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Dynamics of tubulin structures in *Xenopus laevis* oogenesis

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SUMMARY

The distribution of tubulin and/or tubulin-containing structures was examined in separate classes of *Xenopus laevis* oocytes and in germinal vesicles isolated from them. Although a monoclonal antibody has been used, the technique applied on paraffin sections does not allow clear-cut definition of the state of tubulin present (monomeric, dimeric or polymerized form); however, the probable existence of assembled microtubules is indicated by supplementary techniques, i.e. histology and immunoperoxidase staining. Immunofluorescence reveals maximum tubulin concentration in the Balbiani body and in a ring-shaped formation around the nucleus in young oocytes. The Balbiani body disintegrates in the course of vitellogenesis, granules formed from its periphery migrate into the cytoplasm and gradually fill the entire cytoplasm as radial cords. In the ring-shaped formation around the nucleus strongly fluorescent cords and fibres are formed, particularly on the future vegetal-half-facing part of the nucleus. Reorganization of tubulin may be related to the establishment of a structure directing two-way shifts (1) of cytoplasmic organelles from the Balbiani body to the cytoplasm, and (2) of yolk proteins containing endosomes derived from the endocytically active oolemma to the yolk platelets. A distinct fluorescent fibrillar network is found inside the isolated germinal vesicles, near the nucleus membrane. Peripheral nucleoli, often present in nuclear membrane protuberances, seem to be surrounded by this material, which is oriented along the surface, and as a basket towards the inside of the nucleus. It is assumed that the structures may participate in the transport of nucleoli from the nucleus to the cytoplasm via the nuclear envelope.

INTRODUCTION

The significance in early embryogenesis of microtubules, microfilaments and intermediate filaments representing cytoskeleton and cytomusculature has become evident. They play a crucial role both in the egg/sperm interaction, cortical reaction, cytokinesis, and in morphogenetic processes such as gastrulation, neurulation and succeeding events (see Burgess & Schroeder, 1979; Cohen, 1979; Weatherbee, 1981; for reviews). The amphibian oocyte shows an apparent radial symmetry about the animal–vegetal axis. This symmetry is reflected by the internal arrangement of organelles; it seems very probable that cytoskeletal structures are responsible for the spatial distribution of e.g. yolk, cortical and pigment granules, lipid droplets or mitochondria (Ball & Singer, 1982; Gall, Picheral & Gounon, 1983). Interaction

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of egg and sperm triggers a number of temporally linked processes such as the cortical reaction, migration of pronuclei, grey crescent formation, dorsoventral polarity determination, and cytokinesis (Elinson, 1980; Gerhart, Ubbels, Hara & Kirschner, 1981; Kirschner, Gerhart, Hara & Ubbels, 1980; Ubbels, Mácha, Paleček & Koster, 1983). All these events, both intracytoplasmic and cortical, involve a precisely organized and collaborating contractile system and a stable supporting matrix (Gall *et al.* 1983).

Different techniques have been applied to demonstrate microtubular proteins in eggs and embryos of many species. In the majority of cases they are already present in unfertilized eggs in unpolymerized form as a pool and remain at relatively constant amount throughout development of the embryos, e.g. in sea urchin (Harris, Osborn & Weber, 1980; Schatten & Schatten, 1981), *Spisula solidissima* (Suprenant & Rebhun, 1984), *Chaetopterus pergamentaceus* (Eckberg & Yuan-Hsu Kang, 1981), *Drosophila melanogaster* (Green, Brandis, Turner & Raff, 1975).

