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Germinal vesicle breakdown in the *Xenopus laevis* oocyte : description of a transient microtubular structure

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Summary. During progesterone-induced meiotic maturation of *Xenopus* oocytes *in vitro*, 7 morphological stages were defined. Using cytological analysis, nuclear breakdown was divided into three stages. Stage 1 corresponded to basal germinal vesicle breakdown. Stage 2 was characterized by the advent and development of a fibrillar network formed by microtubules at the basal part of the nucleus. Below, a lamellar microtubule organizing center (MTOC) was present. Numerous vesicles of smooth endoplasmic reticulum were proximal to both of these structures. During its formation and modification (stages 2 and 3), the fibrillar network migrated towards the animal pole. A prometaphase I stage was observed before the formation of the metaphase I spindle.

Introduction.

In amphibians, steroid hormones (progesterone and other Δ^4-3-ketosteroids), synthesized in the follicular envelope under gonadotropic stimulation (LH activity), are responsible for the initiation of maturation (see review in Masui and Clarke, 1979).

Nuclear membrane breakdown and subsequent stages of meiotic maturation are initiated after the appearance in the oocyte cytoplasm of the maturation promoting factor or MPF. It has been reported that the formation of MPF activity is accompanied by numerous physiological or biochemical events within the oocyte, for instance : increase in protein synthesis, increase of cAMP-independent protein phosphorylation, and decrease in membrane potential (see review in Masui and Clarke, 1979). However, the correlations between biochemical events and cytological changes are unknown.

Meiotic maturation of the amphibian oocyte was cytologically analyzed in *Bufo bufo* asiaticus by Tchou-Su and Wang Yu-Lan (1958) who reported that the breakdown of the nuclear membrane begins basally (towards the oocyte center) after gonadotropic stimulation *in vitro* (see fig. 5 ; Tchou-Su and Wang Yu-Lan, 1958). Similar

observations in other amphibian oocytes were carried out by Dettlaff, Nikitina and Stroeva (1964), Dettlaff and Skoblina (1969) and Brachet, Hanocq and Van Gansen (1970). Chromosome condensation and changes in the nucleoli during the early phase of steroid-induced maturation were described in *Xenopus* oocytes by the latter authors who also reported the accumulation of a fibrillar material in the basal area of the germinal vesicle when the nuclear membrane begins to break down. The present article sequentially analyzes the cytological events in progesterone-induced maturation of *Xenopus laevis* oocytes *in vitro* and reports the presence of a unique transient microtubular structure which appears after germinal vesicle breakdown (GVBD).

Material and methods.

Xenopus laevis females from South Africa (De Rover, Holland) were bred and maintained under laboratory conditions.

They were anaesthetized with MS 222 (1 g/l ; Sandoz). The ovaries were removed and transferred to medium A : 88 mM NaCl ; 0.33 mM $Ca(NO_3)_2$; 1 mM KCl ; 0.41 mM $CaCl_2$; 0.82 mM $MgSO_4$; 2 mM Tris ; pH 7.4 (Merriam, 1971). Penicillin (50 000 IU/l) was added to the medium. Stage VI oocytes (Dumont, 1972) were collected after digestion by dispase (0.4 mg/ml ; grade II from Boehringer), a protease from *Bacillus polymixa*, for 4 hrs at laboratory temperature and by collagenase (1 mg/ml ; type I from Sigma) for 1 hr at 25-28 °C. Maturation was induced by treatment with 1 μM progesterone.

The appearance and development of the maturation spot at the animal pole of the oocyte were studied individually at regular time intervals of 10 to 15 min under a dissection microscope ; 10 oocytes were analyzed for each female tested.

For cytological analysis with a light microscope, the oocytes were fixed in Smith's solution, dehydrated, embedded in paraffin and cut into serial sections of 7 to 10 μ which were stained with Feulgen reagent and light green.

For electron microscopy, the oocytes were fixed in 3 p. 100 glutaraldehyde in 0.075 M phosphate buffer at pH 7.2 for 2 hrs ; fragments of the oocytes containing the germinal vesicle were then post-fixed for 1 hr in 2 p. 100 osmium tetroxide in distilled water and placed for several hours in 0.5 p. 100 aqueous uranyl acetate at 4 °C. They were dehydrated in ethanol and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate for 30 min and with lead citrate for 10 min before examination with a Zeiss EM-10 B electron microscope.

