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Ultrastructural Observations of Human and Mouse Oocytes Treated with Cryopreservatives[1]

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ABSTRACT

The effects of the cryopreservative agents dimethylsulfoxide (DMSO) and propanediol (PROH) on mature human and mature mouse oocytes have been examined with transmission electron microscopy. Treatment of CD-1 mouse oocytes and human preovulatory oocytes in a stepwise manner with either DMSO or PROH up to 1.5 M appears to trigger the exocytosis of 70–80% of the cortical granules in all oocytes. Successive stages in premature dehiscence, including a loss in granule electron density, fusion of the granule-limiting membrane with the oolemma, and extrusion of the cortical granule core into the perivitelline space, have been observed in all human oocytes studied. In addition, all human DMSO- and PROH-treated oocytes exhibited crypt-like invaginations and clusters of endocytic vesicles that subtend the oolemma. The presence of these crypts and pinocytotic vesicles in treated oocytes may suggest a mechanism for the retrieval of cortical granule membrane that is inserted into the original plasmalemma during exocytosis. The paucity of cortical granules in treated mouse and human oocytes as it potentially relates to an impaired ability to elicit the cortical reaction at fertilization is discussed.

INTRODUCTION

The cryopreservation of human eggs and embryos is a technology that is quickly becoming integrated into many in vitro fertilization (IVF) programs world wide. The routine use of superovulation regimens in IVF necessitates the production of large numbers of oocytes and embryos to achieve high pregnancy rates. Since most IVF programs restrict the number of oocytes inseminated or embryos replaced in utero, a dilemma arises regarding the fate of the excess healthy eggs and embryos. While the post-thaw survival rate of embryos is generally much higher than that of oocytes, the freezing of excess oocytes may be a more attractive alternative in light of various legal, ethical, and religious objections (Trounson, 1986). Moreover, oocyte preservation could additionally provide a means for the storage of gametes in an oocyte donation program or of oocytes from women who face chemo or radiation therapy (van Eum et al., 1987).

That the cryopreservation of oocytes is feasible has been demonstrated by the successful survival and fertilization in vitro of cryopreserved oocytes of mammals such as the mouse (Tsunoda et al., 1976; Parkening and Chang, 1977; Whittingham, 1977; Wood, 1985), hamster (Selman and Anderson, 1975; Tsunoda et al., 1976; Fleming et al., 1979; Parkening and Chang, 1977; Chuong and Coulam, 1986), rat (Kasai et al., 1979), and rabbit (Tsunoda and Sugie, 1977; Al-Hasai et al., 1986; Diedrich et al., 1986). Moreover, Diedrich et al. (1986), using dimethylsulfoxide (DMSO), and Quinn et al. (1986), using propanediol (PROH), have achieved fertilization in vitro of cryopreserved human oocytes. To date, however, only two births have been reported from cryopreserved uninseminated human eggs (Chen, 1986; van Eum et al., 1987).

Considerable attention has been focused on the deleterious effects of low temperature on mammalian

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oocytes in light of the observed low post-thaw survival rate. The most serious effects appear to be the instability of the meiotic spindle, resulting in chromosomal abnormalities (Whittingham, 1977; Magistrini and Szollosi, 1980; Wood, 1985; Chen, 1986; Mohr, 1986; Glenister et al., 1987), a high incidence of polyspermy in cryopreserved oocytes fertilized in vitro (Whittingham, 1985; Wood, 1985; Glenister et al., 1987), and reduced fertilization rates (Tsunoda et al., 1976; Whittingham, 1977; Wood, 1958; Chuong and Coulam, 1986). However, an issue that remains to be addressed is that of the possible deleterious effects caused by the cryopreservative solutions themselves. A common assumption made by clinicians and scientists alike has been that routine cryoprotective agents, such as DMSO and PROH, cause minimal or no structural damage to oocytes. Since cryopreservation is a two-step process involving not only the exposure of oocytes to extremely low temperatures, but also the addition and removal of cryoprotectant, some assurance that the cryopreservative is nontoxic to oocytes would appear warranted. Moreover, the high incidence of structural and/or developmental abnormalities associated with cryopreserved oocytes reported elsewhere therefore may not be caused entirely by low temperature (Schalkoff et al., 1987).

The present study was undertaken to investigate any possible adverse effects that two routinely used cryopreservative agents, DMSO and PROH, may have on the fine structure of mature human and mouse oocytes. To separate cryopreservative and low temperature effects, unfrozen oocytes were subjected to a regimen of incremental addition and removal of cryopreservative mimicking that used with several freezing protocols.

Of particular interest in this study was the ultrastructural morphology of the cortical granules in cryopreservative-treated oocytes. Cortical granules are a common feature of the cortex of the oocytes of many species of mammals (Selman and Anderson, 1975; Nicosia et al., 1978; Schuel, 1978; Longo, 1985; Okada et al., 1986; Schalkoff and Powers, 1986; Szollosi et al., 1986) and function as a mechanical block to the entrance of supernumerary sperm at fertilization or following parthenogenetic activation. The induction of cortical granule exocytosis in mammalian oocytes (Whittingham and Siracusa, 1978) and in nonmammalian oocytes (Schalkoff and Hart, 1986) following parthenogenetic activation with calcium ionophores suggests that an increase in intracellular calcium may be the universal trigger for this secretory event. The failure of oocytes injected with calcium chelators such as [ethylenebis(oxyethylenedinitrilo)]tetraacetic acid (EGTA) to elicit this exocytotic response (Zucker and Steinhardt, 1978) would further support a concept of a calcium-induced granule release.

Cortical granule exocytosis typifies a merocrine type of secretion. Upon parthenogenetic activation or the binding of the initial spermatozoan with the oolemma, the cortical granules fuse with the oolemma, rupture, and release their contents into the perivitelline space (Schalkoff and Hart, 1986). As a result of fusion between the granules and oolemma, the limiting membrane of the granules is encorporated into the plasmalemma, forming a mosaic surface (Donovan and Hart, 1986). The subsequent induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs following exocytosis suggests that a component of the cortical granule exudate functions to block sperm-binding sites at the level of both the zona pellucida and the oolemma (Wolf and Hamada, 1977; Sathananthan and Trounson, 1982). The major glycoprotein of the zona, ZP3, has been shown to undergo a decrease in sperm-binding capacity by a trypsin-like protease identified in cortical granules (Gwatkin et al., 1973; Bleil and Wassarman, 1980a,b; Bleil and Wassarman, 1983). Furthermore, the inhibition of the cortical granule exudate-dependent decrease in sperm penetration in zona-intact eggs (Wolf and Hamada, 1977) and an increased incidence of polyspermy in oocytes incubated with soybean trypsin inhibitor (Gwatkin et al., 1973) would appear to strengthen the hypothesis that cortical granules play a critical role in insuring monospermic fertilization. In light of the increasing interest in cryopreservative technology in IVFand the well-documented role of cortical granules in preventing polyspermy, the objective of this study was to determine if exposure of mature oocytes to DMSO or PROH induced any ultrastructural changes.

MATERIALS AND METHODS

Ovulation Induction Protocol

A total of 31 oocytes was collected for this study from women undergoing the gamete intrafallopian transfer (GIFT) procedure for infertility treatment.

