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GERMINAL VESICLE BREAKDOWN IN THE MOUSE OOCYTE

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SUMMARY

Germinal vesicle breakdown in mouse oocytes *in vivo* and *in vitro* has been examined by electron microscopy. *In vitro* oocytes were studied immediately after release from follicles and at various times (0·5–11 h) in culture. Approximately 30 min after oocyte release, chromatin condensation begins along the convoluted nuclear envelope (NE). Chromatin granules are common in all condensing chromosomes. Heterochromatin, visible from early condensation until chromosomes are of uniform density, often is observed near the kinetochores. The nucleolus breaks down after peripheral incorporation of separate nucleolus-associated bodies composed of 25-nm diameter fibrils. These bodies are later found free in the cytoplasm. As chromosome condensation progresses, the NE becomes highly convoluted, then discontinuous, finally forming NE doublets. Spindle formation begins with the appearance near the NE of small medium-dense areas from which microtubules emanate. No centrioles are present. Dark granules and mitochondria move centrally in the oocyte and surround the spindle. Peripheral cortical granules and large aggregations of multivesicular bodies are present at all stages. The Golgi apparatus is not well developed. Very little rough endoplasmic reticulum is present, although free ribosomal clusters are common. There are no significant ultrastructural differences between eggs maturing *in vivo* and *in vitro*.

INTRODUCTION

Under the influence of gonadotrophins, nuclei of mouse oocytes usually progress beyond the germinal vesicle stage prior to ovulation. Germinal vesicle breakdown is also induced when follicular oocytes are liberated into a suitable culture medium (Donahue, 1971). The early nuclear changes accompanying this resumption of meiosis have been examined at the light-microscope level by Donahue (1968) who found that maturation proceeds fairly synchronously in groups of cultured oocytes, with the majority reaching metaphase I after 9 h. The present paper presents electron-microscopic observations on chromatin condensation, dissolution of the nucleolus, breakdown of the nuclear envelope, spindle formation, and associated cytological changes occurring as cultured mouse oocytes progress from the germinal vesicle (GV) stage to metaphase I.

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MATERIALS AND METHODS

Full-sized follicular oocytes with few or no investing follicular cells were liberated from CF-1 (Carworth, Inc.) female mice (*Mus musculus*), 8–12 weeks old, into a chemically defined Krebs–Ringer culture medium and cultured at 37 °C in 5 % CO_2 under oil for up to 11 h (Donahue, 1968). The stage of the oestrus cycle was not determined. Since 1 h elapsed between liberation and the initiation of culture, samples were taken at the time of liberation (*t*-1 h) and 0·5 h later (*t*-0·5 h). Subsequent samples were taken after oocytes had been cultured for 0·5, 1·0, 1·5, 2·5, 3·5, 6·5, and 10–11 h. The sample size at each time was 30, 10 of which were prepared as whole-mounts and scored by light microscopy to determine the nuclear stages attained by the group; the remaining 20 were processed for electron microscopy.

In order to study GV breakdown *in vivo*, females were given an intraperitoneal injection of 15 units of pregnant mare's serum (PMS), followed 46 h later by an intraperitoneal injection of 15 units of human chorionic gonadotrophin (HCG), according to the method of Fowler & Edwards (1957) for superovulation. Oocytes were liberated from enlarged ovarian follicles 45 h after PMS or 1–10 h after HCG and fixed. In addition some oocytes from PMS only and PMS-HCG stimulated animals were cultured as described above. In all, 330 oocytes were collected from hormonally and non-hormonally stimulated females.

Fixation consisted of a primary fixation in 3 % glutaraldehyde in 0·1 M phosphate buffer, followed by a 15-min postfixation in 2 % osmium tetroxide in 0·1 M phosphate buffer. Oocytes were then rapidly dehydrated through a graded ethanol series and embedded in Epon 812 by a modification of the procedure described by Luft (1961).

Thin sections for electron microscopy were cut with a diamond knife on a Reichert microtome. Contrast was enhanced by treating the sections with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Sections were examined in a Philips EM 200 electron microscope.

Thick sections cut adjacent to many of the thin sections were mounted on glass slides and stained with a combination of methylene blue and azure II (Richardson, Jarett & Finke, 1960). A Zeiss photomicroscope was employed for 35-mm photography using a × 100 objective for both bright-field and phase contrast.

Observations

Immediately after their release from ovarian follicles, oocytes exhibit a large (approximately 30 μm in diameter) germinal vesicle with a spherical and usually single nucleolus which is finely fibrillar, very electron-dense, and possesses no granules (Fig. 1). Chromatin is non-condensed but a few areas of chromatin-like material are seen near the nucleolus. These denser localizations may correspond to the chromocentres of heterochromatic areas. Another type of electron-dense aggregate is often observed near the nucleolus and consists of twisted fibrils approximately 25 nm in diameter separated by spaces of 15 nm (Fig. 2). The nuclear envelope (NE) is nearly smooth and exhibits pores at regular intervals.

Large aggregations of what appear to be multivesicular bodies (Sotelo & Porter, 1959) are commonly seen in mouse oocytes. Often these are in the vicinity of the germinal vesicle. After maturation commences, aggregates of multivesicular bodies are rarely near the nucleus but are usually found at the periphery of the oocyte. Clusters of membrane-bound dark granules and cortical granules are also seen near the periphery ot the oocyte. The Golgi apparatus is observed occasionally as stacks of a few parallel short lamellae scattered throughout the cytoplasm. Parallel arrays of fibrous lamina are a common feature of the ooplasm and may represent 'yolk' accumulations (Szollosi, 1971). Ribosomes are seen, frequently in clusters, but rough endoplasmic reticulum is rare.

The first indication of germinal vesicle breakdown is the appearance of undulations in the nuclear envelope (Fig. 3), which can be seen in the earliest oocytes (fixed as early as 8 min after liberation from the follicle). Occasionally, microtubules appear to be directed toward the NE. Subsequently, chromatin condensation begins and is first observed as dense regions along the inner margin of the nuclear envelope. Concomitantly, the nucleolus is seen at the periphery of the nucleus (Fig. 4).