Results.

Under a dissection microscope, the maturation spot localized in the pigmented animal hemisphere appeared at variable times after hormonal stimulation, depending on the female tested. However, the development of the spot between GVBD and extrusion of the first polar body was comparable for the different females (figure 1).

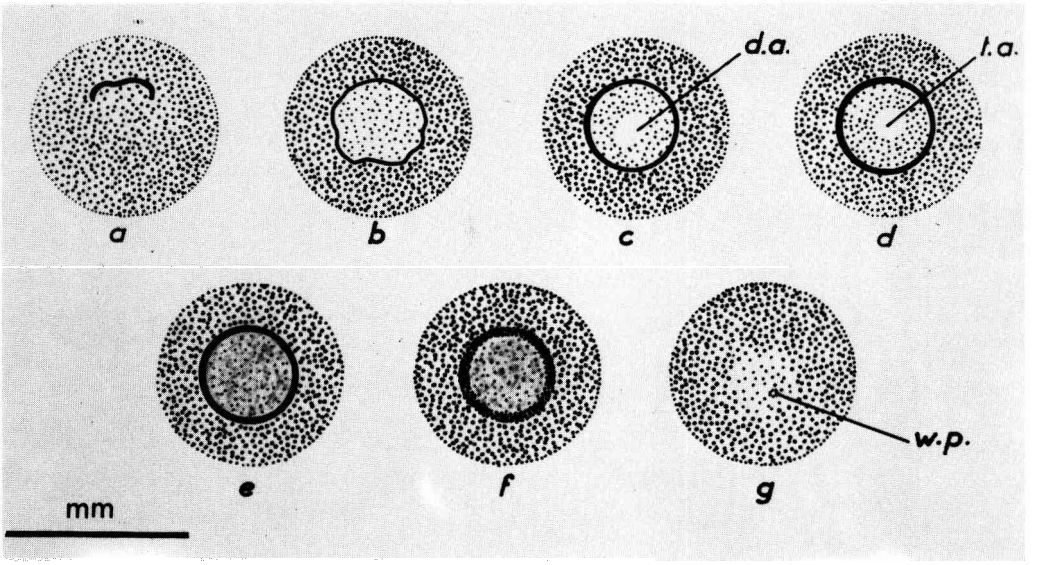


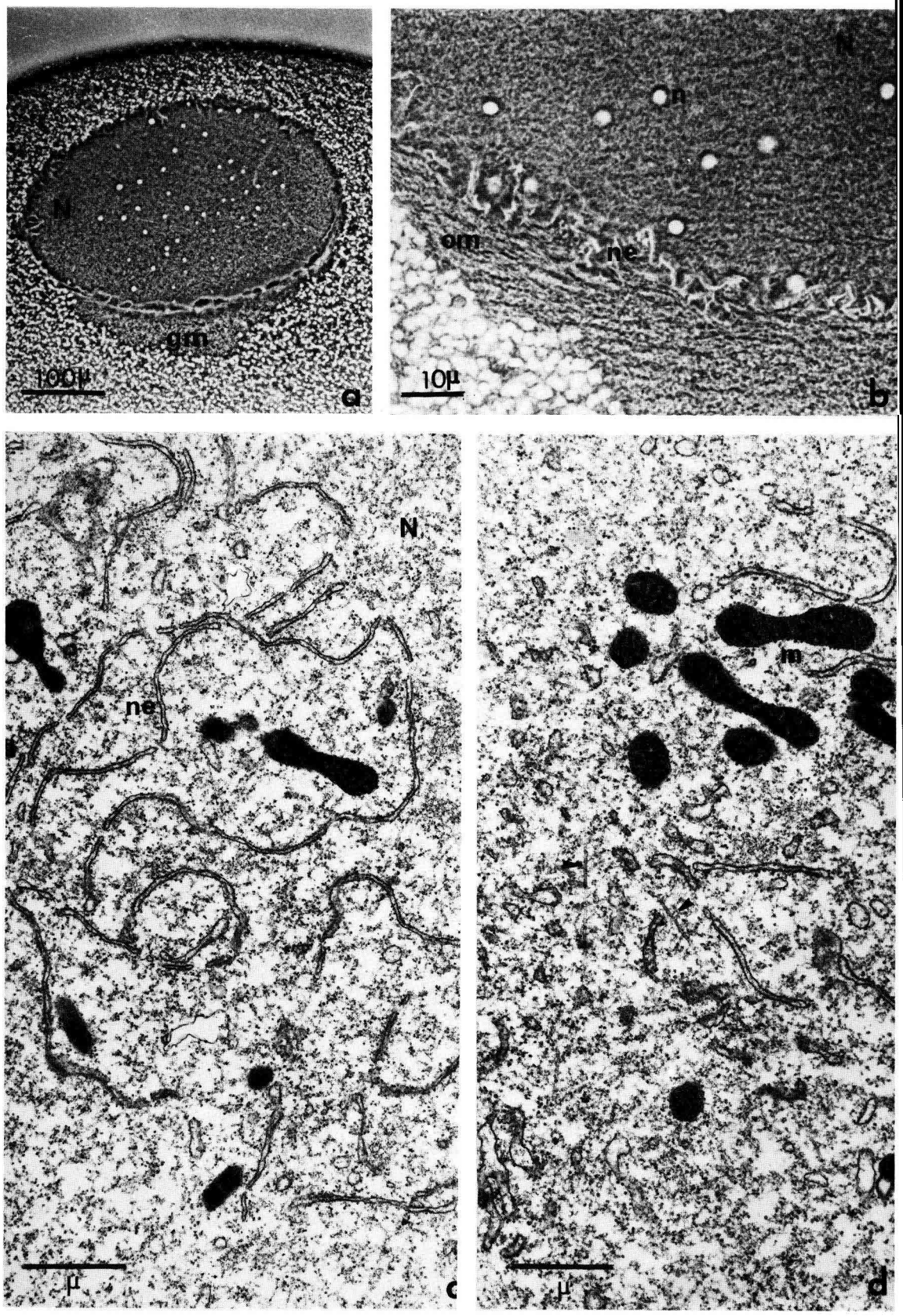
FIG. 1. — *External chronology of GVBD established from observations of oocytes from six females.* Seven stages are described. *Stage a* : at the pole of the uniformly pigmented animal hemisphere, irregular, dark pigment deposits are first seen ; *Stage b* : the pigment deposits form a continuous, irregular border delimiting a slightly depigmented homogeneous area ; *Stage c* : the maturation spot, surrounded by a typical pigmented ring, is well organized. A totally depigmented area is seen in the center of the spot ; *Stage d* : the central depigmented area becomes translucid ; this stage is always transient ; *Stage* e : the pigment ring remains well defined but the pigmentation of the central area becomes fuzzy and opaque ; *Stage f* : the peripheral pigmented ring thickens slightly and begins to disperse. *Stage g*: the well-defined spot disappears and a more or less depigmented area is observed at the animal pole. A brilliant white point, situated near the pigment ring, was observed in 80 p. 100 of the oocytes between stages e and g.

This development of the maturation spot was used to select oocytes for defining a cytological chronology of GVBD and maturation spindle formation, established after observing 148 oocytes from five females with a light microscope.

