LUTEINIZING HORMONE-ACCELERATED REDISTRIBUTION OF LYSOSOME-LIKE ORGANELLES PRECEDING DISSOLUTION OF THE NUCLEAR ENVELOPE IN RAT OOCYTES MATURING IN VITRO

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ABSTRACT

Maturation of the mammalian oocyte is characterized in part by dissolution of the nuclear envelope, or germinal vesicle breakdown (GVB). By fluorescence microscopy after vital uptake of acridine orange (AO), redistribution and perinuclear accumulation of organelles corresponding to lysosomes occur before GVB in rat oocytes undergoing meiotic maturation in vitro. In follicle-enclosed oocytes explanted during the preovulatory gonadotropin surge (GS) and individually cultured as such in chemically defined medium at -22°C, lysosomes aggregated into disperse clusters after 30 min; by 60 min, perinuclear concentration of lysosomes and their essential disappearance from the cortical ooplasm were observed. GVB occurred within 120 min. In contrast, follicle-enclosed oocytes explanted before the GS displayed a generally homogeneous distribution of lysosomes and an intact GV for up to 5 h in culture.

In oocytes aspirated from follicles before the GS, partially denuded of granulosa cells, and cultivated without added hormone, most lysosomes concentrated around the GV within 60 min, with GVB occurring generally by 120 min. Luteinizing hormone (LH) added in vitro to the isolated preparation at 3 or 30 × 10^-8 M sharply accelerated these events. The effects of LH, not seen with 1.5 × 10^-8 M hormone, were blocked by anti-LH IgG. Up to 60 × 10^-8 M follicle-stimulating hormone or 80 × 10^-8 M prolactin were ineffective in accelerating lysosome redistribution or GVB.

After GVB, lysosomes became once again uniformly dispersed and unresponsive, even to 60 × 10^-8 M added LH, a finding consistent with tachyphylaxis of target cells by independent criteria. The present data, all statistically significant at P < 0.05, demonstrate that mobilization of lysosomes before GVB is a specific response to factors that promote resumption of meiotic maturation of rat oocytes.

KEY WORDS rat oocytes · meiotic maturation · nuclear envelope · luteinizing hormone · lysosome translocation · acridine orange · fluorescence microscopy

Maturation of the mammalian oocyte is arrested at the diplotene stage of the first meiotic division. Meiosis resumes before ovulation upon exposure to appropriate hormone levels and proceeds to...
metaphase of the second meiotic division, remaining at this stage until fertilization or parthenogenetic activation takes place (44). Dissolution of the nuclear envelope, or germinal vesicle breakdown (GVB), is an early and unequivocal indicator of resumed meiotic maturation.

Mammalian oocytes mature 'spontaneously' when removed from their ovarian follicles, partially demuded of follicular cells, and cultured in vitro without hormonal additives (18, 22, 41). For oocytes retained within intact follicles that had been explanted and cultured in vitro, interruption of meiotic arrest requires prior exposure in vivo to oocytes retained within intact follicles that had been explanted and cultured in vitro without hormonal additives (18, 22, 41). For oocytes retained within intact follicles that had been explanted and cultured in vitro, interruption of meiotic arrest requires prior exposure in vivo to preovulatory levels of endogenous gonadotropins or addition of these hormones to the incubation medium (27, 58). This approach minimizes disturbance of the physical relationships between the ovum and surrounding follicular cells whose processes penetrate the zona pellucida, forming tight intercellular junctions at the oocyte surface (4, 63). However, the complex system of cells and their secretory products enveloping the follicular oocyte precludes direct microscopic observation of events leading to GVB in the living state. Therefore, it is also advantageous to study isolated oocytes, partially divested of follicle cells, maturing in vitro under defined conditions.

Previous studies in our laboratory have shown by Nomarski interference cinemicrography that isolated rat oocytes maturing in vitro in hormone-free medium exhibited perinuclear accumulation of organelles before GVB (28). In the same study, GVB was accelerated in the presence of luteinizing hormone (LH). Moreover, in a preliminary report we demonstrated, by fluorescence microscopy of oocytes vitally stained with acridine orange (AO), that the organelles which assume a perinuclear position before the onset of GVB possess the dimensions and metachromatic fluorescence of lysosomes (20). In the present investigation, we examine the redistribution of such organelles in oocytes maturing in vitro under two sets of conditions: (a) immediately upon aspiration from intact follicles that had been explanted before and during the preovulatory gonadotropin surge and cultured in chemically defined hormone-free medium, and (b) isolated oocytes, partially divested of follicular cells and incubated in chemically defined medium, with or without added LH, follicle-stimulating hormone (FSH), or prolactin (PRL). In selected experiments, parallel observations were also made by Nomarski interference-contrast microscopy (NIC).

**MATERIALS AND METHODS**

**Preparation of Oocytes**

Sprague-Dawley female rats, 3–5 mo of age, were maintained five per cage in a temperature- and light-controlled room with illumination between 0500 and 1900 each day. Regularity and stages of estrous cycles were confirmed by daily vaginal smears for three consecutive cycles before experimental use. Approx. 70% of these animals exhibited regular 4-d cycles and 30% had regular 5-d cycles. Within the same strain maintained at corresponding temperature and lighting conditions in this institution (11), onset of the preovulatory LH surge occurred between 1400 and 1500 on the afternoon of proestrus, with maximal values between 1600 and 1800 h for 4-d cyclic rats (11; cf. also references 6 and 46). In 5-d cyclic series the corresponding peak may take place slightly earlier (46). Timing of sacrifice in the present experiments led to results that were consistent with predicted stage of the estrous cycle.