In oocytes collected at 1·5 h after culture, early chromosome condensation can be seen in thick Epon sections with the light microscope (Fig. 5). The fine structure of chromosomes of this stage is characterized by a 'patchy' appearance, i.e. areas of light to medium electron density (Fig. 6) and by the ubiquitous presence of electron-dense chromatin granules within the chromosomes (Figs. 6, 8, and 10). Very dense or heterochromatic regions of chromatin containing a few peripheral chromatin granules are also observed associated with condensing chromatin. This is the predominant condensation form of chromatin seen. The kinetochores (centromeres) are not visible at this time. The nucleolus has now progressed to a reticulated form characterized by regions of high and medium electron density, and does not appear to contain chromatin granules. The twisted fibrils of 25 nm diameter observed in the germinal vesicle are now peripherally associated with the condensing nucleolus (Fig. 6). (After the chromosomes have completely condensed, bodies composed of these fibrils are found free in the cytoplasm and are not associated with the chromosomes or the spindle.) Another type of intranuclear condensation also associated with heterochromatin is a rounded body composed of granules approximately 17 nm in diameter (Fig. 7). No breaks are seen in the NE, which now consists of 2 leaflets with no pores (Figs. 6, 7). Occasionally, small areas of medium electron density from which microtubules emanate occur in the cytoplasm near the nuclear envelope.

During slightly later stages in germinal vesicle breakdown the chromosomes appear to be more electron-dense (Fig. 8). Large numbers of granules are still embedded in the condensing chromosomes. These granules vary from 40 to 90 nm in diameter and seem to be surrounded by a clear halo. Occasionally, regions of varying density free of chromatin granules are observed and may represent condensed regions of the nucleolus. Even at the level of the light microscope, condensing chromosomes are quite markedly associated with the nuclear envelope (Fig. 9). Kinetochores with associated microtubules are now seen for the first time, often in the vicinity of the heterochromatic regions of the condensing chromosomes (Fig. 10). In the cytoplasm near the nuclear envelope microtubules emanate from large aggregations of moderately electron-dense material representing diffuse centriolar satellites or microtubule organizing centres (MTOC's) (Pickett-Heaps, 1971) (Figs. 8, 10). However, no centrioles have been observed in this material. The microtubules pass through breaks in the highly convoluted nuclear envelope into the nucleoplasm (Fig. 10). As nuclear envelope breakdown proceeds, the convolutions in the nuclear envelope are more pronounced, resulting in the formation of quadruple membrane complexes (Fig. 11) formed by the close apposition of the nucleoplasmic surfaces of 2 nuclear envelope segments. These segments of the NE eventually break down into cisternae indistinguishable from those

of the endoplasmic reticulum (Szollosi & Calarco, 1970; Szollosi, Calarco & Donahue, in preparation).

Near the completion of chromosome condensation, occurring close to 3 h in culture, the bivalents are V-shaped and telocentric. Often a portion of the condensed chromosome remains in contact with a fragment of the nuclear envelope. Condensation of chromosomes proceeds to the point where the heterochromatic portions of the chromosomes cannot be distinguished because of their uniform density. Dense chromatin granules are seen at the outer margins of the chromosomes. The nucleolus is no longer evident. Mitochondria are present in greatest numbers in the nuclear area although the time of numerical increase of mitochondria in this area is quite variable and is not related to any exact stage of chromatin condensation. It may begin as early as 2·5 h or not until after 6·5 h in culture. Clusters of membrane-bound dark bodies also occur in this central nuclear region of the oocyte (Fig. 11).

Typically after 3 h of culture the chromatin is circularly arranged, highly condensed, and still located in the middle of the egg. This circular orientation persists although the chromosomes have lost all connexions with the nuclear envelope, which now consists only of quadruple membrane complexes (Fig. 12).

The circular arrangement of the bivalents is altered during the movements of the chromosomes on the first meiotic spindle. The first ultrastructural indication of this is the wider V-shape of the bivalents. Two kinetochores are frequently seen on one homologue and they probably arise during chromosome condensation as one per chromatid (Calarco, 1971). During this time of kinetochore 'repulsion' no MTOC's are seen in the central 'nuclear' area.

Metaphase I (Fig. 13) is reached as early as 4·5 h in culture. The metaphase I spindle is barrel-shaped and remains centrally located within the oocyte. 'Poles' are formed by several patches of MTOC's distributed around the broad end of the spindle. The telocentric bivalents are co-oriented (Rieger, Michaelis & Green, 1968) on the metaphase spindle preparatory to the anaphase I separation of homologues. The aggregation of mitochondria around the spindle is quite marked and clusters of dense granules are also commonly interspersed with the mitochondria. No nuclear envelope complexes are seen.

Approximately 5 % of oocytes liberated into culture do not resume meiosis (Donahue, 1968). Four oocytes, apparently arrested at the germinal vesicle stage, have been examined. There were no obvious differences between these and other germinal vesicles that would account for their arrest. In 2 of these oocytes (13 and 17·5 h in culture) the germinal vesicle had migrated to the periphery of the oocyte.

Oocytes obtained from hormonally stimulated mice are in the germinal vesicle stage when examined shortly before HCG and up to 3 h after HCG injection. These GV oocytes are morphologically indistinguishable from those obtained from non-hormonally stimulated animals, and both groups behave similarly in culture, exhibiting well condensed chromosomes after 3 h, and metaphase II after longer culture periods.

Three effects possibly due to the hormonal treatments were observed. The 'stickiness' of the investing follicular cells was increased. Chromosome condensation may be

more rapid *in vivo*, requiring about 1 h instead of 3 h. In addition, there was some indication that the germinal vesicle or condensed chromosomes migrated to the periphery of the egg earlier, i.e. prior to anaphase.

DISCUSSION

The full-sized follicular oocytes obtained in the present study presumably correspond to the antral oocytes described by Oakberg (1968). In Oakberg's study the nucleoli of these oocytes showed no [^{3}H]uridine incorporation, suggesting that ribosomal RNA is not being synthesized. In the present study the nucleoli possess no granular component. The association of agranular nucleoli with the absence of [^{3}H]uridine incorporation has also been reported in early embryonic stages of amphibians (Karasaki, 1965; Hay & Gurdon, 1967) and mice (Hillman & Tasca, 1969). The passage of [^{3}H]uridine from the nucleolus to the cytoplasm in younger oocytes (Oakberg, 1968) may represent the ribosomes we observed in the cytoplasm.

