BIOCHEMICAL STUDIES OF MAMMALIAN OOGENESIS: PROTEIN SYNTHESIS DURING OOCYTE GROWTH AND MEIOTIC MATURATION IN THE MOUSE

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SUMMARY
Using oocytes isolated from juvenile and adult mice, we have examined the qualitative patterns of protein synthesis during growth and during meiotic maturation of these oocytes. Oocytes were cultured in a defined medium in the presence of [35S]methionine and radioactively labelled proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography. The results of these studies demonstrate that: (i) the patterns of protein synthesis are very similar in individual oocytes which are at the same stage of growth or of meiotic maturation, indicating a high degree of biochemical homogeneity in a given population of isolated mouse oocytes, (ii) the linear increase in protein content of growing mouse oocytes (with respect to oocyte volume) is accompanied by significant qualitative changes in the size classes of proteins synthesized, and (iii) meiotic maturation (germinal vesicle dissolution and nuclear progression to the second metaphase) is characterized by several discrete qualitative changes in the pattern of protein synthesis in the oocyte, especially during the period following germinal vesicle breakdown. Experiments carried out with oocytes cultured in the presence of drugs which have been shown to inhibit meiotic maturation at specific stages of nuclear progression suggest that: (i) protein synthesis is not required for germinal vesicle breakdown to take place; (ii) mixing of the oocyte's nucleoplasm and cytoplasm must occur in order for those changes in the pattern of protein synthesis which characterize meiotic maturation to take place; and (iii) failure of nuclear progression to proceed beyond the circular bivalent stage does not prevent those changes in the pattern of protein synthesis which characterize meiotic maturation from taking place. The latter observations suggest that there are basic differences in the control of meiotic maturation in oocytes isolated from mammalian, as compared to non-mammalian, animal species.

INTRODUCTION
During the process of oogenesis, oocytes of many animal species undergo meiotic arrest prior to the completion of chromosomal reduction and it is in this state that they undergo tremendous growth. The length of time that oocytes remain in this arrested state and the nature of the stimulus which reinitiates meiosis are species-dependent (Baker, 1972a; Schuetz, 1974; Smith, 1975). For example, the resumption of meiosis in starfish and frog oocytes requires the interaction of a diffusible molecule (1-methyladenine and progesterone, respectively), probably produced by the surrounding follicle cells, with the oocyte's plasmalemma (Kanatani, 1975; Smith, 1975).
Oogenesis in the mouse really begins with the formation of primordial germ cells in the 8-day embryo (Fig. 1). As early as day 12 of embryogenesis, after migration of the primordial germ cells is complete, a few oogonia enter the first meiotic prophase. By day 16 of embryogenesis nearly all oocytes are in pachytene and by day 18 some have entered diplotene. At parturition a few oocytes have already entered late diplotene, or 'dictyate', the so-called 'resting stage', and by day 5 post partum the mouse ovary is populated with thousands of small (approximately 20 μm in diameter) oocytes arrested at the dictyate stage of meiosis, where they remain until just prior to ovulation, a period extending from several weeks to more than a year. Commencement of oocyte growth is apparently regulated within the ovary, the number of oocytes entering the growth phase being a function of the pool of non-growing oocytes (Krarup, Pedersen & Faber, 1969; Peters et al. 1973). The oocyte and its surrounding follicle grow coordinately, progressing through a series of definable morphological stages (Pedersen & Peters, 1968). The oocyte completes its growth (to approximately 80 μm in diameter) in the adult mouse before the formation of a follicular antrum; consequently, the majority of follicle growth occurs after the oocyte has stopped growing (Brambell, 1928). Growth is continuous, ending in the ovulation of a mature oocyte, or degeneration (atresia) of the oocyte and its follicle (Pedersen, 1969).

In the sexually mature female mouse, fully grown oocytes in Graafian follicles resume meiosis and complete the first meiotic division just prior to ovulation. The resumption of meiosis can be mediated by a hormonal stimulus in vivo (Baker, 1972) or simply by the release of oocytes from their ovarian follicles into a suitable
culture medium in vitro (Biggers, Whittingham & Donahue, 1967; Donahue, 1968; Sorensen, 1973; Wassarman & Letourneau, 1976a). The oocytes undergo nuclear progression from dictyate to metaphase II and remain at this stage of meiosis in the oviduct, or in culture, until fertilization or parthenogenetic activation takes place. The period of time during which meiosis progresses from dictyate to metaphase II is termed the period of 'meiotic maturation'. The process of meiotic maturation is characterized by dissolution of the nuclear (germinal vesicle) membrane, condensation of diffuse dictyate chromatin into distinct bivalents, separation of the homologous chromosomes and emission of the first polar body, and arrest of meiotic progression at metaphase II. Mouse oocytes matured and fertilized in vitro have developed into viable foetuses following transplantation to the uteri of foster mothers (Cross & Brinster, 1970).

The period of meiotic maturation, which occurs relatively late in oogenesis, is a vitally important time in early animal development, for it is during this period that the terminal events of meiosis take place, that certain morphogenetic substances localized in the oocyte's germinal vesicle are dispersed into the cytoplasm, and that, perhaps, a portion of the developmental programme laid down in the oocyte during the extended period of oogenesis is activated (Smith, 1975). Because oocyte maturation is fundamental to further animal development, the biochemistry of this period of oogenesis has been studied extensively, especially in echinoderms and amphibians; consequently, much is known about macromolecular synthesis during meiotic maturation of oocytes from lower species (Smith & Ecker, 1970; Smith, 1975). On the other hand, very little biochemical information is currently available concerning meiotic maturation of mammalian oocytes.

This report describes two experimental approaches which we have taken in order to gain some insight into certain biochemical aspects of growth and meiotic maturation of the mammalian oocyte. One approach utilized oocytes isolated at different stages in their growth phase from juvenile mice (21 days of age or less). We have shown previously that the mean diameter of isolated oocytes increases linearly with the age of the donor mice and that oocytes recovered from mice younger than 15 days of age remain in the germinal vesicle stage, whereas, those from mice 15 days or older resume meiosis at a frequency which increases with the age of the mice (Sorensen & Wassarman, 1976). These oocytes have provided an in vitro system with which to carry out biochemical studies of the control of meiotic maturation in the mammal. The second approach involves the use of fully grown oocytes isolated from adult mice, together with several drugs which have been shown to block meiotic maturation of mouse oocytes in vitro at discrete stages prior to the second meiotic metaphase (Wassarman, Josefowicz & Letourneau, 1976).

