60 min, the radioautographic label has often spread from the one restricted region across a good part of the nucleolar surface, so that, in some instances, only a small

portion of the spheroidal nucleolar surface remains unlabeled (Fig. 10). In preparations incubated for more than 60 min, all the spheroidal nucleoli are labeled over their whole surface; this is so in all the spheroidal forms examined, even those from very mature oocytes (cf. Macgregor, 1967).

In contrast to the spheroidal forms, after short periods of incubation (15, 30, and 45 min) the ring or necklace nucleoli show symmetrical labeling throughout their entire length (Fig. 11). As would be expected, similar patterns are observed following prolonged periods of incubation.

The uridine- ^3H is being incorporated into RNA, since RNase treatment of spread preparations prior to radioautography results in removal of essentially all the isotope; DNase treatment, on the other hand, has no such effect.

Nucleolar Ultrastructure

Electron micrographs of spheroidal nucleoli from immature or mature oocytes of either species show an eccentric fibrous core consisting of fibrils embedded in a homogeneous matrix (Figs. 12, 17, and 19). In mature oocytes, this core is surrounded by a cortex or hull composed of fibrils and granules measuring about $20\text{ m}\mu$ in diameter (Figs. 17 and 19). There is a less extensive cortex in the immature spheroids than in the mature ones and many of the former are constituted solely of core material (Fig. 12). In immature oocytes, the nucleoli are attached to the nuclear envelope by fibrillar strands (Fig. 12) and the fibrous core seems always to be associated with that side of the nucleolus closest to these strands and the nuclear envelope. In very young oocytes, the nucleoli are flattened

against the nuclear membrane; they gradually round up into spheroids before their disconnection from the nuclear envelope.

As the nucleoli become extended into necklace forms, the fibrous component is no longer restricted to one area. It becomes pulled out to spread irregularly throughout most of the nucleolus (Figs. 13 and 14). Ultimately, when extended, the ring nucleoli contain a central fibrillar core which is apparently continuous throughout their length like the string running through and connecting the beads of a necklace (Fig. 18). This core is encased by a granular sheath composed of a material similar to that forming the hull of the spheroidal nucleoli. This outer granular layer is not very thick except when the section cuts through a bead of the necklace (Fig. 14). When the ring nucleoli are reclumping to form spheroids for the second