The ovulation induction protocol consisted of clomiphene citrate on Days 2–5 at a dosage of 50–100 mg/day (depending on body weight) followed by 150 IU human menopausal gonadotropin (HMG) starting ten days before the anticipated day of ovulation. HMG was continued until the two leading follicles were each 15.0 mm in diameter. Human chorionic gonatropin (hCG, 5000 IU) was then administered and oocytes were recovered via laparoscopy 36 h later. As a general rule, only mature oocytes (a maximum of 7) were placed into the oviduct(s) during each GIFT procedure; any excess mature oocytes were used for this ultrastructural study.

Prior to any manipulation of the excess healthy oocytes, their cumulus cells were removed by gently swirling the oocytes in a Falcon culture dish (Falcon Plastics, Los Angeles, CA) containing a modified Dulbecco's phosphate-buffered saline (PB1 medium) with 0.05% hyaluronidase on a warming pad for 2–3 min. After two rinses in hyaluronidase-free PB1, 7 oocytes were selected as controls and immersed immediately in fixative, and 24 were subjected to either the DMSO or PROH cryopreservative treatment described below.

The oocytes of virgin Swiss albino mice were used in a correlative study to examine any ultrastructural effect(s) of cryopreservatives. Six-to ten-week-old females were superovulated with i.p. injections of 10 IU pregnant mare's serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO) and 10 IU (hCG, Sigma), given 48 h apart. Approximately 18–20 h following hCG administration, the mice were killed by cervical dislocation. The oviducts were surgically removed and placed in Falcon culture dishes containing PB1 medium and hyaluronidase. Mature (polar body stage) oocytes were flushed from the oviducts and the cumulus cells were removed enzymatically. After two rinses with PB1 medium, the oocytes were either fixed immediately (for controls) or subjected to the same regimen of DMSO or PROH treatment as the human oocytes.

PB1 Medium and Cryopreservative Solutions

PB1 was used throughout these studies for the collection and rinsing of oocytes and for the dilution of DMSO and PROH. Previous studies have shown that PB1 is an optimal medium for the suspension of oocytes and embryos (Whittingham, 1974). The cryopreservative solutions were prepared by diluting tissue culture grade DMSO (Sigma) and 1, 2-propylene glycol (propanediol; Sigma) with PB1 medium at pH 7.4 to give final concentrations of 0.5 M, 1.0 M, and 1.5 M. To minimize any osmotic stress to the oocytes and to insure proper infiltration of the cryopreservative, DMSO and PROH solutions were added at room temperature in a stepwise manner as follows: 0.5 M (5 min), 1.0 M (5 min), 1.5 M (20 min), 1.0 M with 0.2 M sucrose (5 min), 0.5 M with 0.2 M sucrose (5 min), fixative.

Preparation of Oocytes for Transmission Electron Microscopy

Control and treated human and mouse oocytes were fixed at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer with 4% sucrose and 0.5% tannic acid at pH 7.4 for 90 min. Oocytes were washed twice for 10 min each in 0.1 M cacodylate buffer with sucrose and postfixed in 1% OsO_4 in cacodylate buffer with sucrose for 1 h. After two buffer rinses, oocytes were dehydrated in a graded series of ethanol and infiltrated over a 2-h period with 1:3, 1:1, and 3:1 mixtures of Polybed 812-Araldite epoxy resin:acetone (Polysciences, Inc., Warrington, PA). Each oocyte was placed in an individual beem capsule with fresh 100% Polybed 812-Araldite resin and polymerized for 48 h at 65°C. Gold and silver ultrathin sections were cut with a Dupont diamond knife (Delaware Diamond Knives, Wilmington, DE) on an LKB ultramicrotome (LKB, Rockville, MD) and picked up on carbon-coated, formvar-covered slot grids (Polaron, Cambridge, MA). Sections were stained for 5 h with freshly prepared 3% uranyl acetate and for 90 min with Reynolds lead citrate (Reynolds, 1963) and viewed with a Phillips 200 transmission electron microscope operated at 60 kV.

Cortical Granule Counts and Statistical Analysis

Cortical granule counts were made with a 7× ocular lens from 35 mm photographic negatives of treated and control mouse and human oocytes. Five areas from each of 3 sections of treated and control oocytes were used to determine mean cortical granule numbers in this study. Each of these areas (fields of view) were photographed at the same magnification. Cortical granule counts in control and treated oocytes were analyzed statistically using an unpaired *t*-test at a significance level of 0.05%.

RESULTS

Human preovulatory oocytes in this study measured 100–125 μm in diameter and were characterized by a well-defined zona pellucida and perivitelline space. Transmission electron microscopy revealed that the oolemmal surface was organized into numerous, finger-like folds, the microvilli. These projections measured 0.1–0.3 μm in diameter and approximately 0.6 μm in length.

The cortical ooplasm of human preovulatory oocytes contained a large population of intact cortical granules (Fig. 1). Quantitative analysis of these cortices indicated there were 17.02 ± 3.46 (mean ± SEM) cortical granules per field of view. Each cortical granule consisted of a homogeneous, electron-dense core; a well-defined limiting membrane surrounded some of the cores (Fig. 1a), whereas the membrane was obscured around other cores (Fig. 1b). Granules measured 250–500 nm in diameter and were arranged as a single layer (Fig. 1a) or as double layers (Fig. 1b) beneath the oolemma. Cortical granules within the ooplasm were located between the microvilli beneath flattened patches of the oolemma. The distance between the inner face of the oolemma and the outer aspect of the cortical granule membrane was approximately 50 nm. This gap was not measurable in some granules, because they appeared to contact the oolemma (Fig. 1b). The premature release of granule contents was seen in only 1 of the 7 control oocytes in this study.

The cortical ooplasm of human preovulatory oocytes contained other organelles in addition to cortical granules. Hypertrophied dictyosomes, swollen cisternae of the Golgi complex, which in concert with the endoplasmic reticulum form the granules, were observed in all oocytes. Clusters of mitochondria surrounded the dictyosomes in the cortex (Fig. 1a). Other cortical organelles included smooth and rough endoplasmic reticulum, coated pits, coated vesicles, and occasional microtubules.

The meiotic spindle of human oocytes assumed a peripheral position within the ooplasm and consisted of several distinct chromosomes and associated microtubules (Fig. 2). The ooplasm immediately surrounding the spindle was devoid of organelles (asterisks, Fig. 2). The polar body typically contained mitochondria, microtubules, and aggregated chromatin that was not organized into a spindle.

The cortical region of preovulatory oocytes overlying the second meiotic spindle was particularly abundant in cortical granules. Quantitative analysis showed there were 41 ± 2.92 cortical granules per field of view, and these organelles were arranged in 4–5 densely packed layers (Fig. 2). The delicate microvillous projections into which the oolemma was folded elsewhere in the cortex were replaced by thickened, ridge-like extensions in this region. Cortical granules were noted in these extensions as well as in the periphery of the polar body.

