Behaviour of annulate lamellae during the maturation of oocytes in the newt, 
Cynops pyrrhogaster

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SUMMARY

The distribution of annulate lamellae, electron-dense masses, rough endoplasmic reticulum, and Golgi complexes in longitudinal sections of newt oocytes at several stages of progesterone-induced maturation was recorded with an electron microscope equipped with a drawing device. Annulate lamellae in full-grown oocytes occur in close contact with electron-dense masses and the nuclear envelope and elsewhere. Stacks of annulate lamellae increase in number for 6 h after progesterone treatment. Meanwhile, they segregate into three groups. The largest group, comprising about 75% of total stacks, forms a row parallel to and just beneath the oocyte cortex of both the animal and vegetal hemispheres, a second group is distributed in the middle area of the vegetal hemisphere, and a third group appears near the yolk-free cytoplasm formed at the vegetal side of the germinal vesicle during the maturation. About 6 h after progesterone treatment the annulate lamellae begin to disappear at their places of localization and none is found a few hours after germinal vesicle breakdown. No immediate fine-structural change in the cortical area follows the disappearance of subcortical annulate lamellae. The possible origins and fates of annulate lamellae in the maturing newt oocytes are discussed.

INTRODUCTION

Annulate lamellae, which are stacks of pitted membranes with finely fibro-granular material attached to them, occur in oocytes as well as in many other cell types of various animal species (see Kessel, 1968a; Maul, 1977a, for reviews). Their role is still not clear. It has been suggested, however, that they may function in the transfer of genetic specificity from the nucleus to the cytoplasm (Swift, 1956), as supported by observations showing their probable nuclear origin (Kessel, 1968a, b; Kessel & Beams, 1969; Bal, Jubinville, Cousineau & Inoue, 1968; Gulyas, 1971) and the probable existence of RNA on the lamellae (Ruthmann, 1958; Kessel & Beams, 1969; Conway, 1971).

Despite the possibly crucial importance of the above suggestion, the temporal-spatial distribution of annulate lamellae in amphibian oocytes during oogenesis has been reported only to a small extent. According to Balinsky & Devis (1963),
in oocytes of *Xenopus laevis* they become observable at the earliest vitellogenetetic stage, continue to be apparent throughout the growth phase, and then disappear on completion of maturation by transformation into masses of vesicles. These authors considered that the vesicles form a subcortical morphogenetic substance by spreading underneath the egg cortex.

The spatial distribution of annulate lamellae in full-grown oocytes of *Rana pipiens* has been noted by Kessel (1968a). It was found that they are abundant in the polar region of the animal hemisphere but rare in the vegetal polar region, and that those located nearer the cortex are smaller in size than those located more internally. This suggests the movement of the lamellae towards the cortex and their subsequent fragmentation. As with *Xenopus* there is extensive vesiculation and disappearance of annulate lamellae at the completion of maturation in oocytes of *Rana*.

These previous reports have been concerned with limited areas, mainly of the animal hemisphere, in growing and full-grown oocytes or in oocytes after germinal vesicle breakdown; and no detailed and systematic examination of annulate lamellae has been performed for oocytes in the middle of the maturation process. The present study fills in this gap by covering roughly the whole period of maturation and all regions of the oocyte. The results obtained show that it is in this phase of oogenesis that dynamic changes occur in the number and location of annulate lamellae in the oocytes.

**MATERIALS AND METHODS**

**Biological**

Oocytes of *Cynops pyrrhogaster* were used throughout the experiments. The full-grown control oocytes were obtained from dormant females taken from a field in mid-winter. Maturation-inducing stimuli were carefully avoided for these females except for the warmth of the sun on a shallow pond before collecting them.