The dissolution of nucleoli during GV breakdown involves incorporation of the intranuclear bodies composed of 25-nm diameter twisted fibrils. After the nucleolus has disappeared these fibrils are found free in the cytoplasm and can still be seen in metaphase II oocytes. It would be of interest to determine if nucleolar dissolution involves similar fibrils in cleavage stages of the mouse. Recently, 'coiled bodies' of similar dimensions have been reported in the nucleus of the growing mouse oocyte (Chouinard, 1970).

The chromatin granules seen in early condensation stages appear to be smaller and less distinct than in later stages. The later-stage granules are similar in structure to the perichromatin granules reported by Watson (1962) in mouse and rat cells but are generally larger, ranging up to 90 nm in diameter. Bloom (1970) has postulated 3 stages in mitotic chromatin condensation in *Ambystoma* somatic cells. The chromatin granules of the mouse oocyte are similar to Bloom's stage-3 granules, but there is no clear evidence available from our study for the relation of the granules to chromatin coiling.

The disappearance of nuclear pores occurs concomitantly with the initial stages of chromatin condensation, raising the possibility that non-condensed chromatin is necessary for pore integrity. No intermediate stages in the loss of nuclear pores have been observed.

Much of the chromatin condensation is observed along the nuclear envelope as might be expected if chromosomes are attached to the NE. Attachment of chromosomes to the NE has in fact been reported in mouse spermatocytes (Woolam, Millen & Ford, 1967) and various cell lines (Comings & Okada, 1970). By prometaphase, the chromosome-NE association is no longer observed in the present study.

The very dense regions of the condensing chromosomes are interpreted to be heterochromatin and quite probably are the regions of centromere heterochromatin. Pardue & Gall (1970) have shown that mouse satellite DNA will hybridize with chromocentres in interphase nuclei and with centromeric heterochromatin in mitosis and meiosis. In fact, centromeres first appear near these heterochromatic regions in the germinal vesicle (Calarco, 1971).

The membrane-bounded dark granules seen in the peripheral regions of the GV oocyte and in the central part of the egg during spindle formation may be similar to the osmiophilic bodies described in Hela cell mitosis (Robbins & Gonatas, 1964). In Hela cells these dark bodies are reportedly derived from multivesicular bodies and are acid-phosphatase positive. Their locations suggest their involvement in spindle formation and/or function. Similar structures ('dense bodies') are seen in growing mouse oocytes (Odor & Blandau, 1969).

The maturation division in the mouse occurs without the aid of centrioles. The intriguing formation of a functional meiotic spindle with 'satellite-like' material or MTOC's at the 2 poles is being presented in another paper (Szollosi, Calarco & Donahue, in preparation).

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REFERENCES

BLOOM, W. (1970). Electron microscopy of chromosomal changes in Ambystomal somatic cells during the mitotic cycle. *Anat. Rec.* **167**, 253–276.

CALARCO, P. (1971). The kinetochore in oocyte maturation. In *Oogenesis* (ed. J. D. Biggers & A. W. Schuetz), (in the Press). U.S. Government Printing Office.

CHOUINARD, L. A. (1970). The extranucleolar bodies of the growing oocyte in the prepubertal mouse. *J. Cell Biol.* **47**, 34A.

COMINGS, D. E. & OKADA, T. A. (1970). Association of nuclear membrane fragments with metaphase and anaphase chromosomes as observed by whole mount electron microscopy. *Expl Cell Res.* **63**, 62–68.

DONAHUE, R. (1968). Maturation of the mouse oocyte *in vitro*. I. Sequence and timing of nuclear progression. *J. exp. Zool.* **169**, 237–250.

DONAHUE, R. (1971). The relation of oocyte maturation to ovulation in mammals. In *Oogenesis* (ed. J. D. Biggers & A. W. Schuetz), (in the Press). U.S. Government Printing Office.

FOWLER, R. E. & EDWARDS, R. G. (1957). Induction of superovulation and pregnancy in mature mice by gonadotrophins. *J. Endocr.* **15**, 374–384.

HAY, E. D. & GURDON, J. B. (1967). Fine structure of the nucleolus in normal and mutant *Xenopus* embryos. *J. Cell Sci.* **2**, 151–162.

HILLMAN, N. & TASCA, R. J. (1969). Ultrastructural and autoradiographic studies of mouse cleavage stages. *Am. J. Anat.* **126**, 151–174.

KARASAKI, S. (1965). Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J. Cell Biol.* **26**, 937–958.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.* **9**, 409–414.

OAKBERG, E. F. (1968). Relationship between stage of follicular development and RNA synthesis in the mouse oocyte. *Mutation Res.* **6**, 155–165.

ODOR, D. L. & BLANDAU, R. J. (1969). Ultrastructural studies on fetal and early postnatal mouse ovaries. II. Cytodifferentiation. *Am. J. Anat.* **125**, 177–215.

PARDUE, M. L. & GALL, J. G. (1970). Chromosomal localization of mouse satellite DNA. *Science, N.Y.* **168**, 1356–1358.

PICKETT-HEAPS, J. (1971). The autonomy of the centriole: fact or fallacy? *Cytobios* **3**, 205–214.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208–212.

RICHARDSON, K. C., JARETT, L. & FINKE, E. H. (1960). Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**, 313–323.

RIEGER, R., MICHAELIS, A. & GREEN, M. M. (1968). *A Glossary of Genetics and Cytogenetics*. New York: Springer-Verlag.

ROBBINS, E. & GONATAS, N. K. (1964). The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* **21**, 429–463.

SOTELO, J. R. & PORTER, K. R. (1959). An electron microscope study of the rat ovum. *J. biophys. biochem. Cytol.* **5**, 327–342.

SZOLLOSI, D. (1971). Changes of some cell organelles during oogenesis in mammals. In *Oogenesis* (ed. J. D. Biggers & A. W. Schuetz), (in the Press). U.S. Government Printing Office.

SZOLLOSI, D. & CALARCO, P. (1970). Nuclear envelope breakdown and reutilization. *7th Int. Conf. Electron Microsc.*, Grenoble, pp. 275–276. Paris: Soc. Française de Microscopie Electronique.

WATSON, M. (1959). Staining of tissue sections for electron microscopy with heavy metals. *J. biophys. biochem. Cytol.* **4**, 475–478.