MATERIALS AND METHODS

Oocyte collection, culture, and examination

Fully grown oocytes were obtained from adult (8–12 weeks of age), randomly bred, female Swiss albino mice (CD-1, Charles River Laboratories) by puncturing ovaries with fine steel needles under a dissecting microscope, essentially as described by Donahue (1968) and Rafferty
R. M. Schultz and P. M. Wassarman (1970). Oocytes containing an intact germinal vesicle (GV) and free of cumulus cells were harvested using a mouth-operated micro-pipette and washed in culture medium (Biggers, 1971) containing 100 μg/ml dibutyryl cyclic AMP (dbcAMP, Sigma) (Stern & Wassarman, 1974). Cell culture was carried out in either plastic dishes (Falcon) or embryological watch glasses in 50-250 μl of medium under paraffin oil at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Growing oocytes were obtained from juvenile Swiss mice, i.e. less than 21 days of age, using essentially the method described by Mangia & Epstein (1975). Ovaries dissected from animals killed by cervical dislocation were first washed thoroughly with culture medium containing 100 μg/ml dbcAMP and then incubated at 37 °C for 15-20 min in medium containing dbcAMP (100 μg/ml) and 0.5 mg/ml of testicular hyaluronidase (type V, Sigma), 100 units of collagenase (type III, Sigma), and 0.5 mg/ml of egg white lysozyme (grade I, Sigma). After incubation, the ovaries were gently punctured using fine steel needles under a dissecting microscope and oocytes containing an intact GV and free of follicle cells were harvested using a mouth-operated micro-pipette. The oocytes harvested from each ovary were transferred immediately to fresh medium containing dbcAMP (100 μg/ml) and were washed thoroughly to eliminate further contact with the enzyme mixture.

Air-dried chromosome spreads were prepared essentially by Tarkowski's procedure (1966). Chromosomes were stained with Giemsa (Harleco no. 620) at a 1:50 dilution with 0.1 M phosphate buffer, pH 6.8, for 30 min. Light microscopy was performed on isolated, unfixed oocytes and on fixed preparations using a Zeiss Photomicroscope II equipped with Nomarski differential-interference optics. In some cases, oocyte diameters (exclusive of zona pellucida) were measured with an ocular micrometer attached to an inverted microscope.

Analysis of protein content of growing oocytes

The protein assays were carried out using the method devised by Schultz & Wassarman (1976a, b). Oocytes were isolated and washed in phosphate-buffered saline containing 3 mg/ml polyvinylpyrrolidone as previously described. The sample (bovine serum albumin or oocytes disrupted by repeated freezing and thawing) was incubated at 60 °C for 30 min in a microfuge tube (400 μl capacity, Beckman) in 0.5 M sodium bicarbonate, pH 9.8, and 14% in sodium dodecyl sulphate, at a final volume of 50 μl. One microCurie of [PH]dansyl chloride (18 Ci/mmol, New England Nuclear) in 20 μl of acetone was added to the sample and the tube was vortexed immediately. After incubation of the reaction mixture at 37 °C for 30 min, 10 μl of 37 mM dansyl chloride were added and the sample was incubated at 37 °C for an additional 60 min. Fifty microgrammes of succinylated bovine serum albumin (10 μl) were added to the tube, followed by an equal volume (90 μl) of ice-cold 25% CCl₃COOH. The precipitate was transferred to a glass test tube with 10% CCl₃COOH. The precipitate was collected on a Whatman GF/A filter and was washed 7 times with 125-μl portions of ice-cold 10% CCl₃COOH. The pellet was then solubilized by incubation for 1 h at 60 °C in 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulphate (SDS), and 62.5 mM Tris-HCl, pH 6.8.

Electrophoretic analysis of oocyte proteins

Oocyte proteins labelled with [3H]dansyl chloride were prepared for electrophoresis in the following manner. Dansylation was conducted as previously described except that 20-25 μCi of [PH]dansyl chloride were added and the addition of non-radioactive dansyl chloride was omitted. Twenty-five microgrammes of succinylated bovine serum albumin were added to the tube, followed by addition of an equal volume of ice-cold 25% CCl₃COOH. The precipitate was transferred to a glass test tube with 10% CCl₃COOH. The precipitate was collected on a Whatman GF/A filter and was washed with 10% CCl₃COOH and then 95% ethanol. The radioactivity trapped on the filter was measured by liquid scintillation counting using standard procedures.

Oocyte proteins labelled with [35S]methionine were prepared for electrophoresis in the following manner. Oocytes labelled with [35S]methionine (200 μCi/ml, New England Nuclear) were washed thoroughly, harvested in a volume of less than 5 μl, and frozen and thawed 3 times in a tube containing 45 μl of 100 mM NaHCO₃, pH 9.8, and 3.5 μl of 20% SDS. The mixture was heated at 65 °C for 30 min, 10 μl of BSA (25 μg) were then added, followed by
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60 μl of ice-cold 25% CCl₃COOH. The precipitate was pelleted by centrifugation, washed 3 times with ice-cold 10% CCl₃COOH, and then solubilized by heating at 65 °C for 30 min in 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 2-3 (w/v) SDS, and 62 mm Tris-HCl, pH 6-8.

Protein samples were applied to vertical SDS-polyacrylamide slab gels 15 cm wide, 13 cm long, and 0.15 cm thick. The same radioactivity was usually applied to each well in nearly equal volumes. Electrophoresis was carried out as described by O'Farrell (1975) using a separation gel that was a continuous 9–15% acrylamide gradient (exponential) and a 4%, 10-cm-long, stacking gel. Samples were subjected to electrophoresis for approximately 7 h at room temperature at a constant current of 20 mA per gel (the tracking dye, bromphenol blue, migrated at the rate of 2.2 cm/h) and the gels were then processed for fluorography according to the procedure described by Bonner & Laskey (1974). Molecular weights were estimated by comparing relative mobilities of oocyte proteins with those of several proteins of known molecular weight as described in the figure legends.

