ABSTRACT OF THESIS

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Title of Thesis ..... Ultrastructural and cytochemical studies of oogenesis in Drosophila melanogaster with special reference to the formation of yolk.

Ovarian chambers of Drosophila melanogaster in previtellogenic and vitellogenic stages have been studied by high resolution electron microscopy and various methods of cytochemistry. The development of nurse cells, follicle cells and the oocyte was examined in each stage and the interrelationships between these cells were analyzed mainly with a view to clarifying the process of yolk formation.

In previtellogenic chambers, the cytoplasm of the oocyte and nurse cells contains similar organelles although in the latter the organelles are fewer in number. Among the organelles common to both the cell types there are cytolysosomes of variable size and shape. Those in the oocyte are most probably derived from the nurse cells by way of the ring canals.

The presence of cytolysosomes in nurse cells is an indication that focal degradation of cytoplasmic organelles occurs prior to the initiation of active vitellogenesis. These cytolysosomes exhibit in part a yolk-like appearance. This observation confirms earlier findings of other workers that oocytes in some insects contain yolk-like material prior to active vitellogenesis. The present finding lends no support to Mahowald's (1972) assumption that previtellogenic oocytes synthesize yolk proteins in situ.

The beginning of vitellogenesis is marked by the appearance of a number of ultrastructural elements in the cortical ooplasm. These are the pits of the oolemma, the coated vesicles and the tubules. A fuzzy layer of carbohydrate material (corresponding to what has been called the cell coat in the literature) appears to be restricted in Drosophila oocyte to the pit regions of the oolemma. Such fuzzy layer also lines the inside of the vesicles and tubules. In the cortical ooplasm there are also found a number of small yolk platelets, while in deeper regions, the yolk platelets appear progressively larger in size. The various entities of the cortical ooplasm are interpreted as part of the mechanism (pinocytosis) involved in the uptake of proteins from the haemolymph.

The mature yolk platelets (alpha 1) of stage 9-12 ovarian chambers consist of (a) the main body, (b) the superficial layer with an associated body embedded therein, and (c) the limiting membrane. In stage 13-14 chambers, the number of alpha-1 yolk platelets decreases progressively as the number of alpha-2 yolk platelets increases. A direct transformation of the alpha-1 type yolk platelets into the alpha-2 seems likely. This transformation also coincides with the appearance of glycogen in close association with the yolk platelets. The glycogen particles later aggregate in the form of beta-spheres. The occurrence of catalase activity in stage 13-14 oocytes indicates that glycogen formation may occur by gluconeogenesis.

The follicle cells which surround the nurse cells and

Use other side if necessary.

the oocyte are small and of uniform size until vitellogenesis begins (stage 8). Following the initiation of vitellogenesis, the follicle layer comes to contain two types of cells: the columnar follicle cells in the region overlying the oocyte and the thin follicle cells over the nurse cells. During stages 9-10, the columnar follicle cells are involved in the formatio of the vitelline membrane, while from stage 11 to 13, these cells produce the material of the endochorion. The columnar follicle cells of stages 9-13 contain a number of Golgi apparatus with several electron dense granules (the vitelline and endochorion precursors). Following the completion of the endochorion, a third layer - the exochorion - is deposited. The method of origin of this layer is unknown. There is no evidence in the present study to indicate that the columnar follicle cells contribute any material - enzymes or yolk precursors - to the enlarging ooplasm.

Autoradiographic studies show that the rate of incorporation of labelled uridine in nurse nuclei is low in previtellogenic chambers, while it is very high in nurse nuclei of stage 9-10 chambers. My observations indicate that the ribosomes of the oocyte are derived mainly from the nurse cells. The presence of RNA and proteins in the amorphous material of the perinuclear cytoplasm in nurse cells has been demonstrated by the use of autoradiography Enzyme extraction experiments with RNase and selective staining technique for ribonucleoproteins have further shown that the amorphous material and their later manifestation - the fuzzy bodies - contain chromatin-like material. The presence of DNA within these bodies requires confirmation by more direct methods. The fuzzy bodies eventually reach the ooplasm after passing through the ring canals.

Evidence from incorporation studies with 3-H-lysine indicate that in vitellogenic chambers, the columnar follicle cells are the only cells which are involved in the formation of the egg coverings. A distinct labelling of the yolk platelets is obtained only in <u>in vivo</u> conditions and with a certain time lapse after the injection of the tracer. This finding indicates that yolk proteins are synthesized in some extraovarian tissue and taken into the ovary. Various considerations discussed in this thesis make Cummings and King's (1970) view on the origin of yolk platelets untenable.

It has been established that extracellular tracers such as ruthenium red and lanthanum nitrate find free access to the oocyte surface by passing through the follicle layer. The possible sites of accumulation of blood proteins within vitellogenic oocytes as well as the kinetics of pinocytotic uptake have been studied by employing peroxidase as an extracellular tracer. All structural entities of the cortical ooplasm, i.e. coated vesicles, tubules and small yolk platelets, are labelled with peroxidase. Further, when oocytes are fixed at longer intervals of time after injection of the tracer, a progressively larger number of yolk platelets comes to contain peroxidase.

The cytochemical results of the Gomori method indicate that acid phosphatase occurs in the early phases of yolk formation. It is also shown that the enzyme is transferred to the yolk platelets by means of Golgi derived vesicles which fuse with the limiting membrane of the yolk platelets This method of transfer of acid phosphatase in the yolk platelets is similar to that generally held for the origin of lysosomal enzymes in phagolysosomes It is suggested that the acid phosphatase (and probably other hydrolytic enzymes) in the yolk platelets may be involved in a selective hydrolysis of non-yolk proteins taken up into the oocyte together with specific yolk precursors.

ULTRASTRUCTURAL AND CYTOCHEMICAL STUDIES OF OOGENESIS IN *DROSOPHILA MELANOGASTER* WITH SPECIAL REFERENCE TO THE FORMATION OF YOLK.

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I. SUMMARY

Ovarian chambers of *Drosophila melanogaster* in previtellogenic and vitellogenic stages have been studied by high resolution electron microscopy and various methods of ultrastructural cytochemistry. The development in nurse cells, follicle cells and the oocyte were examined in each stage and the inter-relationships between these cells were analyzed mainly with a view to clarifying the process of yolk formation.

The oocyte nucleus in stage 1-2 ovarian chambers exhibits a dispersed chromatin with recognizable synaptonemal complexes and a rounded nucleolus with both fibrillar and granular components. From stage 3 onwards, the chromatin of the oocyte nucleus condenses into a compact ring-shaped body called the karyosome. At the same time, the nucleolus of the oocyte looses the peripheral granular constituents and comes to form a rounded fibrillar body termed the endobody.

In previtellogenic chambers, the cytoplasm of the oocyte and nurse cells contains similar organelles although in the latter, the organelles are fewer in number. Apart from typical mitochondria, free ribosomes and Golgi apparatus, the other organelles present in both cell types are rickettsia-like bodies and several cytolysosomes of variable size and shape. The cyto-lysosomes in the oocyte are most probably derived from the nurse cells by way of the ring canals.

The presence of cytolysosomes in nurse cells is an indication that focal degradation of cytoplasmic organelles occurs prior to the initiation of active vitellogenesis. These cytolysosomes exhibit in parts a

yolk-like appearance. This observation confirms earlier findings of other workers that oocytes in some insects contain yolk-like material prior to active vitellogenesis. The present evidence lend no support to Mahowald's (1972a) assumption that previtellogenic oocytes synthesize yolk proteins *in situ*.

The beginning of vitellogenesis is marked by the appearance of a number of ultrastructural elements in the cortical ooplasm. These are the pits of the oolemma, the coated vesicles and the tubules. A fuzzy layer of carbohydrate material (corresponding to what has been called the cell coat in the literature) appears to be restricted in *Drosophila* oocyte to the pit regions of the oolemma. Such fuzzy layer also lines the inside of the vesicles and tubules. In the cortical ooplasm there are also found a number of small yolk platelets, while in deeper regions, the yolk platelets appear progressively larger in size. The various entities described in the cortical ooplasm are interpreted as part of the mechanism involved in the uptake of proteins from the haemolymph. It is also assumed that the small yolk platelets of the cortical ooplasm originate by fusion of coated vesicles and tubules.

The mature yolk platelets (alpha-1 yolk platelets) of stage 9-12 ovarian chambers consist of (a) the main body, (b) the superficial layer with an associated body therein, and (c) the limiting membrane. In stage 13-14 ovarian chambers, the number of alpha-1 yolk platelets decreases progressively as the number of alpha-2 yolk platelets increases. A direct transformation of the alpha-1 type yolk platelets into the alpha-2 seems likely. This transformation of the yolk platelets also coincides with

the appearance of glycogen in close association with the yolk platelets. The glycogen particles later aggregate in the form of beta-spheres. The occurrence of catalase activity in stage 13-14 oocytes indicates that glycogen formation may occur by gluconeogenesis.

In nurse nuclei of previtellogenic chambers at stages 1 to 4, the chromatin material is in the form of large diffuse masses which correspond to individual chromosomes, while the nucleolus is rather compact and centrally located. As ovarian chambers approach vitellogenesis and nurse nuclei enlarge progressively in size, the chromatin disperses throughout the nucleus and becomes variously intertwined with the nucleolar masses. By the time the nurse nuclei, especially the posterior ones, reach their maximum dimensions (stage 10), the nucleolar masses come to occupy most of the volume of the nucleus and form a thick network close to the inner surface of the nuclear envelope.

The nurse nuclei in stage 11-13 ovarian chambers undergo structural modifications which are indicative of degeneration. This occurs through a progressive breaking up of the chromatin material and a loosening of the nucleolar masses. Further, the rupture of the nuclear envelope enables the nuclear contents to escape into the cytoplasm of the nurse cell.

From early previtellogenic stages amorphous material is present in the perinuclear cytoplasm of every nurse cell. In ovarian chambers approaching vitellogenesis, the amorphous material appears to move away from the perinuclear cytoplasm and gradually transform into fuzzy bodies. These eventually reach the ooplasm after passing through

the ring canals.

In each ovarian chamber, a layer of follicle cells surrounds the nurse cells and the oocyte. This layer maintains an uniform thickness all around the chamber until vitellogenesis begins (stage 8). Following the initiation of vitellogenesis the follicle layer comes to contain two types of cells; the columnar follicle cells in the region overlying the oocyte and the thin follicle cells over the nurse cells. During stages 9-10, the columnar follicle cells are involved in the formation of the vitelline membrane, while from stage 11 to 13, these cells produce the material of the endochorion. The cytoplasm of the columnar follicle cells of stage 9-13 ovarian chambers contains a number of Golgi apparatus with several electron dense granules within (the vitelline and the endochorion precursors). Both the vitelline and the endochorion precursors reach the basal portion of the columnar follicle cells from where they are released into the interspace between the follicle layer and the oocyte. Following the completion of the endochorion, a third layer, the exochorion is laid down. The method of origin of this layer is unknown. There is no evidence in the present study to indicate that the columnar follicle cells contribute any material - enzymes or yolk precursors - to the enlarging ooplasm.

Autoradiographic studies show that the rate of ^{3}H-uridine incorporation in nurse nuclei is low in pre-vitellogenic chambers while is very high in nurse nuclei of stage 9-10 ovarian chambers. A rate of transcription comparable to that of nurse nuclei is also detectable in follicle cell nuclei of corresponding stages. However, a

greater output of RNP molecules may be expected from nurse nuclei in view of their greater degree of ploidy than the follicle cell nuclei. My observations indicate that the ribosomes of the oocyte are derived mainly from the nurse cells.

The presence of RNA and proteins in the amorphous material of the perinuclear cytoplasm in nurse cells has been demonstrated by the use of autoradiography. Enzyme extraction experiments with RNase and selective staining technique for ribonucleoproteins have further shown that the amorphous material and the fuzzy bodies contain chromatin-like material. The presence of DNA within these bodies requires confirmation by more direct methods.

Evidence from incorporation studies with ^{3}H-lysine indicate that in previtellogenic chambers, the columnar follicle cells and the region of the various egg coverings are highly labelled within an hour of incubation in the tracer. The observations confirm that columnar follicle cells are the only cells in the egg chamber which are involved in the formation of materials which make up the egg coverings. A distinct labelling of the yolk platelets is obtained only in <u>in vivo</u> conditions and with a certain delay after the injection of the tracer. This finding along with several others from various techniques is taken to indicate that yolk precursors (proteins) are synthesized in an extraovarian tissue and taken into the oocyte by pinocytosis at specialized regions of the oolemma, i.e. the pits.

The results of the electrophoretic and isoelectro-focusing analyses indicate that a number of proteins fractions have similar electrophoretic mobilities and pI values in

the ovary and the haemolymph. When fat body extracts are analyzed by these techniques a protein composition similar to that of the ovary is also evident. However, the fact that incorporation of labelled aminoacids into fat bodies is negligible makes it unlikely that synthesis of yolk proteins occurs in this tissue. Various considerations discussed in this thesis make Cummings and King's (1970) views on the origin of yolk platelets in Drosophila extremely unlikely.

The possible route of transport of yolk precursors into vitellogenic oocytes has been revealed by the use of electron opaque extracellular tracers such as ruthenium red and lanthanum nitrate. It has been established that molecules of the size of these tracers find free access to the oocyte surface by passing through the basement lamina and the intercellular spaces of the follicle layer. The possible sites of accumulation of blood proteins within vitellogenic oocytes as well as the kinetics of pinocytotic uptake have been studied by employing peroxidase as an extracellular tracer. All structural entities of the cortical ooplasm, i.e. the coated vesicles, tubules and small yolk platelets are labelled with peroxidase. Further, when oocytes are fixed at longer intervals of time after injection of the tracer, a progressively larger number of yolk platelets come to contain peroxidase.

The cytochemical results of several techniques indicate that carbohydrates occur in several locations in the vitellogenic chambers. These are, (a) the follicle-oocyte border, which comes to contain PTA-positive glycoproteins during the period of active vitellogenesis, (b)

the fuzzy layer of the pits and coated vesicles in the cortical ooplasm, to which concanavalin and PTA-glycoproteins bind, and (c) the superficial layer of the yolk platelets with the embedded associated body.

The cytochemical results of the Gomori method indicate that acid phosphatase occurs in the early phases of yolk formation. It is also shown that the enzyme is transferred to the yolk platelets by means of Golgi - derived vesicles which fuse with the limiting membrane of the yolk platelets. This method of transfer of acid phosphatase to the yolk platelets is similar to that generally held for the origin of lysosomal enzymes in phagolysosomes. It is suggested that acid phosphatase (and probably other hydrolytic enzymes) in the yolk platelets may be involved in a selective hydrolysis of non-yolk proteins taken up into the oocyte along with specific yolk precursors.

II. REVIEW OF THE LITERATURE

Within the class Insecta, there are two major types of oogenesis depending on the nature of the mechanism used to synthesize RNA. In the panoistic ovary, the oocyte nucleus is the source of all RNA contained within the mature egg. In the meroistic ovary, on the other hand, the RNA needed for the egg is synthesized by a cluster of nurse cells which have a common origin with the oocyte. The meroistic type may be further divided into (a) telotrophic ovary, in which the nurse cells are localized at the anterior end of the ovariole, and (b) polytrophic ovary in which a group of nurse cells is intimately associated with the growing oocyte within the developing chamber.

Each ovary in Drosophila melanogaster is composed of a variable number of polytrophic ovarioles which are held together by a common peritoneal sheath. A thin epithelial sheath is also present around each ovariole. In both the epithelial and peritoneal layers the cells possess an internal network of myofilaments which make them capable of rhythmic contractions. At the base of the ovary the two layers fuse and become continuous with the lateral oviducts.

An ovariole is composed of the germarium at the anterior end and this is followed by a series of chambers that follow one another in a developmental sequence: the posterior chambers are more advanced in development and of larger size than the anterior ones. The germarium as well as the developing chambers are covered with an acellular layer which has been termed tunica propria (King, 1970) or basement lamina (Bonhag and

Arnold, 1961), and adjacent chambers are connected through interfollicular stalks.

King (1970) has reported that during the period of maximum egg production, the overall ovarian morphology remains largely unchanged. This indicates that the development of the egg chambers is continuous and fairly well coordinated with the formation of cystocytes in the germarium.

The whole course of ovarian development has been divided into fourteen different stages by Cummings and King (1969) who used the following criteria to distinguishing the stages, (a) the dimensions and position of the egg chambers within the ovariole, (b) the size of the oocyte relative to the nurse cells, (c) the differing nuclear morphology of the oocyte and the nurse cells, (d) the presence or absence of egg coverings, and, (e) the nature of cytoplasmic organelles and inclusions present in the oocyte.

The first seven chambers are previtellogenic stages: stage 8 marks the beginning of vitellogenesis and between this and stage 12 the oocyte grows at a remarkably fast rate. During stages 13 and 14 there is no appreciable increase in the volume of the oocyte. During stage 9, the formation of the vitelline membrane begins by apposition of vitelline membrane precursors in the interspace between the surface of the oocyte (oolemma) and the basal membrane of the follicle cells. When this is completed, by about stage 11, another layer, viz. the endochorion is deposited. At this time the nurse cells begin to degenerate and by the end of stage 13 they have disappeared.

The relative duration of each stage in oogenesis of Drosophila melanogaster has been estimated by David and Merle (1968) who found that previtellogenic stages last comparatively longer than the vitellogenic ones.

The cluster of two or three oogonia at the anterior portion of each germarium is thought to be the stem cell line which gives rise to the cystocyte groups situated in the intermediate region of the germarium (Koch and King, 1966). Oogonia differ from cystocytes in that they divide indefinitely and by means of complete cytokinesis so as to produce a cystoblast and an oogonium at each division. On the other hand, cystoblasts undergo only four consecutive mitoses and with incomplete cytokinesis to result in the formation of a 16-cell cluster. Koch and King (1966) speculated that the oogonia are prevented from being involved in a cystoblast pattern of differentiation because they adhere to the apical end of the germarium and only those cells which migrate posteriorly become cystoblasts.

The pattern of cellular interconnections which comes about within the cluster of sixteen cells is that which one would expect on the basis of four consecutive divisions with incomplete cytokinesis. The cellular bridges formed in the process have been termed ring canals (King, 1970) or fusomes (Meyer, 1961), and are thought to be formed in association with the microtubules of the mitotic spindle which remain after the completion of mitosis. A stream of cytoplasmic organelles passes from one cell to another through the ring canals. This movement appears to be accomplished by the rhythmical

contractions posteriorwards of the epithelial and peritoneal sheaths (Mahowald, 1972a).This, however, does not seem to be the only way of transferring material between contiguous cells, since Woodruff and Telfer (1973) have demonstrated that in in vivo conditions an electrophoretic diffusion can occur in the chamber through the ring canals. Meyer (1961) had previously reported that a ring canal is a dynamic structure which is capable of regulating the flow of material from nurse cells to the oocyte. This regulation, however, would require further specializations that have not been observed so far in the ultrastructure of the canal rim in Drosophila (Mahowald, 1972a).

Intercellular communications have been reported in a wide variety of organisms and most commonly in germ cells, as for instance in Dytiscus marginalis (Steinert and Urbani, 1969), in chicken (Skalko et al., 1972), in Habrobracon juglandis (Cassidy and King, 1969), in Bombyx mori (King and Akai, 1971) and in Drosophila (Koch and King, 1969). All these authors agree with Fawcett's (1961) interpretation that intercellular bridges ensure synchrony in the development of the connected cells.

From a three-dimensional reconstruction of the 16-cell clusters, it appears that two of the cells have four ring canals, another two have three ring canals, four others have two ring canals and that the remaining eight possess one ring canal each. Koch and King (1969) observed that the oocyte is always one of the two cells possessing four ring canals. They speculated that the presence of the four ring canals represents the factor

which induces two out of the sixteen cells to diverge from nurse cell metabolism and become pro-oocytes.

Synaptonemal complexes appear only in cells possessing four ring canals. Koch et al. (1967), however, noticed that by the time the stage 1 chamber is formed only one of the pro-oocytes maintains the synaptonemal complexes. These authors assumed that the cell with the largest part of its plasma membrane in contact with the ovarlying follicle cells becomes the definitive oocyte. The validity of this interpretation, however, has more recently been questioned by Mahowald and Strassheim (1970). These authors suggested that the larger area of contact between the oocyte surface and the follicle layer, rather than being a causal factor in the differentiation of the oocyte, may simply be a consequence of the greater accumulation of material in this cell.

The meiotic behaviour of the oocyte nucleus in Drosophila melanogaster has recently been examined at the light microscope level by Davring and Sunner (1973). The oocyte nucleus differs from nurse cell nuclei in that its chromosomes condense as early as stage 3 into a compact structure, the karyosome (King et al., 1956). The fact that the chromosomes of the oocyte are condensed into a single dense body is taken as an indication that they are not actively engaged in RNA synthesis, at least not to the same extent as the lampbrush chromosomes in panoistic ovaries (Telfer, 1965). At stage 14, the nuclear envelope of the oocyte nucleus breaks down and the nuclear content is released into the cytoplasm as a karyosphere. The synaptonemal complexes also become packed within the karyosome of the oocyte, and by stage 7 the complexes

are no longer visible. King (1970) has assumed that the period when the complexes are visible represents the zygotene stage and that from stage 7 onwards the oocyte nucleus is in a modified diplotene stage.

Previtellogenic stages are characterized by a similar rate of growth in all sixteen cells (Cummings and King, 1969). Apart from the usual organelles like mitochondria and free ribosomes, the cytoplasm of the oocyte and nurse cells during this period contain A, B, and C bodies. The A bodies probably correspond to the Epsilon granules of Ullmann (1965) which have been identified later as rickettsia-like bodies by King (1970). The B and C bodies are reported to be PAS-positive organelles with a multiple membranous structure (King and Mills, 1962). The C bodies are assumed to be derived from the aggregation of B bodies. Later, King (1970) has suggested that B and C bodies are lysosomes or lysosomal derivatives. A number of authors (Steinert and Urbani, 1969; Anderson, 1969; Mahowald, 1972a)have also noticed the presence of several multivesicular bodies in the ooplasm of insects just prior to active vitellogenesis.

During the period of active vitellogenesis, the plasma membrane of the oocyte presents numerous infoldings and microvilli. Several ultrastructural entities have also been described in the subcortical ooplasm of vitellogenic oocytes by a number of workers (Roth and Porter, 1964; Anderson, 1969). Roth and Porter (1964) have suggested that in Aedes aegypti the proteoid body of the mature yolk spheres, which appear at the beginning of vitellogenesis is formed through fusion of progressively larger vesicles of the cortical ooplasm. According to

these authors, fusion also occurs between mature yolk spheres as indicated by the differently oriented profiles of the crystalline cores of some of the large yolk spheres of Aedes aegypti.

Vesicles have also been observed in the cortical ooplasm of either pre- or vitellogenic oocytes of Periplaneta americana (Anderson, 1964). Their role in the process of uptake of yolk precursors was further demonstrated by using peroxidase as an extracellular tracer.

In Drosophila melanogaster, Mahowald (1972b) has reported that the morphology of the oocyte surface at the time of active vitellogenesis closely resembles that previously described in Aedes aegypti and Periplaneta americana. Although female-specific proteins have not yet been demonstrated in Drosophila, Mahowald (1972a) has suggested that, on the basis of ultrastructural similarities between the oocytes of Drosophila and those of other insects, uptake of proteins by pinocytosis is a method probably common to all insects.

Up till now there has been only one detailed study (Beams and Kessel, 1963) of an oocyte which is capable of synthesizing its own yolk proteins, i.e., that of crayfish. The oocyte in this instance has an extensive endoplasmic reticulum which contains densely packed material almost undistinguishable from yolk. This morphological analysis led Beams and Kessel (1963) to assume that the endoplasmic reticulum is the site of synthesis and packaging of yolk proteins. Recently Ganion and Kessel (1972) have confirmed this early report by their EM autoradiographic studies using labelled leucine

in *in vivo* and *in vitro* conditions. When peroxidase was injected into the haemocoel of the crayfish, it was taken into multivesicular bodies and not into yolk platelets. Ganion and Kessel (1972) explained this finding as indicating that the contribution of the blood proteins to yolk was negligible compared with the bulk of material synthesized by the endoplasmic reticulum. By comparison, the extent and the morphology of the endoplasmic reticulum in the oocytes of *Drosophila melanogaster* are such that any involvement of this organelle in the synthesis of yolk proteins appears doubtful (Mahowald, 1972b).

The ultrastructure of mature yolk spheres has been studied in a variety of animals, both during oogenesis and early embryonic development. Karasaki (1963) has reported that in amphibian oocytes a fully formed yolk sphere consists of three components, namely, a main body which exhibits a crystalline structure of characteristic periodicity, a superficial layer of a finely granular texture and a limiting membrane with a typical unit membrane profile. The crystalline organization of the main body has been demonstrated in oocytes of various animals (Karasaki, 1967).

Spornitz and Kress (1972) have noted the presence of a typical periodicity in the crystalline core of the yolk platelets in *Rana esculenta* and *Rana temporaria*. A crystalline core has also been observed in yolk spheres of oocytes in some insects such as *Aedes aegypti* (Roth and Porter, 1964).

King (1970) has observed various classes of yolk spheres in oocytes of *Drosophila melanogaster*. Of these, the alpha-1 spheres make their appearance in the

ooplasm at stage 8; they are reported to be membrane-enclosed bodies showing a homogeneous internal structure. They contain proteins, lipids and PAS-positive material. By stage 13, the alpha-2 yolk spheres become visible in the ooplasm; they too are limited by a typical membrane and consist of a population of rod-shaped bodies embedded in a homogeneous matrix. The beta-spheres are also formed during stage 13 and are reported to be composed of "clustered morula-shaped subunits" which are not enclosed by an external membrane. The beta-spheres do not contain proteins but are rich in saturated lipids and PAS-positive material extractable by amylase (King et al., 1966).

Recently, Massover (1971) has reported a new category of yolk platelets in amphibian oocytes. He calls them nascent yolk platelets, and has added that, although they exhibit the three basic components as previously reported by Karasaki (1963), the superficial layer is asymmetrically positioned with respect to the main body and has a granular texture within which empty membrane-less spherules are embedded. Massover (1971) has also considered the possibility that these spherules originate from cytoplasmic vesicles whose membrane is lost during their fusion with the growing yolk platelets.

The superficial layer of the yolk platelets does not show a crystalline structure, but nevertheless it appears to include some of the chemical components of the main body (Karasaki, 1967).

Favard and Favard-Sereno (1969) have reported that the superficial layer of the yolk platelets in amphibian oocytes is positively stained by a variety of cytochemical techniques that react specifically with

carbohydrates. In these oocytes, they consider fusion between polysaccharide-containing vesicles and yolk spheres to be the mechanism by which the superficial layer acquires polysaccharides.

As stated already, only one of the sixteen cells of the ovarian chambers in Drosophila melanogaster becomes the oocyte, while the other fifteen cells undergo a completely different line of development.

From very early stages, nurse cell chromosomes undergo a number of endomitotic reduplications. In Drosophila melanogaster Jacob and Sirlin (1959) have estimated the relative ploidy of nurse nuclei in various stages of oogenesis, taking as reference for diploid values the DNA content of follicle cells. King (1970), however, considers the degree of ploidy so obtained to be an underestimate because according to Schultz (1956), the follicle cells themselves show a certain degree of ploidy. In early stages of development of the ovarian chambers the reduplication of DNA occurs synchronously in all nurse nuclei, but after stage 6 the nuclei are out of step with each other in their rate of increase of DNA (Jacob and Sirlin, 1959). These workers as well as Brown and King (1964) have noted that the nuclear volume is fairly well correlated with the degree of ploidy till stage 9, and that there is an anterio-posterior gradient for these parameters with the dominant end towards the developing oocyte.

While endomitotic doublings of DNA give rise to polytenic chromosomes in the salivary glands of Drosophila, the process gives a different result in nurse cells. In the latter, the chromosomes lose their

coherence and become dispersed throughout the karyoplasm (King, 1970). In both instances, the process leads to a large increase in the amount of DNA template available for synthesis of RNA for use in the subsequent life of the cell (Telfer, 1965).

In polytenic chromosomes the perfect register of all nucleolar organizer loci, results in the formation of a large nucleolus whereas, in the nurse cell nuclei with dispersed chromosomal threads it is difficult to visualize a well defined nucleolus (Painter and Biesele, 1966; Dapples and King, 1970).

In the past decade, there have been a number of autoradiographic studies to detect the synthesis of RNA molecules in nurse cell nuclei and their transfer to the ooplasm. In Drosophila melanogaster, it has been demonstrated that there is a flow of RNA from the nurse cell cytoplasm towards the ooplasm (Sirlin and Jacob, 1960). A similar observation has also been made in Musca domestica (Bier, 1963).

In vitellogenic follicles of cecropia moth ovaries, Pollack and Telfer (1969) have found that with long incubations in tritiated uridine the label appears in the ooplasm, whereas with short incubations, the silver grains are restricted to the nurse cells.

The processing of ribosomal RNA in nurse cells has been studied biochemically by Hughes and Berry (1970) in the ovary of the insect Antharea polyphemus. They have shown that ribosomes formation is a relatively slow process in nurse cells compared with wing cells. After a chase period of four hours, following a pulse of sixty minutes in radioactively labelled uridine, a conspicuous amount

of radioactivity appears in the ooplasm. On the other hand, no label can be detected in ovarian chambers devoid of the nurse cells. They concluded that ribosomes are synthesized in nurse cells and then transferred to the ooplasm where they persist as monosomes for the rest of the period of oogenesis. It is generally believed that the bulk of the RNA synthesized during oogenesis is not used by the oocyte itself but it is stored for use during early embryonic development (Brown and Dawid, 1968).

The ultrastructural characterization of the RNP transfer from the nurse cell nucleus to the cytoplasm is another aspect that has received much attention in recent years. Three basic mechanisms are known to control the nucleo-cytoplasm exchanges : (a) passage of RNP through the nuclear pores, (b) budding off of the nuclear envelope and (c) temporary interruptions of the nuclear envelope.

Ultrastructural evidence for the transport of material through the nuclear pores was first presented for the nurse cells of the insect Rhodnius by Anderson and Beams (1956). In salivary glands of Chironomus larvae, Beermann (1964) has described electron dense material within the nucleus close to and penetrating the nuclear pores. Monneron and Bernhard (1969) have presented evidence which indicate that perichromatin granules transform into tiny fibrils as they approach the nuclear envelope. Furthermore, they have suggested that, since only fine fibrils are visible at the nuclear envelope level, no granules as such migrate through the nuclear pores.

An alternative way of nucleocytoplasmic

transport seems to be represented by the formation of blebs on the outer membrane of the nuclear envelope which are then released into the cytoplasm (Scharrer and Wurzelmann, 1969). Blebbing of the nuclear envelope seems to be a wide spread phenomenon in embryonic cells where it may occur as a nuclear response to a temporary need for nuclear products (Wischnitzer, 1973).

A unique way of transferring nuclear material to the cytoplasm has been reported in the silk glands of Bombyx mori. This is by means of temporary interruptions of the nuclear envelope. It has been assumed that this relates to the genesis of ribosomes or that simply represents an extrusion of excess nuclear material (Tashiro et al., 1968).

Follicle cells are cells of mesodermal origin which come to cover the 16-cell clusters of cystocytes during their migration through the germarium. It is only in the intermediate region of the germarium that clusters of sixteen cells are present, and it is only at this location that the pre-follicle cells adhere to the cystocyte surface. King and Koch (1963) have, therefore, speculated that the cystocyte plasma membrane has properties different from those of the 8-cell clusters.

As soon as the first egg chamber leaves the germarium, a layer of approximately eighty cells, all of them more or less of the same size, surrounds the 16-cell cluster (King and Vanoucek, 1960). These workers have estimated that about five hundred prefollicle cells are present within the germarium; some of these also must function as generative cells in order to provide a follicle layer for any 16-cell cluster that passes down from the

germarium. The above mentioned workers also speculate that this capacity belongs to those cells which remain attached to the external tunica propria of the germarium. On the other hand, the follicle cells of the developing egg chamber must also be capable of subsequent divisions so as to provide enough cells to allow the follicle layer to keep pace with the increasing dimensions of the growing chamber. At stage 6, however, a maximum number of about one thousand and two hundred cells is reached; cell divisions then cease and further development of the egg chamber is accomplished by a stretching of the follicle layer (King and Vanoucek, 1960).

When vitellogenesis commences, the follicle cells acquire an uneven distribution. Moreover, the follicle cells overlying theoocyte become columnar, while those overlying the nurse cells become extremely thin. A third group of follicle cells is derived from the anterior pole of the stage 9-10 chamber and, by migration between adjacent nurse cells, they reach the oocyte surface. At this location they are called border cells.

Two major functions have been attributed to the columnar follicle cells: (a) the formation of the egg coverings such as the vitelline membrane and the endochorion (King, 1970; Mahowald, 1972b),and (b) the secretion of a follicular product which has some role in the uptake of blood proteins (Anderson, 1971).

Ultrastructural as well as autoradiographic evidence from several investigations have indicated that only follicle cells are involved in the synthesis of vitelline membrane and endochorion precursors. This assumption is strengthened by the findings in "ty" and

"fes" mutants. In these mutants where the columnar follicle cells extend anteriorly much beyond the limit of the oocyte, the vitelline membrane is also formed between the nurse cells and the extended layer of follicle cells (Falk and King, 1964; King et al., 1961). It thus appears that once the follicle cells reach a certain level of maturity they can secrete a vitelline membrane against the plasmalemma of any adjoining cell.

At the anterior end of the oocyte, the border cells secrete both the vitelline membrane and that portion of the endochorion which forms the micropylar apparatus (King, 1970). In a more lateral position, the chorionic appendages are formed by follicle cells which migrate in-between degenerating nurse cells; there, they form a cylinder of endochorion which encloses a strutwork of endochorionic pillars and septa. When the secretory activity of the follicle layer is almost ended, a third layer - the exochorion - the origin of which is still unclear, is apposed to the external side of the endochorion (Quattropani and Anderson, 1969).

Adjacent follicle cells are joined by specialized cell junctions: a typical zonula adherens is situated along the lateral border. These kinds of junctions are present in all stages of oogenesis, while during the period when the follicle cells are engaged in the synthesis of the endochorion, the adjacent cells are linked by septate junctions (Mahowald, 1972a).

The apical side of the columnar follicle cells is in contact with the tunica propria. An histochemical analysis has shown that the latter is composed of glycoproteins or neutral mucopolysaccharides (Bonhag

and Arnold, 1961). It is believed that, in addition to the mechanical function of maintaining the shape of the growing chamber, the tunica propria plays a role as a dialytic membrane regulating the flow of material from the blood to the underlying intercellular spaces (Bennett, 1963).

In cecropia moths , it is believed that follicle cells participate in the process of absorbing proteins from the external medium. The first indication of their involvement in such a process came from the work of Anderson and Telfer (1969) who found that in vitellogenic follicles of this moth incubated in vitro in a medium containing blood and tritiated amino acids, the radioactivity appears initially in the follicle cells, and, later in the small yolk spheres in the cortical ooplasm. They interpreted this finding as indicating that a follicle cell product, secreted in the adjoining interspace, combines with blood proteins and that the whole complex is then taken up by the oocyte. Melius and Telfer (1969) also reached the same conclusion from their in vivo experiments with cecropia moths. Anderson and Telfer (1969) also showed that incubation of vitellogenic follicles in saline solution rather than in blood produced the same pattern of labelling thereby demonstrating that follicle cells do not require blood proteins as such to stimulate their absorptive and secretory functions.

The role played by the product of the follicle cells in cecropia moths has been further elucidated by Anderson (1971) by incubating oocytes that had been previously denuded of their layer of

follicle cells, in a medium of female blood and labelled leucine. In these conditions, the label appears only in the yolk-free ooplasm and not in yolk spheres themselves. The follicle layer, if isolated and incubated *in vitro* with labelled amino acids, is capable of proteins synthesis and the labelled product is released into the incubation medium. When denuded oocytes are incubated in a medium containing labelled follicle product, the oocytes become labelled. In ovarian chambers denuded of follicle cells, the oocyte is incapable of taking up proteins to the same extent as in normal conditions. It is assumed that a prior concentration of blood proteins brought about by binding with the assumed follicle cell product in the intercellular spaces of the follicle layer is an indispensable step for uptake into the oocyte (Anderson, 1971).

Chia and Morrison (1972) claim to have visualized the follicular product which they say makes the bulk of yolk spheres in oocytes of *Musca domestica*. Their observations, however, are open to criticism since they do not seem to have recognized the vitelline membrane precursors which also have the same electron density as the product they envisage to be the yolk precursors.

Telfer (1954) was apparently the first to demonstrate, by using immunological techniques, the antigenic identity of many blood proteins with those in the ovary in cecropia moths. He observed that antigen 7, which is present as blood protein from the larval stage, exhibits a sharp decrease in concentration at the time of initiation of the vitellogenesis. Furthermore, when

the ovaries were transplanted into male abdomen, which lacked the specific proteins, the oocytes failed to undergo yolk formation indicating that the ovary *per se* is incapable of synthesizing yolk proteins. Finally, the observation that, in ovariectomized females, antigen 7 increases its concentration over the level present in control females, constituted convincing evidence in support of the view that antigen 7 is the yolk precursor protein. From these experiments, Telfer (1954) concluded that a tissue other than the ovary synthesizes the yolk precursors and that the ovary is capable of absorbing them from the blood. Since antigen 7 occurs at a concentration twenty times greater in the ovary than in the blood, it was taken to indicate the existence of a mechanism capable of active uptake against a gradient. So, despite the fact that every protein present in the blood can get into the ovary, only one component, namely antigen 7 in the cecropia moth, can actually increase its concentration over that in the blood. This would indicate that the process of yolk formation is particularly adapted for the selective absorption of specific yolk proteins (Telfer, 1965).

Since these early studies on cecropia moth ovary, immunological techniques have been employed successfully for the study of oogenesis in many other animals. On the basis of the evidence obtained so far, it would appear that the selective uptake of proteins from the blood is a process of rather general occurrence whereby developing eggs are provided with the proteins required for the formation of yolk spheres.

In *Periplaneta americana*, Bell (1970)

has demonstrated the presence of two antigens common to female blood and ovary, but absent in male blood.

The haemolymph of many species of Lepidoptera, when analyzed electrophoretically reveals the presence of 23 protein bands; some of these also stain for glycoproteins and lipoproteins. Two lipoproteins have been identified as vitellogenins because of their variation in concentration in various developmental stages (Whitmore and Gilbert, 1974).

A specific female protein could also be demonstrated in Bombyx mori by determining its increase in concentration in the haemolymph of ovariectomized females as well as by checking its absence or low concentration in males (Doira and Kawaguchi, 1972).

In the honey bee, Engels (1972) has shown that about 60-80% of the haemolymph in the female consists of female specific proteins and that proteins having similar electrophoretic mobilities also occur in the ovary.

There is also evidence to suggest that, as yolk formation progresses, the yolk proteins are complexed into larger molecular aggregates. The work of Brookes and Dejmal (1968) in Leucopharea, for instance, shows that the sedimentation profile of the yolk fluid from mature oocytes is different from that of early vitellogenic follicles.

Evidence from autoradiographic experiments are also consistent with the assumed extra-ovarian synthesis of yolk proteins (Bier, 1963).

Although the extraovarian origin of yolk proteins has been fairly well documented, there is still much speculation and controversy as to where and how these

proteins are synthesized. This may be either because different experimental approaches have been used to examine the problem or it may simply reflect a natural variation in organisms adapted for different environmental conditions. Roth and Porter (1964) have proposed that in the adult Aedes aegypti, the cells of the mid gut epithelium are a likely candidate for yolk protein synthesis. In fact, only this tissue exhibits a high synthetic activity when tested for protein synthesis during the period of active vitellogenesis; the fat bodies are hardly labelled even after thirty hours of exposure to radioactive amino acids. Later, it has been found (Bodnaryk and Morrison, 1968) that the fat bodies in the adult female of the house fly produce an antigen which is immunologically identical to a specific blood protein. Since the same antigen was not detected in the larval fat bodies, they assumed that this protein was synthesized in the adult stage in order to meet some requirements of the ovarian development. This conclusion, however, does not prove the capacity of the fat bodies to synthesize yolk proteins because the protein in question may simply be channelled through the fat bodies after being synthesized elsewhere. There is, in fact, some evidence to support the view that fat bodies are capable only of absorption of proteins from the haemolymph and not to synthesize proteins from aminoacid precursors. This evidence was obtained by Locke and Collins (1968) who examined this question in the larva of the insect Calpodes ethlius. In Drosophila melanogaster also there are indications that the fat bodies are capable only of sequestering proteins from the haemolymph and not of in situ synthesis (Thomasson

and Mitchell, 1972).

Brachet (1942) was apparently the first to demonstrate, at the light microscope level, the presence of acid phosphatase in yolk platelets of amphibian oocytes. Yao (1950) confirmed these observations in the oocyte of Drosophila melanogaster where the acid phosphatase activity is preferentially located in the cortical ooplasm of early oocytes.

When the Gomori (1939) technique for the detection of acid phosphatase was adapted for the electron microscope in early 1960s by Holt and Hicks (1961), it became possible to trace the origin and the fate of lysosomes in many animal tissues. With this method, cytologists have been able to confirm the validity of the lysosome concept as first formulated by Berthet et al. (1952), and also to characterize the ultrastructure of those organelles involved in the synthesis and transfer of lysosomal enzymes. This method has also been applied successfully to the localization of acid phosphatase in oocytes of many animal species. Cone and Eschenberg (1966) have observed that, in the cortical ooplasm of the oocyte of the hemipteran Gerris remigis, acid phosphatase is associated with small yolk granules, and, as vitellogenesis nears completion, mature yolk spheres are no longer acid phosphatase-positive. They have suggested that acid phosphatase operates a highly selective hydrolysis which would allow preservation of those constituents which characterize the mature yolk spheres while the extraneous constituents would be degraded. Hopkins and King (1966) have observed lysosomes in the cortical ooplasm of the oocyte of Bombyx terrestris and queried

whether the presence of these bodies could be associated with a selective hydrolysis of certain blood proteins. In a recent review article on the occurrence of lysosomes in insects, Lockshin (1969) has stressed the importance of lysosomal enzymes in relation to the selectivity of uptake: he has suggested that blood proteins are converted into yolk proteins by an enzymatic hydrolysis operated by lysosomes.

In biochemical studies of unfertilized eggs of Drosophila melanogaster, acid phosphatase activity has been found to be quite high; this activity decreases sharply in the larval stages and again rises steeply in the pupal stage (Mulherkar et al., 1972). These workers have suggested that the cyclic appearance of acid phosphatase in two different periods of Drosophila development may correspond to the activation of different molecular forms of the same catalytic activity. In contrast, catheptic activity was not detected in eggs and does not appear until the third instar larval stage.

In a recent review article on lysosomes, Pasteels (1973) has stated that, if we consider the lysosome in its broadest sense, the yolk platelets may be regarded as lysosomes, but this author could not provide an explanation as to how the enzyme found its way into the yolk platelets. He further assumed that the lytic enzymes which come to be included in the yolk platelets during vitellogenesis remain in an inactive state. This, however, contrasts with the frequently reported positive reaction for acid phosphatase in the yolk platelets of oocytes of several organisms. In order to explain this, Pasteels (1973) has speculated that the fixation procedure, normally

used for the cytochemical detection of acid phosphatase activity affects the inactive proenzyme molecule in such a way as to liberate an active form which would not otherwise be present *in vivo*. Pasteels' (1973) conclusion is that the hydrolytic enzymes are transformed into an active form only during the period of yolk utilization. As far as I am aware, there is no evidence to substantiate the explanation offered by Pasteels. In this context it is of interest to note the widely held view that prefixation of tissues employed for the localization of enzymes, results, if anything, in some loss of enzymatic activity rather than increased activity (Daems *et al.*, 1969).

III. THE MAIN OBJECTIVES OF THE PRESENT INVESTIGATION

In many insects, yolk proteins are known to be immunologically identical to sex-specific blood proteins. A number of investigators have shown that the antigenic identity between blood and yolk proteins can be attributed to the involvement of pinocytosis in the formation of yolk. However, in spite of structural similarities that vitellogenic oocytes of *Drosophila* bear with those of other insects which are known to form yolk platelets by pinocytosis, it is still held by some workers that in this insect yolk precursors are synthesized by the oocyte itself. One of the main objectives of the present work was to clarify this point and with that end in view a detailed study of oogenesis was carried out. This study was to include the structural and functional aspects of every cell type in the ovarian chamber. These are the nurse cells, follicle cells and the oocyte. An examination was also to be made of various intracellular structures in the above mentioned cells that could be expected to play a role in the formation of yolk platelets.

The methods employed were mainly ultrastructural and cytochemical procedures in conjunction with electron microscopy.

The possible route of substances into vitellogenic oocytes was also to be examined at the electron microscope level by using electron opaque extracellular tracers and autoradiography.

IV. MATERIAL AND METHODS

1. The rearing of *Drosophila*

Drosophila melanogaster (Oregon-K strain) were grown in half pint glass bottles containing the usual corn-meal-molasses-yeast medium. Adult flies were constantly maintained at 25°C and periodically changed from bottle to bottle to allow the growth of newly born larvae in freshly prepared medium. For collection, the flies were anaesthetised with ether vapour and kept under anaesthesia until dissection of the ovaries was carried out under a stereo-microscope.

2. Fixation and embedding for electron microscopy

(a) fixation in glutaraldehyde and osmium tetroxide. Ovaries of 2-3 day old flies were collected in *Drosophila* Ringer solution and transferred to the fixative kept at a low temperature (4°C). After about 10-15 minutes, the ovaries were carefully separated into single ovarioles and transferred to fresh fixative at 4°C where they remained for a period of 2 hours. The fixative was prepared by mixing equal volumes of 5 % formaldehyde and 5 % glutaraldehyde both made in 0.1 M cacodylate-HCl buffer at pH 7.2.

After fixation the ovarioles were rinsed overnight in the buffer and then post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 3 hours at 4°C. The ovarioles were then briefly washed in the buffer, dehydrated through a graded series of alcohols and taken through propylene oxide into a freshly prepared mixture of Epon-Araldite. The mixture consisted of the following:

Epon 812	7.5	ml
Araldite epoxy resin	6.0	ml
Araldite hardener	18.0	ml
Araldite accelerator	0.63	ml

After infiltration overnight in the Epon-Araldite mixture at room temperature, single chambers were isolated from the ovarioles and placed in gelatin capsules containing the epoxy resin mixture. Polymerization was carried out in an oven at 58-60 °C for 3 days.

After trimming the blocks, sections were cut on a Porter-Blum MT-1 ultramicrotome using glass knives. Silver-to-pale gold sections were collected on copper grids covered with a carbon coated Formvar membrane. The sections were then stained for 15 minutes in 1 % uranyl acetate in 25 % ethyl alcohol. The solution was freshly prepared just before use from a stock solution of 2 % uranyl acetate in distilled water. After the uranyl acetate staining, the grids were rinsed in 50 % ethyl alcohol and taken up for staining in Reynolds lead citrate (Reynolds, 1963). The lead citrate solution was placed in drops on a layer of wax contained in a Petri dish, and the grids with sections facing down were floated for 15 minutes with the usual precautions to prevent the formation of lead carbonate. After the final staining, the grids were washed first in 0.01 N NaOH solution and then in distilled water and subsequently dried. The double stained sections were examined in a AEI-EM6 electron microscope operating at 60 Kv.

(b) fixation in potassium permanganate. A few ovaries were dissected in Ringer solution and transferred

to a 0.6 % solution of potassium permanganate, prepared in 0.1 M veronal acetate buffer at pH 7.4. The period of fixation was 2 hours at 4°C. After fixation, the ovaries were transferred without any rinse to 70 % ethyl alcohol and gradually brought up to absolute alcohol. The embedment of the ovaries was done as already described.

(c) Glycol methacrylate embedding procedure. For cytochemical studies involving extractions with Pronase and RNase and for the selective staining procedure for ribonucleoproteins, the ovaries were embedded in glycol methacrylate (GMA). This is a water-soluble embedding medium which offers the advantage of preserving the tissue in such a way as to enable digestion by enzymes (Leduc and Bernhard, 1967).

For enzymatic studies, the ovaries were fixed for 60 minutes at 4°C in 5 % glutaraldehyde in 0.1 M Sorensen's buffer and the infiltration with 70 %, 80 % and 97 % GMA in distilled water was carried out at 20 minutes intervals. The ovaries were then transferred for a period of 20 minutes to a 1 : 1 mixture containing 97 % GMA solution and the pure embedding medium. The pure embedding medium consists of the following:

7 parts of 97% GMA plus three parts of non destabilized butyl methacrylate with 2% Luperco added.

Subsequently, the material was taken to the pure embedding medium for one hour, and then into partially polymerized embedding medium where it remained for about 2 hours. At this stage, the ovarioles were separated and individual chambers or groups of chambers were placed

in gelatin capsules which were subsequently filled with the partially polymerized embedding medium and left uncapped for 30 minutes to allow the removal of air bubbles. The capsules were then closed with gelatin caps and care was taken at this stage to trap as little air as possible. This was done because the presence of air inhibits polymerization by ultraviolet light. The polymerization of GMA was carried out in a cold room at 3-5 °C under an UV source (3650 Å wavelength). Under these conditions, polymerization was complete in 3 days.

As mentioned earlier, silver-to-pale gold sections were collected on gilded nickel grids, covered with Formvar-carbon coated membranes.

3. Extraction by enzymes

The gilded nickel grids carrying the GMA sections were floated on enzyme solution for periods ranging from 1 to 3 hours at 37 °C. After this treatment, the grids were washed, dried and double stained in uranium and lead as previously described. The following concentrations of enzymes and durations of incubation were used:

(a) Ribonuclease was used at concentrations of 0.2 % and 0.5 % in distilled water and the pH of the solutions was adjusted to 6.4-6.6 with 0.01 N NaOH. The treatment was prolonged for periods ranging from 1 to 3 hours.

(b) Pronase was used in 0.1 M Sorénsen's phosphate buffer at pH 7.4. The concentrations used were 0.01-0.05 % and the sections were treated for 30 minutes to 1 hour. The control sections were floated on the buffer solutions.

4. Selective staining for ribonucleoproteins in ultrathin sections.

The method employed was that of Bernhard (1969) which involves the bleaching of DNA-containing regions by the chelating agent EDTA. The ribonucleoproteins are unaffected by this treatment and after staining with lead citrate show up as dense entities in striking contrast to the bleached areas.

For this procedure, the ovaries were fixed in glutaraldehyde and embedded in GMA as previously described. The sections were stained in 0.05 % aqueous uranyl acetate for 1 minute, treated with 0.02 M EDTA in distilled water for 2 minutes and again stained in 0.5 % lead citrate for 1 minute.

5. Autoradiography at the light and electron microscope levels.

Electron microscope autoradiography was used in the present study to investigate the functional aspects of nurse nuclei and to determine the chemical nature of the amorphous material observed in nurse cytoplasm. Further, by using this technique, the kinetics of incorporation of labelled amino acids into vitellogenic oocytes was also investigated.

Tritiated precursors of biological macromolecules were introduced into the ovaries by two different methods: (a) incubation <u>in vitro</u> and (b) microinjection into the haemocoel.

(a) Incubation <u>in vitro</u>. The ovaries were removed from the abdomen of 2-3 day old flies and quickly transferred to tissue culture medium 199 (Bio-cult.

Laboratories, Glasgow). Tritium labelled precursors were added to the medium to provide a radioactive concentration of 100 μCi/ml. All radioactive precursors used in this study were obtained as sterile aqueous solutions at a concentration of 1 Ci/ml from the Radiochemical Centre, Amersham. Uridine-5-^{3}H (specific activity 31 Ci/mM) and L-lysine-4,5-^{3}H-monohydrochloride (specific activity 4.6 Ci/mM) were used in these experiments.

The ovaries were cultured in the radioactive medium for 30, 60 or 120 minutes at room temperature (22°C). In some experiments with uridine, the ovaries were first cultured in the radioactive medium and then transferred to a non-radioactive medium containing uridine at a concentration 50 times greater than in the radioactive medium.

At the end of the culture period, the ovaries were transferred to the fixative at 4°C for 2 hours. Fixation artifacts are known to occur in EM autoradiographic experiments (Monneron and Moule, 1969) using labelled uridine. To avoid this it has been suggested (Jacob, 1971) that the material should be first fixed in glutaraldehyde and not in osmium tetroxide. It has also been suggested that the aldehyde fixed material should be washed repeatedly in cold buffer for 2-3 days in order to remove all unincorporated precursors. Fixation artifacts are also liable to occur in autoradiographic studies of protein synthesis. Several authors (Caro and Palade, 1964; Ashley and Peters, 1966; Peters and Ashley, 1967, 1969) have pointed out that the use of glutaraldehyde causes a non-specific binding of free amino acid molecules to

the tissue structure. To overcome this difficulty the use of formaldehyde freshly prepared from paraformaldehyde has been recommended by several workers (cf. Jacob, 1971). In view of this, the ovaries cultured for studying protein synthesis were fixed in freshly prepared 5 % formaldehyde and washed for several days in the buffer. In all culturing experiments, the aldehyde fixed tissues were post-fixed for 3 hours in 1 % osmium tetroxide made up in 0.1 M cacodylate buffer at pH 7.2. The material was then dehydrated in ethyl alcohol and embedded in Epon-Araldite mixture as described earlier.

(b) Microinjection of tracer into the haemocoel. Early in the course of this study, it was observed that the just dissected ovaries are capable of rhythmical contractions but they lose this capability after a few minutes stay in Ringer solution. In view of the possibility that rhythmical contractions may play a role in the movement of material from nurse cells to the oocyte, it seemed desirable to expose the ovaries to radioactive precursors in as near physiological conditions as possible. It was with this end in view that the microinjection experiments were performed.

The apparatus for microinjection consists of a micropipette connected by a length of polythene tubing to a microsyringe. The micropipettes were made from 1 mm diameter glass tubing using a De Fonbrune microforge. The micropipette was fixed to a Singer hand micromanipulator, Mark I (Barer and Saunders-Singer, 1951) to allow fine control over its movement. The syringe, the length of tubing and the micropipette were

all filled with liquid paraffin.

The amount of label (10 μl) to be injected was measured out with a microsyringe (Hamilton precision syringe), placed in a watch glass and allowed to dry at room temperature. An equal amount of Ringer solution was then added to the watch glass and drawn into the micropipette. The injection was carried out on etherized flies which were placed ventral side up on the stage of a stereo-microscope. The micropipette was introduced into the haemocoel at the level of the 7-8th abdominal segment. The tip of the micropipette was calibrated so that an equal amount of tracer could be injected into each fly. Each fly received 0.5 μl of the Ringer solution containing radioactive precursors at the concentration of 1 μCi/μl. The micropipette was then very slowly withdrawn from the abdomen and care was taken to ensure that no flow back of fluid occurred through the wound. The micro-injected flies were kept in glass vials containing standard Drosophila food and maintained at a constant temperature of 25°C. The majority of flies survived the injection and went on depositing eggs from which larvae hatched in due course.

At various intervals after injection, the flies were killed and the ovaries were fixed in glutaraldehyde or formaldehyde (depending on the tracer used) and after a prolonged wash, fixed again in osmium tetroxide. Finally the material was embedded in Epon-Araldite mixture as usual.

6. Emulsion coating, autoradiographic exposure and photographic processing

Thick sections (0.5 to 1.0 μ) were cut first for light microscope autoradiography. This was done to check the orientation of the tissue and to establish that there is sufficient incorporation of label to permit autoradiography at the electron microscope level. Thick sections were collected from the microtome trough with a hairloop and transferred to a gelatin coated slide to ensure proper adhesion to the glass. The slides were then taken to the dark room where they were coated by dipping in liquid Ilford L4 emulsion. After allowing the coated slides to dry, they were placed in a light proof box for autoradiographic exposure. The atmosphere in the box was kept dry by keeping a few crystals of Drierite ($CaSO_4$) in the box which was stored in a refrigerator at 4 °C for periods of time ranging from 6 to 30 days.

After exposure, the slides were developed in Kodak D19b developer for 3 minutes at 18-20 °C after which they were rinsed in distilled water for 30 seconds and fixed for 8-10 minutes in Fix-Sol fixative (Johnsons of Hendon) diluted 1:10 with distilled water. Subsequently, the slides were washed in running cold water, dried, stained in 3 % fuchsin in 70 % ethyl alcohol and examined in a phase contrast microscope.

For electron microscope autoradiography, ultrathin sections (about 1000 Å thick) were cut and collected on nickel grids covered with a carbon coated Formvar membrane. The grids were then mounted on glass slides by attaching their edge to a stretch of double faced sticky tape fixed at the centre of the glass slide.

A second layer of carbon was deposited on the sections to avoid any possible interaction between the tissue and the emulsion. The slides were then taken to the dark room and coated with a monolayer of Ilford L4 emulsion according to the technique of Caro and Van Tubergen (1962). Ten gm. of the emulsion were weighed in a glass beaker to which 20 ml of distilled water was added. The mixture was then melted in a water bath at 45 °C for 10 minutes with gentle stirring. Subsequently the emulsion was chilled in an ice bath for 2 minutes in order to start the process of gelling. The gel was then allowed to warm up to room temperature for 30 minutes. A copper wire loop, 4 cm in diameter, attached to a glass rod was dipped into the emulsion and withdrawn slowly so as to form a thin and even film of emulsion across the loop. The thickness and homogeneity of the film were checked at the safe lamp by looking at the interference colours. A deep purple interference colour is thought to be indicative of an optimum monolayer of closely packed silver halide crystals (Salpeter and Bachmann, 1972).

After coating, the grids were stored in light tight boxes for periods of time ranging from 1 week to 2 months. After suitable periods of autoradiographic exposure the grids were developed in Kodak D19b developer for 3 minutes, washed in distilled water for 30 seconds and then fixed in freshly prepared twice filtered Kodak F-24 fixative for 8 minutes. The slides were washed under running distilled water for 30 minutes; the grids were then removed from the slides and allowed to dry. Finally, the grids were double stained in uranyl acetate and lead citrate, both prepared in aqueous solutions.

7. Analysis of autoradiograms

The information obtainable from an electron microscope autoradiograph is derived from the distribution of developed silver grains which is related to the distribution of the underlying radioactive sources. In order to determine whether a particular silver grain can be considered as due to the radioactivity present in a structure closely associated with it, the resolution of the autoradiography has to be taken into account: for recent reviews on this see Jacob (1971) and Salpeter and McHenry (1973). Autoradiographic resolution may be defined as the distance from a radioactive source within which 50 % of the silver grains originating from that source are contained. Basically, the resolution depends upon two factors which may be described as (a) the photographic error and (b) the geometric error. The first is related to the size of the silver halide crystals. The geometric error is concerned with the relationship between the source of radiation and the detector or the silver halide crystals. These two errors account for the final autoradiographic error which is equal to the square root of the sum of the squares of the two errors. For details see Bachmann and Salpeter (1965).

The resolution obtained in the present study has been calculated according to the formula derived originally by Bachmann and Salpeter (1965) and the value is 160 nm. The silver grains in my preparations were ascribed to a nearby structure if the latter was contained within a circle of 160 nm radius drawn around the centre of the grain.

In order to study the kinetics of uptake

of yolk proteins, numerous autoradiographs of vitellogenic chambers were examined and the density of silver grains was determined in each one of them. This was done by superimposing a grid of small square over the autoradiographs and counting the number of small squares within the area occupied by the yolk platelets. From a knowledge of the magnification of the micrograph, the total number of small squares was converted into actual area measured in square microns. Further, the number of autoradiographic grains was counted over such areas and the result expressed as grains per unit area or grain density. Finally because of the inevitable variation that occurs in sampling, the standard error was calculated for data obtained in each experiment. The diagram in Text-Fig. 7 reports the mean density of grains plotted against time of exposure to the tracer and each value was considered $\pm$ 2 standard error. The standard error was determined according to the following formula:

$$\Sigma x^2 - \frac{\Sigma x^2}{n} = \Sigma x^2 \qquad V\bar{x} = \frac{\Sigma x^2}{n(n-1)} \qquad SE\ \bar{x} = \sqrt{x}$$

8. Ruthenium red technique

Ovaries dissected from 2-3 day old flies were fixed for 2 hours at 4 °C in glutaraldehyde containing ruthenium red. The fixative was made up as follows:

25 % glutaraldehyde	2.0	ml
0.2 M cacodylate buffer, pH 7.2	5.0	ml
distilled water	3.0	ml
Ruthenium red	0.1	gm

After fixation the ovaries were washed in several changes of 0.1 M cacodylate buffer and left

overnight in the last change of buffer. The following day they were post-fixed for 2 hours at 4 °C in osmium tetroxide containing ruthenium red. This fixative had the following composition:

2 % Osmium tetroxide in distilled water	5.0 ml
0.2 M cacodylate buffer, pH 7.2	5.0 ml
Ruthenium red	0.01 gm

Because ruthenium red is known to be unstable in solution with osmium tetroxide, this fixative was freshly prepared each time just before use.

9. Lanthanum technique

Ovaries dissected from 2-3 day old flies were fixed in 5 % glutaraldehyde containing 1.5 % lanthanum nitrate in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. The ovaries were then rinsed twice and left overnight in the same buffer containing 1.5 % lanthanum nitrate. On the next day they were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C and dehydrated and embedded in Epon-Araldite.

10. Colloidal thorium dioxide (Thorotrast) technique on fixed material

Ovaries dissected from 2-3 day old flies were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. They were then rinsed and left overnight in the buffer. The following day they were placed in a 1 % solution of thorium dioxide (supplied as thorium dioxide EM by TAAB Laboratories) in 30 % acetic acid for 1 hour at room temperature.

Then the ovaries were briefly washed in 30 % acetic acid for 2-5 minutes and subsequently taken through a graded series of decreasing concentrations of acetic acid to distilled water. Finally, the ovaries were post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, dehydrated and embedded in Epon-Araldite mixture.

11. Colloidal thorium dioxide (Thorotrast) technique *in vivo*.

2-3 day old flies were injected with a 1 % solution of thorotrast made up in *Drosophila* Ringer. On the average, 0.5 μl was injected into each fly with the microinjecting device described earlier. After 1, 2, 6, 15 or 30 hours, the ovaries were dissected out and fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer for 2 hours. They were then rinsed in the buffer and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 2 hours at 4 °C. After dehydration in a graded series of alcohols the ovaries were embedded as usual in Epon-Araldite mixture.

12. Horseradish peroxidase technique *in vitro*.

The ovaries were dissected from 2-3 day old flies and placed in a solution of *Drosophila* Ringer containing peroxidase (Sigma) at the concentration of 0.5 mg/ml for periods of 15,30, 60 or 120 minutes. After exposure to peroxidase, the ovaries were washed in the Ringer and fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer,pH 7.2, for 2 hours at 4 °C. They were then washed in the buffer and transferred to a freshly made solution of diaminobenzidine (Koch-Light Laboratories) having the following composition:

	3,3-diaminobenzidine tetra-HCl	10 mg
0.1M	Tris-HCl buffer at pH 7.6	10 ml
	1 % H_2O_2	0.1 ml

After 30 minutes in this reagent at room temperature, the ovaries were washed in several changes of Tris-HCl buffer and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 3 hours at 4 °C. After dehydration, the ovaries were embedded in Epon-Araldite mixture.

13. Horseradish peroxidase technique in vivo.

In order to study the kinetics of pinocytotic uptake, peroxidase was injected into the haemocoel of 2-3 day old flies. This was done by injecting peroxidase into the flies with the microinjecting device previously described on page 31. In these experiments the peroxidase was used at a concentration of 5 mg/ml in Drosophila Ringer and, on the average, 0.5 μl of this solution was injected into each fly. After periods of 15 or 30 minutes and 1, 2, 4, 8, 16 or 24 hours, the ovaries were removed and fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. Following the fixation step, the ovaries were thoroughly washed in the buffer and transferred to the diaminobenzidine medium for 30 minutes at room temperature. In this case, the diaminobenzidine solution had a composition similar to that of the in vitro experiments with peroxidase. Subsequently the ovaries were post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, dehydrated and embedded in Epon-Araldite as usual.

14. Method for the demonstration of endogenous peroxidase activity.

In one experiment, the ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. They were thoroughly washed in the buffer and subsequently transferred to a solution of diaminobenzidine of the following composition:

3,3-diaminobenzidine tetra-HCl	10	mg
0.1M Tris-HCl buffer at pH 7.6	10	ml
1 % H_2O_2	0.1	ml

The ovaries were kept for 30 minutes in this solution and then washed in several changes of Tris-HCl buffer. They were then post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 3 hours at 4 °C, and this was followed by dehydration and embedment in Epon-Araldite mixture.

As control of the enzymatic reaction, some glutaraldehyde-fixed ovaries were incubated in the same medium as before, but devoid of hydrogen peroxide.

In another experiment, the ovaries were fixed in 5 % formaldehyde for periods ranging from 3 hours to overnight in Drosophila Ringer at pH 7.2. After a thorough wash in the buffer, the ovaries were incubated in the diaminobenzidine medium for 60 minutes at 37 °C. The latter consisted of the following:

3,3-diaminobenzidine tetra-HCl	20	mg
0.1M 2-amino-2-methyl-1,3-propandiol buffer, pH 9.0	9.8	ml
2.5 % H_2O_2	0.2	ml

The final pH of the solution was adjusted at pH 9.7 with 0.1 N NaOH.

As controls, some formaldehyde-fixed ovaries

were incubated in the diaminobenzidine medium devoid of hydrogen peroxide. The specificity of the reaction was also tested by incubating some ovaries in the complete medium with 3-amino-1,2,4-triazole added at a concentration of 10^{-2} M. This reagent is a specific inhibitor of the catalase activity. Therefore, the lack of DAB reaction products in the controls indicates a true enzymatic activity in the experimental material. Because of the known presence of catalase activity in peroxisomes (Novikoff et al., 1972), the incubation medium reported above was used to locate these organelles in ovarian chambers.

Control ovaries were post-fixed, dehydrated and embedded in Epon-Araldite mixture as in the case of the experimental ones.

15. Periodic acid - Silver methanamine (PASM) technique

The ovaries were fixed in 5 % Glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. They were then washed in the buffer, dehydrated and embedded in Epon-Araldite without post-osmication.

Pale gold sections were cut with an ultramicrotome and collected on gilded nickel grids, that were covered with a Formvar membrane reinforced with a thin layer of carbon. The grids were floated, sections side down, on a solution of 1 % periodic acid in distilled water for 30 minutes. After this oxidation step, the grids were thoroughly washed in distilled water and allowed to dry. The silver methanamine solution was prepared and used as follows:

Five ml of 5 % silver nitrate were added to 45 ml of a fresh 3 % solution of hexamethylene tetramine (hexamine). Five ml of fresh solution of sodium borate (2 %) was then added to the mixture. These reagents were mixed in the dark. The solution of silver methanamine was then filtered and transferred to a Petri dish placed in an incubator at 60 °C. Then the grids were floated on the silver methanamine solution and incubated for 30 minutes. The grids were then removed from this solution, briefly washed in pre-heated distilled water and placed again for 30 minutes at 60 °C in another Petri dish containing fresh silver methanamine solution. After this step, the grids were washed for few minutes in distilled water and transferred to a solution of 5 % sodium thiosulphate for 5-10 minutes. Finally, the grids were washed in several changes of distilled water and allowed to dry.

As controls, some grids were incubated in silver methanamine solution without prior oxidation with periodic acid. Structures which exhibit silver deposits only after periodic acid oxidation and silver methanamine treatment were taken as stained specifically.

16. Periodic acid - thiosemicarbazide - silver proteinate (PA-TSC-SP) technique

The ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer for 2 hours, rinsed in the buffer and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 3 hours at 4 °C. They were then dehydrated and embedded in Epon-Araldite mixture as previously described. The sections were oxidized for

30 minutes by floating on a 1 % solution of periodic acid in distilled water. After this oxidation step, the grids were thoroughly washed in distilled water and allowed to dry. Then they were taken to a 1 % solution of thiosemicarbazide in 10 % acetic acid for periods of 30 or 60 minutes and 48 or 72 hours at room temperature. After the thiosemicarbazide treatment, the grids were taken through two changes of 10 % acetic acid, each of 15 minutes duration. Then, following two more changes of similar duration in 5 % acetic acid, they were brought to distilled water and allowed to dry. The grids were subsequently floated for 30 minutes on a 1 % solution of silver proteinate (TAAB Laboratories) in distilled water, washed in distilled water and allowed to dry. The grids were examined in the electron microscope without further staining.

17. Phosphotungstic acid (PTA) technique.

The ovaries were fixed for 2 hours at 4 °C in 5 % glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.2. After a thorough wash in the buffer they were embedded in glycol methacrylate according to the procedure described on page 27.

Silver-to-pale gold sections, cut from polymerized blocks of glycol methacrylate, were mounted on gilded nickel grids that were covered with a carbonized Formvar membrane. The grids with the sections facing down were then floated for 2 minutes in a 1 % solution of phosphotungstic acid in 10 % chromic acid. After this treatment, the grids were briefly washed in distilled water and allowed to dry.

18. Concanavalin A technique on fixed material

The ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. After fixation, they were washed in the buffer and subsequently incubated for 1 hour at room temperature in concanavalin A (Sigma) at concentration of 1 mg/ml in 0.1 M cacodylate buffer at pH 7.2. After this incubation, the ovaries were washed in several changes of the buffer and subsequently transferred to a solution of peroxidase at concentration of 1 mg/ml for 1 hour at room temperature. After a brief wash in the buffer, they were transferred to a freshly made solution containing 10 mg of diaminobenzidine (Koch-Light Laboratories) in 10 ml of 0.1 M Tris-HCl buffer, pH 7.6 with 0.01 % hydrogen peroxide. Following 30 minutes of incubation in this reagent at room temperature, the ovaries were washed in Tris-HCl buffer and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 for 3 hours at room temperature. After dehydration, the ovaries were embedded in Epon-Araldite mixture.

19. Concanavalin A technique *in vivo* and *in vitro*.

In order to study the inward flow or movement of fragments of the plasma membrane of vitellogenic oocytes during the process of pinocytotic uptake, the ovaries were exposed before fixation to a concanavalin-peroxidase conjugate. The conjugation of peroxidase to concanavalin A was carried out according to the method of Avrameas (1969).

Five mg of concanavalin and 12 mg of peroxidase

were dissolved in 1 ml of 0.1 M phosphate buffer at pH 6.8. While the solution was gently stirred at room temperature, 0.05 ml of 1 % aqueous solution of glutaraldehyde in distilled water was added drop-by-drop to it. The solution was left for 2 hours at room temperature and then dialysed overnight at 4 °C against phosphate buffer saline (PBS). The latter consisted of 0.1 M phosphate buffer at pH 6.8 with 0.2 N NaCl added. At the end of this step, a precipitate formed in the dialysis tube and this was removed by centrifugation at 20,000 rpm for 30 minutes at 4°C. The pellet was discarded and the supernatant was applied to a column of Sephadex and eluted with 0.1 M phosphate buffer at pH 7.2 to get rid of unconjugated concanavalin and peroxidase molecules. The fractions obtained from this elution were read in a UV reader at 2800 Å. Subsequently, the fractions were read again on a spectrophotometer at a wavelength (4200 Å) known to correspond to the absorbance of the concanavalin molecule. Those fractions which gave overlapping peaks when read at the two wavelengths mentioned above were taken to contain the concanavalin-peroxidase conjugate and therefore were selected for further use. The conjugate was then concentrated to a final volume of 0.5 ml by a prolonged (2 days) dialysis against dry Sephadex.

The ovaries were exposed to the conjugate either *in vitro* or *in vivo*. In the first case, they were incubated in the conjugate solution for periods of 10, 20 or 30 minutes. After this treatment, the ovaries were

fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4°C. They were then washed in the buffer, post-fixed in 1 % osmium tetroxide, dehydrated and embedded in Epon-Araldite mixture.

The in vivo experiments were carried out by injecting 0.5 µl of concanavalin-peroxidase conjugate into the haemocoel of the flies. The injected flies were then maintained at 25 °C in glass vials containing standard Drosophila food, for periods ranging from 10 minutes to 12 hours. When the required time of exposure to the conjugate was over, the flies were anaesthetized and the ovaries were dissected from the abdomen. The ovaries were then fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. After washing thoroughly with the buffer, they were post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 3 hours. They were finally dehydrated in a graded series of alcohols and embedded in Epon-Araldite mixture, as usual. Thin sections cut from these blocks were examined in the electron microscope without further staining.

20. Osmium tetroxide-zinc iodide (OZI) technique

The ovaries were fixed in the osmium tetroxide-zinc iodide solution for 4 hours at room temperature in the dark. The osmium tetroxide-zinc iodide complex was made by preparing first, a zinc iodide solution, and then allowing it to react with osmium tetroxide. The zinc iodide solution was prepared by mixing 15 gm of metallic zinc powder with 5 gm of grounded iodine; subsequently, the mixture was slowly added to 200 ml of distilled water.

The solution was left at room temperature for a few minutes and then filtered when a pale yellow colour started to appear. The pH of the clear yellow solution was adjusted to pH 7.0 with 0.1 N NaOH. The osmium tetroxide-zinc iodide complex was made by mixing 8 ml of the filtered solution of zinc iodide with 2 ml of unbuffered 2 % osmium tetroxide in distilled water.

After fixation in the osmium tetroxide-zinc iodide complex, the ovaries were washed in several changes of distilled water, dehydrated in alcohol and embedded in Epon-Araldite mixture. The ultrathin sections were examined in the electron microscope without further staining.

21. Cytochemical detection of acid phosphatase.

The ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 0 °C. The ovaries were then washed in the buffer and left overnight in it at 4 °C. The following day they were placed in 0.1 M acetate buffer at pH 5.2 for 15 minutes at room temperature. After this step, the ovaries were transferred to the Gomori medium and incubated at 37 °C. The Gomori medium was prepared and used as follows: two solutions, hereafter referred to as A and B respectively, were freshly prepared the day preceding the incubation. Solution A consisted of 0.12 gm of lead nitrate dissolved in 50 ml of 0.05 M acetate buffer at pH 5.2. The solution B consisted of 3 % sodium b-glycerophosphate in distilled water. The solutions were mixed in a relative proportion of A/B = 10/1 (v/v) and the mixture was left overnight in an incubator at 37 °C. As a result of this

treatment, a white precipitate formed and a clear solution was prepared by filtering the mixture after cooling it to ice-cold temperature.

The filtrate was then warmed up to 37 °C and the glutaraldehyde-fixed ovaries were incubated in it for 40 minutes. After the incubation, the ovaries were thoroughly washed in 0.1 M acetate buffer at pH 5.2 and transferred to 0.1 M cacodylate buffer, pH 7.2, and left in it either overnight or for several days. After this short or long wash in the buffer, the ovaries were post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 3 hours, dehydrated in alcohol and embedded in Epon-Araldite mixture. Sections were examined in the electron microscope either with or without further staining.

As controls, some glutaraldehyde-fixed ovaries were incubated in the substrate-free Gomori medium (devoid of sodium b-glycerophosphate) at 37 °C for the same length of time as the experimental tissues.

22. Cytochemical detection of thiamine pyrophosphatase.

The ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 0 °C. The ovaries were then washed in the buffer and left overnight in it. The next day they were incubated at 37 °C for 60 minutes in the Novikoff and Goldfischer's (1961) medium. This medium consists of the following:

Lead nitrate	2	mM
Manganese chloride	5	mM
Tris-Maleate buffer, pH 7.2	75	mM

Thiamine pyrophosphate (TPP) 5 mM

The medium was freshly prepared every time just before use. At the end of the incubation period, the ovaries were briefly washed in the Tris-Maleate buffer and then passed through several changes of 0.1 M cacodylate buffer, pH 7.2. The ovaries were then post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 3 hours, dehydrated in alcohol and embedded in Epon-Araldite mixture. Thin sections were examined in the electron microscope either unstained or after staining with uranyl acetate and lead citrate.

As controls, some glutaraldehyde-fixed ovaries were incubated in the Novikoff and Goldfischer's (1961) medium without the thiamine pyrophosphate.

23. Cytochemical detection of acyltransferases

The ovaries were fixed in 5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at 4 °C. The ovaries were then washed in several changes of cacodylate buffer and left overnight in it. The following day they were preincubated in a solution of potassium ferricyanide at the concentration of 15 mg/ml for 30 minutes in order to oxidize the endogenous substrates. After this oxidation step, the ovaries were taken through several changes of the buffer and then incubated for 2 hours at 37 °C in the complete medium whose composition was as follows:

potassium ferricyanide	1.5 mg/ml
manganous chloride	5.0 mg/ml
palmytil-CoA	2.0 mg/ml
α-glycerophosphate	5.0 mg/ml

cacodylate buffer, pH 7.2 0.05 M

After the incubation, the ovaries were washed in several changes of the buffer and subsequently fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 for 2 hours at 4 °C. They were finally dehydrated in an ascending series of alcohols and then embedded in Epon-Araldite mixture.

As controls, some glutaraldehyde-fixed ovaries were incubated in the same medium but without palmytil-CoA.

24. Electrophoresis technique.

(a) Apparatus used. For the electrophoretic analysis (Davis, 1964) of several tissues from Drosophila flies an analytical apparatus (Shandon) with polyacrylamide gels was used. The apparatus consists of two cylindrical buffer reservoirs made of inert, non-conductive material, which were held in a vertical manner by suitable holders. A number of glass tubes are accommodated in corresponding holes of the top reservoir. Rubber grommets are fitted in these holes to ensure a water tight contact with the tubes. Electrodes, made of platinum wires, are lodged in each reservoir; they are placed in such a way as to have the same distance from each glass tube. When the apparatus is assembled, the glass tubes are positioned vertically and their ends are immersed in the buffer solutions contained in the two reservoirs. This makes possible an actual electrical continuity between the two electrodes only through the glass tubes.