Oocytes maturing *in vivo* were examined in the preliminary studies and the results were confirmed and extended by *in vitro* studies using the procedures of Pilone & Humphries (1975). In the experiments presented, mature females were injected with 30 units of a gonadotrophic hormone (Gonatoropin, Teikokuzoki). About 24 h later the ovaries were removed, treated with 10 μg/ml of progesterone for 1 h, and cultured in the Ringer’s solution. Fragments of ovaries containing several normally maturing oocytes were transferred for fixation at 3 h intervals for 15 h, counting from the start of progesterone treatment. The oocytes maturing too early (Imoh, 1981) were discarded. Under the conditions used germinal vesicle breakdown did not occur before 9 h but occurred in 40% of oocytes at 12 h and in more than 90% at 15 h after progesterone application.
Preparation of material for electron microscopy

Either one of two fixative solutions was used for prefixation. One was the dimethyl sulphoxide-containing (DMSO) fixative developed by Kalt & Tandler (1971). The DMSO fixative preserved the fine structures well but caused considerable oocyte deformation, probably as a result of osmotic dehydration. The other fixative having lower osmotic pressure was thus developed, and this consisted of 1·5 % glutaraldehyde and 2·5 % acrolein in 0·05 M cacodylate buffer pH 7·4 containing 0·5 mM-CaCl₂ (GAC fixative). This fixative gave satisfactory results in preserving both the fine structures and the original shape of the oocytes, and was mainly used. During the prefixation for 6 to 8 h the oocytes were trimmed parallel to the longitudinal plane. They were then washed for about 2 h in the buffer used in the preparation of the fixative with addition of sucrose up to 0·25 M and post-fixed with 2 % osmium tetroxide in the washing buffer for about 12 h. Both fixations were performed in the cold. Fixed oocytes were dehydrated and embedded in an epoxy resin (Kalt & Tandler, 1971).

Electron microscopic analysis

The location of annulate lamellae and a few other organelles was studied in 3 full-grown oocytes from 2 dormant females and in 11 maturing oocytes from 3 other females, using the GAC fixative for prefixation. In order to observe the entire longitudinal plane of the oocyte, in most cases each animal and vegetal hemisphere was covered by one large section, about 1 x 2 mm², with slight overlapping areas. In a few cases the plane was covered by four sections. The trimming depth in the preparation of one sectioning area was carefully made small enough so that the differences in the sectioning planes would be negligible with respect to the distribution of the organelles.

Thin sections on 100-mesh copper grids were stained with uranyl acetate and lead citrate for 1·5 h and 30 min, respectively. The entire area of a section was observed and the location of every stack of annulate lamellae, electron-dense mass, rough endoplasmic reticulum (rough ER), and Golgi complex was recorded on an electron microscope equipped with a drawing apparatus. This apparatus was constructed so as to indicate on a recording paper the centre of the observing field in the electron microscope. Thus, by placing the oocyte surface and the organelles at the centre of the observation field, the contour of the oocyte and the position of the organelles in the section could be traced at a fixed magnification, 150 x. The areas under the copper mesh in the original record were observed in two more adjacent sections made serially. For small areas still unobserved after these corrections, a certain number of organelles expected from their densities in the adjacent areas was added. The number of organelles added, though very limited except for Golgi complexes, was noted in the legend of each figure. Two or four such records for the same oocyte were
combined to give the distribution of organelles in a longitudinal section of the oocyte.

**Measurement of the cytoplasmic area**

The area of oocyte cytoplasm was measured using the above completed figure by weighing the piece of homogeneous paper cut in the shape of the cytoplasm, in comparison with the weight of the same paper of a known area.

**RESULTS**

**Differentiation of annulate lamellae in full-grown and in maturing oocytes**

Figure 1a shows annulate lamellae in contact with an electron-dense mass in the subcortical area of a full-grown oocyte. Similar complexes of annulate lamellae and electron-dense masses are observed very frequently in full-grown oocytes and much less frequently in maturing oocytes. The pattern of contact and the ratio of annulate lamellae to electron-dense masses varies among complexes (Figs. 1b, c, d). There are two types of electron-dense masses, one homogeneous (Figs. 1a, b – lower part) and the other heterogeneous (Figs. 1b – lower part, c) owing to the existence of many electron-dense foci. Smooth or rough ER is observed in close vicinity of, or contiguous with, some of annulate lamellae (Figs. 1a, c).