WATSON, M. (1962). Observations on a granule associated with chromatin in the nuclei of cells of rat and mouse. *J. Cell Biol.* **13**, 162–167.

WOOLAM, D. H. M., MILLEN, J. W. & FORD, E. H. R. (1967). Points of attachment of pachytene chromosomes to the nuclear membrane in mouse spermatocytes. *Nature, Lond.* **213**, 298–299.

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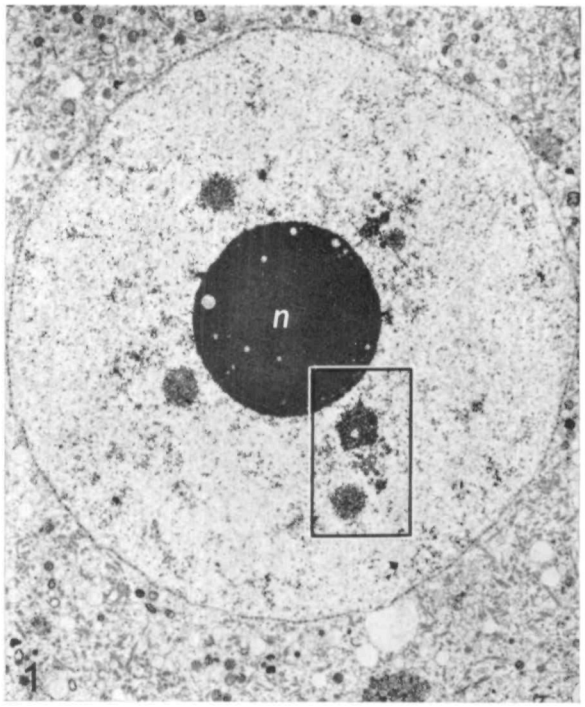
Fig. 1. Intact germinal vesicle immediately after release from an ovarian follicle. Note the nearly smooth nuclear envelope. Chromatin-like material is near the nucleolus (*n*). × 4300.

Fig. 2. Enlargement of area outlined in Fig. 1. Note the body of twisted fibrils (arrow). One 'chromatin' area (*c*) is composed of approximately 17-nm diameter granules. *n*, nucleolus. × 17200.

Fig. 3. Portion of a germinal vesicle after 0·5 h in culture. Pores are present in the slightly undulating nuclear envelope. The agranular nature of the nucleolus (*n*) is apparent. Chromatin granules (40–90 nm in diameter) are present (arrow). × 8740.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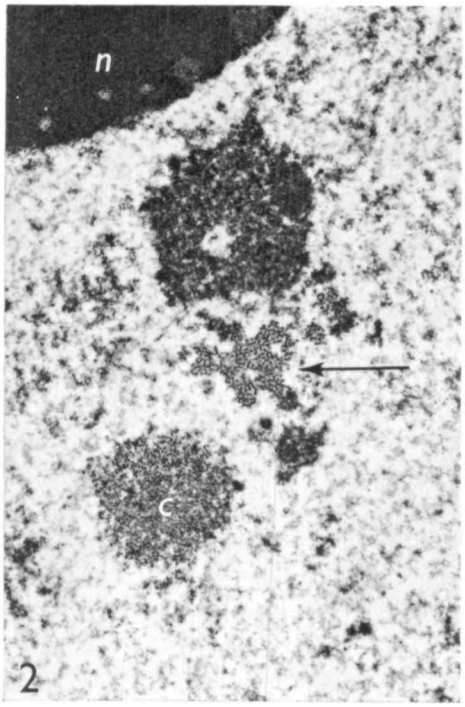
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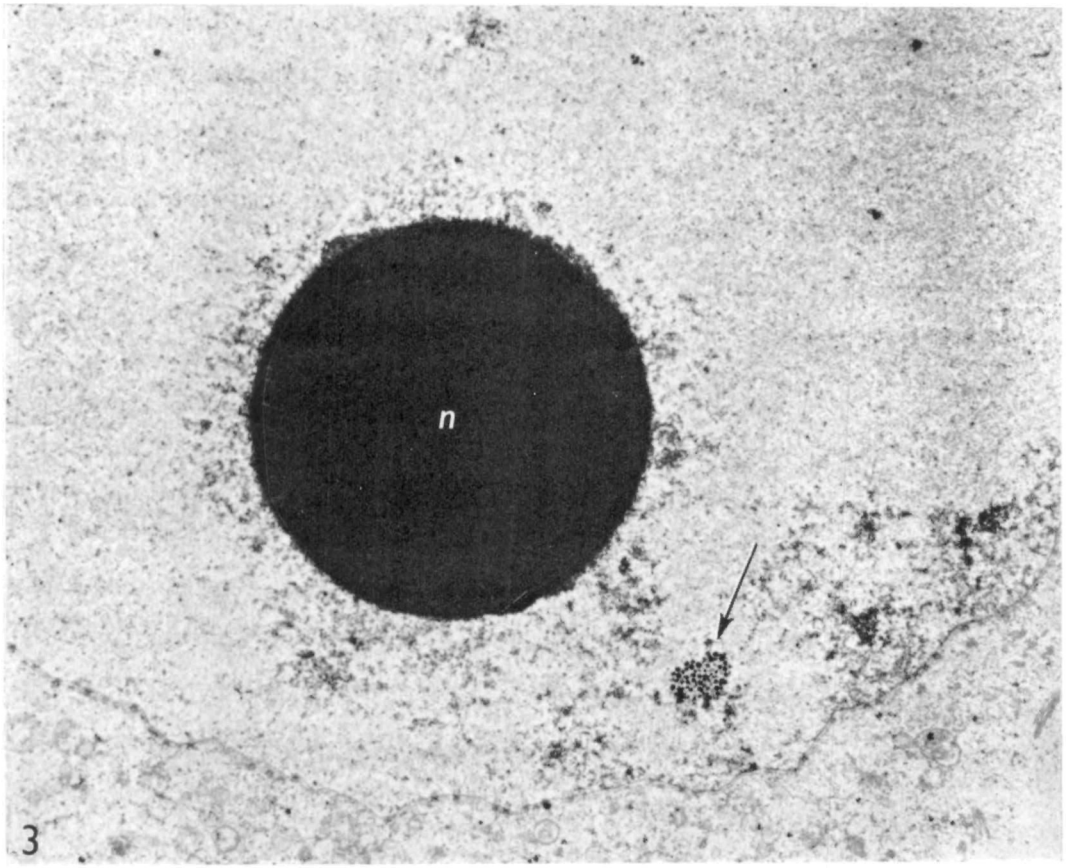
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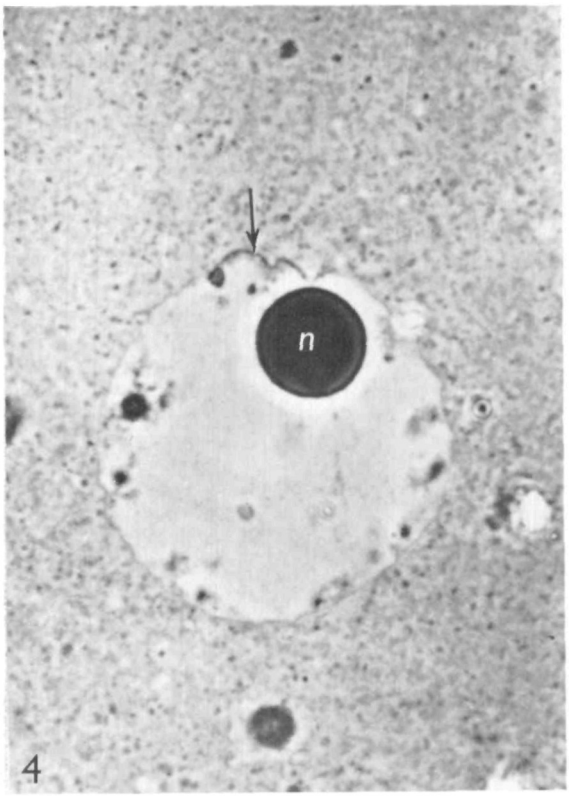
Fig. 4. A 2-μm section through an oocyte cultured for 30 min. The arrow indicates a region of chromatin condensation along the nuclear envelope. *n*, nucleolus. × 1600.