For electron microscopic observation, the oocytes were selected according to external morphological criteria ; thin and thick sections were cut in each case to determine the cytological stage.

a) *Germinal vesicle breakdown.* Three stages were defined based mainly on the following cytological criteria : disappearance of the nuclear membrane, organization in the basal nuclear area (towards the oocyte center) of a fibrillar network ; chromosome condensation, changes in nucleolar structure, accumulation of a granular material beneath the basal end of the nuclear envelope.

Stage 1 (Pl. I*a*). — In the animal hemisphere, the black pigment layer (melanosomes) still appeared homogeneous. The lobed basal part of the nuclear membrane showed numerous fragmentations and curled figures (Pl. I*a*), confirmed under elec-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tron microscopy (Pl. Ic). These remnants of the nuclear envelope lacked pores. Some microtubules were present nearby in the cytoplasm and the nuclear area (Pl. Id). Beneath the lobed basal nuclear envelope, a granular material, lacking yolk platelets and strongly stained with light green, was observed (Pl. Ia). With phase-contrast light microscopy, the upper part of this material appeared slightly oriented tangentially to the basal part of the nuclear enveloppe (Pl. Ib). The lateral and apical segments of the nuclear membrane were still intact.

The radial zones, lacking yolk platelets and stained with light green, were well developed between the nucleus and the apical plasma membrane. Under electron microscopy, these zones appeared to contain numerous mitochondria. The condensing diplotene chromosomes were generally dispersed in the nucleoplasm. Nucleoli of different sizes were seen in the upper part of the nucleus ; vacuolization was observed in hypertrophied nucleoli.