Adjoining the nuclear envelopes in full-grown oocytes there are many stacks of annulate lamellae consisting of a few lamellae and the stacks become much larger in maturing oocytes. In particular, quite large stacks occur both away from (Fig. 2a) and in close contact with the nuclear envelope in the yolk-free cytoplasm which is formed on the vegetal side of the germinal vesicle during maturation (Czofowska, 1969; Brachet, Hanocq & Van Gansen, 1970; Pilone & Humphries, 1975). The continuation of annulate lamellae with rough ER is marked in maturing oocytes (Figs. 2a, b).

The probable transformation of annulate lamellae into electron-dense masses is observed in the middle of the vegetal hemisphere in the oocyte prior to germinal vesicle breakdown (Fig. 2c). Electron-dense masses are observed in the corresponding areas in oocytes after germinal vesicle breakdown (Fig. 2d).

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**Fig. 1.** Annulate lamellae (AL) in close contact with electron-dense masses (EDM) observed in full-grown oocytes. These complexes are often located near the oocyte surface (a) and occur in various appearances (b, c, d). Other abbreviations: ER, endoplasmic reticulum; RER, rough ER; FS, fibrous structure; L, lipid droplet; VM, vitelline membrane; Y, yolk platelet. Magnifications: a, 13000 x; b, c, 18000 x; d, 12000 x. Fixatives: a-d, GAC-OsO₄.
Annulate lamellae in newt oocyte
Fig. 2. Differentiation of annulate lamellae in maturing oocytes. In maturing oocytes, annulate lamellae (AL) occur in contact with, or away from, the nuclear envelope (NE) (a) and is connected to rough ER (RER) particularly in the subcortical area (b). Probable transformation of degrading AL (DAL) into electron-dense masses (EDM) is observed in the middle area of the vegetal hemisphere (c), and EDM occur in the corresponding area (d), before (c) and after (d) germinal vesicle breakdown. Arrow indicates fibrous structure containing microtubules. Time after progesterone treatment: a, b, 6 h; c, 12 h; d, 15 h. P, pigment granules; other abbreviations as in Fig. 1. Magnifications: a, b, 22000 x; c, 19000 x; d, 14000 x. Fixatives; a, b, DMSO-OsO₄; c, d, GAC-OsO₄.
Annulate lamellae in newt oocyte

Distribution of annulate lamellae and a few other organelles in full-grown and in maturing oocytes

The location of annulate lamellae, electron-dense masses, rough ER, and Golgi complexes in a longitudinal section of a full-grown oocyte is shown in Fig. 3. It can be seen that more than 45% of annulate lamellae occur in contact with electron-dense masses and that most of the annulate lamellae are located in the peripheral ooplasm, less than 0.4 mm from surface. The electron-dense masses are distributed evenly from the nuclear envelope to the subcortical area. Golgi complexes are located without any apparent relationship with annulate lamellae.

The number of annulate lamellae in the early phase of maturation, after 3–6 h of progesterone application, was considerably more than that in full-grown oocytes (Fig. 4, compare with Fig. 3). As apparent from Fig. 4, many stacks of annulate lamellae form an ill-defined row just beneath the oocyte cortex with the exception of a few places in the marginal and animal polar areas where the row is broken. It is also evident that several stacks of annulate lamellae remain in the inner cytoplasm of the vegetal hemisphere.

At this early maturation stage, the number of electron-dense masses is less than that of the control oocytes, though the pattern of the masses' distribution is retained. Annulate lamellae in contact with the masses are observed only rarely. On the other hand, there is marked continuation of annulate lamellae with rough ER, in particular in the subcortical area (Figs. 2b, 4). It should be noted, however, that a greater number of annulate lamellae are free of rough ER and that rough ER isolated from annulate lamellae is also abundant.