Fig. 5. A 2-μm section through an oocyte cultured 1·5 h. Note the condensing chromosomes within the germinal vesicle. × 1680.

Fig. 6. An electron micrograph of a thin section from the same oocyte shown in Fig. 5. The nuclear envelope (*ne*) now lacks pores. The condensing chromosomes (*ch*) exhibit many dense granules. The arrows indicate areas where twisted fibrils are peripherally incorporated into the nucleolus (*n*). *c*, chromatin area composed of approximately 17-nm diameter granules; *h*, heterochromatin. × 13270.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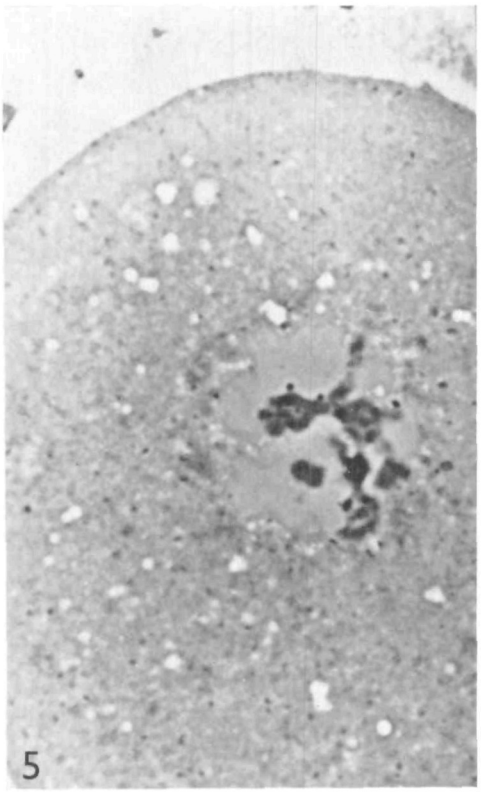
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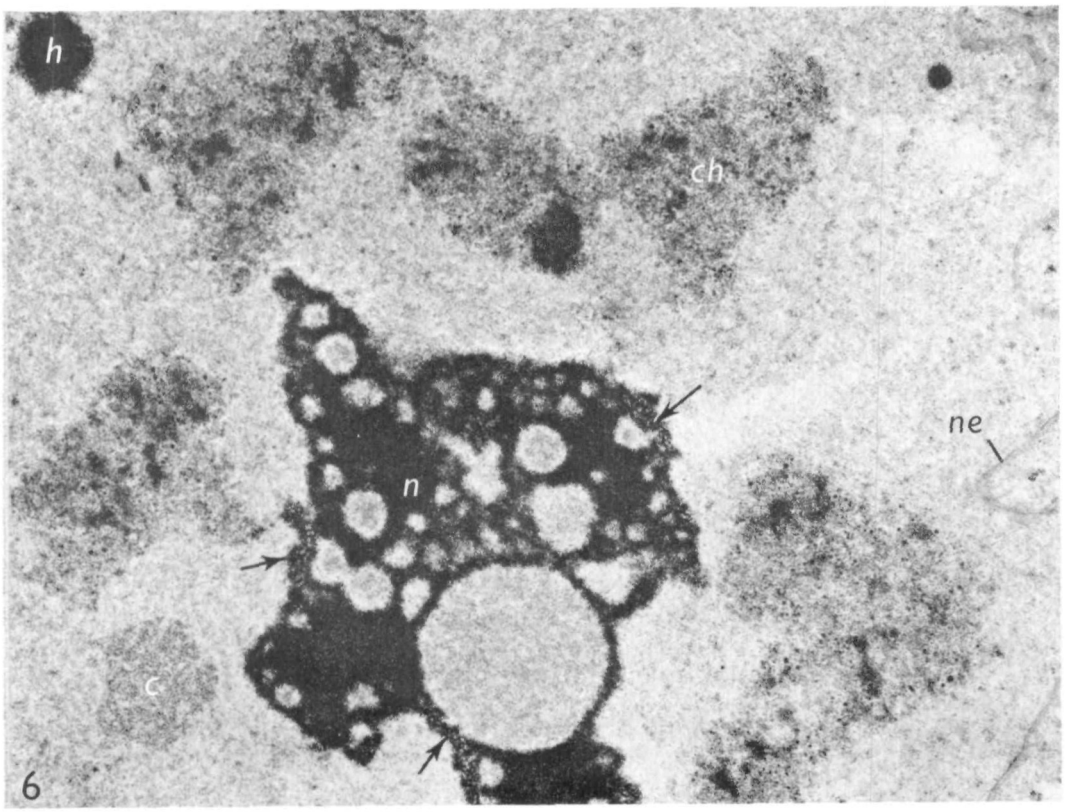
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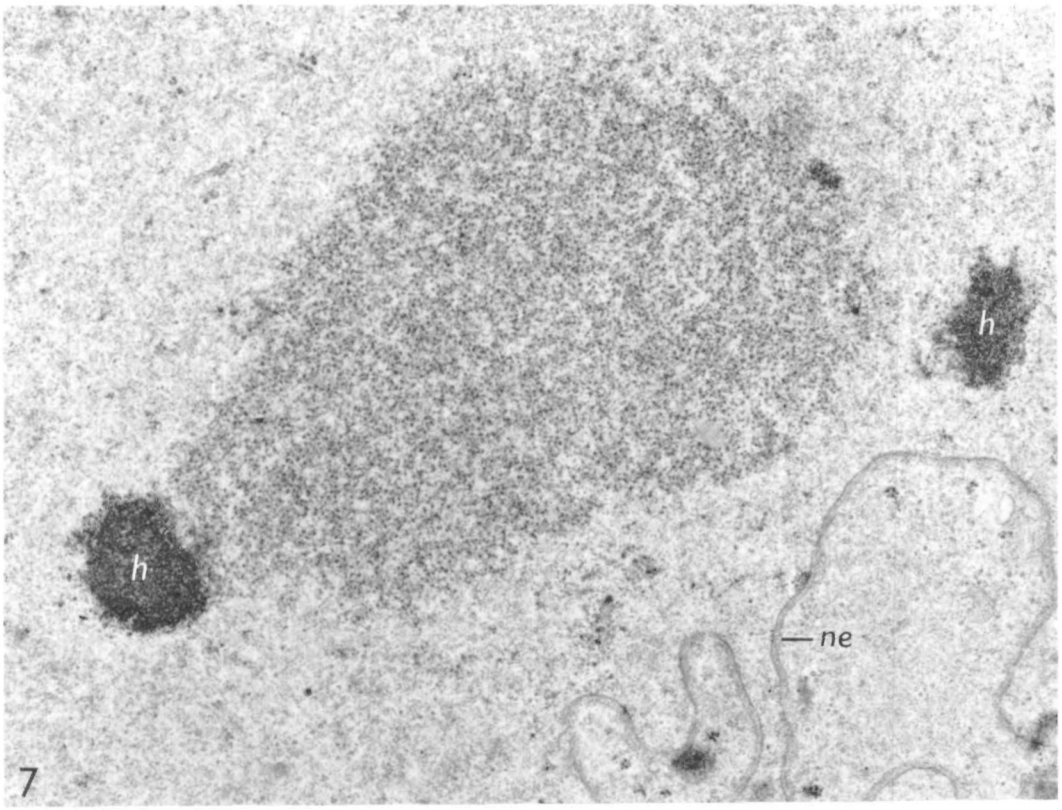
Fig. 7. A second type of intranuclear condensation associated with heterochromatin (*h*) from an oocyte cultured 1·5 h. Serial sections indicate that this body of granules, approximately 17 nm in diameter, is also contiguous with the heterochromatin at the right. Note the absence of pores in the nuclear envelope (*ne*). × 2280.