Media for incubations were either Earle's balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) or mammalian Ringer's, the latter with the following constituents (mM): NaCl, 136.9; KCl, 2.7; Na2HPO4, 6.5; KH2PO4, 1.5; CaCl2, 0.7; and MgCl2, 0.6. Both media, buffered at pH 7.4, contained 0.1% each of glucose and high-purity albumin (Pentex, Miles Laboratories, Inc., Elkhart, Ind.). No differences in results were noted between experiments conducted in either medium, which is hereafter termed chemically defined medium (CDM). Hormonal additives, where indicated, included LH, FSH, and PRL.1

For culture of follicle-enveloped oocytes, intact preovulatory follicles were isolated by microdissection from ovaries of lightly etherized rats chosen at specific times of their estrous cycle. After a rinse in fresh medium, the intact follicles were cultured in sterile 60-mm tissue-culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for up to 5 h at room temperature (~22°C). Preliminary work revealed no striking differences between experiments conducted at this temperature and those carried out at 37°C. Nevertheless, 22°C was selected for the experiments presently reported to promote stability of membrane systems that may be labilized nonspecifically at the customary 37°C (cf. reference 40).

To isolate oocytes, cultured or freshly explanted follicles of ~1-mm diam were punctured with a sharp scalpel, releasing the oocyte and attached follicle cells into the medium. The oocyte was then transferred to fresh medium with a 10-μl capillary pipette. A sufficient portion of the surrounding follicular cells was removed immediately upon aspiration from intact follicles that had been explanted before and during the preovulatory gonadotropin surge and cultured in chemically defined hormone-free medium, and isolated oocytes, partially divested of follicular cells and incubated in chemically defined medium, with or without added LH, follicle-stimulating hormone (FSH), or prolactin (PRL). In selected experiments, parallel observations were also made by Nomarski interference-contrast microscopy (NIC).

1 LH (NIH-LH-S19-ovine), FSH (NIH-FSH-S9-ovine), and PRL (NIH-P-S-10-ovine) were all gifts from the National Institute of Arthritis, Metabolic, and Digestive Diseases, National Institutes of Health, United States Public Health Service.
Preparation and Characterization of IgG

Either of oocytes from follicular culture without exogenous hormone or of isolated oocytes with or without added hormone and incubated individually for times to be specified in a moist chamber at \(-22^\circ\text{C}\). Thereafter, 7 \(\mu\)l of a freshly prepared solution of 100 \(\mu\)g/ml AO (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) were added to give a final concentration of \(-40 \mu\text{g/ml}\). Two 200-\(\mu\text{m}\) nylon fibers were then placed at either side of the oocyte to support a No. 1 coverslip. The preparation was immediately examined with a Leitz Ortholux fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) using Ploem vertical illumination (excitation 490 nm, barrier 510 nm). Color photomicrographs were taken on Kodak Ektachrome 35-mm film (160 or 200 ASA). Upon vital uptake of AO, lysosomes fluoresce intense red-orange, in contrast to green orthofluorescent cytoplasm and nucleus (1, 2, 65). Because of rapid quenching of the characteristic fluorescence properties of AO, it was essential to observe and photograph the preparation within 1 min after initial exposure to ultraviolet radiation.

Alternatively, fresh, unstained oocytes were examined by NIC, which permitted continuous observation of the living cell over a period of several hours. Half-tone photomicrographs were taken on Kodak Tri-X Pan 35-mm film (400 ASA).

Experimental Protocols and Quantitation of Observations

From the paired ovaries of a rat in the desired phase of the estrous cycle, an average of eight oocytes was isolated. At this time, the oocyte was examined with phase-contrast optics to ascertain that manifest damage had not occurred during isolation and to confirm an intact nuclear envelope. This whole procedure was accomplished within 10 min.

The oocyte was then transferred in \(-2 \mu\text{l}\) of medium to a slide containing a 15-\(\mu\text{l}\) drop of medium with or without added hormone and incubated individually for times to be specified in a moist chamber at \(-22^\circ\text{C}\). Thereafter, 7 \(\mu\text{l}\) of a freshly prepared solution of 100 \(\mu\)g/ml AO (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) were added to give a final concentration of \(-40 \mu\text{g/ml}\). Two 200-\(\mu\text{m}\) nylon fibers were then placed at either side of the oocyte to support a No. 1 coverslip. The preparation was immediately examined with a Leitz Ortholux fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) using Ploem vertical illumination (excitation 490 nm, barrier 510 nm). Color photomicrographs were taken on Kodak Ektachrome 35-mm film (160 or 200 ASA). Upon vital uptake of AO, lysosomes fluoresce intense red-orange, in contrast to green orthofluorescent cytoplasm and nucleus (1, 2, 65). Because of rapid quenching of the characteristic fluorescence properties of AO, it was essential to observe and photograph the preparation within 1 min after initial exposure to ultraviolet radiation.

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Preparation and Characterization of IgG

The IgG fractions of rabbit anti-ovine (anti-o)LH serum and corresponding nonimmune serum were isolated by precipitation with ammonium sulfate, pH 7.0, to 45% saturation (26). The precipitate was dissolved in a minimum volume of 10 mM phosphate-buffered saline (PBS; pH 7.5, containing 15 mM sodium chloride). After a second ammonium sulfate precipitation, the redissolved preparation was dialyzed against repeated changes of PBS for 1–2 d or until no ammonium ions were detectable in the dialysate by Nessler's reagent. IgG concentration of the product was standardized at 12 mg protein/ml (29).

To define the specificity of the IgG, double immunodiffusion (36) and immunoelectrophoresis (13) were carried out by standard procedures, using LH and its component \(\alpha\)- and \(\beta\)-subunits, FSH, and PRL at appropriate concentrations.