Cytoskeletal proteins are present in amphibian eggs and embryos as well. Tubulin was detected and characterized in oocytes and eggs of *Xenopus laevis* (Dumont & Wallace, 1972; Pestell, 1975; Paleček, Ubbels & Mácha, 1982), *Rana pipiens* (Smith & Ecker, 1968), *Pleurodeles waltlii* (Gounon & Collenot, 1974; Moreau & Gounon, 1977), *Discoglossus pictus* (Campanella & Gabbiani, 1980), and the axolotl (Raff, 1977; Raff & Raff, 1978). Accumulation of unpolymerized tubulin takes place in parallel with vitellogenesis and its amount reflects the size of the oocyte. The oocyte tubulin has usually been found to be very similar to somatic tubulin and no interspecific differences have been established (Dales, 1972). Comparing isolated tubulins of eggs and tissues of pigmented and albino axolotls Raff (1977) found only minute differences in electrophoretic, and in particular in colchicine-binding properties. *De novo* synthesis of tubulin in sea urchin oocytes represents about 4 % of the total protein synthesis (Cognetti, Di Liegro & Cavarretta, 1977). This endogenously synthesized monomeric tubulin (Raff & Raff, 1978; Raff *et al.* 1975) is used later during activation and cleavage of the egg (Cohen & Rebhun, 1970). By analogy with yolk accumulation a participation of the non-germ cells in tubulin synthesis might be suspected. However, by using defolliculated eggs of *Pleurodeles* it was shown that follicular cells take no part in tubulin synthesis, although another mode of transfer was not excluded (Moreau & Gounon, 1977).

Although free assembly-competent tubulin is present in abundance in unfertilized eggs, comprising about 1 % of total soluble proteins in sea urchins and *Xenopus* (Coffe, Foucault, Raymond & Pudles, 1983; Pestell, 1975), it was not clearly demonstrated in the form of cytoskeletal microtubules until recently (Otto & Schroeder, 1984). At the same time the dynamics of microtubule assembly *in vivo* is not very clear. It is assumed that *in vivo* tubulin is stimulated to polymerize after activation of the egg by the penetrating sperm. Microtubule-organizing centres are activated at this stage, and they may be seen to stimulate microtubule formation even after transfer to unfertilized oocytes (Heidemann & Kirschner, 1978; Raff,

1979). Formation of the meiotic spindle may also be induced hormonally in oocytes via plasma membrane receptors (Hirai, Le Gascogne & Baulieu, 1983). Compartmentalization of free tubulin in the cell takes place mainly by means of association with membrane and other lipidic complexes (Caron & Berlin, 1979; Klausner *et al.*, 1981). Free oocyte tubulin may polymerize *in vitro* forming micro- or macrotubular complexes (Kuriyama, 1977; Suprenant & Rebhun, 1984).

Tubulin was detected by immunofluorescence (Campanella & Gabbiani, 1980) in the periphery of coelomic, uterine, unfertilized and fertilized eggs of *Discoglossus pictus*. However, the authors were unable to detect microtubules in electron microscopy preparations. In *Xenopus laevis*, after the disintegration of the nuclear membrane during maturation of the egg, a fibrillar network lying near the basal part of the nucleus has been observed (Brachet, Hanocq & Van Gansen, 1970; Ubbels, personal communication). Electron microscopy (Huchon, Crozet, Canteno & Ozon, 1981) confirmed that this network is made up of bundles of microtubules. It was assumed that they play a role in the assembly of the chromosomes and the control of their migration toward the animal pole of the egg.

In this study we have examined the dynamics of tubulin-containing structures in growing oocytes of *Xenopus laevis* by means of immunofluorescence. Histological sections were then stained by classical techniques and compared with pictures obtained after immunofluorescence. To elucidate the possible role of microtubules in the nuclear membrane, germinal vesicles were isolated and observed after immunofluorescent staining.

MATERIALS AND METHODS

Oocytes

Fragments of *Xenopus laevis* ovaries, containing oocytes of all classes I–VI, according to Dumont (1972), were fixed in Bouin–Hollande fixative for 24 hours, embedded in paraffin wax and 4 to 6 μm sections were prepared by standard procedures.

Germinal vesicle preparation

Germinal vesicles were isolated according to Ford & Gurdon (1977) in modified Barth saline, buffered with HEPES (MBS–H).