Fig. 8. At a slightly later stage in GV breakdown, the chromatin appears denser, and large (40–90 nm diameter) granules are embedded in it. The nuclear envelope is quite convoluted. The arrows denote microtubule organizing centres (MTOC). × 11 760.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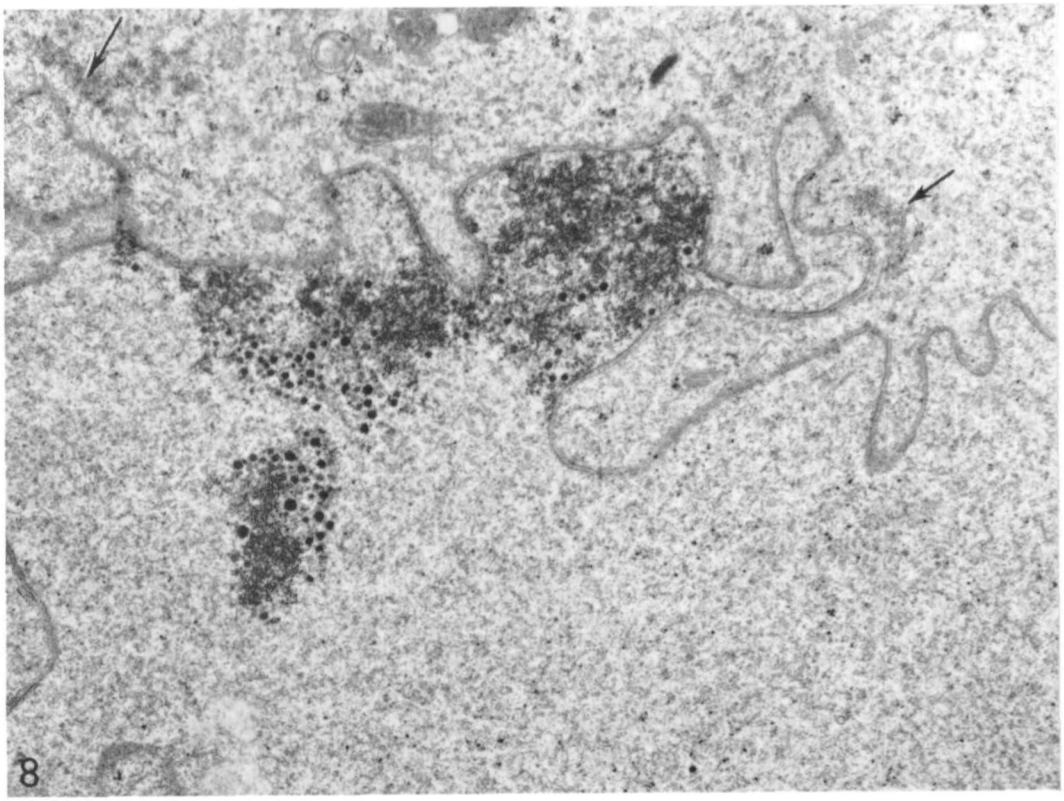
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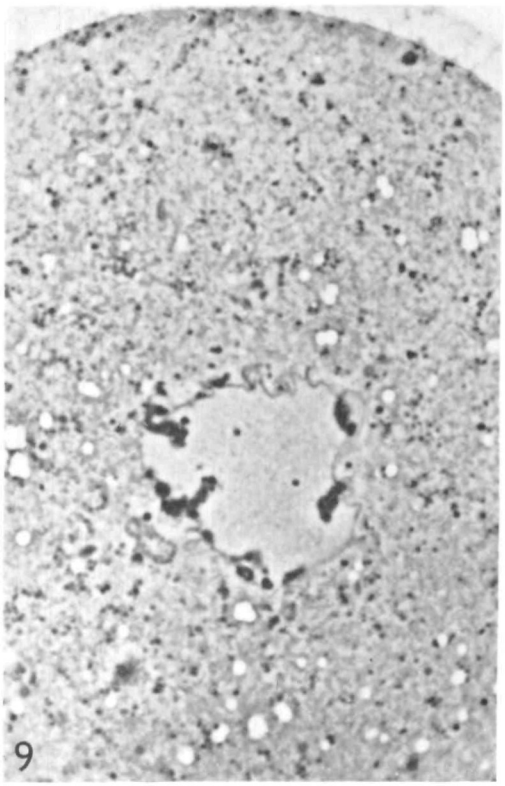
Fig. 9. A 2-μm section showing well defined chromosomes associated with the nuclear envelope. × 1600.