RESULTS

Meiotic maturation of mouse oocytes in vitro

Meiotic maturation takes place spontaneously when oocytes from adult mice (3 weeks of age or older) are released from their ovarian follicles into a suitable culture medium (Biggers et al. 1967; Donahue, 1968; Sorensen, 1973; Wassarman & Letourneau, 1976a). This process, which takes approximately 15 h to complete in vitro, is characterized by dissolution of the nuclear (germinal vesicle, GV) membrane, condensation of diffuse dictyate chromatin into distinct bivalents, separation of homologous chromosomes and emission of the first polar body, and arrest of meiotic progression at metaphase II; some of these events are shown in Fig. 2. The time sequence of meiotic maturation in vitro can be approximated as follows: GV breakdown takes place within 1–5 h, metaphase I is reached in 5–10 h, and metaphase II is reached in 10–16 h. Under the experimental conditions used in this study, approximately 80% of the oocytes placed in culture underwent GV breakdown within 3 h and, of these, approximately 70% subsequently emitted first polar bodies.

Protein synthesis during growth of mouse oocytes

Oocytes recovered from mice 21 days of age or younger ('juvenile mice') undergo meiotic maturation in vitro at a frequency which increases with the age of the mice and, consequently, with the size of the oocytes. These results are summarized in Fig. 3 and confirm previous observations made in this laboratory (Sorensen & Wassarman, 1976a).

In order to determine the relationship between oocyte size and protein content, we have devised an assay utilizing [3H]dansyl chloride which has enabled us to measure as little as 50 ng of protein (Schultz & Wassarman, 1976a, b). The results of this assay are linear in the range of 50–5000 ng of protein and are related linearly to the number of oocytes reacted with [3H]dansyl chloride (Fig. 4A). Measurements made with oocytes isolated from juvenile mice indicate that the protein content of growing mouse oocytes is related linearly to oocyte volume, with each doubling of volume accompanied by a doubling of protein (Fig. 4B). This method has also been used to determine that fully grown oocytes (from adult animals) contain approximately
Protein synthesis in mouse oocytes

Fig. 3. Relationship between oocyte size, meiotic competence, and the age of the donor mice. Mean diameters of oocytes recovered from 9-, 12-, 15-, 17-, 19-, 21-day-old and adult mice (●—●) were determined from a minimum of 200 oocytes at each age. The percentage of germinal vesicle (GV) breakdown (○—○) was determined after culturing the oocytes for 16 h as described in Materials and methods.

30 ng of protein and that, of this, approximately 4.5 ng are contributed by the zona pellucida. Brinster (1967) had previously estimated, based upon measurements using Lowry, Rosebrough, Farr & Randall’s (1951) method, that the fully grown oocyte contains 28 ng of protein.

To begin to determine whether the increase in protein content during oocyte growth is accompanied by the stage-specific synthesis of different size classes of proteins or by the synthesis of the same set of proteins at all stages, perhaps at differing relative rates, we have examined electrophoretically the molecular weight classes of proteins synthesized in mouse oocytes at several stages of growth. Such an analysis is of particular interest since the mouse oocyte undergoes significant ultrastructural changes during growth (see Discussion) and since the oocyte becomes competent to resume meiosis at a specific stage of growth (Fig. 3; Sorensen & Wassarman, 1976). Oocyte proteins were labelled with [35S]methionine during a 5-h incubation period, were separated on SDS-polyacrylamide gels (9.5–15 % polyacrylamide, exponential

Fig. 2. Photomicrographs of mouse oocytes during culture in vitro and of Giemsa-stained chromosome spreads from mouse oocytes during condensation of dictyate chromatin into compact bivalents in vitro. Shown are oocytes in dictyate and metaphase II of meiosis (A and B, respectively) (Nomarski differential-interference optics) and oocyte chromosomes at various stages of condensation (C–E). A and B, ×310 approx.; C–E, ×2380 approx.
gradient), and were detected by fluorography. Fluorograms showing the size classes of proteins synthesized in oocytes isolated from mice 9, 12, 14, 15, 19, and 21 days of age are presented in Fig. 5A–H. Each lane of a given gel shows the electrophoretic profile of proteins synthesized in an individual oocyte. Therefore, a comparison of the lanes on a single gel should provide an estimate of the biochemical homogeneity of a population of oocytes of any particular size. Such a comparison indicates that, within the limits of resolution of our electrophoretic system, the oocytes are, indeed, behaving as a homogeneous population of cells. This is virtually the first demonstration using biochemical criteria which points to the homogeneous nature of isolated mouse oocytes cultured in vitro.

Oocytes isolated from mice younger than 15 days of age fail to undergo spontaneous meiotic maturation when cultured in vitro and remain arrested in dictysate of the first meiotic prophase; on the other hand, oocytes isolated from mice 15 days of age or older undergo meiotic maturation at a frequency which increases with the age of the mice (Fig. 3). Accordingly, a comparison of the electrophoretic profiles of proteins synthesized in oocytes isolated from 9-, 12-, 14-, and 15-day-old mice (Fig. 5A–D) with those from 19- and 21-day-old mice (Fig. 5E–H) could reveal not only

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Fig. 4. A, relationship between the number of mouse oocytes per assay and the amount of protein. Assays were performed as described in Materials and methods. Each point represents the average of 4 determinations and the range of values for these determinations is indicated by the bars. B, relationship between the amount of protein per oocyte and oocyte volume. Assays were performed as described in Materials and methods. Each point represents a single determination. Oocytes were obtained from adult, 7-, 14- and 21-day-old mice. The line shown is the theoretical line calculated for a 1:1 correspondence between the amount of protein per oocyte and oocyte volume.
Protein synthesis in mouse oocytes

changes associated with oocyte growth, but with meiotic maturation as well. An indication that the changes in the patterns of protein synthesis observed are, indeed, associated with oocyte growth and not with meiotic maturation, comes from our finding that the fluorograms of 19- and 21-day-old oocytes which have undergone GV breakdown are virtually identical to those of 19- and 21-day-old oocytes which remained in dictyate during the entire period of culture (Fig. 5E-H).