A suitable voltage is applied to the electrodes with a power source (Vokam-Shandon) capable of a voltage range up to 400 volts.

(b) Preparation of the gel. The acrylamide solution was freshly prepared each time from a stock solution of 7 % acrylamide in gel buffer. The acrylamide solution had the following composition:

Acrylamide (B.D.H.)	6.82 gm
Methylene-bis-acrylamide (B.D.H.)	.184 gm
b-mercaptoethanol	.078 ml
Temed (B.D.H.)	.030 ml
gel buffer	up to 100 ml

The gel buffer was prepared by dissolving 4.575 gm of Tris in 90 ml of glass distilled water and by adjusting the pH of this solution to pH 8.9 with concentrated HCl. The final volume of the solution was then brought to 100 ml with glass distilled water.

The acrylamide solution was filtered and stored at room temperature for a maximum period of two weeks. For each electrophoretic analysis, 20 ml of this solution were degassed with a water pump; then 0.2 ml of 7 % ammonium persulphate in distilled water was added to allow polymerization of the solution.

The tank buffer was prepared by dissolving 2.88 gm of glycine in 1 litre of glass distilled water. The pH of the buffer was then adjusted to pH 8.3 with dry Tris.

(c) The loading of the gels. The glass tubes with rubber stoppers at the bottom end placed in a vertical position on a plastic holder. The tubes were filled with the acrylamide solution to about 1 cm from the top. Then, with a fine pipette, water was carefully added until the glass tube was completely filled. When

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chemical polymerization of the acrylamide solution was completed (in about 30 minutes), a sharp interface appeared between the gel and the water layer at the top. The water layer is essential for polymerization as otherwise, polymerization will be inhibited by exposure to air. Furthermore, the presence of water at the top of the polymerizing gel ensures the formation of a flat surface which in turn allows an even migration of proteins within the gel.

The polymerized gels were used soon after polymerization and care was also taken to avoid exposing them to direct sunlight. The rubber stoppers were removed and the glass tubes with the gels were fitted on to the top reservoir. Then the tank buffer was poured into the two reservoirs until the ends of the tubes were completely immersed. Any air bubbles that were trapped on the top of the gels were removed by gentle flushing with a pipette.

(d) Preparation of the sample. Haemolymph was extracted from larvae or adults by immersing them in a few drops of 20 % sucrose solution. The haemolymph could be released into the sucrose solution by rupturing the animals with dissecting needles. The diluted haemolymph was then taken up with a micropipette and stored in an ice-bath.

Ovaries from 2-3 day old flies were dissected in Drosophila Ringer and stored in an ice-bath until a sufficient number was collected. For each experiment, about 1 mg of ovaries was homogenized in a 0.5 ml capacity microhomogenizer with 20 μl of 20 %

sucrose - 0.4 M NaCl solution. Subsequently, the homogenate was centrifuged in a refrigerated MSE centrifuge at 10,000 rpm at 4 °C for 10 minutes. The clear supernatant was taken up with a microsyringe and applied directly on the gel surface as previously described.

Fat bodies were collected in much the same way as the ovaries. However, because of the presence of numerous free cells of fat bodies in the haemolymph, especially in the adult flies, care was taken during dissection to collect as many cells as possible in the minimum possible volume of Ringer solution.

(e) Operating conditions. Once the electrophoretic apparatus was assembled, the gels were loaded with the sample solution, by using a precision microsyringe (Hamilton). Usually 20 μl of the sample in the sucrose-NaCl solution was applied to each tube. Then a small amount of bromophenol blue in sucrose solution was added as track dye to every tube.

The electrodes of the electrophoretic apparatus were connected with the corresponding outputs of the power pack. In this type of electrophoretic analysis, the bottom reservoir was connected to the anode (+) while the top reservoir was connected to the cathode (-). The power pack was switched on and maintained at a constant voltage of 50 volts for about 15 minutes. This operation was intended to allow smooth penetration of the sample into the acrylamide gel. At the same time, the application of a low voltage would prevent the sample solution flowing out of the tube due to convection movements. The initial application of a

low voltage serves to some extent the same function as a small spacer gel.

At the end of the 15 minutes period, the power pack was switched to constant current and a current of 3 mA per tube was maintained throughtout the following run. When the band of the bromophenol blue had reached 1 cm from the bottom of the tube, the power pack was switched off. The gels were then removed from the glass tube by inserting a syringe needle into the sides of the tube while the latter was gently rotated. At the same time, water was injected into the space between the tube and the gel and as a result of this procedure, the gels came out smoothly.

(f) Staining of the gels. The gels freed from the glass tubes were subjected to the following treatments as required. For staining proteins the gels were fixed for 1 hour in 12 % TCA at 65 °C. They were then washed briefly in distilled water and transferred to a 0.1 % solution of Coomassie blue in 45 % ethyl alcohol - 10 % acetic acid. The gels were stained in this solution for a period of 2 hours at 65 °C. Then, the gels were destained for 2 hours at 65 °C in a solution of 45 % ethyl alcohol - 10 % acetic acid. The destained gels were stored indefinitely in 7 % acetic acid. The protein bands are stained blue.

For staining lipoproteins, the gels were incubated for 2 hours at 60 °C in a 3 % solution of Crimson (Scarlet R, Gurr) in 56 % ethyl alcohol. After this treatment, the gels were transferred to a freshly prepared Crimson solution and kept overnight at room

temperature. The gels were destained and stored in 10 % acetic acid. The lipoproteins bands appear red after this procedure.

For demonstrating glycoproteins, the gels were preincubated in a 1 % solution of periodic acid in 3 % acetic acid. They were then washed in several changes of distilled water and transferred to a staining solution of 0.5 % reduced acidified basic fuchsin (Schiff reagent). After this staining step, the gels were destained and subsequently stored in a 1 % solution of metabisulphate in distilled water. The glycoprotein bands appear violet in colour.

For haemoglobins, the gels were fixed for 1 hour at room temperature in a 1 % solution of acetic acid. After a brief wash in distilled water, they were incubated for a few minutes in a 0.2 % solution of benzidine in 0.5 % acetic acid to which 0.2 ml of 30 % hydrogen peroxide had been added. The gels could be stored for long periods in 7 % acetic acid. The haemoglobin bands appear black.

25. Isoelectrofocusing technique.

(a) Preparation of the gel. The apparatus used for isoelectrofocusing was the same as that used for disc electrophoresis. The gels were also prepared in very much the same way. In this case, however, the composition of the gel and the operating conditions of the power pack were different. The gel was prepared and used according to the method of Miner and Heston (1972).

A.	Acrylamide	7.0	gm
	Methylene-bis-acrylamide	0.2	gm
	Urea	30.0	gm
	25 % Triton	1.0	ml
	distilled water	90.0	ml
B.	Riboflavin	2.0	mg
	distilled water	50.0	ml

For each isoelectrofocusing analysis, 25 mg of ammonium persulphate was dissolved in 5 ml of solution B and 2 ml of this was mixed with 18 ml of the solution A. Then 0.5 ml of ampholine carrier with a range of pH from 3.5 to 10.0 was added to this mixture. The presence of both ammonium persulphate and riboflavin in the acrylamide solution ensures an even polymerization of the gel. This was accomplished by exposing the glass tubes to a lamp for at least 30 minutes.

The tank buffers were 0.24 N sulphuric acid for the upper reservoir and 0.48 N NaOH for the lower reservoir. The cathod (-) was connected to the lower reservoir, while the upper reservoir was connected to the anode (+).

After the isoelectrofocusing apparatus was completely set up, the gels were loaded with 25 μl per tube of a freshly prepared ampholine solution. The latter consisted of 0.05 ml of ampholine (pH range 3.5-10.0) diluted in 2 ml of distilled water with 100 mg of sucrose and a few grains of methyl green added as track dye. Subsequently, the gels were prerun for about 30 minutes at an initial voltage of 200 volts. At the end of this period, the sample was applied on to the gel surface by

using a microsyringe. When the voltage had arisen to 400 volts, the power pack was shifted to constant voltage and left to run overnight. Throughout this period the isoelectrofocusing apparatus was kept in the cold room at 4 °C.

The sample (ovaries and haemolymph of 2-3 day old flies) for this analysis were prepared in a solubilizing solution. This solution had the following composition:

Potassium carbonate	50 mM
Urea	8 M
b-Mercaptoethanol	10 %
Triton X-100	5 %

The isoelectrofocusing was completed when the index of the mA meter marked a current value close to zero. This condition, in fact, indicates that all proteins in the sample had reached their isoelectric point and would therefore no longer be available for conducting electric charges within the gel.

(b) Autoradiography on dried gels. In one experiment, 2-3 day old flies were injected with 0.5 μl each of ^{14}C - protein hydrolysate (Radiochemical Centre, Amersham) with a radioactive concentration of 1 μCi/ μl. The injected flies were then maintained at 25 °C for 24 hours in glass vials containing standard Drosophila food. At the end of this period, ovaries and haemolymph were extracted from these flies. Both tissues were prepared in the solubilizing solution and used for isoelectrofocusing without further treatment.

After isoelectrofocusing was completed,

the gels were removed from the glass tubes and stained in Coomassie blue. One gel from each of the tissue analyzed was then taken up for autoradiography. The gel was first placed on a block of dry ice until it was well frozen. The frozen gel was then put in an apparatus for longitudinal slicing. The apparatus (Fairbanks et al., 1965) consists of four pieces of plexiglass which when assembled together form a gel holder with a central cavity just sufficient to hold the cylindrical gel.

A cutting device, consisting of three stainless steel wires, was inserted at the top of the gel holder. As this device was drawn through the central cavity, the gel was cut into four longitudinal slices. The two inner slices were then arranged on a wet filter paper and the entire array was placed in a large funnel. The latter was then attached to a conical flask which was connected to a water pump. The filter paper, with the gel slices up, was covered with a polythene bag which was kept in place by a soft rubber gasket.

When vacuum was applied to the glass flask, the polythene bag exerted an uniform pressure over the gel slices. Further, the funnel was heated by a lamp placed at a short distance from the filter paper. Under the combined effect of the mechanical pressure and the heat, the slices were finally dried to extremely thin strips which adhered to the filter paper.

The radioactivity in the gels was detected by autoradiography of the dried slices using Kodirex film. The whole set up was clamped tightly between masonite plates with spring clips, wrapped in aluminium

foil and stored in a refrigerator at 4 °C for a week.

The exposed films were processed according to the following procedure: development was carried out for 4 minutes at 20 °C in Dx-80, diluted 1 : 4 with distilled water. The stop bath was Kodak indicator Stop bath and this was used for 10 seconds at a dilution of 1 : 25 with distilled water. The autoradiographs were fixed for 12-14 minutes in Fix-sol (Johnsons) diluted 1 :3 with distilled water. The preparations were then washed for 30 minutes in running water and allowed to dry.

V. OBSERVATIONS

Part I - Ultrastructural, cytochemical and autoradiographic observations on growing ovarian chambers.

Ovarian chambers in various stages of development are shown in Figs. 1-4. The classification of the chambers is according to criteria described by Cummings and King (1969). The morphological features they described apply particularly well to chambers after the onset of yolk formation, i.e. from stage 8 onwards. It is therefore possible to distinguish consecutive stages of vitellogenic chambers without much difficulty. The situation is, however, different in the case of previtellogenic chambers were Cummings and King's (1969) account is of doubtful value. It is only when ovarioles have been cut through the entire length of their anterior region and a careful comparison made between the successive chambers that a precise staging is possible. Identification of chambers in stages 3 to 5 has thus been made in favourable sections of ovarioles where such a comparative study of successive chambers was possible. The ultrastructural observations that follow have been made from chambers that were staged unambiguously.

For studying the early stages, entire previtellogenic ovarioles were embedded but in the case of vitellogenic stages single chambers were isolated before embedment in Epon-Araldite. The actual staging was carried out on thick sections of the Epon-Araldite blocks that were subsequently used for preparing ultrathin sections.

General note to micrographs

All micrographs presented in this thesis are electron micrographs except when stated otherwise. As described in the material and methods section, the material for ultrastructural studies was usually fixed in glutaraldehyde followed by osmium tetroxide and embedded in Epon-Araldite mixture. Details regarding fixation and embedding procedures are therefore given in the explanation to the figures only if the methods employed differ from those mentioned above. The staining of the ultrathin sections was usually done in uranyl acetate and lead citrate unless stated otherwise.

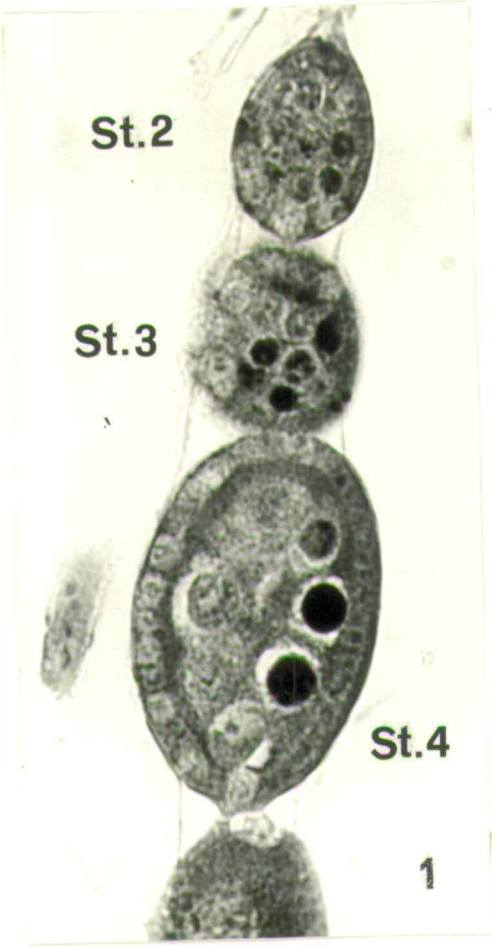
Figs. 1-4. Light micrographs of tangential sections of ovarian chambers at different stages, taken from ovaries embedded in paraffin and stained in iron haemotoxylin.

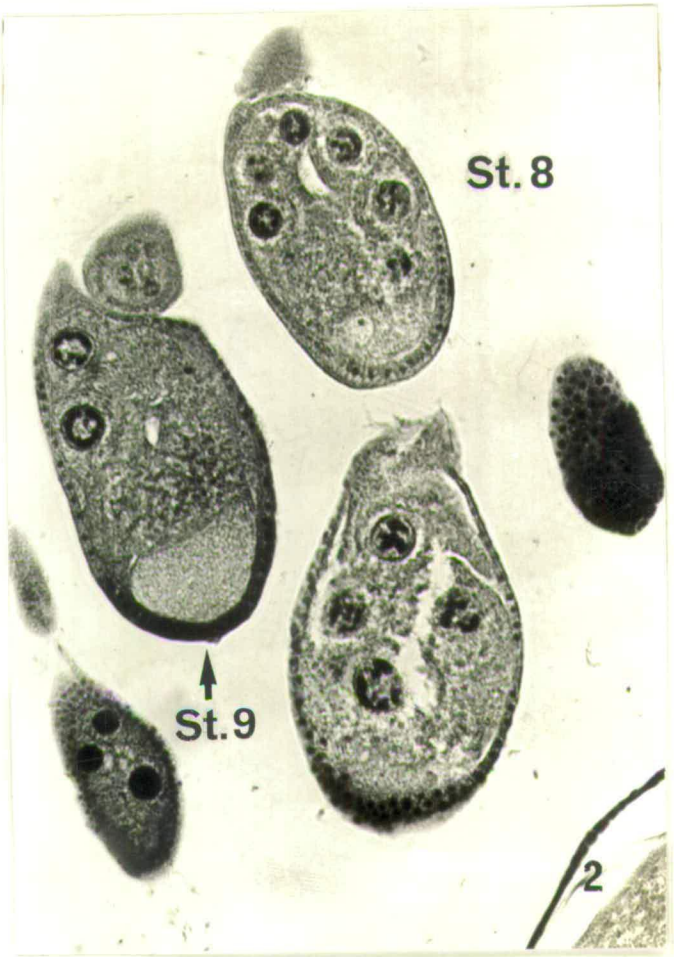
Fig. 1. Early previtellogenic stages: stage 2 (St.2), stage 3 (St.3), stage 4 (St.4). Note that all cells of the same chamber have more or less the same size. A layer of follicle cells, all of the same size, surrounds the chamber. x 1,000.

Fig. 2. Vitellogenic stages: in the ovarian chamber at the top of the picture (St. 8) the oocyte occupies a small part of the posterior end of the chamber. In the ovarian chamber on the left (St.9) the oocyte occupies about one third of the length of the chamber. x 500.

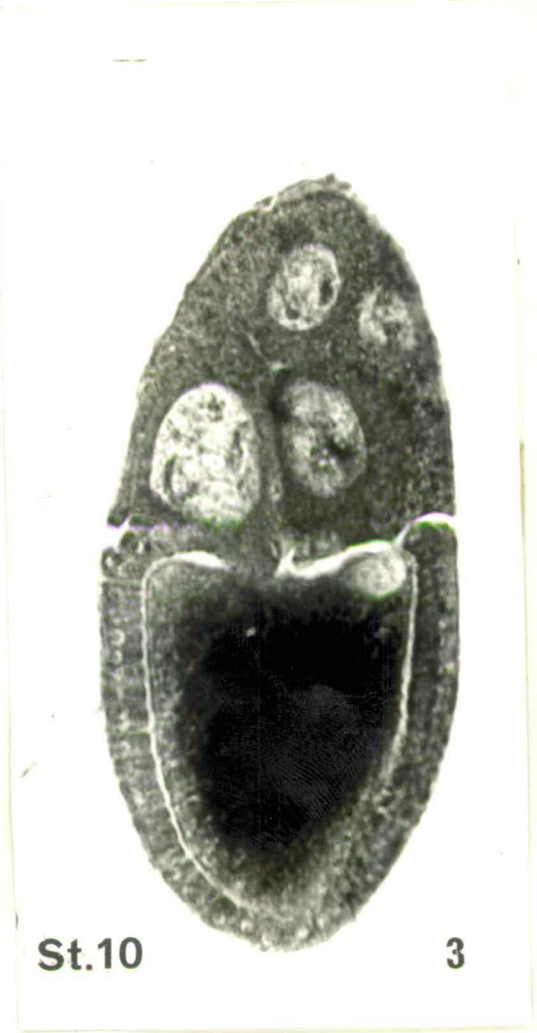
Fig. 3. Stage 10 ovarian chamber (St.10): the oocyte occupies the entire posterior half of the chamber, while the nurse cells occupy the anterior half. x 1,000.

Fig. 4. Stage 12 ovarian chamber (St.12): the oocyte extends for up to about two thirds of the chamber and the nurse cells form a cap-like region at the anterior end of the chamber. x 1,000.


St.2
St.3
St.4
1


St. 8
St.9
2


St.12
4


St.10
3

The abbreviations to the micrographs are as follows:

A, rickettsia-like bodies
Ab, Associated body
Am, Amorphous material
Bf, bundle of fibrils
bl, basement lamina
C, centriole
chr, chromatin material
Cr, canal rim
cv, coated vesicles
Cy, cytolysosomes
db, dense bodies
eb, endobody
EC, epithelial cell
ena, endochorionic precursors
ER, endoplasmic reticulum
ES, epithelial sheath
Fb, fuzzy bodies
fb, fibrillar components
FC, follicle cells
Fc, fibrous coat
fl, fuzzy layer
G, Golgi apparatus
gJ, gap junctions
gr, granular components
ib, intercellular bridges
ien, inner endochorion
J, desmosomes
Ks, karyosome
L, lipid droplets
Lf, lipofuchsin granules
Ly, lysosomes
m, mitochondria
mb, main body
mf, myelin figures
mfi, microfilaments
mt, microtubules
mp, microperoxisomes
mvb, multivesicular bodies
mVm, mottled vitelline m.
N, nucleus
nb, nuclear blebs
NC, nurse cells
ne, nuclear envelope
no, nucleolus
nor, nucleolar remnants
np, nuclear pores
nv, nucleolar vacuoles
oen, outer endochorion
OO, ooplasm
ool, oolemma
p, pits
pi, endochorionic pillars
py, micropyle
R, ring canals
Rb, rounded bodies
rb, residual bodies
RER, rough endoplasmic reticulum
SC, synaptonemal complexes
Sl, superficial layer
SJ, septate junctions
t, tubules
v, vesicles
Vb, vitelline bodies
Vm, vitelline membrane
vp, vitelline precursors
w, wispy material
α_2, alpha-2 yolk
β, beta-spheres
y, yolk platelets
α_1, alpha-1 yolk

1. The oocyte

(a) The nucleus. The oocyte is one of the two cells in the 16-cell clusters situated in the anterior region of the germarium that exhibit synaptonemal complexes in the nucleus (Figs. 17 and 19). When the stage 1 chamber is formed the oocyte is the only cell with persisting synaptonemal complexes and it also occupies the most posterior part of the chamber.

The oocyte nucleus of ovarian chambers in stages 1 and 2 exhibits a round shape and measures about 5 microns in average diameter (Fig. 5). It also occupies most of the oocyte volume since the average diameter of the entire oocyte at these stages is about 7 microns in diameter. The chromatin material is more or less evenly distributed throughout the nucleus and synaptonemal complexes are also frequently observed. Amidst the chromatin material there also occur patches of dense granules which measure 400 A in diameter as shown in Fig. 5. The nucleolus in oocyte stage 1 and 2 chambers is fairly large in size and has a fairly compact organization. These features together with its high electron density make this organelle a prominent constituent of the nucleus at these stages; it usually has an eccentric location in the nucleus.

In the oocyte nucleus in stage 3 chamber the chromatin tends to aggregate and the nucleolus shows a tendency to have a loose organization of its constituents. A stage 4 oocyte nucleus is shown in Figs. 6-9. The chromatin material is now organized as a ring-shaped structure in which the synaptonemal complexes are still evident (Fig. 6); this structure has been referred to

Fig. 5. The oocyte from a stage 1 ovarian chamber. Synaptonemal complexes (SC) are observable within the chromatin material (chr). The nucleolus (no) consists of fibrillar and particulate regions. Note that the small area of the ooplasm contains ribosomes, mitochondria (m), a centriole (C) and a Golgi apparatus (G). Patches of dense granules (double arrows) are visible in the nucleus. x 22,500.

Figs. 6-9. Serial sections of the oocyte nucleus from a stage 4 ovarian chamber.

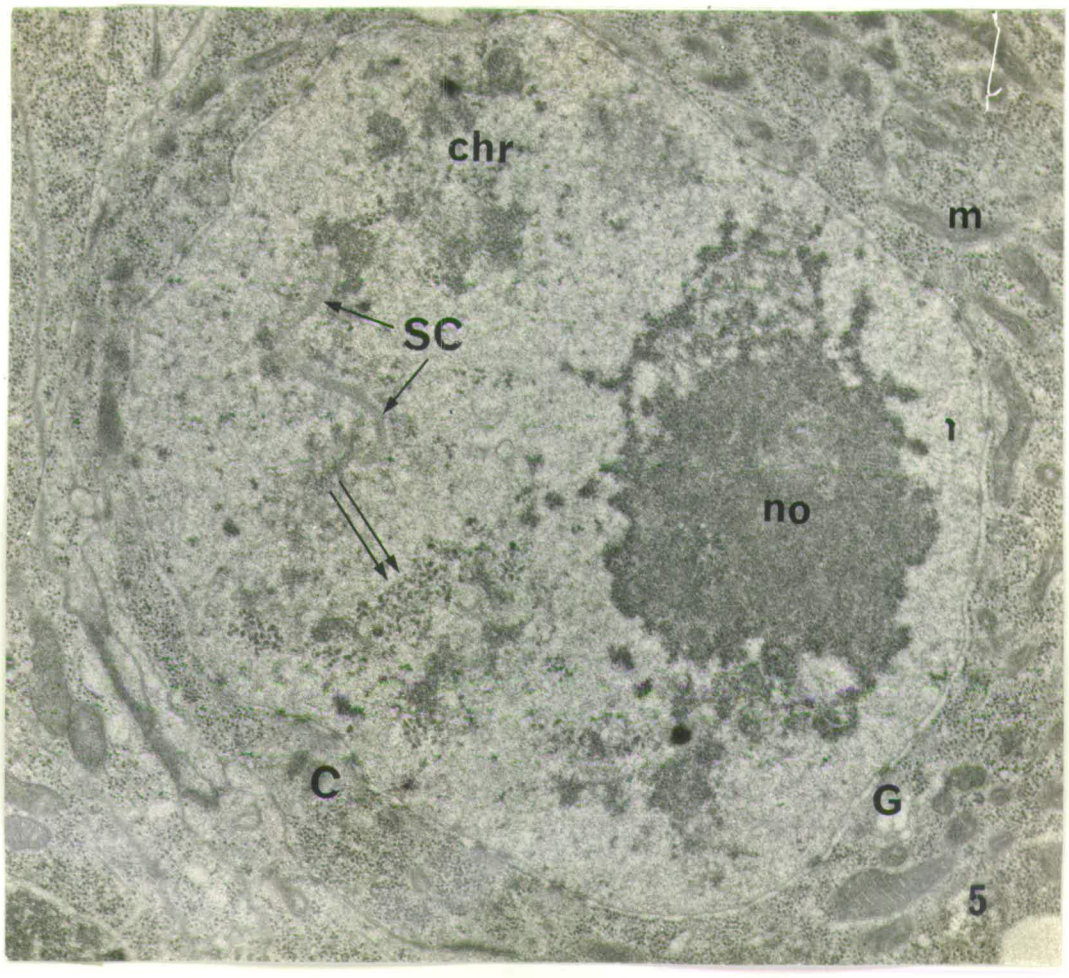
Fig. 6. The chromosomes are gathered into a rounded body called the karyosome (Ks). Synaptonemal complexes (SC) are visible within the karyosomal mass. x 22,500.

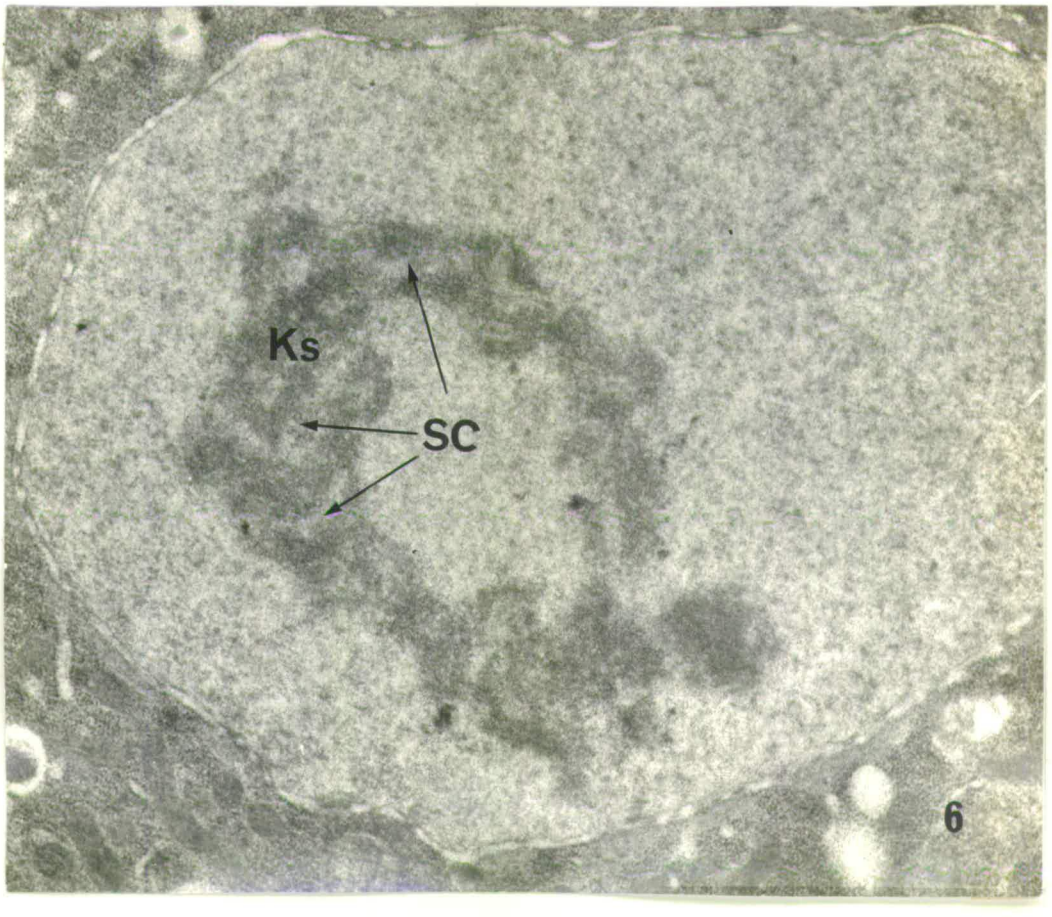
Fig. 7. Intimate association between the karyosomal material (Ks)(still recognizable by the presence of synaptonemal complexes (SC) within), and the endobody (eb). x 22,500.

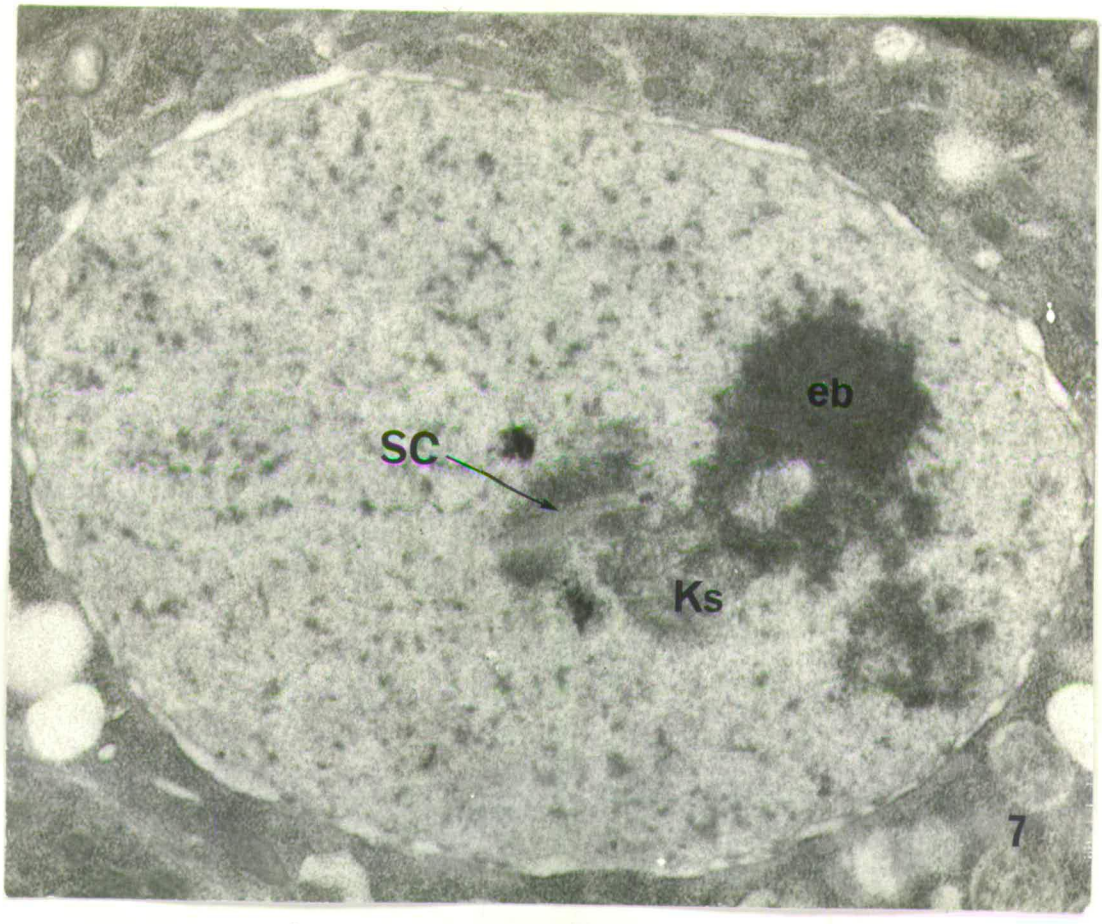
Fig. 8. Karyosomal material (Ks) and endobody (eb) can still be seen closely associated, but the synaptonemal complexes are no longer discernible. x 22,500.

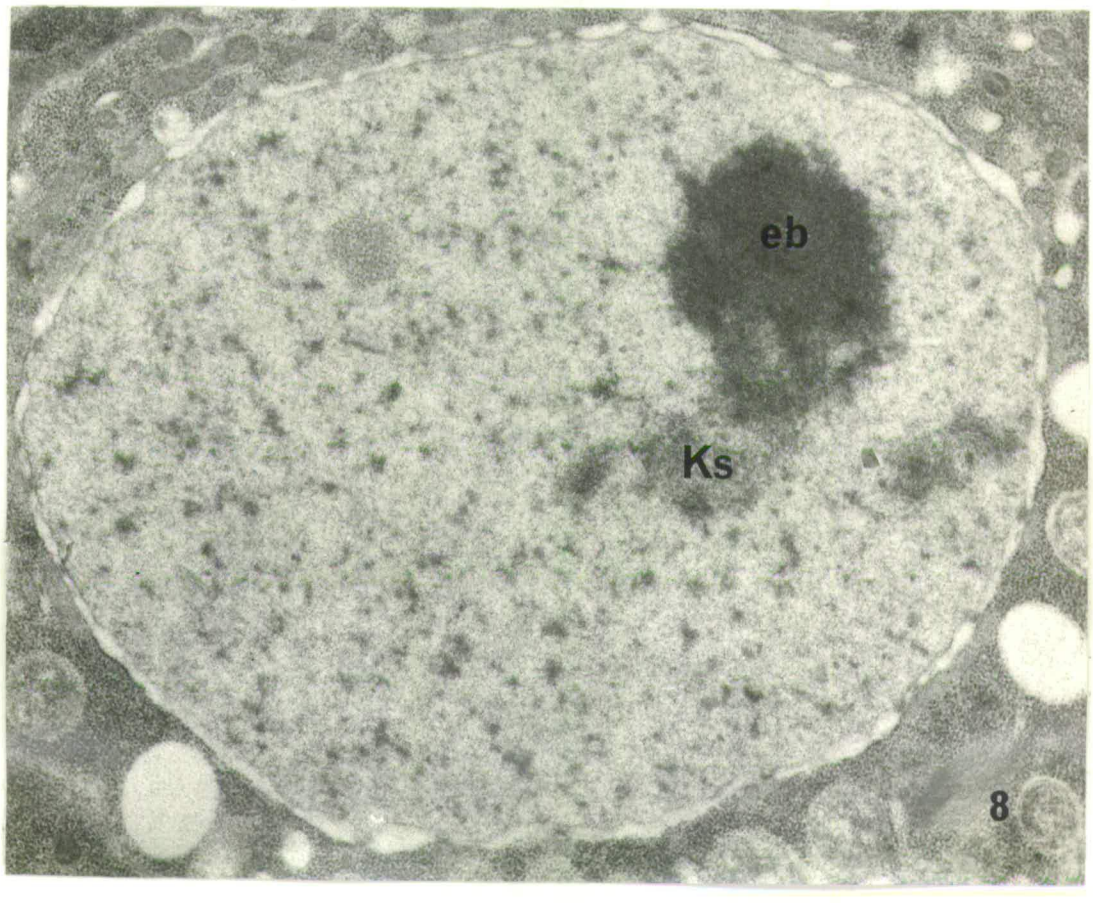
Fig. 9. Small clumps of fibrillar material (see arrows) are visible in regions surrounding the endobody. The ooplasm forms a small even fringe around the nucleus; several rickettsia-like bodies or A bodies (A) can be seen. x 22,500.

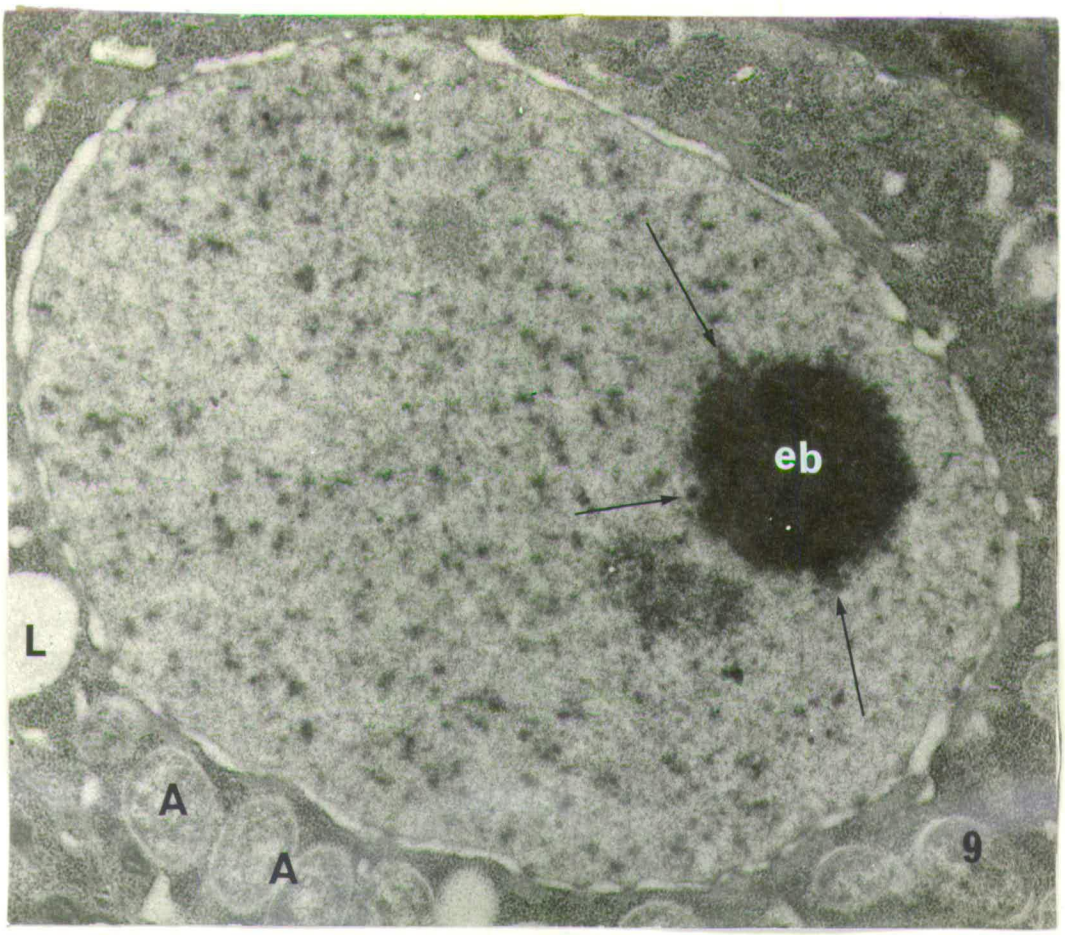
Fig.10. The oocyte nucleus from a stage 5 ovarian chamber. Note that the endobody (eb) and the karyosome (Ks) interlock with each other. Remnants of synaptonemal complexes (SC) are visible within the karyosomal mass. Fibrillar clumps (see arrows) can be seen within the nuclear sap and at the margin of the endobody. x 30,000.

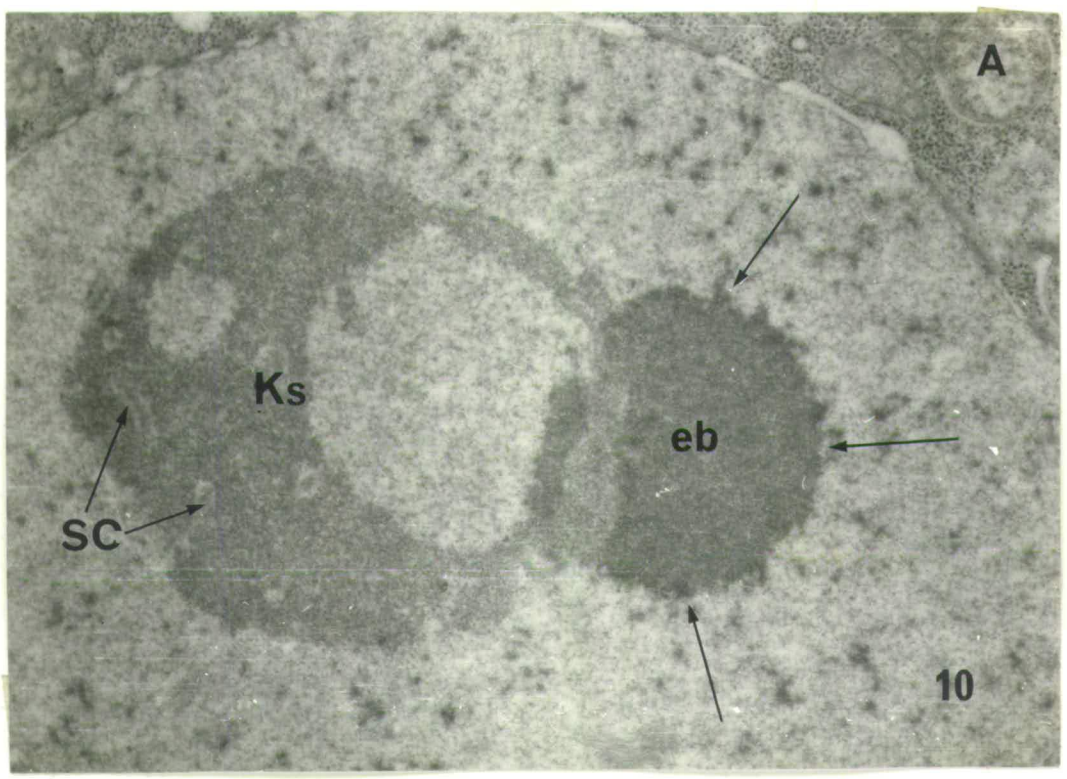

chr
m
SC
no
C
G
5


Ks
SC
6


eb
SC
Ks
7


eb
Ks
8


eb
L
A
A
9


A
Ks
eb
SC
10

as karyosome in the literature. The nucleolus at this stage is not so conspicuous as in earlier stages and appears as a small fibrillar mass continuous with the karyosome (Figs. 7 and 8). The granular constituents of the nucleolus are no longer discernible and these have presumably become dispersed in the nuclear sap. A nucleolus of purely fibrillar nature has been observed in certain stages of oogenesis in Musca domestica by Bier et al. (1967) who referred to it as the endobody. Although the morphology of the nucleolus has changed, compared with earlier stages, it continues to have a peripheral location in the nucleus.

The intimate association between the chromatin material (karyosome) and the nucleolus (the so called endobody) is clearly seen in Fig. 10 which represents an oocyte nucleus in a stage 5 chamber. The relationship between the karyosome and the endobody appears to continue in oocyte stage 7, although the endobody is now smaller than before. By the use of cytochemical technique which enables one to distinguish between RNA and DNA containing structures (Bernhard, 1969), I have confirmed the nucleolar (ribonucleoprotein) nature of the endobody and the chromosomal (deoxyribonucleoprotein) nature of the karyosome (Figs. 11 and 12).

The appearance of the karyosome, which was more or less unchanged during stages 4-7 begins to change in stage 8, and in stage 9 it appears as a fibrillar mass with a loose texture (Fig. 13). The synaptonemal complexes are no more evident as clearly as before. A characteristic feature of the oocyte nucleus at this

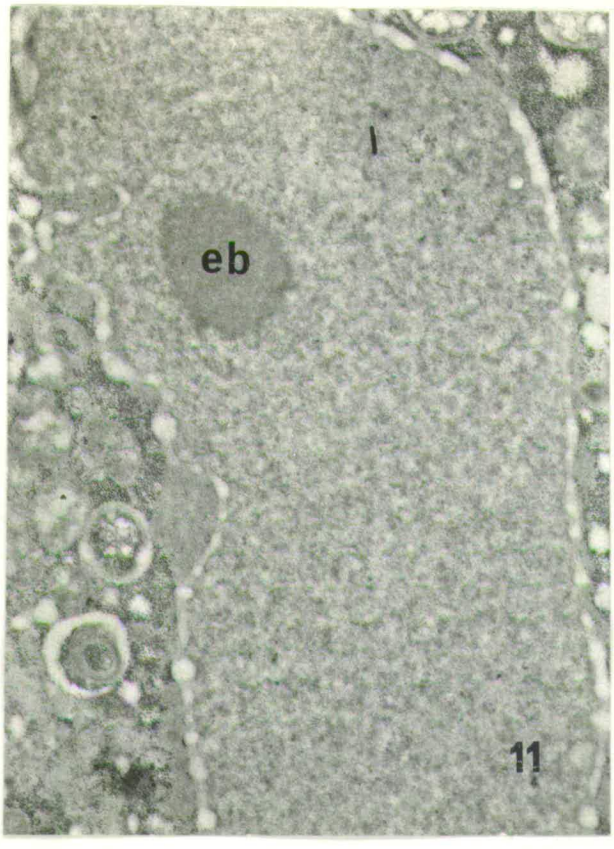
Figs. 11-12. Stage 7 ovarian chamber fixed in glutaraldehyde and embedded in glycol methacry-late. Sections containing the oocyte nucleus were stained with 0.05 % aqueous uranyl acetate for 1 minute, treated with 0.02 M EDTA for 2 minutes and then counterstained with lead citrate.

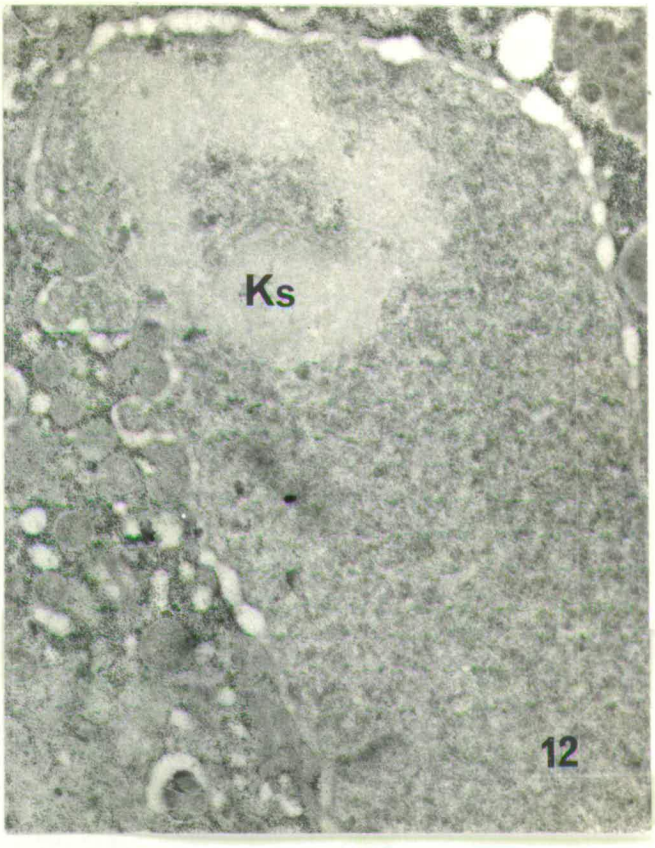
Fig. 11. Section of the oocyte nucleus passing through the endobody (eb). Note that after EDTA treatment the endobody is not bleached. x 15,000.

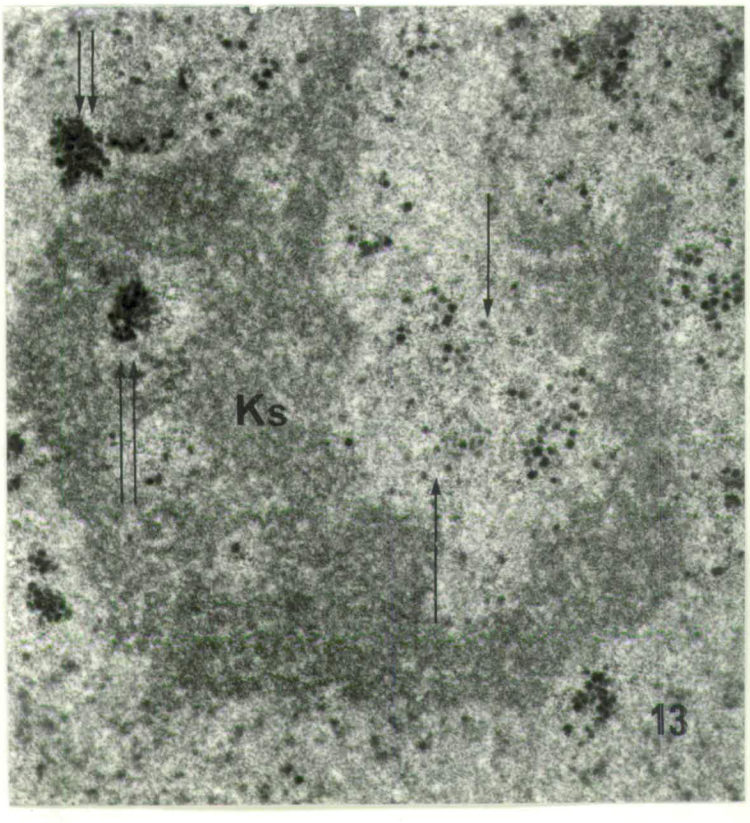
Fig. 12. Section of the oocyte nucleus passing through the karyosome (Ks). Note that after EDTA treatment a ring-shaped area corresponding to the karyosome appears bleached. x 15,000.

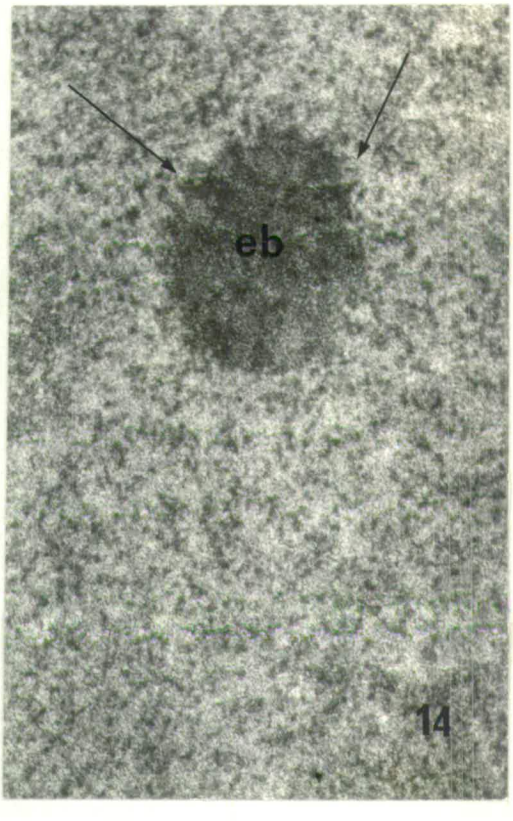
Fig. 13. An oocyte nucleus from a stage 9 ovarian chamber. Note that the karyosome (Ks) at this stage exhibits a looser structure than in previous stages (see Fig. 10). Two types of granules are present in sections passing through the karyosome. The large granules (700 A) occur in clusters, while the small granules (400 A) are more scattered. x 20,000.

Fig. 14. An oocyte nucleus from a stage 9 ovarian chamber. The endobody (eb) consists of only fibrillar constituents. Some of these constituents are present in regions of the nucleus near the endobody. x 20,000.


l
eb
11


Ks
12


Ks
13


eb
14

stage is the presence of granules. Some of the granules are large (about 700 A in diameter) and tend to occur in clusters amidst and around the karyosome (Fig. 13). The second type of granules is of smaller size (about 400 A in diameter), less dense, and are scattered in that region of the nucleus containing the karyosome. In stages 9 and 10 the region of the oocyte nucleus containing the endobody is, however, characterized by the presence of small clumps of finely fibrillar material (Fig. 14). This material, in fact, was first observable in stage 4 in sections of the nucleus passing through the endobody (Figs. 7 and 8). The granular constituents described above, which occur in association with the karyosome, were noticeable for the first time only in stage 9, as already mentioned. A careful examination of a large number of sections in stages 9 and 10 gives the impression that the large granules are most probably derived from the karyosome (Fig. 15). The source of the small granules is less clear, although their presence in the vicinity of the karyosome makes a similar origin likely.

A peculiar feature that has been observed frequently in stage 10 is the appearance of nuclear protrusions into the cytoplasm (Fig. 16). These protrusions are bounded by both the membranes of the nuclear envelope, and images such as those in Figs. 16 and 18 give the impression that they are in the process of being detached from the nucleus. At the same time a new stretch of nuclear envelope appears to be forming as though to bridge the gap at the base of the protrusion. Some of the largest protrusions extend up to about 2 microns from the nucleus and measure about 0.6-0.8 microns at their

Fig. 15. The oocyte nucleus from a stage 10 ovarian chamber. Note that a number of large sized granules are in close association with the karyosome (Ks). x 20,000.

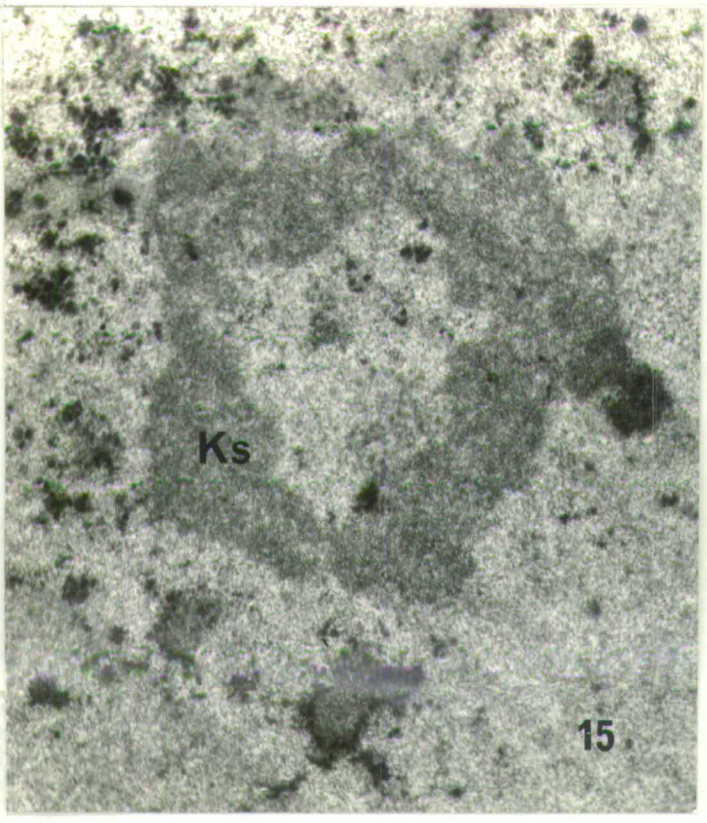
Fig. 16. Part of the oocyte nucleus from a stage 10 ovarian chamber. Note the presence of several nuclear blebs (nb) formed by both membranes of the nuclear envelope. Some of these blebs are found free in the cytoplasm close to the nuclear envelope (see arrows). Clusters of large granules (see double arrows are visible in the nucleus. x 16,000.

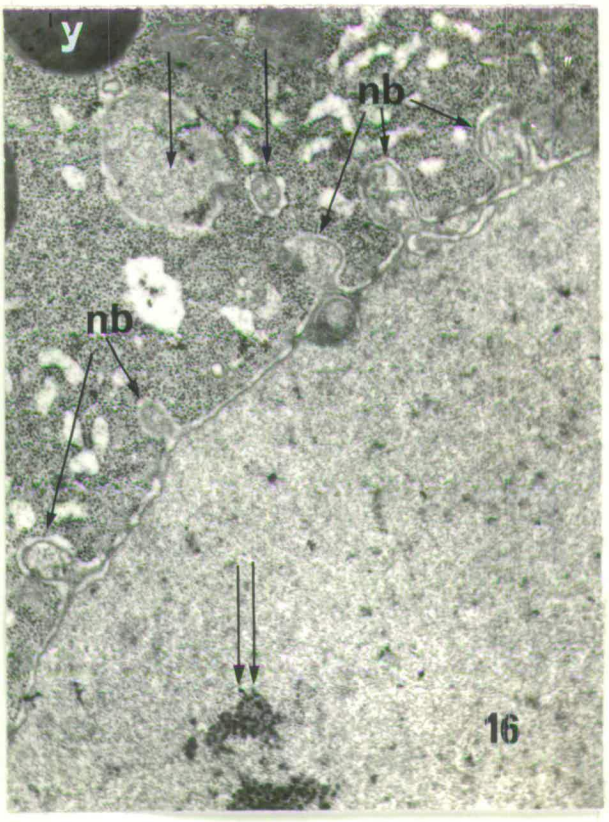
Fig. 17. Part of a 16-cell cluster in the germarium. Two of the cells visible in the micrograph possess synaptonemal complexes in their nuclei; they are thus identified as pro-oocytes. Unbound ribosomes, rickettsia-like bodies (A) and a ring canal (R) are visible. x 10,000.

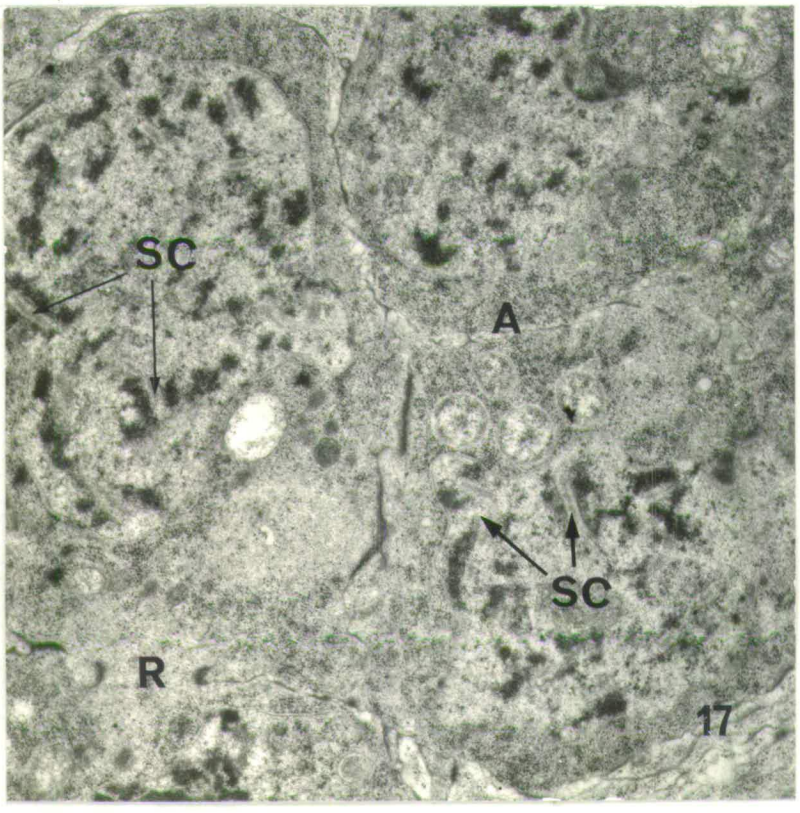
Fig. 18. A high resolution electron micrograph of the nuclear bleb in the perinuclear ooplasm of a stage 10 ovarian chamber. Note the formation of new stretches of nuclear envelope at the base of the bleb (see arrows). Fibrillar material is visible inside the nuclear bleb. x 32,000.

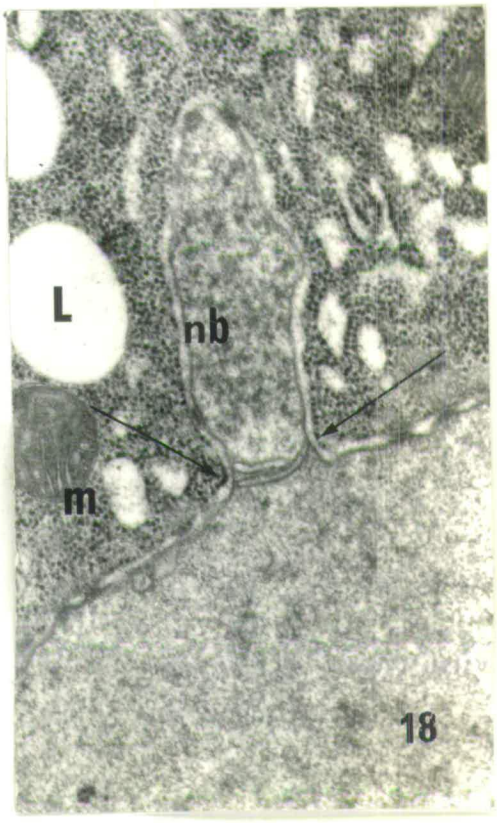
Fig. 19. Part of a pro-oocyte at the anterior region of the germarium. Note the abundance of ribosomes. Also note mitochondria (m), centriole (C) and Golgi apparatus (G) in the perinuclear cytoplasm. Microtubules (mt) are oriented along an axis perpendicular to the ring canal (R). x 20,000.

Fig. 20. Nurse cell-ooplasm border of a stage 6 ovarian chamber. Note the presence of numerous rickettsia-like bodies (A) in the ooplasm (OO). Such bodies are extremely scarce in the nurse cytoplasm (NC). x 15,000.


Ks
15


y
nb
nb
16


SC
A
SC
R
17


L
nb
m
18

greatest width. Occasionally they appear to contain the same type of material as in the nuclear sap but, perhaps in somewhat higher concentration. In these stages (stages 9 and 10), there are also observable large vesicles in the oocyte cytoplasm close to the nucleus as shown in Fig. 16. The size and content of these vesicles closely match those of the nuclear protrusions. It is, however, surprising that such vesicles were never observed in the deeper regions of the cytoplasm although an intense search was made for them in a large number of sections.

(b) The ooplasm. There is no observable difference in the cytoplasm of the two pro-oocytes in the 16-cell cluster at the anterior region of the germarium (Fig. 17). As stated earlier, the area of the cytoplasm is very limited in all these cells, the bulk of which is occupied by the nucleus (Fig. 5). The cytoplasm of the pro-oocytes contains a large number of free ribosomes. Although they sometimes occur in groups, no images suggestive of polysomes were observed. There are also no suggestions of the presence of endoplasmic reticulum at this stage. Apart from the abundant ribosomes, the other constituents of the cytoplasm are the Golgi apparatus, mitochondria, microtubules, centrioles and the rickettsia-like bodies. In several favourable sections it has been noted that the microtubules are preferentially oriented along an axis perpendicular to the ring canal (Fig. 19).

In stage 1 chamber, where the oocyte is identifiable as such with certainty, the picture of the cytoplasm is essentially the same as that described

Fig. 21. Part of the ooplasm from a stage 6 ovarian chamber. The fuzzy bodies (Fb) are seen to consist of bundles of fibrils about 1000 A thick (see arrows). Fine fibrils (about 60 A in thickness) can be discerned within the bundles. x 20,000.

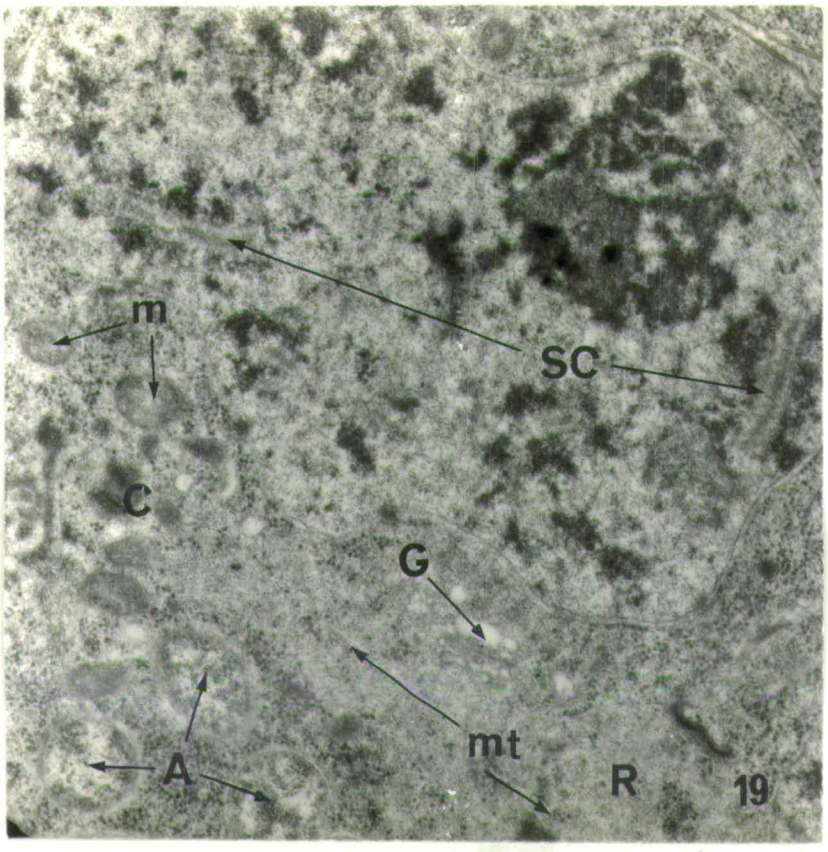
Fig. 22. Part of the ooplasm from a stage 7 ovarian chamber. Note the cytolysosomes (Cy) of various size and consistency. No cytoplasmic organelles are visible within them. x 12,000.

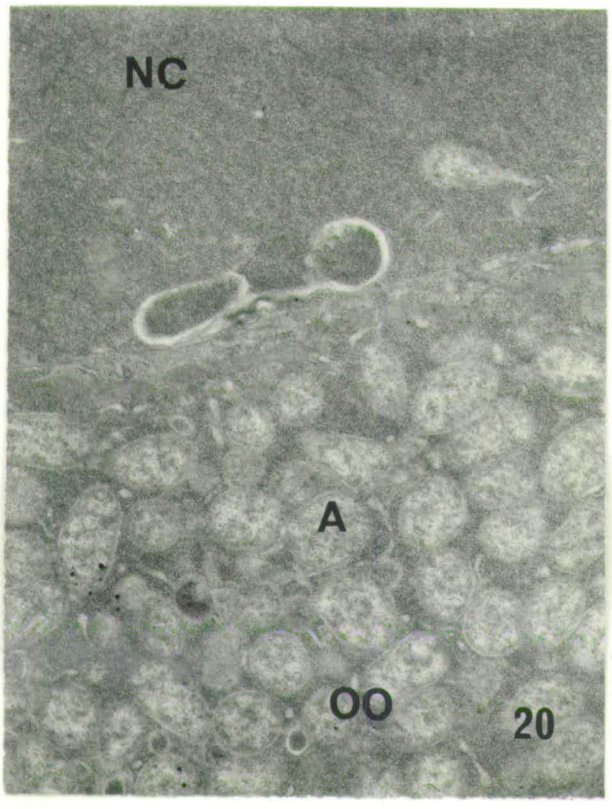
Fig. 23. A high resolution micrograph of the central region of the ooplasm from an early stage 8 ovarian chamber. Amidst what look like yolk platelets (y) there are a number of lysosomal derivatives which range from multivesicular bodies (mvb) to myelin figures (mf). x 32,000.

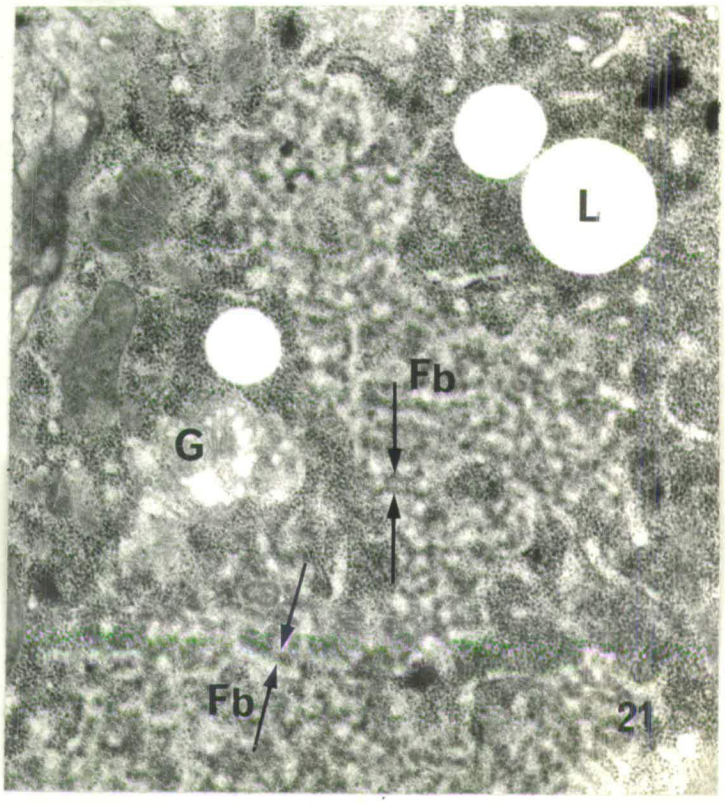
Fig. 24. Ooplasmic inclusions from an early stage 8 ovarian chamber. Note that these inclusions consist of two distinct regions with a yolk-like appearance (y) and a myelin configuration (mf). x 20,000.

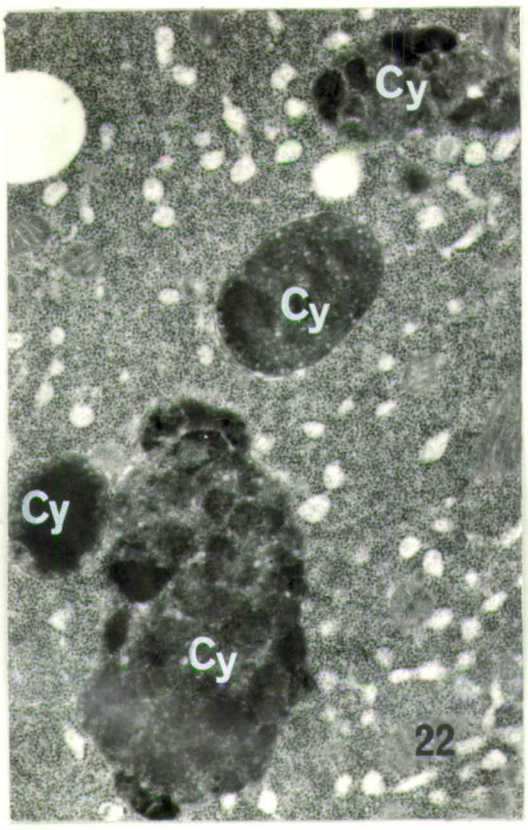
Fig. 25. Yolk platelets (y) in the central ooplasm of an early stage 8 chamber. The yolk platelets appear to be derived from cytolysosomes (Cy). x 32,000.

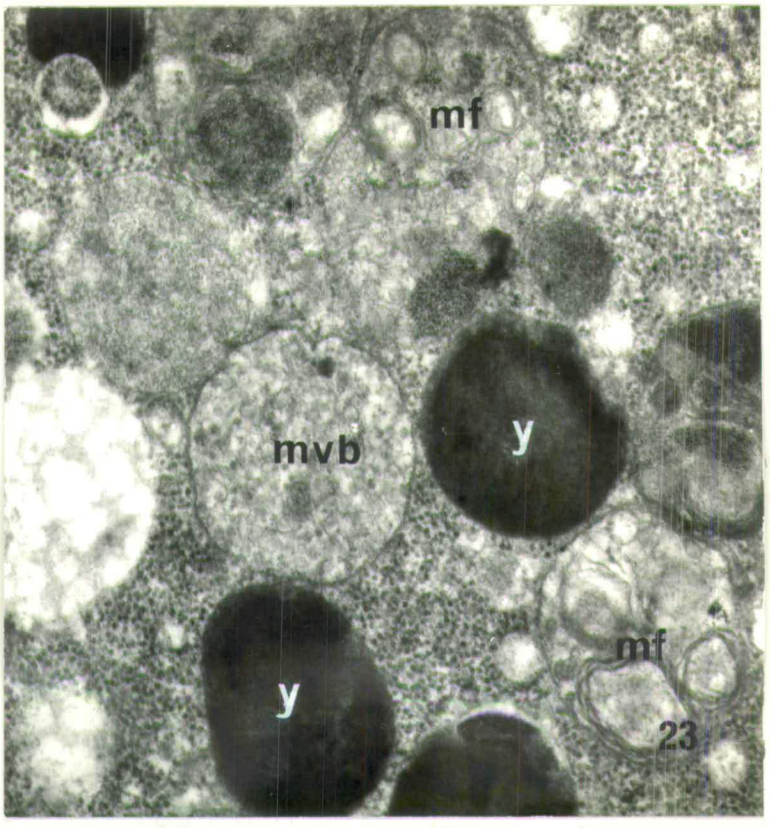
Fig. 26. Part of the central ooplasm from an early stage 8 ovarian chamber. Yolk platelets (y) may be seen amidst myelin figures (mf) containing a number of concentric whorls of membranes. The central yolk platelet is closely associated with lysosomal derivatives (Cy). x 32,000.

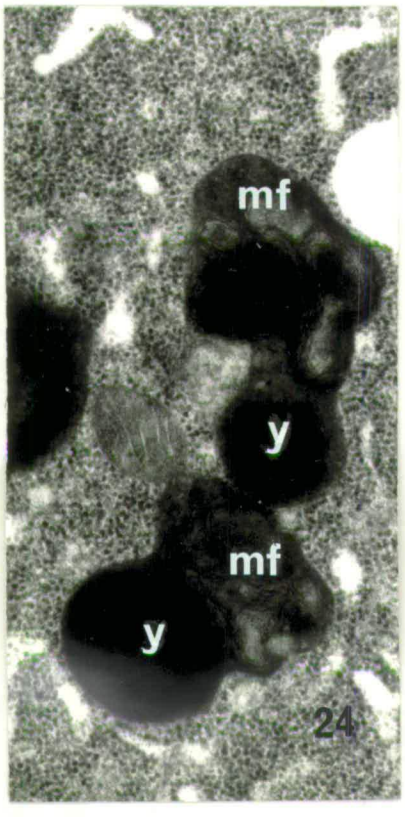

m
SC
C
G
mt
A
R
19

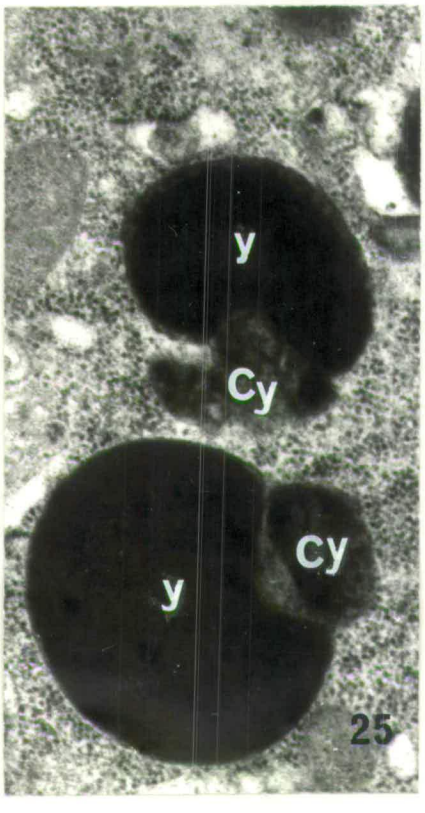

NC
A
OO
20

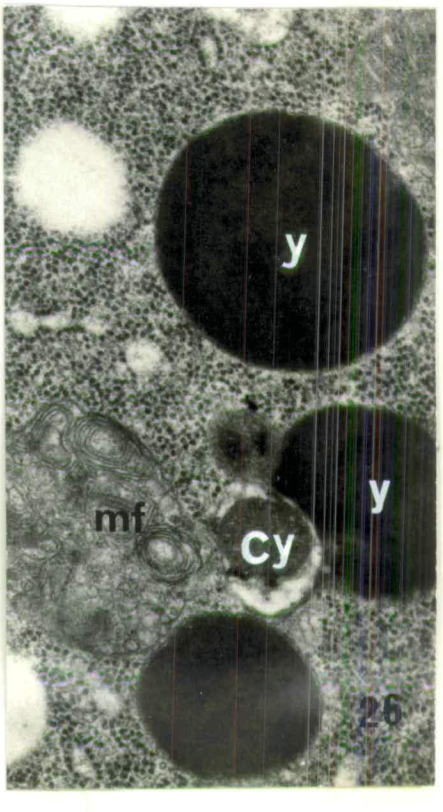

L
Fb
G
Fb
21


Cy
Cy
Cy
Cy
22


mf
mvb
y
y
mf
23


mf
y
mf
y
24


y
Cy
Cy
y
25


y
y
mf
Cy
26

above. The same situation continues through stages 2 and 3.

As stage 4 is reached, the volume of the cytoplasm relative to the nucleus has increased and the number of the rickettsia-like bodies has increased enormously (Fig. 20). Mitochondria are also abundant and several lipid droplets become visible for the first time. Another constituent which has also appeared for the first time in noticeable numbers in the developing oocyte is an irregular amorphous looking, membrane limited, dense body. These bodies may measure up to 4 microns in length and about 2 microns at their greatest width, and some of them appear to contain what looks like mitochondria. The overall morphology presented by these bodies closely resembles that of certain structures referred to as cytolysomes by Novikoff and Essner (1962) and as cytolysosomes by other workers.