RESULTS

Effects of Endogenous Gonadotropins

Intact preovulatory follicles were explanted from ovaries of rats sacrificed at various stages of their estrous cycle and cultured for up to 5 h at \(-22^\circ\text{C}\). Oocytes were aspirated from the individually cultured follicles at 30-min intervals, partially denuded of surrounding follicular cells, vitally stained with AO, and examined immediately to record the distribution of organelles which exhibit lysosome-like properties, in relation to stage of maturation. Among oocytes isolated from cultured follicles that had been obtained from rats at diestrus or between 0900 and 1000 during the morning of proestrus (and thus before the onset of the preovulatory gonadotropin surge; cf. references 6 and 11), meiotic maturation had not resumed, as evidenced by an intact nuclear envelope. Such oocytes displayed a homogeneous distribution of organelles with the characteristic metachromatic fluorescence of lysosomes; a few random patches of such organelles were scattered throughout the ooplasm. Lysosomes were absent from the nucleoplasm which, together with the prominent nucleolus, fluoresced yellow-green (Fig. 1A). The nuclear envelope remained intact and no appreciable change in lysosome distribution was seen during the 5-h incubation period. NIC also revealed a similar homogeneous distribution of cytoplasmic organelles and an intact nuclear envelope throughout the culture period in preparations explanted before the LH surge (Fig. 2A).

\(^2\) Gifts of Professor C. H. Li; see reference 31 for preparation.
In contrast, when follicle-enclosed oocytes were obtained from rats sacrificed between 1300 and 1500 of the afternoon of proestrus (and thus during the onset of the preovulatory gonadotropin surge; cf. references 6, 11, and 46) and cultured in vitro, lysosomes of oocytes aspirated from follicles after 30 min in culture were aggregated in clusters that were broadly distributed throughout the cytoplasm (not shown). Similar preparations from follicles that had been cultured for 60 min revealed appreciable accumulation of patches of lysosomes around the nuclear envelope, with remarkable paucity of the organelles in the peripheral ooplasm (Fig. 1B). Clumping and perinuclear aggregation of cytoplasmic organelles, together with irregularities in the nuclear envelope, were also observed by 60 min with NIC (Fig. 2B). After 90 min, undulations of the latter were more pronounced (Fig. 2C). By 120 min, GVB was evident by either technique of observation. Promptly on completion of GVB, clumps of lysosomes viewed by AO fluorescence were particularly conspicuous in the region previously occupied by the intact nuclear envelope and also close to the nucleolus (Fig. 1C).

From ~30 min after GVB until the end of the culture period (5 h), lysosomes were once again evenly dispersed throughout both the cytoplasm and the area formerly occupied by the nucleus. This second redistribution of lysosomes was more homogeneous, with substantially fewer aggregates and patches (cf. Fig. 1M), when compared to the disposition of lysosomes in oocytes that had not resumed meiotic maturation.

The staging evident in the redistribution of lysosomes and the apparent repercussions in nuclear envelope dissolution have been outlined in Table I. It is self evident that individual oocyte responses were not identical, and thus each of the arbitrary designations, as part of a continuum, encompasses a range of morphologic variation. Nevertheless, the distribution of lysosomes and the accompanying qualitative changes conformed to these staging parameters.

Table II summarizes the effects of exposure to endogenous gonadotropins before cultivation of explanted follicles. It is clear that explanation of follicular oocytes during the LH surge on the afternoon of proestrus is accompanied by correlates of meiotic maturation in contrast to the relative quiescence of diestrous or early proestrous oocytes. A total of 79 diestrous or early proestrous oocytes was individually processed and examined (Table II). In terms of stage of organellar distribution, these differed statistically from 92 of their counterparts, taken from follicles that had been explanted during the LH surge, at all times of in vitro cultivation ($P < 0.05$).

**Isolated Diestrous or Early Proestrous Oocytes Cultured In Vitro in Hormone-Free Medium**

The behavior of organelles with properties of lysosomes before and during GVB was examined in isolated oocytes undergoing 'spontaneous' maturation in vitro in hormone-free medium. To avoid preovulatory levels of endogenous gonadotropins that could initiate meiotic maturation of the follicle-enclosed oocyte before its isolation, the ovaries were obtained either at diestrus or between 0900 and 1030 during the morning of proestrus. Oocytes were then aspirated from freshly explanted follicles, partially denuded of follicular cells, and incubated for up to 3 h at ~22°C, as previously outlined. Before culture, they were examined by phase-contrast optics to confirm the presence of an intact nuclear envelope. Although obtained before the onset of the preovulatory gonadotropin surge, 1 out of 10 such oocytes displayed irregularities in the nuclear envelope which indicated impending GVB and resumption of meiotic maturation. When vitally stained with AO and examined with the fluorescence microscope, considerable perinuclear accumulation of clusters of lysosomes was observed in these specimens. Such oocytes were not carried further in the incubation experiments.

During the first 30 min in culture, lysosomes remained homogeneously distributed throughout the ooplasm as individual organelles with only occasional random patches (Fig. 1D). However, after 40–50 min, virtually the entire lysosomal population had aggregated into patches, some close to the intact nuclear envelope (Fig. 1E). By 60 min, the nuclear envelope was virtually surrounded by lysosomes while the peripheral ooplasm was essentially devoid of these organelles (Fig. 1F). When corresponding preparations were examined with NIC, irregularities in the nuclear envelope were observed after 60 min, with GVB occurring generally within 2 h (cf. also Fig. 2B and C). After GVB, lysosomes were once again homogeneously distributed throughout the entire
cell, including the region formerly occupied by the nucleus (e.g., Fig. 1M).

Upon extended cultivation of several oocytes under these conditions, extrusion of the first polar body was observed within ~7 h after completion of GVB.

**The Influence of LH Added In Vitro on Isolated Diestrous or Early Proestrous Oocytes**

In this series of experiments, isolated oocytes were obtained at either diestrus or between 0900 and 1000 during the morning of proestrus and incubated at ~22°C in CDM with $3 \times 10^{-8}$ M LH. This concentration approximated serum levels of LH at the peak of the preovulatory gonadotropin surge (6, 11).

After 5 min of cultivation under these conditions, lysosomes had aggregated into patches that were randomly distributed throughout the ooplasm (Fig. 1G and H), as compared to corresponding controls (i.e., partially denuded, isolated oocytes).