Examination of the fluorograms shown in Fig. 5A-H reveals 2 major kinds of changes which take place in protein synthesis during oocyte growth: (i) the appearance or disappearance of particular size classes of proteins, and (ii) an increase or decrease in the relative rates of synthesis of particular size classes of proteins. These changes are more readily seen in Fig. 6 which is a composite of our results. The following are examples of the most prominent changes in protein synthesis that occur during growth of mouse oocytes and which have been observed consistently in our experiments. Band 1 which is barely detectable at day 9, intensifies by day 12 and is resolved into a doublet. At day 14 this doublet is less clearly resolved and by day 15, and at all subsequent times, appears as a single band. The rate of synthesis of band 3 relative to that of band 2 increases throughout growth; band 3 is quite diffuse at day 9, but is resolved into a doublet by day 12. At day 14 this doublet is quite diffuse and by day 19 it has become a single discrete band nearly as intense as band 2. Band 4, which is barely detectable at day 9, increases in intensity throughout growth. The rate of synthesis of band 6 relative to that of band 5 decreases and approaches equality at about day 14. Finally, at day 9 the region between bands 7 and 10 is diffuse, although there is a suggestion of a band (band 9) just above band 10; by day 14 this region is more clearly resolved and bands 8 and 9 are observed. It should be noted that band 8 disappears during the first 5 h of meiotic maturation in oocytes which have undergone GV breakdown (Fig. 5F, H). Although other minor changes have been detected in our fluorograms, they have not been seen consistently and are, therefore, not discussed.

Protein synthesis during meiotic maturation of mouse oocytes

Analysis of protein synthesis using juvenile oocytes. In order to determine whether changes in protein synthesis take place during the process of meiotic maturation, we have compared [35S]methionine-labelled proteins of oocytes isolated from 12- and 15-day-old mice ('incompetent oocytes') with proteins of oocytes isolated from 19- and 21-day-old mice ('competent oocytes'). Oocyte proteins were labelled with [35S]methionine during a 20-h incubation period, were separated on SDS-polyacrylamide gels (8-3-15 % polyacrylamide, exponential gradient), and were detected by fluorography. Fluorograms showing the size classes of proteins which are synthesized in these oocytes are presented in Fig. 7. Once again, each lane of a given gel shows the electrophoretic profile of proteins synthesized by an individual oocyte and a comparison of the lanes on each gel demonstrates the homogeneity of the oocyte population. Since the oocytes from 12- and 15-day-old mice did not undergo meiotic maturation during the incubation period, while the oocytes from 19- and 21-day-old mice did, differences in their protein profiles could reflect changes in protein synthesis
The major reproducible changes in protein synthesis which take place during meiotic maturation are observed as a decrease in the intensity of band A and the appearance of bands B, C, D and/or D'. At this level of resolution it is not clear whether bands D and D' are related to the single band which migrates to a very similar position on the gel which is seen in oocytes arrested in meiosis. In addition to these changes, the region between bands A and B becomes significantly more diffuse during meiotic maturation.
Protein synthesis in mouse oocytes

Fig. 5. Separation of 35S-labelled proteins from juvenile mouse oocytes. The oocytes were collected, labelled with [35S]methionine for 5 h, processed, and the proteins from single oocytes were subjected to electrophoresis as described in Materials and methods. The SDS separating gel was 13 cm long; the stacking gel was 1 cm long. The gradient was a 9.5-15% exponential acrylamide gradient. A volume of 12 ml of 15% acrylamide was used in the bottom chamber of the gradient mixer; the total volume of the gel was 30 ml. Oocytes from 9-, 12-, 14-, 15-, 19- and 21-day-old mice were used for gels A-D and E-H, E, F both 19-day, G, H both 21-day. The samples electrophoresed in gels F and H were obtained from oocytes that had undergone GV breakdown; in all other cases, an intact GV was present. The lanes in gels A-H contained 5400, 6425, 4075, 4900, 12 850, 11 425, 9350, and 7750 cpm, respectively. The gels were exposed for a length of time calculated empirically according to the formula: 112 000/cpm per sample = days of exposure. The extent of development under these conditions was fairly uniform, though differences did occasionally occur.
Analysis of protein synthesis using adult oocytes. In order to determine when the changes in protein synthesis associated with meiotic maturation (Fig. 8) are initiated, we have compared the protein patterns of adult oocytes which were labelled with $[^{35}\text{S}]$methionine at different times during culture. These results are presented in

Fig. 6. This figure is a composite of the fluorogram shown in Fig. 5. Individual lanes from the gels in Fig. 5 were cut out and aligned next to each other to facilitate analysis of the qualitative changes in the patterns of protein synthesis that are associated with oocyte growth. The molecular weights were obtained by interpolation of a standard curve constructed with phosphorylase a (mol. wt 92,000), bovine serum albumin (66,000), ovalbumin (45,000), chymotrypsinogen (25,000), myoglobin (17,000), and cytochrome c (12,500). The numbers 1–10 refer to bands of a particular size class and are discussed in the text.

Fig. 9. It is clear that the major changes in protein synthesis take place following the breakdown of the GV (i.e. after the first 5 h of culture) up to the time of the formation of the metaphase I spindle. For example, it should be noted that the disappearance of band 8 (see Fig. 8) is the only reproducible change that occurs during the first
Protein synthesis in mouse oocytes

5 h of meiotic maturation. The increase in the relative rates of synthesis of bands B and C is most striking during the 5- to 10-h period of meiotic maturation, while band A decreases in intensity and bands D and/or D' appear during this time. During the 10-h to overnight period, each of these changes associated with meiotic maturation is further enhanced. These results indicate that the major changes in protein synthesis which are observed take place following GV breakdown. It should be noted that each lane seen in Fig. 9 is one of many such lanes on individual slab gels. Examination of each lane of a given gel reveals that every oocyte that had progressed to a specific stage of maturation during the labelling period exhibited the...
same electrophoretic pattern. Minor differences in the quantitative aspects of the patterns have been observed and are probably due to the asynchrony of meiotic maturation in vitro.