The ooplasm of stage 5 chamber shows increased numbers of the various organelles and inclusions described in stage 4. Certain fuzzy looking areas, however, become observable at this stage for the first time. This stage also marks the beginning of the formation of vesicles which appear to be the forerunners of the endoplasmic reticulum. The vesicles are scattered throughout the ooplasm but there seems to be some preferential location in the vicinity of the fuzzy areas mentioned. A continued increase in the concentration of the various cytoplasmic entities is noticeable in the ooplasm of stages 6 and 7. An increase is also noticeable in the size of some of the cytolysosomes and the areas occupied by the fuzzy material. In some

sections of stage 6, the area of such material may be as much as 48 μ^2 out of about 170 μ^2 of the ooplasm. In high resolution picture (Fig. 21) the fuzzy material resolves into meandering bundles of fibrils 1000 A thick within which extremely fine fibrils, measuring about 60 A in diameter, can be made out. An idea of the tremendous numbers of rickettsia-like bodies occupying certain areas of the ooplasm even in stage 6 can be gained from the electron micrograph shown in Fig. 20.

By stage 7 there is also a bewildering variety of cytolysosomes some of which are shown in Fig. 22. The largest of these has a very irregular outline, contains dense and light material and presents a highly heterogenous internal structure. The smallest one has a fairly homogeneous area in the middle which is surrounded by a narrow periphery made up of vesicles of more or less uniform size, 0.5-1 micron in diameter. The third morphological type of cytolysosome has a matrix of low density within which occur numerous small vesicles; the overall appearance of this body recalls the entities referred to as multivesicular bodies in the literature.

The stage 8 ooplasm presents a far greater variety of lysosomal inclusions than ever before. Typical example of these are shown in Figs. 23-26. In some of these inclusions myelin figures of different sizes are an almost universal constituent; such inclusions are generally electron translucent. What is probably a later version of a multivesicular body is seen in the centre of the electron micrograph in Fig. 23. What looks like

a free yolk platelet is present in Fig. 26; it has a limiting membrane and an electron dense main body. A type of inclusion which appears to combine in itself features of the yolk platelets and lysosomal inclusions is quite common at this stage (Figs. 24-26). All above mentioned lysosomal inclusions are usually located in the deeper portions of the ooplasm. Another noteworthy feature in the ooplasm of stage 8 chamber is the high incidence of Golgi apparatus: these organelles occupy a subcortical position and some of them are in contact with yolk platelets. Some of the most interesting observations in stage 8 oocytes relate to events which occur at the level of the plasma membrane or oolemma facing the overlying layer of follicle cells. The oolemma is now thrown into numerous folds or microvilli which interdigitate with similar folds of the plasma membrane of adjoining follicle cells (Figs. 27 and 28). The modification of the oolemma is much less pronounced along the border facing the nurse cells (Fig. 27). The folds or microvilli mentioned above are not in any sense long and disposed regularly as for instance in the intestinal epithelium. The microvilli are rather short and appear to be intertwined so that in some sections the follicle-oocyte border appears as a vesiculated area. Close to the basal region of the microvilli of the oolemma there are present numerous small vesicles and tubules (Figs. 27-28). These vesicles are more or less uniform in size (measuring on the average 1500 A in diameter) and even in low power pictures they can be seen to have some ultrastructure. This is best studied in high resolution pictures where the vesicles clearly

exhibit three layers (Figs. 29 and 29a to c). The outermost layer - about 200 A thick - has a spiked appearance which gradually merges with the matrix of the ooplasm. Because of the presence of the spiked layer, these vesicles are referred to as coated vesicles. The innermost layer of the vesicles is about 300-400 A thick and seems to consist of a rather fuzzy material similar to that present at certain locations or pits (described below) on the surface of the oolemma. In favourable sections (Fig. 30), the outermost layer is seen to be composed of a regular array of rounded granules (about 150 A in diameter) placed at an average centre-to-centre distance of about 200 A from each other. Some of the vesicles, however, do not show the spiked outer layer, and from an examination of several electron micrographs, one gets the impression that it is mainly those vesicles lying further away from the microvilli that lack the outer spiked layer. The tubules of the cortical ooplasm have the same ultrastructure as the vesicles just described but without the external spiked layer.

The surface of the oocyte shows another interesting modifications known as the pits which are present in between the microvilli (Figs. 29 and 29c). The pits consist of depressions of the oolemma and contain a dense fuzzy material which bears a close resemblance to the innermost layer of the vesicles and tubules already described in the cortical ooplasm. In some instances, the pits are situated at a distance of up to 0.5 microns from the surface of the oolemma and appear to possess a long neck; the neck region is, however, devoid of the fuzzy material. It has also been noted in higher power

Fig. 27. Portion of the ooplasm (OO) from an early stage 8 ovarian chamber along the border with the follicle cells (FC) and the nurse cells (NC). Numerous folds or microvilli (mv) are present along the follicle-oocyte border, but are absent at the nurse-oocyte border. Note the tubular entities (t) in the cortical ooplasm. x 20,000.

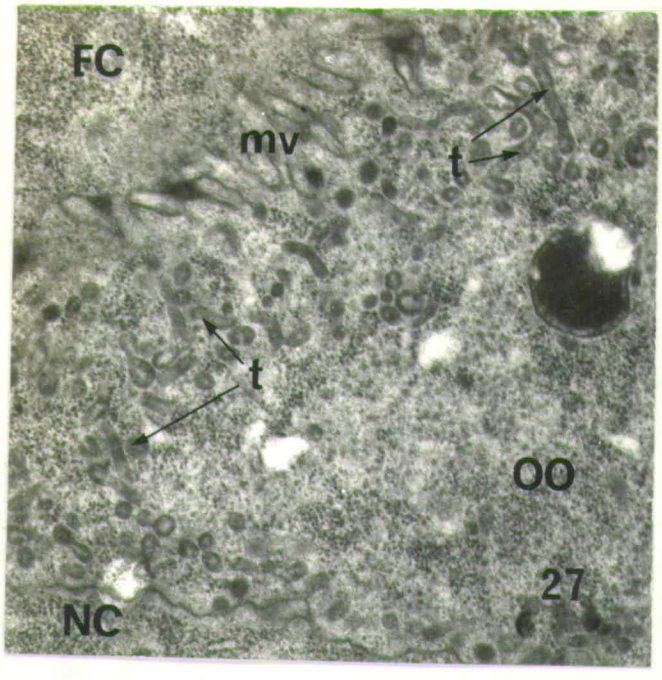
Fig. 28. The cortical ooplasm from a stage 8 ovarian chamber. Note the presence of several coated vesicles (cv) close to the basal region of the microvilli. Other vesicles (v), which are apparently not coated, are present in deeper regions. Two s small yolk platelets (y) and mitochondria (m) are also visible. x 20,000.

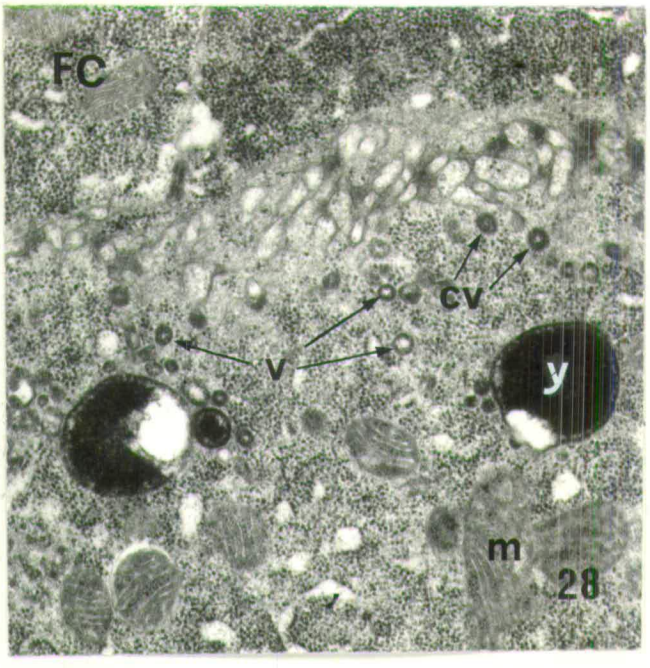
Fig. 29. Part of the oocyte-follicle border from a stage 10 ovarian chamber. By this stage the vitelline membrane (Vm) is almost completed. The cortical ooplasm exhibits a number of pits (p) and coated vesicles (cv), at the base of the microvilli. (vp): vitelline membrane precursors in the follicle cells (FC). x 20,000.

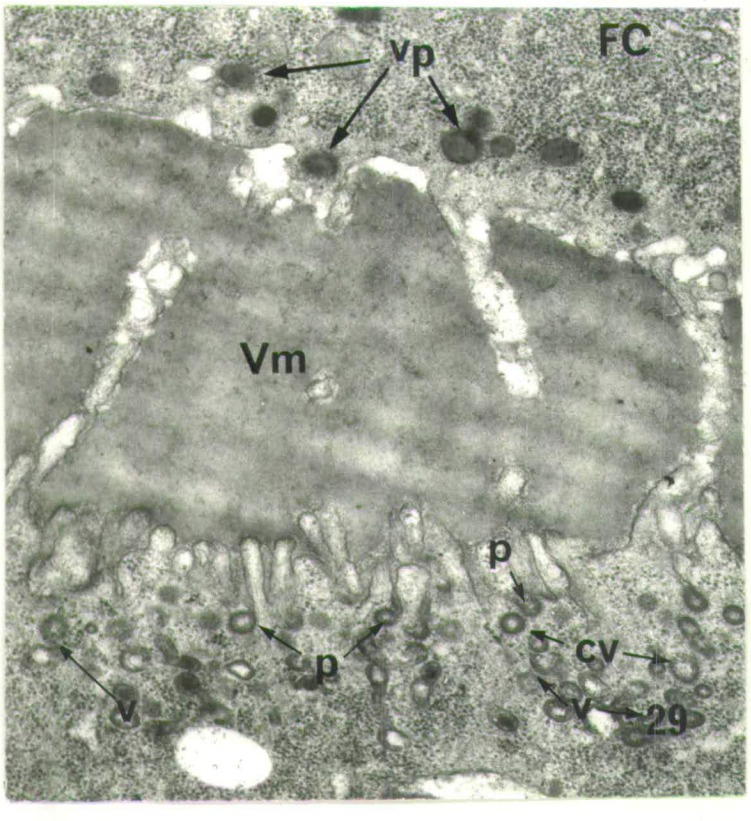
Figs. 29a and 29b. Higher magnifications of the regions of the pits (p). A thick (about 300 A) layer of fuzzy material lines the interior of the pits. On the outside, the pits have a 200 A spiked layer. The unit membrane which forms the intermediate layer of the pits is continuous with the oolemma. x 40,000.

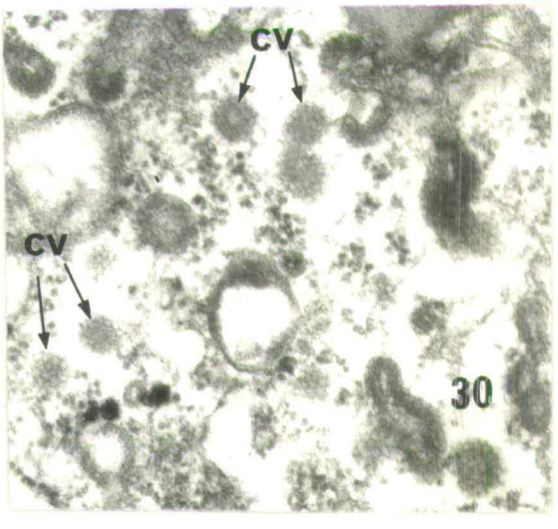
Fig. 29c. A coated vesicle situated close to the region of a pit. Note that the coated vesicle exhibits features quite similar to those of the pit. The vesicle is not in contact with the unit membrane of the oolemma. x 40,000.

Fig. 30. Part of the cortical ooplasm showing several tangential sections of the peripheral spiked layer of the coated vesicles (cv). The spiked layer resolves into a number of granules about 150 A in diameter, and arranged regularly with a centre-to-centre distance of about 200 A. x 32,000.


FC
mv
t
t
OO
NC
27


FC
cv
v
y
m
28


vp
FC
Vm
p
p
cv
v
v
29


cv
cv
30


29a


28b


29c

pictures that the region of the proper pit embedded in the ooplasm has a spiked appearance as in the case of some vesicles (the coated vesicles) already described.

In the region of the cortical ooplasm immediately below the microvilli and the other entities so far described, there are observable larger vesicles measuring about 0.6 microns in average diameter. Three such instances are shown in Figs. 31-33. From the disposition of the smaller vesicles around the larger ones, one gets the impression that the larger vesicles are formed by extensive fusion of the smaller ones. It would also appear that the tubules mentioned earlier in the cortical ooplasm are also involved in the phenomenon of fusion (Fig. 33). While the small vesicles have a rather empty appearance, apart from the fuzzy layer on the inner side, the larger vesicles contain somewhat denser material which fills them to varying degrees (Figs. 31-33); these vesicles are referred to as the earliest formed yolk or the smallest yolk platelets in the cortical ooplasm. In the deeper regions of the ooplasm of stage 8 chambers there are also usually found spherical bodies with a homogeneous content which is extremely electron dense (Figs. 25-26); these have been referred to in earlier pages and are regarded as yolk platelets which originate, at least in part, from cytolysosomes.

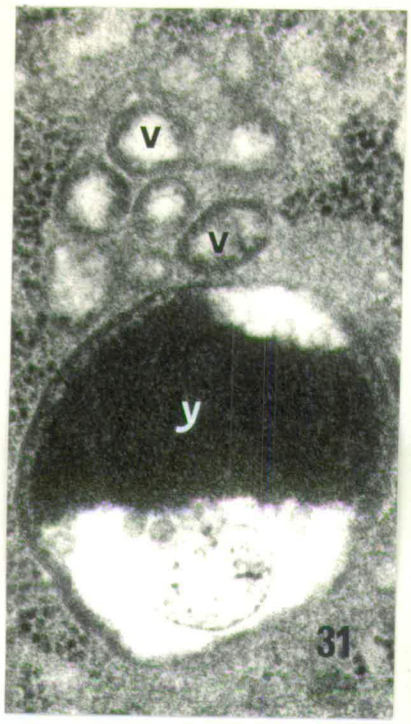
Vitelline membrane formation along the follicle-oocyte border begins at stage 9. The overall morphology of the oocyte surface at this stage remains essentially the same as described in stage 8 chambers, see Fig. 34. Noticeable differences, however, occur in

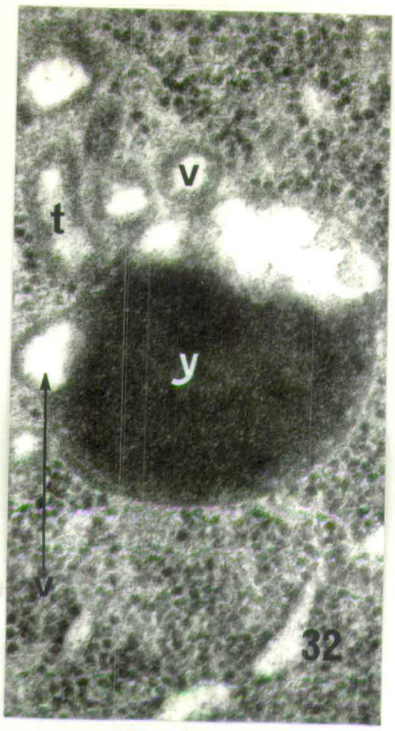
Fig. 31. High power micrograph of the cortical ooplasm from an early stage 8 ovarian chamber. Several non-coated vesicles (v) are in the proximity of growing yolk platelet (y). A fuzzy layer is discernible in both the vesicles and the yolk platelet. Material of homogeneous consistency fills part of the lumen of the yolk platelet (y); the rest is seemingly empty. x 80,000.

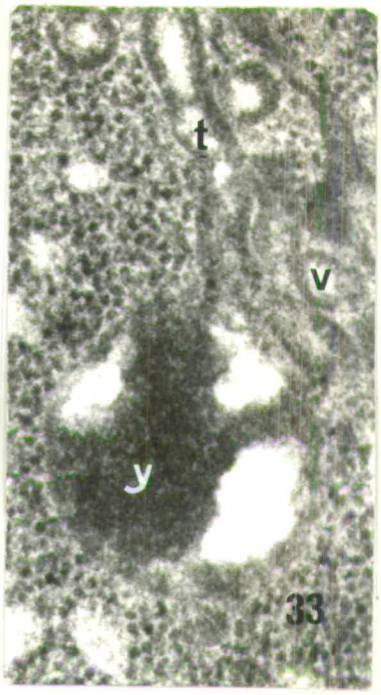
Fig. 32. Another high power micrograph of the cortical ooplasm from an early stage 8 ovarian chamber. Note that a non-coated vesicle (v) has fused with the limiting membrane of the yolk platelet. A tubule (t) is also visible in the proximity of the yolk platelet (y). x 80,000.

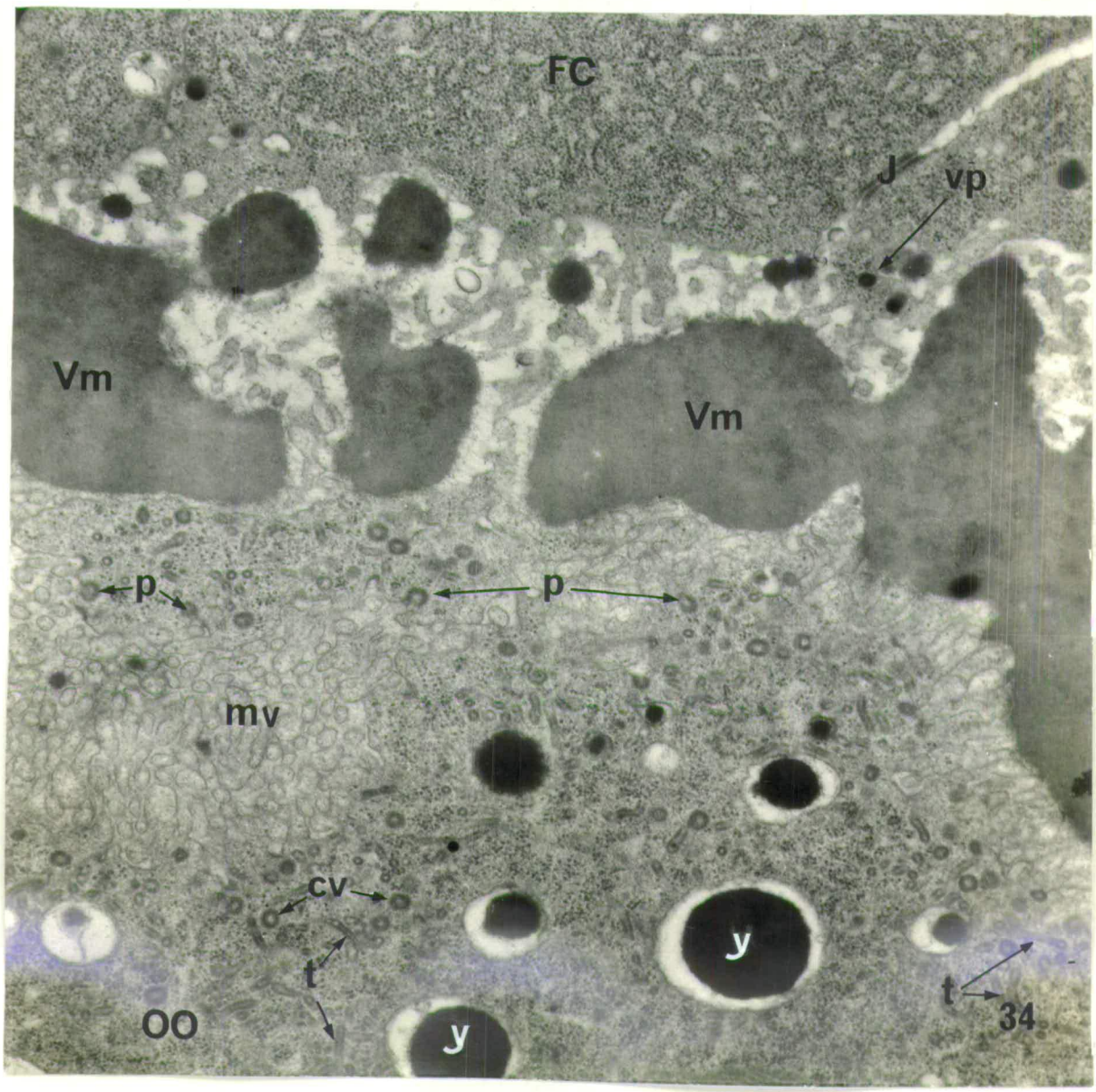
Fig. 33. Also a high power micrograph of the cortical ooplasm of an early stage 8 chamber. A tubule (t) is seen in actual continuity with a growing yolk platelet (y). Note an inner fuzzy layer in both the tubule and the yolk platelet. x 80,000.

Fig. 34. Follicle-oocyte border from a stage 9 ovarian chamber. Follicle cells (FC), vitelline membrane (Vm) and cortical ooplasm (OO) are shown in this micrograph. Note that large yolk platelets (y) are present in deeper regions of the cortical ooplasm. A cell junction (J) is discernible between two adjacent follicle cells. The pits (p) in the oolemma, as well as the coated vesicles (cv) and tubules (t) in the cortical ooplasm are also indicated. x 18,000.


v
v
y
31


t
v
y
v
32


t
v
y
33


FC
J
vp
Vm
Vm
p
p
mv
cv
t
y
OO
y
t
34

the morphology of the yolk platelets present. Several yolk platelets in stage 9 ooplasm exhibit a peculiar appearance as shown in Figs. 35 and 36. In addition to the limiting membrane and the main dense body, this type of yolk platelets have an asymmetrically located superficial layer of lower density than the main body; this is the yolk platelet described as alpha-1 by King et al. (1966). The ultrastructural appearance of this cap-like area (superficial layer) sitting on the main body varies according to the fixative and embedding medium used. Following fixation in glutaraldehyde and embedment in glycol methacrylate the cap region appears to contain a prominent empty looking body - what I have called the associated body - and a number of much smaller spherules (Fig. 35). In preparations made after osmium fixation and embedment in Epon-Araldite mixture, the associated body does not seem quite empty (Fig. 36).

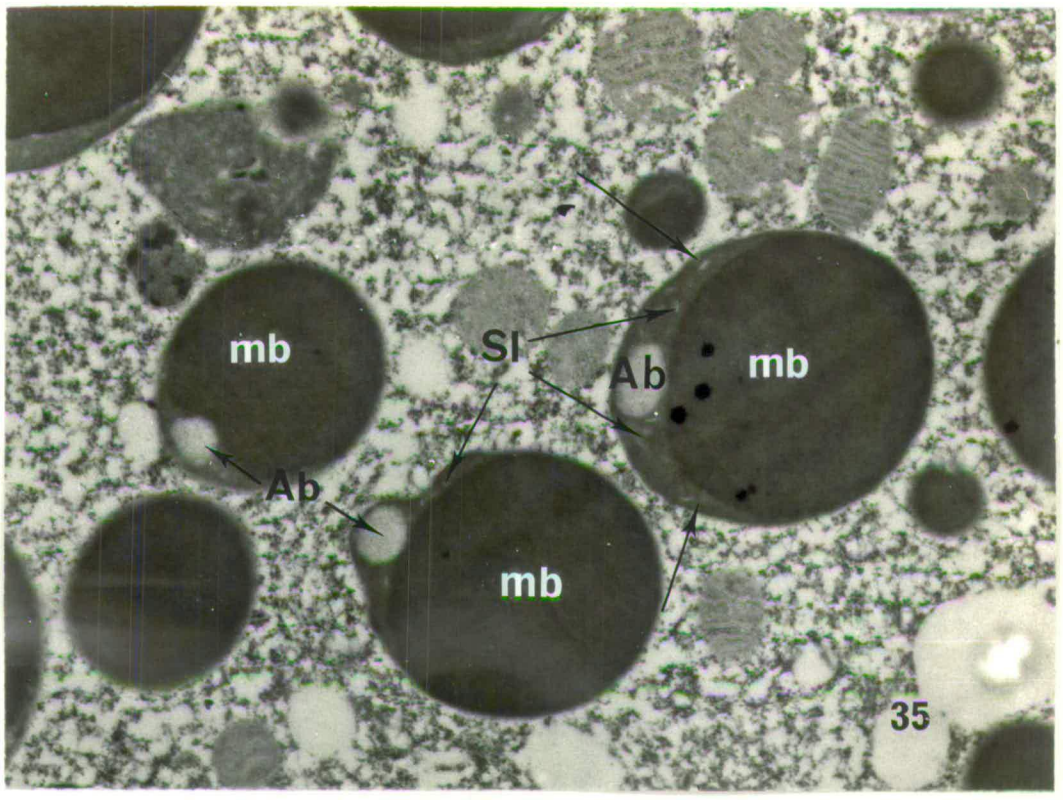
In stage 9 oocytes, some changes occur at the oocyte-nurse cells border which are somewhat similar to those described in previous stages along the follicle-oocyte border. However, the modifications along the nurse cells border are not so exaggerated as those described along the border with the follicle cells. A type of inclusion seen occasionally in the ooplasm along the border with the nurse cells is shown in Fig. 37. The nature of this inclusion is not clear. Typical multivesicular bodies have also been encountered in the ooplasm close to the nurse cells border.

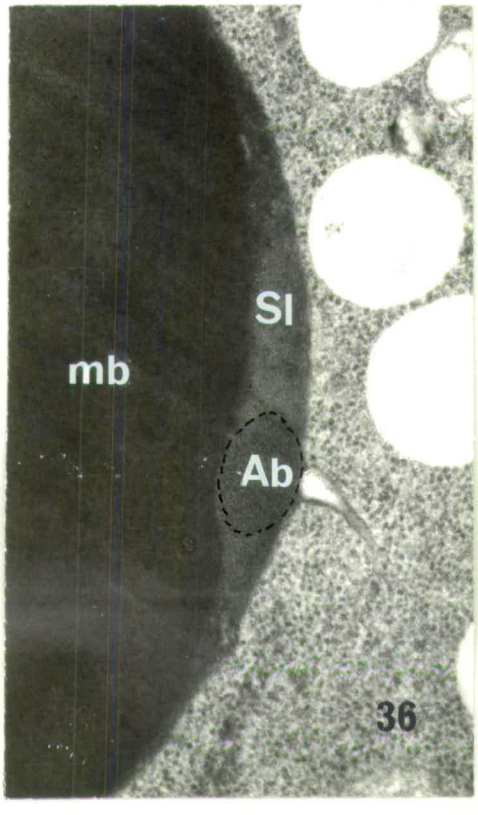
No significant differences have been noted in the ooplasm during stages 10-12 except that from stage 11 onwards there is an accumulation of mito-

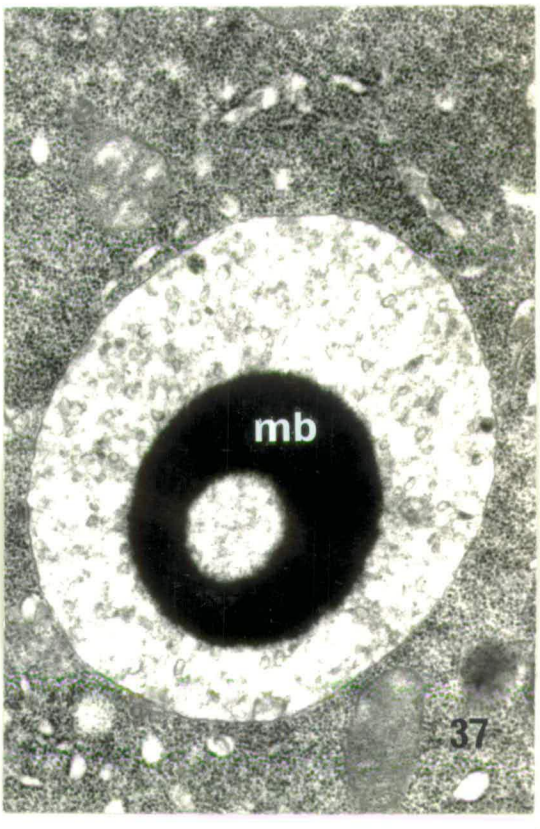
Fig. 35. Part of the central ooplasm from a stage 9 ovarian chamber taken from an ovary fixed in glutaraldehyde and embedded in glycol methacrylate. The alpha-1 yolk platelets consist of an homogeneous main body (mb) surrounded by a cap-like region of the superficial layer (Sl) within which a prominent empty looking associated body (Ab) and small spherules (see arrows) can be made out. x 8,000.

Fig. 36. High power micrograph of part of a alpha-1 yolk platelet from the central ooplasm of a stage 9 ovarian chamber, showing the cap-like region of the superficial layer (Sl). Note that the associated body (Ab) stands out less clearly than in Fig. 35. x 32,000.

Fig. 37. A type of inclusion found in the ooplasm of a stage 9-10 chamber along the border with the nurse cells. It has a ring-shaped central mass (mb) and a wide surrounding area with sparse material. The whole inclusion is bounded by a membrane. x 20,000.


mb
Sl
Ab
mb
Ab
mb
35


Sl
mb
Ab
36


mb
37

chondria and electron dense bodies of irregular shape in the region of the ooplasm facing the nurse cells (Fig. 38). These bodies contain patches of ferritin-like particles in regular arrays and bear a superficial resemblance to what has been called lipofuchsin granules in the literature.

An important observation in stage 13 and later oocytes relates to the frequency with which one encounters different types of yolk platelets. In stage 13, in addition to the yolk platelets described in earlier stages, there are also present inclusions of the type shown in Figs. 39 and 40b. The one shown in Fig. 39 is limited by a membrane and has several dense areas within; this inclusion corresponds in appearance to the alpha-2 yolk platelet described by King et al. (1966). The inclusion shown in Fig. 40b consists of numerous particles and have no limiting membrane; in these features, this inclusion resembles the beta-spheres of King et al. (1966). These particles measure about 410 A in diameter, and by the use a cytochemical method (Thiery, 1967) I have confirmed that the particulate components are glycogen (Fig. 40a). It can be clearly seen from Fig. 41a that glycogen makes its first appearance in close association with the type of yolk platelets referred to as alpha-1. What appears to be a later stage in the process is shown in Fig. 41b where the accumulated glycogen tends to form a separate entity. This seems to be the mode of formation of the beta-spheres. In addition, the yolk platelet associated is no longer of the alpha-1 type, but rather acquires characteristics of the alpha-2 yolk platelets.

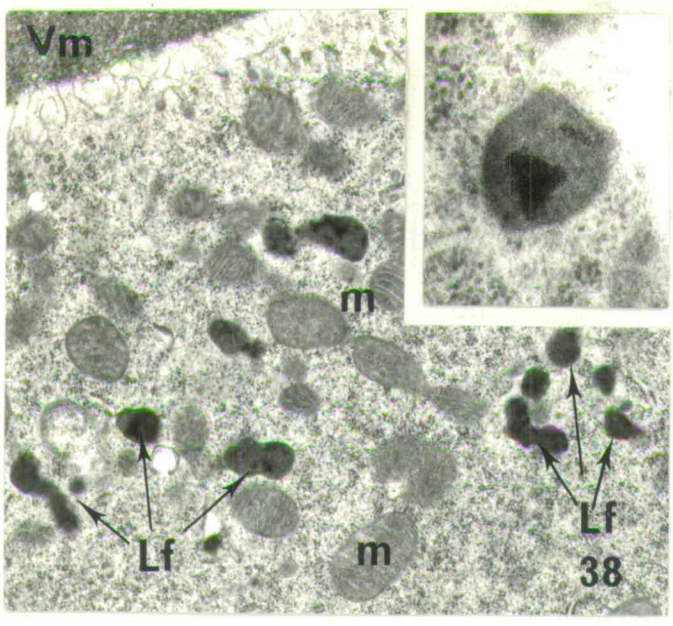
Fig. 38. Part of the ooplasm from a stage 11 ovarian chamber along the border with the nurse cells. Amidst the mitochondria (m) there are present several lipofuchsin granules (Lf) with typical clusters of ferritin-like particles within (see insert). x 12,000.

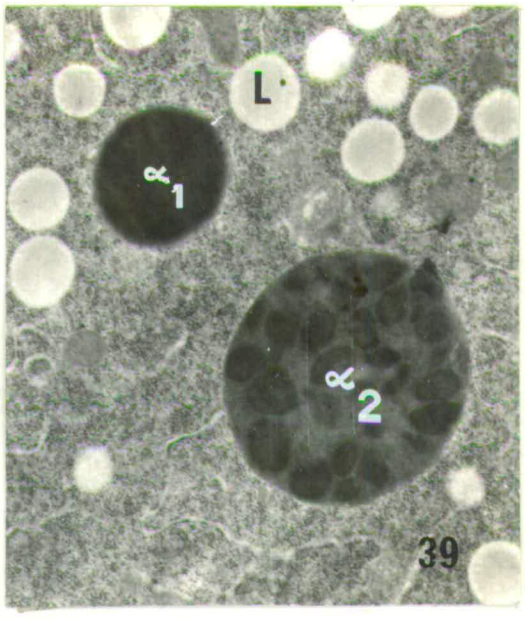
Fig. 39. Part of the central ooplasm from a stage 13 ovarian chamber. Two types of yolk platelets are visible: the one on the left (alpha-1: α_1) has a homogeneous main body, while that on the right (alpha-2: α_2) contains a number of dense areas embedded in a less dense matrix. (L) lipid droplets are also present. x 12,000.

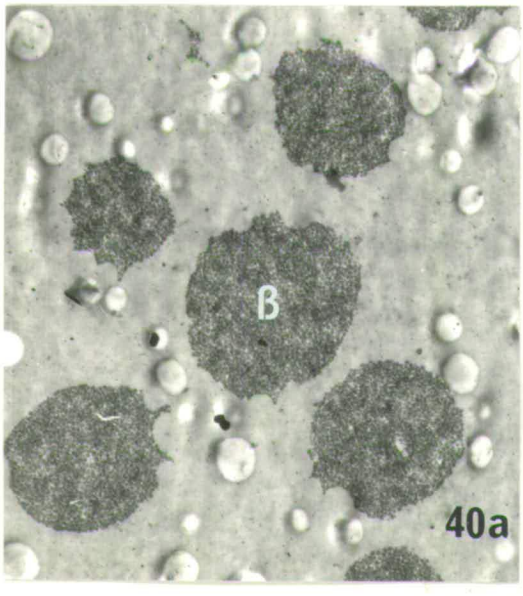
Fig. 40a. Part of the central ooplasm from a stage 14 chamber treated according to Thiery (1967) to demonstrate the presence of glycogen. Note that only the beta-spheres (β) are stained after this treatment. x 3,000.

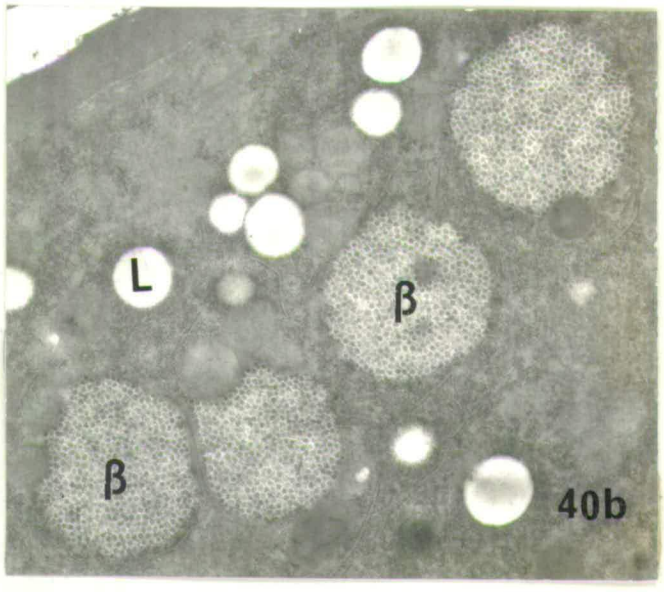
Fig. 40b. Part of the cortical ooplasm from a stage 14 untreated ovarian chamber. Beta-spheres (β) and lipid droplets (L) are present. x 8,000.

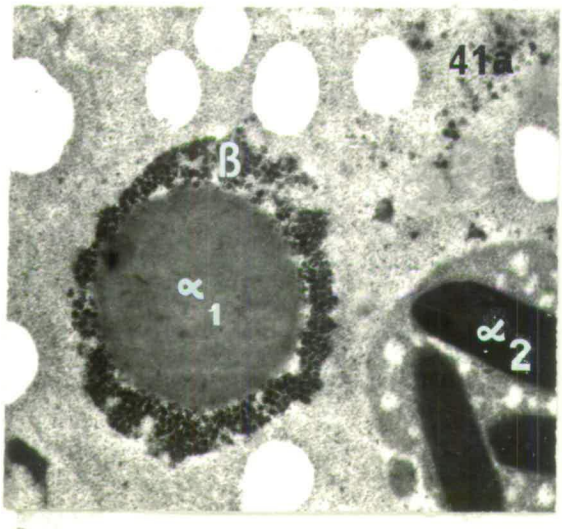
Figs. 41a and 41b. Early phases of glycogen formation in a stage 13 ovarian chamber. Fig. 41a shows glycogen at the periphery of a homogeneous yolk platelet (alpha-1: α_1). Fig. 41b shows that glycogen acquires a beta-spheres-like appearance in the proximity of an alpha-2 (α_2) yolk platelet. These micrographs are from an ovarian chamber treated with diaminobenzidine to demonstrate the presence of peroxisomes. In these preparations glycogen shows a higher contrast than in Fig. 40b. x 12,000.

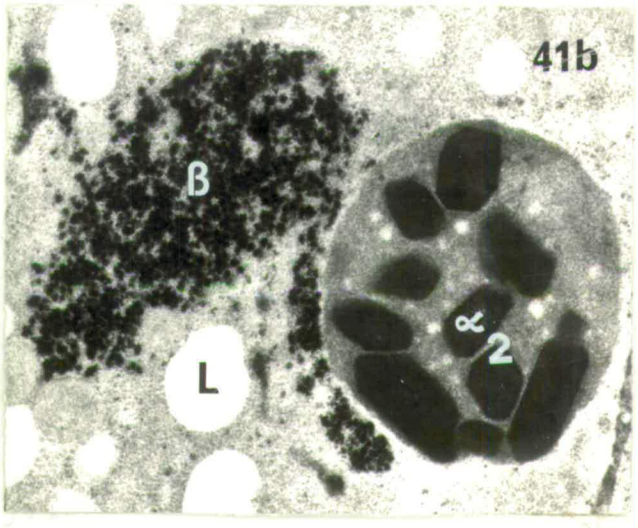

Vm
m
Lf
m
Lf
38


L
α1
α2
39


β
40a


L
β
β
40b


41a
β
α1
α2


41b
β
L
α2

By stage 14, the predominant types of yolk platelets in the ooplasm are the ones shown in Figs. 40b and 41b.

2. The nurse cells

(a) The nucleus. In the anterior pole of the germarium, 14 out of the possible 15 nurse cells can be easily distinguished from the two pro-oocytes of the 16-cell cluster because the former lack synaptonemal complexes in their nuclei (Figs. 17, 42 and 43a).The nurse nuclei are spherical, have an average diameter of 5 microns and present several ultrastructural features of interest. One of this concerns the nucleolus which at this stage is usually located at the periphery of the nucleus. The overall shape of this organelle tends to be rather elongate extending for up to 3 microns. The internal area of this organelle appears mainly fibrillar or fibrillo-granular but the peripheral region consists predominantly of granules. The fibrils measure about 50 A in diameter and the granules about 200 A (the cytoplasmic ribosomes have an average diameter of about 250 A). Patches of chromatin-like material are observable in some sections of nucleoli. The chromatin appears to be distributed as irregular masses of varying electron density throughout the nucleus. Amidst the chromatin masses there are present particles with an average diameter of 400 A. The nurse nucleus at this stage is surrounded by a typical nuclear envelope consisting of two membranes separated by a 250 A wide perinuclear space. The outer membrane of the envelope is studded with ribosomal particles. Nuclear pores can be discerned without difficulty and their frequency of occurrence

has been calculated. They occur with a centre-to-centre distance of 1600 A and on this basis about two thousand nuclear pores may be expected to be present in a nucleus which has a diameter of 5 microns.

Another interesting feature is the presence of several patches of amorphous material in the perinuclear cytoplasm adjoining the envelope (Figs.43aand 43b). In high power micrographs (Fig. 43b) the masses seem to be made up of fibrils which are 50-60 A thick.

The stage 1 chamber occupies the basal part of the germarium. The overall appearance of nurse nuclei at this stage is very much the same as that described above. However, the nucleus now comes to occupy a more central position within the cell.

In stage 2 chamber, the nurse nuclei are slightly larger than before with an average diameter of 6-7 microns. The nucleolus has also increased in size and maintains a peripheral location within the nucleus. Besides the increase in size, the nucleolus comes to exhibit an irregular shape: an examination of several sections, however, shows that it is still a single entity. The chromatin masses are more prominent and patches of such material are encountered more frequently in association with the nucleolus. The large granules (400 A) described in the previous stage seem to be more numerous and sometimes they occur in clusters, close to the chromatin material. As regards the nuclear envelope, there seems to be a slight decrease in the centre-to-centre distance of the nuclear pores which is now only about 0.13 microns.

In sections passing through the nurse cells

Fig. 42. Part of a 16-cell cluster at the anterior pole of the germarium from an ovariole fixed in glutaraldehyde and embedded in glycol methacrylate. Synaptonemal complexes (SC) are visible in the pro-oocyte nucleus. Two nurse nuclei (N) are present in the uppermost part of the micrograph. x 15,000.

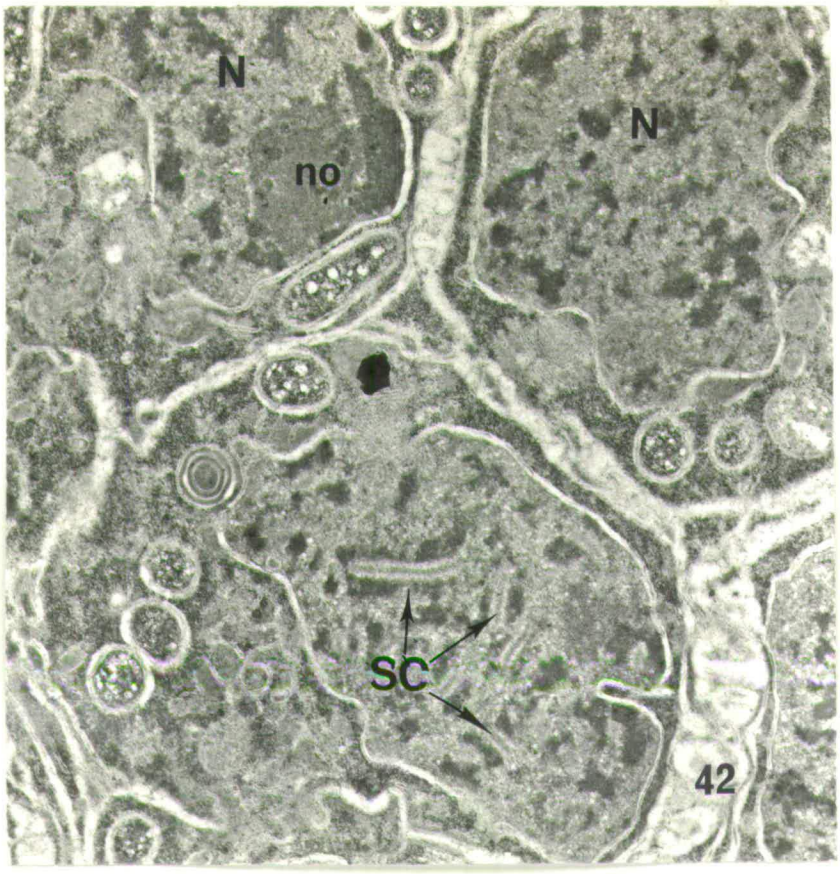
Fig. 43a. A nurse nucleus from a 16-cell cluster at the anterior pole of the germarium. The chromatin material (Chr) is scattered throughout the nucleus. The nucleolus (no) is compact and consists of fibrillar (fb) and granular (gr) components; also present within the nucleolus are patches of chromatin-like material (ichr). Clumps of amorphous material (Am) are closely apposed to the outer membrane of the nuclear envelope. x 20,000.

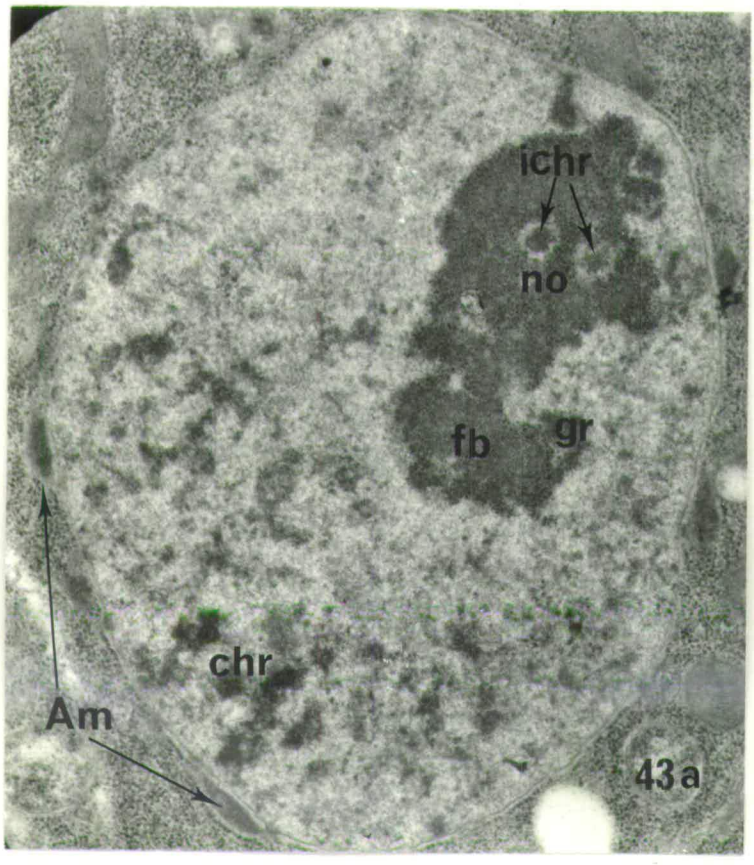
Fig. 43b. Part of the perinuclear cytoplasm from a nurse cell at the anterior pole of the germarium. Note the amorphous material (Am) and the Golgi apparatus (G). A ring canal (R) is also visible. (ne) nuclear envelope; (N) nurse nucleus. 30,000 x.

Figs 44-46. Ovarian chambers fixed in glutaraldehyde and embedded in glycol methacrylate. Sections are treated with 0.02 M EDTA as described before in Fig. 11.

Fig. 44. Part of the nucleolus from a nurse nucleus of a stage 3 chamber. The chromatin material (chr) is clearly evident within the nucleolus (no) in the form of several bleached areas. x 20,000.

Fig. 45. A posterior nurse nucleus from a stage 4 ovarian chamber. Note the presence of four bleached areas corresponding to the chromosomal masses (chr). The nucleolus (no) in this section appears separated into various clumps. x 8,000.


N
no
N
SC
42


ichr
no
fb
gr
chr
Am
43a

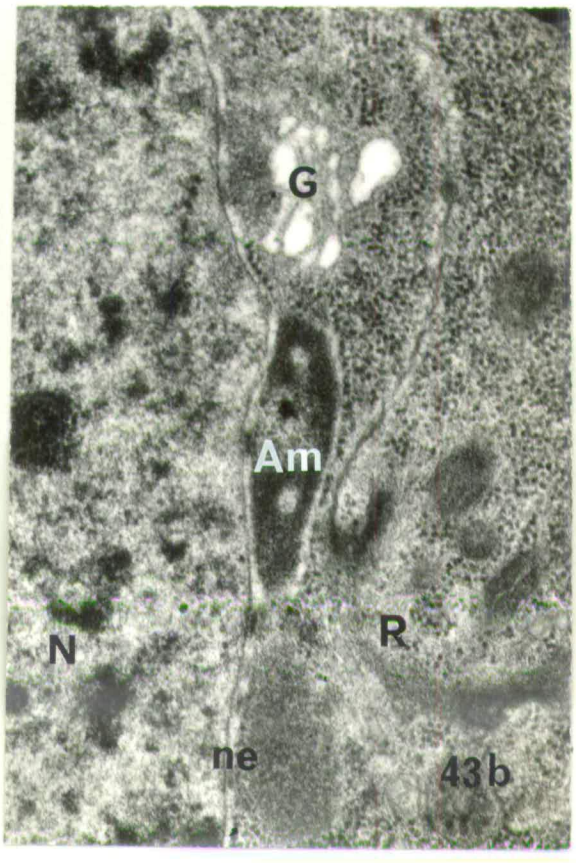

G
Am
N
R
ne
43b

Fig. 46. An electron micrograph of a nurse nucleus from a stage 4 ovarian chamber. Note that the interchromatin regions contain numerous electron dense granules. These are not bleached after EDTA treatment and are therefore ribonucleoprotein particles. x 20,000.

Fig. 47. Part of the perinuclear cytoplasm from a stage 4 nurse cell. The amorphous material (Am) is now more extensive than before and contains lacunae-like areas within. A layer of fine fibrils is now beginning to appear close to the nuclear envelope. (chr) chromatin; (no) nucleolus. x 32,000.

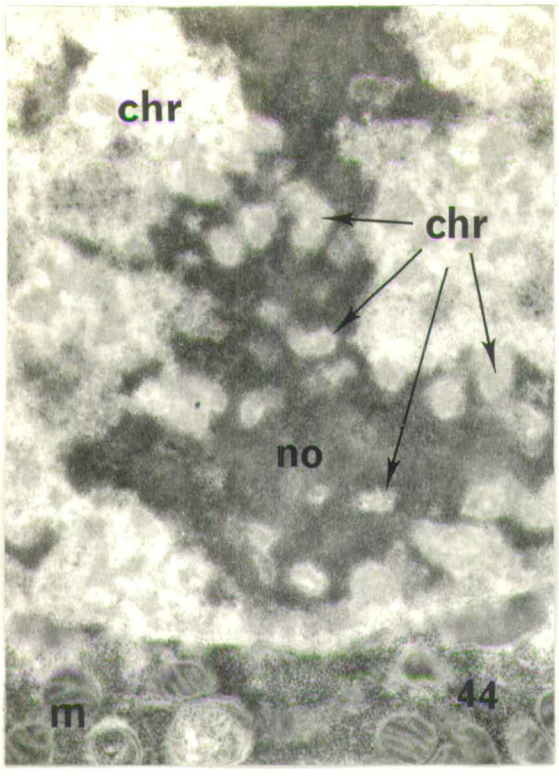
Fig. 48. The perinuclear cytoplasm of a nurse cell from a stage 5 ovarian chamber. The clumps of amorphous material (Am) have lacunae-like areas within. One of the clumps is not in contact with the outer membrane of the nuclear envelope. x 20,000. The chromatin material (chr) appears diffuse.

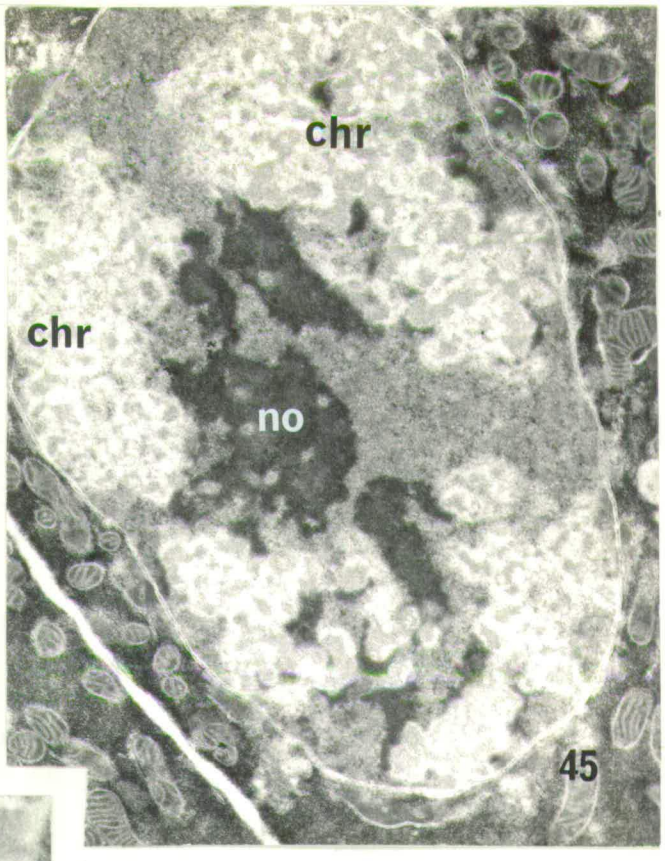
Fig. 49. The perinuclear cytoplasm of a nurse cell from a stage 8 ovarian chamber fixed in glutaraldehyde and embedded in glycol methacrylate. Note a finely fibrous layer (Fl) about 4000 A thick, which is continuous around the nucleus. x 32,000.

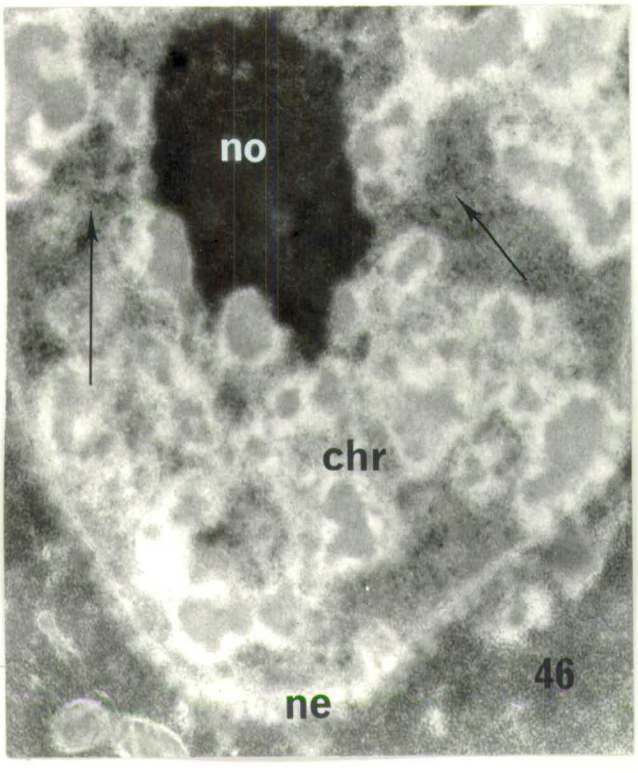
Fig. 50. Part of a nurse nucleus from a stage 10 ovarian chamber. Note that clumps of chromatin material are spatially arranged in a polytene-like fashion (see dotted line). The nuclear envelope (ne) has an undulated appearance. x 12,000.

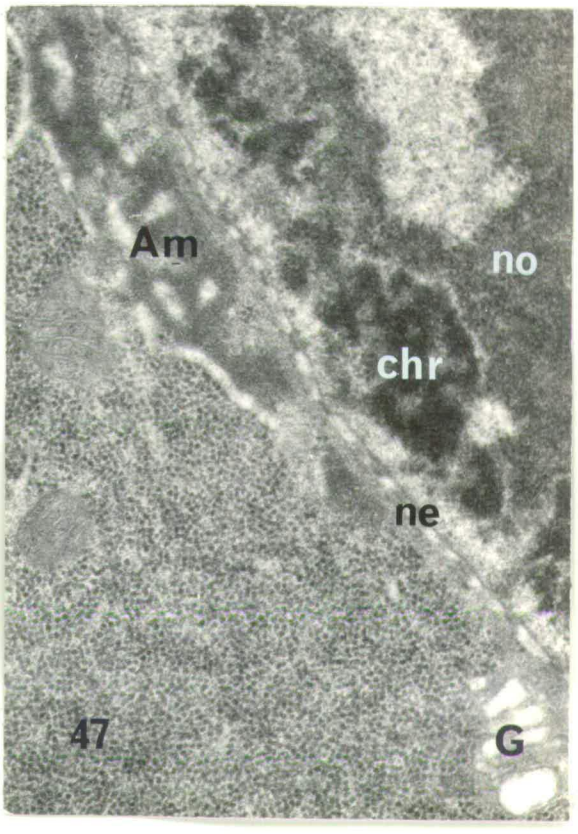
Fig. 51. A nucleolar mass from a stage 10 nurse nucleus. The fibrillar (fb) and granular (gr) constituents are discernible in this mass. Small clumps of chromatin material (Chr) are located within and at the periphery of the nucleolar mass. x 7,500.

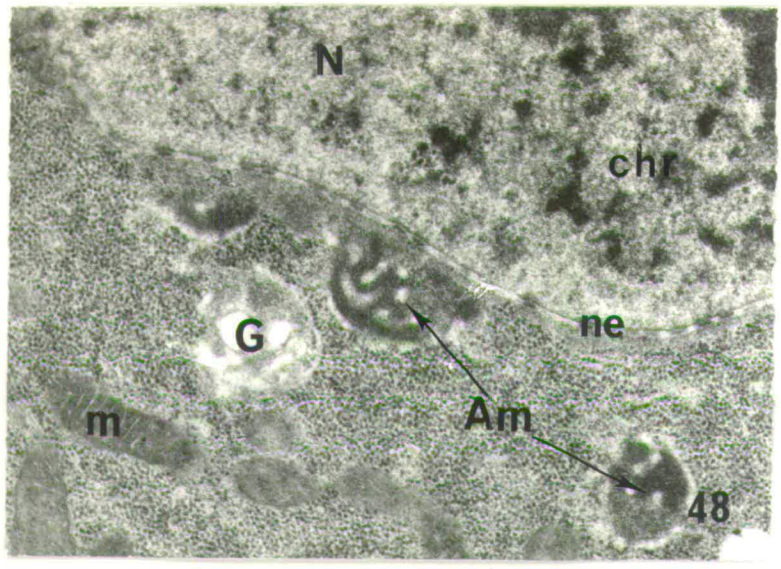
Fig. 52. Part of a nurse nucleus from a stage 10 chamber fixed in glutaraldehyde and embedded in glycol methacrylate. The section was treated with 0.02 M EDTA as described in Fig. 11. The chromatin (Chr) is visible as bleached areas within the nucleolus and also dispersed throughout the nucleus. Several vacuoles (nv) are present within the nucleolar mass. x 6,000.

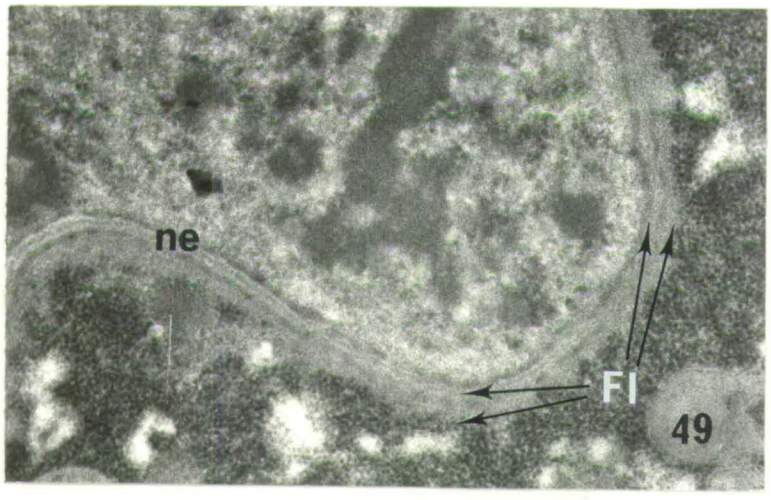

chr
chr
no
m
44

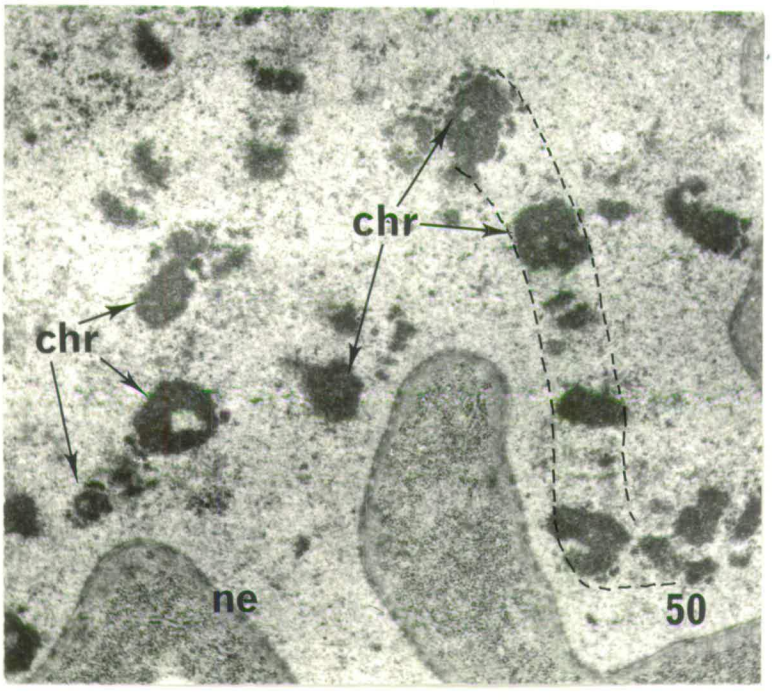

chr
chr
no
45

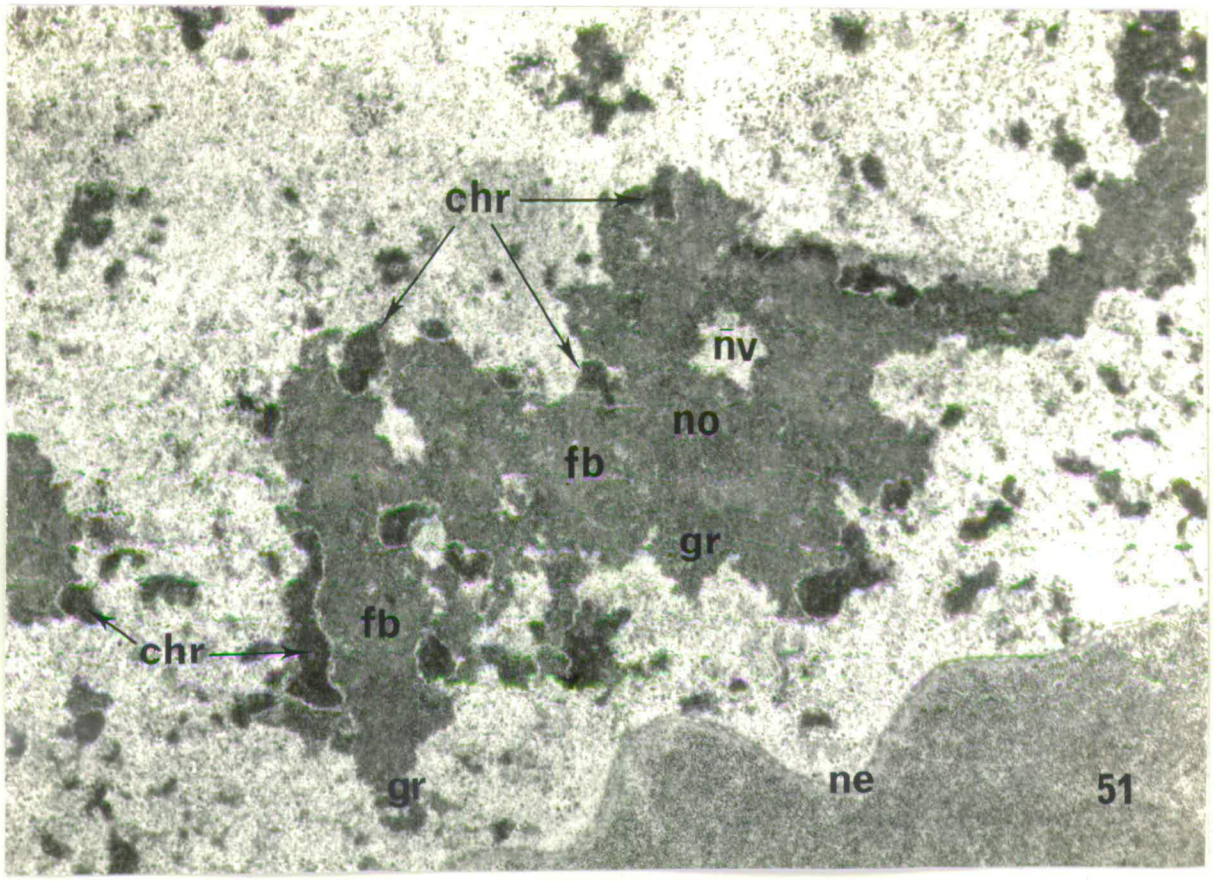

no
chr
ne
46

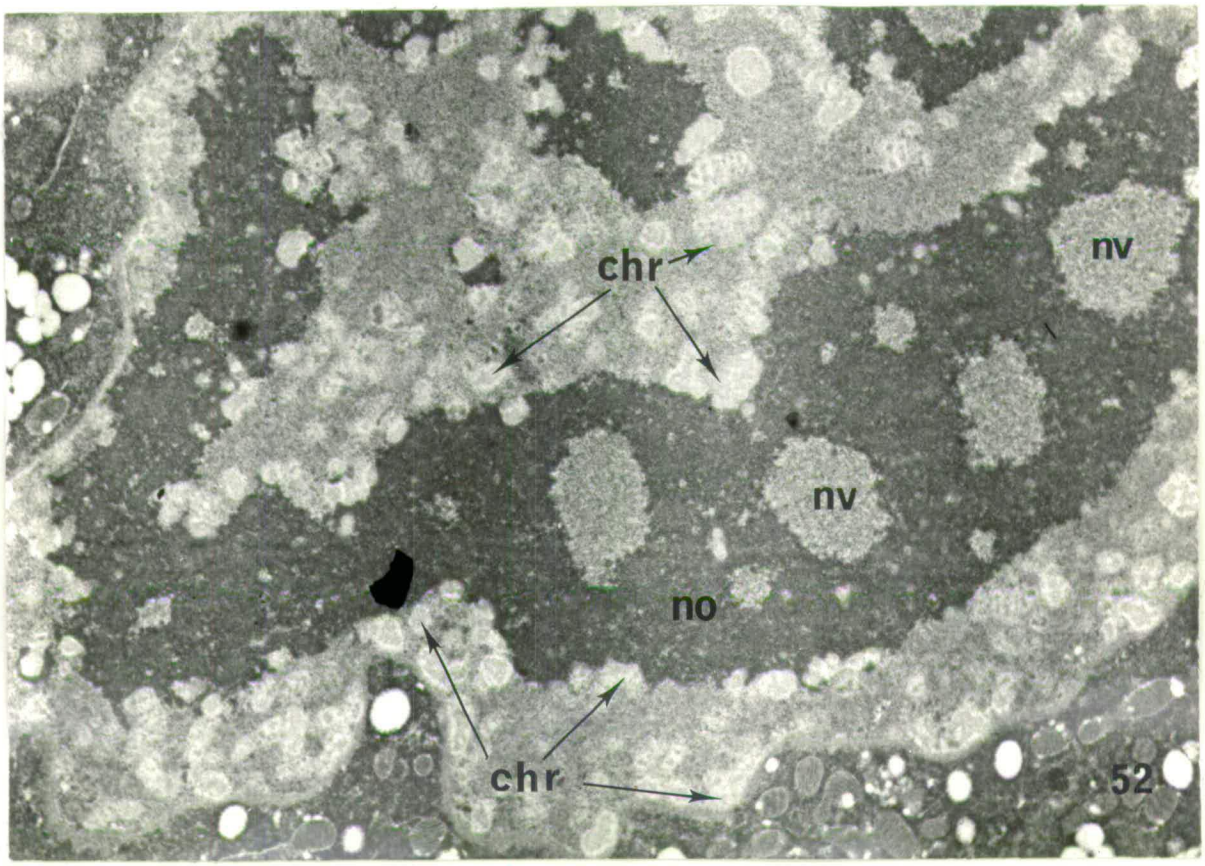

Am
no
chr
ne
47
G


N
chr
G
ne
Am
m
48


ne
Fl
49


chr
chr
ne
50


chr
nv
no
fb
gr
chr
fb
gr
ne
51


chr
nv
nv
no
chr
52

of stage 2 chamber, masses of amorphous material have been more frequently observed than before in the perinuclear cytoplasm, but they are still closely apposed to the outer nuclear membrane. Their average dimensions have also increased to about 0.4 microns.

The nurse nucleus in stage 3 ovarian chambers is larger; the average diameter now ranges from 8 to 10 microns. The distribution of the nuclear pores is, however, as in the previous stage. A nucleolar mass of a stage 3 nurse nucleus is shown in Fig. 44. This preparation is from a glycol methacrylate embedded and EDTA treated chamber. At this stage, the nucleolar mass has a more irregular outline than before and the patches of intranucleolar chromatin, (as shown by the number of bleached areas within the nucleolus) appear far more numerous than in previous stages. The chromatin material tends to be more diffuse than before but four distinct masses representing four paired chromosomes are visible.

A posterior nurse nucleus in a stage 4 chamber is shown in Fig. 45. In this case also, the chamber was embedded in glycol methacrylate and treated with EDTA according to the procedure of Bernhard (1969) for distinguishing between ribonucleoproteins and deoxyribonucleoproteins. Within this nucleus the four bleached areas are clearly visible, and the nucleolar material still retain their high electron density characteristic of ribonucleoproteins. Fig. 46 illustrates a similarly treated nurse nucleus of another stage 4 chamber. The interchromatin granules which measure about 400 A in diameter are apparently unaffected by the EDTA treatment. The average nuclear diameter of nurse cells varies from

10 to 12 microns and the frequency of pores in the nuclear envelope is unchanged. Another notable feature in stage 4 chambers is the further increase in the amount of amorphous material located close to the nuclear envelope in the perinuclear cytoplasm. Such material has also become more heterogeneous with lacunae-like areas within as seen in Fig. 47. On occasions (Fig. 64) images have been seen which suggest an actual continuity between the amorphous material and the nuclear pores. Similar images have, in fact, been observed in all stages from stage 3 (Fig. 65).

As stated earlier, the chromosomes in the nurse cells are visible more or less as distinct masses during stage 4. This was somehow not evident in previous stages when the chromatin material appeared to be dispersed in the nucleus. The situation again alters in stage 5 when the chromatin masses are no longer visible; instead, the chromatin material is scattered throughout the nucleus (Fig. 48).

In stage 5 chamber, the posterior nurse nuclei measure up to 12 microns in average diameter, and from now onwards there seems to be a progressive increase in the amount of nucleolar material which appears in sections as distinct masses of various shapes and sizes and dispersed throughout the nucleus.

The masses of amorphous material lying in the perinuclear cytoplasm at this stage are often at a distance from the nucleus and may be situated up to about 1 micron from the nuclear envelope. The ultra-structural organization of the masses is now very peculiar (Fig. 48). The fibrillar constituents of this entity

appear to be organized as thick strands, surrounding lacunae-like areas. Occasionally, the entity in question has different appearance in that, part of it appears fuzzy and part appears as more compact areas (Figs 67 and 68). While these changes take place in the amorphous masses that were formed earlier, one can still obtain images suggestive of the actual formation of such masses by passage of material from the nucleus through the pores as observed in Figs. 64 and 65. In these electron micrographs the material of the amorphous masses in the cytoplasm is clearly continuous through the nuclear pores with similar material in the nucleus. These pictures are very suggestive of the passage of nuclear material into the cytoplasm which occurs in nurse cells during stages 3 to 5.

The outer membrane of the nuclear envelope is now devoid of ribosomal particles and is in fact beginning to be separated from cytoplasmic constituents by a layer of extremely fine fibrils; in places, the layer is about 3000 A thick (Fig. 47).

Stage 6 and 7 nuclei exhibit similar features as those described in stage 5. During these stages, there is further increase in the size of the nuclei and nucleoli. The average diameter of nurse nuclei in stage 7 is about 14 microns. The nucleolar material appears to be dispersed throughout the nucleus, although there seems to be a preferential peripheral location.

By stage 8, the nurse nuclei are larger than before and there is also a noticeable anterio-posterior gradient in the size of the nurse nuclei, the posterior most one being the largest with an average diameter of about 18 microns. This gradient in the size

of nurse nuclei is first noticeable in stage 7. The nucleolar material in stage 8 continues to appear as large masses and some of the largest measure up to 4 microns in average diameter. Within such large masses there are also seen a number of vacuole-like areas which are more common in the nurse cells situated nearer the oocyte. What little material is present within these areas resembles the material of the nuclear sap or diffuse chromatin.

It is difficult to visualize if there has been any change in the relative proportion of the fibrillo-granular components of the nucleolus. From a study of a large number of sections it would appear that the granular looking areas are becoming more prominent. The distribution of the chromatin fibrils also merits mention. In places, they appear as very diffuse aggregates of low electron density and in other places the aggregates are denser and larger. The geometrical arrangement of such dense fibrillar aggregates gives the impression of a continuous linear array of banded chromosomal threads (for example, see Fig. 50). The nuclear envelope at this stage (stage 8) begins to be slightly undulated, but the overall shape of the nucleus is still spherical.

The entities in the perinuclear cytoplasm previously referred to as masses of amorphous material are met with only rarely in stage 8. More will be said of this in the section dealing with the nurse cell cytoplasm. The layer of extremely fine fibrils between the nucleus and the cytoplasmic constituents which started to appear in stage 5 chambers is now more continuous and measures nearly 4,000 A in width (Fig. 49). The distribution

of nuclear pores on the nuclear surface was examined in stages 8 to 10 and the pore frequency was found to be practically unchanged from that in stages 2-3.

At stage 9, the size of the nucleus has increased dramatically, especially in some of the most posterior ones in the chamber. The nucleus now is also lobate with an average diameter of 35 microns.

The nuclei of the posterior most nurse cells (viz. those which lie closest to the oocyte) in stage 10 ovarian chambers are highly lobate and have attained an average diameter of about 45-50 microns as measured in light microscope preparations. This is the maximum dimension attained by the nurse nuclei during the whole course of oogenesis. The nucleolar masses in the posterior nurse cells are as described earlier in stage 8 but they are situated closer to the nuclear periphery. Figs. 51 and 52 are low power electron micrographs of a spread out nucleolar mass containing several vacuoles of low density. The vacuoles are also larger than in previous stages (up to 2 microns in average diameter in stage 10 chambers). The distribution of the chromatin material is much the same as in stage 8 and in some sections the chromatin clumps still retain a polytene-like organization (Fig. 50).

The layer of fine fibrils surrounding the nucleus is now continuous and very prominent, and although the significance of this layer is not known, it may be related to a high rate of transfer of material from the nucleus to the cytoplasm. If this is so, it must be noted that the masses of amorphous material which were described earlier and which were more clearly shown to be derived

from the nucleus are no longer visible in the perinuclear cytoplasm of stage 10 nurse cells.

Another inclusion seen sometimes in the nucleus and in the cytoplasm of nurse cells in stage 10 chambers is shown in Figs.54 and 53 respectively. This inclusion is made up of a bundle of parallely oriented fibrils about 60 A thick. The bundle is not enclosed within a limiting membrane.

As stated earlier, the most posterior nurse nuclei in stage 10 chambers are the largest in the chamber. In succeeding stages, the nuclei suffer a drastic diminution in size and the nuclear envelope is thrown into numerous highly convoluted folds (Fig . 55). The ultrastructure of the nurse nuclei within a given chamber in each of these stages is highly variable, but is, on the whole, suggestive of a phase of disintegration.

In stage 11, there is a further loosening of the nucleolar structure and disorganization of its constituent elements (Fig. 56). This is carried even further in stage 12 when the amorphous areas of the nucleolus begin to clump together. Within the clumped areas the chromatin-like material is still evident and it gives the nucleolus a mottled appearance. Simultaneously clusters of large particles become visible scattered in the nucleus (Fig. 57). Such clusters begin to appear even in some of the stage 11 nurse nuclei (Fig. 56). The particles in question measure about 600 A in diameter and the clusters occupy areas as large as 2 μ^2. In high power pictures, these large particles seem to consist of subunits. The chromatin in stage 12 nucleus is represented by extremely diffuse material, amidst which

Fig. 53. Part of the cytoplasm of a stage 10 nurse cell. Note a bundle of parallely oriented fibrils. Each fibril is about 60 A thick. x 12,000.

Fig. 54. Part of a stage 10 nurse nucleus. A bundle of fibrils similar to the one shown in Fig. 53 is evident. x 12,000.

Fig. 55. A stage 11 nurse cell. Note the highly convoluted nuclear envelope. x 12,000.

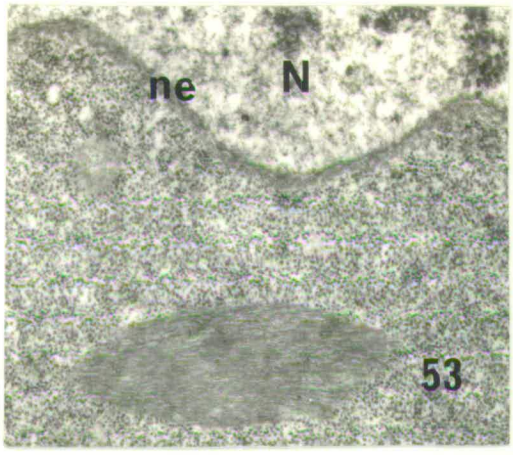
Fig. 56. Part of a posterior nurse nucleus from a stage 11 ovarian chamber. The nucleolar mass exhibits a loose structure. Small clumps of chromatin material (Chr) are evident within the nucleolar mass. Note the presence of interchromatin granules (see arrows). x 12,000.