Immunofluorescence

Isolated germinal vesicles were processed by a technique described previously (Paleček & Hašek,1984). They were washed for 20 seconds with potassium phosphate buffer, pH 7·0, complemented with 0·1M-NaCl, 1 mM-EGTA, 1 mM-$MgCl_2$ and 10 % dimethylsulphoxide, and extracted for 1 min in 0·15 % TRITON-X-100 in the above solution without dimethysulphoxide (EB - extraction buffer). After washing in EB for 10 min they were fixed in 0·3 % glutaraldehyde, washed in phosphate-buffered saline (PBS–pH 7·2) and treated with 1 mg $NaBH_4$ per ml of PBS for 8 min. After additional washing in PBS they were incubated with monoclonal antitubulin serum TU–01 (Viklický, Dráber, Hašek & Bártek, 1982) for 1 hour at 37 °C in a dark moist chamber, washed in PBS and postincubated in SWAM FITC (Sevac, Prague, ČSSR) for 45 min at 37 °C in a dark moist chamber. After final washing in PBS they were mounted in 50 % glycerol in PBS (pH 7·4) and observed in a fluorescence microscope (Fluoval, Carl Zeiss, Jena; maximum excitation at 405 nm).

Sections

Sections of oocytes were deparaffinized and transferred through alcohols to PBS. Monoclonal antitubulin serum TU–01 was applied following identical procedures as above. For the details see Paleček & Romanovsky (1985).

Staining techniques

After immunofluorescence, sections were stained successively with 1 % azocarmine in 1 % acetic acid for 10 min, 10 % orange G + 2·5 % aniline blue in 1 % acetic acid for 5 min and 0·5 % aniline blue in water for 7 min, dehydrated and mounted. Staining revealed areas where specific immunofluorescence was present as blue, contrasting with dark orange yolk granules.

Control of antiserum specificity

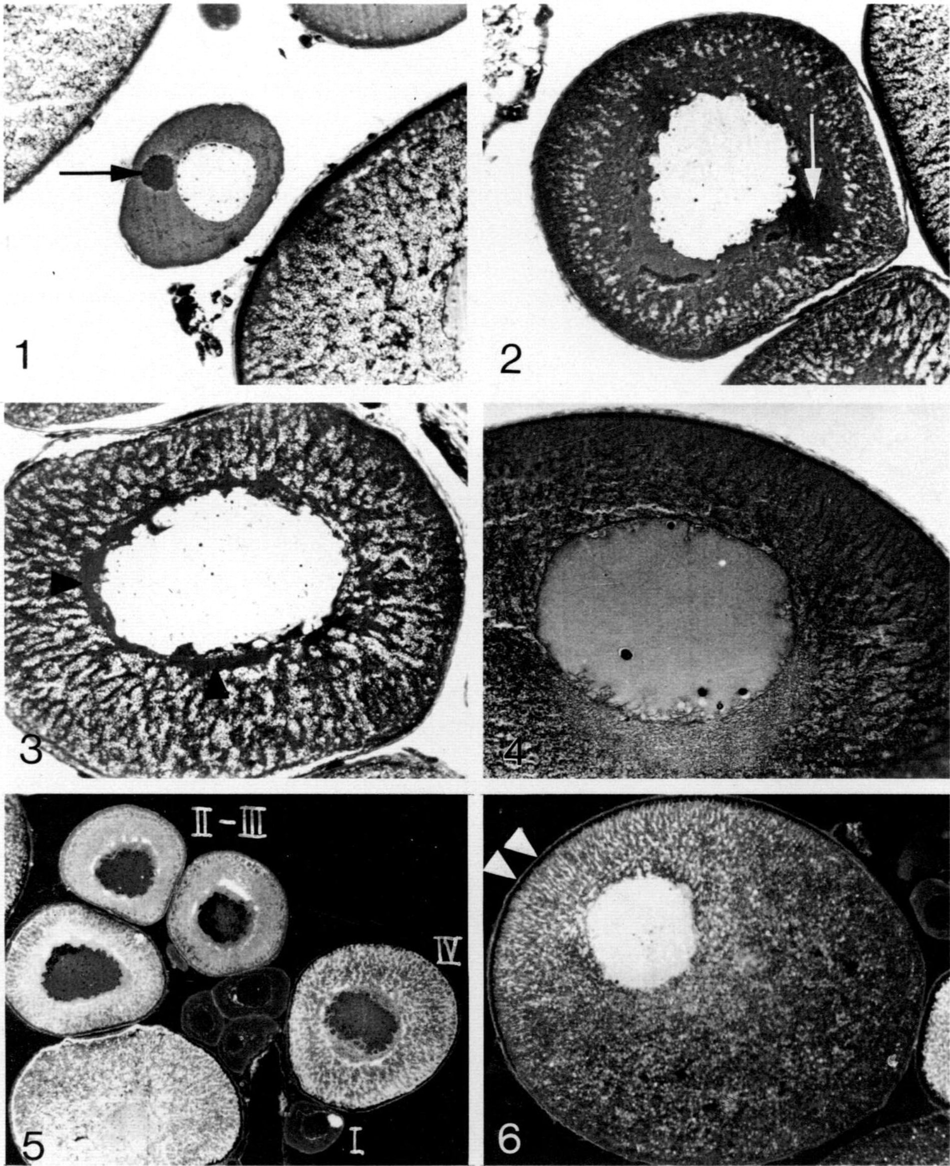
The samples for one-dimensional gels were prepared by the method originally described by Laemli (1970). The ovarian oocytes were isolated, equal amounts of 2 % SDS and 0·5 % mercaptoethanol added and immediately heated in a boiling waterbath for 1 min; homogenized and centrifuged. One-dimensional slab gels were run using standard procedures (Laemli, 1970). Gels were calibrated with standards of known relative molecular mass. Western transfer analysis was run according to the method described by Towbin, Staehelin & Gordon (1979) using a monoclonal antitubulin antibody TU – 01 (Fig. 15).