The distribution of organelles in an oocyte 9 h after progesterone treatment shows two major features (Fig. 5). First, no annulate lamellae are detectable in the animal hemisphere, as so far examined, at the side opposite to yolk-free cytoplasm. Secondly, the segregation of annulate lamellae into three groups can be observed; a first group just beneath the oocyte cortex forming a row and hence referred to as subcortical annulate lamellae, a second group situated in a broad middle area of the vegetal hemisphere, and a third group near the yolk-free cytoplasm. When the number of stacks of annulate lamellae in each group is counted from Fig. 5, it is seen that the first, second and third groups contain 75%, 10% and 10% of total stacks, respectively. Similar figures have been observed in another oocyte at the same stage. The subcortical annulate lamellae apparently approach the oocyte cortex during maturation (compare Figs. 4 and 5). Fibrous structures as shown in Fig. 1a may play a role for the translocation, but the point is not fully studied.

The amount of rough ER with or without associated annulate lamellae is not large in oocytes after the arrival of the germinal vesicle at the vicinity of the animal pole. The electron-dense masses, located apart from the germinal vesicle, are also reduced in number. The pattern of distribution of Golgi
Fig. 3. Distribution of organelles in a longitudinal section of a full-grown oocyte from female A. This oocyte, though from a dormant female, has already entered the earliest stage of maturation judging from the inward movement of extrachromosomal nucleoli (Imoh, 1981). The keys are; AL, annulate lamellae; RER, rough ER; EDM, electron-dense mass; Golgi, Golgi complexes. In the trace, the number and the location of organelles is accurate, except that three stacks of annulate lamellae and a few dozen Golgi complexes have been added artificially to cover some unexamined areas, as their occurrence was expected in these areas judging from each organelle's density distribution at particular regions surrounding the unobserved areas and also from that in whole oocyte section. The sizes of the symbols indicate only relative largeness. In this figure, 86 stacks of AL and 78 EDM were counted, and 40 of them occur as complexes. Six small stacks of rough ER occur with or without contact to AL in the peripheral area. Areas of cytoplasm and the germinal vesicle were 2.89 and 0.20 mm², respectively, giving values of 29.8 stacks of AL and 27.0 EDM/mm² cytoplasm (plotted in Fig. 8). The future animal pole is at the top of the figure, judged by the distribution of pigment granules.
Annulate lamellae in newt oocyte

Fig. 4. Distribution of organelles in a longitudinal section of an oocyte from female C at 6 h after exposure to progesterone. Labelling of organelles as in Fig. 2. The yolk-free cytoplasm (area enclosed by a dotted line) has developed on the vegetal side of the germlinal vesicle. Judging on the same basis as noted in the legend of Fig. 3, a few dozen Golgi complexes but no other organelles have been added to unexamined areas of the figure. In the figure, 141 stacks of AL and 21 EDM were counted. The number of RER consisting of a few lamellae is 121 in total, 29 of them associating with AL. Note the cortical location of RER.

complexes in maturing oocytes is difficult to interpret in relation to that of annulate lamellae.

Absence of annulate lamellae in oocytes near completion of maturation

Following the stage shown in Fig. 5, the number of subcortical annulate lamellae as well as those in deeper cytoplasm seemed to decrease more or less evenly everywhere in the ooplasm, and neither annulate lamellae nor rough ER was observed in oocytes near the end of the maturation process (Fig. 6). Several electron-dense masses, however, exist in the middle area of the vegetal hemisphere (Fig. 2d).

The vesiculation of annulate lamellae was not observed and no mass of vesicles was found in oocytes during or after germlinal vesicle breakdown,
except for the mass derived from the vesiculation of the nucleoplasm which occurs after the breakdown of the nuclear envelope (Imoh, unpublished observation). Thus, apart from the animal polar area, the number of subcortical vesicles is limited (Figs. 7a, b). The oocyte cortex did not show any appreciable change in the fine structure before (Fig. 7c) and after (Fig. 7d) the disappearance of annulate lamellae from the adjacent area.

Changes in the number of annulate lamellae during maturation

The numbers of stacks of annulate lamellae and of electron-dense masses per unit area of ooplasm were calculated, as noted in the legend of Fig. 3, for 3 full-grown oocytes from females A and B and for 11 maturing oocytes from females
Annulate lamellae in newt oocyte

Fig. 6. Distribution of organelles in a longitudinal section of an oocyte from female C, 15 h after exposure to progesterone. Electron-dense masses (open circle) are located mostly in the middle of the vegetal hemisphere but AL and RER have completely disappeared. The distribution of Golgi complexes (dots) is similar to that in Fig. 5, a few dozen of them being added for unexamined areas judged as in Fig. 3.