Stage 2. — The density of the pigment granules decreased in a localized area of the animal pole corresponding to the formation of the white spot (late stage a, stage b). The basal segment of the nuclear membrane was no longer visible ; only a few remnants were seen by electron microscopy. At the basal part of the disintegrating nucleus a fibrillar network was observed. When this formation was well developed (late stage 2), it included two parts (Pl. Ib, c) : (i) a dense layer of fibrous structures tangent to the basal limit of the disintegrating nucleus delimited the lower border of the network, and (ii) bundles of fibers perpendicular to this layer and oriented towards the animal pole. A dense, lamellar-shaped layer was clearly seen at the basal end of the nuclear area (Pl. IIIc, d) by electron microscopy. This structure was formed of dense material, including many electron-dense granules about 50 nm in diameter. Numerous tangentially-oriented microtubules were present at the upper part of the material, indicating that the dense, lamellar-shaped layer might be a microtubule-organizing center (MTOC). Perpendicularly above, many microtubules were oriented towards the animal pole (Pl-IIIe, f). During early stage 2, the bundles of filaments perpendicular to the lower border were short and not very dense (Pl. IIa). The distance from the base of the network to the plasma membrane decreased as maturation progressed (early stage 2 : about 200 μ ; late stage 2 : about 100 μ). The granular material underlying the microtubular network was more abundant than in stage 1 and formed a cone extending towards the oocyte center (Pl. IIb). This material appeared as a dense area of smooth endoplasmic reticulum vesicles (Pl. IId). The nuclear envelope was still

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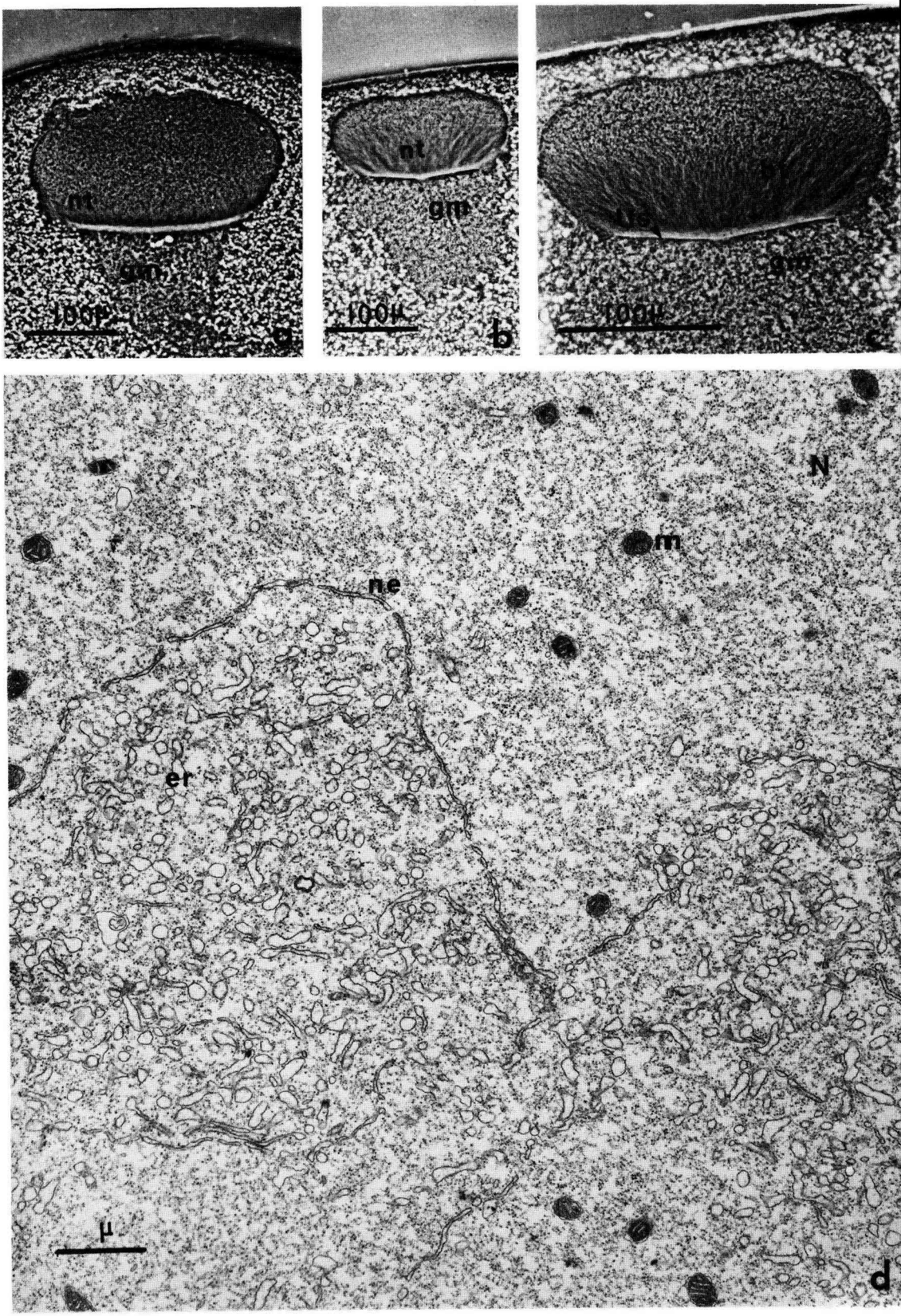
PLATE I