Fig. 8. This figure is a composite of the fluorograms shown in Fig. 7. Individual lanes from the gels in Fig. 7 were cut-out and aligned next to each other to facilitate analysis of the qualitative changes in the patterns of protein synthesis that are associated with meiotic maturation. The molecular weights were calculated as described in Fig. 6. The letters A–D refer to bands of a particular size class and are discussed in the text.

A comparison of the types of protein present in adult mouse oocytes as detected by [3H]dansylation with those that are synthesized during the first 5 h of meiotic maturation (Fig. 10) reveals that all of the abundant proteins present (e.g. structural proteins) are also synthesized. There are other proteins that are synthesized, but which are not detected by the [3H]dansylation procedure; presumably these have not ac-
Protein synthesis in mouse oocytes

Fig. 9. Separation of $^{35}$S-labelled proteins from adult mouse oocytes at several stages of meiotic maturation. The oocytes were collected, labelled with $[^{35}S]$methionine for the given time period, processed, and the proteins from single oocytes were subjected to electrophoresis as described in Materials and methods. A, the oocytes were labelled from 0 to 5 h and only those oocytes that had undergone GV breakdown were processed. B, the oocytes were cultured in medium from 0 to 5 h and those that had undergone GV breakdown were then cultured in the presence of $[^{35}S]$methionine for an additional 5 h before processing. C, oocytes were cultured in medium for 10 h and those that had undergone GV breakdown were cultured in the presence of $[^{35}S]$methionine for an additional 10 h (overnight, on) before processing. D, oocytes were cultured continuously in the presence of $[^{35}S]$methionine from 0 on (20 h) and those that had emitted a polar body were processed. Individual lanes from separate slab gels were cut out and aligned next to each other to facilitate analysis of the qualitative changes in the patterns of protein synthesis that occur during specific stages of meiotic maturation. Gels A–D had 8050, 5725, 10425, and 25000 cpm, respectively. Formation of the gradient gel was carried out as described in Fig. 5. The exposure times and molecular weight estimations were calculated as described in Fig. 5. A–D' refer to bands of a particular size class and are discussed in the text.
Fig. 10. Comparison of $^{35}$S-methionine-labelled mouse oocyte proteins. $A$, 30 adult mouse oocytes were dansylated and subjected to electrophoresis as described in Materials and methods. Approximately 300,000 cpm were applied to the gel which was exposed for 5 days. $B$, adult mouse oocytes were labelled for 5 h with $^{35}$S-methionine, processed, and subjected to electrophoresis as described in Materials and methods. Approximately 35,000 cpm were applied to the gel which was exposed for 2.5 days. Formation of the gradient gel was carried out as described in Fig. 7. Individual lanes from the separate slab gels were cut out and aligned next to each other to facilitate comparison. Under the conditions of dansylation, the relative mobilities of the dansylated proteins are not affected (Schultz & Wassarman, 1976b).
Protein synthesis in mouse oocytes

Fig. 11. Effect of drugs on meiotic maturation of mouse oocytes in vitro. Shown is the percentage of oocytes in dictyate (GV), in the circular bivalent stage (GVBD), or in metaphase II (PB) after culture for 16 h in medium or in the presence of dbcAMP (100 μg/ml), puromycin or Colcemid (10 μg/ml) or CCB (5 μg/ml). Light micrographs (Nomarski differential-interference optics) of oocytes are shown following in vitro culture under various conditions. × approx. 250 (metaphase I, × approx. 1000).
cumulated during oocyte growth to a level sufficient for detection by this method. A comparison of the [3H]dansylated proteins obtained with oocytes at dictyate and at metaphase II reveals the same electrophoretic patterns (not shown); therefore, although maturation bands B, C, D and D' appear, they are not accumulated to the level necessary for detection by this method.

**Effect of drugs on protein synthesis in mouse oocytes during meiotic maturation**

Meiotic maturation of mouse oocytes *in vitro* can be inhibited by several drugs at specific stages of nuclear progression (Wassarman et al. 1976). Oocytes are arrested at the GV stage by dibutyryl cyclic AMP (dbcAMP, 100 μg/ml), at the circular bivalent stage by either puromycin (10 μg/ml) or Colcemid (10 μg/ml), and at metaphase I by cytochalasin B (CCB, 5 μg/ml); emission of the first polar body is blocked by each of these drugs (Fig. 11). Since meiotic maturation of mouse oocytes *in vitro* is accompanied by distinct changes in the pattern of protein synthesis, especially during the period from the circular bivalent stage to metaphase II, we have examined the effect of these inhibitors of meiotic maturation on these changes.

Dibutyryl cyclic AMP, at a concentration of 100 μg/ml, is an effective inhibitor of meiotic maturation of mouse oocytes *in vitro* (Stern & Wassarman, 1973; Cho, Stern & Biggers, 1974; Wassarman & Turner, 1976; Wassarman et al. 1976); less than 5% of the oocytes placed in culture resume meiosis, as evidenced by the retention of an intact GV even after 16 h. The inhibitory effect of dbcAMP is reversible, although the degree of reversibility decreases over extended periods of culture. The results shown in Fig. 12 indicate that the major changes in protein synthesis which normally accompany oocyte maturation (Fig. 9) fail to occur when oocytes are cultured continuously in the presence of dbcAMP. For example, protein bands B, C, D and/or D' ('maturation bands') fail to appear, and bands A and 8 (Fig. 8) do not decrease in intensity. The electrophoretic profile observed with oocytes cultured in the presence of dbcAMP is virtually indistinguishable from that obtained with oocytes which fail to undergo spontaneous maturation in the absence of inhibitors. These results are apparently not simply due to the lack of polar body emission in the presence of dbcAMP, since oocytes which fail to emit a polar body under normal culture conditions (i.e. no inhibitor present) still exhibit those protein changes characteristic of meiotic maturation. Furthermore, addition of dbcAMP to the culture medium after GV breakdown has taken place (in the absence of dbcAMP) does not prevent the changes in protein synthesis associated with meiotic maturation from taking place. These results strongly suggest that dissolution of the oocyte's GV, which results in the mixing of nucleoplasm and cytoplasm, is a prerequisite for the changes in protein synthesis associated with meiotic maturation to take place.