C, D and E. The results have been summarized in Fig. 8. It is apparent that the numbers of stacks of annulate lamellae and electron-dense masses at a given stage of maturation are comparable for oocytes from either the same or different females.

Figure 8 shows that the number of stacks of annulate lamellae increases about 50% during the first 6 h of maturation, after which time it decreases, becoming zero by 15 h after progesterone treatment. On the other hand, the number of electron-dense masses decreases for the first 6 h and then becomes constant. When the number of annulate lamellae begins to decrease, around 6 h after progesterone application, a pale spot appears at the animal polar area. The spot is due to the approach of the germinal vesicle to the cortex and not due to germinal vesicle breakdown, as all normally maturing oocytes have an intact nuclear envelope at that time. Two oocytes analysed and plotted at 12 h in
Annulate lamellae in newt oocyte

Fig. 8. Changes in the number of annulate lamellae and electron-dense masses during maturation of newt oocytes. For the calculation of the number of the organelles per unit area of cytoplasm, see text and the legend of Fig. 3. Open symbols about the solid line show the number of stacks of annulate, and the filled symbols about the broken line the number of electron-dense masses. Control oocytes at 0 h were full-grown oocytes from dormant females A (small circles) and B (small rectangles). Maturing oocytes were from females C (circles), D (triangles), and E (rectangles). The number of electron-dense masses was not recorded for two oocytes from female C (at 3 and 9 h) nor for two oocytes from female E (at 6 and 12 h).

Fig. 8 had intact nuclear envelopes, and those at 15 h had completed germinal vesicle breakdown.

DISCUSSION

Origin of annulate lamellae in maturing newt oocytes

The results obtained clearly revealed that the number of stacks of annulate lamellae increases during the early phase of the maturation process (Figs. 3, 4) and that the increase is reproducibly observed and significant irrespective of

Fig. 7. The fine structure of cortical area in oocytes near completion of maturation. In a and b this is shown at a distance of about 0.5 mm from the animal pole (a) and at the vegetal polar area (b) in an oocyte after germinal vesicle breakdown. In c and d it is shown in the right marginal zone of the oocyte shown in Fig. 5 at about 0.2 mm below (c) and above (d) the equatorial line, where nearby AL are still present and have disappeared, respectively. FC, follicle cell; GP, glycogen pool; other abbreviations as in Figs. 1 and 2. Magnifications: a, b, 11000 x ; c, d, 23000 x . Fixatives: a–d, GAC-OsO₄.
which oocytes or females are examined (Fig. 8). These facts show that the observed increase represents a net increase in the number of stacks of annulate lamellae per oocyte rather than a fluctuation in their regional concentration.

The fragmentation of pre-existing stacks as suggested for the maturing oocytes of *Rana* (Kessel, 1968a) will cause an increase in the number of the stacks at the expense of their sizes. This possibility could not be excluded, but a full-grown oocyte of *Cynops* does not seem to contain enough annulate lamellae to give rise to all the annulate lamellae in a maturing oocyte (compare Figs. 3 and 4). The results rather suggest that the formation of new stacks of annulate lamellae occurs.

The germinal vesicles of full-grown and maturing oocytes always bear many stacks of annulate lamellae. As suggested by Swift (1956), they are probably the precursors of the cytoplasmic annulate lamellae, though when and how they detach from the nuclear envelope is not established, and how many stacks of annulate lamellae are given off by them during maturation is totally unknown.