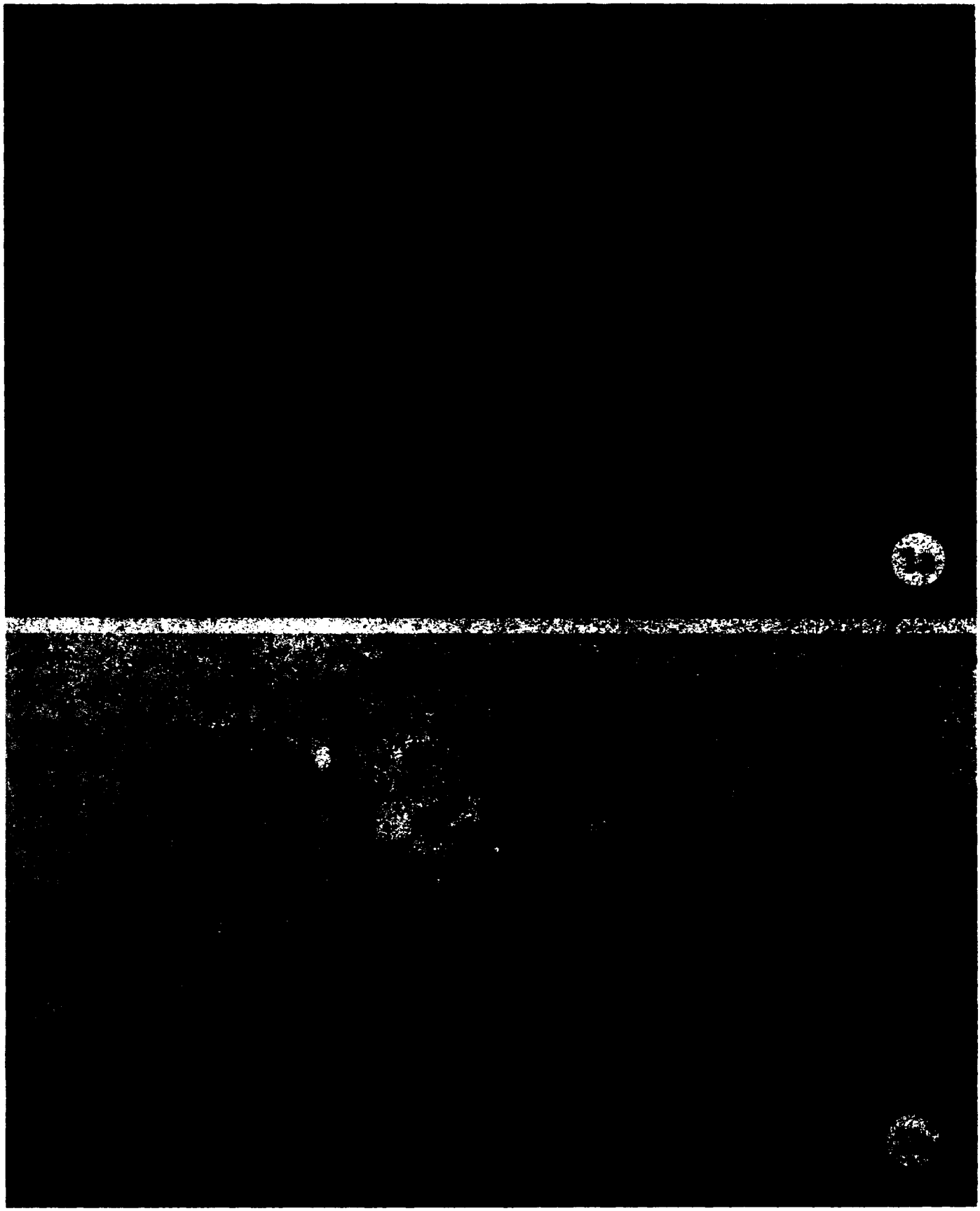
Transmission electron microscopy revealed that the cryopreservative treatment of human oocytes did not appear to affect the structural integrity of the meiotic spindle (Fig. 3, inset). Distinct chromosomes and associated microtubules were observed in all oocytes exposed to DMSO or PROH. The spindle was located in the peripheral ooplasm and was characterized by a surrounding area devoid of organelles. A normal complement of organelles was present elsewhere in the cortex in all oocytes.

The cortical ooplasm of human preovulatory oocytes exposed to DMSO or PROH contained few cortical granules. A striking contrast in granule number relative to controls was seen in the cortical region overlying the meiotic spindle (Fig. 3). This area contained only 20% of the complement of cortical granules in control oocytes (8 ± 1.23). In addition, other areas of the cortex not overlying the meiotic spindle contained only 4.64 ± 1.88 granules per field of view. These granule complements in treated oocytes were statistically different from those of control oocytes at a level of 0.05% ($p = 0.0005$).

An additional striking ultrastructural feature of all DMSO- and PROH-treated oocytes in this study was a premature exocytosis of cortical granules, both in the region overlying the spindle (Fig. 3) and elsewhere in the cortex (Figs. 4 and 5). Fluffy cortical granule exudate and electron-dense granule cores were often observed adjacent to the oolemmal surface or within the perivitelline space in all oocytes.

Three successive stages in the premature dehiscence of cortical granules were noted in human cryopreservative-treated oocytes (Fig. 5). The initial step appeared to be a loss in the homogeneity of the

FIG. 1. Cortical ooplasm of a human preovulatory oocyte. Cortical granules *(CG)* lie subjacent to the oolemma *(OL)* and are arranged in single *(a)* or multiple *(b)* layers. Hypertrophied dictyosomes of the Golgi apparatus *(arrows, a)* in close association with mitochondria *(M)* are a common feature of the preovulatory oocyte cortex. *ZP*, zona pellucida; *MV*, microvilli; *OO*, ooplasm. (*a*, ×22,875; *b*, ×18,960).