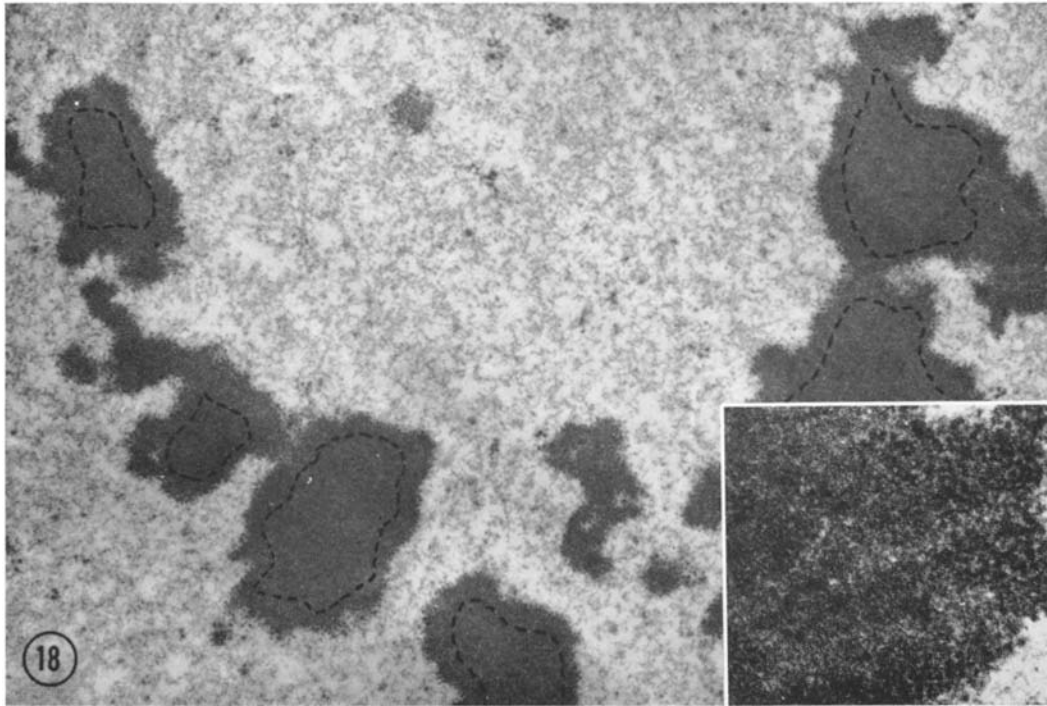


FIGURE 18 This nucleus is from an oocyte of *T. viridescens*, of approximately 1.3 mm in diameter. It has been fixed in the same way as those in Figs. 12–15. One-half of a necklace nucleolus is shown. Note that the fibrous moiety (delimited by dotted lines) forms the internal component of each bead and presumably is continuous throughout the length of the ring. The peripheral granular portion forms a rather thin layer around this fibrous internum. Compare the shape of this nucleolus with that of the nucleoli in Fig. 5. The insert shows part of a bead of a ring nucleolus at higher magnification. In this insert, the granular material to the right merges almost imperceptibly into the fibrous portion on the left. $\times 20,000$. Insert, $\times 62,000$.

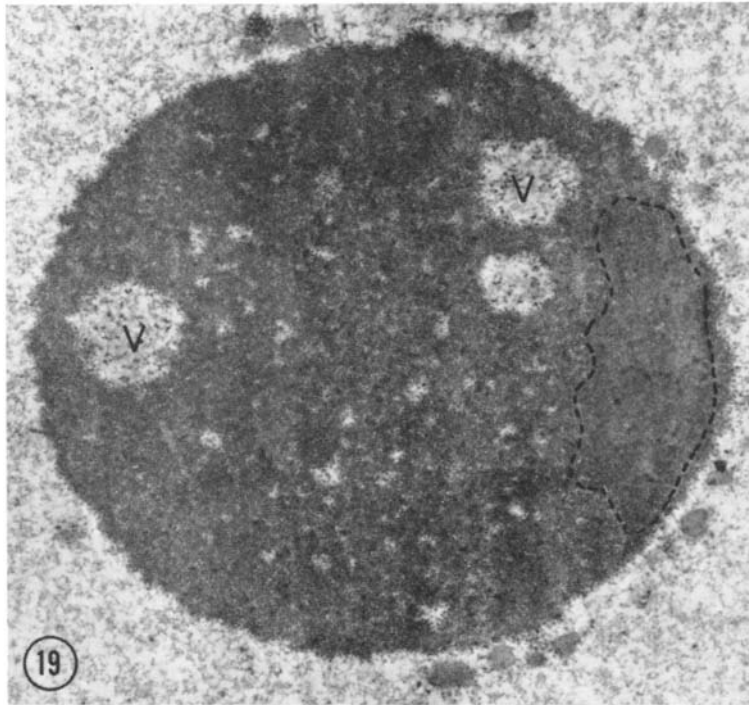


FIGURE 19 This nucleus is from *A. mexicanum* and was fixed in the same way as those in Figs. 12–15; it comes from a mature oocyte of 1.9 mm diameter. The nucleolus has reclumped from a ring into a spheroid that consists of an extensive granular hull and an internal fibrous core. The latter is outlined by a dotted line. Compare the shape of this nucleolus with that of those in Fig. 7. Note the vacuoles (v) which occur within the nucleolar substance and the separated granules that are present both within the vacuoles and near the external surface of the nucleolus. $\times 18,000$.

time (Figs. 15 and 16), the fibrillar component gradually reaggregates in one restricted area and the granular material clumps together to form the hull (Figs. 17 and 19). Vacuoles often form in the rings as each one clumps together; these vacuoles remain even after the spheroids are completely reconstituted (Figs. 17 and 19).

It is of interest to note that the ring nucleoli and the immature spheroids are often connected by “streams” of granules to channels associated with the nuclear pores (Figs. 20–22). These streams of particles are found in intact oocytes as well as isolated nuclei, thereby suggesting that the phenomenon is not the result of osmotic forces introduced during nuclear isolation.