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**Figure 1** Fluorescence photomicrographs of oocytes vitally stained with acridine orange. (A-C) Oocytes examined immediately upon aspiration from follicles that had been explanted at specified times during the estrous cycle and cultured at ~22°C in hormone-free CDM (see text). (A) Illustrates the homogeneous distribution of organelles, both as clusters and dispersed granules, with the dimensions and metachromatic-fluorescence properties of lysosomes. The oocyte was observed within minutes of aspiration from a follicle that had been explanted at 1000 on the morning of proestrus (and thus before the onset of the preovulatory gonadotropin surge), and cultured for 1 h thereafter. Note intact germinal vesicle (GV), nucleolus (N), and surrounding granulosa cells (gc). Bar, applicable to Fig. IA-G and I-L, 20 μm. × 510. This morphology is representative of oocytes examined throughout the 5-h follicular culture period (not illustrated; see Table II). (B) Oocyte maintained under same conditions as Fig. 1A, except that follicles were explanted at 1400 during the afternoon of proestrus (and thus during the onset of the preovulatory gonadotropin surge). Lysosomes are densely accumulated around GV and depleted from the peripheral cytoplasm. × 510. (C) Preparation similar to Fig. 1B, but follicular culture carried out for 2 h; meiotic maturation is correspondingly more advanced, as exemplified by loss of GV. Clumps of lysosomes are localized in area formerly occupied by intact GV, and they surround Nl (arrow). × 510.

(D-F) Oocytes aspirated from follicles at diestrus or between 0900 and 1000 during the morning of proestrus, partially divested of follicular cells, and cultured at ~22°C in hormone-free CDM (see text). All × 510. (D) Homogeneous distribution of lysosomes (as in Fig. 1A) in isolated oocyte incubated for 30 min. (E) After 45 min in culture, most lysosomes are aggregated into clumps, some of which are near the GV. (F) By 60 min, perinuclear accumulation of lysosomes is more pronounced, while depletion of these organelles from the peripheral cytoplasm is evident. Perinuclear accumulation becomes more accentuated up to the time of completion of GVB at ~2 h (not illustrated; see Table III). (G, H, and J-L) Isolated oocytes similar to those illustrated in Fig. 1D-F, but exposed in vitro to $3 \times 10^{-8}$ M LH (see text). (G) Isolated oocyte examined after 5 min in culture in the presence of LH; lysosomes are aggregated into clusters, with some in close proximity to GV. × 510. (H) Same oocyte, but photographed in focal plane above GV to illustrate extensive clumping of lysosomes in cytoplasm. Bar, applicable to Fig. 1H-K, 30 μm. × 274. (I) In contrast, lysosomes are homogeneously distributed as pinpoint-fluorescing organelles in an isolated oocyte that had been incubated for 5 min in hormone-free medium. Photography was in focal plane above GV, as in Fig. 1H. × 274. (J) By 7 min of exposure of another oocyte to LH, aggregates of lysosomes are concentrated around GV. This is also observed when the same oocyte is photographed in a focal plane slightly above GV (Fig. 1K), both × 274. (L) Within 15 min of LH exposure in vitro, perinuclear accumulation of lysosomes is especially prominent. × 510. (M) Oocyte aspirated from a follicle that had been explanted at 1730 during the afternoon of proestrus (and thus during the preovulatory gonadotropin surge) and had undergone GVB in vivo, as ascertained by phase-contrast microscopy before start of culture period. The isolated oocyte was then incubated in vitro for 1 h in CDM in the presence of $3 \times 10^{-8}$ M LH. Lysosomes are homogeneously distributed throughout ooplasm, including the area formerly occupied by the nucleus. The mottled appearance and irregular outlines of remaining Nl may indicate its impending dissolution. × 510. In comparison to the appearance of oocytes examined before beginning of GVB (see Fig. 1A and D), lysosomes in Fig. 1M are more diffusely distributed. This morphology is representative of oocytes that have completed GVB, whether 'spontaneously' or in response to endogenous or exogenous gonadotropin (see text). (N) The effects of exogenous LH in accelerating the time-course of lysosome redistribution were blocked by preincubation of both the LH and oocyte (during its isolation before culture) with rabbit anti-oLH IgG, followed by incubation of the oocyte in CDM containing the pretreated LH ($3 \times 10^{-8}$ M) for 30 min (see text). Under these conditions, the lysosomes remained homogeneously distributed. × 510.
FIGURE 2. Oocytes examined by Nomarski interference-contrast microscopy (NIC) after aspiration from follicles that had been explanted at specified times during the estrous cycle and cultured in hormone-free CDM at −22°C (see text). (A) Illustrates homogeneous distribution of cytoplasmic organelles in an oocyte observed within minutes of aspiration from a follicle that had been explanted at 1000 on the morning of proestrus (and thus before the onset of the preovulatory gonadotropin surge), and cultured for 1 h. Note intact germinal vesicle (GV) and nucleolus (NI). Bar, 20 μm. × 860. (B) Oocyte examined upon aspiration from a follicle that had been explanted at 1430 on the afternoon of proestrus (during the onset of the preovulatory gonadotropin surge), and cultured for 1 h in CDM. Clusters of cytoplasmic organelles are evident, as are undulations of the GV (arrows). × 770. (C) Oocyte from a cultured follicle of the same ovarian preparation as Fig. 2B, incubated for 90 min before aspiration and NIC microscopy. A more advanced stage of incipient GVB is apparent, as revealed by accentuated undulations of the GV (arrows). × 770. Although perinuclear accumulation of cytoplasmic organelles is demonstrated preceding GVB (Fig. 2B and C), a significant portion of organelles identified by NIC remains in the peripheral ooplasm.