RESULTS

In small oocytes (stage 1) extrachromosomal nucleoli, the mitochondrial mass (Balbiani body) and a sparse fibrillar meshwork arranged close to the nucleus are stained blue after histological staining with aniline blue (Fig. 1). Vitellogenic oocytes (stage-II and later) exhibit distinct blue staining of a layer located around the nucleus and disintegrating Balbiani body (Fig. 2). As oogenesis progresses the circular layer grows thicker and radial bundles connected with this layer are formed in the cytoplasm. Blue staining remains in the vicinity of the nuclear membrane and its invaginations (Fig. 3). In stage-VI oocytes a fine network of blue fibres may be observed close to the area of the nuclear membrane facing the future vegetal half of the egg (Fig. 4).

The general picture of tubulin-containing structures in oocytes of different sizes and in the stage-V oocyte, as revealed by immunofluorescence, is shown in Fig. 5 and 6. In stage-I to -II oocytes the mitochondrial mass (Balbiani body), connected with a fine fibrillar ring-shaped formation around the nucleus, exhibits a strong positive reaction (Figs 5, 7). At the beginning of vitellogenesis the mitochondrial mass disintegrates; granules are formed from its periphery, which migrate throughout the cytoplasm. Part of the mitochondrial mass close to the nucleus remains compact. Clusters of strongly tubulin-positive material are formed near the nuclear membrane, which gradually contribute to the ring-shaped strutcture situated around the nucleus (Figs 5, 8, 9, 10).

In oocytes of stage-III to -IV cytoplasm, radially oriented bundles exhibiting strong fluorescence are formed, beginning in the area of the Balbiani body (Fig. 9), and gradually filling the entire periphery of the oocyte. In the ring-shaped