Colcemid, at a concentration of 10 μg/ml, a dose sufficient to prevent the formation of the metaphase I spindle, blocks meiotic maturation at the circular bivalent stage (Fig. 11). On the other hand, in the presence of CCB (5 μg/ml), mouse oocytes resume meiosis, undergo nuclear progression to metaphase I with the formation of a spindle, and then arrest without emitting the first polar body (Fig. 11). The protein patterns shown in Fig. 12 indicate that the major changes in protein synthesis which
Protein synthesis in mouse oocytes

Fig. 12. Separation of $^{35}$S-labelled proteins from adult mouse oocytes cultured in the presence or absence of several inhibitors of meiotic maturation. The oocytes were collected, labelled with $[^{35}$S]methionine for 20 h, processed, and the proteins from single oocytes were subjected to electrophoresis as described in Materials and methods. A, oocytes that had not undergone spontaneous GV breakdown. B, oocyte cultured in the presence of dbcAMP (100 μg/ml). C, oocyte cultured in the presence of Colcemid (10 μg/ml). D, oocyte cultured in the presence of CCB (5 μg/ml). Individual lanes from separate slab gels were cut out and aligned next to each other to facilitate analysis of the patterns of protein synthesis. Gels A–D had 24,575, 29,850, 25,400 and 26,450 cpm, respectively. The exposure time and molecular weight estimates were calculated as described in Fig. 5. The formation of the gradient gel was as described in Fig. 5. A–D' refer to bands of a particular size class and are discussed in the text.
accompany oocyte maturation in the absence of inhibitors also take place in oocytes cultured continuously in the presence of either Colcemid or CCB. These results, when considered in light of the results of experiments carried out in the presence of dbcAMP, suggest that once mixing of the oocyte's nucleoplasm and cytoplasm occurs, a programme of changes in protein synthesis is initiated and that the progress of these changes is unaffected by incomplete nuclear progression.

Table 1. The effect of puromycin on meiotic maturation of mouse oocytes in vitro

<table>
<thead>
<tr>
<th>Culturing conditions*</th>
<th>% GVBD</th>
<th>% PB</th>
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<tr>
<td>O → ON</td>
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<td>P → ON</td>
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<td>O → 5 h; 5 h → ON</td>
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<td>P → 10 h; 10 h → ON</td>
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<td>O → 10 h; 10 h → ON</td>
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- Oocytes were cultured for various lengths of time in medium alone (M) or in medium containing puromycin (P, 10 μg/ml). In all cases examined, the presence of puromycin in the culture medium resulted in a greater than 95% inhibition of protein synthesis, as measured by the incorporation of [35S]methionine into TCA-insoluble material. In those cases which involved transfer of oocytes from puromycin-containing medium to medium alone, the oocytes were washed thoroughly with plain medium. After overnight (ON) culture the oocytes were examined microscopically and scored for the presence or absence of a germinal vesicle (GV) or polar body (PB). The data shown for each treatment represent the average of a minimum of 2 replicates, with a total of 100 or more oocytes.

The experiments described above have been extended to include an examination of the effect of puromycin on meiotic maturation of mouse oocytes in vitro. Puromycin, at a concentration of 10 μg/ml, dramatically reduces protein synthesis in mouse oocytes, but fails to block GV breakdown and chromosome condensation (Stern, Rayyis & Kennedy, 1972; Wassarman & Letourneau, 1976; Wassarman et al. 1976). Consequently, in the presence of puromycin, meiotic arrest takes place at the circular bivalent stage (Fig. 11). Since our experiments suggest that the period between the circular bivalent stage of meiosis and metaphase I is the time when changes in protein synthesis are initiated (Fig. 9, 12), we have examined the role of these changes using puromycin. Oocytes were cultured in the presence or absence of puromycin (10 μg/ml) for various periods of time and were then scored microscopically for GV breakdown and polar body formation. The results of these experiments, which are summarized in Table 1, lead to the following conclusions: (i) concomitant protein synthesis is not required for GV breakdown to take place; (ii) inhibition of protein synthesis during the first 5 h of meiotic maturation does not prevent the subsequent events associated with nuclear progression from taking place; and (iii) inhibition of protein synthesis...
Protein synthesis in mouse oocytes during the second 5-h period of meiotic maturation (circular bivalent stage to metaphase I) is sufficient to prevent the subsequent events associated with nuclear progression from taking place.

**DISCUSSION**

During the period of oogenesis following germ cell formation, the female gamete carries out 2 vital processes, meiosis and growth. Meiosis achieves at least 2 ends: a tremendous increase in genotypic variation due to crossing-over and recombination, beyond that introduced by fusion of the male and female gametes, and the production of a haploid gamete which permits the maintenance of the chromosomal constancy of a species. Growth of the female gamete, on the other hand, is associated with the accumulation and storage of materials which satisfy much of the nutritional, synthetic, energetic, informational, and regulatory requirements of the early embryo. Through the years, investigations carried out primarily with amphibians and echinoderms have established that the unfertilized egg possesses not only chromosomal DNA destined for the embryo, but also a large store of yolk, ribosomes, mitochondria, enzymes, histones, tubulin, regulatory factors, informational RNA, and other macromolecules, all of which are destined for utilization during early embryonic development (for review of a very extensive literature see Davidson, 1968, and Gurdon, 1974).

In this report we have dealt with certain of the qualitative and quantitative aspects of protein synthesis in the mammalian oocyte during its growth and during the process of meiotic maturation in vitro. These experiments were made possible, in large part, by the development of methods for the isolation of mouse oocytes at various stages of growth (Mangia & Epstein, 1975; Sorensen & Wassarman, 1976).