Oocytes in hormone-free medium), in which lysosomes had remained dispersed as pinpoint-fluorescing granules (Fig. 1I). By 7–10 min, a significant portion of the patches had translocated from the periphery of the cytoplasm toward the nuclear region (Fig. 1J and K); within 15 min, lysosomes were densely clustered around the nuclear envelope (Fig. 1L). In similar oocytes examined with NIC, undulations in the nuclear envelope were observed between 40 and 60 min, with GVB occurring after 75–90 min in culture (cf. Fig. 2B and C). In the presence of a substantial amount of LH in vitro, the time-course of lysosome redistribution and GVB was appreciably accelerated over the chronology seen in corresponding isolated diestrous or early proestrous oocytes cultivated in the absence of LH (Table III; P < 0.05).

The acceleratory influence of LH appears to be exerted primarily at early stages (cf. Table I) of lysosome redistribution (see Tables II and III). Thus, the time-course of stages 1–3 is substantially abbreviated in the presence of adequate concentrations of LH. Moreover, whether in response to the endogenous gonadotropin surge (Table II) or in 'spontaneously' maturing, or LH-treated, isolated oocytes (Table III), stage 4 as identified in vitro is essentially of equivalent duration.

After GVB, lysosomes were once again homogeneously distributed throughout the ooplasm. The appearance of such oocytes resembled that of specimens which had undergone GVB 'spontaneously' in vitro in hormone-free medium and also that of those follicle-enclosed oocytes induced to mature by the endogenous preovulatory gonadotropin surge (cf. Fig. 1M).

Essentially the same patterns and time-course of organellar disposition before and after GVB were observed when the concentration of LH was increased 10-fold to $3 \times 10^{-7}$ M (see Table III).
In contrast, in the presence of only $1.5 \times 10^{-8} \text{M LH}$, no significant differences in these parameters of meiotic maturation were noted, compared to corresponding controls without hormone (Table III).

**Lack of Effect of FSH and PRL**

In contrast to the evident acceleration of meiotic maturation evoked by adequate concentrations of LH acting in vitro (Table III) and presumably also in vivo (Table II), neither the time-course of GVB nor the prior redistribution of lysosomes differed from those of hormone-free control preparations when isolated diestrous or early proestrous oocytes were individually incubated in the presence of either $3, 30,$ and $60 \times 10^{-8} \text{M FSH}$ or $4, 40,$ and $80 \times 10^{-8} \text{M PRL}$ (Table III). These concentrations are $\sim 20$- to 40-fold greater, respectively, than circulating levels in intact rats during the preovulatory gonadotropin surge (6, 11).

**Observations on Isolated Oocytes Cultured In Vitro After GVB, With and Without Added Hormone**

For these experiments, a total of 122 oocytes was obtained in the late afternoon of proestrus (1600-1800) during the endogenous gonadotropin surge. At the time of their aspiration from explanted follicles, these oocytes had resumed meiotic maturation in vivo as verified by the absence of an intact nuclear envelope. Oocytes were examined at 30-min intervals for up to 2 h at $\sim 22^\circ\text{C}$ in CDM with or without the addition of either $3$ or $30 \times 10^{-8} \text{M LH}$, $3$ or $60 \times 10^{-8} \text{M FSH}$, or $4$ or $80 \times 10^{-8} \text{M PRL}$. Between 3 and 8 oocytes were individually incubated and observed for each condition and time-point. Even in the presence of concentrations of LH that were capable of accelerating lysosome translocation before GVB (cf. Table III), the organelles remained homogeneously distributed throughout the ooplasm for the entire culture period (e.g., Fig. 1M). FSH and PRL were similarly ineffective. Because these oocytes all displayed lysosome distribution within stage 0 (see Table I), the tabulated results ($P < 0.05$) are not shown.

**Inhibition of In Vitro Effects of LH Using Specific Antibody**

IgG preparations from rabbit anti-oLH as well as the corresponding nonimmune serum were utilized in an attempt to block the acceleratory

| Numerical designation | Generalized parameters                                      | Illustration* (Fig. No.) |
|-----------------------|------------------------------------------------------------|--------------------------|
| 0                     | Seen only after GVB; homogeneous distribution and diffuse fluorescence of lysosomes, including area adjacent to nucleus; very few aggregates or patches. | 1M                       |
| 1                     | Lysosomes generally distributed as evenly dispersed organelles, including peripheral cytoplasm; only a few random patches. | 1A, D, J, and N          |
| 2                     | All lysosomes aggregated in broadly dispersed patches or clumps, including the peripheral cytoplasm. | 1G and H                  |
| 3                     | Preponderance of lysosomal clusters concentrated in close proximity to intact nuclear envelope; some lysosomes left at peripheral ooplasm. | 1E, J, and K              |
| 4                     | Predominant accumulation of lysosomal clumps around the nuclear envelope; some undulations\‡ seen in the nuclear envelope; periphery depleted of lysosomes except for a few isolated clusters. | 1F and L                  |
| 5                     | Clumps and patches of lysosomes in close association with the nuclear envelope, obscuring outline of the latter; immediately after GVB, clusters of lysosomes in close association to the nucleus. | 1B and C                  |

* Representative examples are given in Fig. 1, by fluorescence microscopy after vital uptake of acridine orange.

\‡ Best observed with Nomarski interference-contrast microscopy. See Fig. 2.
effects of LH on GVB of isolated diestrous or early proestrous oocytes. By means of immunoelectrophoretic analysis, the IgG directed against oLH was found to form single precipitation arcs with both oLH and its isolated β-subunit, whereas there was negligible affinity for the α-subunit (not shown). Immunodiffusion (IgG, 6 mg protein/ml) revealed no cross-reactivity with 100 μg/ml each of LH-α, oFSH, or oPRL (all at 10 μl; not illustrated; cf. also reference 31). In all cases, IgG from nonimmune rabbits was unreactive.

In preliminary experiments, unfractionated serum from LH-immunized rabbits, added at dilutions of 1:100 or less to isolated diestrous or early proestrous oocytes, elicited perinuclear accumulation of lysosomes. However, similar lysosome activation occurred in the presence of equivalent concentrations of nonimmune serum. This apparently nonspecific effect of raw serum was eliminated when the partially purified IgG fraction of the appropriate antiserum was substituted in succeeding experiments.