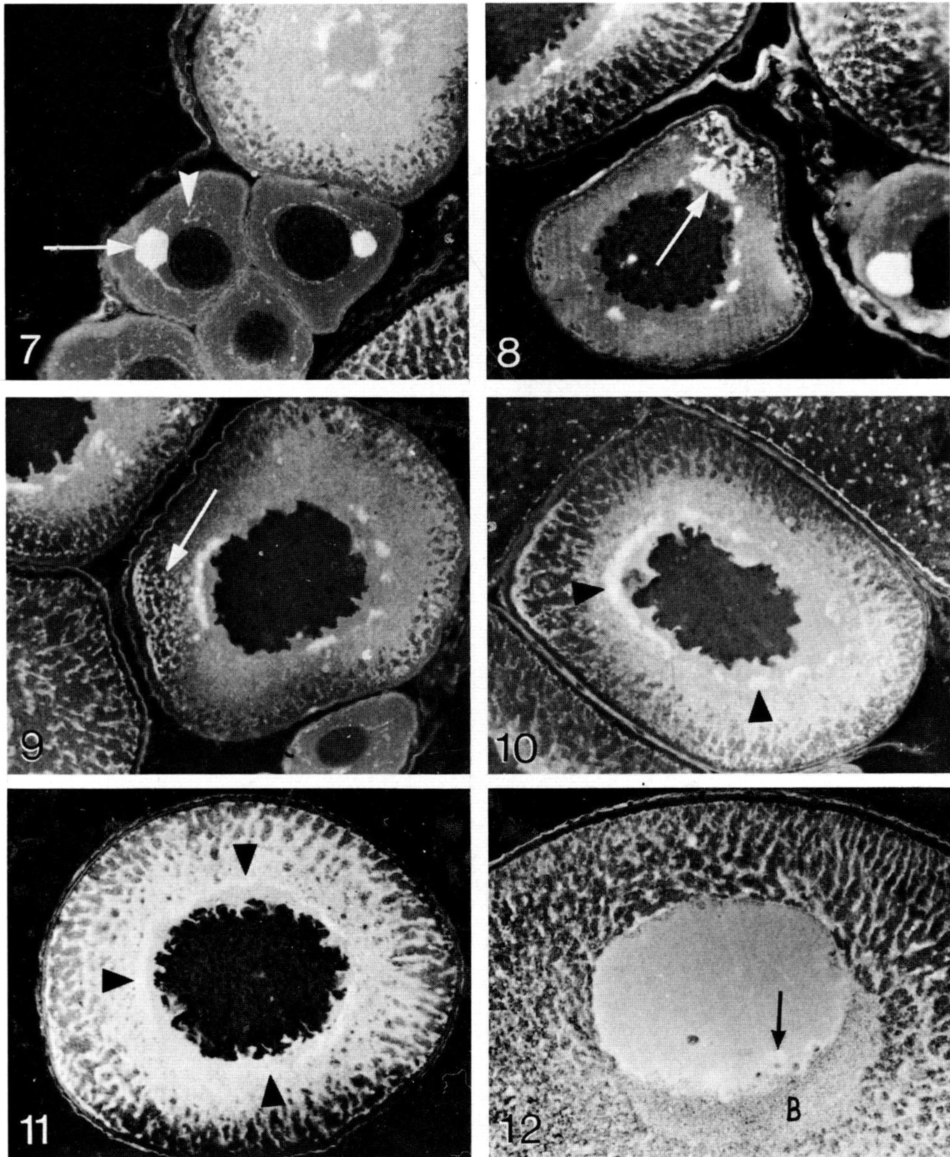
Figs. 1–4 Stage-I, -III, -IV and -VI oocytes. Aniline blue–orange G–azocarmine staining. Stage in Fig. 1 corresponds to those in Fig. 7, in Fig. 2 to that in Fig. 9, in Fig. 3 to that in Fig. 11 and in Fig. 4 to that in Fig. 12. Arrows, Balbiani body; arrowheads, circumnuclear ring. Magnification ×1000.

Fig. 5 Comparison of overall intensity of tubulin-positive fluorescence in a group of oocytes of different stages. Magnification 500 ×.

Fig. 6 Stage-V oocyte. Tubulin-positive fibrils within the cytoplasm oriented mainly towards the future animal cell surface (arrowheads). Magnification ×500.

formation around the nucleus an extremely strongly fluorescent bundle appears (Fig. 10, 11).