Electron microscopy has been used extensively to study the complex ultrastructural changes which occur during growth of the mouse oocyte (for a review of this literature, see Zamboni, 1970, 1972, 1974; Szollosi, 1972; Anderson, 1972). Among the ultrastructural changes observed during growth of the mouse oocyte are (i) the reorganization of the oocyte's endoplasmic reticulum and Golgi complex; (ii) the transformation of the configuration of the oocyte's mitochondria; (iii) formation of extensive 'lattice-like' structures, as well as cortical granules, in the oocyte's cytoplasm; and (iv) the development of microvilli over the surface of the oocyte concomitant with the formation of the zona pellucida around the oocyte's plasmalemma. During the mouse oocyte's growth phase its total volume increases nearly 40-fold, the volume of its nucleus increases six-fold, and the volume of its nucleolus increases 90-fold. Chiefly from autoradiographic and histochemical evidence it is known that the growing mouse oocyte actively synthesizes RNA and protein (Alfert, 1950; Austin & Braden, 1953; Baker, Beaumont & Franchi, 1969; Oakberg, 1968; Moore, Lintern-Moore, Peters & Faber, 1974; Wassarman & Letourneau, 1976b), and recent studies carried out by Mangia & Epstein (1975) on isolated mouse oocytes of different sizes have shown that the levels of 2 enzymes, glucose-6-phosphate dehydrogenase and lactate dehydrogenase, increase throughout most of the growth period and are closely related to cell volume. All of these studies indicate that, like oocytes from lower animal
species, mouse oocytes are metabolically very active during their growth phase in preparation for early embryogenesis.

Our measurements represent the first determination of the actual amounts of protein present in mouse oocytes at various stages of growth. It was found that over the range of oocyte diameters examined, the amount of TCA-insoluble protein per oocyte is linearly related to oocyte volume, with each doubling of volume accompanied by a doubling in the amount of protein. In this context, Mangia & Epstein (1975) reported that the specific activity (expressed as units/oocyte volume) of glucose-6-phosphate dehydrogenase (G6PDH) remained constant, while that of lactate dehydrogenase (LDH) increased, as the diameter of mouse oocytes increased from 40 to 85 \( \mu m \). Since we find that the amount of protein increases linearly with oocyte volume, then it can be assumed that the specific activity of G6PDH, expressed as units/mg protein, remains constant, whereas, the specific activity of LDH increases. This implies that G6PDH accumulates at a constant rate during oocyte growth, while the net synthesis of LDH actually increases as a function of oocyte growth. To ascertain whether similar trends occur for other proteins of a particular size class, we have analysed, using SDS-polyacrylamide gel electrophoresis, the patterns of \(^{[35]}\)S\)-methionine-labelled proteins synthesized in single oocytes at various stages of growth. Oocytes isolated from juvenile mice of a given age have, to within the limits of resolution of our electrophoresis system, the same qualitative and, to a large extent, the same quantitative patterns of protein synthesis. This is the first biochemical demonstration of the homogeneity of a given population of isolated mouse oocytes; the only evidence described previously had been morphological, i.e. oocytes isolated from juvenile mice of a given age are of nearly uniform diameter (Sorensen & Wassarman, 1976).

Accompanying oocyte growth is a progressive change in certain qualitative and quantitative aspects of protein synthesis in the oocyte. It should be noted that a similar spectrum of molecular weight size classes of proteins is present in both juvenile and adult oocytes and that the major changes that occur during oocyte growth are quantitative in nature, involving changes in the relative rates of synthesis of particular size classes of proteins. Most of these changes are completed by day 15 when some oocytes are competent to resume meiosis spontaneously when cultured in vitro. To date, it is not known if these changes are directly responsible for, or are the consequence of, the acquisition of meiotic competence. Since O'Farrell (1975) has shown that most protein bands of a particular molecular weight on SDS gels are composed of many types of proteins of similar molecular weight but different charge, it is not known if the changes in relative rates of synthesis of a particular size class are due to changes in the rate of synthesis of one or more proteins already present in the oocyte or if the change represents the synthesis of a new protein(s) not already present in the oocyte.

It is well documented that protein synthesis is required for the successful completion of meiotic maturation of oocytes from a variety of species, including echinoderms (Brachet & Steinert, 1967; Zampetti-Bosseler, Huez & Brachet, 1973; Houk & Epel, 1974), amphibians (Dettlaff, Nikitina & Stroeva, 1964; Dettlaff, 1966; Brachet,
Protein synthesis in mouse oocytes

1967; Schuetz, 1967; Smith & Ecker, 1969; Merriam, 1972; Brachet et al. 1974), and mammals (Jagiello, 1968; Stern et al. 1972; Wassarman & Letourneau, 1976a). Extensive in vitro studies carried out by Smith & Ecker (1969) and Ecker & Smith (1971) using oocytes isolated from Rana pipiens, have shown that there are changes in both the rate of protein synthesis and the nature of proteins synthesized during progesterone-induced meiotic maturation (see also Smith & Ecker, 1970; Smith, 1972, 1975). Furthermore, these investigators provided strong evidence to support the contention that, in the amphibian oocyte, the morphological events associated with meiotic maturation are under cytoplasmic, not nuclear, control. In order to test the hypothesis that the programme of protein synthesis which functions during meiotic maturation is directed by templates already present in the oocyte's cytoplasm, Smith & Ecker compared the rate of protein synthesis and the nature of the proteins synthesized in nucleated oocytes, with enucleated oocytes, after exposure to progesterone in vitro. They found that those qualitative and quantitative changes in protein synthesis which normally take place in the amphibian oocyte following stimulation with progesterone, also take place in enucleated oocytes. Based on the results of their experiments these investigators concluded that the mixing of the amphibian oocyte's nucleoplasm and cytoplasm is not essential for the successful completion of meiotic maturation or, for that matter, for the activation of the programme of protein synthesis which accompanies nuclear progression.