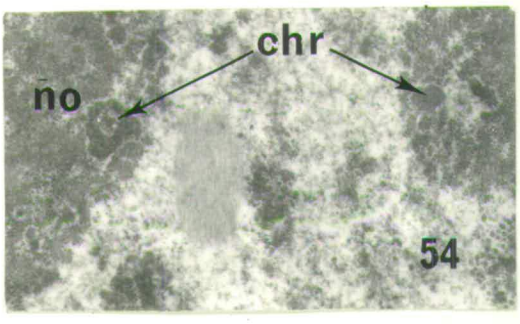
Fig. 57. Part of a posterior nurse nucleus from a stage 12 ovarian chamber. The nucleolar material exhibits a clumped structure and the chromatin patches present give the nucleolus a mottled appearance. Clusters of interchromatin granules (see arrows) are seen in places). x 12,000.

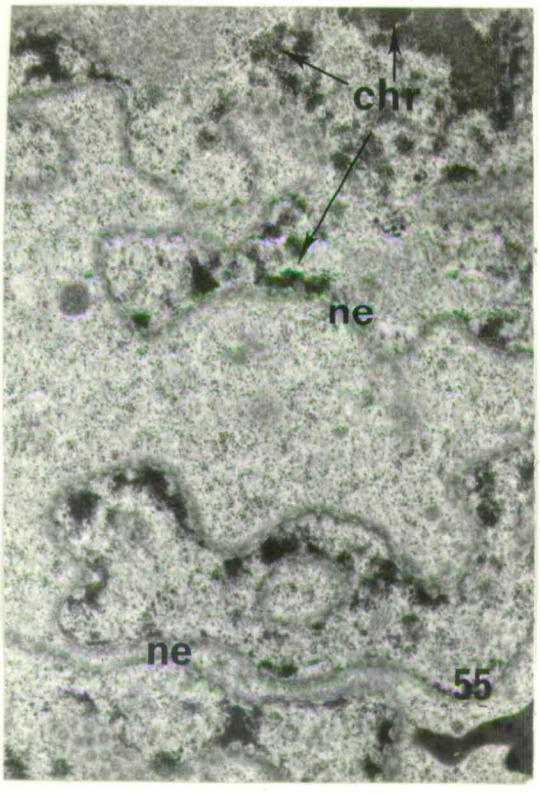
Fig. 58. Part of a posterior nurse cell from a stage 12 ovarian chamber. Note the highly convoluted nuclear envelope (ne). Rounded bodies (Rb) are seen in the cytoplasm. x 12,000.

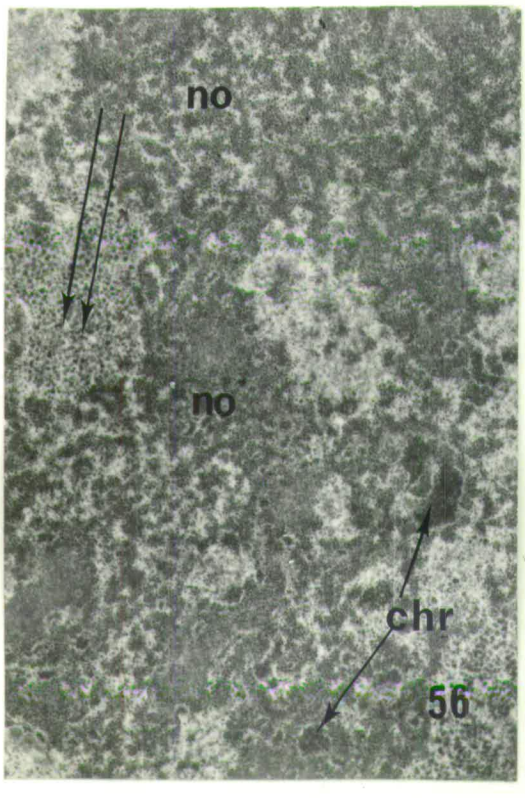
Fig. 59. The perinuclear cytoplasm of a stage 12 nurse cell. Note the presence of rounded bodies (Rb) close to the nuclear envelope (ne). x 8,000.

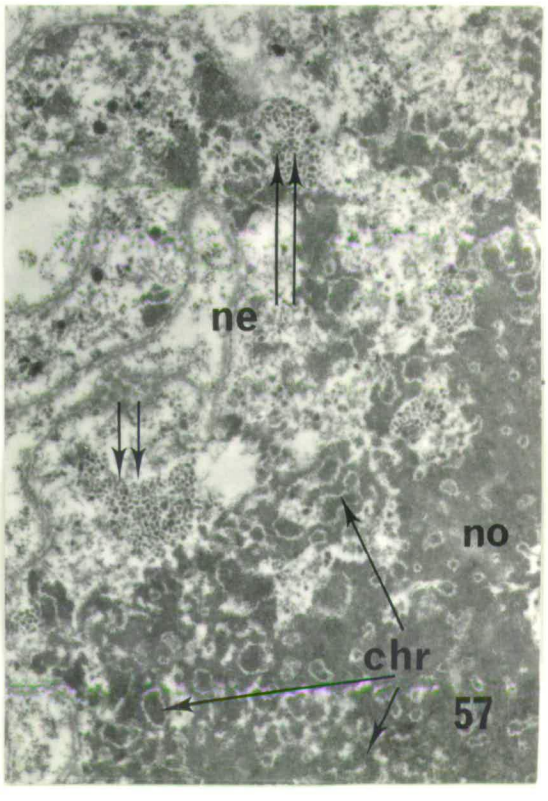
Fig. 60. The region of the ring canals between the nurse cells (NC) and the ooplasm (OO) from a stage 12 ovarian chamber. Two rounded bodies (Rb) are present in the cytoplasmic stream in between the canal rims (Cr). A degenerating nurse nucleus (N) can be seen on the left of the micrograph. x 12,000.

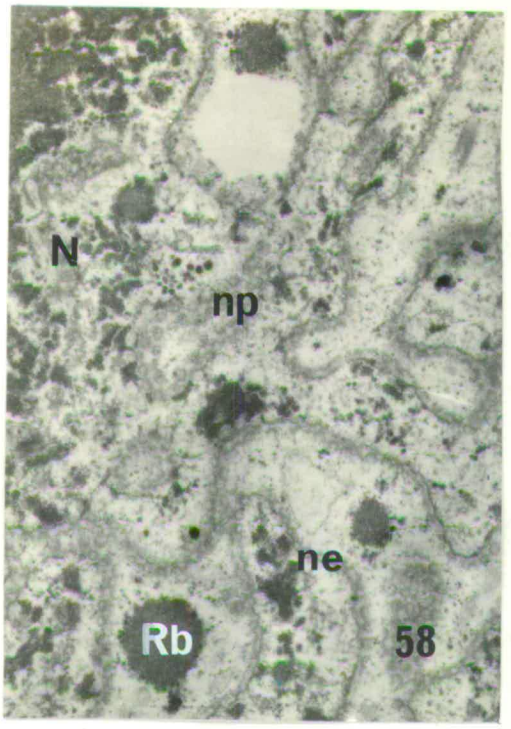

ne
N
53

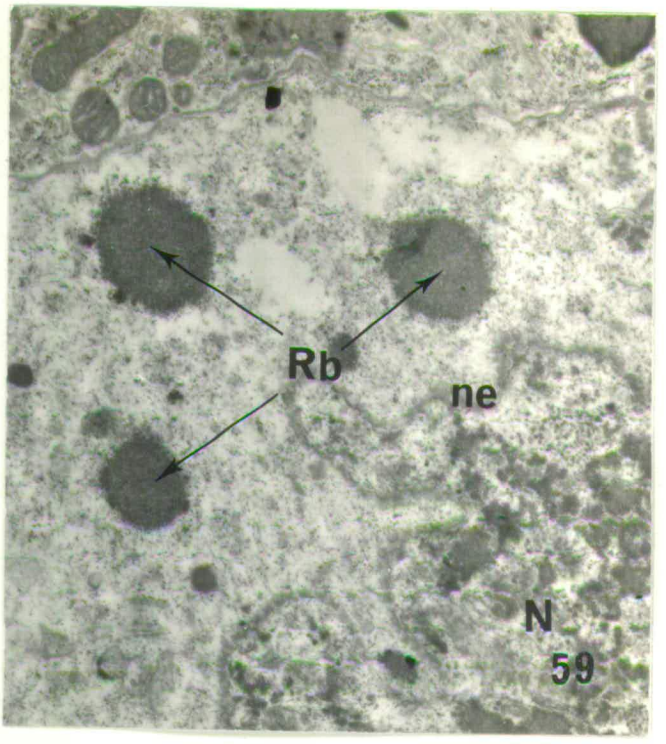

chr
no
54

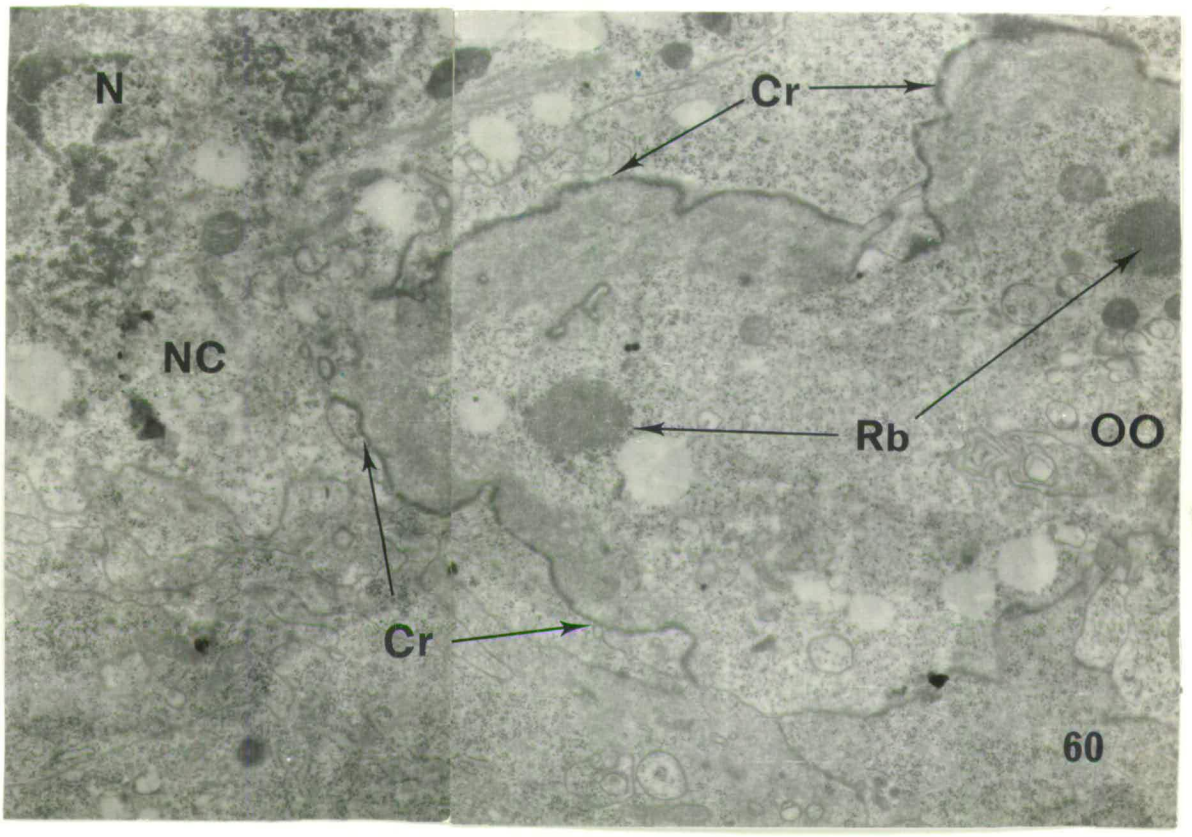

chr
ne
ne
55


no
no
chr
56


ne
no
chr
57


N
np
ne
Rb
58


Rb
ne
N
59


N
NC
Cr
Cr
Rb
OO
60

only dense spherical masses or rounded bodies are visible. In autoradiographic experiments (described later) these bodies are found to be labelled with uridine and they are, therefore, presumably ribonucleoproteins of nucleolar origin.

The dissolution of the nuclear envelope at this stage also results in some of these bodies finding their way into the cytoplasm (Figs. 58-60).

The nucleolar remnant seen in nurse nuclei, especially the posterior ones, of stage 13 chamber, is shown in Fig. 62. It appears to consist of coarse fibrils. This remnant mass also appears to be breaking up further and small aggregates of the constituent elements are noticeable both in the nucleus and in the cytoplasm. (Fig. 61). Some of the aggregates are visible at the level of the nuclear pores (Fig. 61) but in other areas, where the nuclear envelope is broken up, the aggregates pass into the cytoplasm through the gaps. A further indication of the nuclear degeneration is the appearance of membraneous whorls sometimes seemingly continuous with the nuclear envelope (Fig. 62).

(b) The cytoplasm. A few cells of a 16-cell cluster situated at the anterior pole of the germarium are shown in Fig. 63. As stated earlier, the eccentrically situated nucleus occupies the major part of the cell and the relative area of the cytoplasm is therefore very small. Apart from the normal constituents, like mitochondria, microtubules, Golgi apparatus and ribosomes, the cytoplasm contains several rickettsia-like bodies.

Fig. 61. High power micrograph of part of the nucleus shown in Fig. 62 showing the region of the nuclear envelope (ne). Note several nuclear pores (np) which contain nuclear remnants (nor). x 48,000.

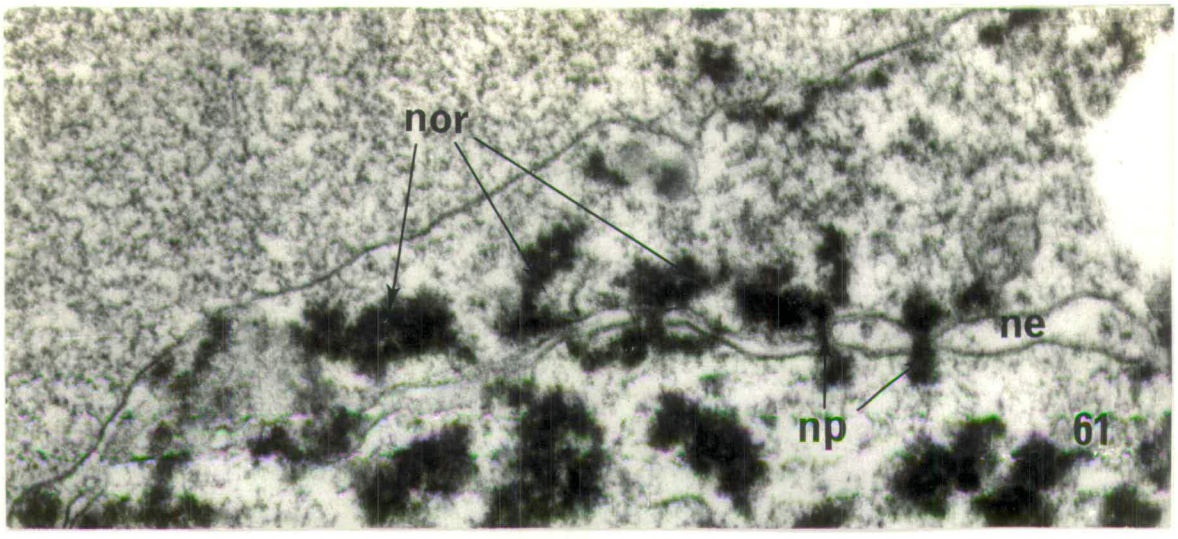
Fig. 62. A nurse nucleus from a stage 13 ovarian chamber. The nuclear envelope (ne) is interrupted in places. Membranous whorls (mf) are evident in areas close to the nuclear envelope. Note that the nucleolar remnants have a fibrous consistency. x 18,000.

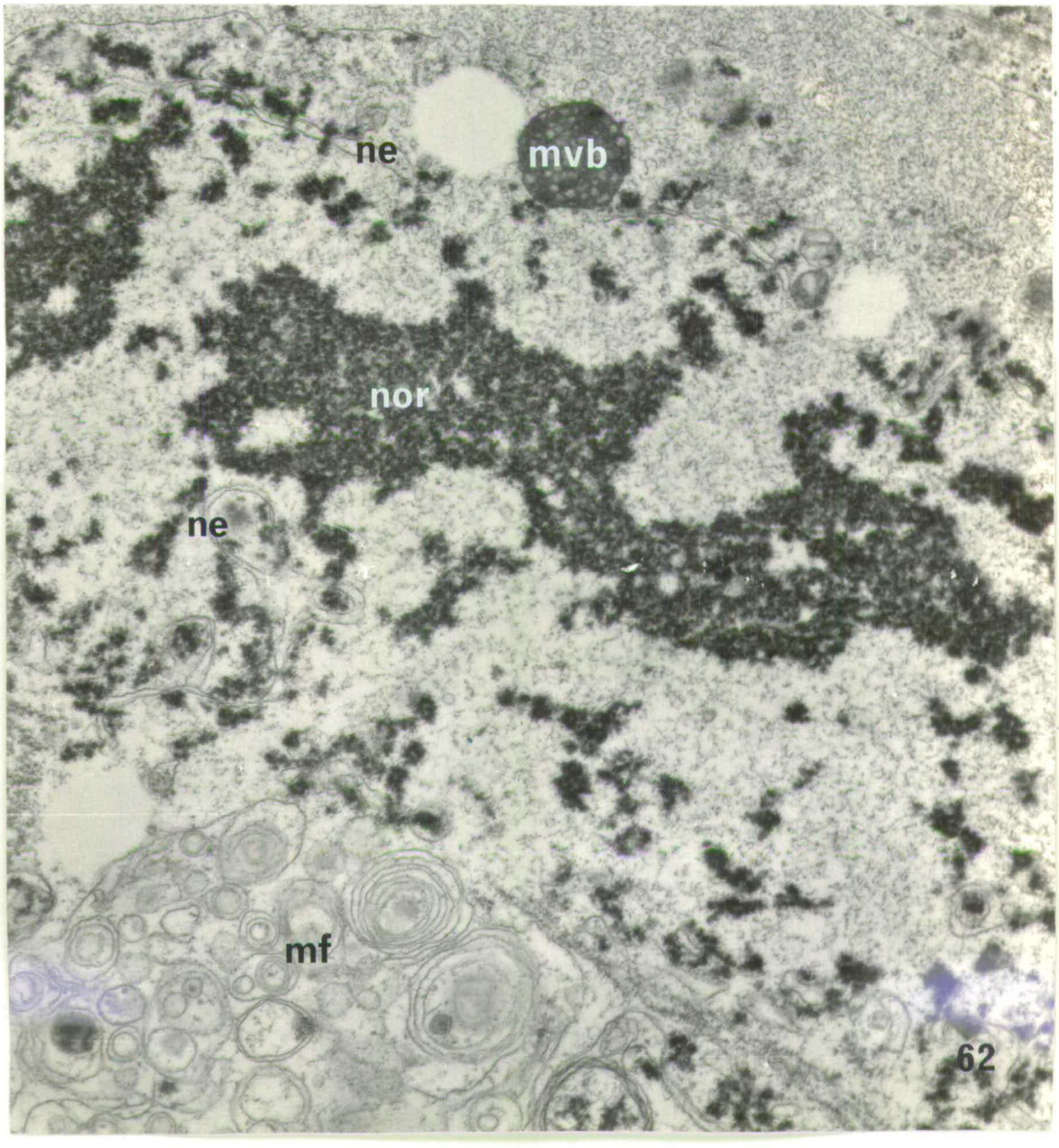
Fig. 63. Part of a 16-cell cluster at the anterior pole of the germarium. Most of the nurse cell is occupied by the nucleus (N) which is centrally located. The cytoplasm contains ribosomes, rickettsia-like bodies (A), mitochondria (m) and a centriole (C). Two ring canals (R) are also evident. x 6,000.

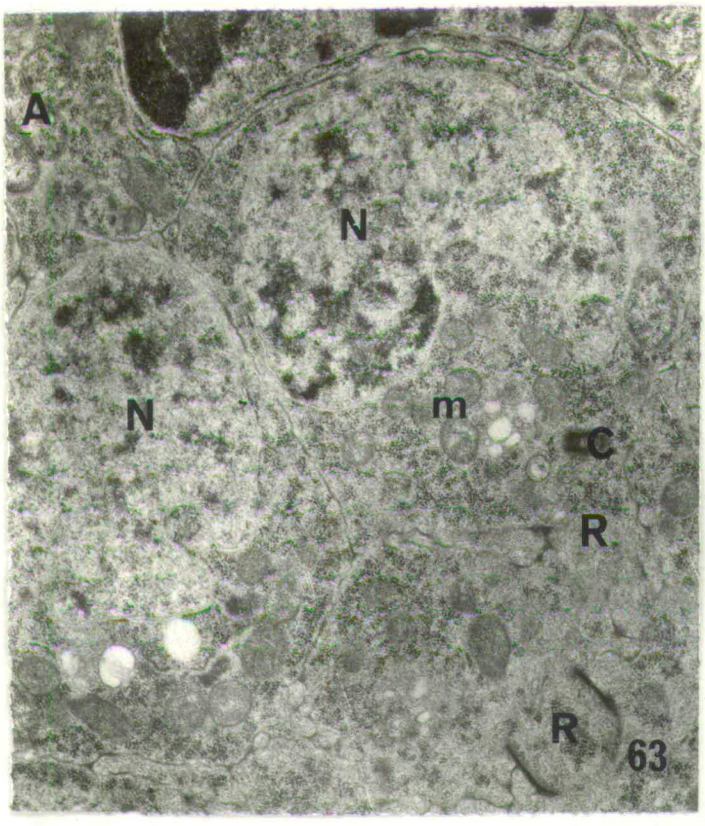
Fig. 64. Part of a nurse nucleus from a stage 4 ovarian chamber. Note the amorphous material (Am) in the cytoplasm associated with the nuclear pores (np). (ne), nuclear envelope. x 60,000.

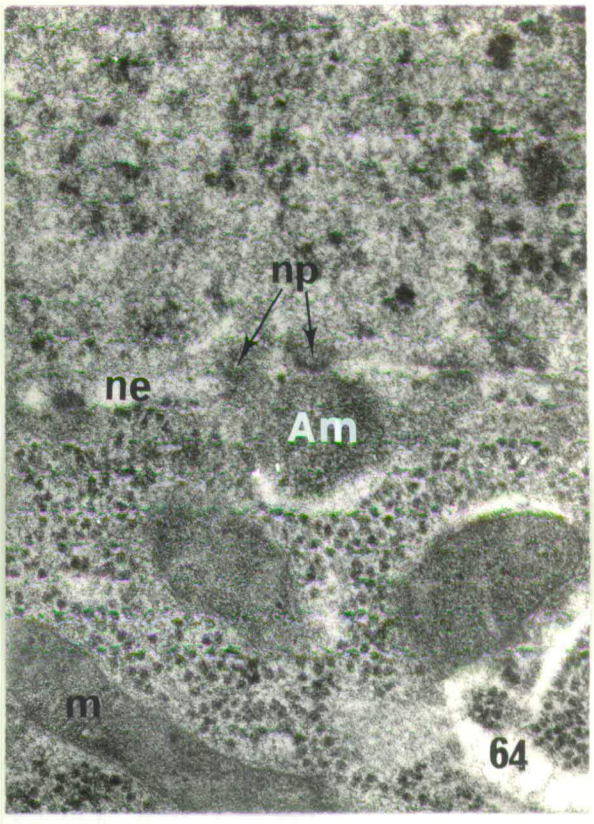
Fig. 65. Part of a nurse nucleus from a stage 3 ovarian chamber. The amorphous material is continuous, through the nuclear pore (np), with material of similar consistency on the nuclear side. x 60,000.

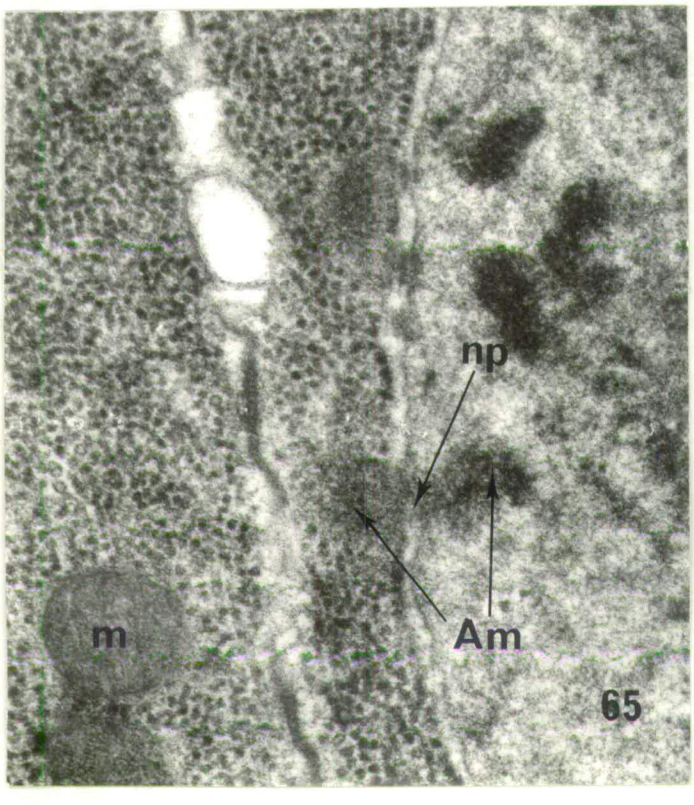
Fig. 66. Cell junction (zonula adherens) between adjacent nurse cells of a 16-cell cluster at the intermediate region of the germarium. Note the dense matrix (arrows) 200 A thick, on the cytoplasmic side of the plasma membrane. Microtubules (mt) are also evident in the cytoplasm. x 60,000.

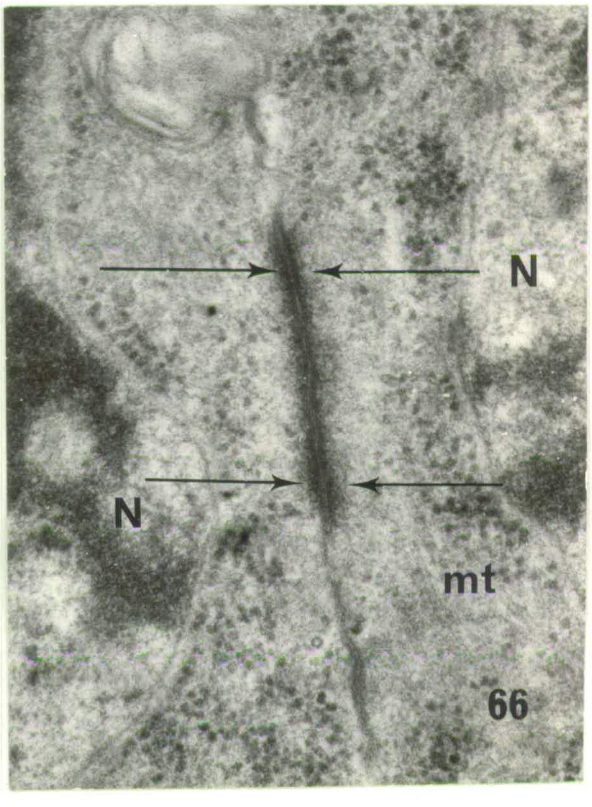

nor
ne
np
61


ne
mvb
nor
ne
mf
62


A
N
N
m
C
R
R
63


np
ne
Am
m
64


np
m
Am
65


N
N
mt
66

A centriole is observable in one of the cells and a ring canal is also present in this section. Even at this early stage in the development, material seems to be passing through the ring canal; in the present instance it is a cluster of ribosomes (Fig. 63). In other sections of these cells, specialized membrane junctions have been encountered between adjacent cells (Fig. 66); they vary in length from 0.14 micron to 0.31 micron. The plasma membrane involved are separated by a 200 A wide space, and an electron dense matrix, about 100-200 A thick is present on the cytoplasmic side of the membranes. The whole structure resembles very closely the type of cell junction described by Farquhar and Palade (1963) as zonula adherens. Lipid droplets are extremely rare and there is no trace of any cisternae of endoplasmic reticulum.

The ultrastructural features of the cytoplasm of nurse cells remain essentially the same up to stage 5, except that when the cell cluster of 16 cells has reached the intermediate region of the germarium there is a noticeable increase in the population of ribosomes. Another noteworthy observation relates to the frequent appearance of amorphous masses in the cytoplasm adjoining the nuclear envelope. This fact was mentioned earlier in the section dealing with the nucleus of nurse cells; it was pointed out then that the amorphous masses gradually undergo changes in their morphology as they move away from the immediate vicinity of the envelope (Figs. 67 and 68).

From stage 6 onwards, these masses seem to have transformed themselves into what may be called

fuzzy bodies (Figs. 67, 69 and 70). As they migrate into the deeper portions of the cytoplasm, they also seem to enlarge in size and come to be surrounded by numerous mitochondria (Fig. 70). Fuzzy bodies have been recorded within ring canals; an instance of this in stage 8 is shown in Fig. 71. Lipid droplets are rare, but ribosomes are much more numerous and more closely packed than in previous stages. A few profiles of endoplasmic reticulum become evident for the first time at stage 6.

Ring canals connecting nurse cells have been observed on numerous occasions and high resolution micrographs of the canals are shown in Figs. 72 and 73. The plasma membrane of adjoining nurse cells, at the site are highly convoluted and form together with some other dense material the rim of the canal. The formation of the electron dense canal rim has been described by Koch and King (1969). A fibrous material, sometimes containing still recognizable microtubules, can be seen projecting into the canal from the canal rim.

The dimensions of the ring canal are fairly constant at about 1.6 microns from germarial stage up to stage 2. There is an enormous increase in the diameter of the ring canal as development progresses further: the average diameter now increases nearly four fold between stage 2 and stage 8.

Nurse cells from stage 1 onwards present a number of small membrane-limited inclusions. These inclusions enclose various cytoplasmic constituents (Fig. 74) as for example, mitochondria and rickettsia-like bodies, which are obviously in the process of degradation. Such inclusions show a progressive increase in size in developing chambers

Fig. 67. Part of the cytoplasm from a stage 6 nurse cell showing an amorphous mass apposed to the nuclear envelope. This entity is undergoing morphological changes so that part of it is amorphous (Am) and part of it is fuzzy (Fb). (m) mitochondria; (ne) nuclear envelope. x 8,000.

Fig. 68. The perinuclear cytoplasm of the cell shown in Fig. 67. The entity in the proximity of the nuclear envelope (ne) contains in part fuzzy areas (Fb) and in part amorphous areas (Am). (m) mitochondria. x 20,000.

Fig. 69. A fuzzy body (Fb) in the cytoplasm of a stage 7 nurse cell. Note the amorphous material (Am) in the central portion of the body. Mitochondria (m) are located at the periphery of the fuzzy body. x 20,000.

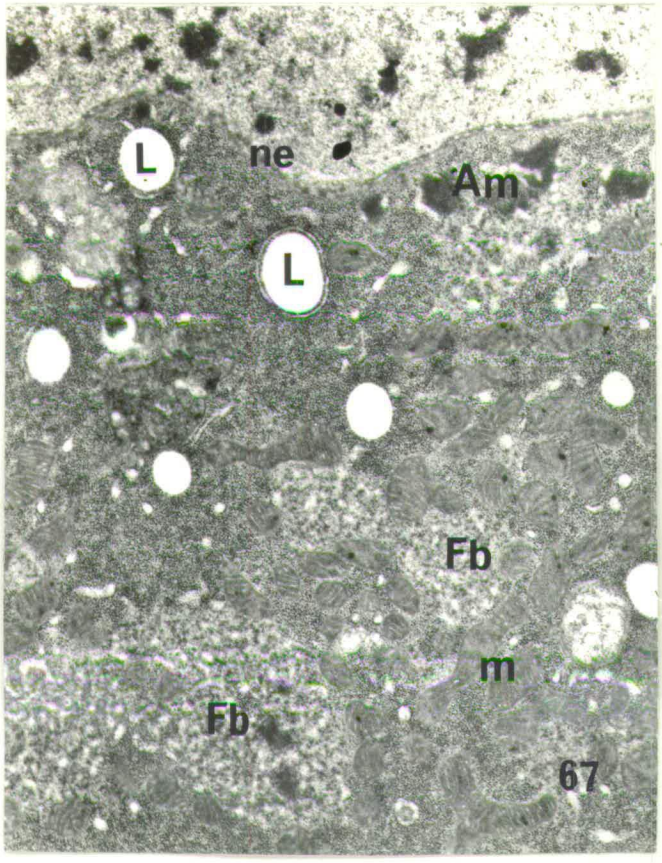
Fig. 70. A large fuzzy body (Fb) in the cytoplasm of a stage 6 ovarian chamber (enlargment of part of Fig. 67). (m) mitochondria.x20,000.

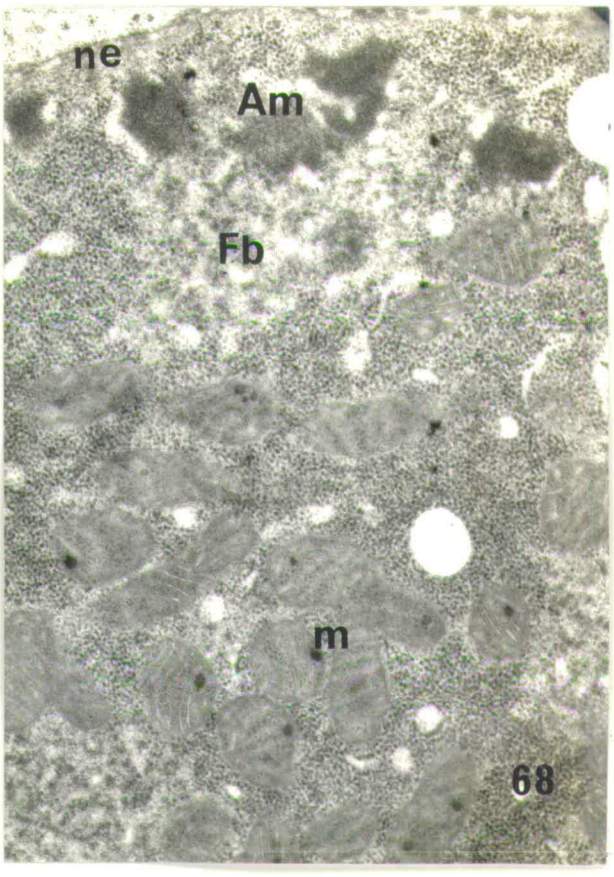
Fig. 71. Part of the cytoplasmic stream in a ring canal between two adjacent cells of a stage 8 chamber. Note the two canal rims (Cr). Fuzzy bodies (Fb), mitochondria (m), a bundle of parallely oriented fibrils (Bf), rickettsia-like bodies (A) and lipid droplets (L) are visible in this region. x 20,000.

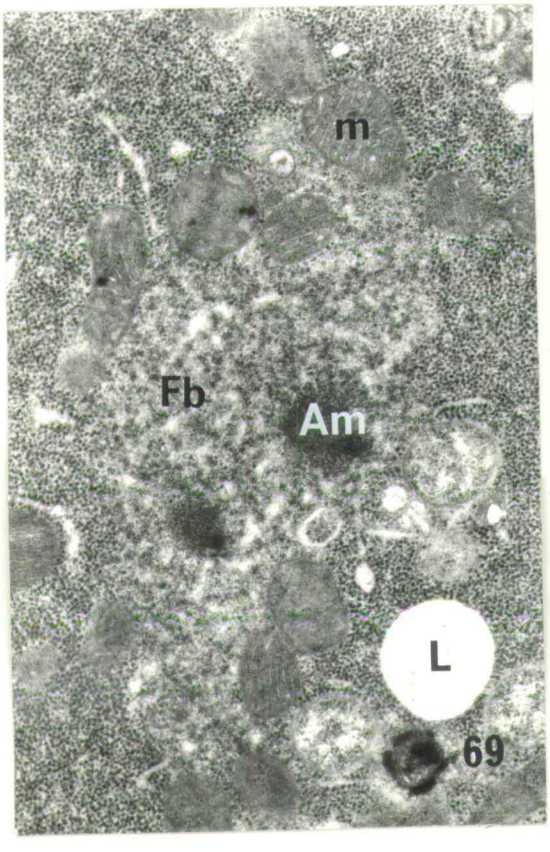
Figs. 72 and 73. Sections taken from different levels of the same ring canal between nurse cells of a stage 8 ovarian chamber.

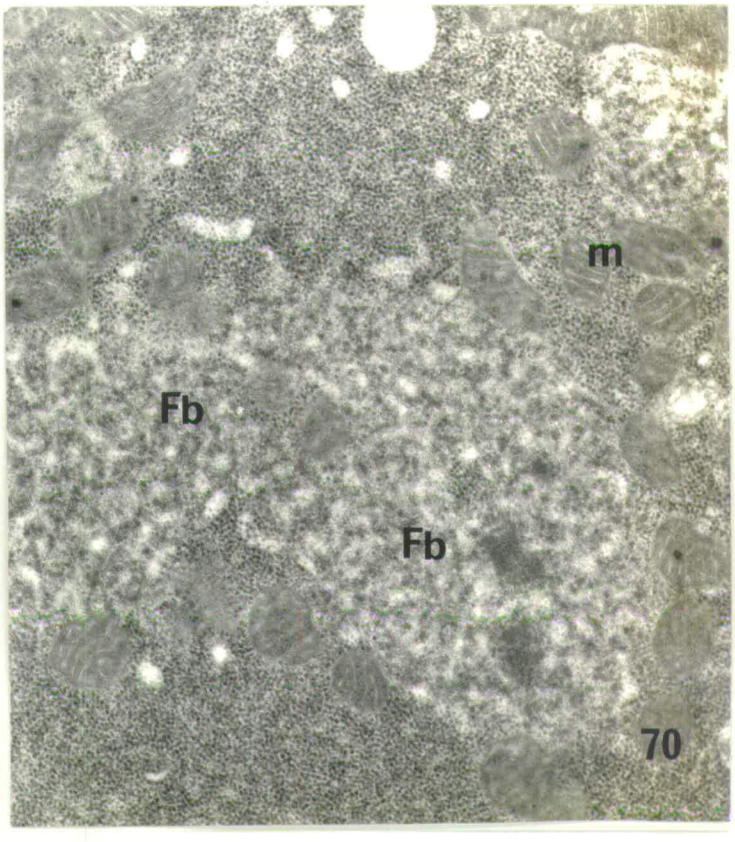
Fig. 72. Tangential section of the ring canal. Fibrous material (Fm) extends from one canal rim (Cr) to the opposite one. The plasma membranes of the adjoining cells are highly convoluted (see arrows). x 20,000.

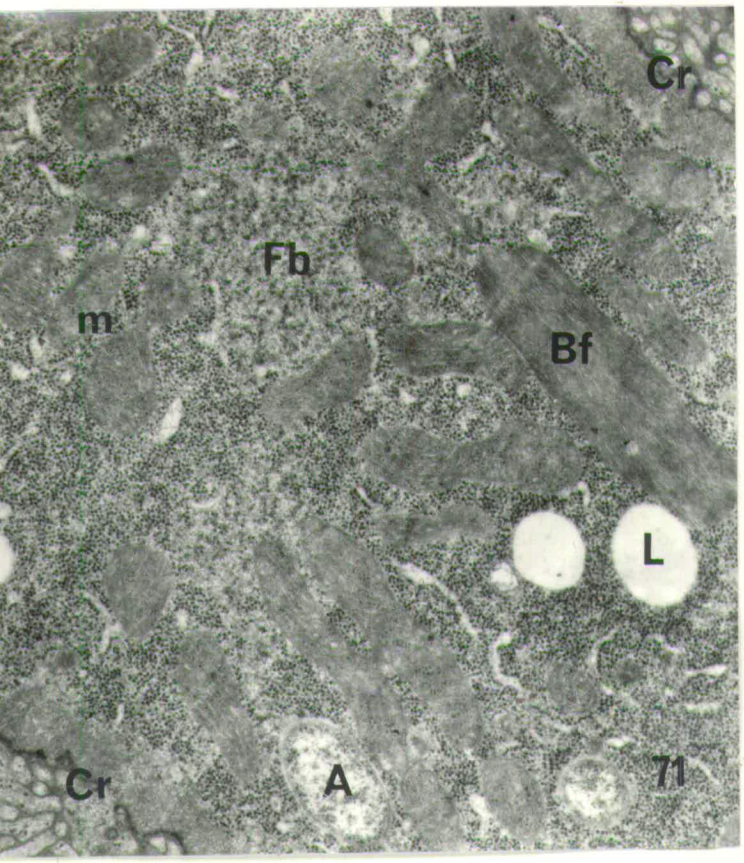
Fig. 73. Another section passing deeper through the same ring canal as above. At this level the fibrous material (Fm) extends for up to about 0.5 microns from the canal rims(Cr). (m) mitochondria. x 15,000.


L
ne
Am
L
Fb
m
Fb
67

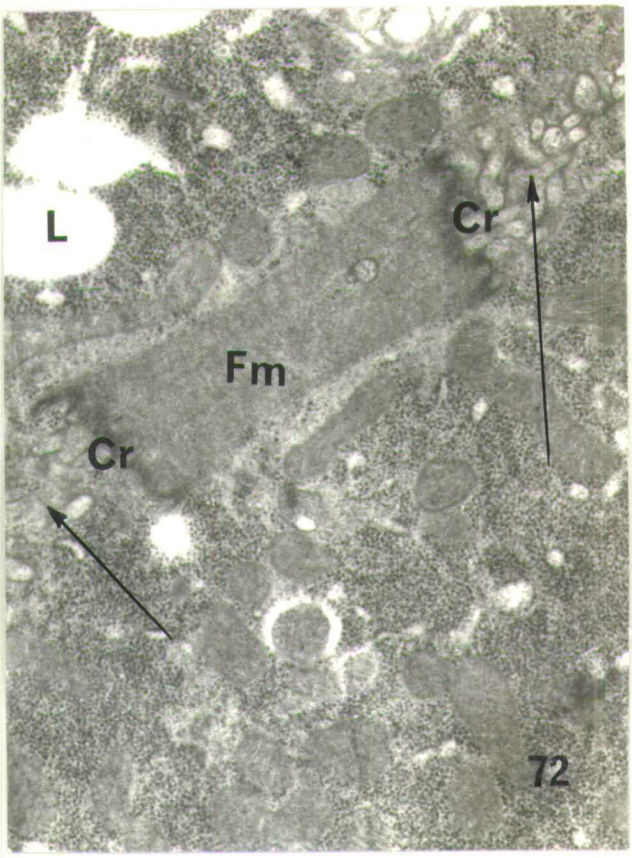

ne
Am
Fb
m
68


m
Fb
Am
L
69

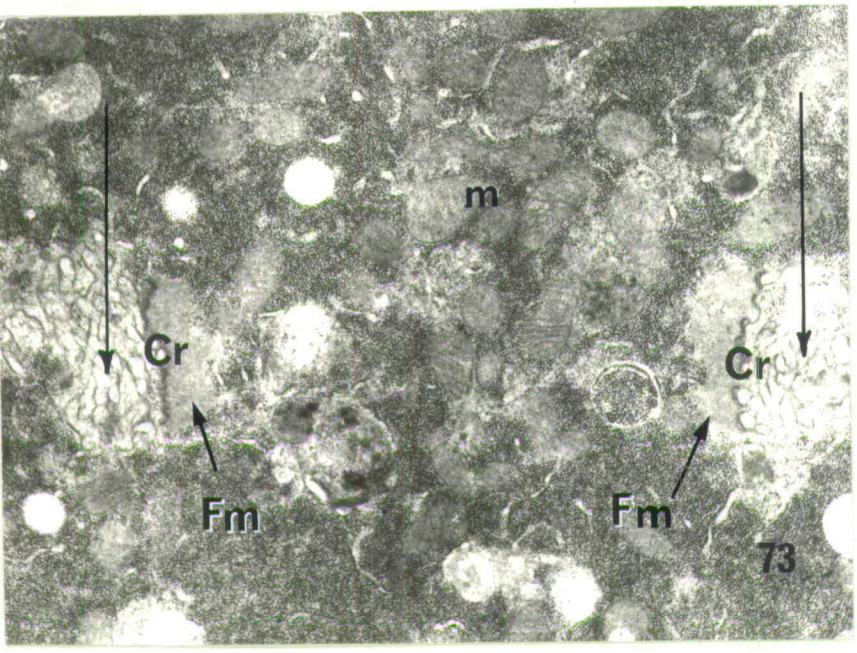

m
Fb
Fb
70



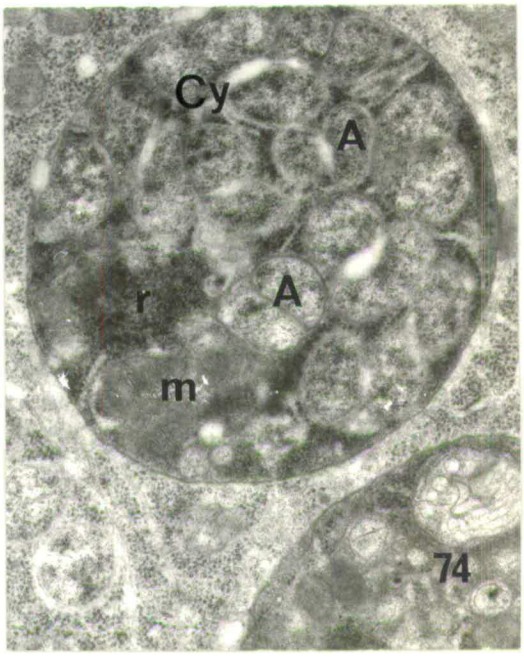
71



72



73



74

and an example of this in stage 5 is shown in Fig. 75. By this stage, the inclusion consists of an assembly of complex membranous systems some of which are typical myelin forms. In places the membranous whorls are seen to enclose numerous minute vesicles. In certain inclusions only myelin figures are visible (Fig. 76). In none of the inclusions just described is it possible to identify any normal cytoplasmic component except perhaps ribosome-like particles. These inclusions, which in all probability are cytolysosomes, have been encountered within ring canals of nurse cells and ring canals between nurse cells and the oocyte.

The Golgi apparatus lies in a perinuclear position in early previtellogenic stages (stages 1-6). In later stages, the Golgi apparatus is more frequently present in deeper regions of the cytoplasm. The Golgi apparatus in these stages do not show the classical picture of parallely oriented cisternae; instead they appear as groups of vesicles of various sizes (Fig. 43b). On several occasions, these organelles have been observed passing through the ring canals between nurse cells as well as the canals between nurse cells and the ooplasm (Fig. 195).

From stage 9 onwards, lipid droplets increase dramatically in number while the concentration of rickettsia-like bodies and mitochondria remains apparently unchanged. The endoplasmic reticulum is not developed to any extent in the nurse cells even by late stages of oogenesis (stages 9-11). This is illustrated in Fig. 162. However, what little of the endoplasmic reticulum is present, may at times show a continuity with the plasma

Fig. 74. Part of a nurse cell cytoplasm from a stage 1 ovarian chamber. Note a large cytolysosome (Cy) containing mitochondria (m), rickettsia-like bodies (A) and a cluster of what look like ribosomes (r). x 20,000.

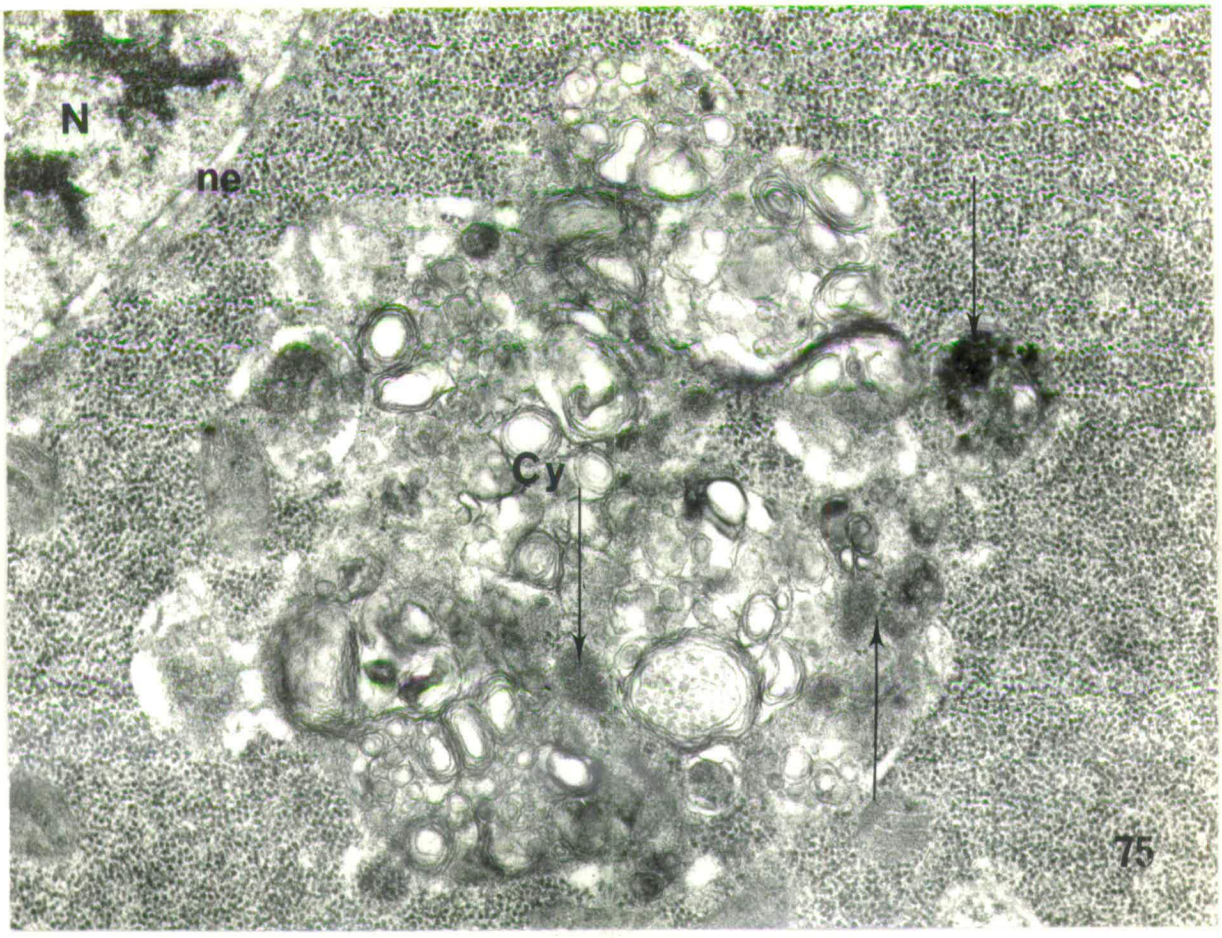
Fig. 75. Part of the nurse cytoplasm from a stage 5 ovarian chamber showing a cytolysosome (Cy) containing a large number of membranous whorls. Dense masses are also discernible within the cytolysosome (see arrows). x 30,000.

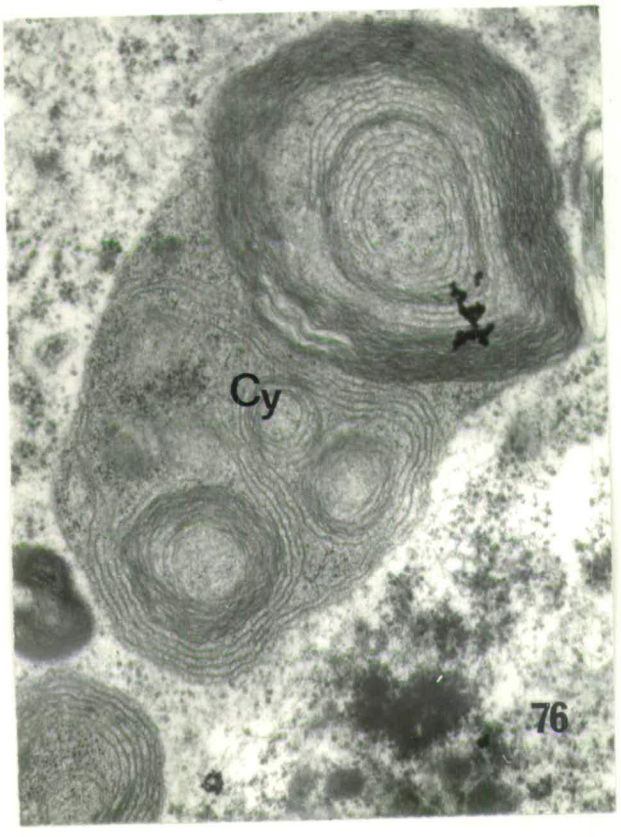
Fig. 76. A later stage of cytolysosome alteration in the nurse cell cytoplasm of a stage 5 ovarian chamber. Only membranous whorls and ribosomes-like particles are visible within the cytolysosome. x 32,000.

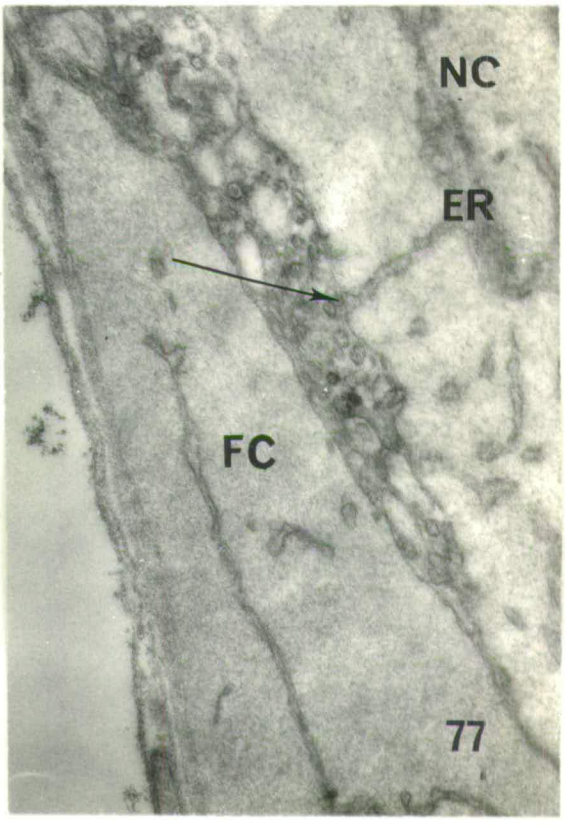
Fig. 77. Part of the follicle-nurse border at the anterior pole of a stage 10 ovarian chamber. This preparation is taken from an ovary fixed in potassium permanganate and embedded in Epon-Araldite mixture. Note the endoplasmic reticulum (ER) is in continuity with the plasma membrane of the nurse cell (see arrow). (FC) follicle cell; (NC) nurse cell. x 32,000.

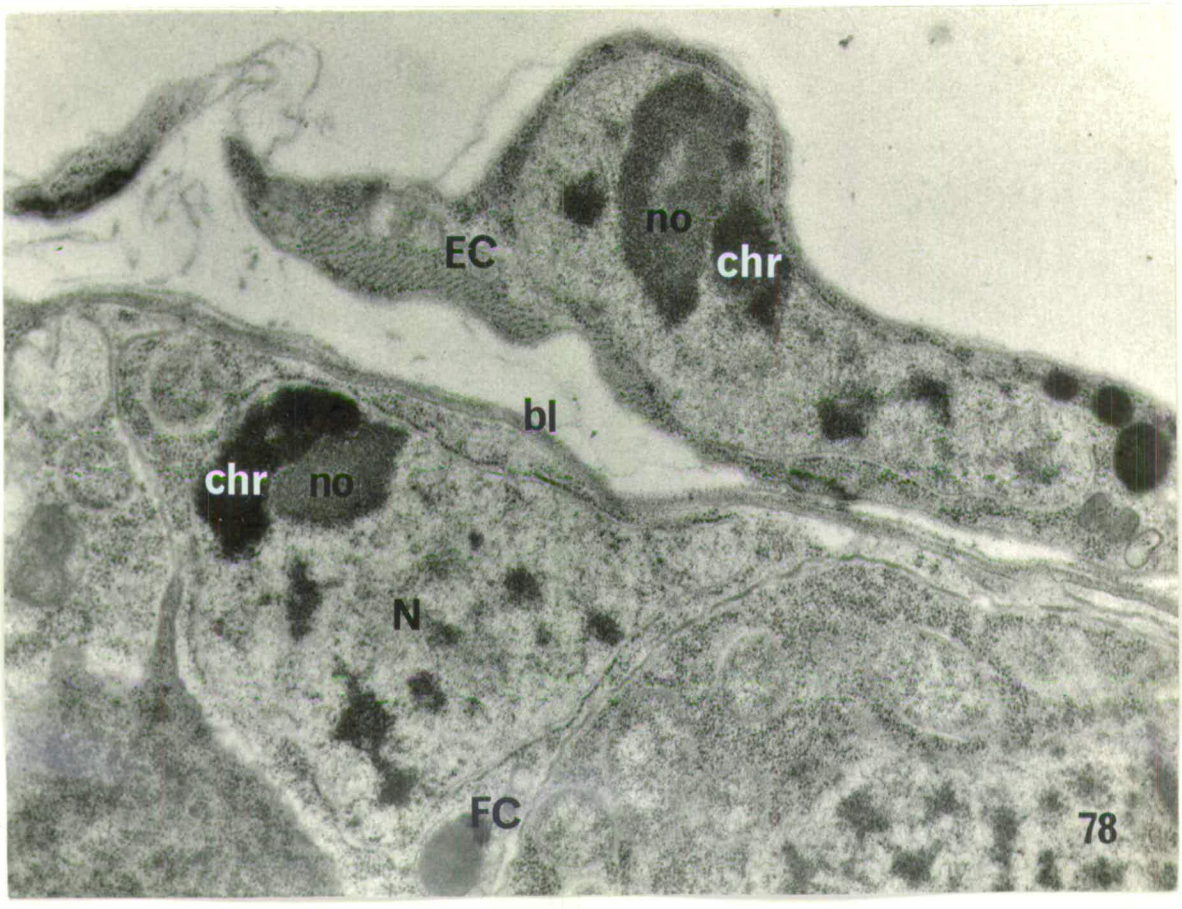
Fig. 78. Part of the anterior pole of the germarium. Note a prefollicle cell (FC) lying underneath the basement lamina (bl). The nucleus (N) occupies most of the area of the cell. The nucleolus (no) contains mainly granular components. A clump of chromatin material (Chr) is in close contact with the nucleolus. The epithelial cell (EC) exhibits a similar nuclear morphology. x 22,500.

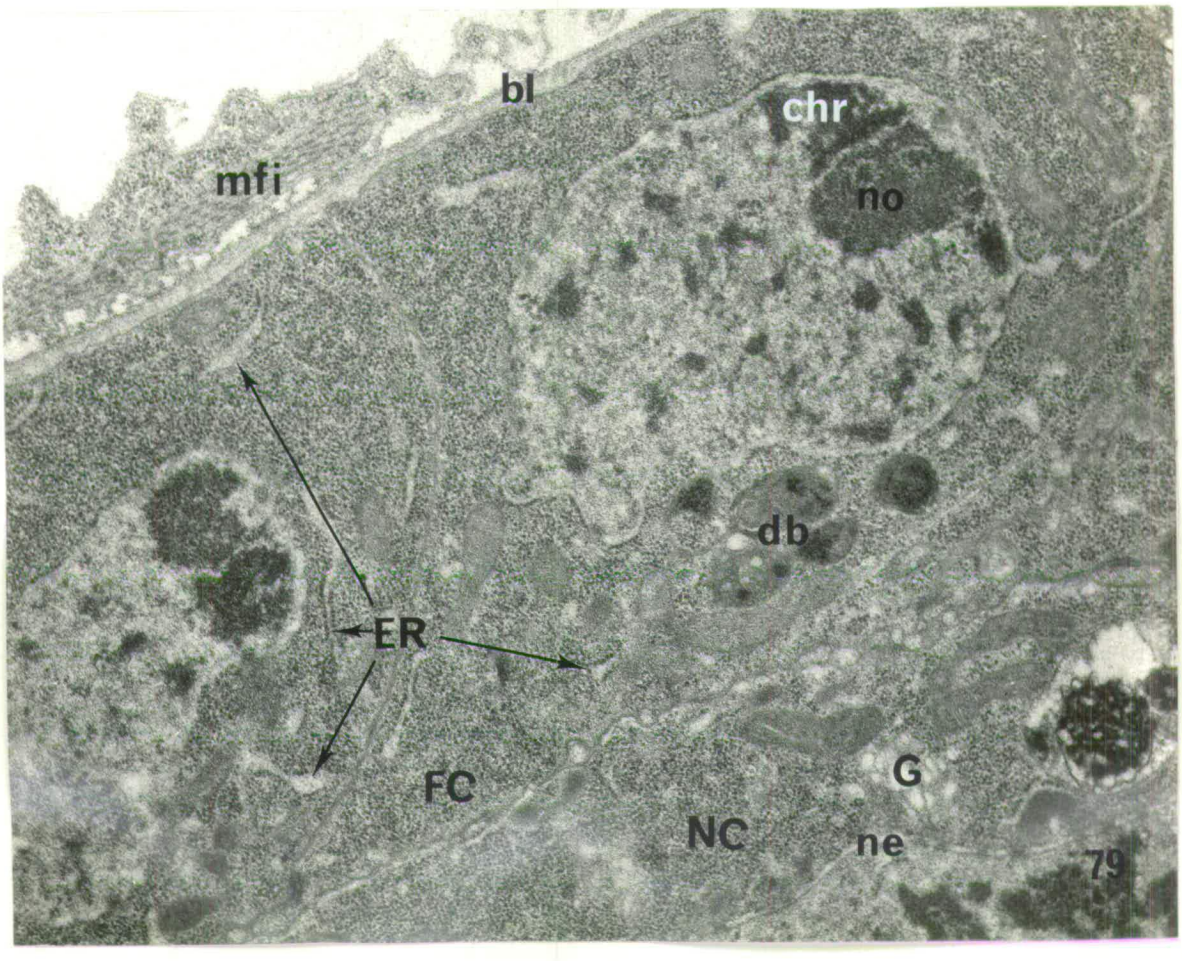
Fig. 79. Part of a stage 5 ovarian chamber showing the follicle layer (FC) and the nurse cytoplasm (NC). Note the presence of a few profiles of endoplasmic reticulum (ER) and various dense bodies (db) in the follicle cell cytoplasm. The nucleolus (no) is eccentrically located. External to the basement lamina (bl), a stretch of the epithelial sheath is visible with myofilaments (mfi) inside. x 22,500.


N
ne
Cy
75


Cy
76


NC
ER
FC
77


EC
no
chr
bl
chr
no
N
FC
78


bl
chr
mfi
no
db
ER
FC
G
NC
ne
79

membrane facing the overlying layer of follicle cells (Fig. 77).

By stage 13, the cytoplasm of all nurse cells has a washed out appearance; only inclusions and whorls of membranes of various sizes are observable (Fig. 62). As mentioned earlier, the nuclear envelope shows wide gaps at this stage and nuclear contents have been observed passing into the cytoplasm. The cytoplasm is totally devoid of any recognizable constituents.

3. The follicle cells

A prefollicle cell in a peripheral location of the germarium is shown in Fig. 78. Most of the volume of the cell is made up of the nucleus, around which the cytoplasm appears as a very narrow fringe. The nucleolus appears as a well defined mass like in any typical interphase cell. The distribution of the chromatin and the morphology of the nucleolus in the prefollicle cells is similar to that of the epithelial cell also seen in Fig. 78. A prefollicle cell which has migrated inwards into the deeper regions of the germarium so as to eventually surround a 16-cell cluster is shown in Fig. 80. Apart from a change in the shape of the cell to an elongate one, the nucleoli contain several patches of chromatin-like material. This is clearly brought out in the glycol methacrylate embedded germarium shown in Fig. 80. At this stage also, the cytoplasm continues to be a narrow fringe around the nucleus. Once the follicle layer is complete in stage 1 chamber, the cells acquire a cuboidal shape and this shape persists up to

stage 7.

Fig. 79 shows follicle cells of a stage 5 chamber. The relative area of the cytoplasm of the follicle cells gradually increases from stage 2 onwards and acquires normal proportions by stage 5. The cytoplasm is already well packed with ribosomes and a few profiles of endoplasmic reticulum have made their appearance. Apart from Golgi apparatus and mitochondria, the other constituents of the follicle cell cytoplasm are dense looking bodies surrounded by a membrane. Some of the dense bodies have a more or less uniform density and show no ultrastructure, while others appear heterogeneous with dense and less dense areas.

The distribution of the chromatin and the structure of the nucleolus in the follicle cells change little during stages 2 to 7 (Figs. 82 and 83). It is already known that follicle cells do not divide after stage 6 and that when the oocyte enlarges, most of the follicle cells migrate posteriorly (King and Koch, 1963). As a result, the anterior part of the chamber, that is the part occupied by the nurse cells, comes to be covered by fewer follicle cells than in earlier stages. In stage 8, the follicle cells at the anterior region of the chamber are consequently stretched and become very thin. The far greater number of follicle cells which overlie the oocyte are closely packed and acquire a columnar shape from stage 8 onwards.

Various types of cell junctions have been observed in the follicle cells during this study. In the cells, especially those surrounding the anterior region of stage 1 chamber, there is present an elaborate

Figs. 80-83. Preparations taken from ovarioles fixed in glutaraldehyde and embedded in glycol methacrylate. While the section shown in Fig. 80 is stained in uranyl acetate and lead citrate, those of Figs. 81-83 are treated with 0.02 M EDTA after uranyl acetate staining.

Fig. 80. A follicle cell from the intermediate region of the germarium. Note the elongate shape of the nucleus (N) and the asymmetrical location of the nucleolus (no). x 20,000.

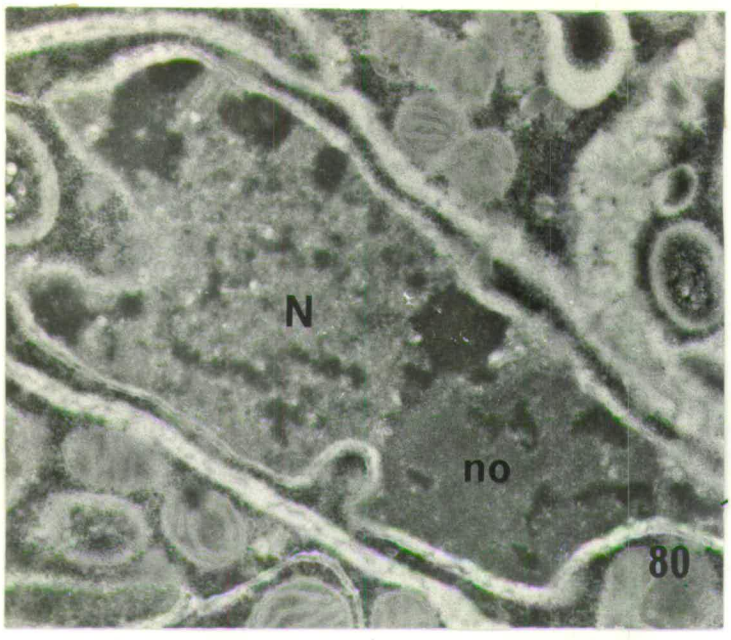
Fig. 81. A cell of the external epithelial sheath. Note the bleached area in the nucleus corresponding to the clump of chromatin material (Chr). x 20,000.

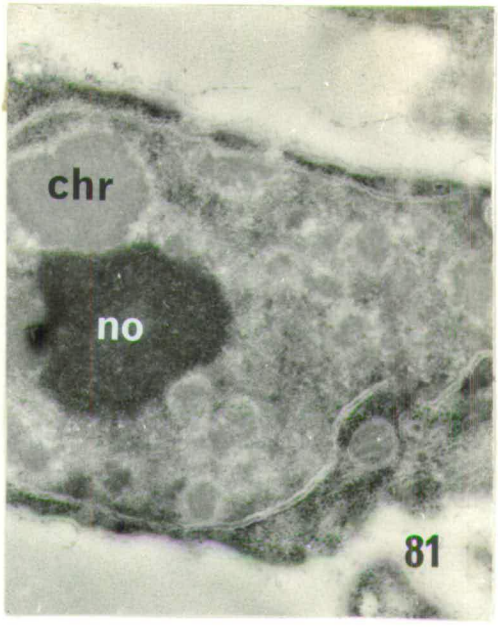
Fig. 82. A follicle cell nucleus from a stage 2 ovarian chamber. Note several bleached areas inside the nucleolus corresponding to the intranucleolar chromatin (Chr). x 20,000.

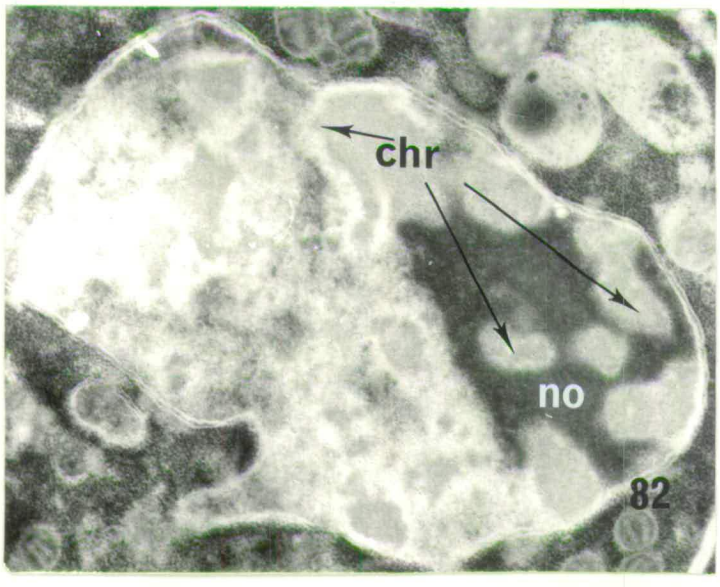
Fig. 83. A follicle cell nucleus from a stage 7 ovarian chamber. The nucleus at this stage is becoming circular. x 15,000.

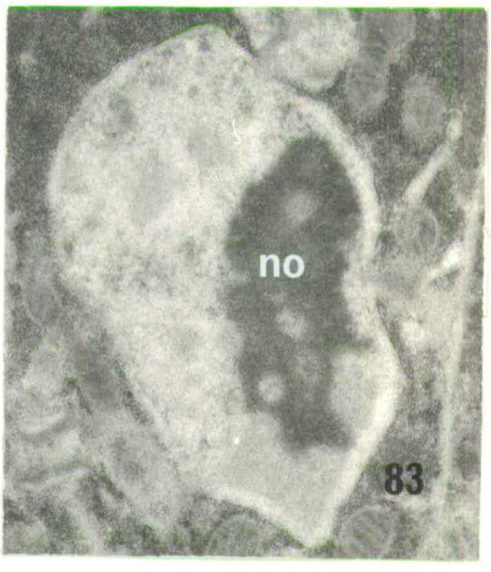
Fig. 84. A transverse section of a columnar follicle cell nucleus from a stage 8 ovarian chamber. The nucleolus consists of mixed fibrillar (fb) and fibrillo-granular (gr) components. The chromatin material (Chr) is scattered throughout the nucleus. x 20,000.

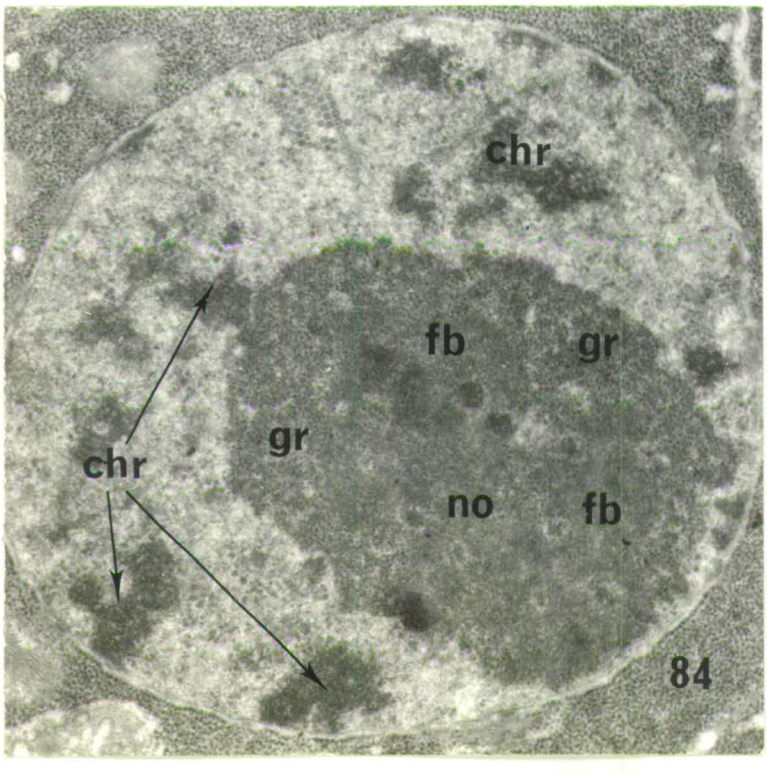
Fig. 85. Enlargment of part of the nucleus shown in Fig. 84. Note the presence of regular arrays of rounded entities. These entities measure on average 400 A in diameter. These intranuclear crystalline arrays are tentatively interpreted as viruses. x 60,000.

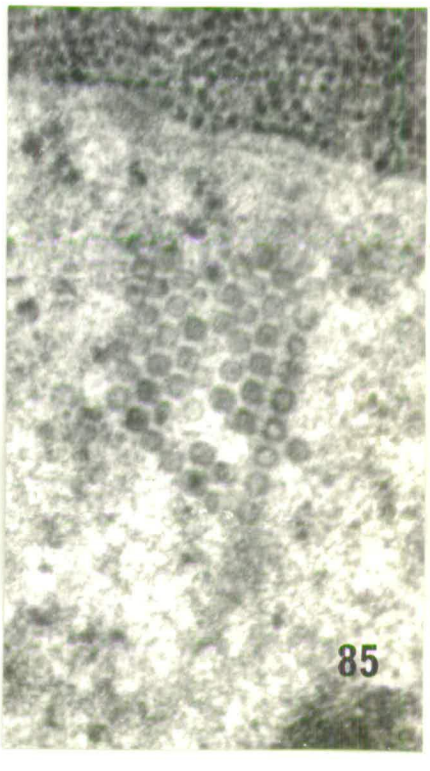

N
no
80


chr
no
81


chr
no
82


no
83


chr
fb
gr
gr
chr
no
fb
84


85

system of cell junctions (Fig. 86). They are similar to the type described as zonula adherens by Farquhar and Palade (1963). In this type of cell junction, the neighbouring plasma membranes of two cells show close approximation to within 200 A along short stretches of about 0.5 microns. Very close to the inner plasma membrane of the two cells, a band of dense material is also evident. No distint thin filaments can, however, be discerned running perpendicular to the dense bands as has been described in certain mammalian tissues, although cross sections of microtubules are evident in the area. From a study of numerous sections of follicle cells in stage 1 chamber, it would appear that the plasma membrane of apposed cells close to the region of the cell junctions, undergo some modification and perhaps subsequent dissolution (Figs. 87 and 88) thereby leading to continuity between cells or the formation of intercellular bridges. This point can be confirmed only through a careful study of serial sections and building a three-dimensional model. All that one can speculate at the present time is that the intercellular bridges arise in the immediate vicinity of the zonula adherens; the modalities of the process remain to be worked out.

Two intercellular bridges connecting three follicle cells are shown in Fig. 90. The high power picture (Fig. 91) shows that the bridge, which is 0.25 microns in average diameter, is a cylindrical structure with a wall 600 A thick and a core of cytoplasm which is continuous with both cells. The wall of the cylindrical bridge consists of an electron dense layer, about 200 A thick, and of an inner, much less dense layer, about

400 A thick.

As far as my observations go, the inter-cellular bridges seem to occur widely between the follicle cells of previtellogenic stages, whereas in vitellogenic stages (viz.: from stage 8 onwards) they are practically restricted to the columnar follicle cells. Zonula adherens, as described earlier, are also observable at the base of the columnar follicle cells (Fig. 34) and also at the base of the follicle cells in previtellogenic stages (Fig. 89).

The other type of junction met with between follicle cells are the gap junctions and the septate junctions as have already been described by Mahowald (1972a). The former, which is of wide spread occurrence between columnar and non-columnar follicle cells, is present as a localized region of the cell surface and is rarely more than 1 micron in length. A septate junction is shown in Fig. 92. It consists of regular septa which are arranged at 150 A intervals across the space between the adjoining cell membranes. Septate junctions first become noticeable between columnar follicle cells in stage 12 chambers when the endochorion is secreted by these cells.

A transverse section of a columnar follicle cell nucleus passing through the nucleolus is shown in Fig. 84. The nucleolus is very prominent and consists typically of intermixed fibrillar and fibrillo-granular areas. Patches of chromatin material are also noticeable mainly in the fibrillar portions.

Striking changes occur in the cytoplasm of columnar follicle cells from stage 8 onwards. These

Fig. 86. Part of the follicle layer overlying the cluster of nurse cells from a stage 1 ovarian chamber towards the anterior end of the germarium. Note the cell junctions (J) in places where the follicle cells interdigitate. (m) mitochondria; (mt) microtubules. x 30,000.

Fig. 87. Part of the same region shown in Fig. 86. A portion of the plasma membrane (see arrow) of one cell close to a cell junction is protruding into the adjoining follicle cell. x 15,000.