Meiotic maturation of Xenopus *oocyte : stage* 1 *of nuclear breakdown.*

a) *General view of the nucleus* ; b) *detail of the basal part of the nucleus* ; phase-contrast images. N : nucleoplasm ; ne : convoluted nuclear envelope ; n : nucleolus ; om : oriented material. c, d) *electron micrographs of the basal nuclear area.* Remnants of the convoluted nuclear envelope (ne) lack pores. Microtubules (arrows) are present in the cytoplasm and the nucleoplasm (N) ; m : mitochondria. × 17 500.


nt
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present apically and laterally (Pl. II*a*, *b*). Apical and lateral segments of the nuclear membrane and the basal fibrillar network still enclosed the nucleoplasm which appeared heavily granulated. This was probably due to the invasion of the nuclear area by cytoplasmic organelles such as mitochondria (Pl. II*d*). Chromosomes in late diplotene or diakinesis were localized inside the network ; since they were still scattered, only a few adjacent to the microtubules were visible (Pl. III*e*). Some large, vesicular nucleoli were still seen then, but most had already disappeared. Feulgen-positive bodies (nucleolar organizers) were also observed near the microtubular network. The peripheral radial zones, containing mitochondria and located between the nucleus and the plasma membrane, began to disrupt when the disintegrating nucleus ascended.

Stage 3 (Pl. III*a*, *b*). — The depigmented maturation spot, surrounded by a thick ring of pigment, was clearly visible. The nuclear membrane had generally disappeared, but some remnants could still be seen above the fibrillar network. The distance from the base of the network to the plasma membrane was then about 60 μ. The yolk platelets and the radial zones were not visible between the fibrillar structure and the oocyte surface. Although the volume of the network decreased, its density increased. The cone of granular material formed an elongated column extending from the center of the oocyte to the network which then became peripheral. Chromosomes in diakinesis were localized at the base of the network inside an homogeneous strand which was strongly stained with light green.

b) *First meiotic spindle organization.* The fibrillar network disappeared and bivalent chromosomes were found near the plasma membrane (about 10 μ) in a spherical homogeneous structure (diameter : 10 μ) which sometimes contained a few fibrils. Furthermore, a second spherical structure, revealed by the light green, was frequently observed near the area containing chromosomes. The granulated material beneath appeared to be dissociated. We have called this stage prometaphase I (Pl. IV*a*).

In metaphase I, the spindle was either parallel or perpendicular to the oocyte surface. When it was perpendicular, the spindle pole localized at the oocyte cortex seemed to correspond to the brilliant white point observed externally (fig. 1). The bivalent chromosomes were aligned at the metaphase plate with the microtubules adjacent to them (Pl. IV*b*,*c*). No asters could be detected at the poles of the barrel-shaped spindle. At the spindle poles, the microtubules were embedded in a dense