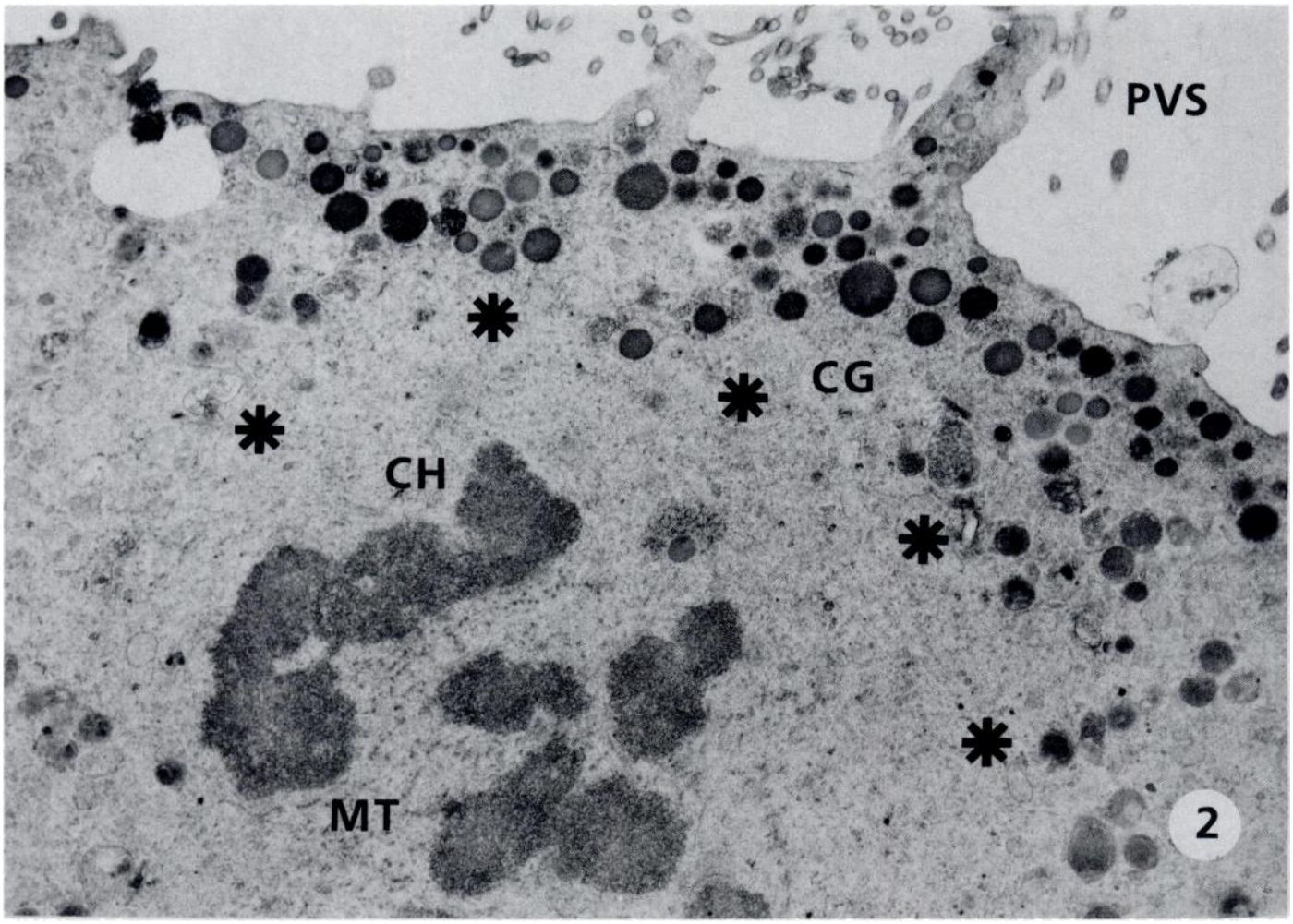


FIG. 2. Cortical ooplasm of a human preovulatory oocyte overlying the second meiotic spindle. This region is characterized by the presence of 4–5 rows of densely packed cortical granules and an area immediately surrounding the chromosomes *(CH)* that is devoid of organelles (*). *MT*, microtubules; *PVS*, perivitelline space. (×17,845.)

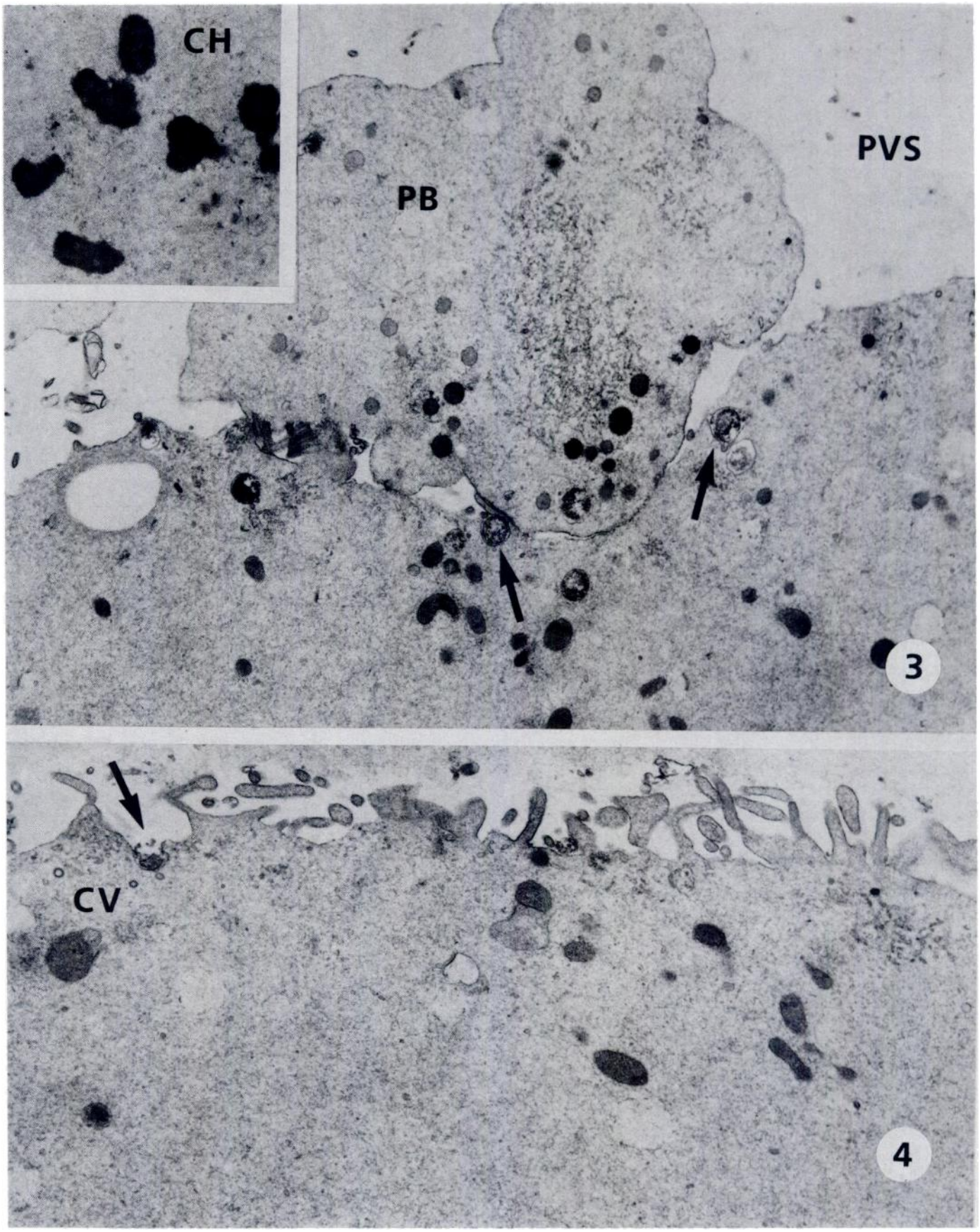
contents of the granule core (Fig. 5a). Cortical granules appeared electron-lucent and contained mottled, fluffy material. Fusion of the cortical granule-limiting membrane with the oolemma (Fig. 5b) and the subsequent opening of the cortical granule and exocytosis of the granule core into the perivitelline space followed (Fig. 5c). The dehiscence of cortical granule contents often occurred between microvilli (Fig. 5, b and c), suggesting that like control oocytes, cortical granules in cryopreservative-treated oocytes were situated beneath flattened patches of the oolemma.

Two other notable ultrastructural features common to the cortex of all DMSO- and PROH-treated human oocytes were clusters of pinocytotic vesicles and large, cavernous crypts (Figs. 5–8). Crypts and pinocytotic vesicular aggregates were seen throughout the cortex, including the area overlying the meiotic spindle. These clusters were observed immediately beneath the oolemma and were occasionally seen at

PLATE I.

FIG. 3. Cortical region and polar body of a human preovulatory oocyte exposed to dimethylsulfoxide (DMSO). The ooplasm overlying the meiotic spindle contains few cortical granules, and many of the granules appear to be undergoing a premature exocytosis of their contents *(arrows)*. The chromosomes *(CH)* and associated microtubules of the spindle of DMSO- or propanediol-treated human oocytes appear normal *(inset)*. (×15,675; *inset*, ×8870.)

FIG. 4. Typical surface of a DMSO-treated human oocyte not overlying the meiotic spindle. Relative to controls, the cortical region of cryopreservative-treated oocytes contains few intact cortical granules. Cortical granule exudate is frequently observed at the oolemmal surface *(arrow)*. *CV*, coated vesicles. (×19,620.)



the base of or within the microvilli (Figs. 5 and 6). A close approximation of pinocytotic vesicles (Fig. 5, b and c) and cortical crypts (Fig. 6) with recently exocytosed granules was also noted. Crypts were seen both in close proximity to the oolemma (Fig. 6a) or more internal (Fig. 6b), and frequently contained flocculent material. The crypts measured 0.5–2.0 μm in diameter and were membrane-delineated (Fig. 6b).