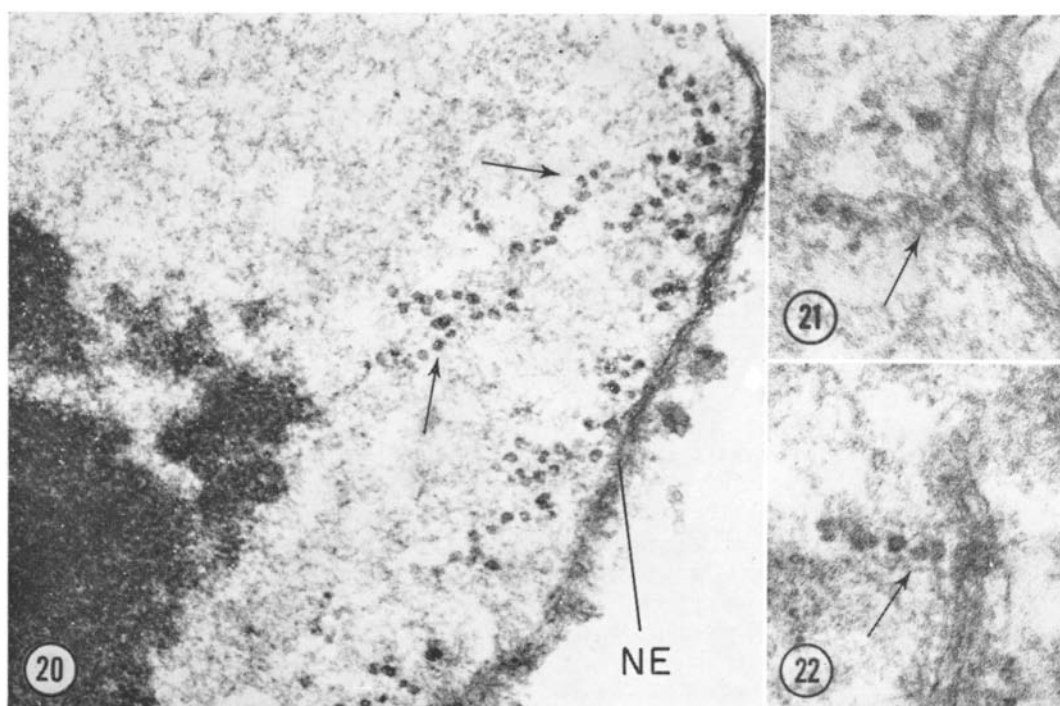
DISCUSSION

Feulgen staining (Brachet, 1940; Painter and Taylor, 1942; Guyénot and Danon, 1953; Gall, 1967, personal communication) and DNase diges-

tion (Miller, 1964; Kezer, 1965) demonstrate the presence of extrachromosomal DNA in the nucleoli of maturing amphibian oocytes. The disposition of this DNA changes when the nucleoli change from spheroids to rings or from rings to spheroids. The sites of RNA synthesis in both ring and spheroidal nucleoli, as indicated by the patterns of labeling in radioautographs after short term incorporation of uridine- ^3H , parallel the DNA distribution. In summary, then, with alterations in nucleolar morphology there are variations in uridine uptake which reflect changes in the DNA localization.

Although there are certain differences in the precise configurations¹ observed, the changes in

¹ Nucleoli of the axolotl often assume “doughnut”-shaped ring forms before becoming extended into lumpy rings; those of *T. viridescens* at a similar stage (as in Fig. 4) have the appearance of linked spheroidal beads.



FIGURES 20-22 Electron micrographs of nuclei isolated from medium-sized oocytes, about 1.7 mm diameter, from the axolotl *A. mexicanum*. The material was prepared in the same way as those in Figs. 12-15. The ring nucleolus in Fig. 20, of which only one edge is visible, is composed of an inner fibrous and an outer granular portion. The latter is connected by a stream of granules (arrows) to the nuclear envelope (NE). These granules apparently run into channels associated with the nuclear pores as shown (at arrows) at higher magnification in Figs. 21 and 22; in both figures, the nuclear envelope is to the right. Fig. 20, $\times 48,500$; Fig. 21, $\times 93,000$; Fig. 22, $\times 91,500$.