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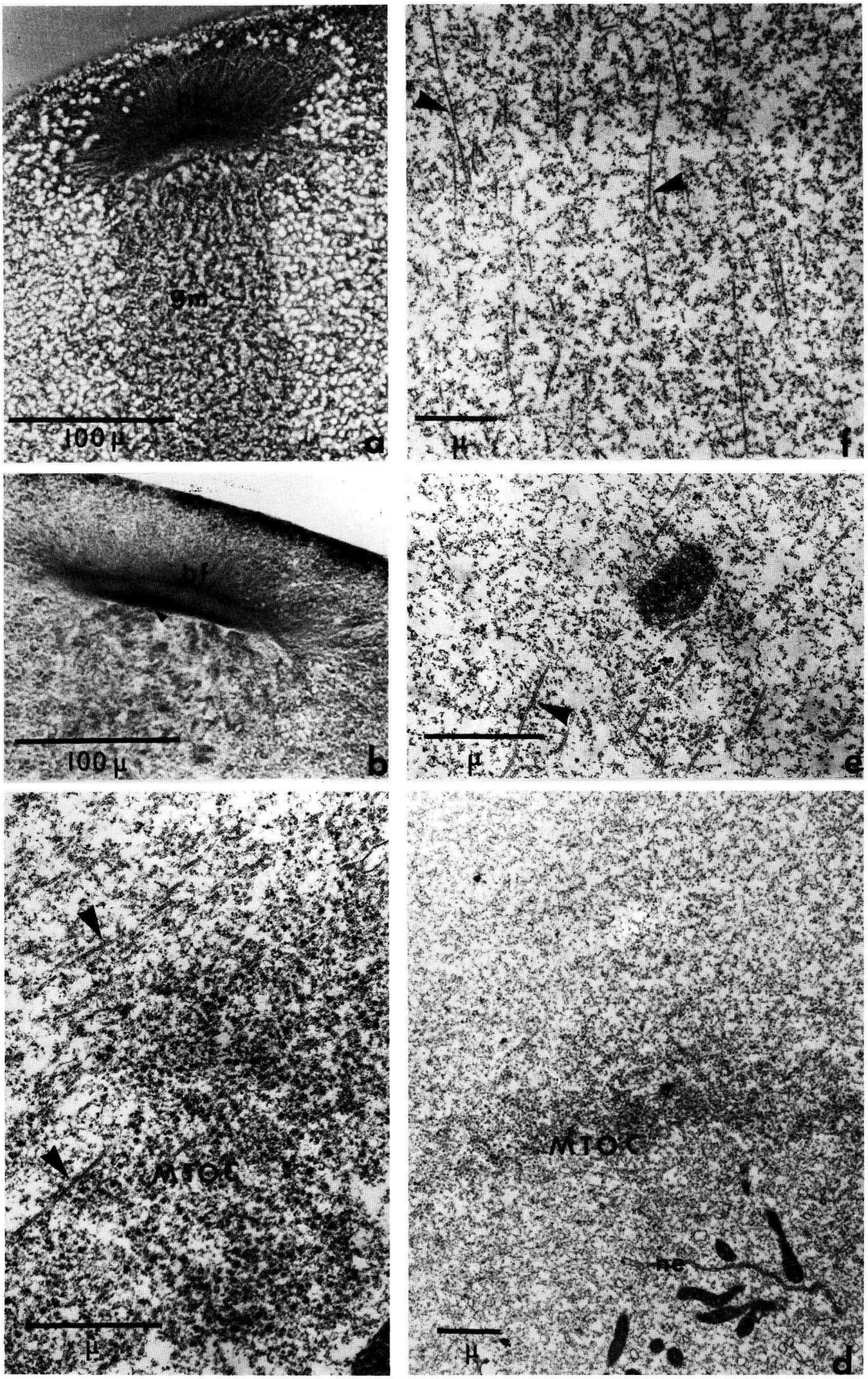
PLATE II

Meiotic maturation of Xenopus *oocyte : stage* 2 *of nuclear breakdown.*

a) *early stage* 2, b) *late stage* 2, c) *late stage* 2: organization of the fibrillar network ; phase-contrast images. bf : bundles of filaments ; gm : granular material ; nt : network of filaments ; tfs : fibrous structures arranged tangentially to the basal segment of the nuclear membrane. d) *electron micrograph of the basal part of the nucleus.* A large area of smooth endoplasmic reticulum (er) is seen in the cytoplasm below the remnants of the nuclear envelope (ne). The granular nucleoplasm (N) contains some mitochondria (m). × 14 000.