Cortical crypts exhibited clustering within the cortex of cryopreservative-treated oocytes (Figs. 7a and b). Crypts in close contact with the oolemma sometimes appeared fused, forming more irregularly shaped cavities (Fig. 7c). An additional morphological modification of crypts appeared to be the budding of the limiting membrane. Clusters of vesicles originating from the crypt membrane (Fig. 7d) and swirl-like, concentric profiles of membrane (Fig. 7e) were observed in 50–75% of the oocytes. A well-defined, electron-dense band of amorphous material, possibly microfilamentous in composition, was additionally observed around 50% of the crypts (Fig. 8).

In a correlative study on the ultrastructural effects of these cryopreservatives, CD-1 mouse oocytes were exposed to an incremental addition and removal of either PROH or DMSO. The mature (polar body stage) mouse oocytes in this study measured 65–70 μm in diameter and displayed a well-defined zona pellucida and perivitelline space (Fig. 9). The oolemma was thrown into numerous microvillous folds, which measured 0.4 μm in height and approximately 0.08–0.1 μm in diameter.

Cortical granules of control oocytes were arranged in a single layer immediately subjacent to the oolemma, and were separated from it by a distance of approximately 40–50 nm. The cortices of oocytes not overlying the meiotic spindle contained 16.67 ± 1.53 cortical granules per measured field of view. These cortical granules measured 200–350 nm in diameter and appeared more heterogenous in composition than the cortical granules of human oocytes; some cortical granule cores appeared uniformly electron-dense, whereas other contained lighter, more flocculent material. A limiting membrane was usually detected around the granules. The cortical ooplasm immediately overlying the meiotic spindle did not contain cortical granules. Like human preovulatory oocytes, hypertrophied dictyosomes of the Golgi complex were a common feature of the mouse oocyte cortex (Fig. 9). Other cortical organelles included yolk platelets, mitochondria, lysosomes, smooth and rough endoplasmic reticulum, microtubules, and concentric lamellar bodies. The premature exocytosis of cortical granules was not observed in any control mouse oocytes.

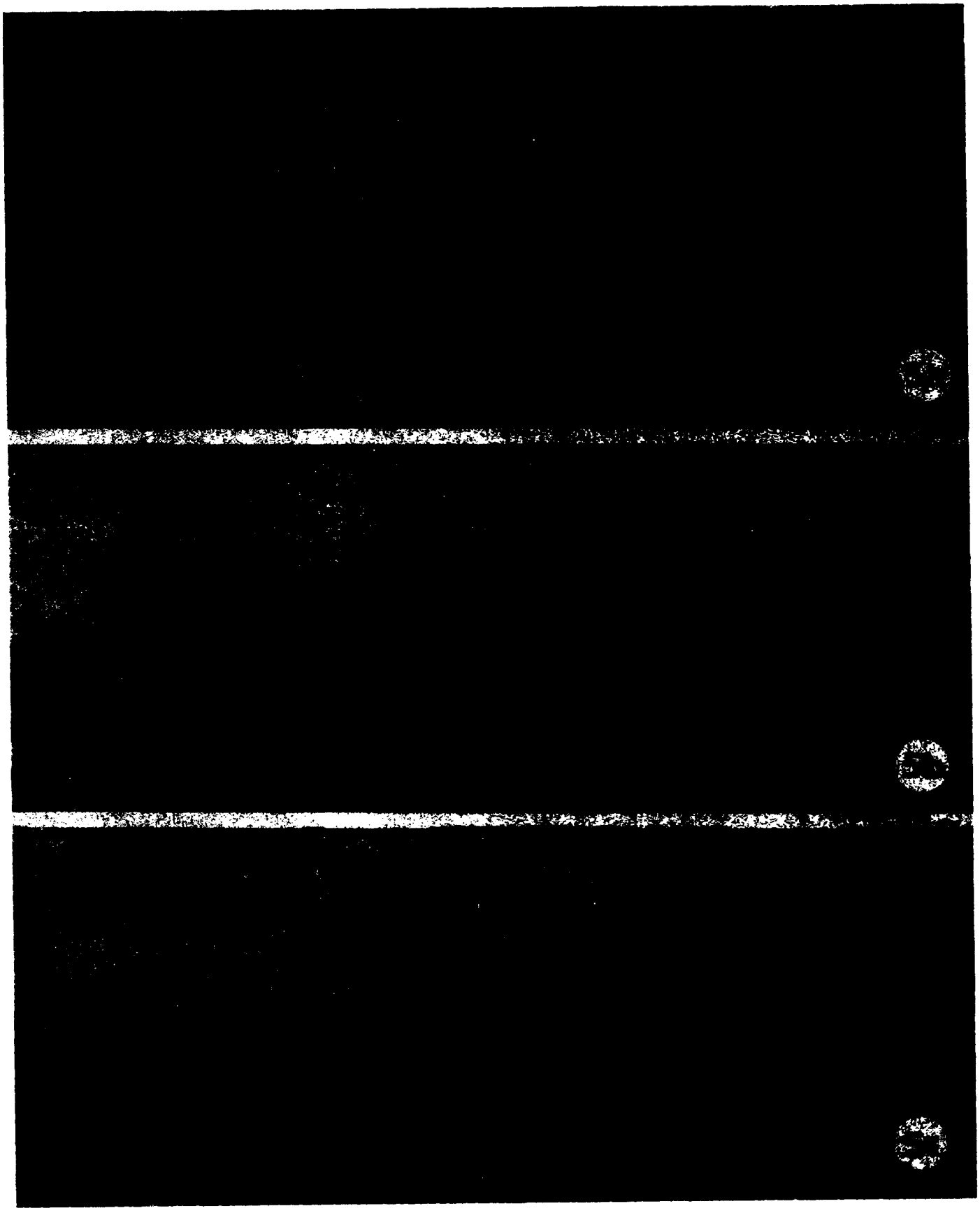
Relative to controls, the cortex of DMSO- or PROH-treated mouse oocytes contained few intact cortical granules (Fig. 10). Each measured field of view in treated oocytes contained 1.82 ± 1.53 granules. This value was statistically different from that of control oocytes at a level of 0.05%. In addition, premature cortical granule dehiscence was noted in 100% of the cryopreservative-treated mouse oocytes in this study. Unlike human oocytes treated in this manner, however, successive stages in cortical granule dehiscence were not seen. Rather, only the last stage in dehiscence, the presence of cortical granule cores at the oocyte surface, was observed. Exocytosed cortical granule cores appeared heterogenous in composition and were usually found adherent to flattened patches of the oolemma. A further contrast in the ultrastructure of human and mouse oocytes treated with DMSO or PROH was the absence of clusters of pinocytotic vesicles and cortical crypts in the latter; these putative endocytotic mechanism were not observed in any of the treated mouse oocytes.

The meiotic spindle of treated mouse oocytes, like that of treated human oocytes, appeared morphologically normal. Distinct chromosomes and associated microtubules within the peripheral ooplasm were always observed. In addition, normal dictyosomes, as well as the complement of other cortical organelles, were always noted in the cortical region.