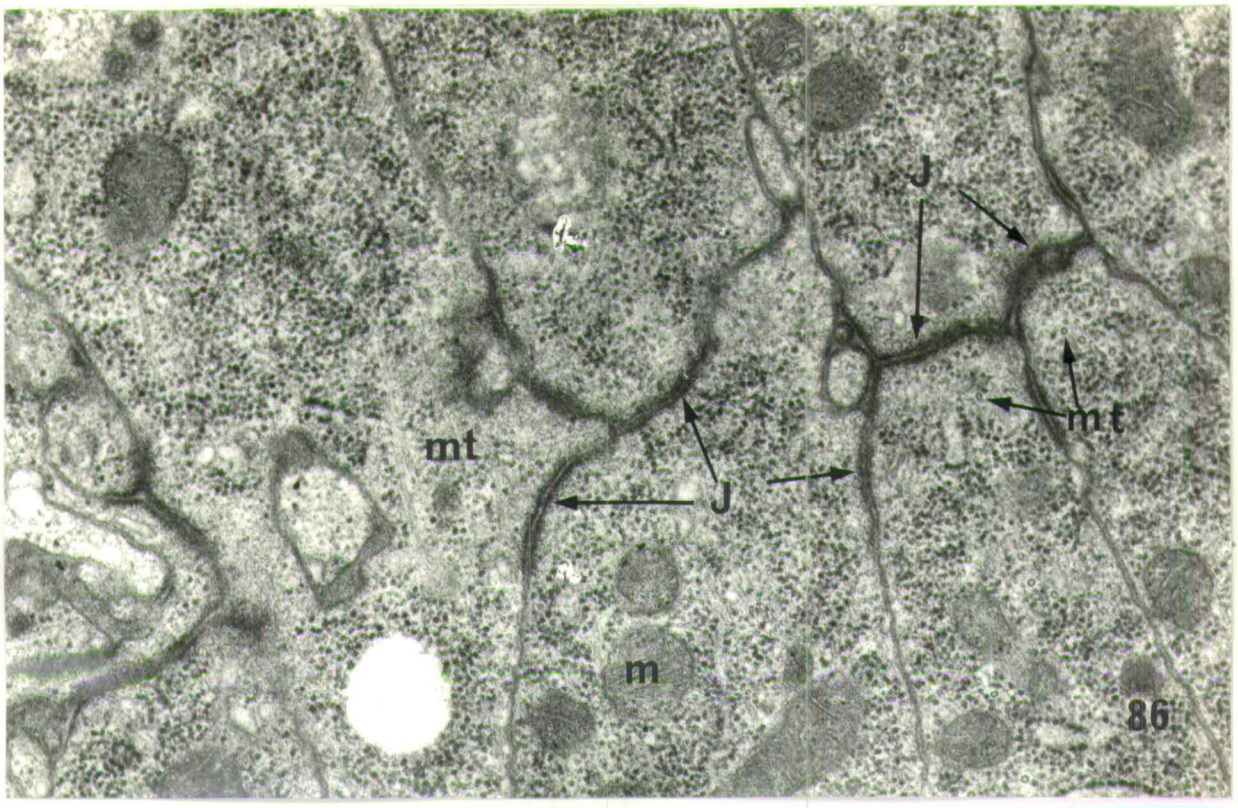
Fig. 88. The plasma membranes of two adjoining follicle cells in the same region shown in Fig. 86. The images at the region of the cell junctions are indicative of dissolution of the plasma membranes. x 20,000.

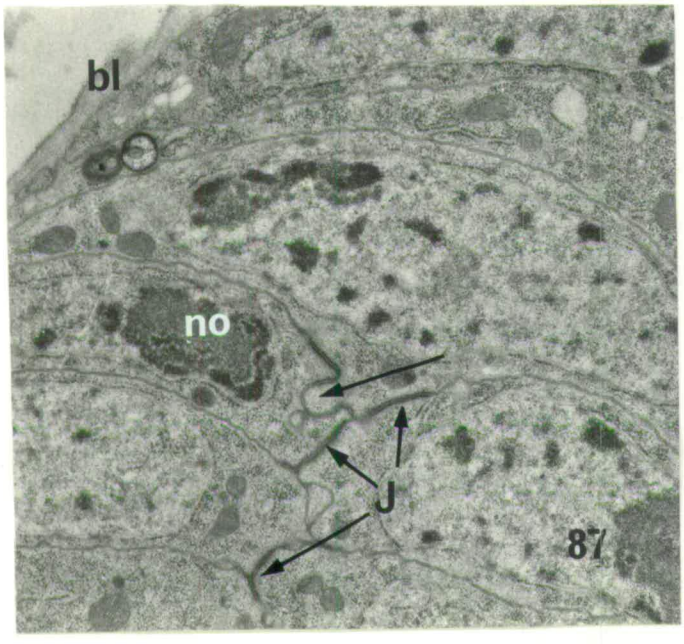
Fig. 89. Part of the follicle layer overlying the ooplasm from a stage 7 ovarian chamber. Cell junctions (J) are visible at the base of each cell. x 20,000.

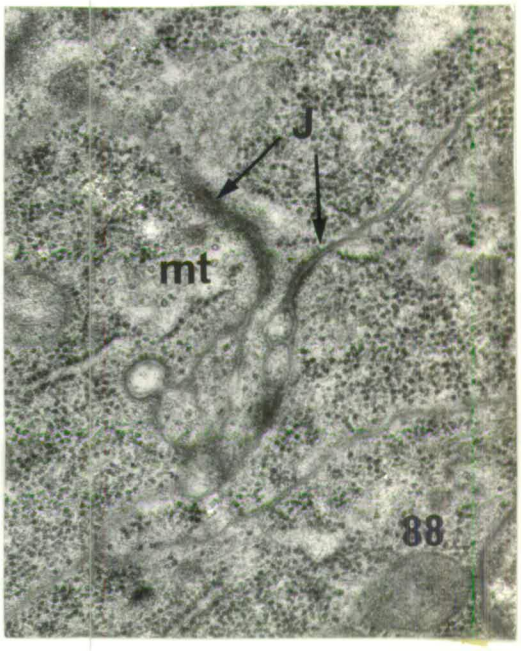
Fig. 90. A cross section of columnar follicle cells from a stage 10 ovarian chamber. Note two intercellular bridges (ib) joining adjacent cells. (no) nucleolus; (Chr) chromatin material. x 12,000.

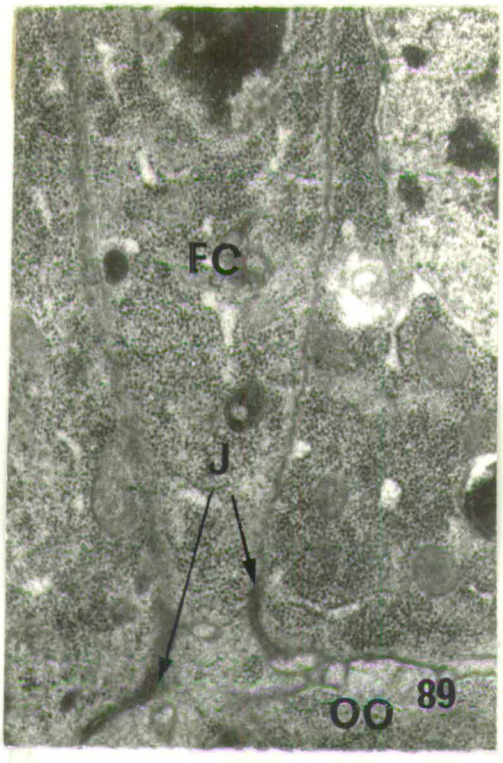
Fig. 91. A longitudinal section of an intercellular bridge (ib) between adjacent follicle cells of a stage 7 ovarian chamber. The wall of the bridge consists of two layers: a 200 A thick dense layer (L) and an inner 400 A thick, less dense layer (l). Note that the plasma membranes of the two adjoining cells join to form the bridge wall (see arrows). x 30,000.

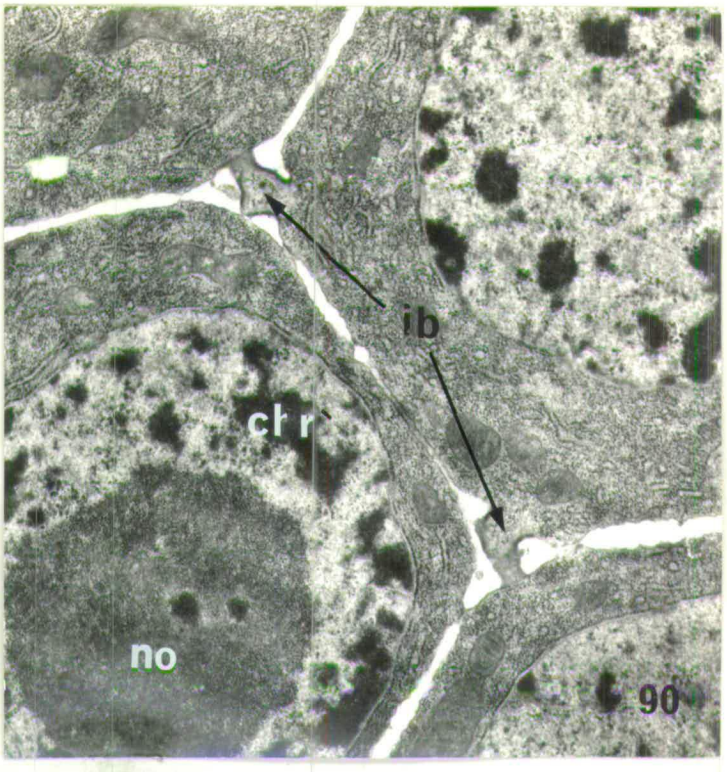
Fig. 92. A section passing through the septate junctions (SJ) joining adjacent follicle cells of stage 13 ovarian chamber. Note the regular septa which separate the plasma membranes of the two cells (see arrows). A number of microtubules are oriented parallel to the septate junctions. x 90,000.

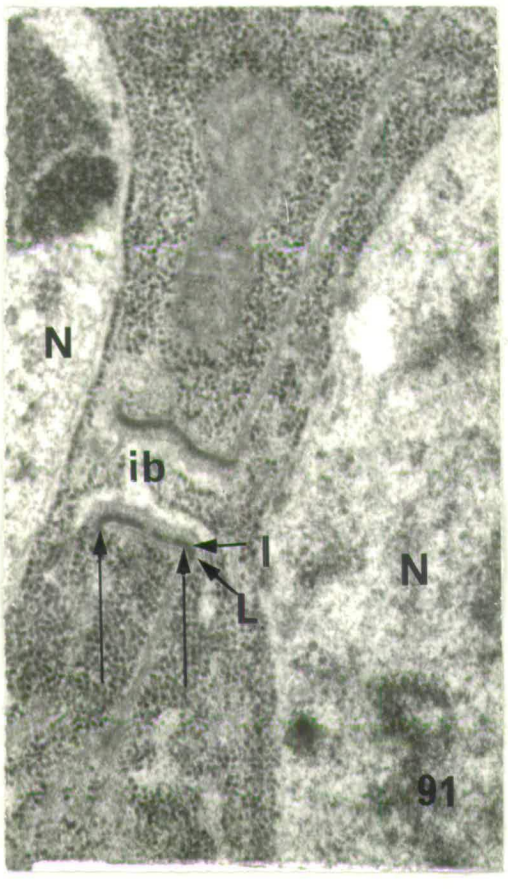

J
mt
mt
J
m
86

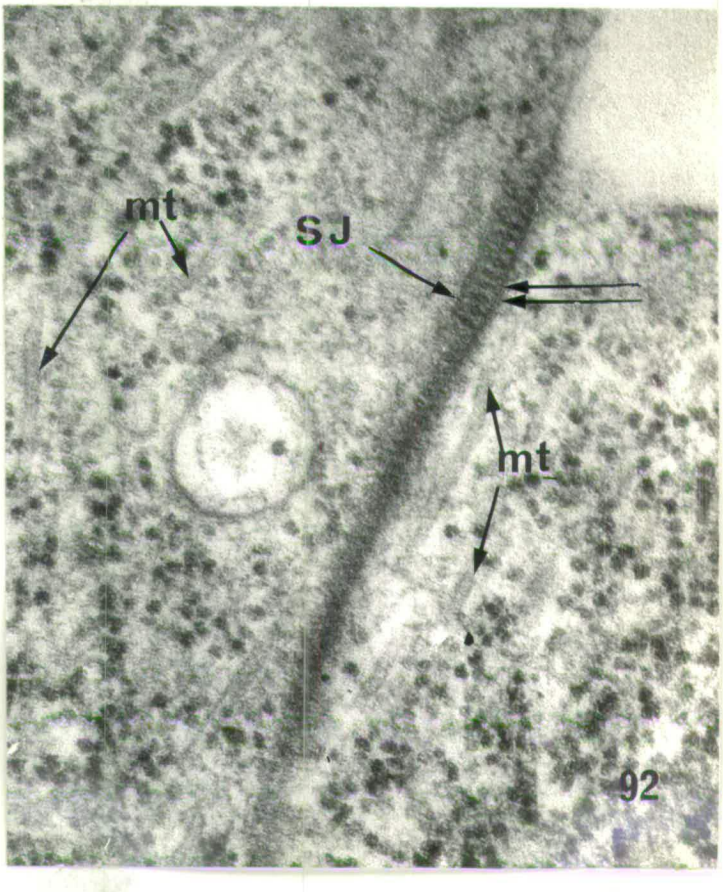

bl
no
J
87


J
mt
88


FC
J
OO
89


ib
no
90


N
ib
l
L
N
91


mt
SJ
mt
92

relate mainly to the endoplasmic reticulum and the Golgi apparatus. A representative picture of the cytoplasm of a columnar follicle cell in stage 8 chamber is shown in Fig. 93. The endoplasmic reticulum appears to be developing into a system of parallel cisternae and the Golgi apparatus is far more extensive than in any of the previous stages. With further development of the chamber, the endoplasmic reticulum comes to form a continuous system and profiles of various shapes (vesicles and cisternae) can be seen throughout the cytoplasm. The endoplasmic reticulum is also studded with ribosomes, but free ribosomes are also observable. In several instances, areas of what appear to be intimate contact between endoplasmic reticulum and the Golgi apparatus have been seen. One such instance is shown in Fig. 93. In favourable sections of such areas, profiles suggestive of the transformation of endoplasmic reticulum to the Golgi apparatus are present, as reported by numerous workers in the past (Morre et al., 1972). The electron micrograph shown in Fig. 94 is presented mainly to show the two types of Golgi-derived vesicles which occur in the present material: one is much more electron dense than the other. In the vicinity of the Golgi apparatus, however, the two types do not seem to remain as two distinct populations of vesicles. Quite often the two seem to fuse as to form compound bodies bounded by a common membrane (see in Fig. 94). In stages 9-10, large number of such compound bodies can be found in the basal part of the cell bordering the oocyte (Fig. 95). The bodies in question then appear to move to the basal border (Fig. 100) and release their

Fig. 93. Part of the cytoplasm from a columnar follicle cell of a stage 8 ovarian chamber. Note the parallel cisternae of the rough endoplasmic reticulum (RER) in close association with the Golgi apparatus (G)(see arrows). x 18,000.

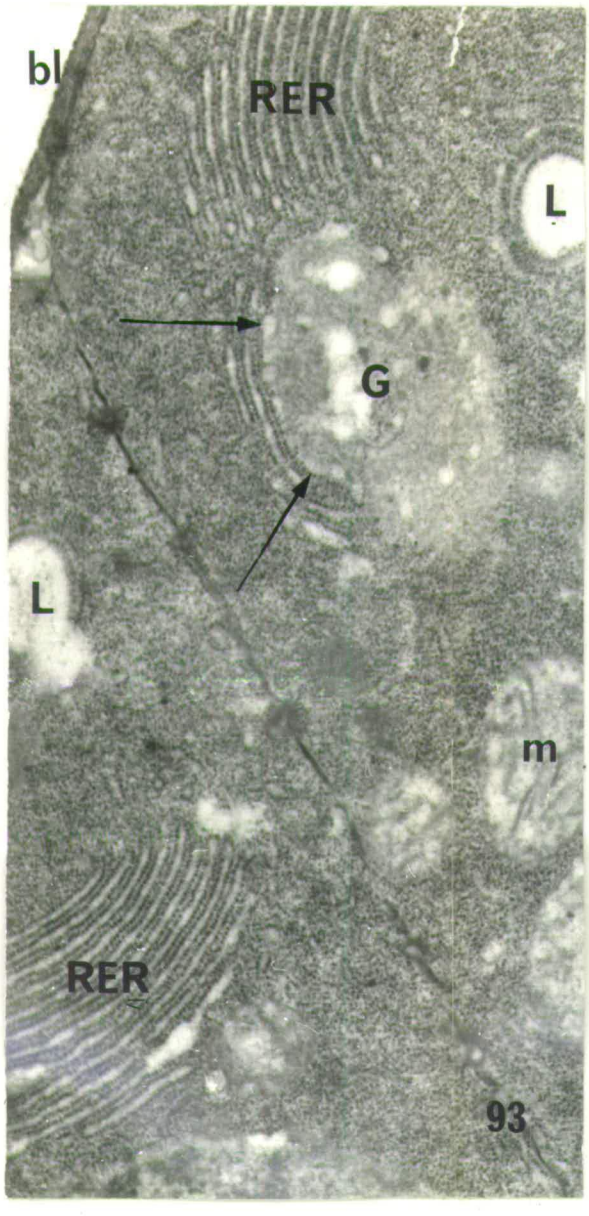
Fig. 94. Part of the cytoplasm of a columnar follicle cell from a stage 10 ovarian chamber. Two types of vesicles can be distinguished in the region of the Golgi apparatus (G): dense (double arrows) and less dense (single arrows). In one instance the vesicle (V) shows both dense and less dense contents: this is probably the result of fusion between the two types of vesicles described above. (m) mitochondria; (db) dense bodies; (ne) nuclear envelope; (N) nucleus. x 60,000.

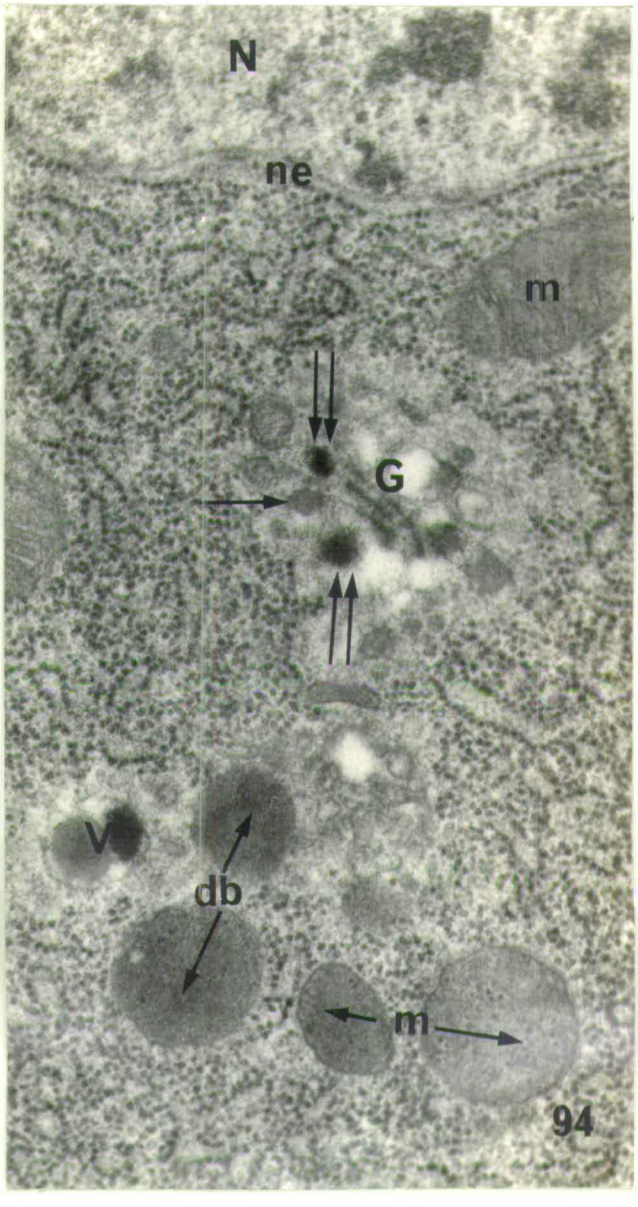
Fig. 95. The basal part of the cytoplasm of a columnar follicle cell of a stage 10 ovarian chamber. Note the large number of vitelline membrane precursors (vp). Two regions (dense and less dense) are distinguishable in several of them. The plasma membrane of the follicle cell is highly convoluted. x 20,000.

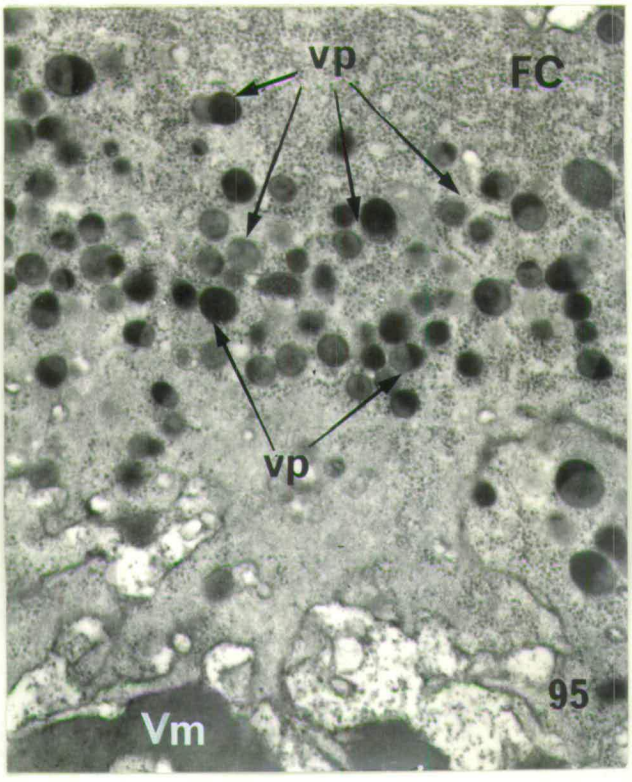
Fig. 96. Low power micrograph of part of a follicle cell cytoplasm from a stage 10 ovarian chamber. Several Golgi apparatus (G) are seen to contain the vitelline precursors;(gJ) gap junctions. x 8,000.

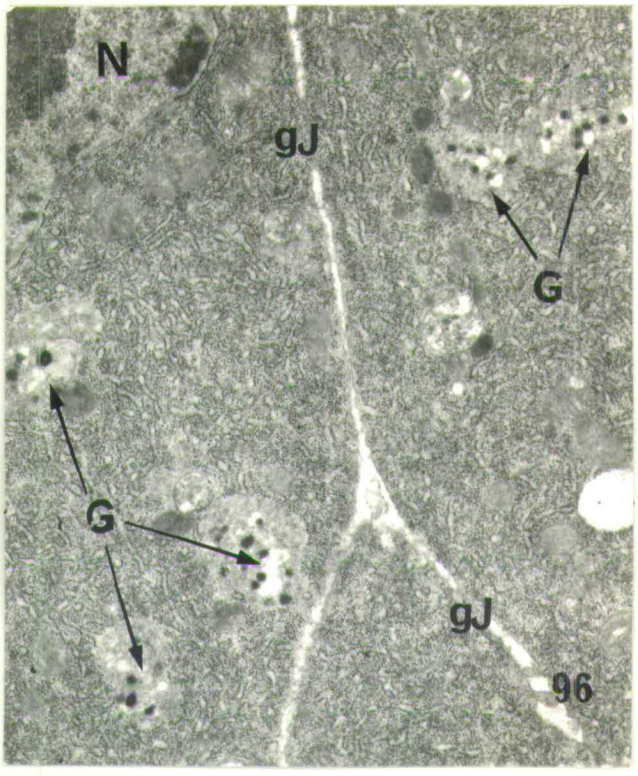
Fig. 97. The basal part of the cytoplasm from a columnar follicle cell of a stage 12 chamber. Note that the endochorionic precursors (ep) exhibit a homogeneous density, whereas in Fig. 95 the vitelline membrane precursors exhibit a dual structure. Endochorionic pillars (p) project from the inner endochorion (ien); (Vm) vitelline membrane. x 18,000.

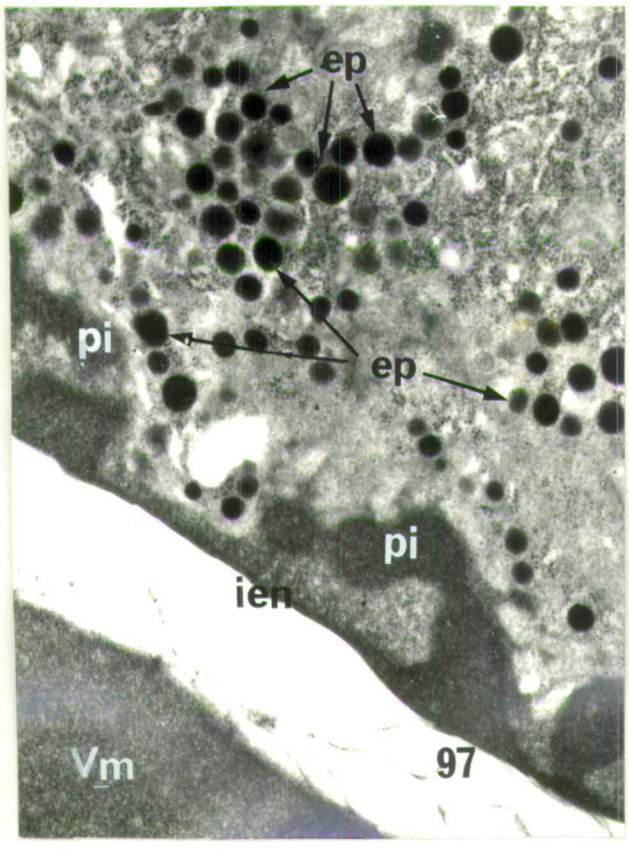
Fig. 98. Low power micrograph of part of the follicle cytoplasm from a stage 12 ovarian chamber. The Golgi apparatus (G) contain several endochorion precursors. The insert shows an enlargement of the Golgi apparatus. x 8,000.

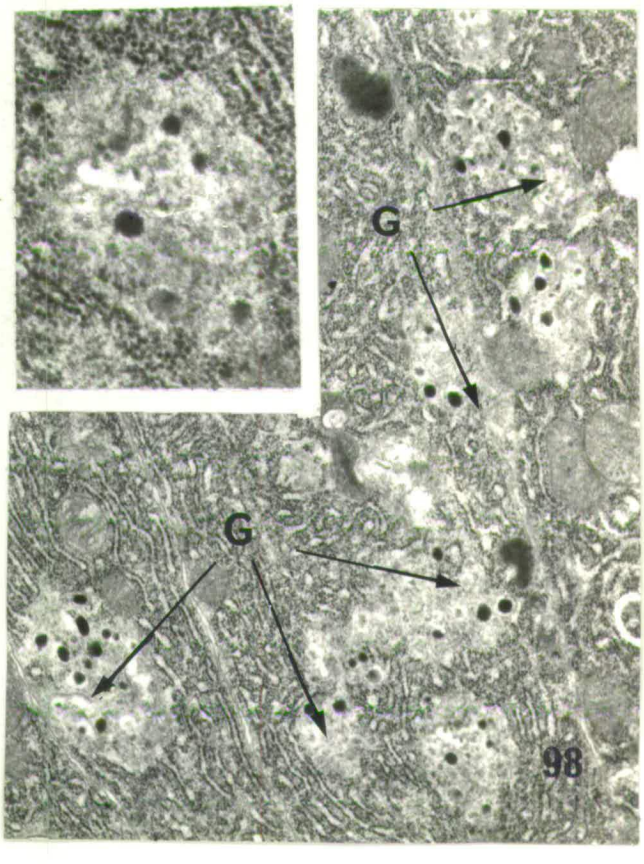

bl
RER
L
G
L
m
RER
93


N
ne
m
G
V
db
m
94


vp
FC
vp
Vm
95


N
gJ
G
G
gJ
96


ep
pi
ep
pi
ien
Vm
97


G
G
98

contents along the border between the follicle cells and the oocyte (Fig. 99). A part of the basal cytoplasm of the follicle cell is also seen in Fig. 99, where in addition to a Golgi apparatus and a few compound bodies, a,vitelline body is visible between the follicle cell and the oocyte. The EM observations strongly suggest that the compound bodies which can be traced back to the Golgi apparatus in stage 9-10 (see also Fig. 96) contain the precursor material for the vitelline membrane. The vitelline membrane precursor material secreted from the follicle cell into the space between the layer of columnar follicle cells and the oolemma gives rise to more or less discrete masses or vitelline bodies. The continued addition of material to such bodies and the formation of new ones finally results in a continuous layer of vitelline membrane. The formation of the vitelline membrane is fairly complete by the end of stage 10 except anteriorly at the level of the follicle cells which adjoin the nurse cells. During stages 11-12, the columnar follicle cells adjoining the nurse cells migrate inwards, as described by Cummings and King (1969) and the secretion of the vitelline membrane by the migrating cells eventually results in the completion of the vitelline membrane along this border also. By the end of stage 12, the vitelline membrane completely surrounds the oocyte except in the region of the four ring canals which connect the ooplasm and the nurse cells.

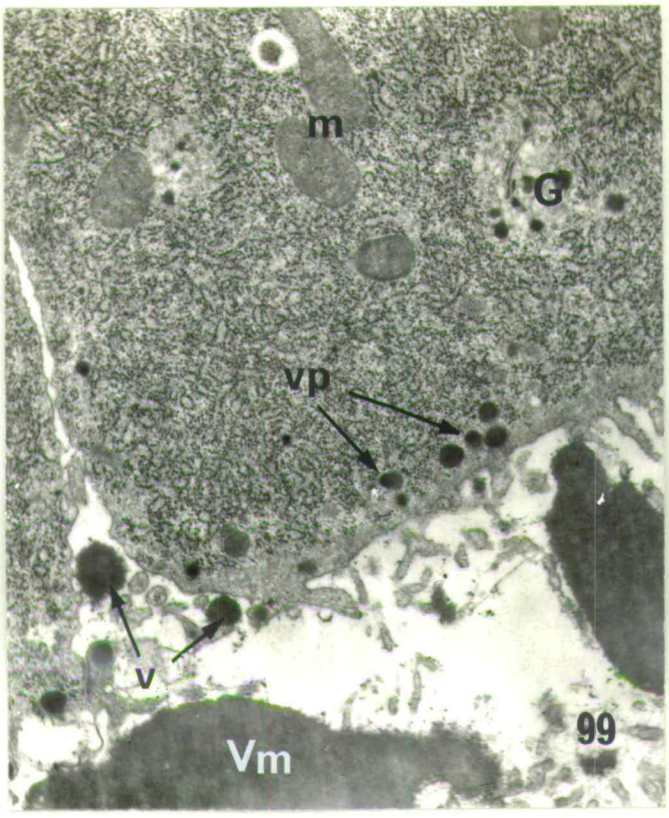
During stage 11, as soon as the formation of the vitelline membrane is completed, the columnar follicle cells begin to secrete the endochorion. The Golgi apparatus of these cells show a number of dense

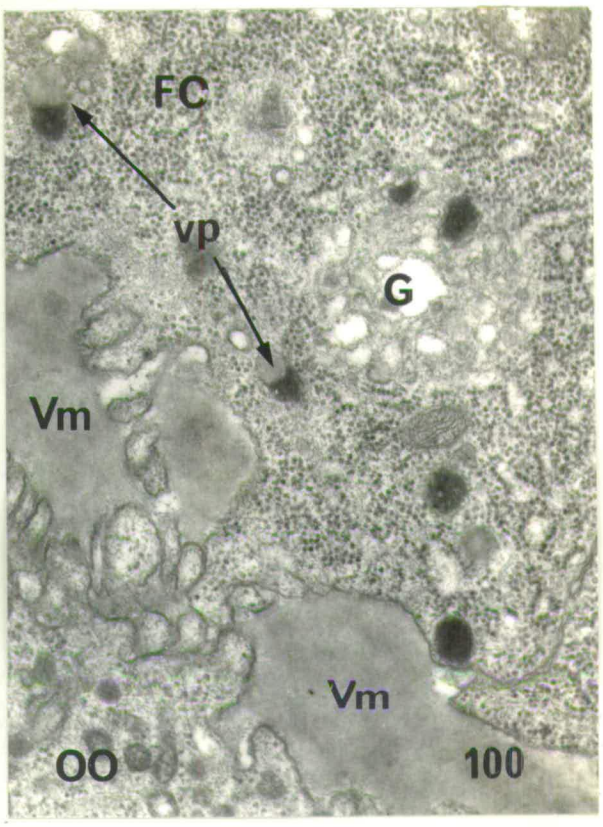
Fig. 99. The follicle-oocyte border from a stage 10 ovarian chamber. Note that a few vitelline membrane precursors (vp) are present along the plasma membrane of the follicle cells and also in the space between the follicle cells. (Vb). (Vm)vitelline membrane. x 12,000.

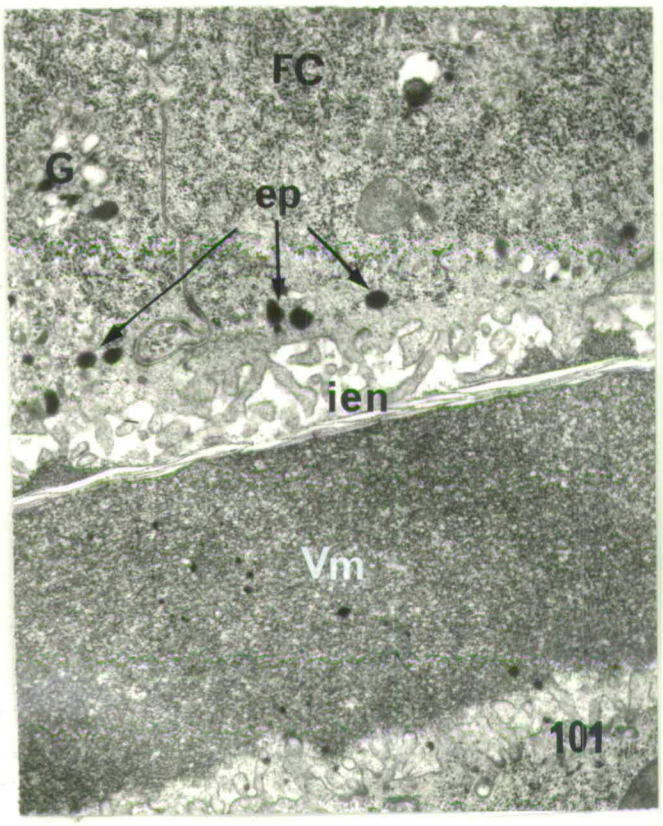
Fig. 100. A Golgi apparatus (G) in the basal portion of the follicle cytoplasm from a stage 9 ovarian chamber. Note the presence of two distinct regions in the vitelline precursors (vp). Vitelline bodies (Vm) are visible between the oocyte (OO) and the follicle cell (FC). x 30,000.

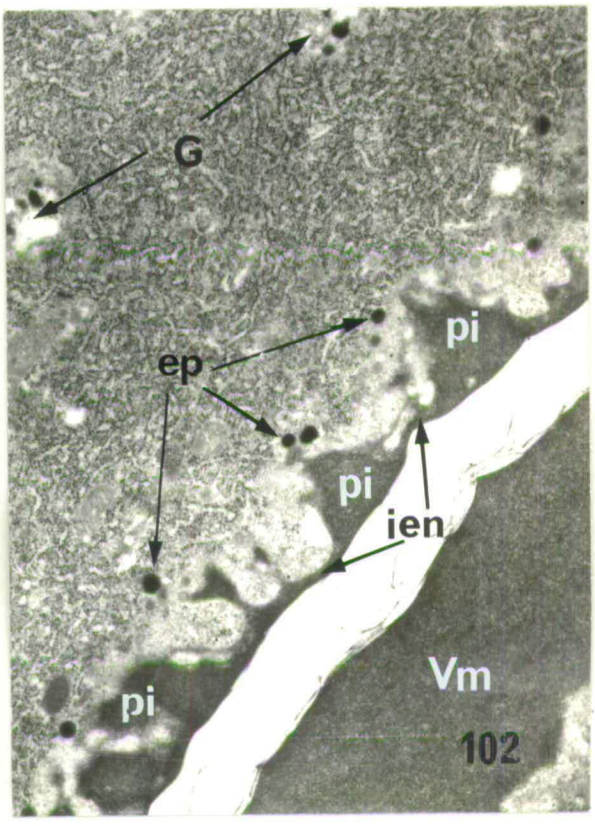
Fig. 101. The follicle-oocyte border from a stage 11 ovarian chamber. The vitelline membrane (Vm) is completed. The endochorionic precursors (ep) are visible in the basal cytoplasm of the follicle cell (FC). The inner endochorion (ien) is being deposited. (G) Golgi apparatus. x 12,000.

Fig. 102. The follicle-oocyte border from a stage 12 ovarian chamber. The endochorionic pillars (pi)have been deposited upon the inner endochorion (ien). Golgi apparatus (G) containing several electron dense vesicles are visible in the uppermost part of the micrograph. (Vm) vitelline membrane; (ep) endochorionic precursors. x 12,000.


m
G
vp
v
Vm
99


FC
vp
G
Vm
Vm
OO
100


FC
G
ep
ien
Vm
101


G
pi
ep
pi
ien
Vm
pi
102

vesicles but, unlike in the earlier stage when the vitelline membrane precursors were formed, these vesicles are of an uniform type (compare Figs. 95 and 97). Low power pictures of cytoplasm of the follicle cells during vitelline membrane precursor formation and the formation of endochorion precursors are presented in Figs. 96 and 98. In both cases it may be noted that the Golgi areas are rich in characteristic vesicles. The migration of the uniformly dense vesicles towards the basal region of the cell and the release of their contents (Fig. 99) appear to be similar to that described earlier during the formation of the vitelline membrane.

At first a more or less continuous layer of endochorionic material is deposited (Fig. 101) and at regular intervals along this, pillar-like thickenings are also formed (Fig. 102). The continued addition of endochorionic material which, as stated earlier, can be traced to the Golgi apparatus results in the formation of another layer which bridges the pillars mentioned above. The final result in stage 14 is a compound layer of endochorion consisting of the first formed inner endochorion and the outer endochorion: the endochorion also has a compartmented appearance due to the presence of the pillars (Fig. 105).

At the anterior end of the ovarian chamber in stage 12, the endochorion forms the dorsal chorionic appendages; the formation of the appendages by the follicle cells which migrate centripetally between the oocyte and the nurse cells has been described by King and Koch (1963). The endochorionic appendages are formed in very much the same way as the rest of the endochorion.

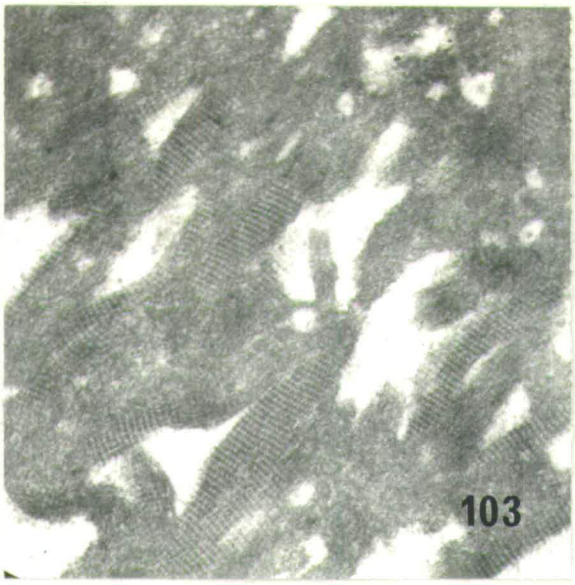
Fig. 103. Part of the dorsal endochorionic appendages from a stage 13 ovarian chamber. Note a characteristic banded structure (250 A) in the endochorionic material. x 32,000.

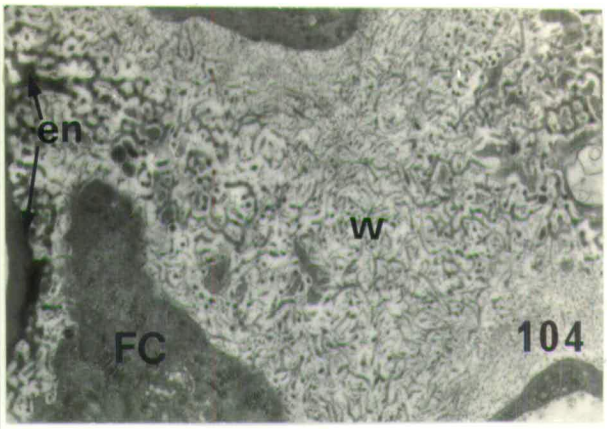
Fig. 104. Part of the follicle layer from a stage 14 ovarian chamber. "Wispy" material (w) fills the interspace between the follicle cell surface (FC) and the endochorion (en). x 8,000.

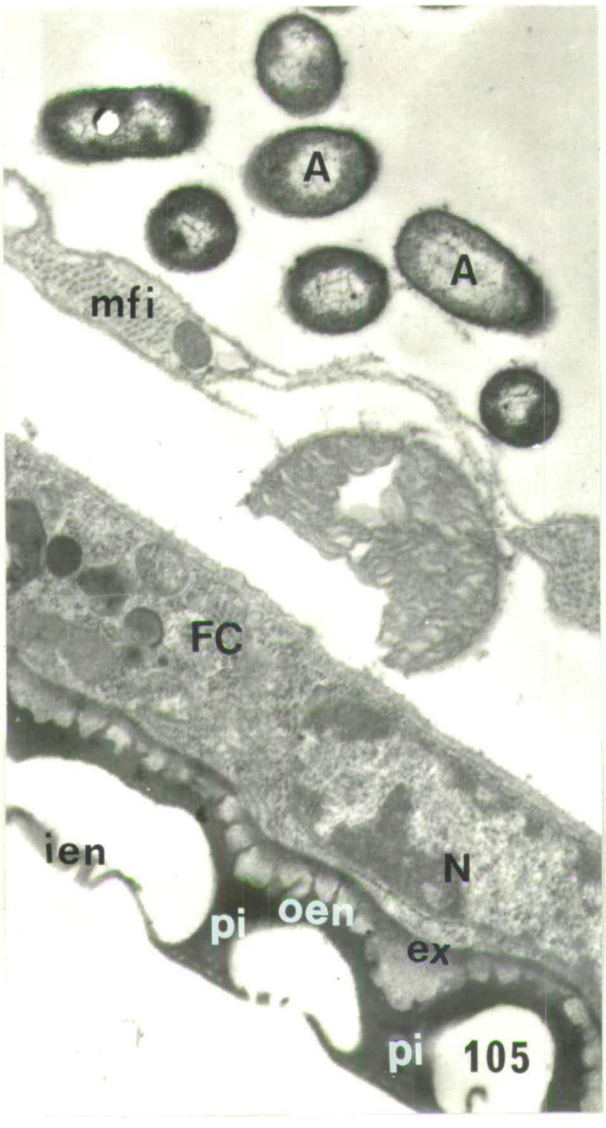
Fig. 105. The complete set of egg coverings in a stage 14 ovarian chamber; (ex) exochorion; (oen) outer endochorion; (p) endochorionic pillars; (ien) inner endochorion. (FC) follicle cell; (N) nucleus of the follicle cell; (mfi) myofilaments of the epithelial sheath. It is interesting to see some rickettsia-like bodies (A) in an extracellular location. It is not known how they actually get there. x 8,000.

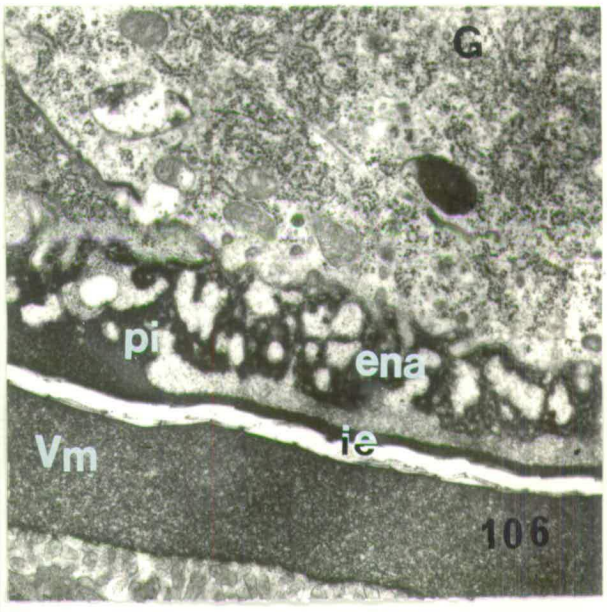
Fig. 106. The anterior region of a stage 13 ovarian chamber showing the site of deposition of ½ endochorionic appendages (ena). (ien) inner endochorion; (pi) endochorionic pillars. x 12,000.

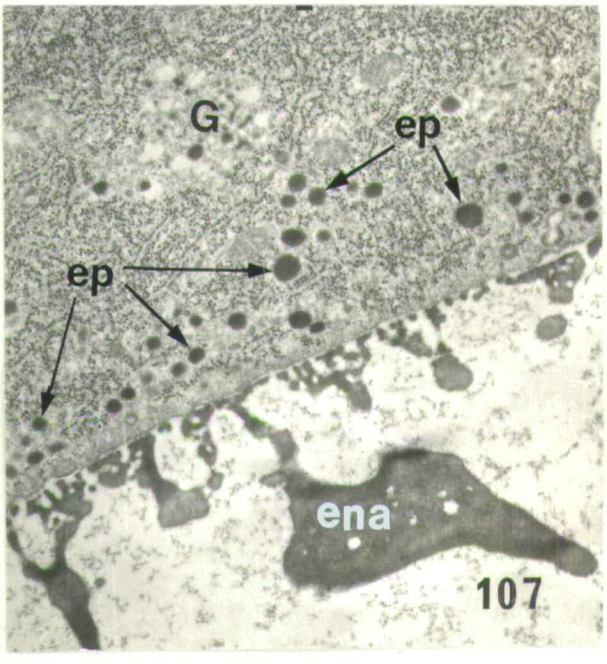
Fig. 107. Part of the cytoplasm of a columnar follicle cell from a stage 13 ovarian chamber. Numerous vesicles, the endochorionic precursors (ep), are visible in the basal part of the cell. (ena) endochorionic appendages; (G) Golgi apparatus. x 12,000.


103


en
w
FC
104


A
A
mfi
FC
ien
N
pi
oen
ex
pi
105


G
pi
ena
ie
Vm
106


G
ep
ep
ena
107

Vesicles derived from the Golgi apparatus, in fact, move towards the basal part of the follicle cell and release their contents into the adjoining space (Fig. 107), by fusing with the plasma membrane. The endochorionic material which makes up the appendages begins to be deposited over the inner endochorion in a restricted region of the anterior part of the chamber (Fig. 106). In this specialized region of the endochorion, it is not possible to distinguish the inner and the outer endochorionic layers. This endochorion consists of an irregular strutwork of dense material as shown in Fig. 103 unlike the compartmented endochorion which surrounds the rest of the oocyte. A longitudinal section of the dorsal chorionic appendages reveals that, in places, the constituent material exhibits a regular periodicity of about 250 A. Such a periodicity has not, to my knowledge, been reported so far in the endochorion of Drosophila.

Another modification of the chorion at the anterior region of the egg relates to the formation of the micropylar apparatus. This region presents some unusual features which seem to be at variance with those presented in the account provided by King and Koch (1963); and this prompted a careful analysis of this region. My observations indicate that an early difference lies in the morphological appearance of the vitelline membrane at this region. As compared with the vitelline membrane in the remaining areas (see Figs. 34 and 100) it may be noted that the appearance of the membrane in the region of the future micropylar apparatus (Fig. 108) is quite different. This part of the vitelline membrane has a rather mottled texture (Figs. 108 and 110) and appears

to project into the ooplasm to a far greater extent than the usual thickness of the rest of the vitelline membrane. However, as shown in Fig. 110 the two portions of the vitelline membrane, i.e. that at the anterior pole of the chamber and that over the rest of the oocyte surface, appear to be continuous. It is also clear from Fig. 110 that the mottled vitelline membrane portion at the site of the future micropylar apparatus is formed by the so-called border cells (those follicle cells which reach the oocyte surface by passing through the interspaces of the nurse cells) and not by the columnar follicle cells which give rise to the rest of the vitelline membrane. Examination of several micrographs of this region of the chamber gives the impression that the vitelline membrane portion with the mottled appearance extends more and more into the ooplasm until it comes to occupy the entire uppermost region of the oocyte (Figs. 109a and 109b).

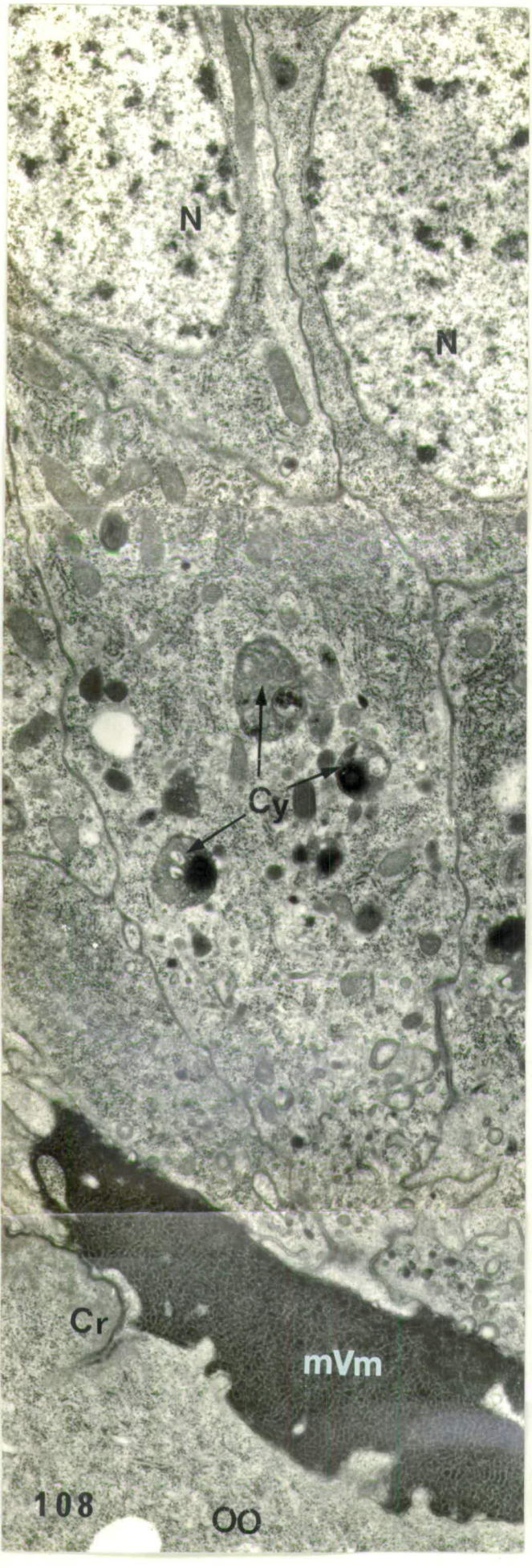
A cross section of the fully formed micropylar apparatus is clearly shown in Fig. 109a. It consists of the two portions of the vitelline membrane which, as already said, are in actual continuity with each other. The part of the vitelline membrane so far described as having a mottled appearance now exhibits a more or less banded appearance with a characteristic periodicity of 250 A. A peripherally located tubular canal, the micropyle, can be clearly seen within the mass of this portion of the vitelline membrane. Both these portions of the vitelline membrane are externally delimited by the endochorionic portion of the micropylar apparatus as seen in Fig. 109a.

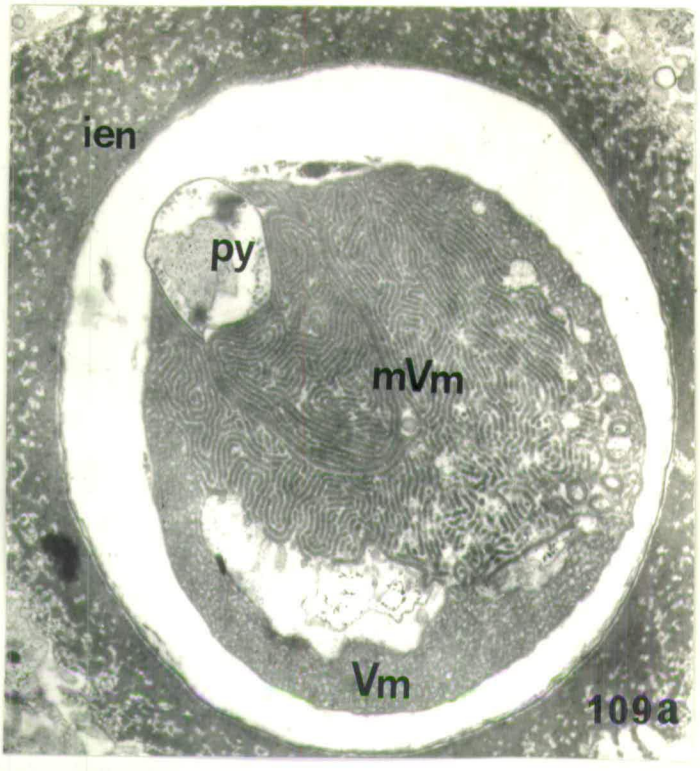
Fig. 108. The border cells at the anterior pole of a stage 11 oocyte. Note the mottled appearance of the vitelline membrane (mVm) at this region. (Cy) cytolysosomes; (N) nucleus; rim of the ring canal (Cr) between the ooplasm (OO) and the nurse cells. x 12,000.

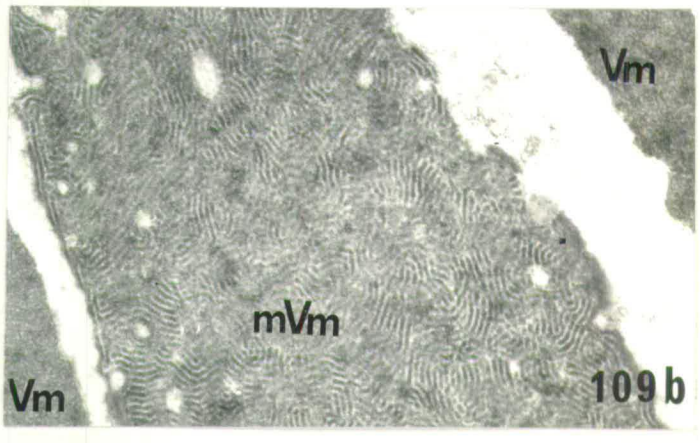
Fig. 109a. A cross section of the micropylar apparatus in a stage 13 ovarian chamber. Note the two parts of the vitelline membrane: (mVm) the one with a mottled-to-banded appearance, and a (Vm) normal looking vitelline membrane; (mp) micropyle; (ien) inner endochorion. x 12,000.

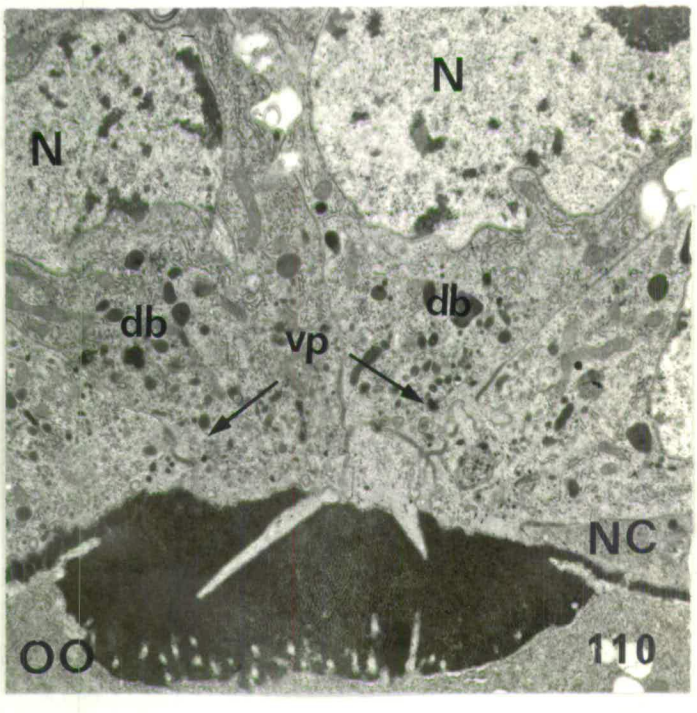
Fig. 109b. A tangential section of the micropylar apparatus in a stage 13 ovarian chamber. The banded-to-mottled looking vitelline membrane (mVm) lies close to the normal looking vitelline membrane (Vm). x 12,000.

Fig. 110. Low power micrograph of the border cells at the anterior region of a stage 12 oocyte. Note the continuity (see arrows) of the mottled vitelline membrane and the rest of the vitelline membrane. The precursors of the mottled vitelline membrane can be seen as dense bodies (vp) in the border cells. These bodies can be traced to the Golgi apparatus of the border cells and in this respect the mode of formation of this stretch of vitelline membrane is similar to that described earlier in the columnar follicle cells for the remaining vitelline membrane. (OO) ooplasm; (db) dense bodies; (NC) nurse cells. x 3,000.


N
N
Cy
Cr
mVm
108
OO


ien
py
mVm
Vm
109a


Vm
mVm
Vm
109b


N
N
db
db
vp
NC
OO
110

By stage 13, another layer, that is the exochorion, makes its appearance closely apposed to the outer endochorion and bordering the follicle cells (Fig. 105). However, I have been unable to find any indications that the exochorion material is produced by the follicle cells. The follicle cells themselves are much smaller at this stage compared with their large size during stages 10-11.

4. Autoradiographic experiments

(a) Experiments with uridine. (i) Nurse cells. As described in the material and methods section, ^{3}H-uridine was used as a tracer for labelling RNA both in <u>in vivo</u> and <u>in vitro</u> conditions. These experiments were carried out in order to compare the synthetic activity of the nucleolus in nurse nuclei of ovarian chambers of different stages. It is known that there is an anterio-posterior polarity in the nuclear volume and the degree of ploidy of nurse cells from stage 7 onwards (Jacob and Sirlin, 1959; Dapples and King, 1970). In view of this, the analyses described below have been restricted to the nurse cells lying closest to the oocyte from stage 7 onwards. In one set of experiments, the tracer was injected into the abdomen of 2-3 day old female flies and after varying intervals of time the ovaries were dissected out, fixed and autoradiographs (ARGs) made for examination in the light and electron microscope. Light microscope autoradiographs (LM-ARGs) of nucleoli representing stage 4 and 10 chambers, fixed 2 hours after injection, are shown in Figs. 111 and 113 respectively. Electron microscope autoradiographs (EM-ARGs)

Fig. 111. A light microscope autoradiograph of part of a stage 4 ovarian chamber from an ovary fixed 2 hours after injection of tritiated uridine. Note the heavily labelled nucleoli. The perinuclear cytoplasm is also labelled (see arrows). The autoradiographic exposure was 7 days. x 1,600.

Fig. 112. A light microscope autoradiograph of part of a stage 10 ovarian chamber from an ovary incubated for 2 hours in 199 medium containing tritiated uridine, followed by a 3 hour chase in cold uridine. Note the high grain density over the follicle cell (FC) and the nurse cells (NC) as compared with the low labelling of the ooplasm (OO). The autoradiographic exposure was 7 days. x 1,600.

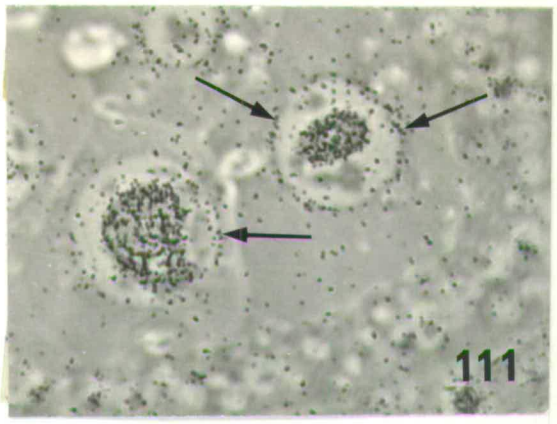
Fig. 113. A light microscope autoradiograph of a posterior nurse nucleus in a stage 10 chamber. The chamber was fixed 2 hours after injection of tritiated uridine. The nucleolus appears very heavily labelled. The autoradiographic exposure was 7 days. x 1,600.

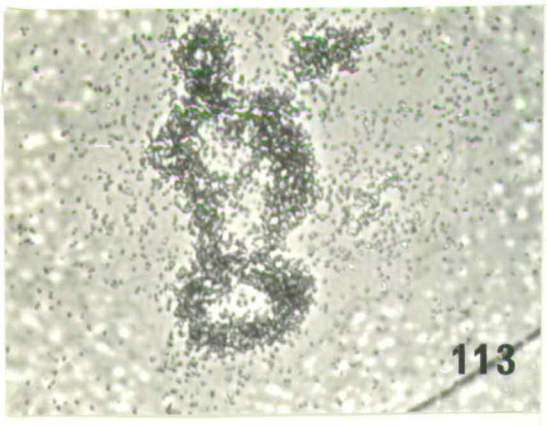
Fig. 114. A light microscope autoradiograph of a stage 9 chamber from an ovary fixed 2 hours after injection of tritiated uridine. Note the heavy labelling of the nurse (NC) and follicle (FC) nuclei. The nurse and follicle cell cytoplasms are also labelled but the ooplasm is almost devoid of silver grains. The autoradiographic exposure was 7 days. x 800.

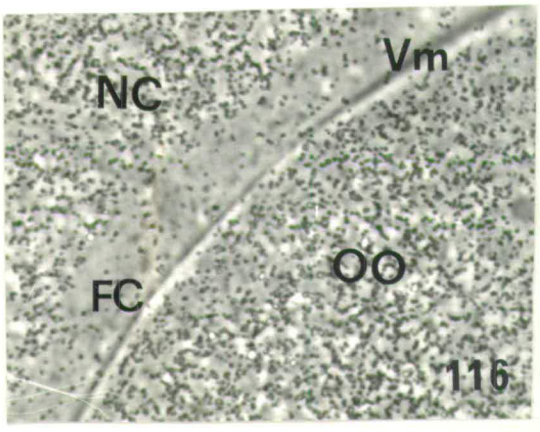
Fig. 115. A light microscope autoradiograph of a stage 9 chamber fixed 6 hours after injection of tritiated uridine. Note the even distribution of silver grains in the ooplasm, follicle cells and the nurse cells. The autoradiographic exposure was 7 days. x 800.

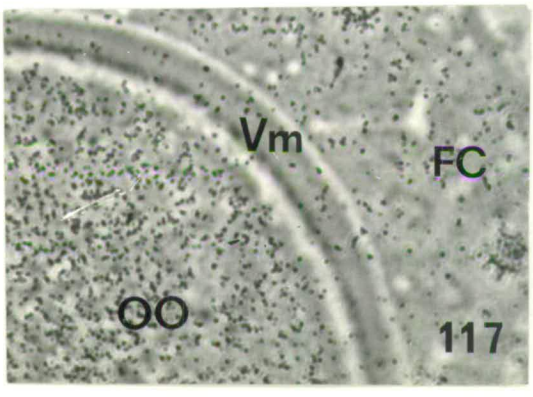
Fig. 116. The nurse-oocyte border from an autoradiographic preparation of a stage 10 ovarian chamber fixed 12 hours after injection of tritiated uridine. Note the high grain density over the ooplasm (OO); by comparison the nurse cell cytoplasm (NC) is not so heavily labelled. (FC) follicle cells; (Vm) vitelline membrane. The autoradiographic exposure was 7 days. x 2,000.

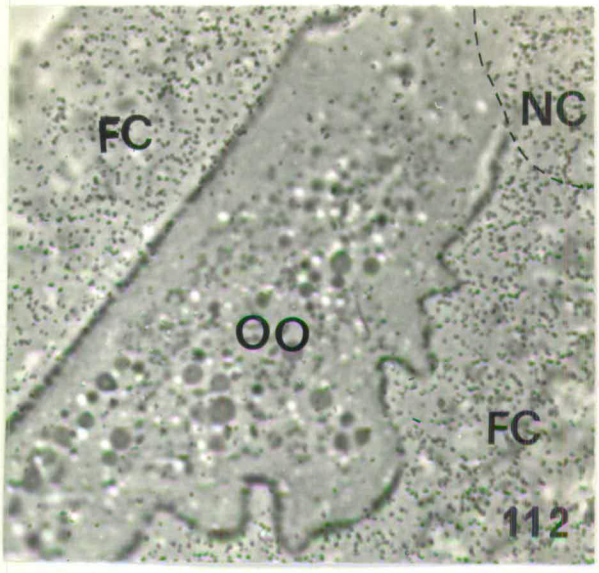
Fig. 117. The follicle-oocyte border from the same ovarian chamber as that shown in Fig. 116. Note the difference in silver grain density between the follicle cell cytoplasm (FC) and the ooplasm (OO). This is in contrast to the difference in grain density between the nurse cells and the ooplasm shown in Fig. 116. (Vm) vitelline membrane. The autoradiographic exposure was 7 days. x 2,000.

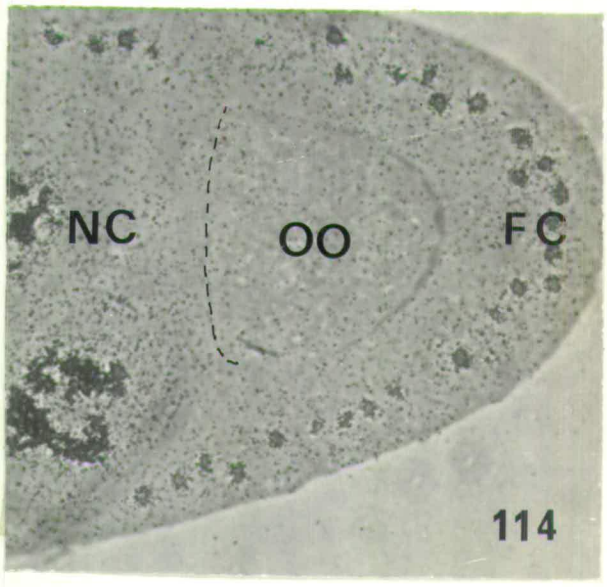

111

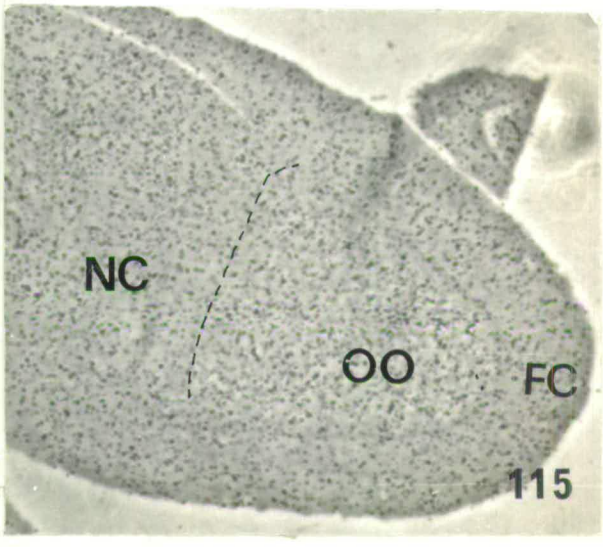

113


NC
Vm
FC
OO
116


Vm
FC
OO
117


FC
NC
OO
FC
112


NC
OO
FC
114


NC
OO
FC
115

of nucleoli in stages 4, 7 and 10 are presented in Figs. 118, 119 and 120 respectively; all these preparations are from ovaries fixed 30 minutes after injection of the tracer and exposed for the same length of time (30 days). From these preparations, it may be noted that there is a considerable increase in the number of grains per unit area (grain density) over the nucleoli in the different stages. As described earlier in the section dealing with the ultrastructural aspects of the developing chambers, there is a noticeable increase in the amount of nucleolar material in the nurse cells during development and the autoradiographs (Figs. 118-120) now show that there is also an increase in the density of grains over these organelles. A large number of autoradiographic preparations of nucleoli from different stages of ovarian chambers were analyzed in a quantitative manner and the data are presented in Text-Fig. 1.

After 30 minutes of <u>in vivo</u> exposure to radioactive uridine, the nurse nucleoli of all stages exhibit the highest grain density as compared with the other regions of the cell. Further, as the chamber develops, the grain density over the nucleolus shows a marked increase (see Text-Fig. 1).

Another observation from the autoradiographic experiments relates to the labelling of the clusters of large particles in the nurse nuclei of stage 12 chambers (Fig. 121). These clusters of large particles (600 A) were described in earlier sections of this thesis and an examination of several preparations has shown that they definitely get labelled after a 2 hour exposure to the tracer <u>in vivo</u>.

Fig. 118. An EM autoradiograph from a stage 4 nurse nucleus. Note the heavy labelling of the nucleolar material (no). The chamber was taken from an ovary fixed 30 minutes after injection of tritiated uridine. The autoradiographic exposure was 30 days. x 8,000.

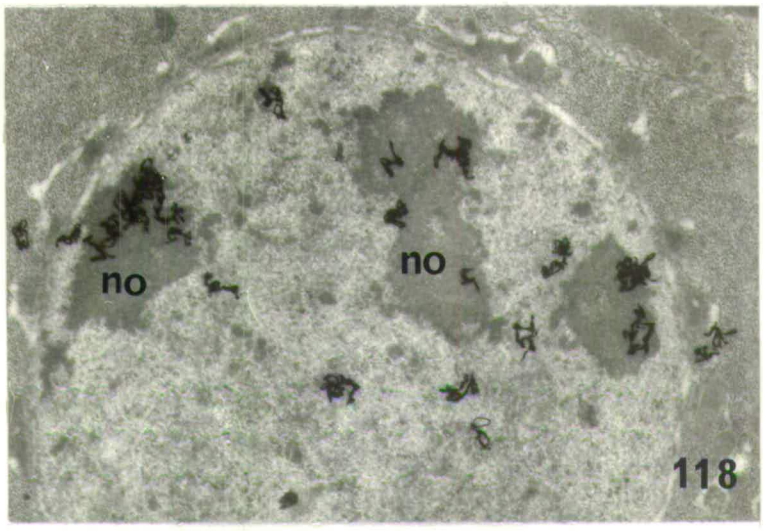
Fig. 119. An EM autoradiographic preparation of part of a stage 7 nurse nucleus. The incubation of the ovary and the autoradiographic exposure are as described in Fig. 118. The grain density over the nucleolus (no) is higher than in the previous figure. x 8,000.

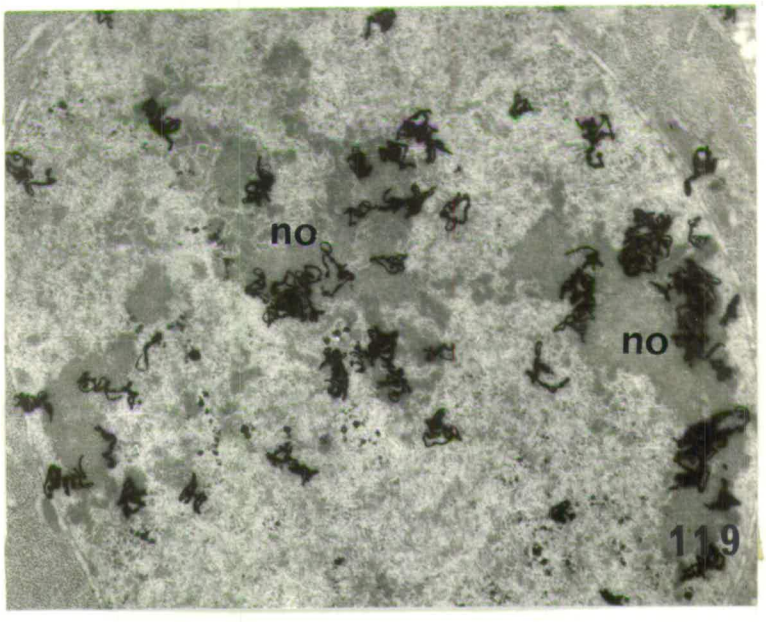
Fig. 120. An EM autoradiographic preparation of part of a posterior nurse nucleus from a stage 10 ovarian chamber. The incubation of the ovary and the autoradiographic exposure are as described in Fig. 118. The grain density over the nucleolus (no) is higher than in the previous two stages. x 3,000.

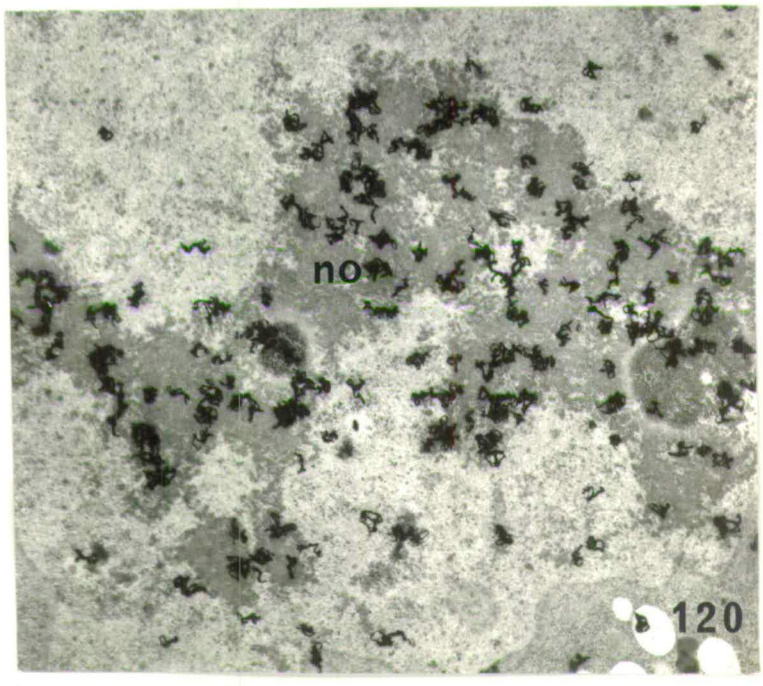
Fig. 121. An EM autoradiograph of part of a nurse nucleus from a stage 12 ovarian chamber. Note the labelling of a cluster of large particles (see arrows). This chamber was fixed 2 hours after injection of tritiated uridine. The autoradiographic exposure was 7 days. x 20,000.

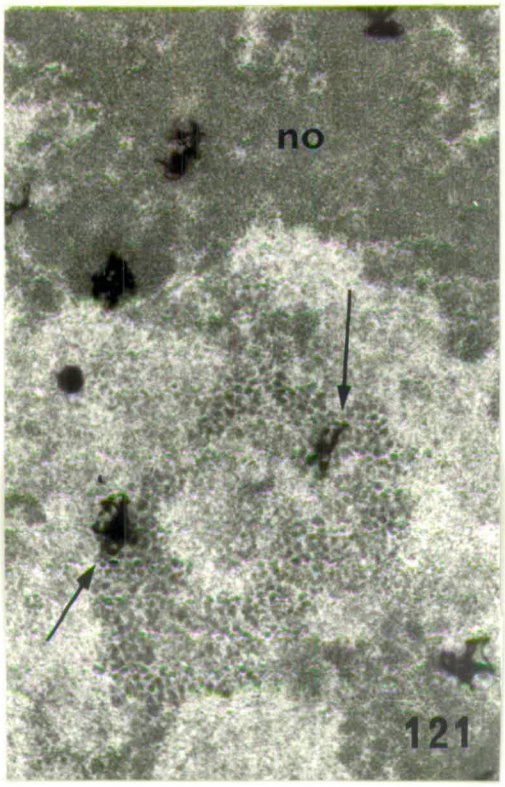
Fig. 122. An EM autoradiographic preparation of the perinuclear cytoplasm of a stage 8 nurse cell. Note the heavy labelling of the amorphous material (Am), near the nuclear envelope (ne). The chamber was taken from an ovary fixed 4 hours after injection of tritiated uridine. The autoradiographic exposure was 7 days. x 12,000.

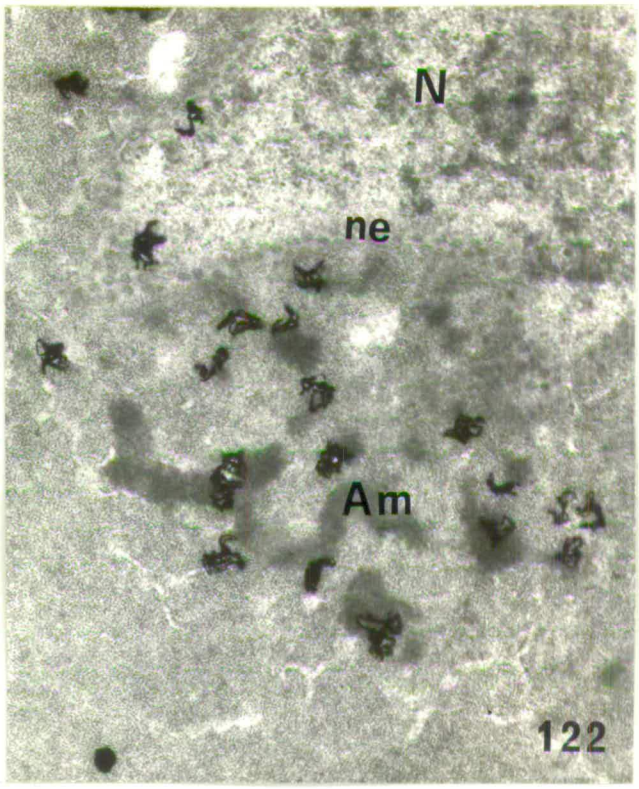
Fig. 123. Another EM autoradiographic preparation showing the perinuclear cytoplasm of a stage 6 nurse cell. Again, note the labelling of the amorphous material (Am) close to the nuclear envelope (ne). The fuzzy bodies (Fb), however, are not labelled. The chamber was taken from an ovary fixed 2 hours after injection of tritiated uridine. The autoradiographic exposure was 30 days. x 8,000.