9m
100 μ
a
f
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100 μ
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MTOC
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d

material. Numerous vesicles of smooth reticulum surrounded this area (Pl. IV*b*), but they did not form large surfaces as those described for stage 2 of germinal breakdown.

Typical anaphases I were observed.

c) *Emission of the first polar body.* Immediately after extrusion, the polar body was localized near the maturation funnel. Chromosomes were grouped beneath the funnel.

d) *Second meiotic spindle.* The second meiotic spindle, narrower than the first one, was « anchored » to the oocyte surface at the level of the maturation funnel which was more or less distinguishable at metaphase II. No aster was detected.

Discussion and conclusion.

Seven stages (a to g), based on external observation of the maturation spot, were defined during the period between the first visible pigment movement and metaphase II. As judged from cytological examination, the period during GVBD was divided into three stages. Just before the formation of the metaphase I spindle, we observed a stage, which we called prometaphase, characterized by the presence of condensed chromosomes in a spherical homogeneous area located near the plasma membrane.

External and internal chronologies have been compared (table). Although there was a correlation between the oocytes from the same female, no exact correspondance could be found when different females were compared. However, the external stages could be used to select oocyte populations.

From the onset of GVBD, microtubules, present in the cytoplasm near the remnants of the basal nuclear envelope, seemed to penetrate the nuclear area. These microtubules might be involved in the process of nuclear envelope breakdown, as seems to be the case in the mouse oocyte (Calarco, Donahue and Szöllösi, 1972).

After nuclear membrane breakdown, a fibrillar network, first reported by Brachet, Hanocq and Van Gansen (1970), was organized at the basal part of the nucleus. The changes in this network, a transitory structure presenting maximal development in stage 2, were analyzed during the maturation process : it appeared to migrate to the oocyte surface of the animal pole since it approached the oocyte periphery during the meiotic process.

PLATE III

Meiotic maturation of Xenopus *oocyte : stages 2 and 3 of nuclear breakdown.*

a, b) *stage* 3 : fibrillar network becomes peripheral (a : phase contrast image). bf : bundle of filaments; gm : granular material ; arrow : chromosomes. c, d) *stage* 2 : electron micrographs of a lamellar-shaped structure defined as a microtubule organizing center (MTOC) situated at the basal part of the nuclear area ; tangentially oriented microtubules (arrows) are present. ne : nuclear envelope. c) × 22 500 ; d) × 10 000. e, f) *stage* 2 : above the MTOC, microtubules (arrows) are oriented towards the animal pole. ch : chromosome adjacent to the microtubules. e) × 21 000 ; f) × 13 600.

10μ
a
1μ
b
1μ
c

TABLE

Correlation between external and cytological stages

External stages

Female	N	a		b		c		d		e		f		g	
		n	cyto-logical stage	n	cyto-logical stage	n	cyto-logical stage	n	cyto-logical stage	n	cyto-logical stage	n	cyto-logical stage	n	cyto-logical stage
1	9			2	st 1										
				1	st 2	2	st 2								
						2	st 3	2	prometa I						
2	69	8	st 1												
		14	st 2	17	st 2	3	st 2								
						15	st 3	5	st 3						
								2	prometa I	3	prometa I				
										2	meta I				
3	23							1	st 3	1	st 3				
								3	prometa I						
								3	meta I	4	meta I			1	meta I
								1	meta-ana I	5	meta-ana I				
														3	gpd. ch.
														1	meta II
4	39	7	st 2												
		1	st 3	3	st 3	1	st 3								
						2	prometa I								
						8	meta I			3	meta I	4	meta I	1	meta I
														2	ana I
														1	gpd. ch.
												2	meta II	4	meta II
5	8									4	prometa I				
										2	meta I				
										2	meta-ana I				

Oocytes fo five females were cytologically analysed. N : total number of analyzed oocytes for each female; n : number of cytologically identified oocytes ; st 1, st 2, st 3 : stages of germinal vesicle breakdown ; prometa I : meiotic prometaphase I ; meta I : meiotic metaphase I ; ana I : meiotic anaphase I ; gpd. ch. : grouped chromosomes ; meta II : meiotic metaphase II.

PLATE IV

Meiotic maturation of Xenopus *oocyte : prometaphase I and first meiotic spindle.*

a) *chromosomes* (arrow) grouped in an homogeneous structure (prometaphase I) surrounded by light-green stained material. b, c) *electron micrographs of the first meiotic spindle.* At the poles, microtubules are embedded in dense material and surrounded by many vesicles of smooth endoplasmic reticulum (er). One chromosome (ch) of the metaphase plate adjacent to the spindle microtubules (arrow). × 28 000.