nucleolar morphology in *T. viridescens* parallel those described for the axolotl² (Callan, 1966), which suggests that similar nucleolar transformations may occur in the nuclei of maturing oocytes of most amphibia. It must be noted, though, that variations in individual animals exist and that in some at least, the changes in nucleolar morphology are not striking. Miller (1966), for example, in his studies on the same species of *Triturus*, has not observed such a sequence of morphological changes in nucleoli from oocytes of varying size. Possibly, therefore, the sequence may be radically

² The oocytes of the axolotl are larger than those of the newt. Similar nucleolar forms occur in oocytes of both genera but corresponding stages in the axolotl are larger than those in the newt. Thus a medium-sized oocyte of the former measures around 1.6-1.8 mm, while in the latter it would be about 1.2-1.4 mm in diameter; both, however, contain ring nucleoli.

altered or perhaps absent under certain environmental or physiological conditions.

It has been demonstrated that radioactive adenine, cytidine, and uridine are taken up by the nucleoli of amphibian oocytes (Gall, 1958; 1959; Ficq, 1961; Gall and Callan, 1961; Izawa et al., 1963; Miller, 1962; Macgregor, 1967). In the experiments reported here, it has been shown that the several patterns of incorporation of uridine-³H into the nucleoli of *Triturus* and *Ambystoma* parallel the intranucleolar distribution of DNA. Further, it has been found that actinomycin D inhibits the incorporation of radioactive nucleotides into nucleolar RNA in amphibian oocytes (Izawa, Allfrey, and Mirsky, 1963). The RNA synthesis occurring in the nucleoli must, therefore, be DNA dependent. The symmetrical labeling of the ring nucleoli, after even very short periods of uridine-³H incorporation, suggests that synthesis may take

place throughout the whole extent of the DNA component, unless there are localized regions of uncoiled or unextended DNA along the length of the necklace. The symmetrical labeling of all the angular and doughnut-shaped nucleoli (Figs. 8 and 11) is consistent with the results of the fine structural studies. These studies show that the fibrous component becomes spread throughout the nucleolar body as soon as the nucleoli become lumpy and irregular in outline (Figs. 13–16); part of this fibrous component is presumably the DNA moiety.

After short periods of incubation in tritiated uridine, the spheroidal nucleoli studied in these experiments show radioautographic label restricted to one part of their border. This suggests that the synthesis of RNA occurs over the DNA-containing core, as has already been discussed above. The spreading of the label, with longer periods of incubation, over the remainder of the spheroid suggests that transport of the synthesized RNA occurs from the core region of the nucleolus to its hull. The cortical granular material probably represents ribosomal precursors since nucleolar RNA in amphibian oocytes has a base composition similar to that of cytoplasmic RNA (Edström and Gall, 1963), and since anucleolate mutants of the amphibian *Xenopus* fail to synthesize ribosomal RNA (Brown and Gurdon, 1964). Moreover, Gall's (1966) studies on labeling patterns and sedimentation values of nuclear RNA from amphibian oocytes suggest that ribosomal RNA precursors are present in the nucleoli. Hence, the fact that particles rather similar to those associated with the cortex and periphery of the nucleoli are at times found in streams connecting the nucleoli with the channels of the nuclear pores, suggests that these may be the ribonucleoprotein particles en route to the cytoplasm via the nuclear pores. These particles, which sometimes appear vesicular (see Fig. 20), are slightly larger than the granules forming the nucleolar cortex. This need not preclude the possibility that they are being synthesized in the nucleoli since material could be added to the granules as they are released from the nucleolar hull. These particles will be described in more detail elsewhere (Lane, 1967, in preparation). Balbiani ring granules have also been observed "passing" through the pores of the nuclear envelope in the salivary glands of *Chironomus* (Stevens and Swift, 1966).

In the experiments reported here, the spheroidal nucleoli of very young oocytes consist entirely of

fibrous core material; there is no accumulation of granules to form a cortex (see Fig. 12). Radioautographic preparations of such nucleoli after short periods of uridine-³H incorporation show completely symmetrical labeling, in contrast with the asymmetrical patterns found in spheroidal nucleoli which possess a granular hull. It is, therefore, probable that this labeling is associated with the *whole* of the fibrous nucleolar core and not just the region of the Feulgen-positive granule. This assumption is supported by the following evidence. 1. In radioautographic preparations made of nuclei incubated in uridine-³H, the smallest nucleolar area ever labeled, even after very short periods of incorporation, is more nearly the diameter of the core (4–5 μ) than the Feulgen-positive granule (less than 1 μ). 2. Ebstein (1967, in preparation) has shown that DNA is found throughout the core area and is not limited to the visible Feulgen-positive granule. This she has done by studying binding of labeled actinomycin D to DNA in cytological preparations. 3. Similarly, it has been shown that, when cores of *T. cristatus* nucleoli are separated from their hulls following uridine-³H incorporation, the radioautographic label is found over the *whole* of the core moiety (Macgregor, 1967).