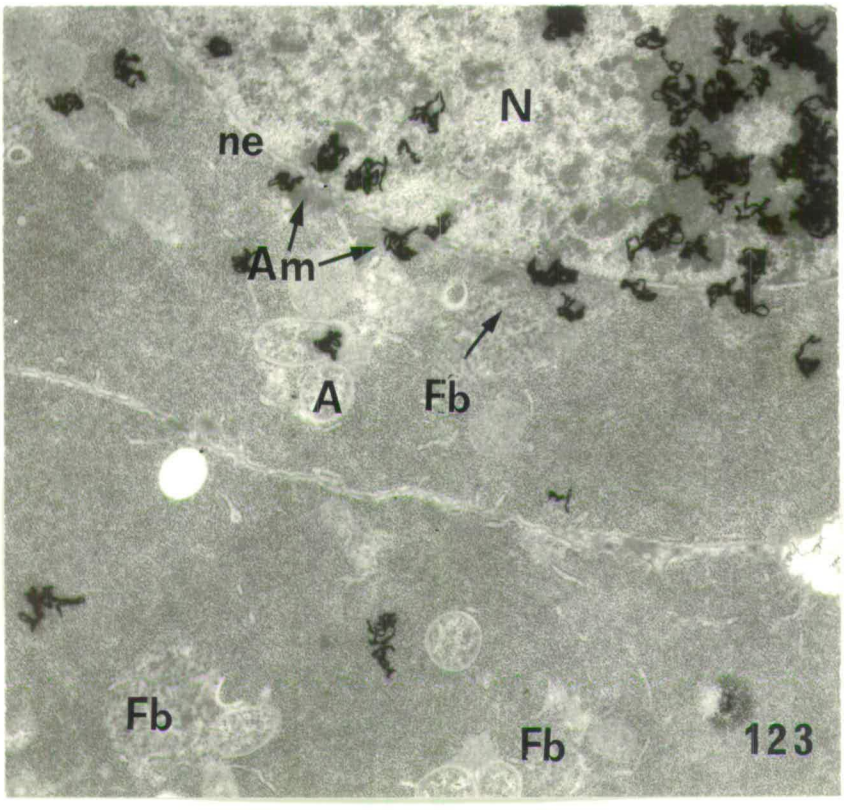

no
no
118


no
no
119


no
120


no
121


N
ne
Am
122


ne
N
Am
A
Fb
Fb
Fb
123

Text-Fig. 1. The grain distribution in nurse cells after 30 minutes of tritiated uridine incorporation in in vivo conditions. (A = total area in μ^2 analyzed; No = number of grains ~~in~~ in the area analyzed; D = grain density, number of grains per μ^2; S-2 to S-10, stages of ovarian development). The autoradiographic exposure was 30 days.

REGION		S-2	S-4	S-5	S-6	S-7	S-8	S-9	S-10
NUCLEOLUS	A	60.2	40.3	15.1	147.8	54.4	117.7	121.3	95.4
	No	21	57	31	235	158	246	462	717
	D	0.34	1.41	2.04	1.58	2.90	2.08	3.80	7.5
NUCLEUS	A	67.1	299.4	81.7	383.5	297.2	260.4	258.7	212.4
	No	9	26	7	59	39	36	66	86
	D	0.13	0.08	0.08	0.15	0.13	0.13	0.25	0.40
CYTOPLASM	A	120.9	198.2	97.1	49.9	230.6	21.2	11.3	83.2
	No	4	8	4	26	27	5	4	38
	D	0.03	0.04	0.04	0.52	0.11	0.23	0.35	0.41

As the data in Text-Fig. 1 show, the label has begun to appear in the cytoplasm of nurse cells 30 minutes after injection of the tracer into the abdomen of flies. The number of grains in the cytoplasm is much higher in preparations made 4 hours after injection of tracer (no data are presented but see later in the section on transport of material from nurse cells to the oocyte). In the preparations made 4 hours after the injection of tracer, it is possible to see that some silver grains are associated with the amorphous masses already described in the perinuclear cytoplasm. A clear illustration of a labelled amorphous mass closely apposed to the nuclear envelope in a stage 8 chamber is shown in Fig. 122. It was mentioned earlier that during further development of the chamber the amorphous masses move away from the nuclear envelope and transform themselves into what was described as fuzzy bodies in the deeper regions of the nurse cell cytoplasm. A search was therefore made for fuzzy bodies in the EM-ARGs of stages 5 to 8 and it was surprising to find no preferential localization of label over these bodies (Fig. 123).

Yet another observation that deserves mention is the finding that the spherical masses or rounded bodies described earlier in stage 12 nurse nuclei are labelled with ^{3}H-uridine. From their morphological features it was assumed earlier that they represent material of nucleolar origin - perhaps remnants of disintegrating nucleoli.

(ii) Transport of material from nurse cells to the ooplasm. The possible movement of label from nurse

cells to ooplasm was examined mainly in LM-ARGs. In one experiment, vitellogenic chambers were incubated at room temperature (22 °C) in medium 199 containing ^{3}H-uridine at a concentration of 100 μCi/ml for 1 and 2 hours. The differences in the intensity of nuclear labelling in stages 4 and 10 are clearly brought out in Figs. 111 and 113. An autoradiograph of a stage 9 chamber cultured *in vivo* for 2 hours is shown in Fig. 114. Another autoradiographic preparation of a stage 9 chamber incubated for 6 hours *in vivo* is presented in Fig. 115. A comparison of these figures (Figs. 114 and 115) shows that after 6 hours exposure to the tracer, there is a significant transfer of label from the cytoplasm of the nurse cells to the oocyte. The grain density over the oocyte after 12 hours exposure to the tracer appears to be greater than that over the nurse cell cytoplasm and follicle cell cytoplasm, see Figs. 116 and 117 respectively. In all experiments mentioned above as well as in experiments conducted *in vitro* (see next paragraph) the oocyte nucleus was found to be practically unlabelled or labelled very slightly.

When ovaries were cultured *in vitro* in radioactive medium containing ^{3}H-uridine at 100 μCi/ml for 2 hours and then chased (as described in the section dealing with material and methods) for a period of 3 hours, there was a negligible amount of label in the ooplasm of stage 10 chambers (Fig. 112). This is in contrast with the heavy labelling observed in the ooplasm of a stage 9 chamber following *in vivo* incubation with tracer (Fig. 115); there was, however, no noticeable difference in the labelling of nurse cell nucleoli

between the experiments performed *in vitro* and those made *in vivo*. These observations suggest that the *in vitro* conditions are probably not quite physiological to enable the transport of labelled RNA from the nurse cells to the oocyte.

(iii) Follicle cells. When ovarian chambers fixed after 30 minutes of *in vivo* exposure to 0.5 μl of ^{3}H-uridine per fly at a tracer concentration of 1 μCi/ μl were examined with EM autoradiography, the highest grain density in the follicle cells was over the nucleolus. A stage-by-stage study has also shown that the grain density over the nucleolus of the follicle cells increases gradually from stage 2 to stage 10 (see Text-Fig. 2). An example of the extent of labelling of two follicle nucleoli from ovarian chambers in stages 5 and 10 exposed 30 minutes to tritiated uridine is given in Figs 125 and 124 respectively.

In an *in vitro* experiment, where the ovaries were cultured for 2 hours in labelled medium contained ^{3}H-uridine at a concentration of 100 μCi/ml and then chased for a period of 3 hours, the columnar follicle cells of stage 10 chamber were well labelled but the ooplasm was practically unlabelled (Fig. 112). The formation of the vitelline membrane begins during stage 9, but it does not become an uninterrupted or continuous layer until the end of stage 10. It is therefore unlikely that this layer would form a barrier for transport of labelled RNA into the ooplasm, if any RNA were released from the follicle cells. At the ultrastructural level, no physical continuity (similar to the ring canals that

Text-Fig. 2. The grain distribution in follicle cells after 30 minutes of tritiated uridine incorporation in *in vivo* conditions. The data are expressed as in Text-Fig. 1.

REGION		S-2	S-4	S-5	S-6	S-7	S-8	S-9	S-10
NUCLEOLUS	A	8.9	9.7	7.1	23.4	8.3	9.0	21.8	40.5
	No	7	26	22	108	50	52	124	255
	D	0.78	2.66	3.08	4.59	5.98	5.73	5.68	6.29
NUCLEUS	A	49.9	20.4	16.0	86.8	78.7	13.5	128.8	262.9
	No	7	9	11	57	10	6	48	91
	D	0.14	0.43	0.68	0.65	0.12	0.44	0.32	0.34
CYTOPLASM	A	135.1	100.0	152.2	268.8	305.4	97.0	224.4	290.0
	No	6	10	6	26	16	7	19	24
	D	0.04	0.09	0.03	0.19	0.05	0.07	0.08	0.08

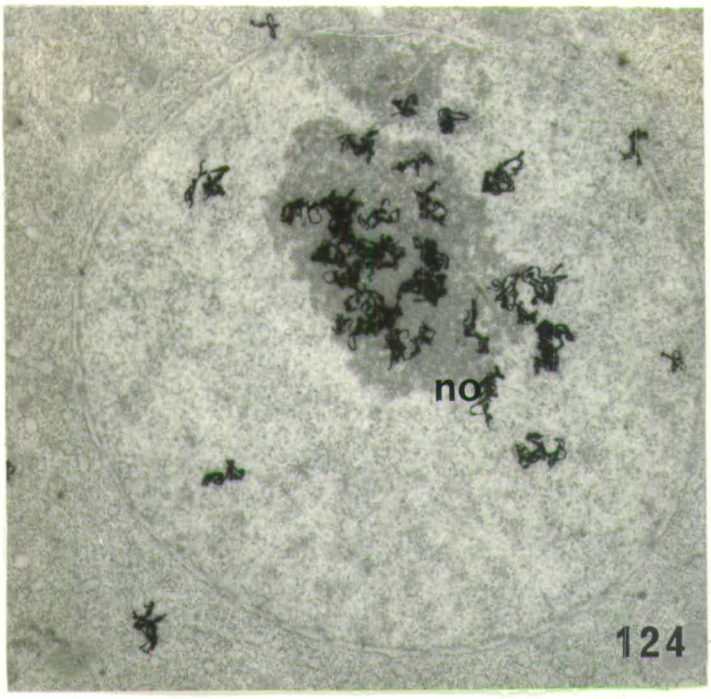
Fig. 124. An EM autoradiographic preparation of a columnar follicle cell nucleus in a stage 10 chamber. The ovarian chamber was fixed 30 minutes after the injection of tritiated uridine. Note the heavy labelling of the nucleolus (no). The autoradiographic exposure was 30 days. x 8,000.

Fig. 125. An EM autoradiographic preparation of a follicle cell nucleus in a stage 5 chamber. The ovarian chamber was fixed 30 minutes after injection of tritiated uridine. The EM autoradiographic exposure was 30 days. x 8,000.

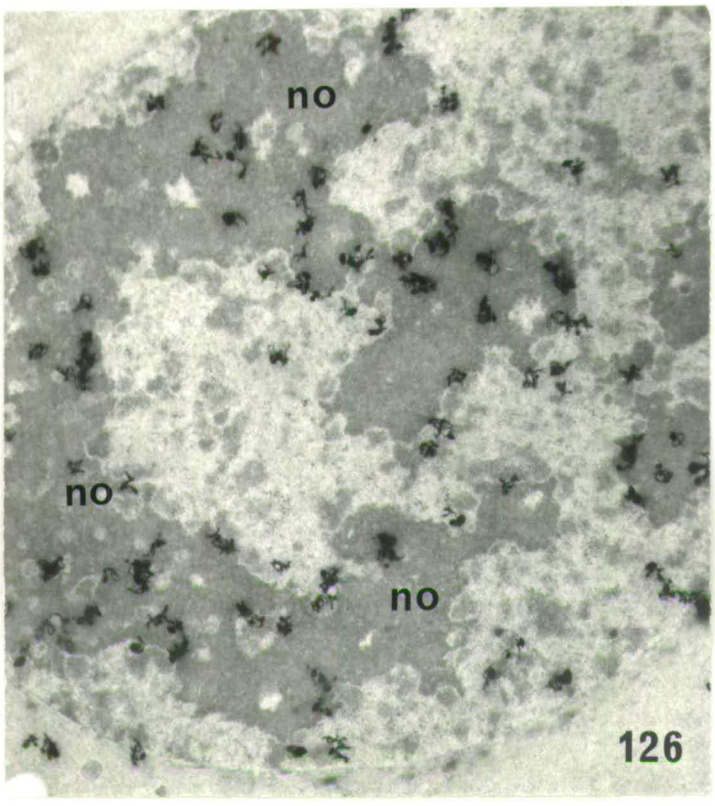
Fig. 126. An EM autoradiographic preparation of a posterior nurse nucleus in a stage 7 chamber. The chamber was fixed 1 hour after injection of tritiated lysine. Note that practically all the grains are over the nucleolar material (no). The autoradiographic exposure was 7 days. x 5,000.

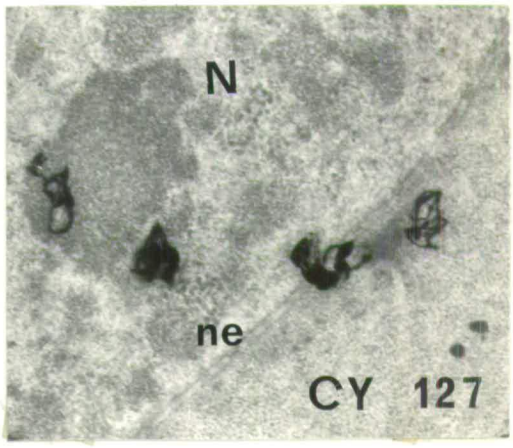
Fig. 127. The amorphous mass situated in the perinuclear cytoplasm of a nurse cell in a stage 7 ovarian chamber. The autoradiographic preparation was made from an ovary fixed 1 hour after the injection of tritiated lysine. (CY) cytoplasm; (ne) nuclear envelope. The autoradiographic exposure was 7 days. x 18,000.

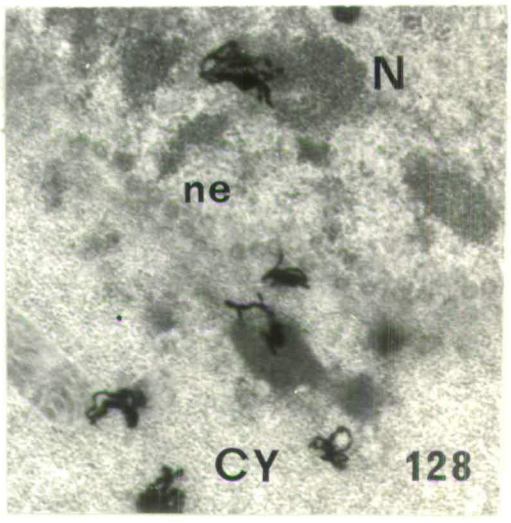
Fig. 128. The amorphous material in the perinuclear cytoplasm of a nurse cell in a stage 4 ovarian chamber. The incubation conditions and the autoradiographic exposure are the same as in Fig. 127. x 18,000.


no
124


no
125


no
no
no
126


N
ne
CY 127


N
ne
CY 128

occur between the nurse cells and the oocyte) has been noted between the follicle cells and the oocyte. In stage 10 chambers fixed 12 hours after injection of tracer into the flies, there is no observable gradient in the follicle cells indicative of a movement of RNA to the oocyte border (Fig. 117); the ooplasm, however, is heavily labelled due to movement of labelled RNA from the nurse cells (Fig. 116).

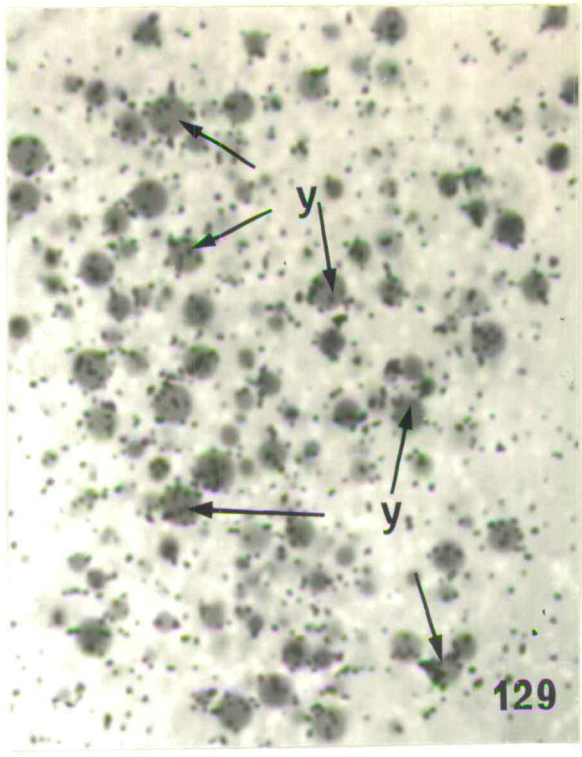
(b) Experiments with lysine. (i) Nurse cells. An EM autoradiograph of a nurse cell nucleus of an ovarian chamber in stage 7, following *in vivo* incubation in tritium labelled lysine, is shown in Fig. 126. In this experiment, the tracer was available to the ovary for 1 hour and it may be seen that practically all the grains overlie the nucleolus. The nurse cell cytoplasm was fairly well labelled, and in certain preparations the amorphous masses lying immediately adjacent to the nuclear envelope in the perinuclear cytoplasm were also labelled (Figs. 127 and 128).

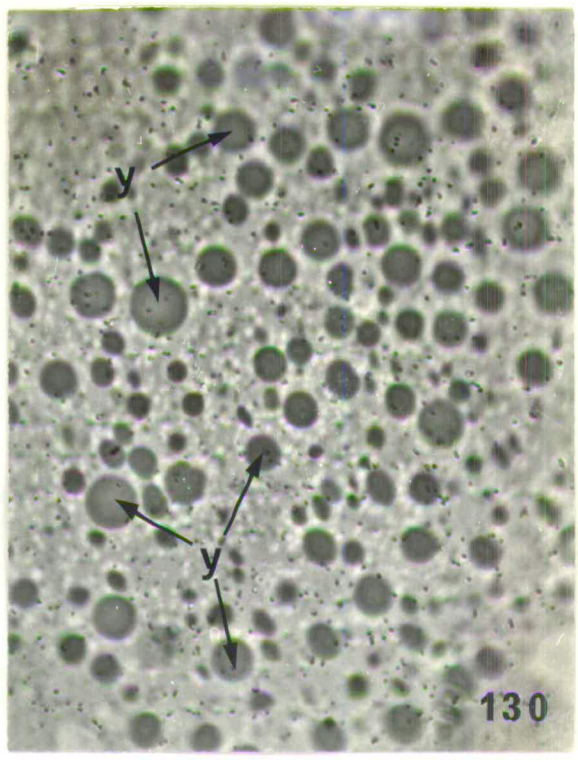
(ii) Follicle cells. In experiments with ^{3}H-lysine, both the nucleus and the cytoplasm of of follicle cells were found to be labelled, but the more important observation in these cells was made in their cytoplasmic region facing the oocyte. In chamber stages 9 or 10 that were fixed after exposure to the tracer for 1 hour *in vivo* or *in vitro*, it was frequently found that there was a predominant localization of label over the vitelline membrane (Figs. 131 and 132). At this time interval, the ooplasm was also labelled but to a much less extent than the follicle cell cytoplasm;

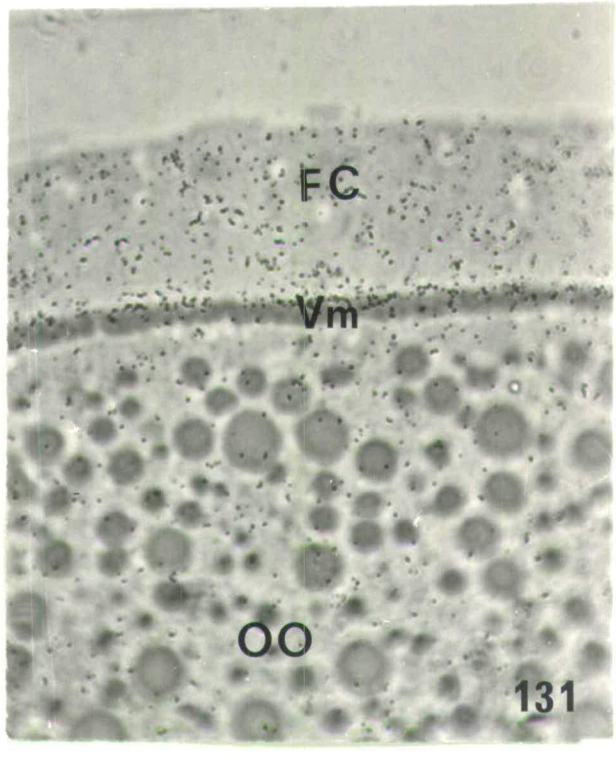
Fig. 129. Light microscope autoradiograph of the ooplasm from a stage 10 ovarian chamber fixed 24 hours injection of tritiated lysine. Note the heavy labelling over the yolk platelets. The autoradiographic exposure was 7 days. x 1,500.

Fig. 130. Light microscope autoradiograph of the ooplasm from a stage 10 ovarian chamber fixed 1 hour after injection of tritiated lysine. The label appears evenly distributed throughout the ooplasm. The autoradiographic exposure was 7 days. x 1,500.

Fig. 131. The follicle-oocyte border from the same ovarian chamber shown in Fig. 130. Note the heavy localization of grains along the vitelline membrane (Vm). The grain density over the follicle cell cytoplasm is higher than that of the ooplasm. The autoradiographic exposure was 7 days. x 1,500.


y
y
129


y
y
130


FC
Vm
OO
131

more will be said of this labelling later. In EM-ARGs of follicle cells, it was also possible to see labelled Golgi regions in several preparations (Fig. 133).

Ovarian chambers in stage 12 that were fixed 1 hour after exposure to the tracer, showed essentially the same pattern of labelling as that described above. The localization of grains over the Golgi apparatus is clearly seen in Fig. 134, and although there are a few grains overlying the vitelline membrane it is obvious that in this preparation the endochorion lying adjacent to it is better labelled. This is even more clearly demonstrated in Figs. 135 and 136. The high resolution preparation shown in Fig. 136 represents a later stage in the formation of the endochorion than that shown in Fig. 135.

The pattern of incorporation of tritium labelled lysine into the oocyte is described later (see pages 138-140).

5. Cytochemical experiments.

Techniques of ultrastructural cytochemistry were employed in this study with a view to characterizing the material that appeared to be transported from the nurse cells to the ooplasm. Ultrastructural evidence suggesting passage of material from the nucleus to the cytoplasm of nurse cells in ovarian chambers up to about stage 7 was described earlier.

After treatment of sections of nurse cells with 0.2 % RNase for 3 hours at 37 °C, there was a slight increase in the electron density of the amorphous masses situated in the perinuclear cytoplasm (Fig. 137).

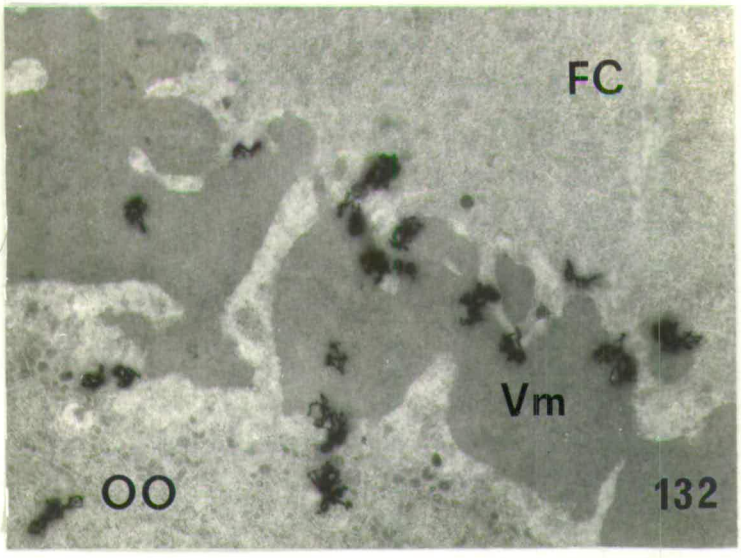
Fig. 132. The follicle-oocyte border from an autoradiographic preparation of a stage 10 ovarian chamber fixed 1 hour after injection of tritiated lysine. The silver grains are clearly localized over the vitelline membrane (Vm). The autoradiographic exposure was 7 days. x 8,000.

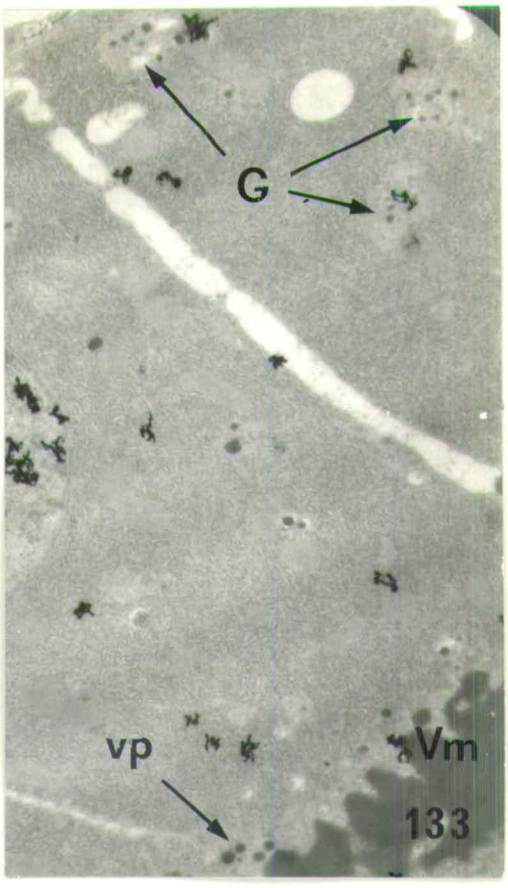
Fig. 133. Autoradiographic preparation of part of the follicle cell cytoplasm from a chamber taken from an ovary fixed 1 hour after the injection of tritiated lysine. The label appears over the endoplasmic reticulum and the Golgi apparatus (G). The autoradiographic exposure was 7 days. x 7,500.

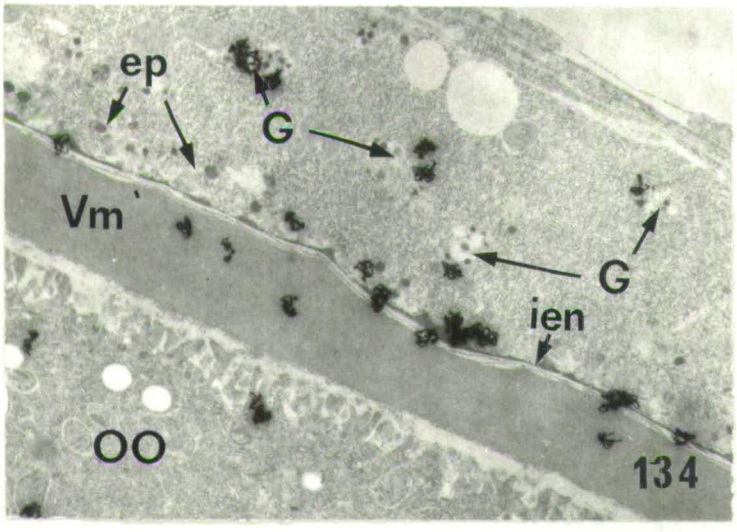
Fig. 134. The follicle-oocyte border from an autoradiographic preparation of a stage 12 ovarian chamber fixed 1 hour after injection of tritiated lysine. Note that the inner endochorion is clearly labelled. The Golgi apparatus (G) in the follicle cell cytoplasm are also labelled. The autoradiographic exposure was 7 days. x 5,000.

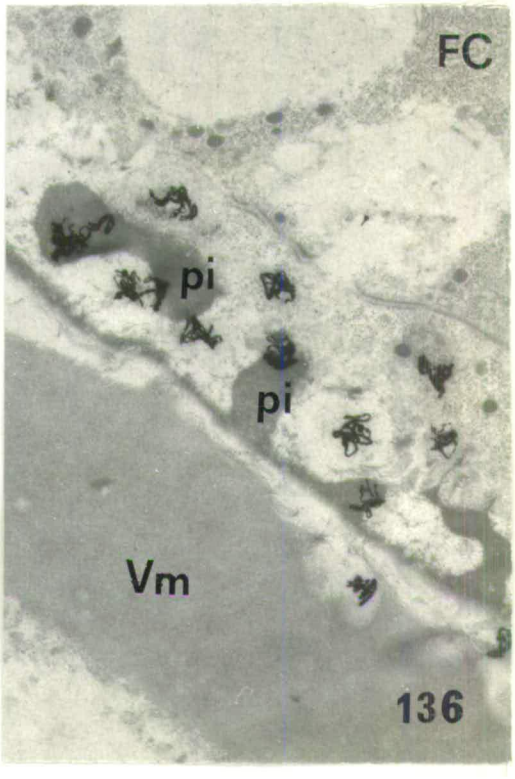
Fig. 135. Autoradiographic preparation of a stage 12 ovarian chamber fixed 1 hour after the injection of tritiated lysine. Note the labelling of the endochorionic pillars (pi) . The autoradiographic exposure was 7 days. x 8,000.

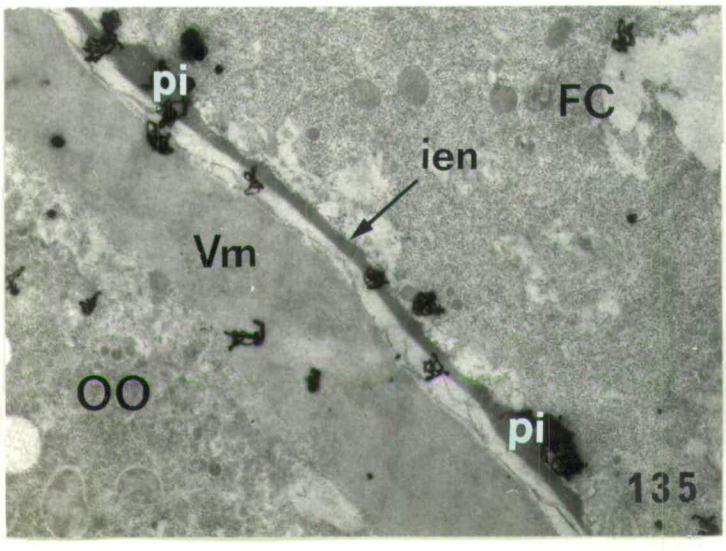
Fig. 136. A late stage in the formation of the endochorionic pillars in a stage 13 ovarian chamber, fixed 1 hour after injection of tritiated lysine. The autoradiographic exposure was 7 days. x 12,000.


FC
Vm
OO
132


G
vp
Vm
133


ep
G
Vm
G
ien
OO
134


FC
pi
pi
Vm
136


pi
FC
ien
Vm
OO
pi
135

The ribosomes were completely removed in the enzyme treated preparations (Fig. 137) while it may also be noted that the chromatin material in the nucleus has increased in density. After treatment with 0.5 % RNase for 1 or 3 hours the chromosomal material became extremely dense and there was also the suggestion that the amorphous material in question too had increased density over that in the controls.

When sections were processed for differential staining of nucleic acids (Bernhard, 1969), the chromatin areas appeared bleached as expected (Figs. 140 and 52). There is also a striking similarity between the chromatin scattered in the nucleus (Fig. 140) and the amorphous material lying just outside the nucleus (Fig. 139) in that both have a sort of variegated appearance. The chromatin material is, however, generally bleached but the amorphous masses are not bleached in their entirety; instead, they show very small bleached areas within them. The suggestion is that the small bleached areas in the amorphous masses may be indicative of chromatin-like material in them.

After treatment of sections with pronase (0.1 % for 1 hour) it was difficult to visualize any cytoplasmic constituents with certainty except perhaps the mitochondria; the masses of amorphous material in question could not be identified in such preparations. It would then seem that the pronase treatment had been too severe.

It was explained earlier how the ultra-morphology of the amorphous masses changes as they move away from the nucleus. These masses appeared as

fuzzy bodies in the deeper regions of the nurse cytoplasm. After treatment with RNase (0.2 % for 3 hours) the increase in density of the fuzzy bodies, compared with those in the control preparations (Figs. 137 and 138) was even more obvious than in the case of the amorphous bodies which are assumed to be the forerunners of the fuzzy bodies. It was, however, difficult to say if there had been extraction of any material from the fuzzy bodies by the enzyme because of the presence normally of lacunae-like areas in these bodies.

One of the most remarkable observations in this series of cytochemical experiments relates to the reaction produced in the fuzzy bodies by the selective staining procedure of Bernhard (1969). Fig. 142 shows the bleached appearance of these bodies. The implication of this observation is that the fuzzy bodies contain DNA and that this becomes more accessible for reaction with EDTA as the amorphous masses become transformed into the fuzzy bodies. In late stages, when the fuzzy bodies reach the ooplasm, they continue to show a similar reaction.

The reaction of the spherical masses or rounded bodies observable in the nurse nuclei of stage 12 and later to the selective staining procedure of Bernhard (1969) indicates that these bodies contain RNA material (Fig. 141).

Figs. 137-142. Ovarian chambers of Drosophila fixed in glutaraldehyde and embedded in glycol methacrylate.

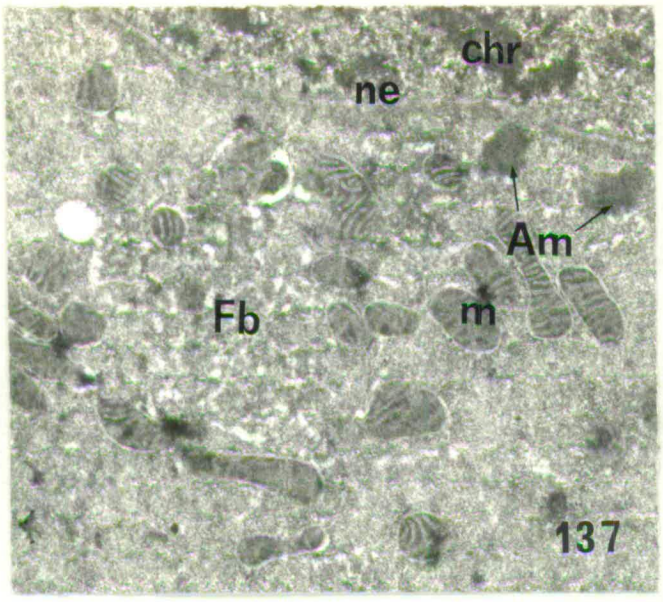
Fig. 137. Part of the perinuclear cytoplasm from a a stage 6 nurse cell. The ultrathin section was treated with 0.2 % RNase; pH 6.6, at 37 °C for 3 hours. The ribosomes are completely extracted. Note that the amorphous material (Am) is not extracted but exhibits an electron density similar to that of the chromatin material (chr); the chromatin has certainly increased in density compared with the control (Fig. 138). The fuzzy bodies (Fb) in tracts exhibit a higher density than the rest of the cytoplasm. (ne) nuclear envelope. x 12,000.

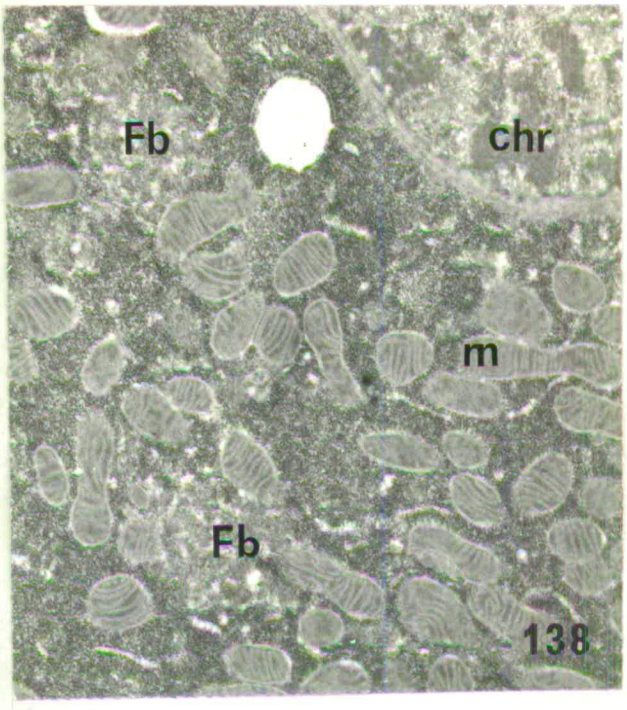
Fig. 138. Control for Fig. 137 to show the perinuclear cytoplasm from the same ovarian chamber as in Fig. 137. The ultrathin section was incubated in distilled water for 1 hour. x 12,000.

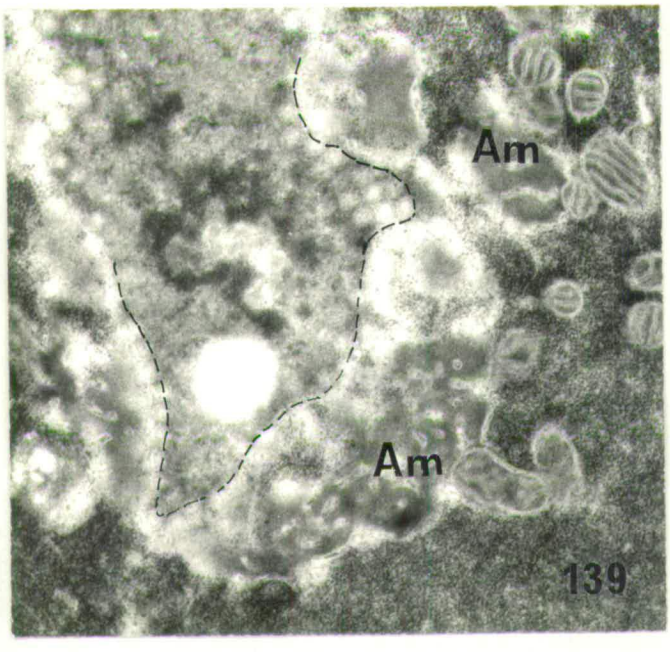
Fig. 139. A tangential section of a stage 4 nurse nucleus to show the region of the perinuclear cytoplasm. The section was stained with uranyl acetate, treated with 0.02 M EDTA for 1 minute and counterstained with lead citrate. The amorphous material (Am) in this case has only a variegated appearance. Dotted line indicates the nuclear envelope. x 20,000.

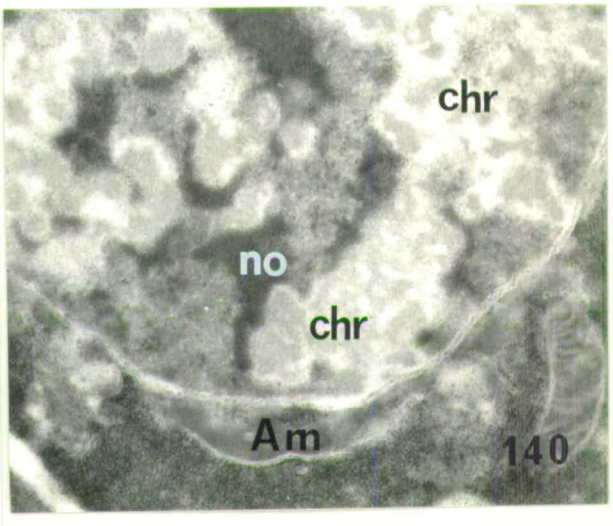
Fig. 140. Part of a nurse cell showing a similar field to Fig. 139. Note the appearance of bleached areas corresponding to the chromatin material (chr); the amorphous material (Am) is only slightly affected after EDTA treatment. x 20,000.

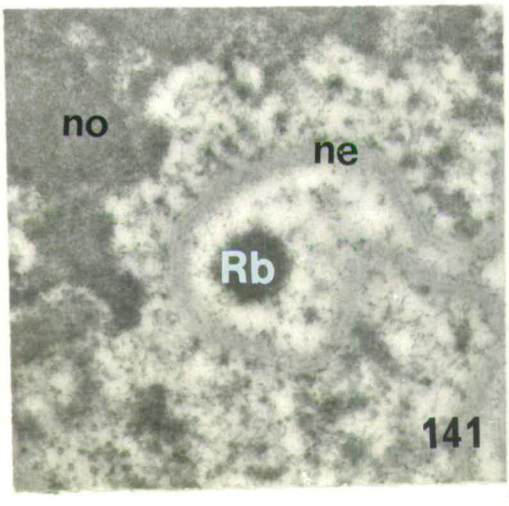
Fig. 141. Part of a nurse nucleus from a stage 12 ovarian chamber treated as in Fig. 139. The rounded body seen in the perinuclear cytoplasm is unaffected by the treatment with 0.02 M EDTA. (ne) nuclear envelope. x 20,000.

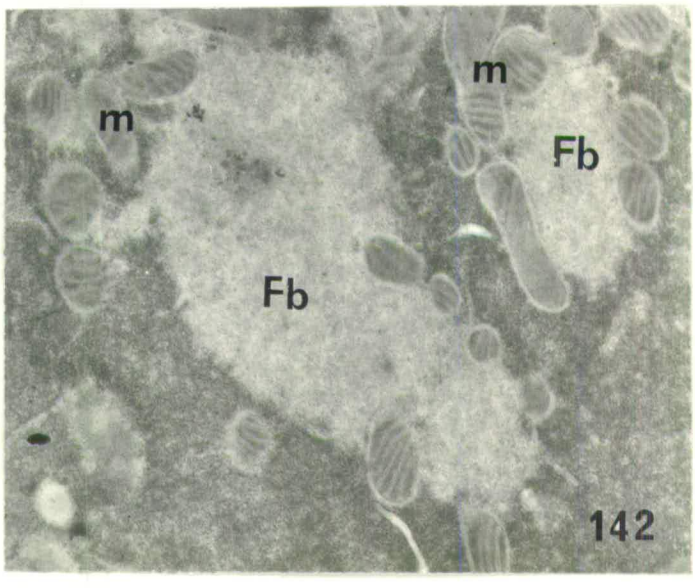

chr
ne
Am
Fb
m
137


Fb
chr
m
Fb
138


Am
Am
139


chr
no
chr
Am
140


no
ne
Rb
141


m
m
Fb
Fb
142

V. OBSERVATIONS

Part II - Mode of yolk formation

This section deals with various experimental approaches that were designed to clarify some of the questions arising from the morphological study. For instance, one may ask whether a type of ovary such as that of Drosophila viz. polytrophic, is capable of endogenous synthesis of material which then accumulate in mature yolk platelets, or whether there is in fact only uptake of proteins synthesized in non-ovarian sites. As will be discussed later there are two schools of thought on the question of the origin of yolk material in Drosophila and the following account aims to clarify the question. In the preceding section it was shown that the ultrastructure of both developing and mature yolk platelets is a rather complex one and it is not inconceivable that material from several sources contribute to the formation of mature yolk platelets. Several experiments will now be described, all of which aim to throw some light on the mode of yolk formation and on the material that come to form the yolk platelets.

1. Possible routes of entry of yolk precursors as studied by the uptake of electron dense extracellular tracers.

(a) Experiments with Lanthanum. Lanthanum is a polyvalent cation which is known to bind to the cell surface and form a deposit detectable with the electron microscope. Lesseps (1967) pointed out that the ionic form of Lanthanum may combine with lipids, proteins and polysaccharides and that no specificity

may be claimed for its staining reaction. In spite of this, however, lanthanum has been widely used as an electron dense tracer to detect intercellular spaces in many animal tissues. This substance has been used in the present study to examine if an actual continuity exists between the oocyte surface and the external medium (haemocoel). In other words, the aim has been to find the route followed by a molecule in order to arrive at the oocyte surface.

When ovarian chambers were fixed in glutaraldehyde containing lanthanum nitrate for a period of 2 hours, the results obtained were as follows. As shown in Fig. 143, which is a low power micrograph of the columnar follicle cell layer in a stage 8 chamber, the intercellular spaces appear filled with lanthanum. As may be seen in higher power micrographs (Figs. 144-145), the intercellular spaces are more or less uniformly dense except in the region where more than two cells meet; in the latter region there is a larger accumulation of lanthanum. Here and there in the intercellular regions, larger spaces sometimes appear (see Fig. 143) which also contain the dense tracer. Deposits of lanthanum are also observable at the follicle-oocyte border of ovarian chambers in stages 8 and 10 (Figs. 144 and 146). This observation may be taken to indicate that, at least until the vitelline membrane is formed as a complete layer around the oocyte, there is continuity between the interspaces of the follicle cells and the surface of the oocyte. The experiments with lanthanum would thus seem to indicate that there is free access from the haemocoel to the oocyte surface

Fig. 142. Part of nurse cell cytoplasm from a stage 6 ovarian chamber treated as in Fig. 139. Note the bleached areas which correspond to the fuzzy bodies (Fb). x 16,000.

Fig. 143. A low power electron micrograph of the follicle layer from a stage 8 ovarian chamber fixed according to the lanthanum nitrate method. Note the dense lanthanum deposits in the follicle interspaces (see arrows). Also see the larger spaces (double arrows) in the intercellular regions. x 5,000.

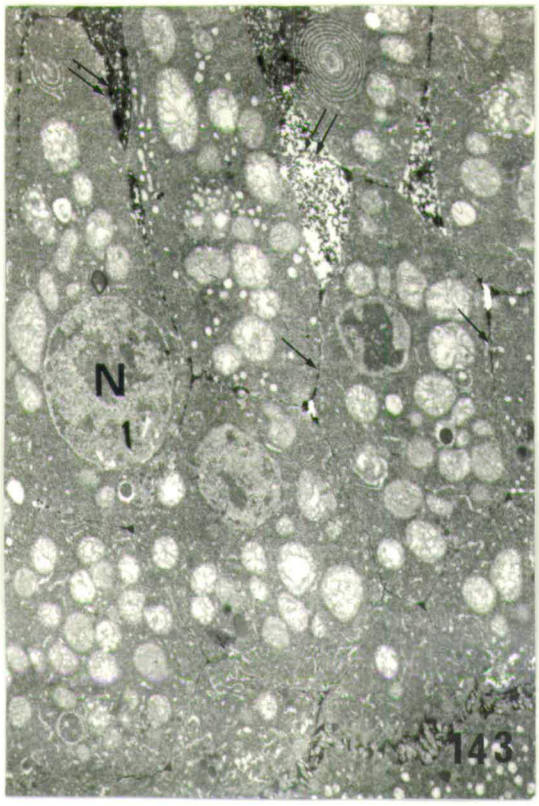
Fig. 144. Enlargement of part of the area shown in in Fig. 143. Note the heavy deposits of lanthanum at the follicle-oocyte border(arrows). (FC) follicle cell; (OO) ooplasm. x 17,000.

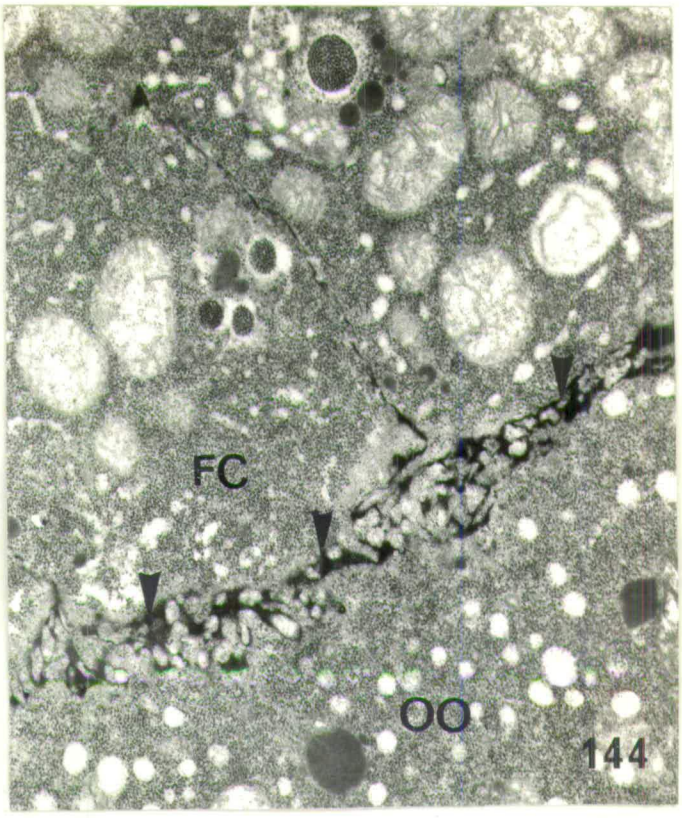
Fig. 145. The follicle layer from a stage 9 ovarian chamber fixed according to the lanthanum nitrate method. Note the presence of heavy deposits of lanthanum in the follicle interspaces (arrows). Deposits are also visible on the basement lamina (bl). x 10,000.

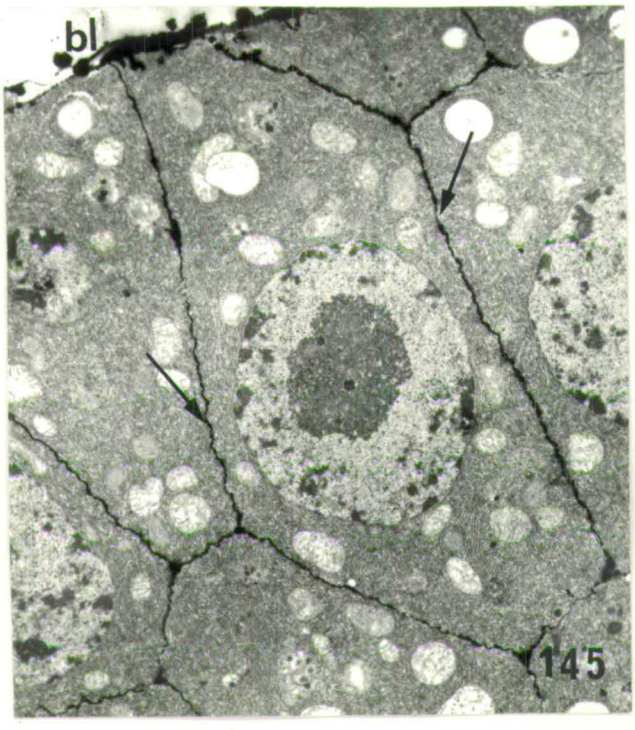
Fig. 146. The follicle-oocyte border from a stage 10 ovarian chamber fixed according to the lanthanum nitrate method. Large spaces (see double arrows) are present between adjacent vitelline bodies. Note the heavy deposits of lanthanum in the follicle interspaces (arrows) and the highly convoluted oolemma (ool). x 10,000.

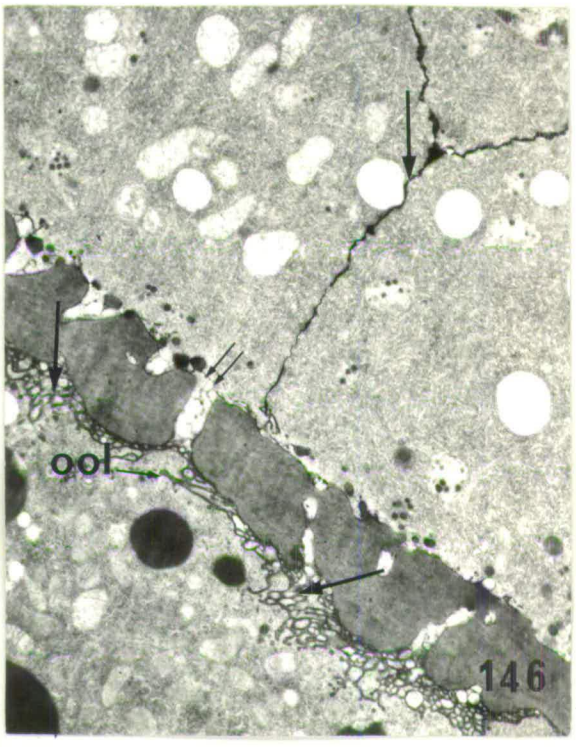
Fig. 147. The follicle layer from a stage 9 ovarian chamber. Ruthenium red method was used. Note that in the interspaces of the follicle layer there are large deposits of ruthenium red (see arrows). x 5,000.

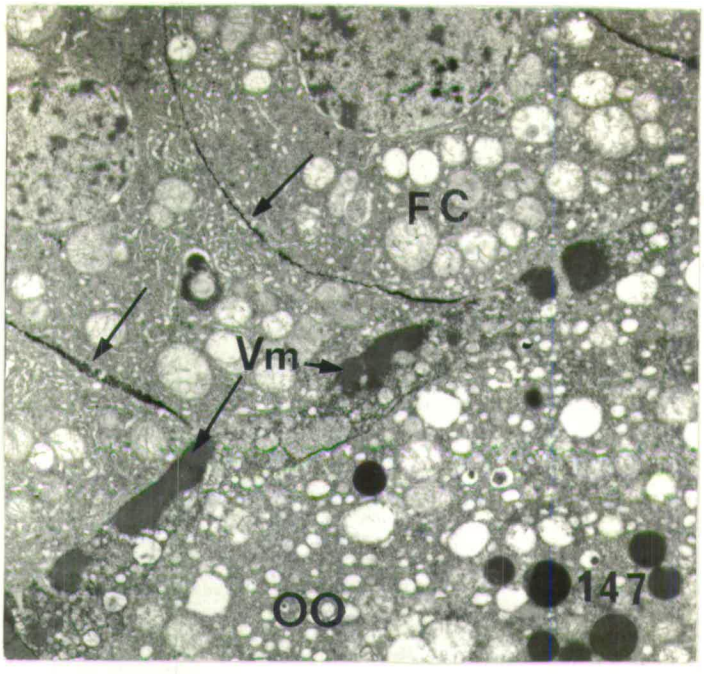
Fig. 148. The follicle-oocyte border from a stage 10 ovarian chamber fixed according to the ruthenium red method. The oolemma (ool) which is thrown into folds is heavily stained with ruthenium red. x 8,000.

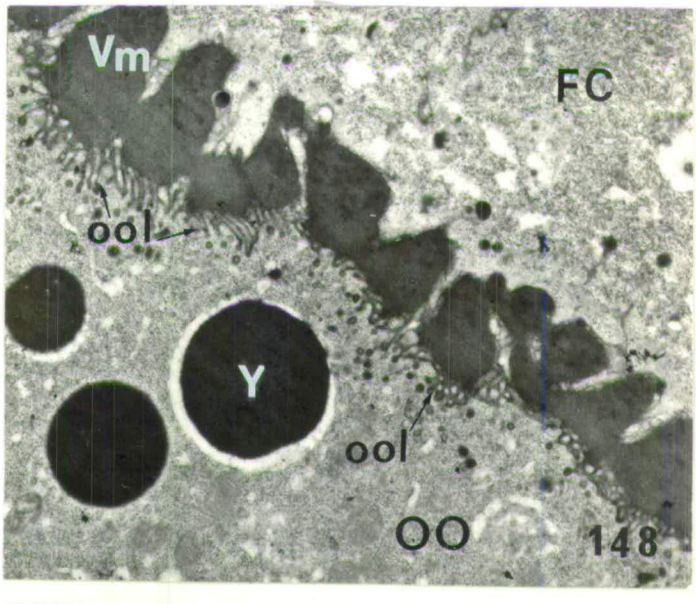

N
1
143

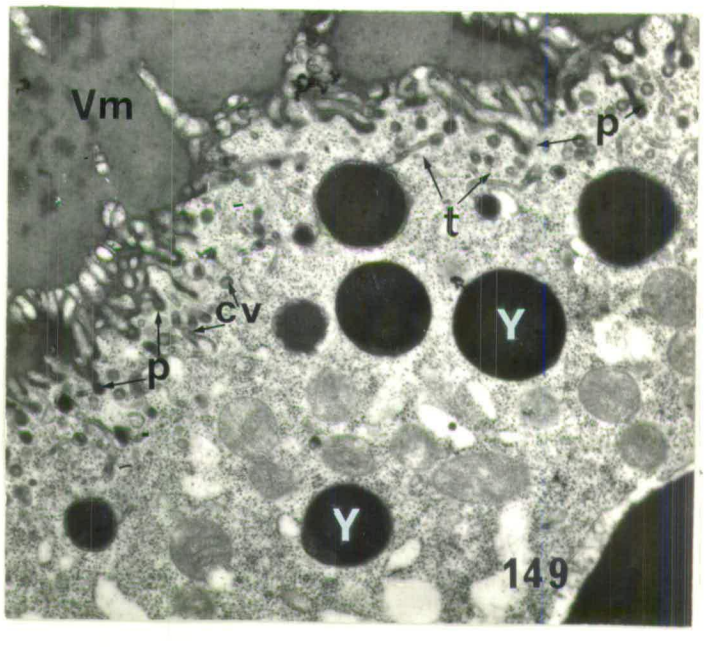

FC
OO
144


bl
145


ool
146


FC
Vm
OO
147


Vm
FC
ool
Y
ool
OO
148


Vm
p
t
cv
Y
p
Y
149

for any molecule of the size of lanthanum. The molecular weight of lanthanum is 433 and its size is about 60 Å.

Indications are also that the external basement lamina does not present a barrier for molecules of this dimension. From the experience of other workers (Martinez-Palomo et al., 1973) it would appear that lanthanum binding is not due to a physical trapping but to a chemical affinity for certain cell surface components. The limitation of this experiment, however, lies in the fact that it does not provide any information on the chemical nature of the surface component to which lanthanum binds. In addition, since lanthanum was used on fixed material, the observed continuity simply indicates the situation in a fixed tissue and no information of a dynamic nature can be gained.

(b) Experiments with Ruthenium red.

Ruthenium red is an inorganic coloured compound which shows up very faintly in electron micrographs after conventional staining. It can, however, be made more electron dense if it is used together with osmium tetroxide. Luft (1971a,b) claimed that the deposits which appear in intercellular spaces of tissues treated with osmium-ruthenium red complex are probably due to acid mucopolysaccharides. Although the exact mechanism of ruthenium red reaction is not known, several accounts have been published in the last few years on the use of such a complex to "stain" the cell coat in various cell systems.

After a period of 2 hours in osmium-

ruthenium red mixture, the ovarian chambers of Drosophila are "stained" in a manner very much the same as that obtained with lanthanum. The deposits of Ruthenium red in the intercellular spaces of the follicle layer are shown in Fig. 147. The deposits on the oocyte surface are shown in Fig. 148. In this micrograph the vitelline membrane formation has progressed to a considerable extent but it is still incomplete and the ruthenium red deposits are present in the microvilli and pits of the oolemma. These structures are better seen in the higher power micrograph shown in Fig. 149. As mentioned above, many of the pits of the oolemma are deeply stained but the pinocytotic vesicles (the coated vesicles described in an earlier section, page 68) and the tubules present in the cortical ooplasm do not appear to contain ruthenium red deposits.

From this experiment, it would seem that the intercellular spaces between the follicle cells form the route for substances to reach the oocyte surface from the haemolymph. The results obtained with ruthenium red also must be interpreted cautiously since the experiment was carried out during the fixation of the tissue. In view of the specificity claimed for ruthenium red to bind polysaccharides, the possibility arises that the material lining the intercellular spaces as well as the follicle-oocyte border includes polysaccharides. Even so, however, it cannot be said whether the polysaccharides form a surface coat over the plasma membranes facing the intercellular spaces or whether they represent some glycoproteins in transit.

Because both lanthanum and ruthenium red find free access to the oocyte surface, the cell junctions (zonulae adherens) of the follicle layer do not seem to constitute a barrier for the penetration of these tracers. In mammalian cardiac cells, this type of junction has been shown (Martinez-Palomo *et al.*, 1973) to be permeable to lanthanum.

(c) Experiments with Thorotrast.

Thorotrast (colloidal thorium dioxide) is a positively charged substance of great electron scattering power. There are several ways of using it; for example, it may be used for staining ultrathin sections of material embedded in butyl methacrylate (Revel, 1964). This author demonstrated that the staining reaction could be abolished by hyaluronidase, and hence put forward the view that the thorotrast staining is due to an acid mucopolysaccharide. Behnke (1968) demonstrated the possibility of using thorotrast as specific staining for mucopolysaccharides after glutaraldehyde fixation, but before post-fixing the tissue in osmium tetroxide. More recently , Brunk (1973) used colloidal thorium dioxide as a tracer in *in vivo* conditions and showed that it is taken up preferentially into residual bodies. Unlike lanthanum nitrate and ruthenium red, thorotrast is a colloid of large dimensions measuring up to 80 A in diameter and I have therefore used thorotrast on ovarian chambers of *Drosophila* to test if the external basement lamina permits the passage of this colloid. With this end in view, two sets of experiments were carried out: in one, thorotrast was used on glutaraldehyde-fixed

material as in the case of other tracers mentioned earlier, and in the second experiment, it was injected into the haemocoel of 2-3 day old female flies.

Ovarian chambers of any stage fixed in glutaraldehyde exhibit electron dense deposits of thorotrast along the outer margin of the basement lamina (Figs. 150 and 152). In no case was any deposit of thorotrast ever observed within the chamber (i.e., the interspaces of the follicle cells or the follicle-oocyte border). Even in chambers that were fixed several hours after the injection of thorotrast, there was no indication of thorotrast in any location within the ovarian chamber. Compared with the *in vitro* experiments, the amount of thorotrast deposited along the external side of the basement lamina after the *in vivo* experiments was found to be very little (compare Figs. 152 and 150).
It was, however, interesting to note that in the *in vivo* experiments, thorotrast was found in certain cells located at the base of the ovary. These are the follicle cells which are known to become detached from mature eggs and accumulate at the base of the ovary when the egg is laid down. Consequently these cells are not covered by the basement lamina and the results have to be interpreted in the light of these facts. The thorotrast which has entered these cells appears to be localized in what are referred to as residual bodies in the literature (Fig. 153). The heavy localization of thorotrast along the surface of these cells after 1 hour of *in vivo* exposure to thorotrast is shown in Fig. 151. The fact that only these cells which are detached from the ovarian chambers show thorotrast

Fig. 149. A high power micrograph of the follicle-oocyte border similar to that shown in Fig. 148. Note that although the pits of the oolemma are stained, the coated vesicles and tubules in the cortical ooplasm are not. (Vm) vitelline membrane; (y) yolk platelets. x 15,000.

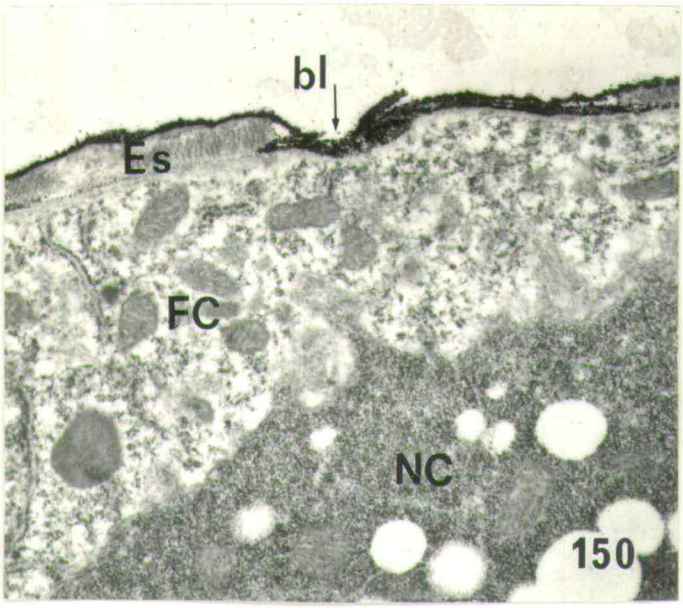
Fig. 150. Part of the anterior region of a stage 10 ovarian chamber fixed after in vitro incubation with colloidal thorium dioxide. Colloidal particles adhere to the surface of the epithelial sheath (Es) and the basement lamina (bl). (FC) follicle cell; (NC) nurse cell; x 12,000.

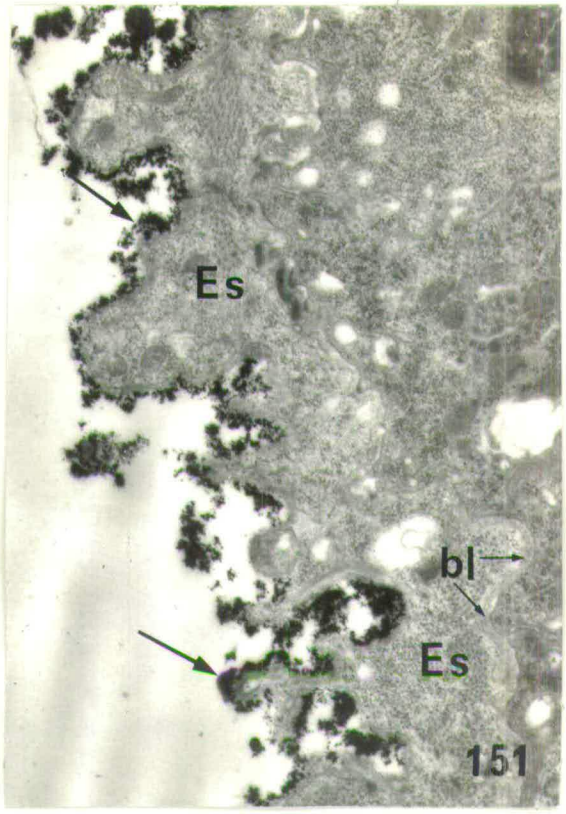
Fig. 151. Follicle cells at the base of an ovary fixed according to the colloidal thorium dioxide method. Note the highly convoluted surface of the follicle cell underneath the epithelial sheath (Es). The basement lamina (bl) is partly visible. Colloidal thorium particles are visible externally to the epithelial sheath (see arrows). x 12,000.

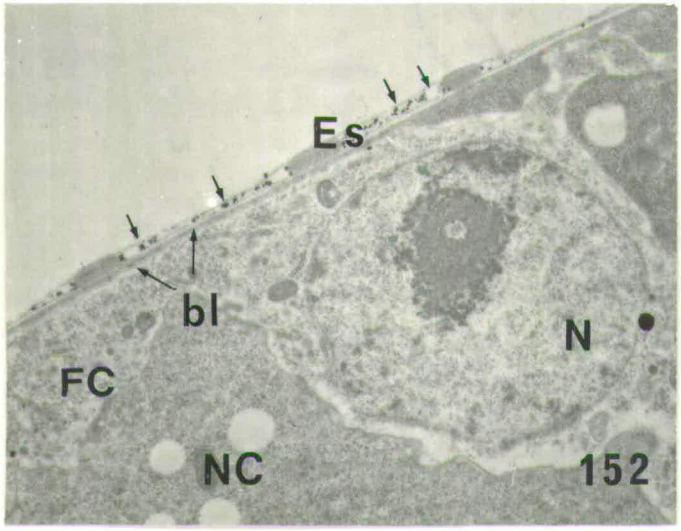
Fig. 152. A field similar to that shown in Fig. 150. The chamber (stage 10) was fixed 30 hours after the injection of thorium dioxide. Note several particles (arrows) within the epithelial sheath (Es) but there is no trace of thorium below the basement lamina. (FC) anterior follicle cell; (NC) nurse cell. x 6,000.

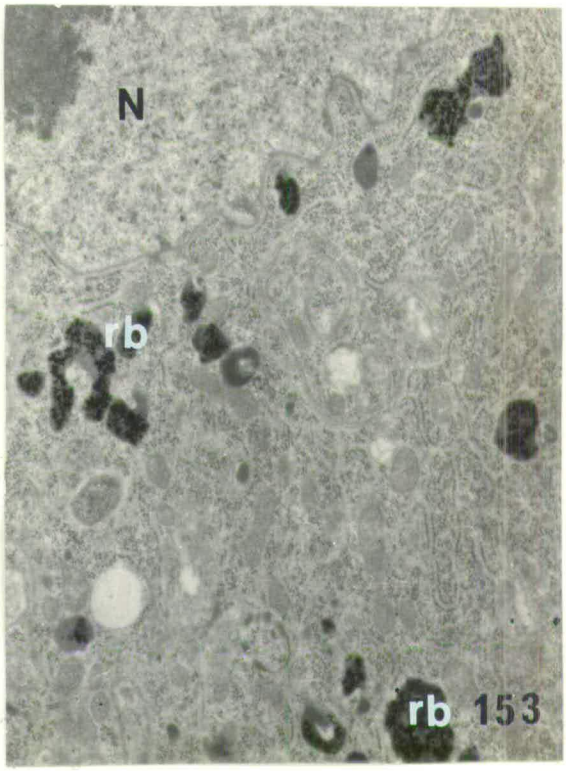
Fig. 153. Part of the cytoplasm of a follicle cell at the base of the ovary. The ovary was fixed 30 hours after the injection of thorium dioxide. Note the heavy deposits of colloidal thorium within the residual bodies '(rb). x 12,000.

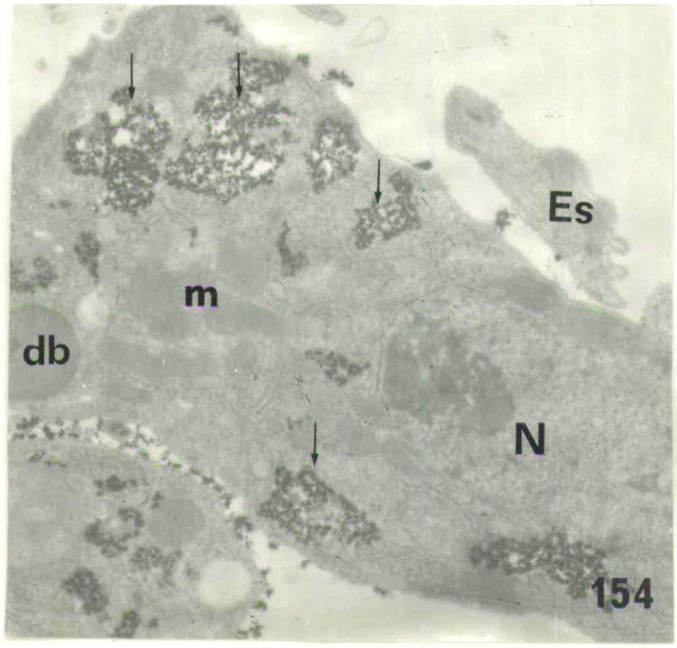
Fig. 154. Lumen cell or plasmatocyte in the lumen between the epithelial sheath and the ovariole. The ovary was fixed 1 hour after the injection thorium dioxide. Note the large number of colloidal particles within several vacuoles (see arrows). (N), nucleus; (m) mitochondria; (db) dense bodies; (Es) epithelial sheath. x 8,000.


bl
Es
FC
NC
150


Es
bl
Es
151


Es
bl
N
FC
NC
152


N
rb
rb 153


Es
m
db
N
154

intracellularly, suggests that, in developing chambers, the basement lamina functions as a barrier to the entry of large molecular aggregates of the dimensions of thorotrast. Other cells, which appear to contain thorotrast intracellularly, are the lumen cells or plasmatocytes which occupy the fluid filled lumen extending between the epithelial sheath and the ovariole. It has been suggested (Koch and King, 1966) that the plasmatocytes scavenge the fluid and neighbouring tissues and also appear to repair the basement lamina at points where it gets torn during growth and discharge of the oocytes. In these cells, thorotrast deposits occupy large areas corresponding to large vacuoles (Fig. 154). It is also worthwhile noting that the amount of thorotrast taken up by the follicle cells at the base of the ovary over a period of 30 hours is considerably lower than that taken up by the plasmatocytes after only 1 hour of *in vivo* exposure.

(d) Experiments with peroxidase *in vivo*.

The use of tracer molecules such as lanthanum and ruthenium red has shown that there is free access or continuity between the haemocoel and the oocyte surface. This is by way of the intercellular spaces in the follicle layer. However, it must be remembered that these tracers were used along with the fixative and the physical continuity that is envisaged must be seen in the light of this experimental condition. Furthermore, it should also be kept in mind that the cell cannot be expected to be capable of active uptake of material under this experimental condition. The

experiments with thorotrast showed that this molecule cannot penetrate the basement lamina of the ovarian chamber even in *in vivo* conditions. It is possible that the molecular aggregates of this tracer are too large to pass through the basement lamina.

The above mentioned limitations make it desirable to attempt to clarify the process of uptake of material in a dynamic way and under physiological conditions with a more suitable tracer. A tracer substance that may be used for this purpose should possess the following essential characteristics: (1) it must have a size that permits passage through the external basement lamina, (2) it must not interfere with the process of uptake itself, (3) it must not have any toxic effects on the tissue, at least for the duration of the experiment, (4) it must not be metabolized by the recepient tissue and must always exist in the injected form, (5) if the tracer substance is an enzyme, its activity should stay constant over a period of several hours at a temperature compatible with the experimental tissue, and (6) finally a cytochemical method must be available to localize the substance at the EM level. A substance which meets practically all these requirements is peroxidase. This was first introduced by Graham and Karnovsky (1966) and it has been repeatedly shown to fulfill the various requirements enumerated above. Peroxidase was therefore injected into female flies and after various periods of time the ovaries were dissected and fixed.

Fig. 155 shows the follicle-oocyte border of an ovarian chamber at stage 8, fixed 30 minutes after the

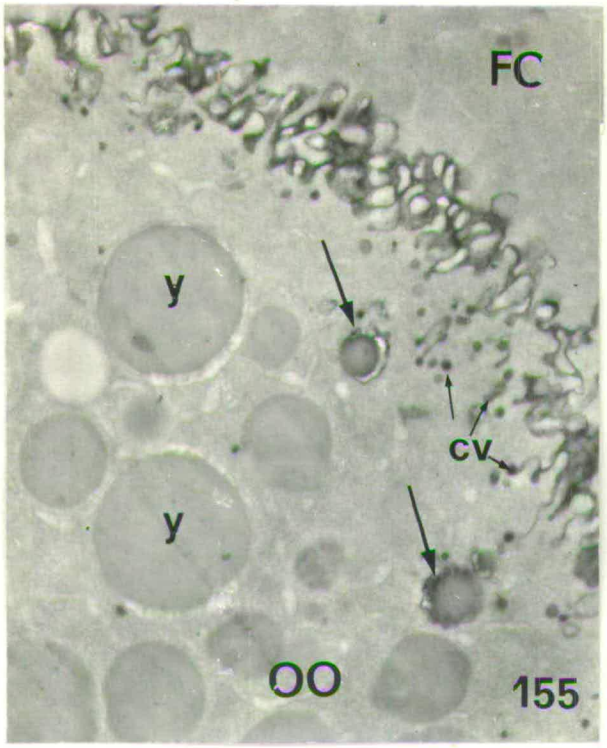
Figs. 155-162. Chambers in various stages of ovarian development fixed at different times after the injection of peroxidase. The injected peroxidase is localized by incubating the fixed chambers in the DAB medium as described in the material and methods section.

Fig. 155. Electron micrograph showing the cortical ooplasm of a stage 8 ovarian chamber fixed 30 minutes after the injection of peroxidase. The follicle-oocyte border contains the tracer. Several coated vesicles (cv) and two growing yolk platelets are labelled (see arrows); the other large yolk platelets (y) are not labelled. (FC) follicle cell; (OO)ooplasm. Section unstained. x 12,000.

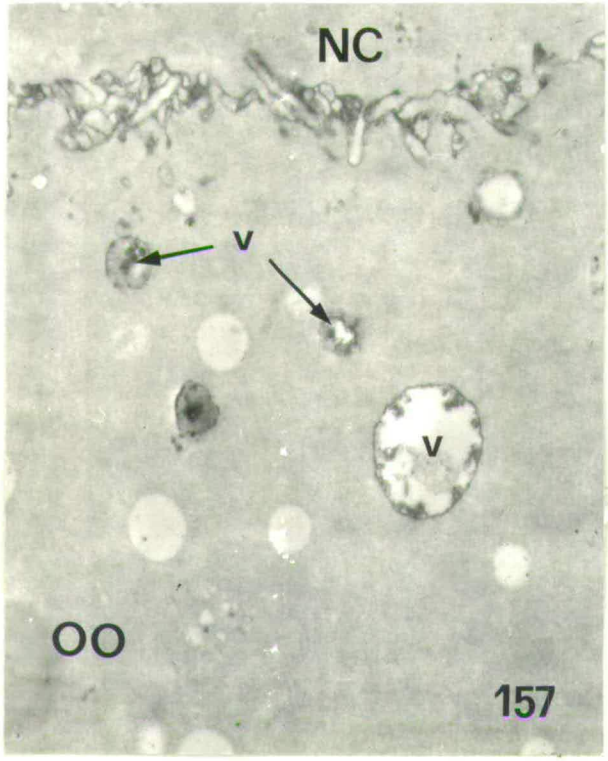
Fig. 156. The cortical ooplasm from a stage 9 ovarian chamber fixed 30 minutes after the injection of peroxidase. Note the absence of the tracer at the oocyte surface. Three yolk platelets (y) contain peroxidase on the margin of the main body and inner side of the limiting membrane (see arrows). Section unstained. x 12,000.