DISCUSSION

Our ultrastructural studies indicate that control human preovulatory oocytes and polar body stage CD-1 mouse oocytes contained an abundance of intact cortical granules immediately subjacent to the

FIG. 5. Successive stages in the premature exocytosis of cortical granules in dimethylsulfoxide (DMSO)- or propanediol (PROH)-treated human oocytes. *a)* Cortical granule contents appear more heterogenous *(arrows)*. (×19,855). *b)* Granule membranes fuse with the oolemmal surface *(arrow)*. (×16,330.) *c)* Cortical granule cores are extruded into the perivitelline space *(arrow)*. (×17,750.) Note the abundance of clusters of pinocytotic vesicles *(PV)* that subtend the oolemmal surface in *a–c*.



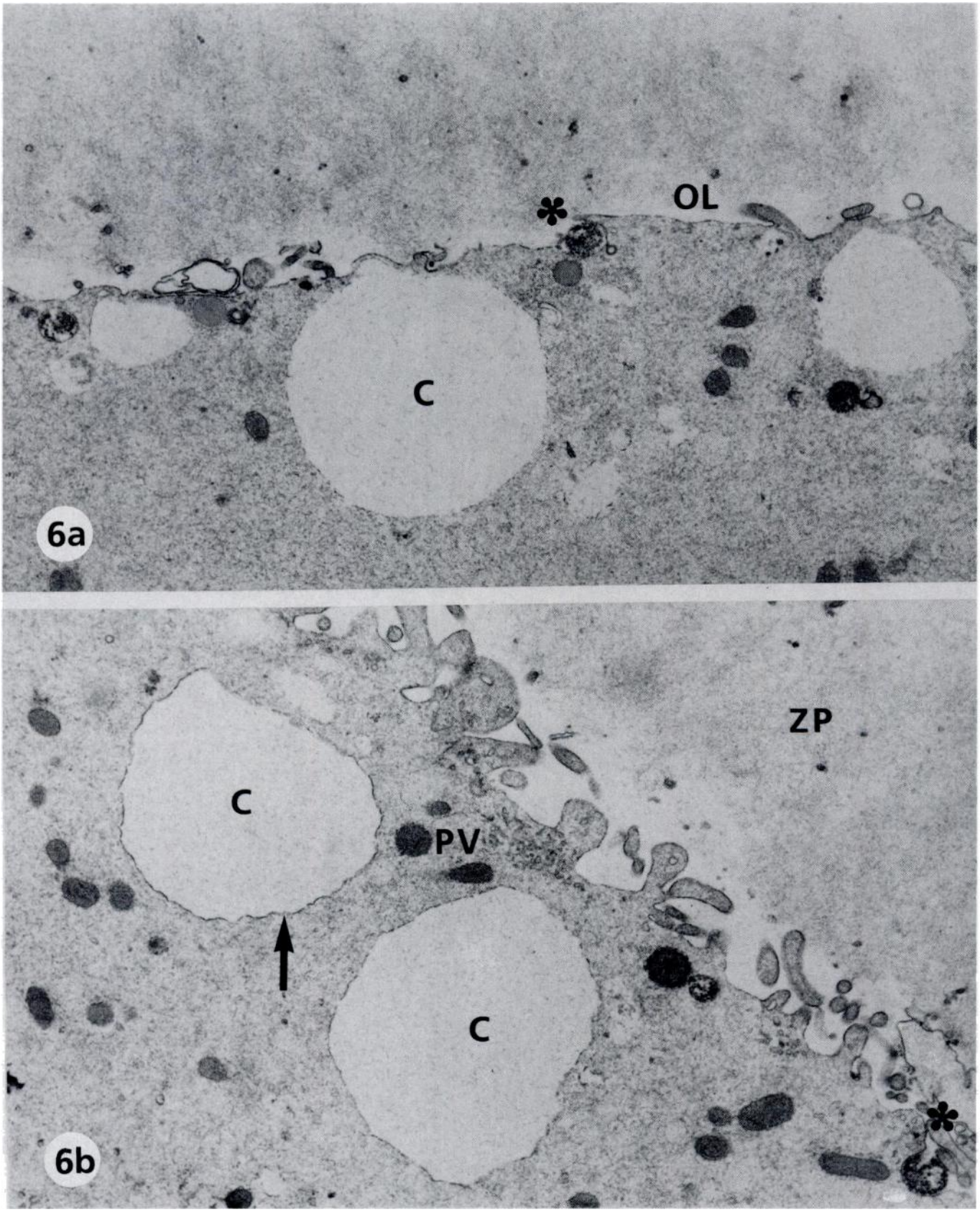


FIG. 6. Large cortical crypts *(C)* characteristic of cryopreservative-treated human oocytes. The crypts appear either in close association with the oolemma *(a)* or more internal *(b)* and frequently contain flocculent material. A well-defined limiting membrane is usually seen *PV*, pinocytotic vesicles; *OL*, oolemma; *, premature exocytosis of granules *(arrow, b)*. (*a*, ×23,200; *b*, ×22,675.)