In stage-V to -VI oocytes radial bundles are distinct in the future animal half cytoplasm but less distinct in the future vegetal half (Fig. 6). A basket of fine fibres surrounds the part of the nucleus facing the future vegetal half of the oocyte; fibres continue as finger-like processes towards the upper part of the nucleus (Fig. 12). The full-grown oocyte shows additional strong fluorescence inside the germinal



Figs 7–12

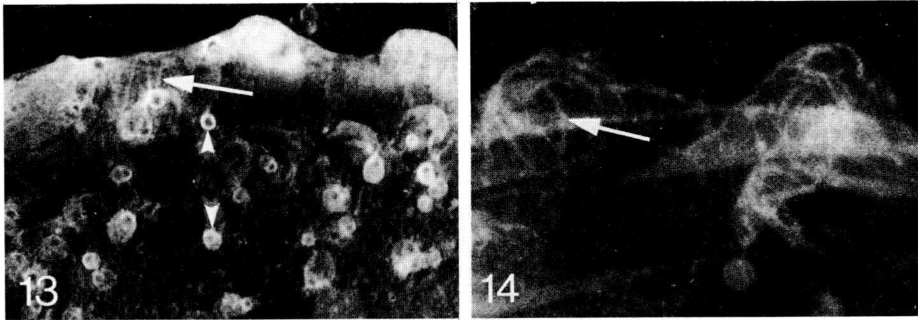


Fig. 13 Isolated germinal vesicle. Tubulin forms a fine fibrillar network in nuclear envelope protuberances (arrow); the network is oriented along the germinal vesicle surface and towards the karyoplasm. Nucleoli (arrowheads) were simultaneously stained by 50 μm fluorescent probe ANS (1-anilinonaphtalene-8-sulphonate). Magnification × 4000.

Fig. 14 Isolated germinal vesicle. Detail of tubulin-positive structures in nuclear membrane protuberances (arrow). Nucleoli were not stained by ANS. Magnification × 8000.

vesicle near the nuclear membrane in sections. Peripheral nucleoli are surrounded by brightly fluorescing material (Fig. 12).

The surface of the isolated germinal vesicle of full-grown oocytes exhibits numerous protuberances; inside some of these structures nucleoli are visible. When immunofluorescence is applied the protuberances reveal the presence of tubulin-containing fibrillar structures oriented both along the surface and towards the inside of the nucleus. Apart from the fluorescence of structures present within the protuberances, bright fluorescence may be observed encircling protruding nucleoli (Figs. 13, 14).

Fig. 7 Stage-I oocytes. Intensive fluorescence of the Balbiani body (arrow) from which fibrillar structures emerge and concentrate around the nucleus. Additional irregularly localized fibres in the cytoplasm (arrowhead). Magnification × 1000.

Fig. 8 Stage-II oocyte. Beginning of migration of tubulin-positive material from the Balbiani body (arrow); gradual increase of tubulin in the vicinity of the germinal vesicle. Magnification × 1000.

Fig. 9 Stage-III oocyte. Continuing disintegration of tubulin-positive material of the Balbiani body (arrow). Fibrillar structures begin to appear near the plasma membrane. Magnification × 1000.

Fig. 10 Stage-III to -IV oocyte. The Balbiani body has disintegrated. A layer of tubulin-positive material is formed around the germinal vesicle (arrowheads). Magnification × 1000.

Fig. 11 Stage-IV oocyte. Circum-nuclear ring of tubulin-positive material (arrowheads) is closed around the germinal vesicle. Fibrillar structures growing out towards the plasma membrane fill the entire cytoplasm of the oocyte. Magnification × 800.

Fig. 12 Stage-VI oocyte. Tubulin-positive fibrillar structures on the future vegetal side of germinal vesicle form a basket. Note the fluorescence around nucleoli, B, near nuclear membrane (arrow). Magnification × 1000.