The dynamics of the functioning nucleolus might be further elucidated if a comparison is made between it in its various forms and the DNA-containing lateral loops of the lampbrush chromosomes. There is a basic similarity between the two which is most evident visually when the nucleoli are extended into rings. This would provide a satisfactory explanation for the reactions of the spheroidal forms: the Feulgen-positive granule would represent the chromomere while the unextended nucleolar DNA moiety in the core, with its associated RNA and protein, would represent the synthetically active loop. If such a comparison is valid, then it is likely that RNA synthesis does occur all over the fibrous core region (which presumably is actually a contracted or coiled loop) rather than over only the Feulgen-positive granule. In addition, most lateral loops are simultaneously labeled along their length, like that of the nucleoli extended into rings as described here; only a few specialized giant loops show sequential labeling (Gall and Callan, 1962).

The ultrastructural details of the spheroidal nucleoli are typical of the nucleoli of other amphibian oocytes, such as those found in *Triturus*

and *Rana* in which a granular component also surrounds a fibrous core (Miller, 1962). The fibrous and granular regions in the nucleoli of the axolotl and *T. viridescens* are not as readily distinguishable one from the other as they are in the spheroidal nucleoli of *Rana* (Miller, 1962) or of *T. cristatus* (Macgregor, 1967). Miller has described the reverse situation—an internal granular component—in very young *Triturus* oocytes, but this has not been observed in either of the species studied here. It seems likely that the nucleolar DNA moiety is represented by the fibrous core and that it is these cores that become extended into the fibrillar rings embedded in ribonucleoprotein granules found in the necklace nucleoli. Electron microscopic cytochemical procedures for the demonstration of DNA are currently in progress to ascertain its exact distribution and appearance within the nucleoli.

Is the change of DNA distribution and the correspondingly altered pattern of uptake of RNA precursor materials in the nucleolar rings, as compared to the spheroids, of any functional consequence? Nucleolar RNA synthesis begins long before the occurrence of ring formation and continues throughout the process of oocyte maturation, and both forms of nucleoli actively incorpo-

rate uridine-³H. The formation of nucleolar rings, therefore, is not associated with a turning on or off of RNA synthesis, although possibly it may reflect a relative decrease or increase in synthetic activity. The presence of vacuoles within the spheroidal nucleoli does not seem to be related to heightened synthetic activity as has been reported elsewhere (Johnson, 1966). Since there is a greater accumulation of granular cortex material in the spheroidal forms than in the rings (compare Figs. 19 and 18), it may be that there are different rates of turnover in the two types of nucleoli. On the other hand, the varying nucleolar forms may simply be a result of physical changes in the nuclear milieu (see Callan, 1966).

At present, then, it can only be noted that the changes in nucleolar morphology are concurrent with changes in DNA distribution which, in turn, are responsible for corresponding alterations in the patterns of uridine-³H incorporation.

I am very grateful to Dr. J. G. Gall for his helpful comments during the course of this work and for critically reading the manuscript.

This investigation was supported by a research grant from the National Institute of General Medical Sciences (GM 12 427).

Received for publication 16 June 1967.