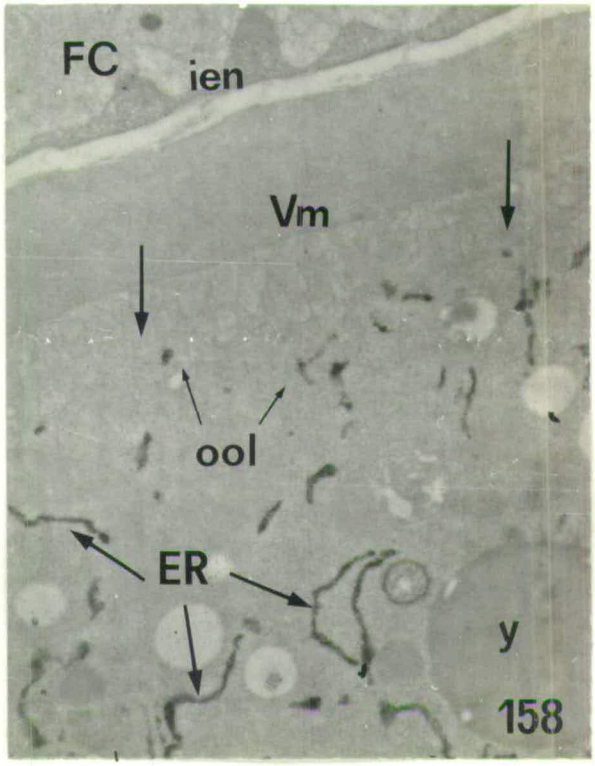
Fig. 157. Electron micrograph of a part of the nurse-oocyte border from a stage 9 ovarian chamber fixed 30 minutes after the injection of peroxidase. The tracer is present on the oocyte surface and also in some vesicles (v) in the ooplasm (OO). (NC) nurse cell; section unstained.

Fig. 158. The cortical ooplasm and overlying areas from a stage 12 ovarian chamber fixed 30 minutes after the injection of peroxidase. Note the peroxidase reaction products in the endoplasmic reticulum(ER). The space between the vitelline membrane (Vm) and the oocyte surface (see arrows) is devoid of the tracer. (FC) follicle cell; (ien) inner endochorion; (ool) oolemma. Section unstained. x 12,000.


FC
y
y
cv
OO
155


Vm
y
y
y
156


NC
v
v
OO
157


FC
ien
Vm
ool
ER
y
158

injection of peroxidase. In this micrograph the reaction products are observable not only along the follicle-oocyte border but also in several micropinocytotic vesicles (coated vesicles) which apparently are not in continuity with the oolemma. In addition, it is also possible to see that small yolk platelets but not the large ones, present in the cortical ooplasm, are labelled by peroxidase. Ovarian chambers in stages 9-10 exposed for the same length oftime to peroxidase, exhibit a similar picture. In this instance, the number of yolk platelets labelled is higher and they are of larger sizes (Fig. 156). However, in the interspace between the follicle cells and the oocyte, which is now very much wider than before, owing to the presence of the vitelline membrane, the amount of peroxidase present is much less. A possible explanation of this is as follows. It is known (Steinmann et al., 1974) that peroxidase does not preferentially bind on the cell surface. I envisage that during stages up to 8, that is, before the formation of the vitelline membrane, the interdigitating membranes of the follicle cells and the oocyte provide a sufficient trapping for the peroxidase that has gained access to this site. On the other hand, when this space widens with the formation of the vitelline membrane during stages 9-10, the peroxidase at this site is no more physically trapped but it is easily washed out during subsequent processing for electron microscopy.

The picture along a stretch of the oocyte-nurse cell border in stage 9 chamber is shown in Fig. 157. In this area of the ooplasm adjoining the nurse cells, there are also present vesicles which appear to

contain peroxidase reaction product after 30 minutes of *in vivo* exposure.

When ovarioles were exposed to peroxidase for 30 minutes and chambers later than stage 10 were examined, no deposits were found either along the follicle-oocyte border or in any yolk platelets of the cortical ooplasm. It was therefore surprising to find in stage 12 and later chambers (stages 13-14), peroxidase reaction products within the endoplasmic reticulum of the oocyte (Fig. 158), and of nurse cells (Fig. 162). It was even more surprising then to find no reaction products in the follicle cells, which at these stages have a much better endoplasmic reticulum. More will be said regarding this interesting observation in a later part of this thesis. The observations reported above are from experiments involving *in vivo* exposure of ovaries to peroxidase for 30 minutes but the results are pretty much the same after exposure of only 15 minutes.

In ovarioles exposed to peroxidase for longer periods (2-24 hours) there was a progressively larger number of yolk platelets which contained the dense reaction products. The distribution of yolk platelets in the ooplasm was at random and there was no indication of any regular layers of yolk platelets. In stage 10 chambers examined in ovarioles that were exposed to peroxidase for 24 hours, practically every yolk platelet contained peroxidase reaction products (Fig. 159). This might be taken to indicate that a period of 24 hours covers the period of vitellogenesis from its inception in stage 8 to stage 10. This observation also confirms that the process of vitellogenesis is

unaffected, as far as it can be made out, by exposure to peroxidase; it is possible, however, that the amount of yolk proteins taken up into yolk platelets is reduced because of their dilution in the haemolymph by the injected volume of peroxidase. Stage 8 chambers examined in ovarioles exposed *in vivo* for 24 hours to peroxidase showed the dense reaction products along the follicle-oocyte border as in similar chambers exposed for only 30 minutes; there was, however, a difference in the results of the two experiments since practically all the yolk platelets in the 24 hours experiments contained the reaction products. The follicle-oocyte border in stage 9 and 10 chambers of ovarioles exposed similarly for 30 minutes or 24 hours showed no reaction products.

As described earlier in the section dealing with the ultrastructure of the ooplasm, the yolk platelets in chambers 10-12 are predominantly of the type referred to as alpha-1 (Fig. 35). When these yolk platelets were examined in ovarioles exposed 24 hours to peroxidase the reaction products were found to be confined to their superficial layer. In sections passing through the associated body of yolk platelets, it was obvious that this body is largely free of peroxidase reaction products (Fig. 160).

An indication of the kinetics of peroxidase uptake into the ooplasm in stage 9 chambers studied in ovaries exposed for varying periods of time is shown in Text-Fig. 3. The Text-Fig. 4 illustrates the kinetics of peroxidase uptake diagrammatically.

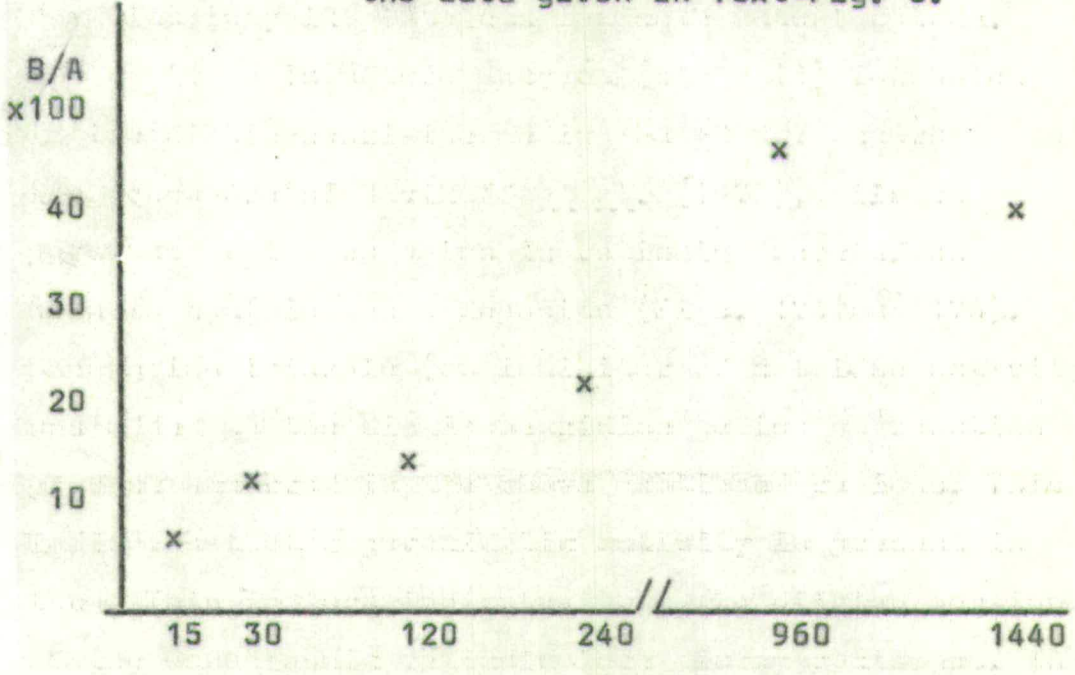
Follicle cells also exhibit some peroxidase

Text-Fig. 3. Uptake of peroxidase into the ooplasm of stage 9 ovarian chambers scored as function of time. Area of labelled yolk platelets (μ^2) is compared with area of the rest of the ooplasm. The latter also includes the area occupied by yolk platelets which do not contain peroxidase.

°° TIME	A	B	B/A x 100
15	348.04	14.96	4.29
30	573.14	73.56	12.83
120	338.53	53.94	15.93
240	311.95	76.64	24.56
960	372.63	167.81	45.03
1440	347.60	139.83	40.22

°° Time in minutes; A, Area of peroxidase free-ooplasm in μ^2 ; B, Area of peroxidase labelled yolk in μ^2 .

Text-Fig. 4. Kinetics of peroxidase uptake into the ooplasm of stage 9 ovarian chambers represented diagrammatically by using the data given in Text-Fig. 3.



reaction products which appear to be localized in multivesicular bodies and residual bodies. This localization is clearly illustrated in the border cells (Fig. 161) which contain multivesicular bodies and residual bodies in larger numbers than in the columnar follicle cells.

Nurse cells do not exhibit peroxidase reaction products for the major part of the oogenesis, that is, up to stage 10. During this period, the tracer is restricted to the intercellular spaces between these cells. Reaction products, however, appear in the endoplasmic reticulum of the nurse cells in chamber stage 12 (Fig. 162) exposed *in vivo* to peroxidase for 1 hour. A similar distribution of reaction products was also observed in stage 13 chambers of control preparations, and the significance of this will be discussed later, see page 182 . Much the same situation was found in stage 13 chambers after 1 hour exposure to peroxidase. It may be recalled (Fig. 158) that the endoplasmic reticulum in the oocyte of stage 12-13 chambers also exhibited the dense reaction products. Such deposits within profiles of endoplasmic reticulum could sometimes be seen in sections passing through the ring canals that connect the oocyte with nurse cells.

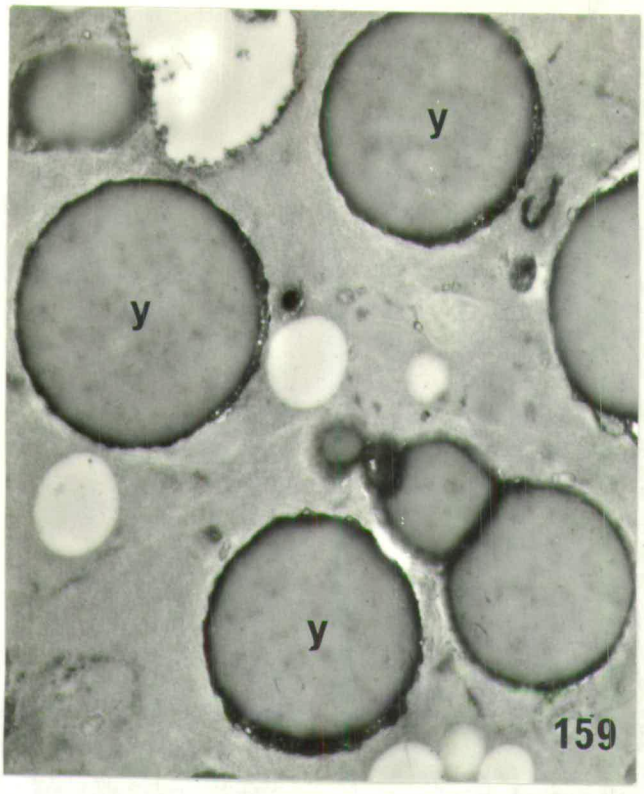
The results of the *in vivo* experiments with peroxidase indicate that in vitellogenic stages 8 to 10, the oocytes are the only cells which absorb proteins from the haemolymph and incorporate them into forming yolk platelets. When the vitelline membrane comes to be interposed between the oocyte and the follicle cells during stages 11-13, no further uptake seems to occur.

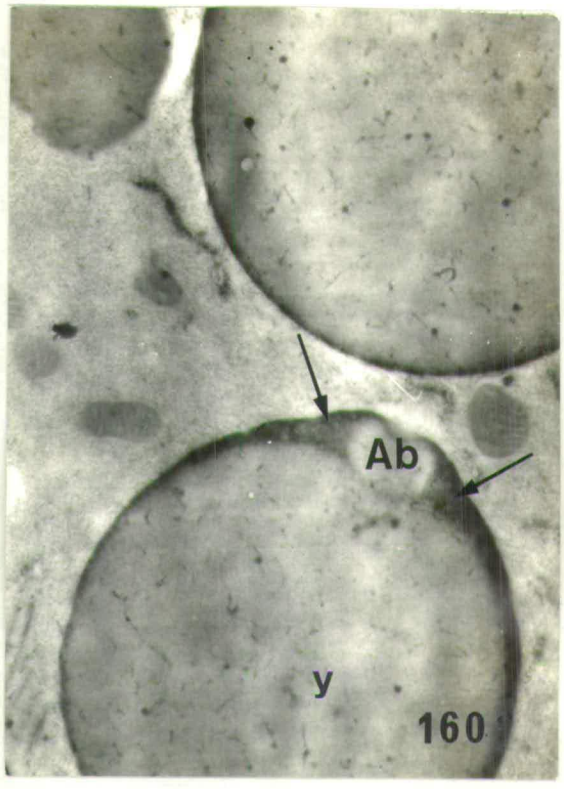
Fig. 159. An electron micrograph of the central ooplasm from a stage 9 ovarian chamber fixed 24 hours after the injection of peroxidase. Note that all the yolk platelets are labelled. The tracer is restricted to the superficial layer of the yolk platelets. Section unstained. x 12,000.

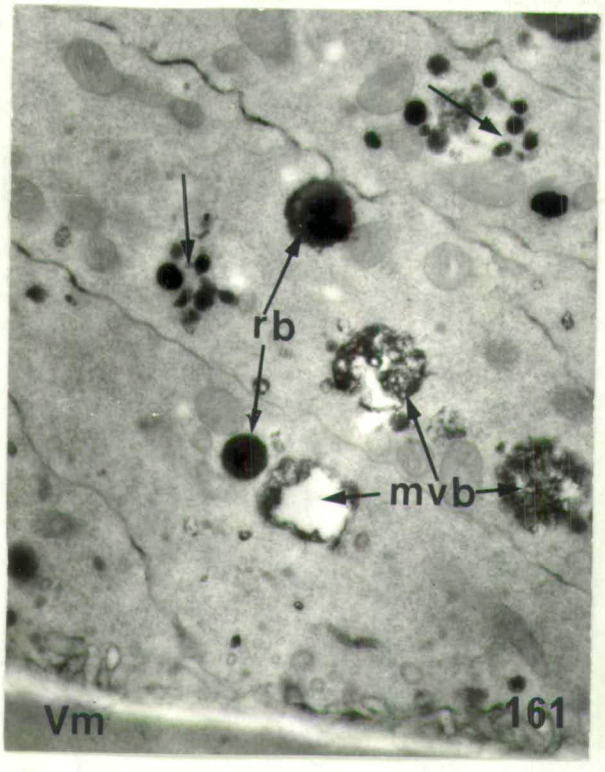
Fig. 160. An alpha-1 yolk platelet from the central ooplasm of a stage 10 ovarian chamber fixed 24 hours after the injection of peroxidase. Note that the tracer is restricted to the superficial layer (see arrows). The associated body (Ab) is devoid of the tracer. Section unstained. x 12,000.

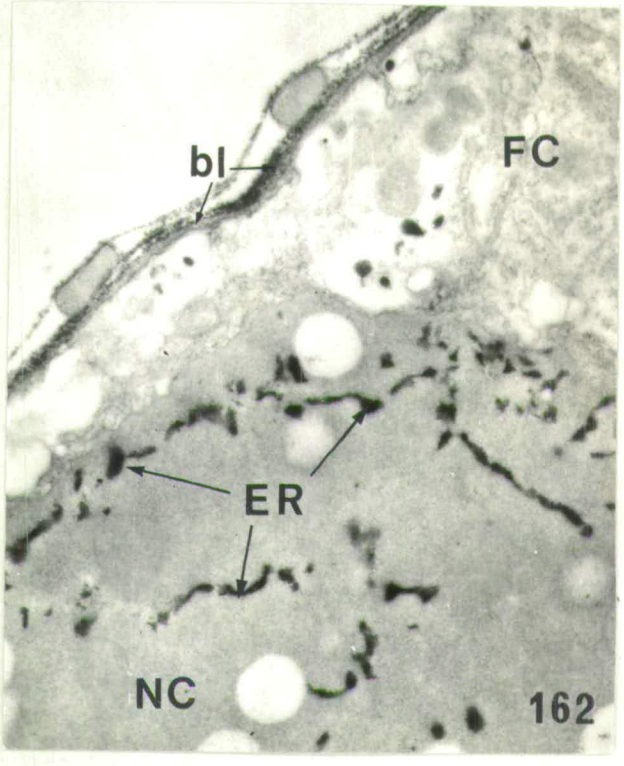
Fig. 161. The border cells at the anterior region of a stage 12 oocyte. The preparation was taken from an ovary fixed 1 hour after the injection of peroxidase. The tracer is present within multivesicular bodies (mvb) and residual bodies (rb). Note that peroxidase reaction products also occur in the endochorionic precursors (see arrows). The significance of this observation is dealt with later in Fig. 171. Section unstained. x 12,000.

Fig. 162. The anterior region of a stage 12 ovarian chamber, fixed 1 hour after the injection of peroxidase. Reaction products are visible in the endoplasmic reticulum (ER) of the anterior nurse cells. (FC) follicle cell; (NC) nurse cell; (bl) basement lamina, Section unstained. x 12,000.


y
y
y
159


Ab
y
160


rb
mvb
Vm
161


bl
FC
ER
NC
162

(e) Experiments with peroxidase *in vitro*.

Ovaries were incubated for 30 and 60 minutes in Drosophila Ringer solution containing peroxidase. Particular attention was paid to developing chambers which were recognized *in vivo* experiments as active in segregating peroxidase into yolk platelets (stages 8-10). It seemed of interest to determine whether pinocytotic uptake could occur also in *in vitro* conditions or if the process of uptake could occur only in *in vivo* conditions. It was hoped that some insight into the question would be gained by comparing the results of these two sets of experiments.

Figs. 163-165 illustrate the ultrastructural features along the follicle-oocyte border of ovarian chambers incubated in peroxidase-containing Ringer solution. The most striking alteration in these preparations, compared with the normal features described earlier is the presence of far more numerous tubular profiles in the subcortical ooplasm (Figs. 164 and 165) which appear within 30 minutes of incubation. It may be recalled that such tubules are present, although in much fewer numbers, in normal preparations (Fig. 34). In Fig. 164 numerous circular profiles are also evident; many of them are apparently cross sections of the tubules but one can also make out several coated vesicles amidst them. Another noteworthy observation relates to the yolk platelets. The number of yolk platelets in this region was unusually low and even the few that were present (Fig. 165) did not show any reaction product. In stage 10 chambers examined in ovaries incubated *in vitro* for 60 minutes, the tubules give

the impression of having fused together to form abnormally large aggregates as shown in Fig. 165. It is of interest to recall here that images bearing a close resemblance to the fused tubules just described were observed by Steinmann *et al.* (1974) in fibroblasts cultured for 1 hour *in vitro* in a peroxidase containing medium. The absence of coated vesicles in the cortical ooplasm of stage 10 chambers treated for 1 hour is another noteworthy feature. The rarity or virtual absence of yolk platelets in the cortical ooplasm was also glaring in stage 10 chambers after 60 minutes of *in vitro* incubation in peroxidase-containing medium.

(f) Further experiments with peroxidase *in vitro*

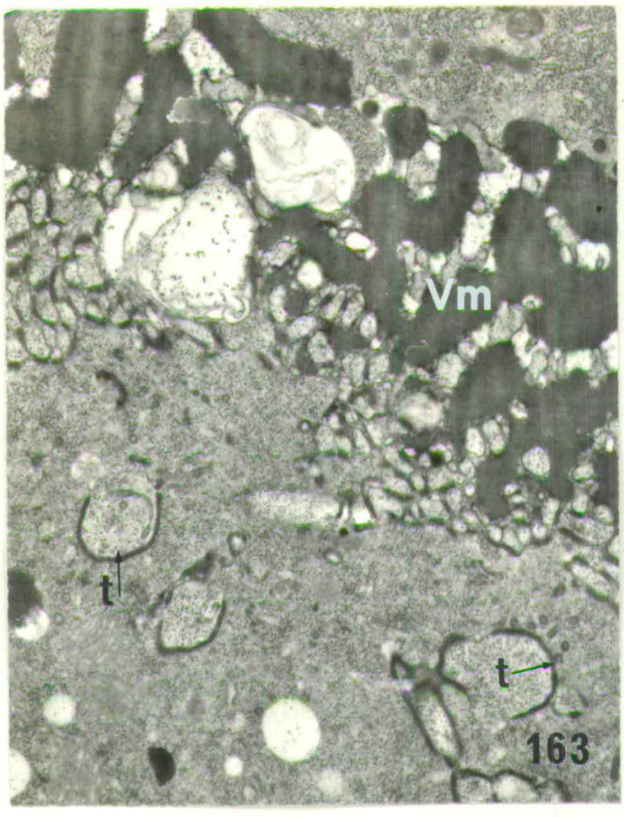
The previous set of *in vitro* experiments showed that there are noticeable differences in the ultrastructure of the cortical ooplasm compared with that after the *in vivo* experiments. These morphological differences also indicated the occurrence of possible alterations in the process of uptake of proteins by the oocyte, and further experiments using various incubation media (see table below) were therefore carried out with a view to clarifying this aspect. The experiments were done for 30 or 60 minutes using ovaries from 2-3 day old flies.

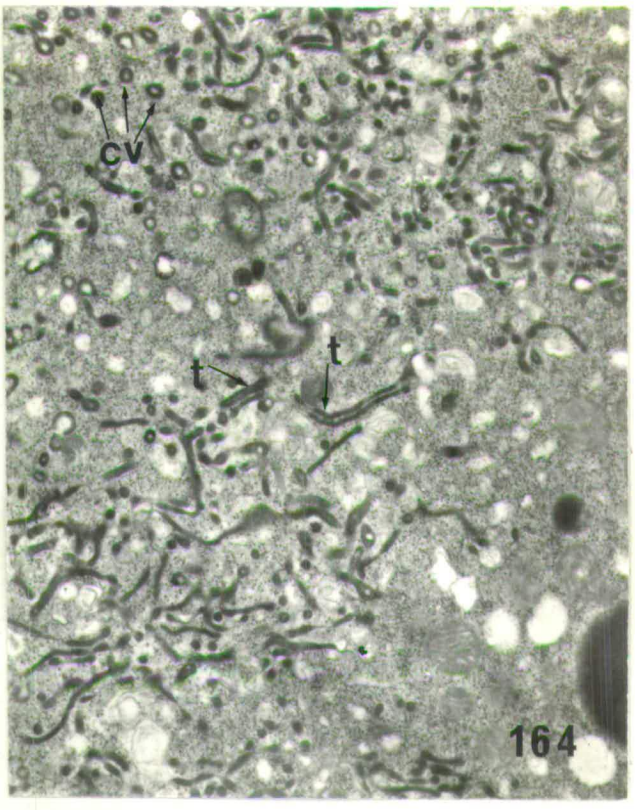
In vivo	In vitro			
Injection	Incubation in one of the following media			
A	B	C	D	E
Ringer Peroxidase -	Ringer Peroxidase -	Ringer Peroxidase Female Haemolymph	Ringer Peroxidase Male Haemolymph	Ringer Peroxidase Fat body

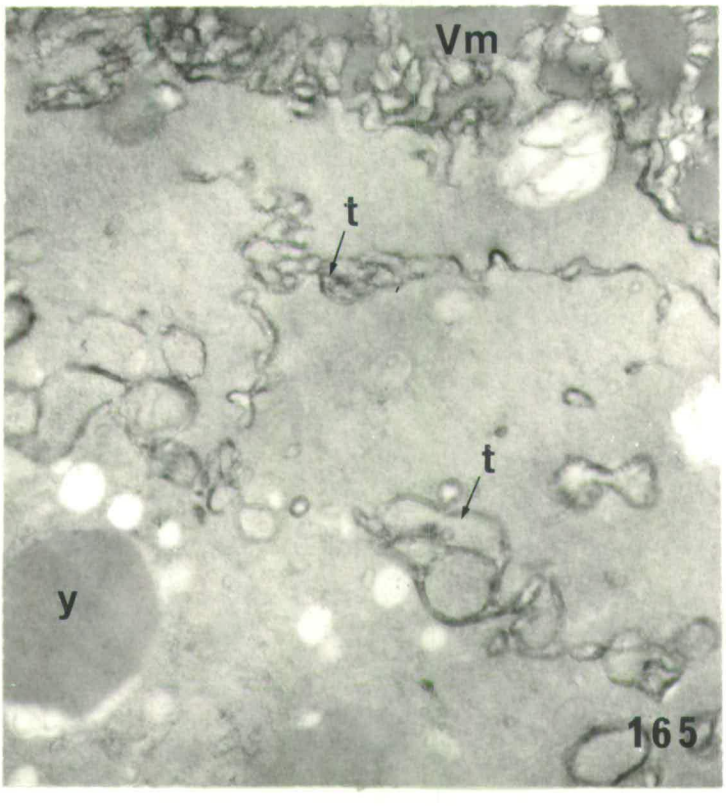
Fig. 163. The cortical ooplasm of a stage 10 ovarian chamber fixed 30 minutes after incubation *in vitro* in *Drosophila* Ringer containing peroxidase. Several fused tubular profiles (t) contain the tracer. x 12,000.

Fig. 164. An electron micrograph of another area similar to that in Fig. 163, from a stage 10 ovarian chamber. Note the large number of tubular profiles containing the peroxidase reaction products. The *in vitro* incubation was 30 minutes. Several coated vesicles are also visible (cv). x 12,000.

Fig. 165. The cortical ooplasm of a stage 10 ovarian chamber fixed 1 hour after incubation *in vitro* in *Drosophila* Ringer with peroxidase. The tubular profiles containing peroxidase reaction products are now fused to form larger aggregates. Section unstained. x 12,000.


Vm
t
t
163


cv
t
t
164


Vm
t
t
y
165

Experiments relating to media A and B have already been considered. The other three incubation media are taken to represent different physiological situations. Medium C can be expected to mimic the conditions obtained in the in vivo experiments. It must, however, be borne in mind that medium C does not possess functional fat bodies, as in in vivo conditions. The D medium provides a situation which is different from that of medium C, in that the haemolymph is derived from the male. It is known (Telfer, 1965) that yolk proteins are sex-specific and that they are either absent or occur in very low concentration in the male haemolymph. Medium E is similar to medium B except that it includes fat bodies and differs from medium C and D in the absence of the haemolymph.

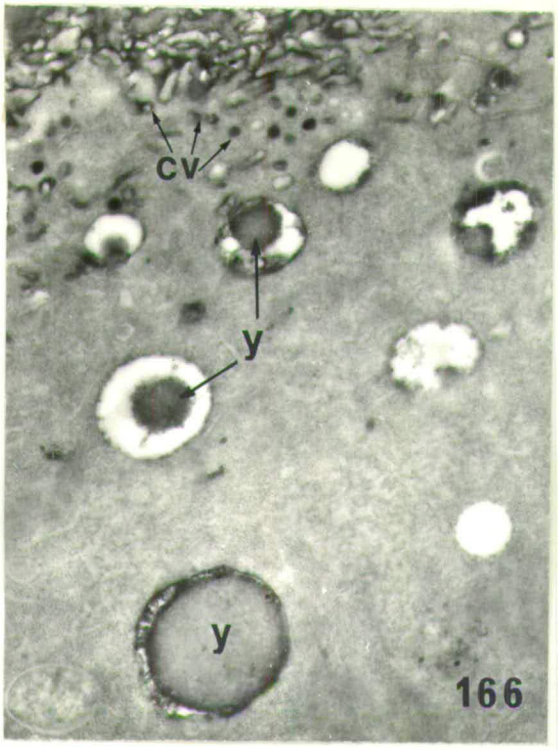
In electron micrographs of the cortical ooplasm of stage 10 chambers prepared after 30 minutes of incubation in medium C, the ultrastructural features (Fig. 166) are similar to those observed after 30 minutes of in vivo incubation (Fig. 156). It may, however, be noted that the relative proportion of the two components of the yolk platelets (the main body and the empty looking area surrounding it) are different; after incubation in medium C the main body is relatively small. Yolk platelets are also formed after incubation in medium D, but on the average, the main body is even smaller (Fig. 167) than that produced in medium C. After incubation in the medium with the fat body extracts (medium E) the ultrastructural features in the cortical ooplasm (Fig. 168) are the same as those observed after straight forward incubation in vitro (medium B) (Fig. 165).

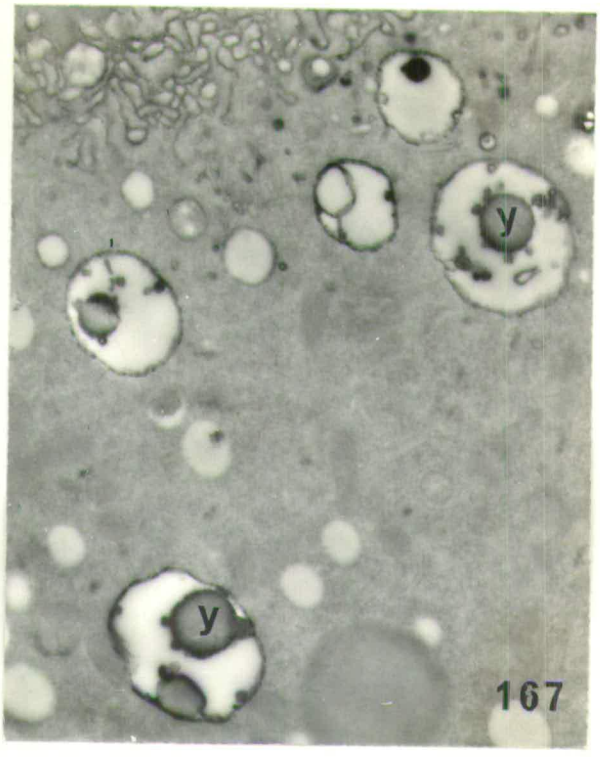
Fig. 166. The cortical ooplasm of a stage 10 ovarian chamber fixed 30 minutes after incubation *in vitro* in female haemolymph with peroxidase added. Note several yolk platelets (y) labelled with peroxidase. The tracer is also present in some coated vesicles (cv) beneath the oocyte surface. Section unstained. x 12,000.

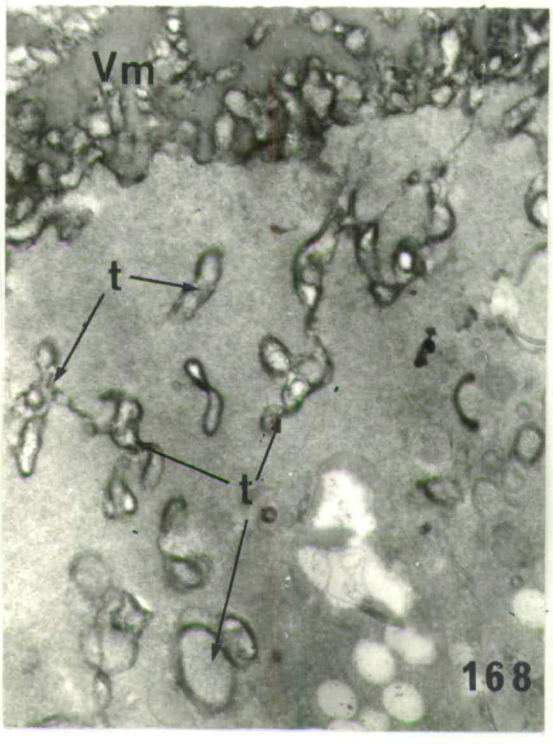
Fig. 167. The cortical ooplasm of a stage 10 ovarian chamber fixed 30 minutes after incubation *in vitro* with male haemolymph containing peroxidase. Section unstained. x 12,000.

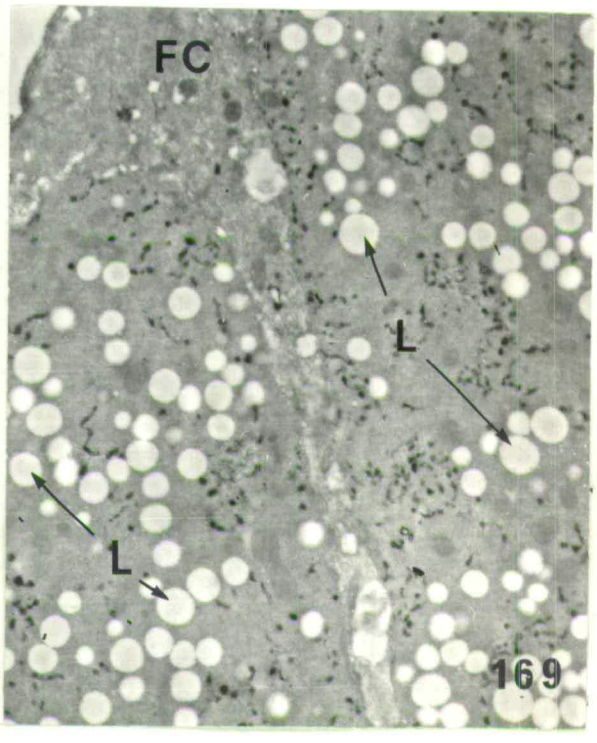
Fig. 168. The cortical ooplasm from a stage 10 ovarian chamber fixed after 30 minutes of *in vitro* incubation in *Drosophila* Ringer with extracts of female fat bodies added. Note that the tubular aggregates contain peroxidase and that no yolk platelets are visible in this preparation. Section unstained. x 12,000.

Fig. 169. Part of the anterior region of a stage 10 ovarian chamber fixed 30 minutes after incubation *in vitro* in *Drosophila* Ringer with extracts of female fat bodies added. Peroxidase reaction products occur in the endoplasmic reticulum. (FC) follicle cell; (L) lipid droplets. Section unstained. x 12,000.


cv
y
y
166


y
y
167


Vm
t
t
168


FC
L
L
169

In both the instances the cortical ooplasm is practically devoid of yolk platelets but shows an abundance of tubular profiles. The various observations made from this series of experiments will be discussed at length later.

(g) Experiments demonstrating the occurrence of endogenous peroxidase.

When ovaries were incubated in the complete diaminobenzidine medium used for the cytochemical detection of peroxidase (H_2O_2 which is the substrate and DAB the coupling agent), without prior exposure to peroxidase, dense reaction products appeared in certain locations in ovarian chambers in late stages of oogenesis. These experiments were initially carried out as control for the peroxidase uptake experiments described earlier. Of the various types of cells in the ovarian chambers, the reaction products were visible in the endoplasmic reticulum of the nurse cells and oocyte and that too in stages 12 and later. The density of the reaction product in the endoplasmic cisternae of both the oocyte and nurse cells was apparently unaffected by varying the pH of the incubation medium from 7.4 to 8.2 but no reaction product was seen at pH 6.0. It is known that, at pH 6.0, the substrate used in this experiment may be oxidized by cytochromoxidase which occurs in mitochondria (Novikoff et al., 1972). At the pH range 7-9, the substrate is specific only to peroxidase activity. Electron micrographs showing the dense reaction product in the endoplasmic reticulum of oocytes in stages 13 and 14 are shown in Figs. 171 and 170 respectively. These figures reveal that the endoplasmic

system in the follicle cells do not contain any reaction product. The electron micrograph in Fig. 171 closely resembles the picture seen in stage 12 chamber which was incubated *in vivo* with exogenous peroxidase (Fig. 158). The preparation shown in Fig. 171 demonstrates that the reaction product in the endoplasmic reticulum of the oocyte is due to endogenous peroxidase. The preparation presented in Fig. 171 was fixed in formaldehyde and this probably accounts for the formation of dense deposits along the endochorion and in the Golgi apparatus of the follicle cells; deposits in such location were not seen in preparations fixed in glutaraldehyde. It has been reported (Hand, 1973) that the type of fixative used may influence the peroxidase reaction. In none of the experiments in this series were yolk platelets ever seen to contain dense deposits. This confirms that yolk platelets do not have any endogenous peroxidase activity and that, in experiments where exogenous peroxidase was used, the resulting deposits in yolk platelets indicate actual uptake.

In mature oocytes (stage 14) incubated in the diaminobenzidine medium at pH 9.7 according to the procedure of Novikoff *et al*. (1972), dilated portions of the endoplasmic reticulum were often seen to contain dense deposits (Figs. 174 and 175). When amino triazole (an inhibitor of catalase activity) was added to the diaminobenzidine medium no reaction product occurred in the above mentioned regions. This indicates that a peroxidatic activity is present in them. This in turn indicates that the dilated portions of the endoplasmic reticulum are microperoxisomes. The

possible significance of the presence of microperoxisomes in oogenesis will be discussed later.

In addition to the above mentioned sites, stage 14 oocytes incubated in the diaminobenzidine medium at pH 9.7 revealed a number of spherical organelles which contained dense reaction products. The presence of these organelles in the ooplasm of stage 11-14 ovarian chambers was mentioned in an earlier section of this thesis (see page 71 and also Fig. 38) when they were described as lipofuchsin granules. The present finding that some of these organelles react positively for peroxidatic activity (indicating the presence of catalase) in stage 13-14 ooplasm may be taken as an indication that the lipofuchsin granules fuse with peroxisomes or catalase-containing portions of the endoplasmic reticulum. The possibility of fusion between lipofuchsin granules and peroxisomes has been previously suggested by Novikoff et al. (1973) in cells of rat liver.

2. Complex carbohydrates and yolk.

Previous evidence have indicated that carbohydrates may occur as constituents of yolk platelets in insect and amphibian oocytes (Favard-Sereno, 1969; Favard and Favard-Sereno, 1969). In recent years, it has been assumed that carbohydrates may play a role as binding substances on plasma membranes for uptake of proteins (Jacques, 1969). There have also been suggestions that carbohydrates may function as inhibitors of certain lysosomal enzymes (Snellman and Sylven, 1974). In view of these observations, it was thought necessary to look for carbohydrates in oogenesis of Drosophila.

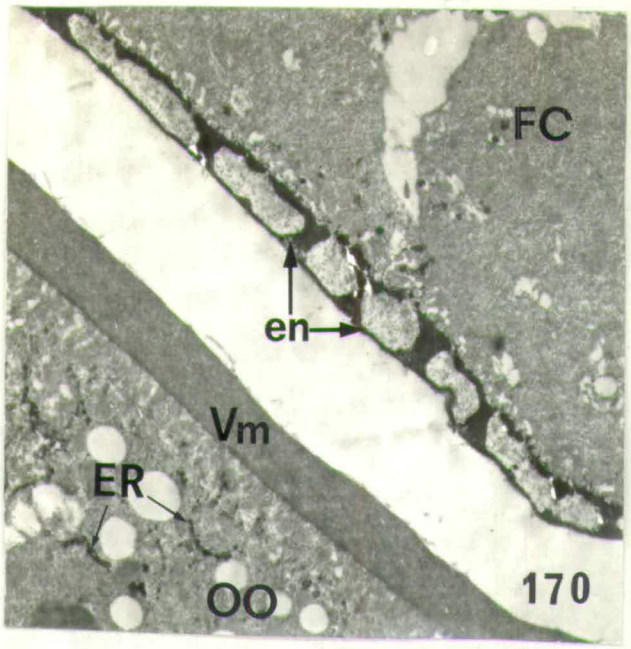
Figs. 170-175. Ultrathin sections from ovarian chambers fixed in formaldehyde and then incubated in the diaminobenzidine medium (DAB) at varying pH values. The chambers were not exposed to peroxidase before fixation. The post-fixation in osmium tetroxide was followed by embedment in Epon-Araldite mixture as usual.

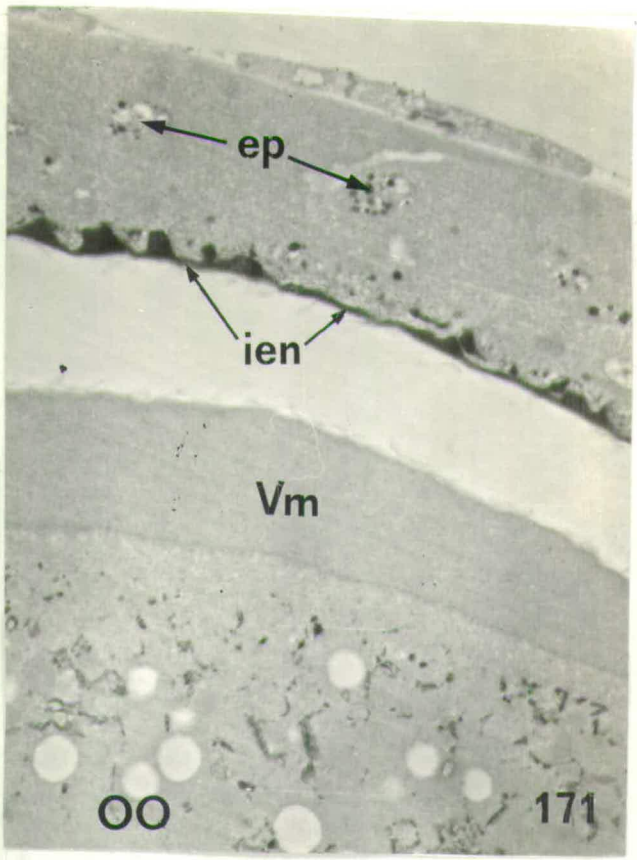
Fig. 170. The follicle-oocyte border from a stage 14 ovarian chamber incubated in the DAB medium at pH 7.4. The endochorion (en) contains the dense reaction product. The endoplasmic reticulum of the ooplasm is also stained (see ER) . (FC) follicle cell; (Vm) vitelline membrane; (OO) ooplasm. Section unstained. x 3,000.

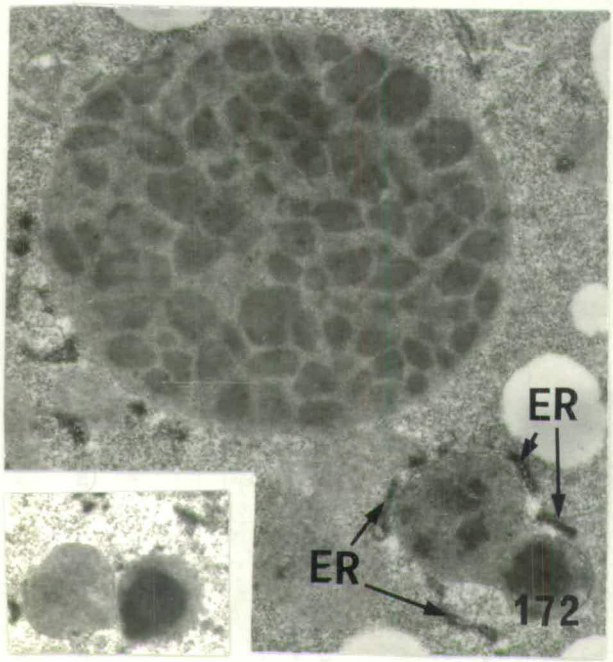
Fig. 171. The follicle-oocyte border from a stage 13 ovarian chamber incubated in the DAB medium at pH 7.4. The inner endochorion (ien) and the endochorionic precursors (ep) in the follicle cell cytoplasm contain the dense reaction product. The endoplasmic reticulum of the ooplasm also contains the reaction product. Compare this micrograph with that in Fig. 158. Section unstained. x 3,000.

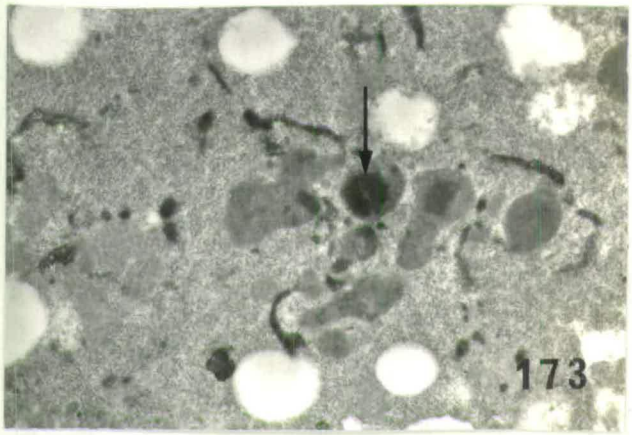
Fig. 172. Part of the central ooplasm from a stage 14 ovarian chamber. The organelle indicated with Lf (lipofuchsin granules) shows a dense product in part. The endoplasmic reticulum (ER) near to the organelle is also densely stained. In the insert, two spherical organelles can be clearly seen of which only one shows a dense reaction product within. The chamber was incubated in the DAB medium at pH 9.7. x 12,000.

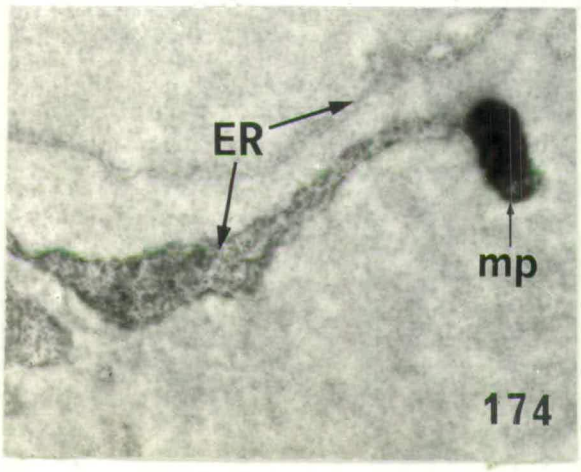
Fig. 173. Part of the central ooplasm from a stage 14 ovarian chamber incubated in the DAB medium at pH 9.7. A cluster of organelles is visible; only the organelle in the centre is densely stained (see arrow). Section unstained. x 8,000.

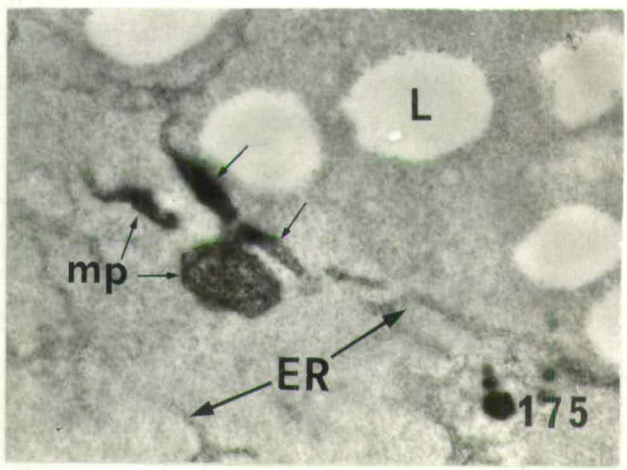

FC
en
Vm
ER
OO
170


ep
ien
Vm
OO
171


ER
ER
172


173


ER
mp
174


L
mp
ER
175

(a) Observations following the use of phosphotungstic acid technique

Before the discovery of alkaline lead stains, phosphotungstic acid (PTA) was used as a general stain in electron microscopy. Subsequently, it proved to be a specific stain for polysaccharides if used at low pH on GMA embedded material (Pease, 1970). Some authors (Glick and Scot, 1970) have, however, questioned the specificity claimed for PTA; they have suggested that it is only the protein moiety of a glycoprotein molecule that is stained by PTA. More recently, Flechon (1974) has reexamined critically the reliability of the PTA technique for polysaccharides. He showed that if the tissue is not subjected to a prolonged wash, and if the staining is carried out at a pH of 1, polysaccharides can be detected with considerable reliability. These conditions were met in the technique employed by Rambourg et al.(1969), and in the present study I have applied this technique to ultrathin sections of ovarian chambers of Drosophila.

In vitellogenic chambers (stages 8 to 10), the most intensely stained region is the oocyte surface below the vitelline membrane (Fig. 178). This region, however, is not stained in younger chambers. The cortical region of the ooplasm of vitellogenic stages contains, as described earlier on several occasions, pinocytotic vesicles and tubules; the tubules were assumed to be formed by the fusion of the vesicles. On careful examination of many high resolution electron micrographs, a very thin layer of stained material could be made out within these entities;

the stained layer was very closely apposed to the limiting membrane (Figs. 176, 182 and 183). The thin layer of stained material most probably corresponds to what was referred to in an earlier section (page 68) as fuzzy material.

It may be recalled that in stages 8-10 the forming and mature yolk platelets contain a body, the so called associated body, and this body also becomes stained with PTA under conditions specific for polysaccharides (Figs. 180 and 181). PTA stained bodies of various sizes are also observable in the follicle cell cytoplasm (Fig. 177) and in the cortical ooplasm (Fig. 181). The ultrastructural preservation obtained after the PTA procedure for demonstrating polysaccharides is generally poor and it has not been possible to identify with certainty the region of the Golgi apparatus in these preparations.

(b) Observations following the use of periodic acid - silver methanamine technique.

The periodic acid - silver methanamine (PA-SM) method for detecting polysaccharides was originally introduced by Gomori (1946). Later it was adapted for electron microscopy by several authors and a detailed procedure was published by Rambourg (1967). His method is based on the oxidation of glycol groups or the equivalent amino or alkyl amino derivatives into dialdehydes by periodic acid. The dialdehydes released reduce the silver methanamine to metallic silver; the location of the silver particles are then assumed to represent the sites of the original dialdehyde donors.

Fig. 174. The central ooplasm of a stage 14 ovarian chamber incubated in DAB medium at pH 9.7. A microperoxisome (mp) continuous with the endoplasmic reticulum (ER) is clearly stained. x 32,000.

Fig. 175. A field similar to Fig. 174. Note that only the portion of the endoplasmic reticulum close to microperoxisome (mp) is stained. Section unstained. x 20,000.

Fig. 176-181. Ovarian chambers fixed in glutaraldehyde and embedded in glycol methacrylate. Ultrathin sections were treated with 1 % phosphotungstic acid (PTA) in 10 % chromic acid for 2 minutes.

Fig. 176. Electron micrograph of part of the cortical ooplasm from a stage 10 ovarian chamber. Note that the inner side or fuzzy layer (fl) of the limiting membrane of the small yolk platelets is densely stained by the PTA treatment. x 32,000.

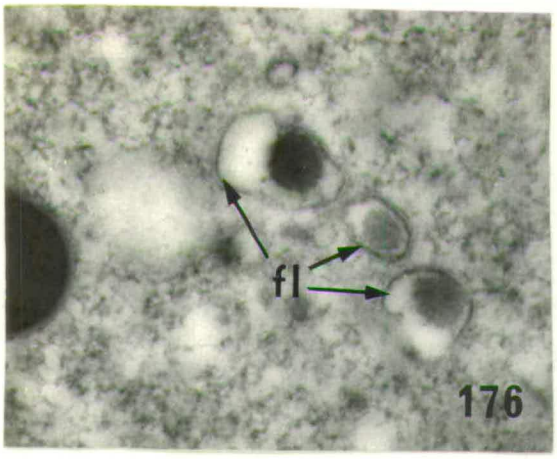
Fig. 177. Part of the follicle cell cytoplasm of a stage 10 ovarian chamber. Dense bodies (db) of different sizes appear heavily stained, especially along the inner margin. Mitochondria and Golgi apparatus (G) are not stained. x 32,000.

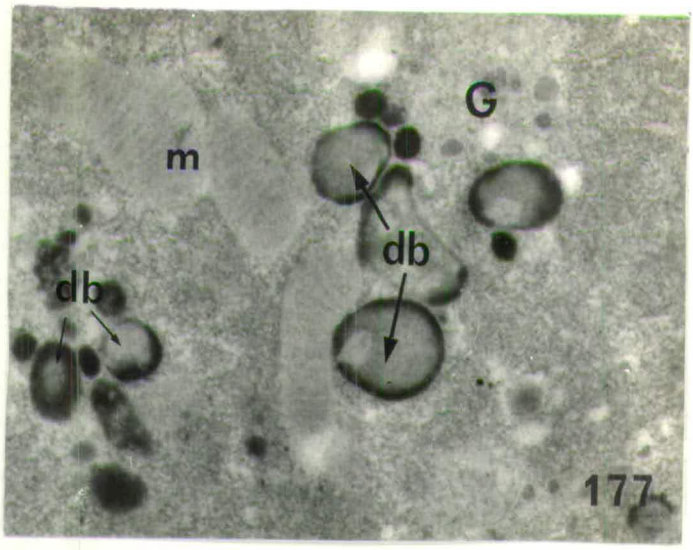
Fig. 178. A micrograph showing the follicle-oocyte border of a stage 10 ovarian chamber. Note a heavy stained layer (see arrows) between the vitelline membrane (Vm) and the oocyte surface. (FC) follicle cell; (OO) ooplasm; (vp) vitelline membrane precursors. x 12,000.

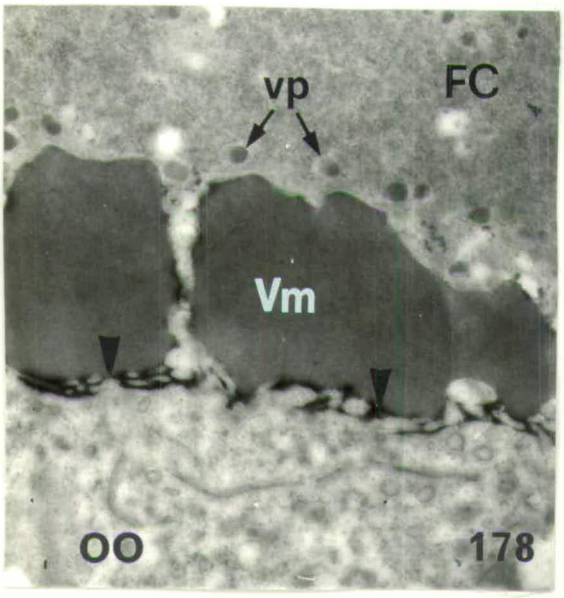
Fig. 179. A control section, that is an ultrathin section of the same region as that in Fig 177 stained with uranyl acetate and lead citrate instead of PTA. Compare with Figs. 176 and 178. x 12,000.

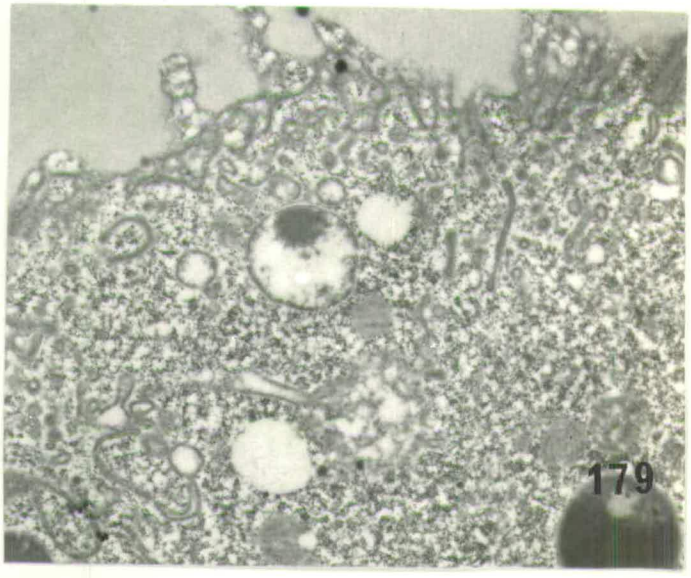
Fig. 180. Part of the central ooplasm from a stage 10 ovarian chamber. Note the dense staining of the associated body in several yolk platelets (see arrows); compare with micrograph of a control preparation (Fig. 35). x 12,000.

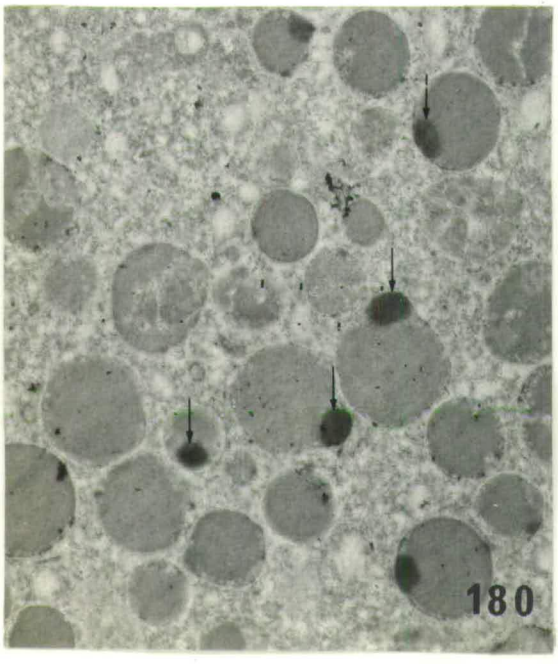
Fig. 181. High power micrograph of a stage 10 ooplasm. The associated body (Ab) in one yolk platelet and three other vesicles in the adjoining ooplasm are heavily stained. x 32,000.

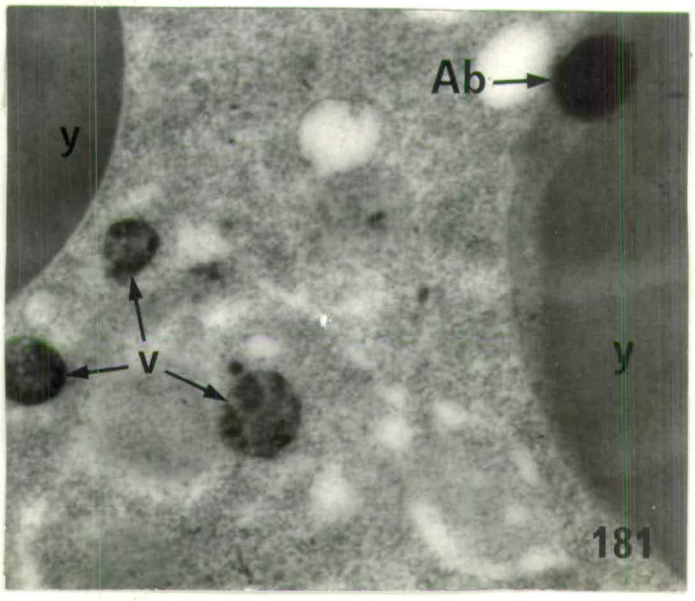

fl
176


m
G
db
db
177


vp
FC
Vm
OO
178


179


180


Ab
y
v
y
181

A major limitation of this method is the possibility of non-specific results brought about by the reduction of the silver methanamine by various groups other than dialdehydes. Therefore, in order to get reliable localization of polysaccharides, it is essential to carry out control experiments without using periodic acid.

When the PA-SM method was carried out according to Rambourg (1967), the central ooplasm in stage 8 chambers showed cytolysosome-like bodies (see Figs. 22 to 26) containing silver deposits distributed irregularly as shown in Fig. 188; they also contain areas which are devoid of silver deposits. In the yolk platelets present in the cortical ooplasm of stages 9 and 10, the silver deposits are confined to the superficial layer as shown in Figs. 184 and 186. It may, however, be noted that, in these instances (Figs. 184 and 186), the silver deposits are very scarce or practically absent in the associated body. It may also be noted that there is deposition of silver along the oolemma of ovarian chambers in stages 9 and 10 (Fig. 189). In the columnar follicle cells of vitellogenic stages there are observable bodies of various sizes with heavy deposits of silver (Figs. 187 and 189). The level of background in the experimental as well as in the control preparations is rather high and this may be due to retention of aldehyde groups of the fixative. It may be possible to get rid of this background by using blocking agents, but the preparations obtained in the present study were considered adequate for the present purpose.

Fig. 182. A growing yolk platelet in the cortical ooplasm of a stage 10 chamber. Note the dense associated body (Ab). The fuzzy layer on the inner side of the limiting membrane of the yolk platelet is also stained with PTA (see arrows). x 12,000.

Fig. 183. High power micrograph of part of the cortical ooplasm. Note the heavily stained fuzzy layer on the inner side of the limiting membrane of the yolk platelet (see arrows). The inner side of the limiting membrane of the coated vesicles is also stained. (mb) main body of the yolk platelet. x 80,000.

Figs. 184-190. Vitellogenic chambers fixed in glutaraldehyde and embedded in Epon-Araldite mixture without post-fixation in osmium tetroxide. Ultrathin sections were treated with periodic acid and then with silver methanamine solution.

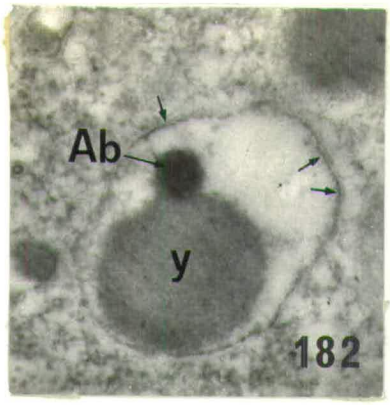
Fig. 184. An alpha-1 yolk platelets from a stage 10 ovarian chamber treated as explained above. Note the heavy deposits of silver over the region of the superficial layer. The associated body (Ab) is not stained. 12,000 x.

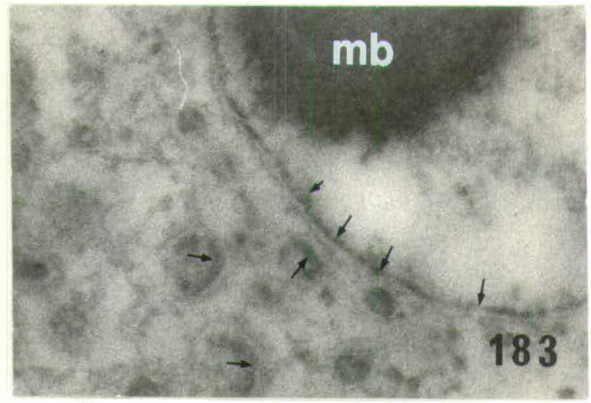
Fig. 185. An alpha-1 yolk platelet from a stage 10 ovarian chamber treated with silver methanamine solution without prior oxidation in periodic acid. The superficial layer contains some silver deposits but the extent of silver deposition is much less than that shown in Figs. 184 and 186. x 12,000.

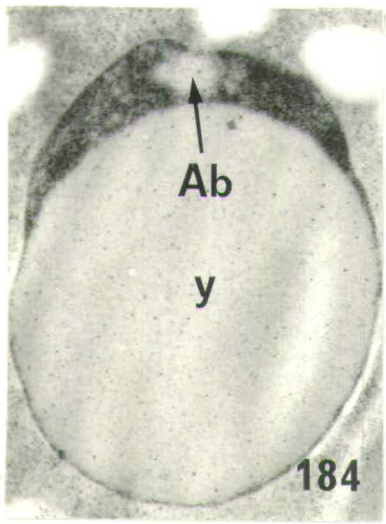
Fig. 186. A similar field to Fig. 184 to show the heavy deposition of silver over the superficial layer of the yolk platelet. Note that the associated body is not stained. x 12,000.

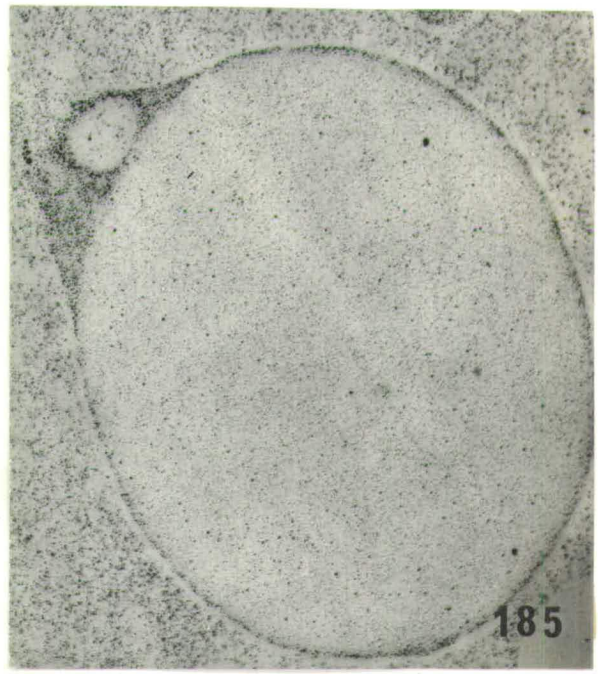
Fig. 187. Part of the follicle cell cytoplasm from a stage 10 ovarian chamber. Note the heavy deposits of silver over the dense bodies (db). There is a high background over the rest of the follicle cytoplasm perhaps due to the retention of the fixative by the tissue. x 12,000.

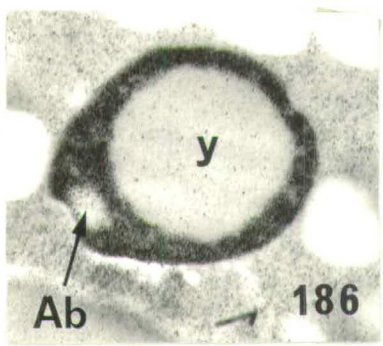
Fig. 188. The central ooplasm from an early stage 8 ovarian chamber. The inclusions present in the ooplasm are those described previously (see Figs 22-26) as intermediate between yolk platelets and lysosomal derivatives. Note that deposits of silver occur only over the lysosomal part of these inclusions (see arrows). The yolk-like area is devoid of such deposits. x 12,000.

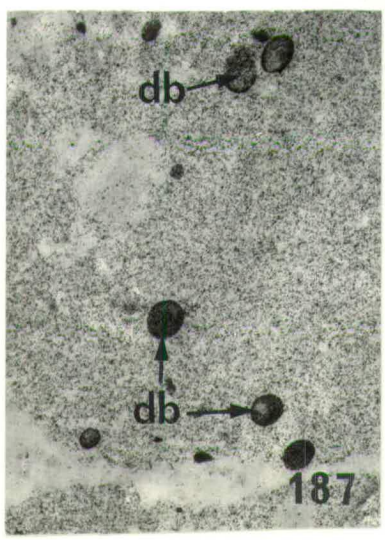

Ab
y
182

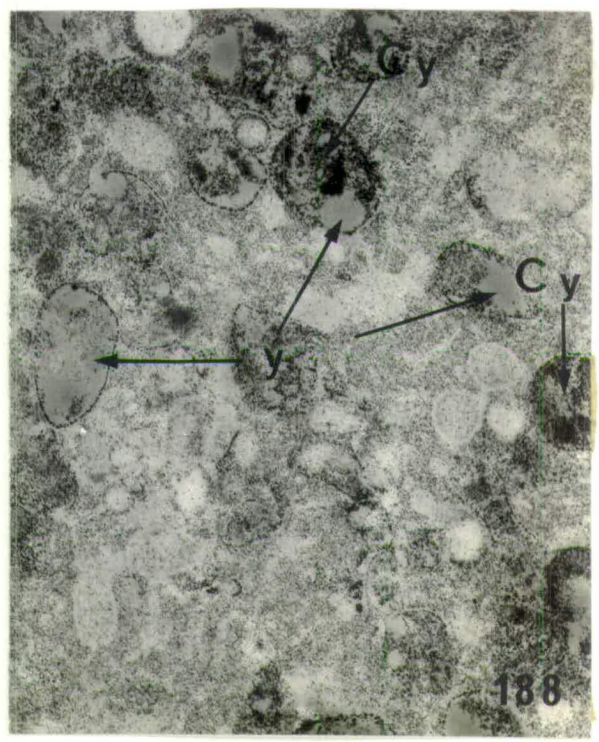

mb
183


Ab
y
184


185


y
Ab
186


db
db
187


C y
C y
y
188

(c) Observations following the use of periodic acid - thiosemicarbazide - silver proteinate technique.

In this method, the aldehyde groups of polysaccharides released by oxidation with periodic acid are coupled with thiosemicarbazide and this is made visible in the electron microscope as deposits of silver after reaction with silver proteinate. According to Thiery (1967), a 30-40 minutes exposure to thiosemicarbazide reveals glycogen, whereas prolonged exposures of 48 and 72 hours reveal mucopolysaccharides and glycoproteins respectively. The duration of the treatment with periodic acid and silver proteinate in all these instances remains constant at about 30 minutes.

When the method was applied to vitellogenic chambers (stages 8 to 12) in the ovary for a period of 30-40 minutes or 48 hours, no silver deposits were noticed in any of the cells in these chambers. When the exposure to thiosemicarbazide was prolonged to 72 hours, the pattern of silver deposition was essentially similar to that after the PTA method especially over the yolk platelets. In the alpha-1 yolk platelets, the silver deposits are confined to the associated body (Figs 190 and 191). In the columnar follicle cells there was a suggestion of labelling only along the membrane of certain rounded bodies in the cytoplasm as was also observed (Fig. 177) in the PTA-treated material.

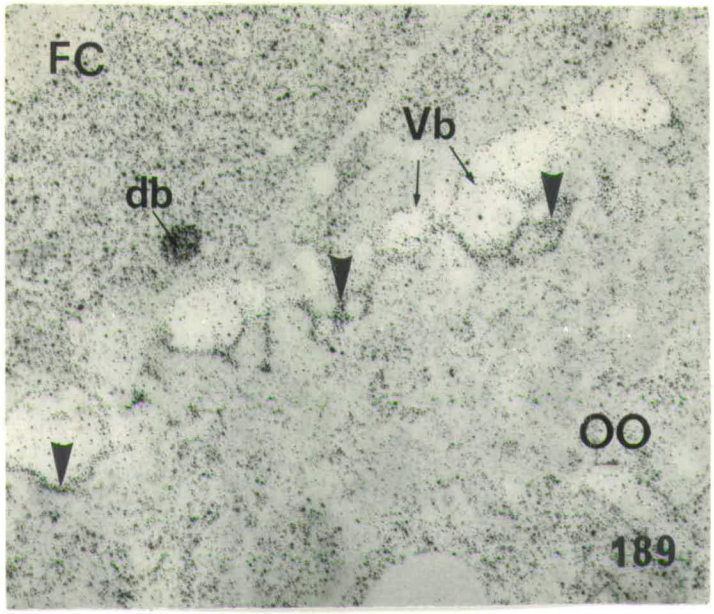
In chamber stages 13-14 exposed to thiosemicarbazide for a short period (up to 1 hour), the only entity exhibiting the silver was the be -spheres (Fig. 40a). The conclusion then is that the beta-spheres

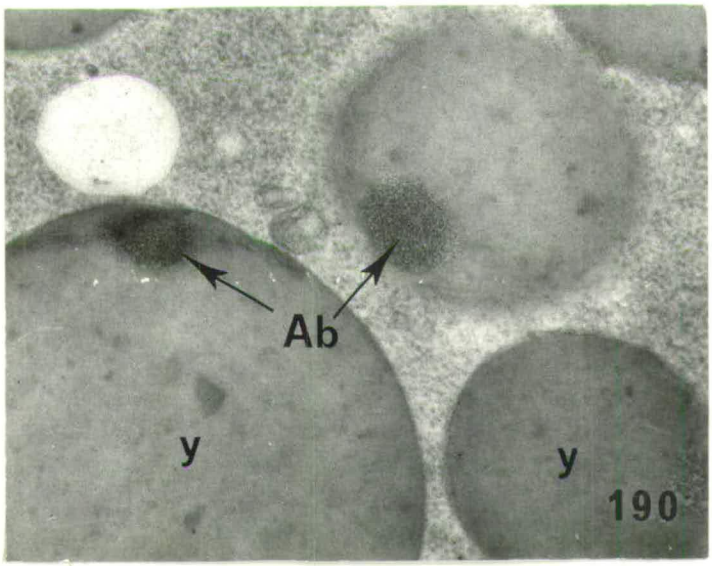
Fig. 189. The follicle-oocyte border from a stage 9 ovarian chamber. Note the silver deposits along the oocyte surface (see arrows). The vitelline bodies (Vb) are not stained by this technique. (db) dense body in the follicle cell cytoplasm. The background in preparation is rather high presumably due to retention of the aldehyde groups of the fixative. x 12,000.

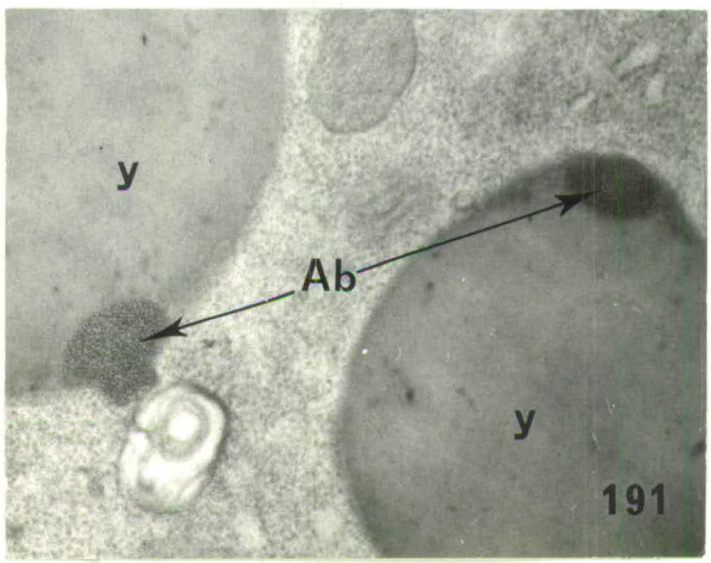
Figs. 190-191. Ovarian chambers double fixed in glutaraldehyde and osmium tetroxide and embedded in Epon-Araldite mixture. Ultra-thin sections were treated according to the procedure of Thiery (1967).

Fig. 190. Alpha-1 yolk platelets in the central ooplasm of a stage 10 ovarian chamber. Note the heavy deposits of silver on the associated body (Ab); the rest of the superficial layer is not stained by this technique. x 32,000.

Fig. 191. An area similar to that shown in Fig. 190. x 32,000.


FC
Vb
db
OO
189


Ab
y
y
190


y
Ab
y
191

contain glycogen. Control experiments made by omitting the periodic acid oxidation step did not yield silver deposits in any location in any of the aforementioned experiments. During the course of this set of experiments I also noted that if the distilled water rinse after periodic acid oxidation was prolonged, no silver deposits occurred. This was presumably because the aldehyde groups were removed by the prolonged wash and, therefore, particular care was taken during this step so as to ensure reproducible results.

3. The Golgi apparatus and yolk platelets.

There are several reports in the literature based on light microscope studies, on the presence of acid phosphatase in the oocytes of several insects (Cone and Eschenberg, 1966; Hopkins and King, 1966; Mahowald, 1972a). It has also become clear that this enzyme is synthesized in the ribosomes associated with the endoplasmic reticulum and concentrated in membrane-bounded bodies in the region of the Golgi apparatus (Novikoff, 1963). The possible association between yolk platelets and the Golgi apparatus was therefore examined at the ultrastructural level in the ovarian chambers of Drosophila melanogaster.

(a) Observations following the use of the Gomori method for the cytochemical detection of acid phosphatase. In 1939, Gomori and Takamatsu independently presented a method for the demonstration of acid phosphatase in biological preparations at the light microscope level. The demonstration of this enzymatic activity is

Figs. 192-201, 203 and 205. Ovarian chambers fixed in glutaraldehyde and incubated in the Gomori medium before the post-fixation in osmium tetroxide. The embedment was in Epon-Araldite mixture.

Fig. 192. Part of the cytoplasm of a follicle cell from a stage 9 ovarian chamber. Lead deposits are visible in the cisternae of the Golgi apparatus(G). x 32,000.

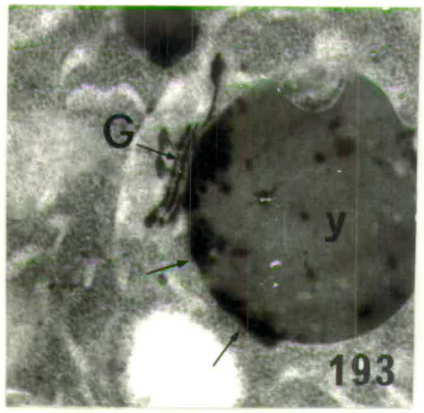
Fig. 193. A growing yolk platelet (y) in the cortical ooplasm of a stage 9 ovarian chamber. Lead deposits are visible over the margin of the yolk platelet (see arrows). The Golgi apparatus (G) closely associated with the yolk platelet also has heavy deposits of lead. x 32,000.

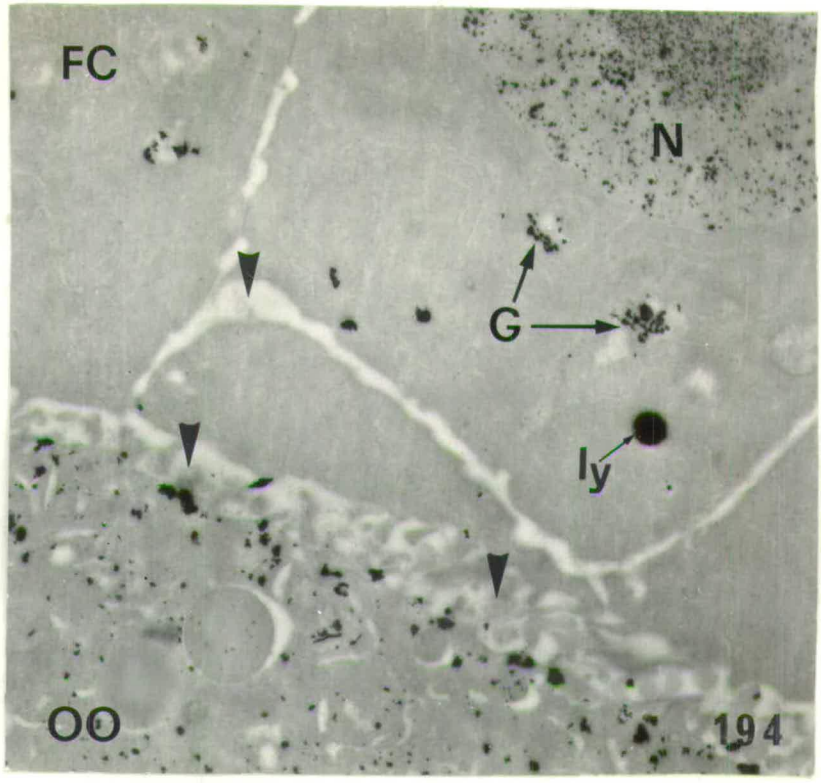
Fig. 194. The follicle-oocyte border from a stage 8 ovarian chamber. The Golgi apparatus (G) and the nucleus of the follicle cells appear very well labelled with lead. Heavy deposits also occur in the ooplasm (OO). Note that lead deposits are not present in the follicle inter-spaces (arrows). (ly) lysosomes. Section unstained. x 8,000.