Fig. 15 Western blots of one-dimensional polyacrylamide gels. *Xenopus laevis* oocytes polypeptides and purified pig brain tubulin were resolved, on 7·5 % one-dimensional gel transferred and blotted with a monoclonal antitubulin antibody TU-01. Lines A, B are total *Xenopus laevis* oocytes polypeptides and line C is the brain tubulin.

DISCUSSION

Clear changes occur in the distribution and localization of tubulin-containing structures in growing oocytes of *Xenopus laevis*. On the basis of the technique used it is not possible to decide with certainty whether and where tubulin is present in the form of a free pool or in polymerized form. In previtellogenic oocytes the major amount of tubulin is concentrated in the Balbiani body. According to several authors this structure contains mitochondria and membranes of the endoplasmic reticulum and the Golgi apparatus (Raven, 1961–for review; Guraya, 1979). Billet & Adam (1976), using electron microscopy, observed a mitochondrial cloud formed by mitochondria and fibrils in bundles. The affinity of tubulin for lipidic complexes is well known from experiments on invertebrate eggs (Caron & Berlin, 1979; Klausner *et al.* 1981), while high-resolution electron microscopy has confirmed that, particularly at cell division, mitochondria make connections with microtubules (Nakamura & Ueda, 1982). In the course of vitellogenesis mitochondria are shifted from the Balbiani body to the cell surface while others stay around the nucleus (Raven, 1961; Tourte, Mignotte & Mounolou, 1984). The reorganization of tubulin observed by us may be related to their directed movements or to movements of other membrane structures detected in the Balbiani body. Disintegration of the Balbiani body is distinctly polarized; tubulin-containing structures localized at the nuclear membrane remain compact and gradually form a ring-like structure around the nucleus. Those directed to the cell surface are disengaged as granules which move to the cortical region of the oocyte. The basic

phases of this process take place at stages 1–III, according to Dumont's (1972) classification.

The tubulin-positive band in stage-III and -IV oocytes appears as a finely fibrillar or unstructured layer. At early stages it contains remnants of the disintegrated Balbiani body. Its position within the oocyte corresponds to the area positive for RNA, where ribosomes are mainly concentrated (Brachet, 1967–for review). In this space yolk reserves are gradually deposited, i.e. yolk granules are localized in the area of the tubulin-positive band. Interspaces among the yolk granules are penetrated by tubulin cords, which are particularly prominent in the future animal half of the oocyte.

As vitellogenesis progresses growth and rearrangements of the cell content take place. Yolk protein is bound by the endocytically active oolemma and transported via endosomes to yolk platelets which grow in size (Wallace & Jared, 1968; Brummet & Dumont, 1977). The transport pathway of the endosomes is probably marked by tubulin-containing structures, which are formed, as vitellogenesis progresses, from the pool present in the tubulin-positive band. This band gradually disappears as a continuous layer and radial tubulin-positive cords take its place, showing a picture typical for stage-V to -VI oocytes. Whether these changes represent rearrangement of structures already present, *de novo* synthesis, or synthesis from the pool of mono- or dimers present in the ring-like layer is not clear.

Changes in the localization of fluorescent material are very similar to those which were observed after the histological staining described above or after the immunoperoxidase reaction (unpublished). The tubulin-containing structures that we describe in this paper are apparently part of a cytoskeletal complex which is taking part in the restructuring of the cytoplasmic space in connection with its growth and vitellogenesis. Although our technique does not allow us to be sure that it is assembled tubulin that undergoes the observed changes, it is plausible to suppose its existence. Moreover, in preliminary experiments (unpublished) using the PAP reaction, distinct fibrillar structures are found in many places where fluorescence shows tubulin to be present.

Immunofluorescence has also confirmed the presence of a distinctly fibrillar network arranged at the surface of the nuclear membrane (Paleček, Habrová & Nedvídek, 1984). Protuberances of the membrane often show a network in the form of a basket, inside which nucleoli are sometimes visible. It seems probable that these tubulin-containing structures participate in the transport of nucleoli from the germinal vesicle to the cytoplasm of the oocyte, which occurs via the nuclear envelope well before its disintegration at the end of the growth phase (Habrová, 1975).

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