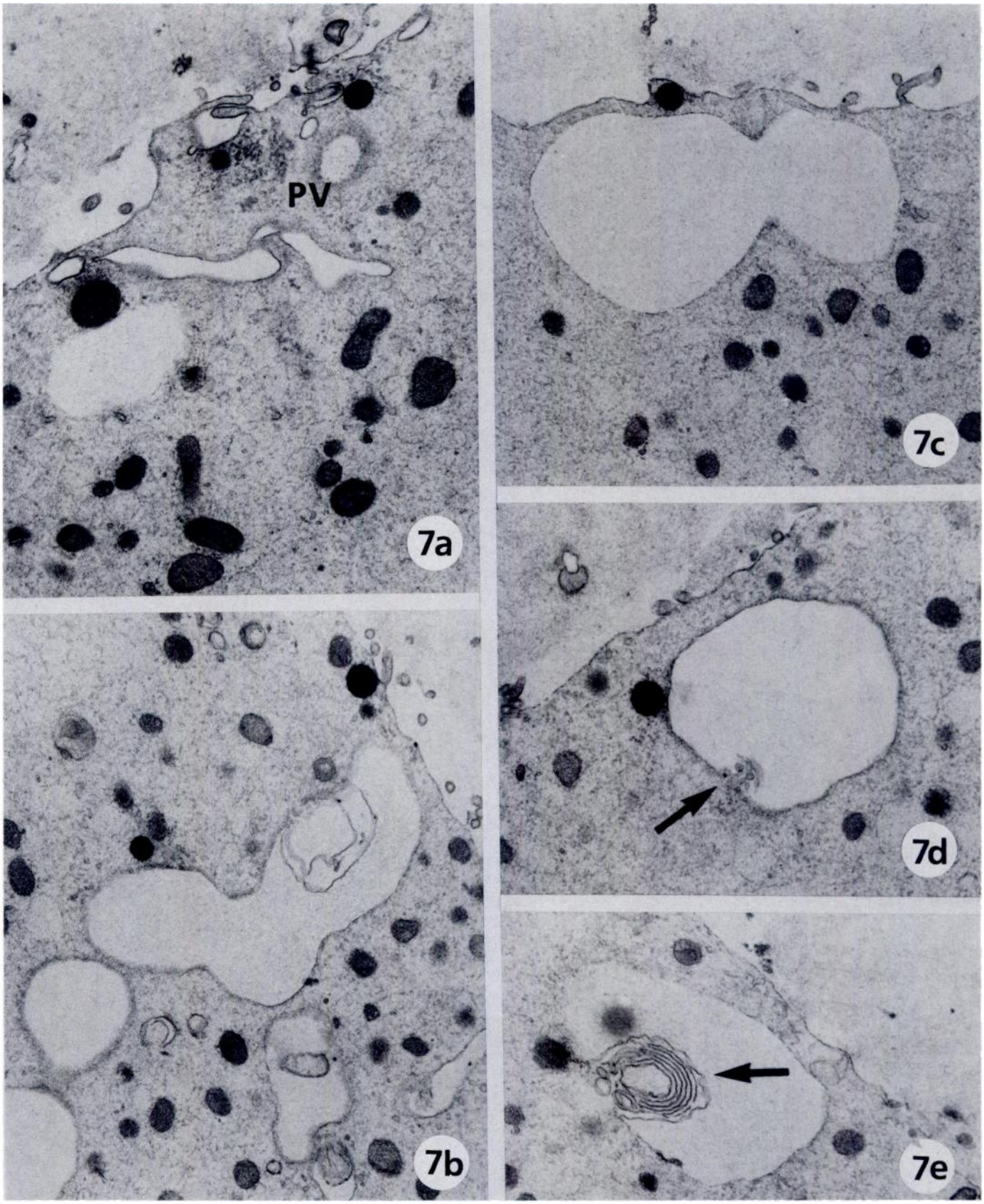


FIG. 7. Features of cortical crypts in cryopreservative-treated human oocytes. *a* and *b)* Crypts sometimes appear in clusters, often close to pinocytotic vesicles *(PV, a)*. *c)* The apparent fusing of neighboring crypts is common. *d* and *e)* Budding of the limiting membrane of the crypts results in the formation of small vesicles *(arrow, d)* or swirl-like profiles *(arrow, e)*. (*a*, ×19,720; *b*, ×18,365; *c*, ×17,035; *d*, ×21,750; *e*, ×23,200.)

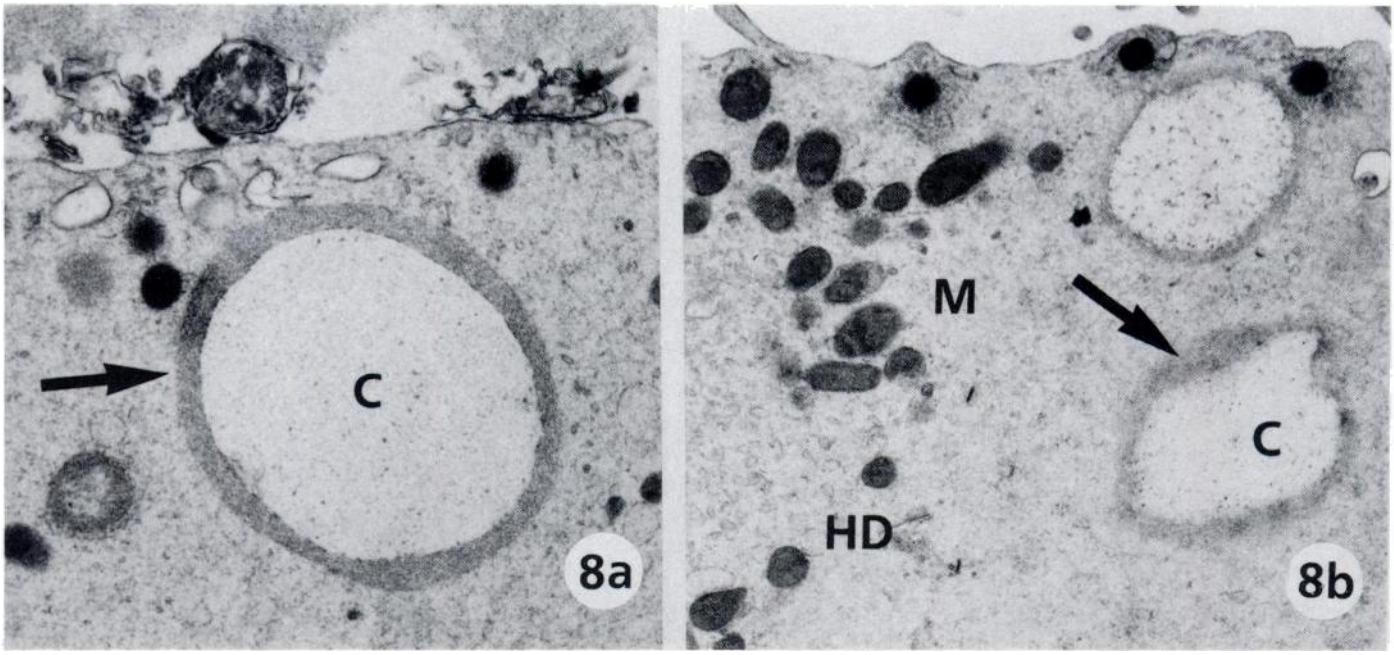


FIG. 8. Further modifications of cortical crypts. A well-defined, electron-dense layer surrounds some of the crypts *(arrows)*. *HD*, hypertrophied dictyosome: *M*, mitochondria. (*a*, ×19,210; *b* ×16,820.)

oolemma. Of the ten mouse and seven human control oocytes examined in this study, only one human oocyte displayed a premature exocytosis of cortical granules. Although the reason for this isolated case of premature dehiscence is unclear, it may be related to the mechanical handling of the oocyte or the enzymatic removal of its cumulus cells. The absence of premature cortical granule exocytosis in the remaining human preovulatory oocytes is in agreement with the earlier ultrastructural studies on human oocytes by Baca and Zamboni (1967), Zamboni et al. (1972), Suzuki et al., (1981), and Santhananthan and Trounson (1982).