Unlike the results obtained with amphibian oocytes, our results indicate that the initiation of those changes in protein synthesis which characterize meiotic maturation of mammalian oocytes is dependent upon the mixing of the oocyte's nucleoplasm and cytoplasm. Whether these changes are due to actual differences in the kinds of protein synthesized following GV breakdown, or due primarily to changes in the relative rates of synthesis of the same group of proteins, cannot be unequivocally determined from our data. Regardless of which situation is the actual case, the overall pattern of protein synthesis changes significantly during meiotic maturation (Figs. 8, 9). Failure of the mouse oocyte's GV to break down, either in medium alone or in the presence of dbcAMP, prevents all of the changes in protein synthesis which normally accompany meiotic maturation (Fig. 12). On the other hand, once the GV has broken down, the inhibition of subsequent morphological events, such as spindle formation and/or polar body emission, apparently does not affect the programme of changes in protein synthesis which has been set in motion (Fig. 12). The importance of the relationship between these changes and the successful completion of meiotic maturation is indicated by the effectiveness of puromycin in blocking nuclear progression when it is applied at the time that these changes are initiated (between circular bivalent stage and metaphase I) (Table 1). However, puromycin has no effect on the rate of resumption of meiosis, only on the extent of maturation, since nuclear progression stops at the circular bivalent stage when oocytes are cultured continuously in the presence of the drug (Fig. 11).

The percentage of juvenile oocytes capable of GV dissolution and polar body emission increases dramatically during oocyte growth (Fig. 3); in a sense, these oocytes are like mutants which are blocked at discrete stages of meiosis. We
have found that the pattern of protein synthesis in juvenile oocytes which are unable to undergo GV breakdown (less than 15 days of age) is very similar to that of an adult oocyte possessing an intact GV. The pattern of protein synthesis in oocytes from slightly older juvenile mice, which are able to undergo GV breakdown but cannot emit a polar body, is virtually indistinguishable from that of fully grown oocytes which are capable of polar body emission. These results, together with the results of experiments carried out with oocytes cultured in the presence of drugs, suggest that the changes in the pattern of protein synthesis are triggered by nuclear membrane breakdown and not by the oocyte's recognition of a discrete stage of nuclear progression, such as spindle formation or migration.

The results described above suggest that, unlike amphibian (Dettlaff, 1966; Schuetz, 1967; Smith & Ecker, 1969) and starfish oocytes (Kanatani, 1975), fully grown mouse oocytes are poised to resume meiosis, so that no new proteins need be synthesized for GV breakdown and chromosome condensation to take place spontaneously in vitro. Such a situation would be consistent with our findings concerning the inhibitory effect of dbcAMP on meiotic maturation in vitro. The fact that dbcAMP reversibly inhibits the onset of nuclear progression in isolated mouse oocytes may be related to the proposed regulatory role for cAMP during the mitotic cell cycle (Burger, Bombik, Breckenridge & Sheppard, 1972; Willingham, Johnson & Pastan, 1972). In this connexion it has been shown that elevated cAMP levels inhibit cell multiplication (Johnson & Pastan, 1972; Bombik & Burger, 1973), that the concentration of cAMP is lower in fast-growing than in slow-growing cells (Sheppard, 1971), that lowered cAMP levels occur during G1 and mitosis relative to S-phase (Willingham et al. 1972; Burger et al. 1972), and that exogenously supplied dbcAMP results in termination of the cell cycle in G1-phase (Zimmerman & Raska, 1972; Willingham et al. 1972; Kaukel, Fuhrmann & Hilz, 1972). It is tempting to suggest that fluctuations in intracellular cAMP levels may play an analogous role in the mammalian oocyte, with elevated intracellular levels of cAMP maintaining mouse oocytes in dictyate of the first meiotic prophase from several weeks to more than a year prior to their undergoing ovulation or atresia. Transfer of oocytes to in vitro conditions may result in a lowering of the intracellular level of cAMP. This in turn could lead to a decreased level of phosphorylation (via cAMP-dependent kinases and/or phosphatases) (Bitensky & Gorman, 1973) and, consequently, to activation of factors already present in the oocyte which are involved in the early events of meiotic maturation (e.g. GV membrane dissolution). Once meiotic maturation is under way, as evidenced by GV dissolution, neither the process of nuclear progression nor the changes in protein synthesis associated with it are affected by dbcAMP. The proposed role for cAMP in the control of meiotic maturation of mouse oocytes may be extended to oocytes from lower species, such as amphibians and echinoderms, despite apparent differences in certain aspects of the control of meiosis. Unlike mouse oocytes, oocytes isolated from frogs and starfish do not undergo spontaneous meiotic maturation in vitro, but instead require stimulation by a specific hormone which interacts with the oocyte's plasmalemma. In addition, the formation of a hormone-receptor complex may itself involve and/or lead to a protein synthesis dependent step(s) which
Protein synthesis in mouse oocytes culminates in the lowering of the intracellular level of cAMP and the onset of meiotic maturation. In this connexion, it should be noted that Smith (1975) has reported that neither cAMP, its dibutyryl analogue, nor theophylline, either singly or in combination, mimicked the effects of progesterone on amphibian oocytes, regardless of whether they were injected into the oocytes or were present in the culture medium; similar findings have been reported by Brachet et al. (1974). The latter observations are consistent with our scheme for the induction of meiotic maturation, since it would require that GV dissolution in the mouse oocyte be dependent at no time upon protein synthesis; such is not the case with oocytes from frogs and starfish. These differences may be reconciled if one considers that the mouse oocyte has proceeded beyond or bypassed the step of hormonal stimulation and the associated dependence upon protein synthesis. The latter can be illustrated as shown in Fig. 13. In this scheme (A) is equivalent to the intracellular level of cAMP, reaction (1) and/or (2) requires concomitant protein synthesis, and B and B' represent the inactive and active forms, respectively, of a factor which is involved in the initiation of GV breakdown; furthermore, it is assumed that high levels of cAMP favour the formation of B, whereas, low levels of cAMP shift the equilibrium towards B'. Accordingly, in frog or starfish oocytes the interaction of progesterone or 1-methyladenine with a membrane receptor would result in a lowering of the intracellular level of cAMP which would in turn lead to the initiation of meiotic maturation. We assume that mouse oocytes have proceeded beyond or have bypassed steps (1) and (2) and that the mere removal of fully grown oocytes from their follicles triggers a lowering of cAMP levels and the onset of meiotic maturation.

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