Fig. 10. A kinetochore (*k*) near an area of heterochromatin (*h*). Microtubules (arrows) are now seen within the germinal vesicle and crossing the nuclear envelope. *mtoc*, microtubule organizing centre. × 31 200.

Fig. 11. Nuclear region of an oocyte cultured 3·5 h. The chromosomes are highly condensed and exhibit occasional peripheral chromatin granules (arrows). *b*, body of twisted fibrils seen associated with the nucleolus in Fig. 6; *dg*, dense granules; *nec*, nuclear envelope complexes. × 13 270.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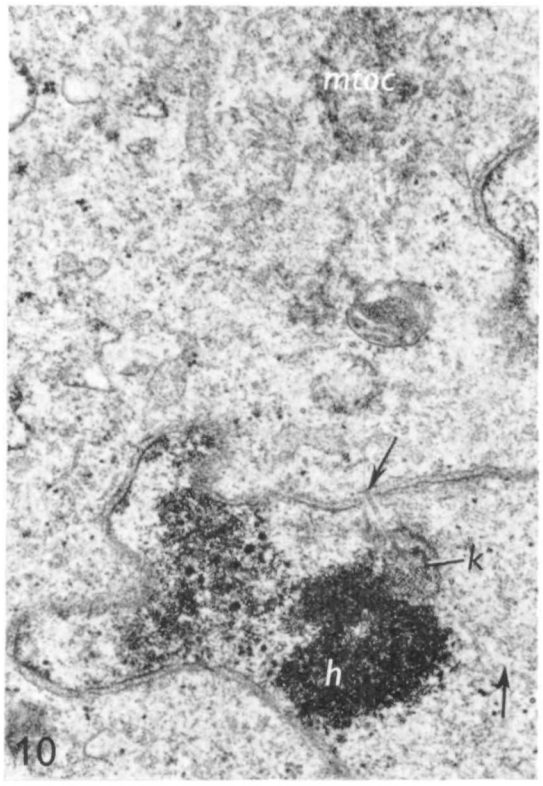
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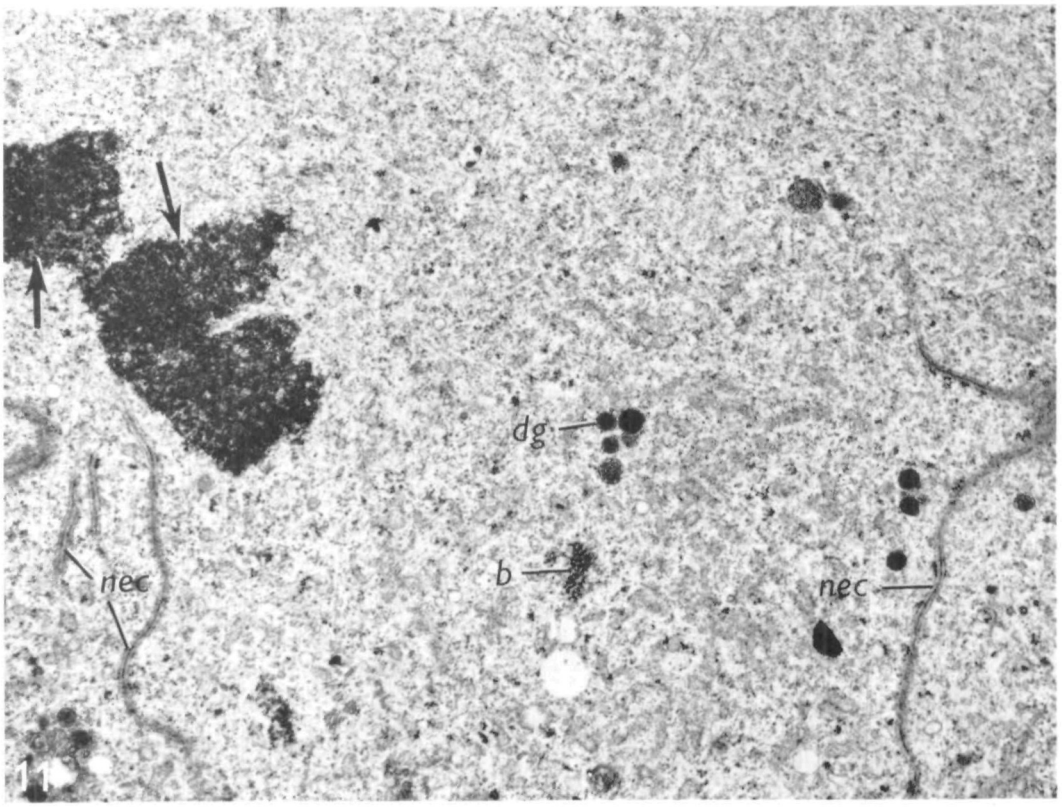
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b
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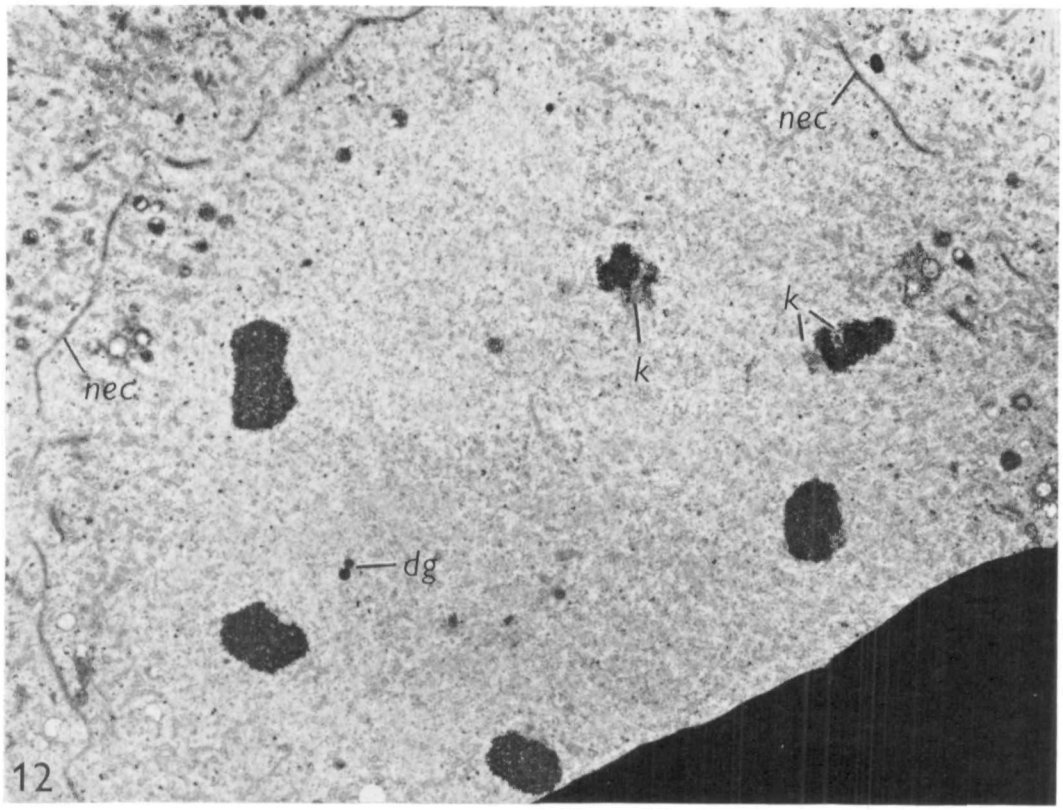
11