Annulate lamellae may be supplied also by formation in the cytoplasm apart from the nuclear envelope. This is suggested by the observation of the frequent occurrence of annulate lamellae in close contact with the electron-dense masses. An almost identical relationship between annulate lamellae and electron-dense masses, which have close structural resemblance to the present electron-dense masses, has been observed in the oocytes of the dragonfly by Kessel & Beams (1969). These authors have concluded that transformation of the electron-dense masses to annulate lamellae takes place in their material. If this conclusion also applies to the present material, then it will be seen from Fig. 8 that most of the increase of the stacks of annulate lamellae might be due to this transformation. The close relationship between the electron-dense masses and annulate lamellae seems to be unique to full-grown or maturing oocytes, as no such complex has been observed in oocytes at earlier stages (Imoh & Terahara, 1982).

Among various mechanisms suggested for the formation of annulate lamellae (see Kessel, 1968a; Maul 1977a), two involving either the nuclear envelope or the electron-dense masses are suggested as probably operating in the maturing oocytes of the newt. In both cases, inclusion of pre-existing annulate structures seems rather unlikely (see Figs. 1, 2a). and the observations may better conform to the possibility of construction of the pore complexes from their constituent molecules (see Swift, 1956; Maul, 1977a, b).

*Localization of annulate lamellae during maturation*

It was shown that marked changes occur in the distribution of annulate lamellae during progesterone-induced maturation *in vitro*. The same changes were observed in the oocytes maturing *in vivo*, though detailed records were not prepared in these preliminary studies. Similar changes have been observed in the *Xenopus* oocytes maturing *in vitro*, except that differentiation of two groups of annulate lamellae other than the subcortical group is not clear (Imoh, Okamoto & Eguchi, 1982).
Annulate lamellae in newt oocyte

It has been observed in the growing oocytes of the newt that the stacks of annulate lamellae first appear at the early vitellogenetetic stage, increase rapidly to about the level of the full-grown oocytes, and increase only slightly thereafter. They are distributed rather evenly in the ooplasm, thus resembling the pattern in the full-grown oocytes (Imoh & Terahara, 1982). Therefore, the dynamic changes in the distribution of annulate lamellae leading to their localization occur only in the maturation period of oogenesis.

Fate of annulate lamellae and their possible role

In sharp contrast to the observations in anuran species (Balinsky & Devis, 1963; Kessel, 1968a), the vesiculation of annulate lamellae and the resulting masses of vesicles were not observed in the present urodelan material. It should be noticed in this respect that cytoplasmic islets consisting of numerous vesicles appear in the subcortical area of the animal hemisphere in the eggs of Cynops soon after their fertilization (Imoh, 1980). No such observation has been reported for fertilized eggs of anuran species. The disappearance of annulate lamellae and the appearance of vesiculated cytoplasm are separate events in the case of Cynops.

Annulate lamellae localized at a particular region of an oocyte seem just to disintegrate, and the material in annulate lamellae become untraceable by electron microscopic analysis. However, the material should still be there and it seems possible that it somehow functions at the corresponding site of the embryo later in embryonic development. The subcortical annulate lamellae may play a role as the morphogenetic determinant (Balinsky & Devis, 1963; Curtis, 1960, 1962; see also Malacinski, Chung & Asashima, 1980).

It is unlikely that annulate lamellae disappear by transforming into rough ER, since the close relationship between these organelles is marked in the early stages of maturation rather than prior to the disappearance of annulate lamellae. The continuation of these organelles, however, conforms to the idea that the material in annulate lamellae may somehow be involved in protein synthesis (Kessel, 1981). In this respect, it is interesting that the observed pattern of changes in the number of stacks of annulate lamellae is similar to that in the content of poly (A) sequences in maturing oocytes of Xenopus (Darnbrough & Ford, 1979; Sagata, Shiokawa & Yamana, 1980). Similarities have also been noted between the changes in the content of poly (A) sequences in the growing oocytes in Xenopus (Rosbash & Ford, 1974; Cabada, Darnbrough, Ford & Turner, 1977) and the above-mentioned changes in annulate lamellae in the growing oocytes in Cynops (Imoh & Terahara, 1982). These behavioural coincidences between annulate lamellae and poly (A) sequences during amphibian oogenesis sugested to us that the material on annulate lamellae may be poly (A)-bearing messenger RNA. This possibility is now being tested by electron microscopic radioautography using tritiated poly (U) as a probe.
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