After a 30-min preincubation of 1 μg oLH/ml with 100 μg/ml of anti-oLH IgG at ~22°C, the resultant uncentrifuged preparation was incorporated into the incubation media of three isolated early-proestrous oocytes at a calculated final concentration of 3 × 10⁻⁸ M LH. After 30 min in culture, all three oocytes exhibited a marginal response, including some clumping of lysosomes with a slight perinuclear accumulation of these organelles (not illustrated). This partial inhibition of LH-accelerated lysosome activation was rendered more complete if, in addition to preincubation of LH with the specific IgG, the oocyte was also exposed to the 100 μg/ml immunoglobulin for ~10 min during isolation and preparation for culture. Under these conditions, lysosomes remained homogeneously distributed during the subsequent 30-min culture period in five oocytes (Fig. 1N). In additional oocytes so treated, one for each time-point of observation, lysosomes had aggregated into randomly distributed clumps by 45 min, followed by perinuclear accumulation within 60 min of continued culture; GVB occurred by 120 min (not shown). This time-course was similar to that which prevailed in isolated oocytes undergoing 'spontaneous' meiotic maturation in hormone-free CDM (cf. Fig. 1D-F; Tables I and II). Thus, although the specific immunoglobulin produced a distinct suppression of the meiosis-acceleratory influence of LH, it did not influence the ‘basal’ sequence of events characteristic of isolated oocytes maturing in vitro in CDM. In a series of trials, it was found that a ratio of 100:1 anti-oLH-IgG:LH (protein) was optimal for maximum inhibition of the effects of the hormone on lysosome redistribution and GVB. In three experiments, LH activity remained unimpaired when IgG from an unimmunized rabbit was substituted for the specific antibody (not illustrated).

**DISCUSSION**

Invagination of the nuclear envelope before its dissolution has been considered to be the first morphological sign of incipient oocyte maturation (47). The present work demonstrates, by fluorescence microscopy after vital uptake of AO, that redistribution of organelles corresponding to lyso-

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**TABLE II**

Distribution of Lysosomes During Meiotic Maturation of Follicular Oocytes Exposed In Vivo to Preovulatory Levels of LH and Cultured in a Chemically Defined Medium*

| Follicular explantation time | Before LH surge | During LH surge |
|-----------------------------|----------------|----------------|
| Time in culture (min)       | No. of oocytes observed | Stage of lysosome distribution | No. of oocytes observed | Stage of lysosome distribution |
| 0                           | 10             | 1              | 12             | 1              |
| 30                          | 7              | 1              | 10             | 2              |
| 60                          | 7              | 1              | 10             | 4              |
| 90                          | 6              | 1              | 9              | 4              |
| 120                         | 8              | 1              | 10             | 5              |
| 150                         | 9              | 1              | 7              | 0              |
| 180                         | 7              | 1              | 8              | 0              |
| 210                         | 8              | 1              | 7              | 0              |
| 240                         | 6              | 1              | 7              | 0              |
| 270                         | 5              | 1              | 6              | 0              |
| 300                         | 6              | 1              | 6              | 0              |
| Totals                      | 79             | 92             |

See Table I for explanation. Using Fisher exact probability test (45) to evaluate the observations for each condition and time-point, stages of lysosome distribution specified were all of statistical significance at P < 0.05.

* Oocytes were examined individually, immediately after aspiration at above-specified times from explanted follicles. See text for details.
† Obtained either during diestrus or on morning of proestrus, 0900-1000.
‡ Obtained during afternoon of proestrus, 1300-1500.
§ Each set of observations represents the composite results of 3–8 independent experiments (see text).
### Table III

*Distribution of Lysosomes During Meiotic Maturation of Isolated Oocytes Cultured in a Chemically Defined Medium in the Presence or Absence of Added Hormones*

| Additives and their concentrations | Time in culture (min) |
|-----------------------------------|-----------------------|
|                                   | 0  | 5   | 10  | 15  | 20  | 30  | 40  | 50  | 60  | 90  | 120 | 150 | 180 |
| None (control)                    | 11 (9)§ | 1 (5) | 1 (6) | 1 (6) | 1 (7) | 1 (5) | 2 (7) | 3 (4) | 4 (5) | 4 (5) | 5 (4) | 0 (3) | 0 (3) | (68)‖ |
| Luteinizing hormone               | 1.5 × 10^-8 M         | 1 (5) | 1 (6) | 1 (6) | 1 (6) | 1 (6) | 1 (4) | 2 (6) | 3 (6) | 4 (7) | 4 (6) | 5 (6) | 0 (6) | 0 (4) | (74) |
|                                   | 3 × 10^-8 M           | 1 (11) | 2 (13) | 3 (14) | 4 (14) | 4 (9) | 4 (11) | 4 (12) | 4 (10) | 5 (10) | 5 (7) | 0 (8) | 0 (7) | 0 (5) | (131) |
|                                   | 30 × 10^-8 M          | 1 (3) | 2 (3) | 3 (3) | 4 (4) | 4 (3) | 4 (3) | 4 (3) | 5 (3) | 5 (3) | 0 (2) | 0 (3) | 0 (3) | (39)  |
| Follicle-stimulating hormone      | 3 × 10^-8 M           | 1 (3) | 1 (4) | 1 (5) | 1 (5) | 1 (3) | 1 (3) | 2 (2) | 3 (3) | 4 (3) | 4 (3) | 5 (3) | 0 (4) | 0 (4) | (45) |
|                                   | 30 × 10^-8 M          | 1 (3) | 1 (3) | 1 (4) | 1 (4) | 1 (3) | 1 (2) | 2 (3) | 3 (3) | 4 (2) | 4 (3) | 5 (3) | 0 (3) | 0 (2) | (38) |
|                                   | 60 × 10^-8 M          | 1 (6) | 1 (5) | 1 (4) | 1 (4) | 1 (4) | 1 (3) | 2 (4) | 3 (4) | 4 (3) | 4 (3) | 5 (3) | 0 (3) | 0 (3) | (50) |
| Prolactin                         | 4 × 10^-8 M           | 1 (4) | 1 (4) | 1 (3) | 1 (3) | 1 (3) | 1 (3) | 2 (3) | 3 (2) | 4 (2) | 4 (3) | 5 (3) | 0 (3) | 0 (3) | (39) |
|                                   | 40 × 10^-8 M          | 1 (3) | 1 (4) | 1 (2) | 1 (2) | 1 (3) | 1 (3) | 2 (3) | 3 (3) | 4 (3) | 4 (3) | 5 (2) | 0 (3) | 0 (2) | (36) |
|                                   | 80 × 10^-8 M          | 1 (4) | 1 (5) | 1 (5) | 1 (6) | 1 (6) | 1 (3) | 2 (3) | 3 (3) | 4 (4) | 4 (2) | 5 (2) | 0 (3) | 0 (3) | (49) |