REFERENCES

1. BRACHET, J. 1940. La localisation de l'acide thymonucléique pendant l'oogénèse et la maturation chez les Amphibiens. *Arch. Biol. (Liège)*. 51:151.
2. BROWN, D., and J. GURDON. 1964. Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc. Natl. Acad. Sci. U. S.* 51:139.
3. CALLAN, H. G. 1966. Chromosomes and nucleoli of the axolotl, *Ambystoma mexicanum*. *J. Cell Sci.* 1:85.
4. CALLAN, H. G., and L. LLOYD. 1960. Lampbrush chromosomes of crested newts *Triturus cristatus* (Laurenti). *Phil. Trans. Roy. Soc. London Ser. B.* 243:135.
5. CARNOY, J. B., and H. LEBRUN. 1897. La vésicule germinative et les globules polaires chez les Batraciens. *La Cellule*. 12:191.
6. EDSTRÖM, J.-E., and J. G. GALL. 1963. The base composition of ribonucleic acid in lampbrush chromosomes, nucleoli, nuclear sap, and cytoplasm of *Triturus* oocytes. *J. Cell Biol.* 19:279.
7. FICQ, A. 1961. Metabolisme de l'oogénèse chez les Amphibiens. In *Symposium on the germ Cells and Earliest Stages of Development*. S. Ranzi, editor. Fondazione A. Baselli, Milano. 121-140.
8. GALL, J. G. 1958. Chromosomal differentiation. In *Symposium on the Chemical Basis of Development*. W. D. McElroy and B. Glass, editors. The Johns Hopkins Press, Baltimore. 103-135.
9. GALL, J. G. 1959. Uptake of nucleic acid precursors by oocytes of the newt (*Triturus viridescens*). *Genetics*. 44:512.
10. GALL, J. G. 1965. Techniques for the study of lampbrush chromosomes. In *Methods in Cell Physiology*. D. Prescott, editor. Academic Press Inc., New York. 37-60.
11. GALL, J. G. 1966. Nuclear RNA of the salamander oocyte. *Natl. Can. Inst. Monograph*. 23:475.
12. GALL, J. G., and H. G. CALLAN. 1961. Patterns of incorporation in amphibian oocyte nuclei. Abstracts, American Society for Cell Biology.
13. GALL, J. G., and H. G. CALLAN. 1962. ³H-

- uridine incorporation in lampbrush chromosomes. *Proc. Natl. Acad. Sci. U. S.* **48**:562.
14. GUYÉNOT, E., and M. DANON. 1953. Chromosomes et ovocytes des Batraciens. Etude cytologique et au microscope électronique. *Rev. Suisse Zool.* **60**:1.
15. IZAWA, M., V. G. ALLFREY, and A. E. MIRSKY. 1963. The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc. Natl. Acad. Sci. U. S.* **49**:544.
16. JOHNSON, J. M. 1966. Observations on contracting nucleolar vacuoles in cultured tobacco cells. *J. Cell Biol.* **31**:52A (Abstr.).
17. JÖRGENSEN, M. 1913. Zellenstudien. I. Morphologische Beiträge zum Problem des Eiwachstums. *Arch. Zellforsch.* **10**:1.
18. KEZER, J. 1965. Observations published in Peacock, W. J. Chromosome replication. *Natl. Can. Inst. Monograph.* **18**:101.
19. LANE, N. J. 1966. Uridine incorporation in amphibian oocyte nucleoli. *J. Cell Biol.* **31**:65A (Abstr.).
20. MACGREGOR, H. C. 1967. Pattern of incorporation of H³-uridine into RNA of amphibian oocyte nucleoli. *J. Cell Sci.* In press.
21. MARÉCHAL, J. 1907. Sur l'ovogénèse des Séla-ciens et de quelques autres chordates. *La Cellule.* **24**:1.
22. MILLER, O. L. 1962. Studies on the ultrastructure and metabolism of nucleoli in amphibian oocytes. In Proceedings of the Fifth International Congress on Electron Microscopy. Philadelphia. S. Breese, editor, Academic Press Inc., New York. **2**:NN-8.
23. MILLER, O. L. 1964. Extrachromosomal nucleolar DNA in amphibian oocytes. *J. Cell Biol.* **23**:60A (Abstr.).
24. MILLER, O. L. 1966. Structure and composition of peripheral nucleoli of salamander oocytes. *Natl. Can. Inst. Monograph.* **23**:53.
25. PAINTER, T. S., and A. N. TAYLOR. 1942. Nucleic acid storage in the toad's egg. *Proc. Natl. Acad. Sci. U. S.* **28**:311.
26. SABATINI, D. D., K. BENSCH, and R. J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19.
27. STEVENS, B. J., and H. SWIFT. 1966. RNA transport from nucleus to cytoplasm in *Chironomus* salivary glands. *J. Cell Biol.* **31**:55.