Fig. 12. The circularly arranged bivalent stage. Note the nuclear envelope complexes (*nec*). *dg*, dense granules; *k*, kinetochore. × 7310.

Fig. 13. Metaphase I. Note the mitochondria surrounding the spindle. *dg*, dense granules; *mtoc*, microtubule organizing centre. × 4560.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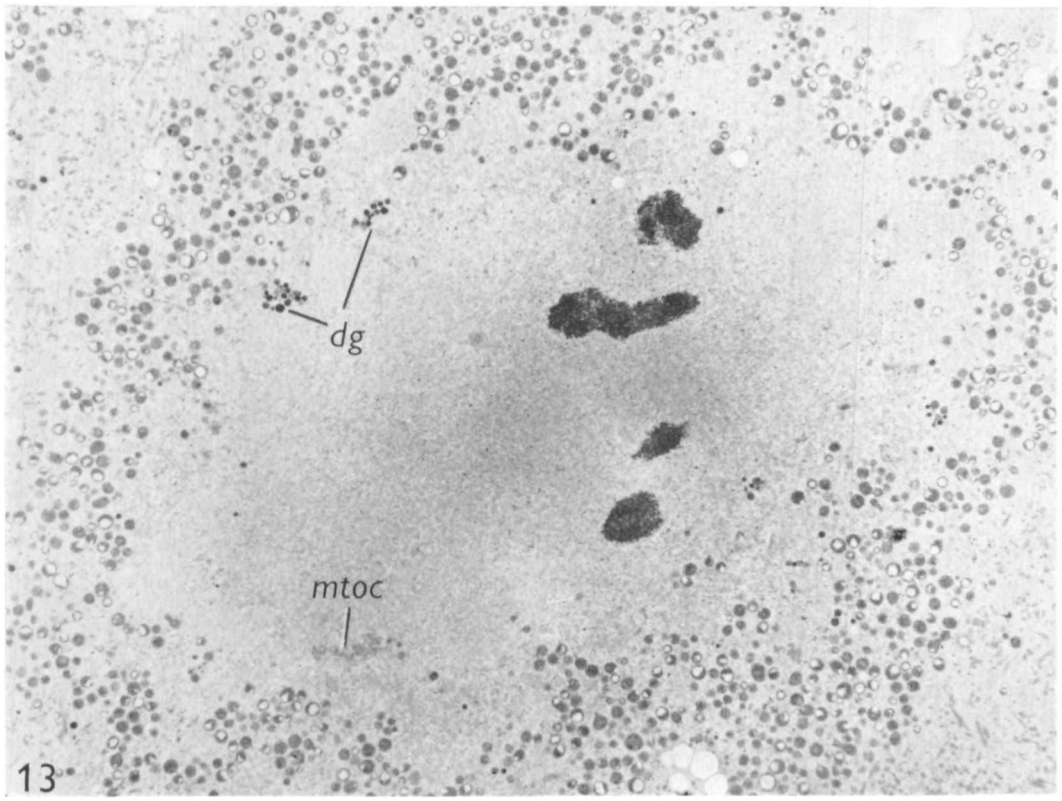
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