* Oocytes were aspirated from follicles that had been explanted before the preovulatory LH surge (diestrus or morning of proestrus; 0900-1030) and incubated and examined individually. See text for details.

† Lysosome distribution stage; see Table I for explanation. Using Fisher exact probability test (45) to evaluate the observations for each condition and time-point, stages of lysosome distribution were all of statistical significance at P < 0.05.

§ Numbers of oocytes observed are shown in parentheses. See Table II and text for explanation of experimental protocols.

‖ Total numbers observed, all time-points.
osomes, resulting in their aggregation into clusters, perinuclear accumulation, and virtual disappearance from the peripheral ooplasm, is an even earlier correlate of resumption of meiotic maturation of rat oocytes.

Oocytes liberated from early proestrous follicles that had been cultivated in vitro in CDM for up to 5 h at ~22°C failed to display GVB. Nor did they exhibit lysosome redistribution from the basal, homogeneously dispersed state. These observations were in sharp contrast to the behavior of corresponding oocytes that had been exposed to the endogenous gonadotropin surge before follicular explantation. In a previous study, completion of GVB was likewise observed in 90% of follicular oocytes ~2 h after the onset of the preovulatory LH surge (57).

To establish the requisite control conditions for experiments with hormones to be added in vitro, temporal and morphologic correlates were determined for resumption of maturation in early proestrous oocytes cultivated in CDM after being partially denuded of surrounding follicular cells. Not surprisingly, in view of the trauma inherent in even the most gentle microdissection, ~10% of the resultant oocytes showed the lysosomal clustering and irregularities of the vicinal nuclear envelope characteristic of impending GVB, and were eliminated from the experimental series. In the remaining diestrous and early proestrous 'control' oocytes so isolated which exhibited no overt damage, the time-course of GVB and antecedent lysosome redistribution were virtually identical to that seen in the follicle-enclosed oocytes that had been explanted during the LH surge. Similar intervals (75–130 min) have been reported for GVB in rat oocytes cultured in the absence of hormone (28, 64).

The time-course of lysosome redistribution and GVB was sharply accelerated on exposure to LH in vitro. The stages of lysosome redistribution that are characteristically abbreviated by LH are those preceding stage 4, as currently defined. Thus, stage 4, which is reached in corresponding hormone-free oocytes no sooner than 60 min, occurs by 15 min in the presence of 3 \times 10^{-8} \text{M LH}. Likewise, this concentration of LH elicited GVB 30 min earlier than in oocytes maturing in hormone-free CDM. The time required for dissolution of the nuclear envelope in oocytes exposed to this concentration of LH (~90 min) was comparable to that for GVB in intrafollicular oocytes of rats injected intraperitoneally with pituitary gonadotropin (59). Similarly, LH has been reported to increase the rate of maturation of rat (28) and hamster (25) oocytes in vitro. It would appear, therefore, that, once lysosome activation is achieved by adequate stimulus, the oocyte is irreversibly committed to resumption of meiotic maturation, as evidenced by GVB.

Specificity of the LH response is supported by lack of acceleratory influence of FSH or PRL, at concentrations 20- to 40-fold higher, respectively, than the circulating levels of these hormones that prevail during the gonadotropin surge, either on lysosome redistribution or on GVB. Although FSH has been found to promote resumption of meiosis in cultured, follicle-enveloped oocytes (58), the FSH preparation used in the latter investigation contained "a small amount of LH-like activity." The FSH used in our experiments, in amounts 10-fold greater than those of the above workers (58), did not cross-react with rabbit antioLH IgG. Therefore, these data suggest that, of the three peptide hormones involved in control of ovarian function, LH is unique in its ability to accelerate both lysosome redistribution and GVB. This conclusion is further supported by the observation that these effects of LH were blocked by anti-oLH IgG (cf. also reference 28), which, by immunoelectrophoretic analysis, exhibited intense specificity toward the unique ß-subunit of LH while being essentially unreactive toward the α. In the present experiments, inhibition was achieved only by pre-exposure of both the oocyte and hormone to the antibody before culture. This result parallels the observation that LH-antiserum, when added after treatment with LH, did not immediately terminate LH-induced steroidogenesis in Leydig-tumor cells (32).

In electron microscope studies of ovarian oocytes of the rat (34, 48), aggregates of multivesicular bodies (mvb) were observed in large follicular oocytes before ovulation. These organelles, which correspond to one class of lysosomes (14), were noted to increase dramatically in numbers with advancing oocyte maturation (34, 48). However, in neither of these studies was systematic analysis undertaken of the cellular disposition of mvb at given phases of meiotic development. Nor was the stage characterized by GVB specifically considered, even though, in fertilized eggs, "... large accumulations of similar vesicles were noted in association with the pronuclei just prior to fusion" (48). In an ultrastructural analysis of the disposition of organelles during GVB of mouse oocytes.
maturing in vitro, clusters of membrane-bounded "dark bodies" were detected in the central nuclear region during chromatin condensation soon after GVB (10). Similarly, during meiotic maturation of amphibian oocytes, large basophilic organelles of uncertain identity were observed to accumulate at the basal surface of the nuclear envelope, where GVB begins (8).