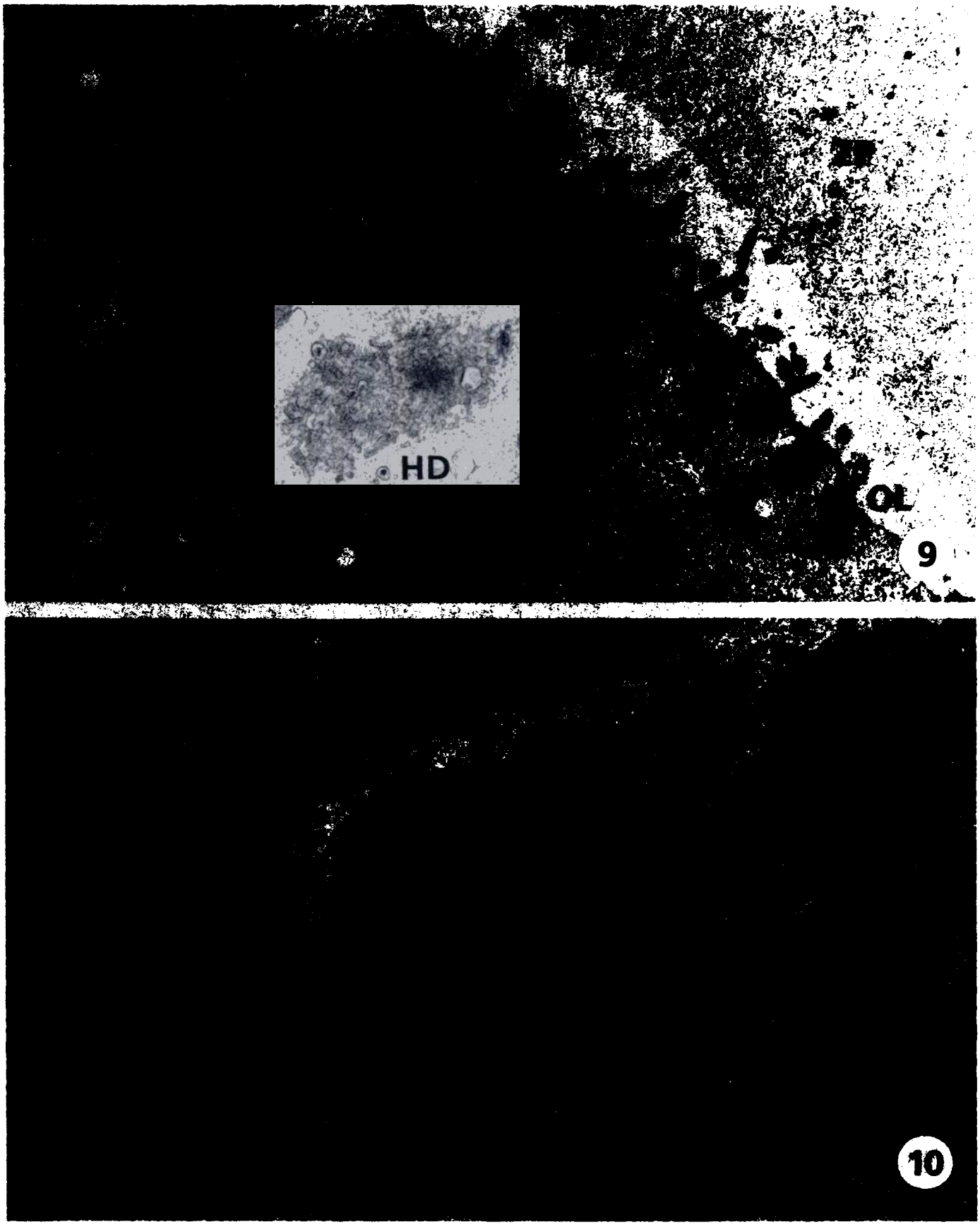
By contrast, all mouse and human oocytes treated with the cryopreservative agents contained few intact cortical granules. Statistical comparisons of cortical granule complements from control and treated oocytes indicated that these values were consistently different at a level of 0.05%. Moreover, human DMSO- or PROH-treated oocytes showed successive stages in the cortical reaction, including a loss in granule electron density, fusion of the granule-limiting membrane with the oolemma, and release of the granule contents into the extracellular space. The observation in mouse oocytes of only the final step in exocytosis, the appearance of granule exudate on the oocyte surface, may reflect a more explosive or faster granule dehiscence upon exposure to the cryopreservatives. Taken together, these data demonstrate that the incremental addition and removal of DMSO or PROH induces profound ultrastructural alterations in mature oocytes.

An additional ultrastructural feature of all cryopreservative-treated human preovulatory oocytes was the presence of large, membrane-limited crypts and clusters of pinocytotic vesicles that subtended the oolemma. The absence of these structures in all control human oocytes suggests that they may represent a restructuring of the oolemmal surface brought about by the premature exocytosis of granules in treated oocytes. A well-documented

PLATE II.

FIG. 9. Cortical ooplasm of a polar body stage mouse oocyte. Numerous cortical granules *(CG)* subjacent to the oolemma *(OL)* and hypertrophied dictyosomes *(HD)* are common features. (×25,240.)

FIG. 10. Cortex of a mouse oocyte subjected to the dimethylsulfoxide (DMSO) treatment regimen. Note the paucity of cortical granules in the cortex and the presence of normal hypertrophied dictyosomes *(HD)*. Exocytosed cortical granule cores are frequently seen at the oocyte surface *(arrow)*. The meiotic spindle and associated microtubules of *DMSO*- or propanediol-treated mouse oocytes appears morphologically normal (not shown). (×24,165.)



phenomenon is the coupling of endocytosis with the exocytosis of secretory granules in various somatic cell types (for reviews, see Steinman et al., 1983; and Willingham and Pastan, 1984) as well as following cortical granule exocytosis in oocytes (Schroeder, 1979; Rebhun et al., 1982; Fisher and Rebhun, 1983; Carron and Longo, 1984; Donovan and Hart, 1986; Sardet and Chang, 1987). Using externally administered fluid phase and adsorptive markers, Fisher and Rebhun (1983) and Carron and Longo (1984) demonstrated that following fertilization and exocytosis, reorganization of the sea urchin egg surface takes place through the internalization of membrane via coated pits, coated vesicles, and large, clear vesicles. As suggested by Donovan and Hart (1986) in the teleost egg, the retrieval of membrane following a secretory burst could therefore serve to maintain a relatively constant cell size. Moreover, a previous ultrastructural report by Sathananthan and Trounson (1985) indicates marked pinocytotic activity at the surface of the fertilized human oocyte. Since the exocytosis of 70–80% of the cortical granules in human cryopreservative-treated oocytes produces a substantial addition of perigranular membrane to the oolemma, the presence of crypts and pinocytotic vesicles may represent a retrieval mechanism used for surface reorganization and remodeling. The observed modifications of the crypt limiting membrane into vesicles (Fig. 7d) and concentric swirl-like profiles (Fig. 7e) might additionally serve to compact the endocytosed membrane further prior to its final processing within the oocyte. In that some granules fused prior to their exocytosis, the large endocytic crypts may represent the accomodation of several fused perigranular membranes, whereas the smaller pinocytotic vesicles may result from the internalization of single patches of perigranular membrane. Moreover, the microfilament-like band surrounding come of the crypts (Fig. 8) may facilitate the resorption of large pits of perigranular membrane. Since microfilaments have previously been observed in human oocytes (Lopata et al., 1980) and appear to be a general requirement for phagocytosis and fluid-phase endocytosis (Wagner et al., 1972; Stossel and Pollard, 1973; Axline and Reaven, 1974; Hartwig and Stossel, 1975), their association with crypts and their apparent role in membrane internalization is not surprising.

In summary, we have found that two cryopreservative agents, DMSO and PROH, can induce profound ultrastructural modifications in mature mouse and mature human oocytes, the most significant of which appears to be the premature exocytosis of cortical granules. As noted earlier, an increase in cytosolic calcium in mammalian oocytes is reported to be a universal trigger for the exocytosis of cortical granules (Whittingham and Siracusa, 1978). A similar secretory process involving the fusion of synaptic vesicles with the surface of the nerve terminal and liberation of neurotransmitter is also known to be triggered by an intracellular calcium rise. This event is mimicked by DMSO and another cryopreservative, polyethyleneglycol (Geron and Meiri, 1985). It has also been demonstrated that the action of DMSO promotes the fusion of membranes by increasing intracellular calcium (Ahkong et al., 1975). It may be possible, therefore, that DMSO and PROH triggered the exocytosis observed in the present study by stimulating a transient calcium rise.

There are no reports of ultrastructural observations on frozen, thawed, and fertilized oocytes. We cannot be certain, therefore, that the reported decrease in the overall fertilization rate is caused by the premature exocytosis of cortical granules that we observed. However, our observations are entirely consistent with these reports and suggest a probable mechanism Moreover, our study provides structural evidence to conclude that DMSO and PROH, as used in the current protocols, are unsatisfactory cryopreservatives for unfertilized mammalian oocytes.

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