The ultrastructure of the fibrillar network in stages 1 and 2 has been analyzed as follows :

— the fibrillar network is composed of microtubular bundles ;

— at the base of this network is a structure of dense material comparable to the MTOC present in numerous cells devoid of a centriole, particularly mammalian oocytes (Calarco, Donahue and Szöllösi, 1972) ;

— in the dense material, small electron-dense granules are present. This observation is not usual ;

— the granular zone, devoid of yolk platelets, beneath the MTOC was composed mainly of smooth reticulum vesicles. An abundant smooth reticulum of this type was reported by Steinert *et al.* (1974) but only during pseudomaturation of *Xenopus* oocytes.

At the onset of GVBD, the diplotene chromosomes were dipersed throughout the nucleoplasm ; they then assembled (diakinesis) at the base of the network during late stage 2 and stage 3. Before the first meiotic spindle was formed, the chromosomes were located near the oocyte surface in an homogeneous area. Contrary to Brachet, Hanocq and Van Gansen (1970), we never observed migration towards the animal pole of « condensed chromosomes... now attached to the maturation spindle ».

Aster formations at the poles of the first meiotic spindle were never observed by either light or electron microscopy. Vesicles of smooth reticulum were always seen near the poles of the first meiotic spindle.

In the amphibian oocyte, a giant cell, nuclear breakdown involves the formation of the fibrillar network ; it is suggested that this unique and transitory structure plays a role :

1) in assembling the chromosomes,

2) in controlling their migration towards the animal pole. The bivalent chromosomes, present at the basal part of the fibrillar network at stage 2, may be pushed to the oocyte periphery when the fibrillar network migrates between stages 2 and 3. When the network disappears, the chromosomes are left near the surface (prometaphase).

The presence of a dense material surrounded by smooth reticulum vesicles has been described at the spindle poles of numerous mitotic cells (see review by Luykx, 1970). This dense material could contain tubulin (Harris, 1975). ATPase activity has been reported in the isolated mitotic apparatus in sea urchin embryos (Miki, 1963 ; Weisenberg and Taylor, 1968 ; Mazia *et al.*, 1972). As suggested by Harris (1975), Ca^{++}-sequestering vesicles of endoplasmic reticulum would be involved in the regulation of the intracytoplasmic Ca^{++} ions necessary for polymerization and depolymerization of the spindle microtubules. Ca^{++}-sequestrating activity was demonstrated recently in the sea-urchin embryo isolated mitotic apparatus containing vesicles (Silver and Cole, 1980).

It is tempting to attribute a similar Ca^{++} sequestering role to the reticulum vesicles in the dense area observed at the base of the microtubule network ; Ca^{++} ions are involved during progesterone-induced meiotic maturation (Cartaud *et al.*, 1980). The exact role of Ca^{++} in assembling and dispersing the fibrillar network is not known. However, preliminary experiments have shown that elevating intracellular Ca^{++} concentration in the presence of the ionophore A 23187 disorganized fibrillar network

formation when applied to the oocyte at the onset of external maturation stage a. The Ca^{++} ionophore does not block the nuclear membrane breakdown basally ; in some oocytes the fibrillar network cannot be observed. Peripheral meiotic spindles are not formed in all cases, and the chromosomes are sometimes grouped deep in the cytoplasm.

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Résumé. Au cours de la maturation méiotique induite *in vitro* par la progestérone dans l'ovocyte de Xénope, 7 stades morphologiques ont été définis. L'analyse cytologique a permis de scinder la période de désintégration du noyau en trois stades. Le stade 1 correspond à la rupture basale de l'enveloppe nucléaire. Le stade 2 est caractérisé par l'apparition et le développement dans la région inférieure du noyau d'un réseau fibrillaire formé de microtubules. Un centre organisateur de microtubules (MTOC) en forme de lame s'étend à sa base. De nombreuses vésicules de réticulum endoplasmique lisse sont situées à proximité de ces structures. Dès sa formation et pendant son évolution (stades 2 et 3) ce réseau fibrillaire migre vers le pôle animal de l'ovocyte. Avant la formation du fuseau de métaphase I un stade de prométaphase I a été observé.

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