By dimensions, mobility, and AO-metachromasia, the organelles that become mobilized to associate with the nuclear envelope upon resumption of meiotic maturation of rat oocytes conform to characteristics of lysosomes (cf. reference 14). Although vital staining with AO does not prove that these organelles are exclusively lysosomes, AO has been used to visualize lysosomes in various cell types (2). Moreover, Robbins et al. noted that in HeLa cell cultures cytoplasmic membrane-bounded vesicles that accumulated AO corresponded to acid phosphatase-positive multivesicular bodies (43). These workers also observed redistribution of acid phosphatase-positive organelles and their increased numbers during mitotic prophase (42).

Lysosomes have been shown to be concentrated in the perinuclear cytoplasm of cells activated by a variety of circumstances, including hormones (51, 54). Even unspecified factors present in the serum that is often added to cell culture media are known to promote lysosomal activation, as well as cell division (21, 23, 55). Likewise, in the present work, whole serum, whether from control or specifically immunized rabbits, promoted perinuclear mobilization of ooplasmic organelles in isolated oocytes within minutes of addition to CDM. This 'nonspecific' effect was eliminated when the purified IgG fraction was substituted.

In evaluating the nature of the organelles responsive to LH, it is instructive to compare parallel observations using AO and NIC microscopy. By these dual criteria, the homogeneous distribution in quiescent oocytes, the perinuclear aggregation before GVB, and the disperse appearance of organelles after GVB were all virtually identical. In contrast, whereas the organelles identified by AO-fluorescence were essentially depleted from the peripheral ooplasm just before GVB, NIC examination of corresponding oocytes revealed a significant, though sparse, remainder in that region. These data demonstrate selectivity in perinuclear mobilization of ooplasmic organelles during oocyte maturation.

In recent years, lysosomes have been implicated in several aspects of gonadotropin action, including ovarian growth and development (16, 19) and follicle rupture (9). Moreover, it is increasingly evident that LH and human chorionic gonadotropin (hCG), in common with other peptide hormones, are in part internalized in their target cells (39, 51, 53), where they accumulate in lysosomes (5, 12, 30, 38). Although it is now widely assumed that intralysosomal degradation of the tropic hormone:receptor complex accounts for loss of surface receptors on maximal stimulation, additional work with immunochemical (38, 39) and radioautographic (3) methods demonstrates that a limited portion of the internalized hCG reaches the nucleus. The significance of such findings requires investigation, especially in light of the potential vectorial role of lysosomes in nucleotropic migration (14, 38, 39, 51, 53).

It has long been recognized that even minimal injury to surface membranes leads to lysosomal activation (15, 50, 62). Moreover, intracellular translocation of lysosomes occurs concomitant with reduction in structural latency of component hydrolases (cf. reference 50). Thus, it is likely that the procedure of aspiration of oocytes from preovulatory follicles promotes lysosomal activation, leading to liberation of proteases that have been implicated in induction of meiotic maturation in some species (24, 37). By analogy, the attenuated fluorescence of lysosomes in oocytes after GVB may represent leakage of AO-binding components (cf. also reference 43). Limited escape of lysosomal products could thus contribute to the availability of cytoplasmic factor(s) implicit in nuclear envelope dissolution (7, 61).

Both LH and hCG have been shown to promote acute labilization of ovarian lysosomes, with enhanced release of marker enzymes above basal values (16, 35, 52), as well as delayed increases in numbers of lysosomes (19) and total cellular hydrolases (17, 19). Recent experiments have also revealed decreased latency of acid phosphatase and cathepsin B, in lysosome-enriched fractions of preovulatory rat ovaries in response to in vitro addition of LH, whereas post-ovulatory preparations, presumably having released a portion of their lysosomal enzyme complement in response to the endogenous gonadotropin surge, were lower in total enzymes and less sensitive to exogenous LH. Similarly, the tachyphylaxis exhibited

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toward LH by lysosomes of late-proestrous oocytes after GVB may reflect depletion of receptors of the oocyte/ follicular cell complex during prior exposure in vivo to preovulatory levels of active hormone.

Cyclic AMP (cAMP), in amounts comparable to those generated in target cells on hormonal destabilization of surface membranes, enhances perinuclear lysosome mobilization and limited escape of hydrolases (49). Although $1 \times 10^{-3} - 2 \times 10^{-3}$ M dibutyryl cAMP, when applied to the external surfaces of isolated oocytes of the mouse (33, 60) or to follicular oocytes of the rat (58), inhibited (33, 60) or failed to promote (58) GVB, microinjection of this nucleotide, at a final concentration essentially equivalent to the lowest level in the above range, into the antrum of follicle-enclosed rat oocytes cultivated in vitro induced meiotic maturation, including GVB; 5'AMP was ineffective (58). The latter workers also observed augmentation of endogenous cAMP levels in LH-treated, isolated Graafian follicles (58). Thus, whether exclusively through mediation of cAMP generated on interaction of the hormone with surface receptors, or via intracellular pathways yet to be defined (cf. references 38, 51, and 53), the action of LH in promoting lysosomal activation may be the means by which resumption of meiotic maturation is triggered. Because LH, whether endogenous or added in vitro, appears to override the meiosis-suppressing effects of inhibitory factor(s) present in the follicular fluid (56), the possibility that limited proteolysis elicited by LH-induced release of lysosomal cathepsins may serve this triggering function demands investigation.

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ADDENDUM

After this manuscript had been submitted for publica-
tion, a paper appeared confirming the original findings of Lopata and co-workers (28) on the accelerator influence of LH on GVB in rat oocytes (Kaplan, R., N. Dekel, and P. F. Kraicer. 1978. Acceleration of onset of oocyte maturation in vitro by luteinizing hormone. Gynic Res. 1:59-63).

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