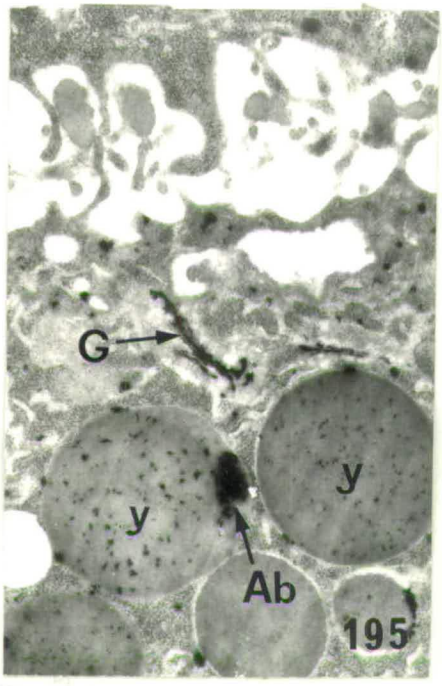
Fig. 195. The cortical ooplasm from a stage 9 ovarian chamber. Lead deposits occur throughout the yolk platelets (y), but the associated body (Ab) shows very heavy deposits of lead. Note the Golgi apparatus (G) near the yolk platelets which also contains a fairly heavy deposits of lead. x 16,000.

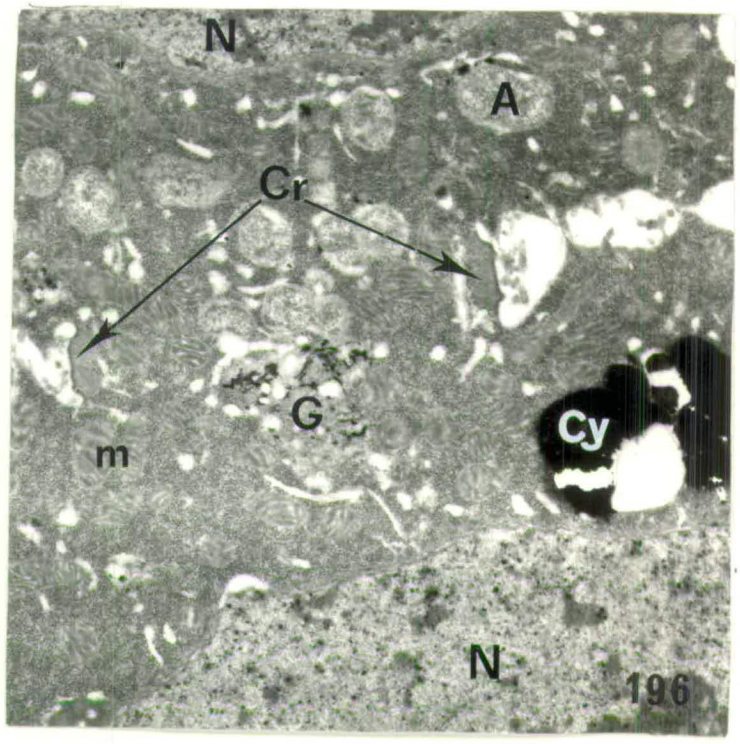
Fig. 196. A ring canal between two adjacent nurse cells from a stage 7 ovarian chamber. A Golgi apparatus (G) is visible within the ring canal (Cr, canal rims). A large cytolysosome (Cy) is loaded with very heavy deposits of lead. (A) Rickettsia-like bodies or A bodies; mitochondria (m); (Cr) canal rims. x 8,000.


G
192


G
y
193


FC
N
G
ly
OO
194


G
y
y
Ab
195


N
A
Cr
G
m
Cy
N
196

obtained by incubating the tissue in a buffered solution at pH 5 and containing compounds that can react with products of the enzymatic reaction. The reaction products so formed are subsequently visualized by their colour. Holt and Hicks (1961) then adapted this technique for electron microscopy where the reaction product is visualized as an electron dense deposit.

The Golgi apparatus in nurse cells, follicle cells and oocyte of previtellogenic chambers show deposits of lead; in nurse cells and the oocyte lead deposits are also present over areas corresponding to cytolysosomes. In follicle cells the primary lysosomes also have very heavy deposits of lead.

The number of the Golgi apparatus in the ooplasm is considerably more after the initiation of vitellogenesis, and it was earlier described (page 67) that this increase in number is brought about by migration of Golgi apparatus from nurse cells to the ooplasm through the ring canals. Association between Golgi apparatus and yolk platelets has been frequently met with during active vitellogenesis (stages 8-10) and a typical instance is shown in Fig. 193. It may be noted that lead deposits not only fill the cisternae of the Golgi apparatus but also are present within the yolk platelets. Another instance like the one above, in the cortical ooplasm of a stage 9 chamber resulting from the Gomori procedure is shown in Fig. 195. Apart from the Golgi apparatus, the reaction products are present in the yolk platelets and the associated body shows particularly heavy deposits of lead. A study of a large number of preparations gives the impression that the

amount of lead deposits, in the yolk platelets is variable and that it is heavier in the smaller yolk platelets (Figs. 197 and 199). Most probably this is related to the stage of growth of the yolk platelets. The smaller the yolk platelet the more intense the acid phosphatase reaction (Fig.199). As the platelet increases in size the lead deposits seem to get displaced towards the periphery as may be seen in Fig. 197. In a fully grown yolk platelet (alpha-1) the acid phosphatase reaction is restricted to the associated body (Fig. 200).

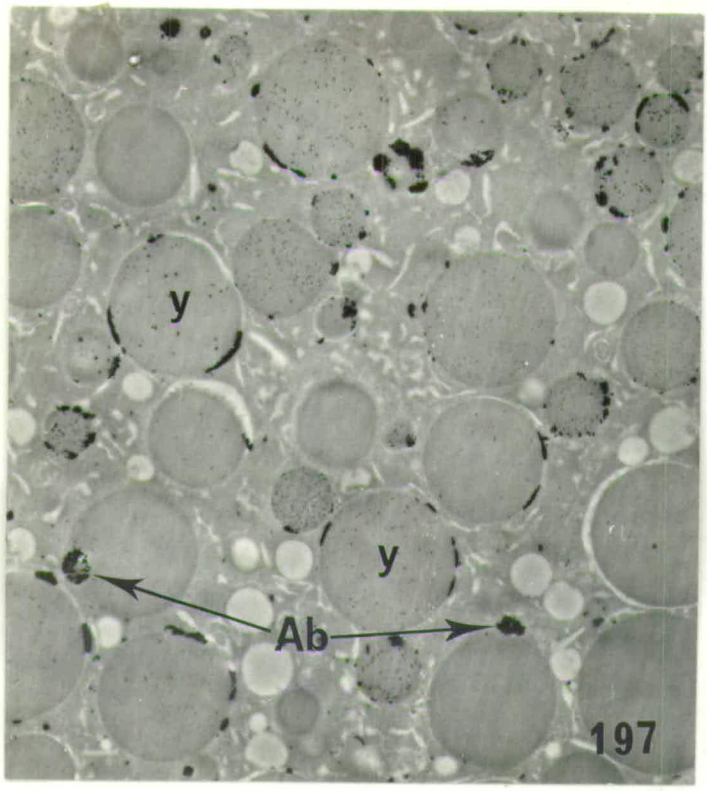
As already stated, yolk platelets have, on several occasions, been observed in intimate association with the Golgi apparatus. This observation provides a clue as to the manner in which the yolk platelets may come to acquire some of their acid phosphatase activity. It is also possible, however, that some of the enzyme may have been brought in from the residual bodies (cytolysosomes) of the nurse cells or fusion of such bodies with the growing yolk platelets. Morphological evidence of the appearance of yolk-like material in association with the cytolysosomes of previtellogenic stages were presented earlier (page 67 and Figs. 22 and 23). A group of residual bodies in the ooplasm of early stage 8 containing very heavy lead deposits in shown in Fig. 198. When such group of organelles are observed in close association with yolk platelets, the lack of structural clarity in the preparations made for enzyme localization, precludes a definite interpretation of these as residual bodies.

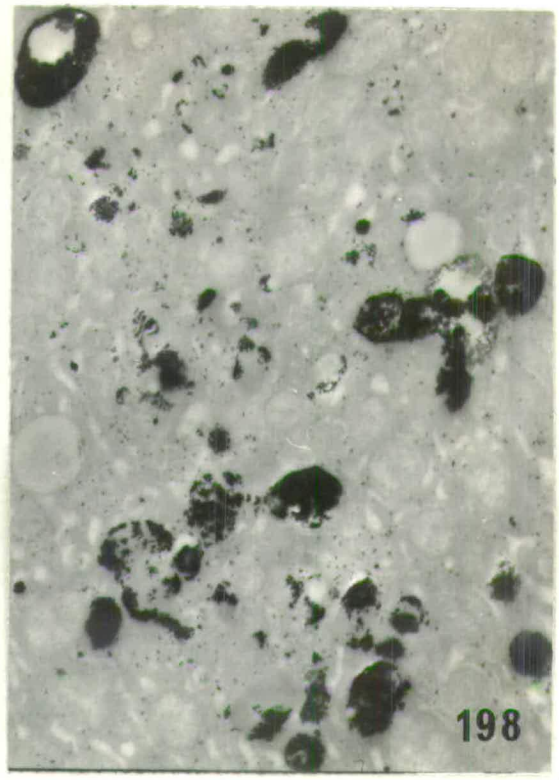
Fig. 197. A low power micrograph of part of the ooplasm from a stage 9 ovarian chamber. Several yolk platelets (y) with lead deposits are visible in the area. Note that generally the amount of lead tends to be greater in the smaller yolk platelets. Some heavy labelled associated bodies (Ab) are also discernible. Section unstained. x 3,000.

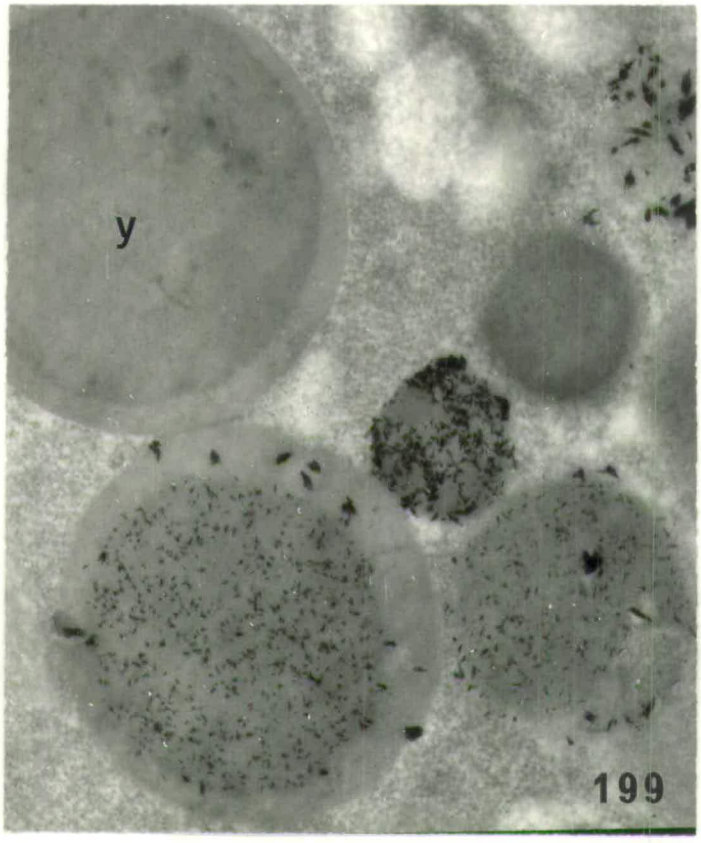
Fig. 198. Part of the ooplasm from an early stage 8 ovarian chamber. The heavy deposits of lead are most probably in cytolysosomes corresponding to those shown in Figs. 22-23. Unstained section. x 8,000.

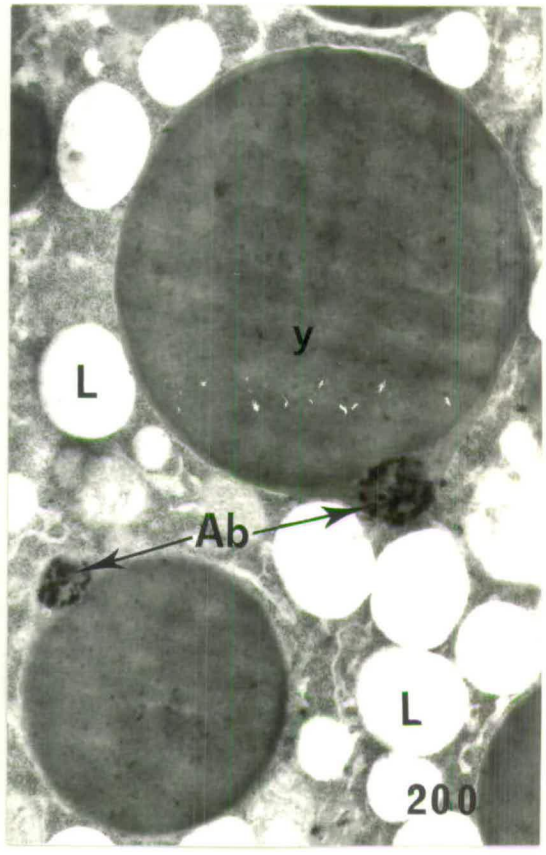
Fig. 199. Yolk platelets of various sizes in the central ooplasm of a stage 9 ovarian chamber. Note the amount of lead deposits in yolk platelets of different sizes. Unstained section. x 20,000.

Fig. 200. Two alpha-1 yolk platelets in the central ooplasm of a stage 10 ovarian chamber. Note the particularly heavy deposits of lead in the associated body (Ab). x 16,000.


y
y
Ab
197


198


y
199


y
L
Ab
L
200

(b) Artifactual localization of lead deposits in preparations processed for acid phosphatase

When ovarian chambers were incubated in the complete Gomori medium, lead deposits appeared in the nuclei of both nurse and follicle cells. The amount of deposits varied from nucleus to nucleus (Figs 194 and 201). A similar observation was also made in control preparations incubated in substrate-free medium.

A number of earlier investigators have noted such deposits (for example, Kessel and Decker, 1972) but the consensus at the present time (Cornelisse, 1974) appears to be that these are artifacts.

Another location where lead deposits appeared in control preparations was along the vitelline membrane (Fig. 203). Such an artifactual deposition of lead was also noted by Kessel and Decker (1972) in oocytes of Rana. In ovarian chambers of Drosophila, however, this particular artifact may be avoided by prolonging the rinse for several days after the incubation in substrate-free medium (this procedure was suggested to me by Dr. A. Jurand).

Another area of artifactual localization in the present material in the interspaces between the various cell types, i.e. between follicle cells (Fig. 201), between the follicle cells and the oocyte (Fig. 205), especially at the anterior region of the oocyte where the vitelline membrane is incomplete, and finally between the follicle and nurse cells (Fig. 201).

During the course of this study, I have found that the occurrence of these deposits in the interspaces between the various cell types is dependent

on the temperature at which the tissue was fixed in glutaraldehyde. If ovarian chambers were fixed immediately after dissection and kept at ice cold temperature (0 °C) throughout the period of fixation the interspaces mentioned above did not show any deposits of lead (Fig. 194). If, on the other hand, fixation was delayed and the temperature of the fixative was above 4 °C, the deposits in question invariably occurred. It would therefore seem that the lead deposits formed are really due to enzymatic activity but that their atypical location is due to displacement or translocation of the enzyme due to faulty fixation.

The absence of lead deposits in the interspaces between cells in tissues fixed properly indicate that there is no movement of enzymes between cells along their plasma membranes. In particular it would appear that the follicle cells do not supply the enzyme to the nurse cells or to the oocyte.

(c) Observations following the use of Novikoff and Goldfischer's method for the cytochemical detection of thiamine pyrophosphatase.

According to Novikoff and Goldfischer (1961) the enzyme thiamine pyrophosphatase may be taken as a cytochemical marker for Golgi apparatus as well as for lysosomes. When ovarian chambers were incubated in the appropriate medium for the detection of this enzyme, no lead deposits were formed either in the nurse cells or in the ooplasm. Lead deposits were, however, formed extensively in what appeared to be lysosomes (Figs. 202, 204 and 206) in follicle cells. A few of the Golgi

apparatus in these cells also appeared to show a positive reaction product, but there were several Golgi profiles where such a reaction could not be detected. In those instances where reaction products were present, they were confined to a few cisternae.

In control experiments the lysosomes were completely devoid of lead deposits and there was no trace of any lead on any Golgi apparatus. Occasionally, some lead deposits were noticed along the vitelline membrane and the interspaces between the various cell types, as described when dealing with the localization of acid phosphatase.

The sites of localization of thiamine pyrophosphatase observed in the ovarian chambers raise several questions. Earlier experiments have shown that Golgi apparatus are present in the ooplasm and that they contain acid phosphatase. It may therefore be asked if the indicated absence of thiamine pyrophosphatase activity in the ooplasm is due to a permeability barrier which prevents access of the substrate or whether it simply reflects a physiological condition with a very low level of the enzyme. The first possibility, although it cannot be ruled out completely, seems unlikely. This is because the substrate for thiamine pyrophosphatase has a molecular weight which is close to that used for acid phosphatase and my experiments (already described) show that the ooplasm is not impermeable to the substrate employed for the demonstration of acid phosphatase. A plausible suggestion then is that, although all cell types in the ovarian chambers are accessible to the substrate for thiamine pyrophosphatase, only the follicle

Fig. 201. The follicle-nurse border from a stage 8 ovarian chamber fixed in glutaraldehyde at a temperature of 4 °C. Note the presence of lead deposits in the interspaces between the follicle cells and between the follicle and nurse cells (FC and NC respectively). Compare with Fig. 194 which is an ovarian chamber fixed in glutaraldehyde at 0°C before incubation in the Gomori medium. (G) Golgi apparatus; (N) nucleus; (no) nucleolus. x 12,000.

Fig. 202, 204 and 206. Ovarian chambers fixed in glutaraldehyde and incubated according to the lead salt method of Novikoff and Goldfischer (1961) to show the presence of thiamine pyrophosphatase.

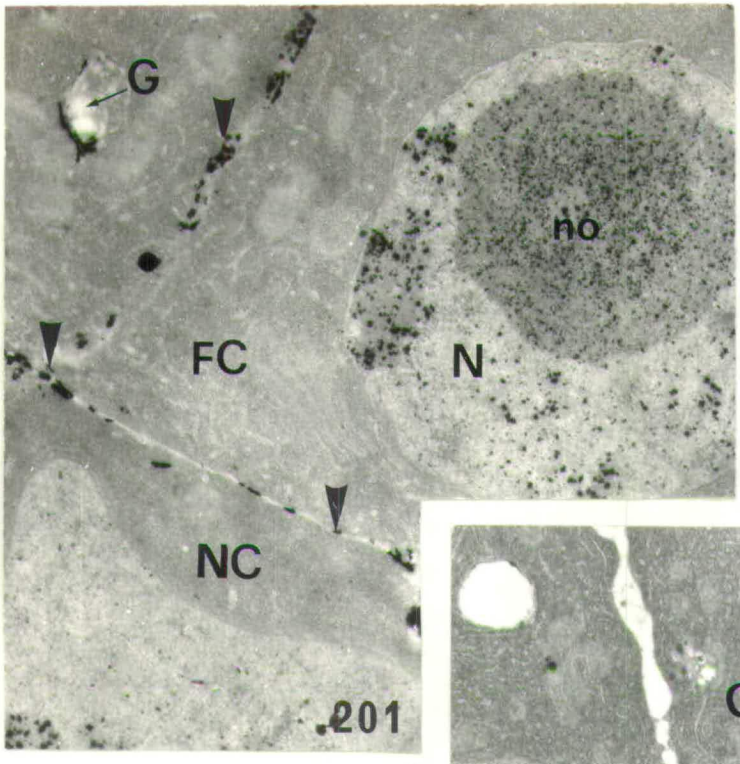
Fig. 202. Part of the cytoplasm of a follicle cell from a stage 10 ovarian chamber. The heavy deposits of lead occur on a lysosome. x 20,000.

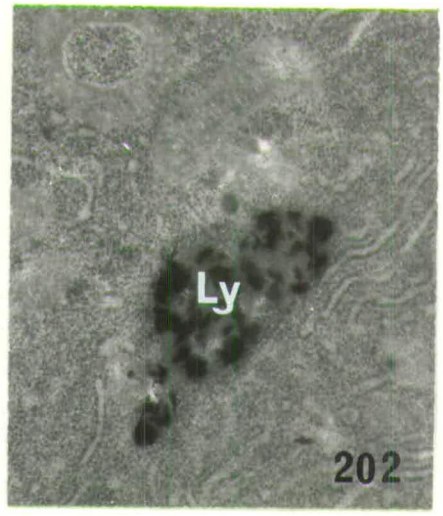
Fig. 203. The follicle-oocyte border from a stage 9 ovarian chamber, incubated in the substrate-free Gomori medium (devoid of b-glycero-phosphate). x 8,000.

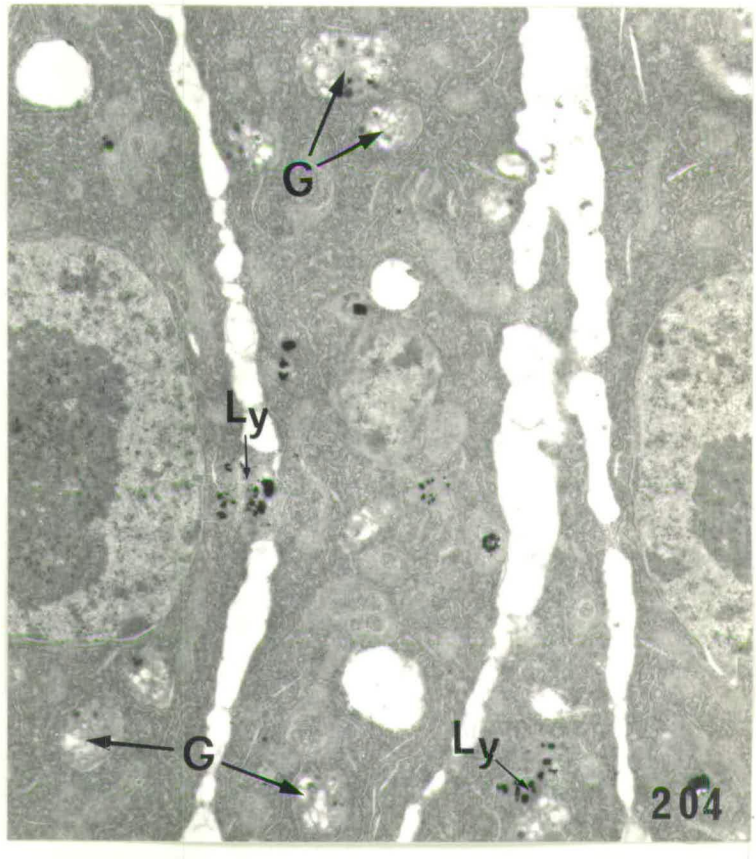
Fig. 204. A low power micrograph of part of the follicle layer from a stage 9 ovarian chamber. Golgi apparatus (G) are visible in several areas of the cytoplasm, but deposits of lead occur only over the lysosomes (Ly). x 5,000.

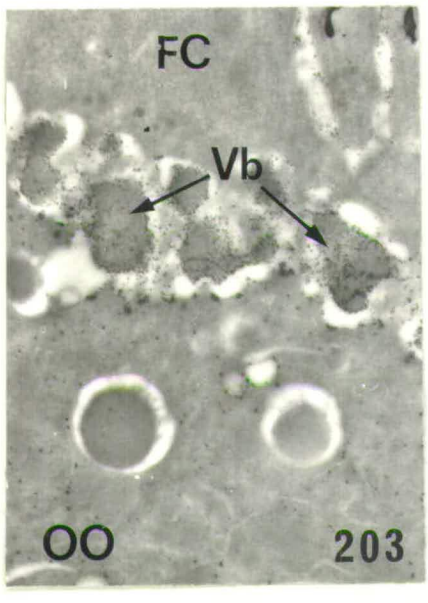
Fig. 205. The follicle-oocyte border from a stage 9 ovarian chamber incubated in the substrate-free Gomori medium (devoid of b-glycerophosphate). Heavy deposits of lead occur along the border between the follicle cells and the oocyte. (FC) follicle cells; (OO) oocyte. x 8,000.

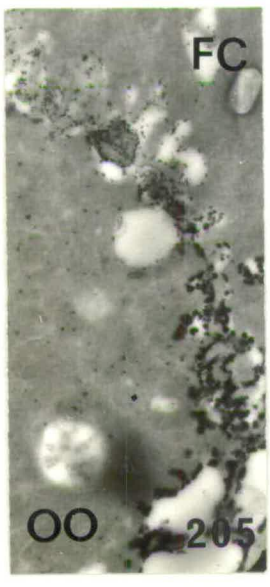
Fig. 206. Follicle cells (border cells) at the anterior region of a stage 10 oocyte. Note that several lysosomes contain deposits of lead. x 5,000.

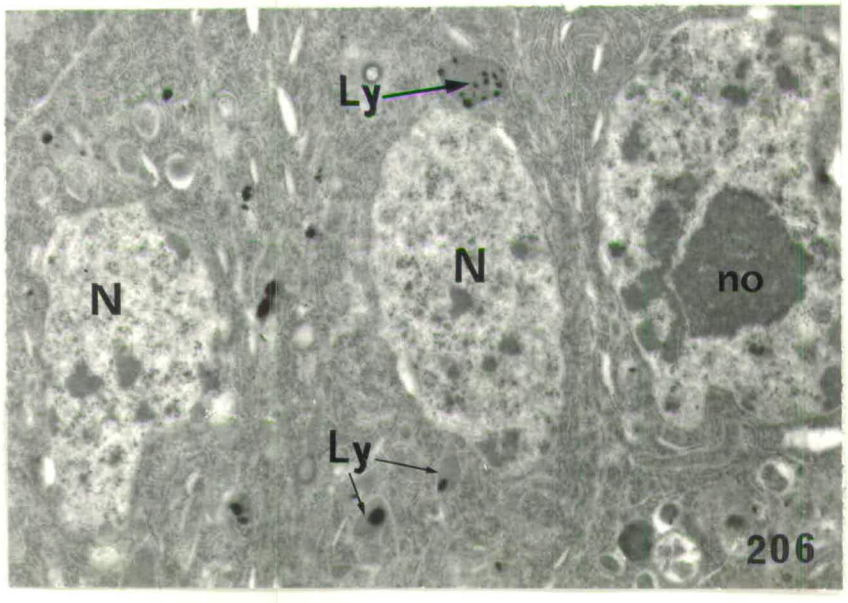

G
no
FC
N
NC
201


Ly
202


G
Ly
G
Ly
204


FC
Vb
OO
203


FC
OO
205


Ly
N
N
no
Ly
206

cells have a sufficient level of thiamine pyrophosphatase activity that is cytochemically demonstrable by the procedure employed.

(d) Observations following the use of osmium tetroxide - zinc iodide technique.

The lack of ultrastructural clarity in preparations of ovarian chambers treated for the cytochemical detection of acid phosphatase makes it sometimes difficult to establish the origin of the enzyme in the yolk platelets. It is well known, on the other hand, that the lysosomal enzymes are released from the Golgi apparatus by means of membrane-bound vesicles. It seemed therefore desirable to try and establish the identity of the Golgi apparatus and the vesicles derived from it by other cytochemical means. One such approach is the osmium tetroxide - zinc iodide method (OZI). Although the mechanism of the reaction involved is not known, it has been shown to be a fairly reliable stain for the Golgi apparatus in various tissues (Elias et al., 1972; Niebauer et al., 1969). This method was therefore applied to ovarian chambers of Drosophila.

In previtellogenic chambers fixed for 4 hours in the osmium tetroxide - zinc iodide complex at pH 7.0, the Golgi apparatus of every cell in the ovarian chamber appeared to contain several electron dense deposits (Fig. 209). As described earlier in the section dealing with the ultrastructure of developing chambers (see page 83), the Golgi apparatus of both nurse and follicle cells during these stages is mainly located in the perinuclear cytoplasm, and consists mainly of vesicles

of various sizes. An instance of such an apparatus in one of the present preparations is shown in Fig. 209. It can be clearly seen that the vesicles only on the forming face of the Golgi apparatus contain electron dense deposits.

The follicle cells and the oocytes in vitellogenic chambers contain a far greater number of Golgi apparatus and they all contain dense OZI deposits (Figs. 207, 210 and 212).

In vitellogenic stages, the Golgi apparatus in nurse cells no longer occupy the perinuclear cytoplasm and they do not increase in number as in the follicle cells. In view of the fact that profiles of Golgi apparatus have been frequently seen within ring canals, it is likely that these organelles are continuously transferred from the nurse cells to the ooplasm. This would account for the decrease in the number of Golgi apparatus in nurse cells and their accumulation in the ooplasm.

In ovarian chambers of early stage 8, OZI deposits are present (Fig. 207) within organelles which were referred to as cytolysosomes in an earlier section (see Figs. 22-26). These entities were then assumed to represent an intermediate stage between lysosomes and yolk platelets.

In vitellogenic chambers (stages 8-10) the OZI deposits are most frequently observed within the superficial layer of the growing yolk platelets in the cortical ooplasm (Figs. 211 and 213). Also in this region, the Golgi apparatus occur frequently in close association with the growing yolk platelets (Figs. 211 and

Figs. 207-214. Ovarian chambers fixed in osmium tetroxide - zinc iodide (OZI) complex at pH 7.0 for 4 hours and embedded in Epon-Araldite mixture.

Fig. 207. The central ooplasm from a stage 8 ovarian chamber. The Golgi apparatus (G) contain heavy deposits of OZI. The organelle on the left of the micrograph exhibits a yolk-like body embedded within an asymmetrical layer which contain scattered OZI deposits. These deposits are probably within the spherules described earlier in Fig. 22. x 12,000.

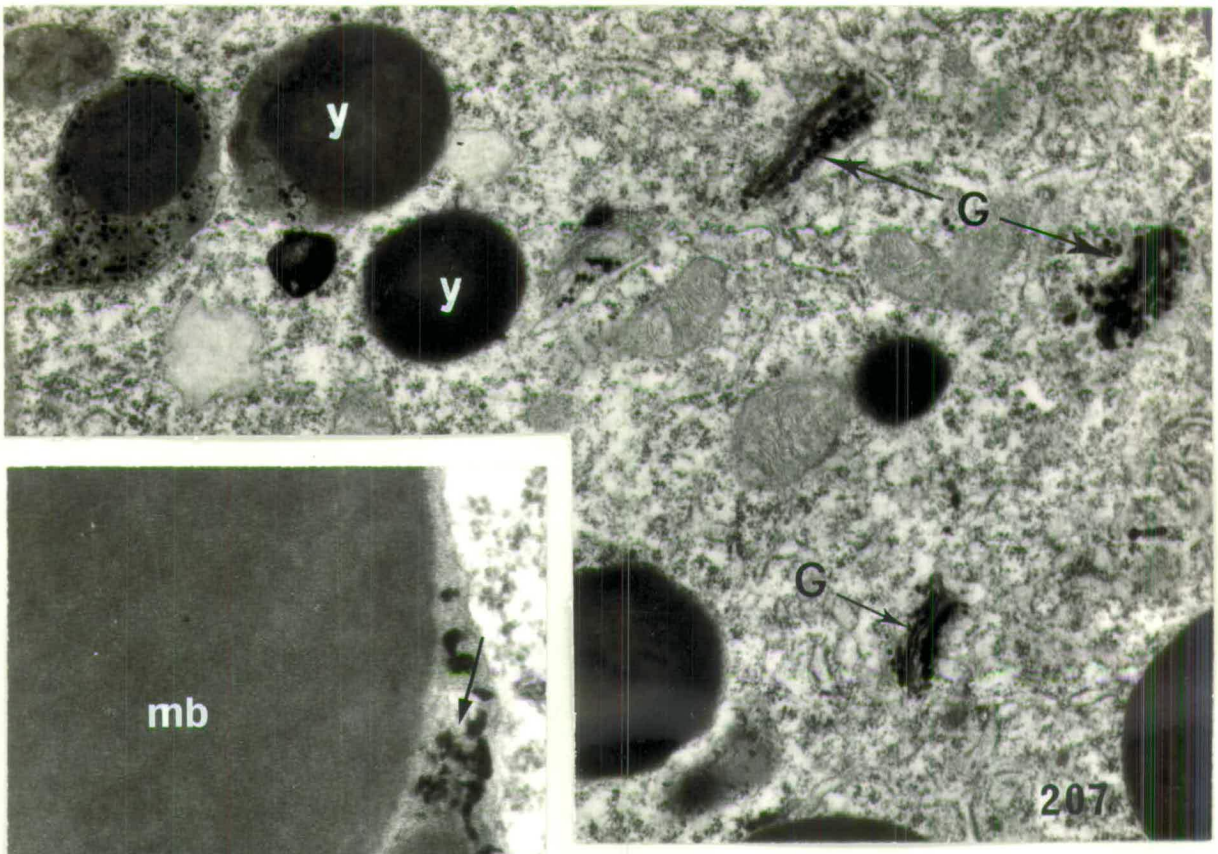
Fig. 208. Part of an alpha-1 yolk platelet from the central ooplasm of a stage 10 ovarian chamber. Note the spherules of the superficial layer with OZI deposits. The associated body (Ab) is free of OZI deposits. x 48,000.

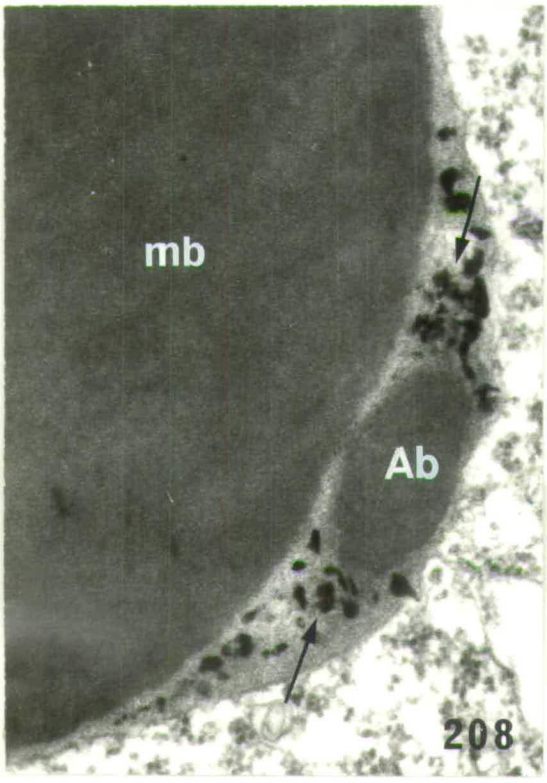
Fig. 209. A Golgi apparatus in the cytoplasm of a stage 4 follicle cell. Only vesicles are present and no trace of cisternae is discernible. OZI deposits occur only in the vesicles at the forming face of the Golgi apparatus (see arrows). Larger vesicles on the maturing side of the apparatus do not contain the deposits. x 40,000.

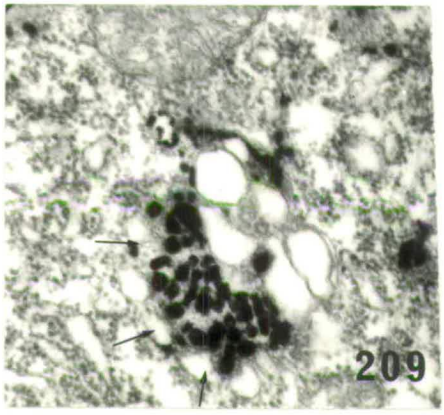
Fig. 210. Part of the cytoplasm of follicle cells from a stage 10 ovarian chamber showing several dense OZI-containing Golgi apparatus (G). (m) mitochondria; (A) rickettsia-like bodies. x 12,000.

Fig. 211. Part of the central ooplasm from a stage 9 ovarian chamber. Several spherules with OZI deposits occur within the superficial layer of the yolk platelets. Note the close association of the Golgi apparatus (G) and the yolk platelet at the top of the picture. x 12,000.

Fig. 212. The cortical ooplasm from a stage 9 chamber. A number of vesicles in the ooplasm possess OZI deposits (see arrows). The coated vesicles and the tubules, however, are free of OZI deposits. x 12,000.


y
y
G
G
207


mb
Ab
208


209

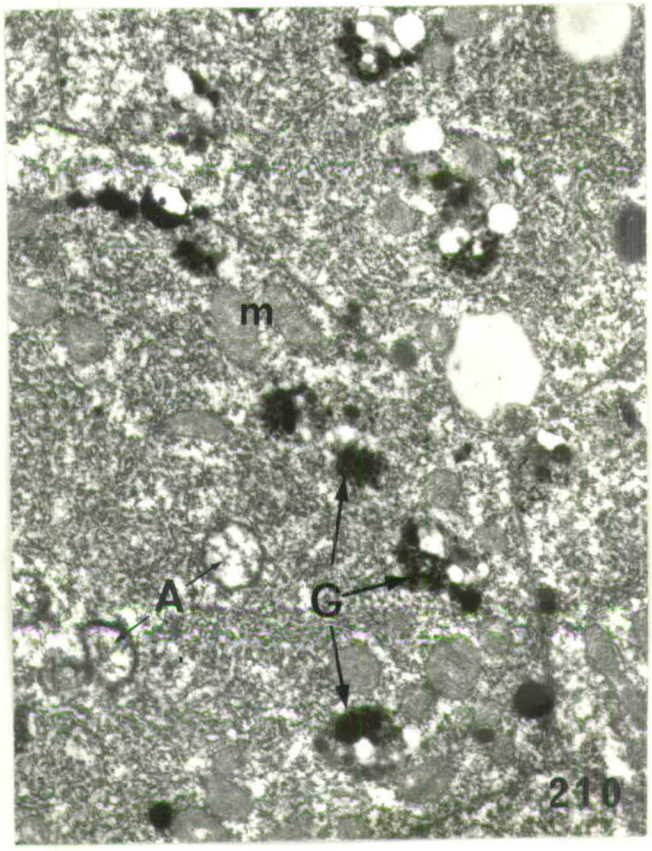

m
A
G
210

Fig. 213. High power micrograph of a stage 9 ovarian chamber showing a growing yolk platelet. Note the spherules with OZI deposits in the superficial layer of the yolk platelet (y). x 48,000.

Fig. 214. The ooplasm of a stage 9 ovarian chamber showing a growing yolk platelet in close association with a Golgi apparatus (G) which contains plenty of OZI deposits. x 20,000.

Figs. 215-219. Ovarian chambers dissected from flies injected with concanavalin-peroxidase conjugate and fixed after varying intervals of time in glutaraldehyde and then in osmium tetroxide.

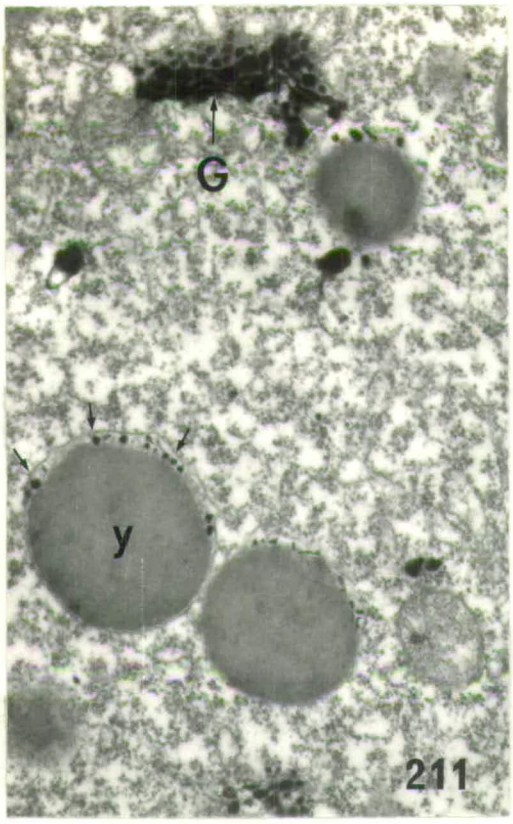
Fig. 215. The oocyte surface from a stage 9 ovarian chamber fixed 30 minutes after the injection of the conjugate. A pit (p) and few coated vesicles (cv) in the cortical ooplasm are clearly labelled. Unstained section. x 20,000.

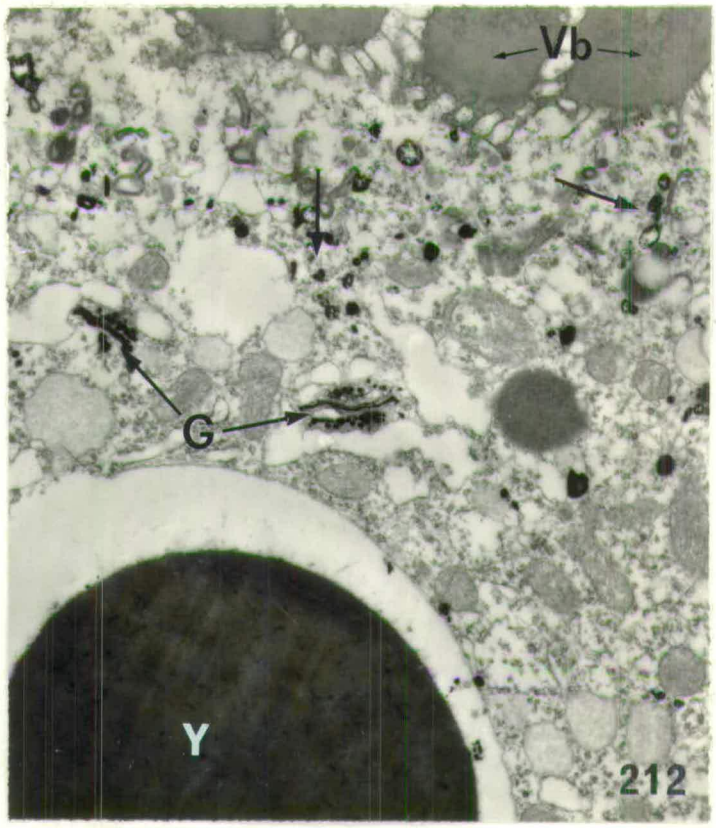
Fig. 216. A similar field to that shown in Fig. 215 from a chamber fixed 30 minutes after the injection of the conjugate. Several tubular profiles of the cortical ooplasm are labelled. Unstained section. x 16,000.

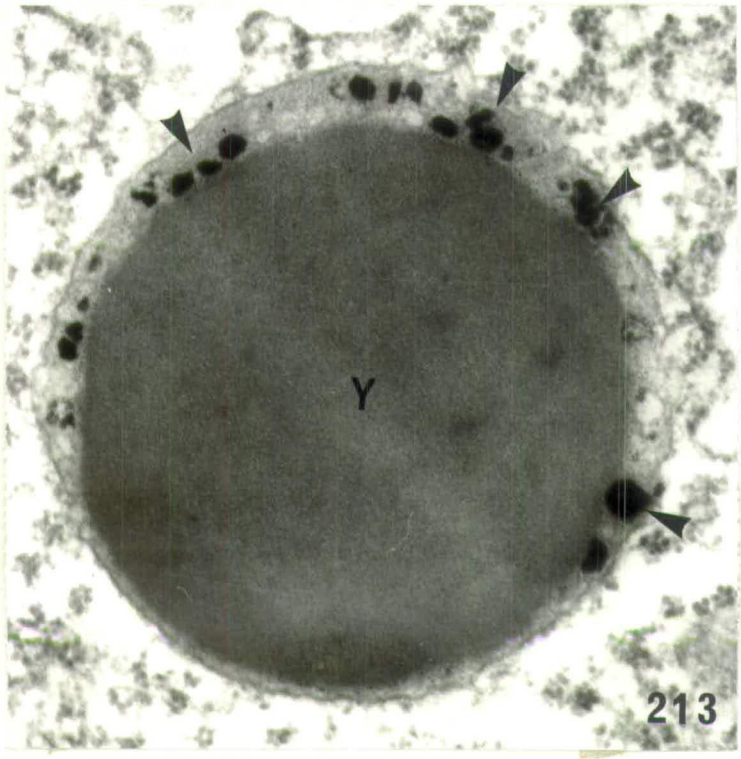
Fig. 217; A large yolk platelet (y) in the central ooplasm of a stage 9 ovarian chamber fixed 4 hours after the injection of the conjugate. Note the labelled superficial layer (Sl). Unstained section. x 32,000.

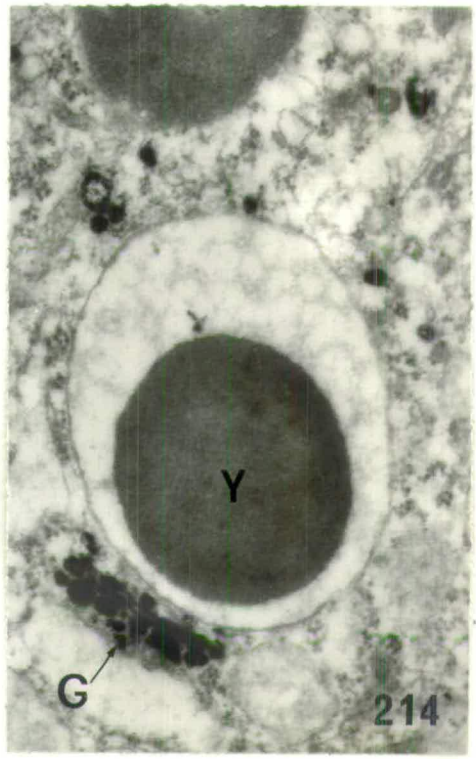
Fig. 218. The cortical ooplasm of a stage 9 ovarian chamber fixed 30 minutes after injection of the conjugate. The label in the small sized yolk platelet is in the space between the limiting membrane and the main body (see arrows) of the yolk platelet. Unstained section. x 8,000.

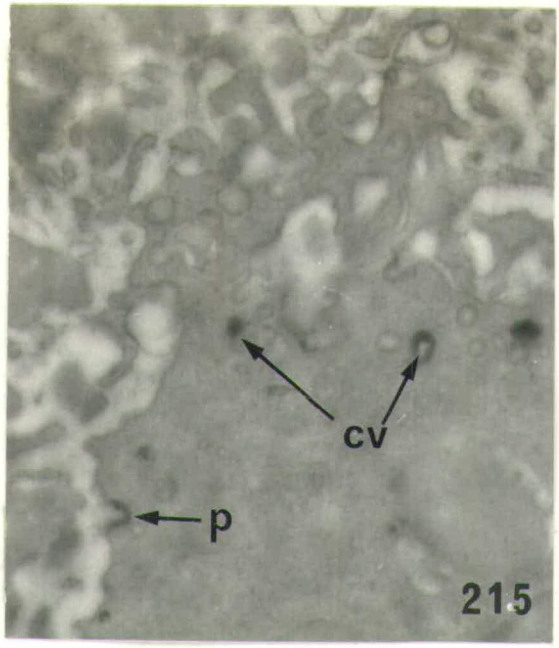
Fig. 219. A field similar to that shown in Fig. 218. x 8,000.

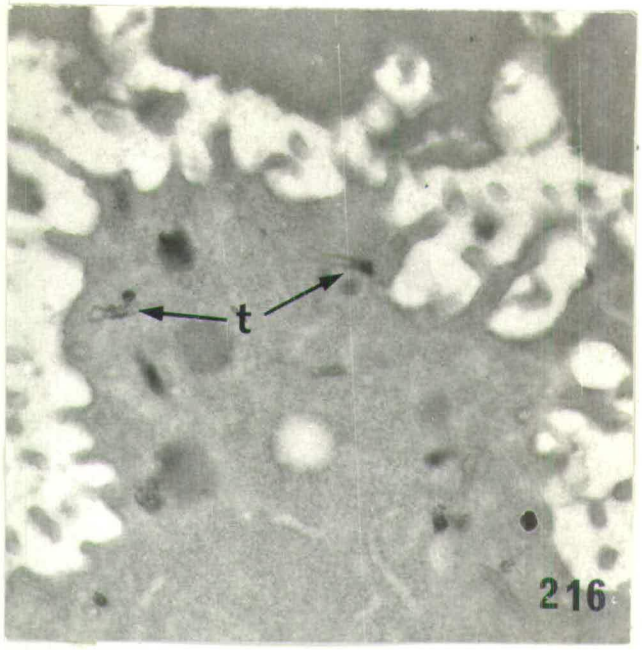

G
y
211

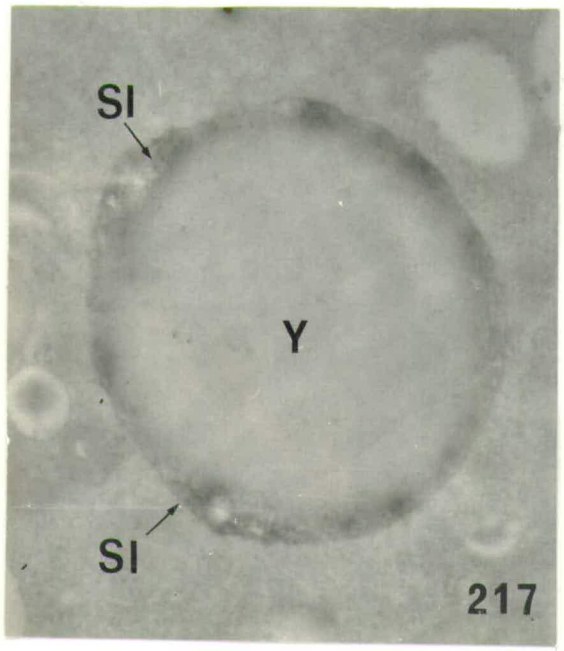

Vb
G
Y
212

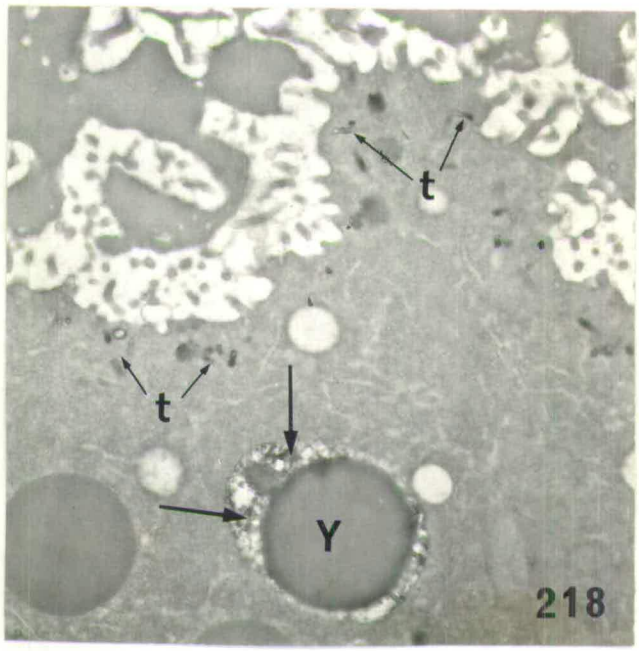

Y
213

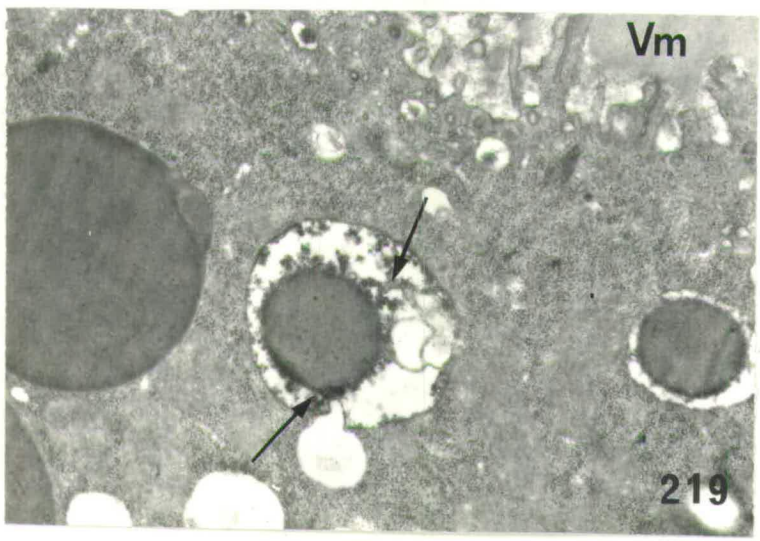

Y
G
214


cv
p
215


t
216


Sl
Y
Sl
217


t
t
Y
218


Vm
219

214).

In fully grown yolk platelets (alpha-1 yolk platelets), which are usually found in the deeper regions of the ooplasm in stages 9-10 chambers, the OZI deposits are restricted to the cap-like region of the superficial layer which contains the associated body. The associated body itself, however, is free of OZI deposits (Fig. 208).

A number of small vesicles containing OZI deposits are present in the cortical ooplasm of vitellogenic chambers. These vesicles, however, are different from the coated vesicles also present in this region (Fig. 212).

4. The fuzzy coat at the pits of the oolemma: its fate and possible origin.

(a) Evidence from studies with concanavalin A (Con-A). Concanavalin is an agglutinin extracted from the jack bean (Concavalia ensiformis) and has been shown to interact only with polysaccharides possessing α-D-glucopyranosyl, α-D-mannopyranosyl and fructofuranosyl residues (Goldstein et al., 1965). Avrameas (1970) has demonstrated that Con-A has two sites both of which can react with sugars or glycoproteins. Bernhard and Avrameas (1971) found that carbohydrates of the plasma membrane surface may bind to one of the sites of the concanavalin molecule and that the remaining site can thus operate as an acceptor of another glycoprotein like peroxidase. The peroxidase so bound may finally be localized in the electron microscope by means of the diaminobenzidine (DAB) method of Graham and Karnovsky (1966). In the normal procedure, the tissue

is exposed *in vivo* to Con-A, fixed in glutaraldehyde and then exposed to peroxidase. This procedure could not be adopted in *Drosophila* ovary, because of the presence of the basement lamina which becomes impermeable to peroxidase after exposure to Con-A and fixation in glutaraldehyde. Therefore, it was necessary to expose the tissue to a concanavalin-peroxidase conjugate in the first place, i.e. before fixation in glutaraldehyde. A method has recently been developed (Nicolson and Singer, 1971) which permits the ultrastructural localization of Con-A binding sites following the use of a ferritin-Con-A conjugate. This procedure was adopted to prepare the Con-A peroxidase conjugate used in the present work. With the use of this conjugate it was hoped to trace any vesicles in the cortical ooplasm that may be derived from the oolemma, and in particular the contribution they made to the yolk platelets.

Fig. 215 shows the cortical ooplasm of a stage 9 ovarian chamber fixed half an hour after the injection of the concanavalin-peroxidase conjugate. In this micrograph it may be noted that the pits of the oolemma are clearly labelled; in addition, the coated vesicles and the tubules in the cortical ooplasm are also labelled (Fig. 216). In several yolk platelets of ovarian chambers similarly exposed *in vivo*, the conjugate can be detected in the area between the main body and the limiting membrane (Figs. 218 and 219).

In chambers exposed to concanavalin for longer periods (2 and 4 hours), yolk platelets larger than those just cited can be found with their superficial layer faintly labelled (Fig. 217).

(b) Evidence from studies on the localization of acyltransferase.

Acyltransferases catalyze the transfer of long chain of fatty acids from acyl-CoA derivatives resulting in the release of CoA. The enzymes are believed to play a role in the synthesis of phospholipids of cellular membranes and cellular sites where the enzymes are localized may be assumed to be involved in membrane biosynthesis. A cytochemical method, based on the precipitation of heavy metal salts at the site of release of CoA, has been recently developed (Higgins and Barrnett, 1971) for the localization of these enzymes. Using this procedure, it has been possible to detect the sites of synthesis of phospholipids in various cells of the ovarian chambers of Drosophila. In view of the intense pinocytotic activity which occurs at the surface of the oocyte during active vitellogenesis, one may expect to find a pattern of acyltransferases localization within this cell indicative of an intense turnover of cell membrane.

After appropriate treatment of the tissue, the reaction products were observed in Golgi complexes of follicle cells and ooplasm (Figs. 220, 221, 222 and 223). In the ooplasm, the deposits are situated very close to the yolk platelets (Figs. 222 and 223). The areas in question are most probably in contact with Golgi complexes. In control experiments done without the substrate, no reaction product was observed within any of these cells.

A suggestion that can be made in the light of the finding of this study of localization of acyl-

transferases is that the Golgi apparatus in the follicle cells and in the ooplasm are involved in membrane formation.

Occasionally, mitochondria in both ooplasm and follicle cells exhibited dense deposits, but deposits in these organelles also appeared in the control experiments without added substrate. This observation suggests that the acyltransferase activity is due to the existence of an endogenous substrate.

As in certain other cytochemical procedures, a major drawback of the method for detecting acyltransferases arises from the presence of endogenous substrates which may give rise to false localizations. It has been claimed (Higgins and Barrnett, 1971) that a preincubation in potassium ferricyanide followed by prolonged rinse would oxidize endogenous substrates and that any subsequent reaction resulting from this cytochemical procedure will indicate the site of CoA released by the enzyme as a result of an exogenous substrate. When this method was adopted, no deposits were found in the mitochondria of oocytes and follicle cells. The deposits described in the Golgi apparatus of ooplasm and follicle cells may, therefore, be considered as genuine indications of acyltransferase activity.

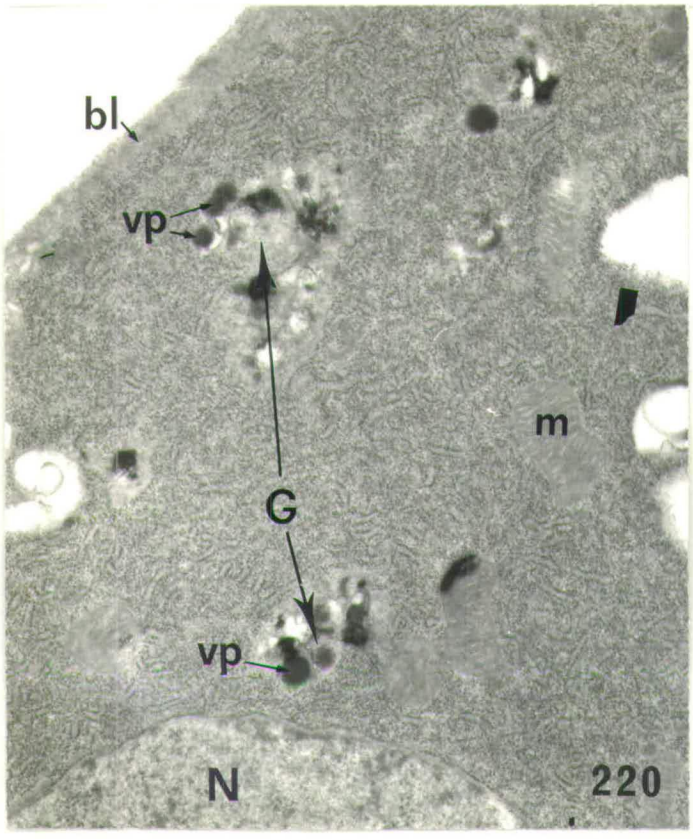
Figs. 220-223. Ovarian chambers fixed in glutaraldehyde and then incubated in a medium containing palmityl CoA, potassium ferricyanide, manganous chloride and alpha-glycerophosphate in cacodylate buffer at 37 °C for 2 hours.

Fig. 220. Part of the cytoplasm of a follicle cell from a stage 10 ovarian chamber. Note the reaction product on the Golgi apparatus (G). Vitelline membrane precursors (vp) are also visible in the Golgi apparatus. (m) mitochondria; (N) nucleus. x 12,000.

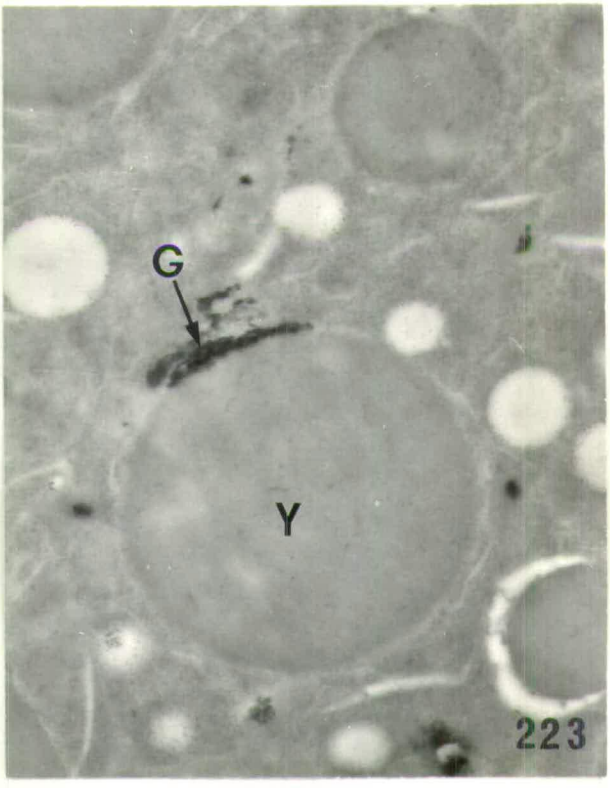
Fig. 221. Part of the cytoplasm of a follicle cell from a stage 7 ovarian chamber showing the reaction product on the Golgi apparatus. Note that reaction product does not occur on the endoplasmic reticulum. x 20,000.

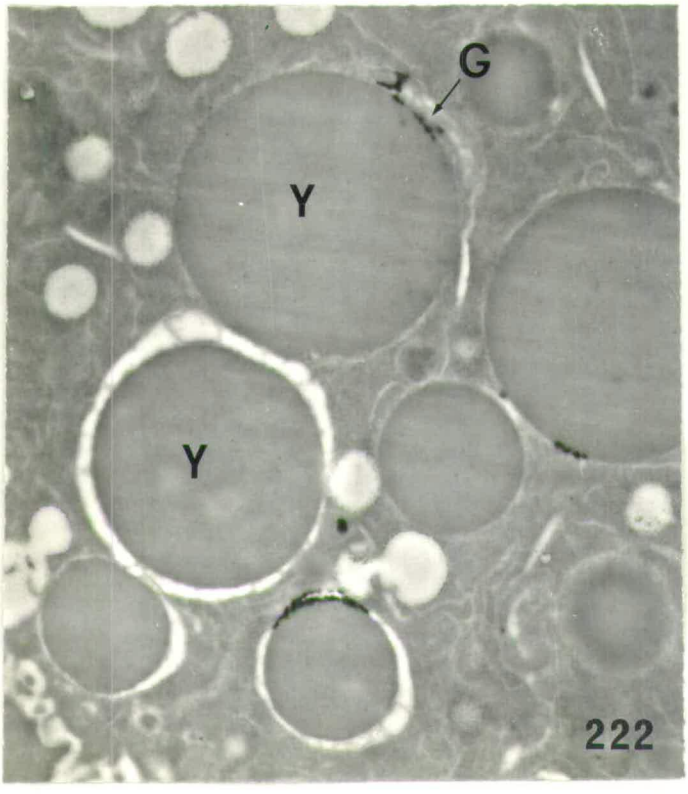
Fig. 222. The central ooplasm from a stage 10 ovarian chamber. The reaction product is at the periphery of the yolk platelets (y). In one instance (see arrow) the reaction product is most probably due to the associated Golgi apparatus (G). Unstained section. x 16,000.

Fig. 223. A field similar to that shown in Fig. 222. The reaction product on the yolk platelet (y) is most probably due to a Golgi apparatus closely associated with it. Unstained section. x 16,000.


bl
vp
m
G
vp
N
220


G
221


G
Y
223


G
Y
Y
222

5. Further evidence for the exogenous origin of yolk material

(a) Pattern of ^{3}H-lysine incorporation into the ooplasm. Evidence from several lines of investigation described so far have indicated that the vitellogenic oocytes may acquire yolk precursors from the haemolymph. This question was therefore examined further by autoradiography. The following account presents the pattern of uptake of tritiated lysine observed in light and electron microscope autoradiographs. Experiments were carried out both in in vivo and in vitro conditions as described in the material and methods section.

In the in vivo experiments the grain density (grains per unit area) was computed as a function of the duration of exposure to the tracer. Two areas were distinguished in the ooplasm; one, the yolk and the other the yolk-free ooplasm, and the grain density was calculated for each. It should be borne in mind that the ovarian chambers were staged at the actual time of fixation. On the basis of the time scale of development worked out by David and Merle (1968), a stage 10 chamber when fixed 20 hours after an injection of tracer would have been in stage 7 at the beginning of the incubation. Similarly a chamber at stage 10 fixed 10 hours after injection of tracer would have been in late stage 8 when the experiment started. This situation is summarized in Text-Fig. 5.

Ovaries dissected from adult flies 1-2 hours after injection of radioactive lysine were first examined. In stage 9-10 chambers (they must have been

Text-Fig. 5. Staging of ovarian chambers in the tritiate lysine incorporation experiment. On the basis of the time scale for ovarian development worked out by David and Merle (1968) it is possible, by knowing the actual stage at the time of fixation, to determine the stage of a chamber at the beginning of incubation. (o) stage 14; (x) stage 10.

STAGE	DURATION (hr)	STAGE AT THE BEGINNING OF INCUBATION					
1	9.56						
2	9.56						
3	9.56						
4	9.16						
5	2.61						
6	8.45						
7	8.69					x	x
8	5.21				x		o
9	5.61			x		o	
10	5.13	x	x	o	o		
11	0.40						
12	1.90						
13	0.79		o				
14	1.98	o					
PERIOD OF INCUBATION ►		1	2	6	10	16	20(hr)

more or less in the same stages at the beginning of the experiment - see Text-Fig. 5) examined 1 hour after injection of the tracer, the grain density in the ooplasm was rather low and the silver grains were distributed randomly (Fig. 224 and Text-Fig. 7). As mentioned earlier, the cytoplasm of the follicle cells of these chambers exhibited a higher density of silver grains (see Fig. 131). In these preparations

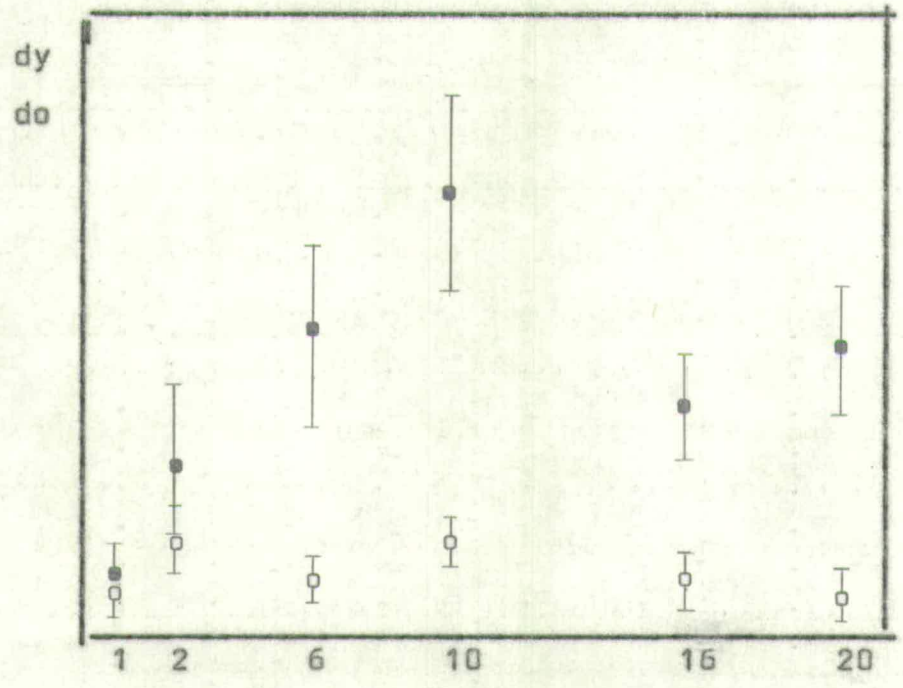
Text-Fig. 6. The distribution of autoradiographic grains in stage 10 ovarian chambers after various periods of time of in vivo incorporation of tritiated lysine. (A), Area in μ^2 ;(G), number of grains counted over that area.

REGION OF THE CELL	VITELLINE MEMBRANE		FOLLICLE CELL		YOLK PLATELETS		OOPLASM	
TIME (hr)	A	G	A	G	A	G	A	G G
1	-	-	-	-	45.5	0	151.2	7
	-	-	-	-	67.2	1	129.8	6
	47.7	3	18.7	0	10.9	1	119.4	5
	-	-	-	-	37.1	3	159.6	8
	-	-	-	-	35.5	0	161.2	4
	65.3	0	38.0	0	4.9	0	85.7	2
	66.0	0	-	-	6.7	0	123.9	21
	46.5	5	32.9	0	10.2	0	103.0	6
	82.5	5	60.4	1	0.6	0	50.4	0
	88.6	8	55.8	3	2.5	1	49.9	0
MEAN	66.1	3.5	41.1	0.8	22.1	0.6	113.4	5.9
2	55.9	30	85.5	7	-	-	58.0	12
	52.8	27	87.3	5	10.2	7	42.3	6
	48.4	25	82.4	4	7.9	8	58.0	17
	58.3	34	109.2	3	2.2	0	27.5	12
	44.0	32	92.8	8	3.9	2	48.8	7
	42.9	14	106.0	5	2.7	2	45.1	17
	45.1	25	120.9	8	1.9	3	28.8	6
	41.8	41	60.4	3	0.5	0	94.0	25
	-	-	-	-	35.0	9	161.7	15
	-	-	-	-	49.1	23	147.6	23
	-	-	-	-	51.4	34	148.1	18
	-	-	-	-	70.1	3	126.6	24
	-	-	-	-	72.9	2	126.6	8
	-	-	-	-	64.0	0	135.5	21
	-	-	-	-	33.4	9	166.1	18
	-	-	-	-	38.7	0	158.0	25
MEAN	48.6	28.5	93.0	5.3	29.6	6.8	98.3	15.8
6	24.8	0	100.8	8	6.6	6	53.1	2
	29.6	2	87.2	11	15.3	7	54.5	0
	-	-	-	-	52.6	18	135.8	11
	22.7	0	157.3	22	-	-	8.4	0
	25.6	2	91.3	14	13.0	7	58.4	5
	24.2	2	88.9	10	24.1	28	59.9	7
	-	-	-	-	63.4	49	122.0	12
	-	-	-	-	35.0	55	153.2	11
	-	-	-	-	60.7	40	127.6	7
	-	-	-	-	72.3	35	115.9	9
MEAN	25.4	1.2	105.1	13	38.1	27.2	88.0	6.4
10	-	-	-	-	82.5	101	114.2	22
	-	-	-	-	62.3	117	137.2	23
	-	-	-	-	81.0	92	118.5	13
	-	-	-	-	69.2	105	130.3	24
	-	-	-	-	59.7	111	139.7	20
	-	-	-	-	81.6	150	117.9	29
	-	-	-	-	95.1	126	101.6	20
	47.3	20	5.5	2	63.3	56	83.4	12
	46.6	8	27.7	8	58.0	38	67.1	12
	36.3	7	17.4	5	77.6	53	68.0	13
	34.3	15	25.1	6	62.0	38	78.0	9
	159.5	42	15.8	2	32.6	17	-	-
	20.8	5	-	-	60.5	58	116.4	13
	20.5	4	-	-	87.0	51	91.9	21
MEAN	52.2	14.4	18.3	4.6	64.8	74.2	104.9	17.7
16	-	-	-	-	56.8	34	142.2	3
	-	-	-	--	63.0	40	136.5	0
	-	-	-	-	65.2	36	133.4	8
	-	-	-	-	84.5	35	114.0	1
	-	-	-	-	53.3	28	145.2	4
	-	-	-	-	56.8	47	142.4	9
	-	-	-	-	59.4	14	139.2	2
MEAN	-	-	-	-	62.7	33.4	136.1	3.8
20	22.4	3	30.5	1	23.5	31	122.4	12
	-	-	-	-	50.8	46	147.7	11
	-	-	-	-	66.3	45	133.5	1
	-	-	-	-	33.5	23	163.2	2
	-	-	-	-	47.7	30	149.0	0
	-	-	-	-	79.1	18	124.4	0
	-	-	-	-	70.5	45	129.0	3
	-	-	-	-	61.1	44	135.6	2
	-	-	-	-	66.2	50	138.5	4
	-	-	-	-	73.4	38	123.7	7
	-	-	-	-	70.6	44	126.1	8
MEAN	22.4	3	30.5	1	58.7	37.6	135.0	4.5

Text-Fig. 7. The density of autoradiographic grains in stage 10 chambers exposed for various lengths of time to tritiated lysine. The values of grain density for each time are obtained from the data given in Text-Fig. 6. The standard error for each value is calculated as described in the material and methods section.

REGION OF THE CELL	YOLK PLATELETS IN OOPLASM	YOLK-FREE OOPLASM
TIME (hr)	dy	do
1	2.08 ± 7.8	4.39 ± 3.0
2	41.70 ± 23.8	19.47 ± 5.0
6	76.62 ± 25.4	6.44 ± 2.6
10	111.99 ± 26.6	16.99 ± 2.4
16	54.11 ± 13.8	2.78 ± 1.8
20	70.00 ± 16.6	3.45 ± 2.0

Text-Fig. 8. Diagrammatic representation of the data given in Text-Fig. 7. The points are plotted with a ± 2 x standard error. (o) dy; (o) do.



the vitelline membrane region was the most intensely labelled area and there seemed to be a gradient of decreasing grain density in the follicle cells towards the basement lamina. In EM autoradiographs of follicle cells the same pattern of distribution of silver grains was evident, and in sections passing through regions of the cell close to the vitelline membrane it could be seen that the silver grains were localized mainly over the Golgi apparatus and the vitelline membrane precursors (Figs. 132 and 134).

In preparations of stage 10 chambers examined 2 hours after injection, the distribution of silver grains in the ooplasm was similar to that after 1 hour of incubation (Text-Figs.7 and 8). Grains were distributed fairly uniformly over yolk platelets and yolk-free ooplasm (see Text-Fig. 7). From 2 to 6 hours after the initial injection of tritiated lysine, the grains begin to appear predominantly over the yolk platelets of these chambers (Text-Figs. 7 and 8). The number of yolk platelets which are labelled increased steadily for up to about 10 hours.

Ovarian chambers of stage 10 when fixed 16 or 20 hours after exposure to the radioactive aminoacid showed most of the yolk platelets labelled (Fig. 226). The overall grain density on the yolk continues to be markedly higher than that on the yolk-free ooplasm (Text-Figs. 7 and 8). There is, however, a noticeable decline (Text-Fig. 8) in the grain density over yolk platelets compared to that at the 10 hour period of exposure. A likely explanation will be proffered in the discussion to account for this drop in grain density

over the yolk.

Ovarian chambers in stage 14 when fixed after 16, 20 or 24 hours exposure to labelled lysine exhibited a grain density (Fig. 227) over yolk platelets (alpha-2 at this stage) which is considerably less than that in stage 10 exposed for similar length of time. The stage 14 oocytes examined in this series of experiments (20 hours exposure to tracer) would have been in stage 8 at the time of injection of the tracer (se Text-Fig. 5), and as such would have covered the entire period of active vitellogenesis in the presence of the tracer. In the few preparations examined, the grain density over the yolk-free ooplasm appeared comparatively higher than that over similar area of stage 10 chambers examined after 20 hours; (the data are not presented because only a limited number of EM-ARGs were available). The higher grain density mentioned above is probably attributable to a greater contribution of ribonucleoproteins from the nurse cells which have reached the oocyte through the ring canals.

The next set of experiments with ^{3}H-lysine was done <u>in vitro</u> for periods ranging from 30 minutes to 3 hours. By the end of 3 hours incubation, the overall morphology of the ovary was clearly altered when examined in light microscope sections, and therefore periods of incubation longer than 3 hours were not tried. The alterations consisted of wider than usual spaces between the follicle cells and between the follicle cells and the oocyte. Morphological alterations were also evident in the nuclei of the nurse cells.

The pattern of labelling in oocytes of

chamber stages 9, 10 and 14 when fixed after up to about 3 hours of *in vitro* culturing in a medium containing ^{3}H-lysine was more or less similar to that already described in the corresponding stages exposed to the tracer *in vivo* for 1-2 hours. The level of labelling was very low and the grains were randomly distributed; there was no indication of any preferential labelling of the yolk platelets. The labelling in the follicle cells, however, showed a gradient as in the *in vivo* experiment (Fig. 131) with the highest grain density along the vitelline membrane region.

(b) Comparative electrophoretic study of various tissues from *Drosophila*

In order to examine whether any of the proteins present in the ovary has an extraovarian origin, it seemed useful to attempt a simple analysis of the soluble proteins of various tissues from larvae and adult *Drosophila*. The method employed was disc electrophoresis on acrylamide gel. The preparation of the tissues and the procedure employed are described in the Material and Methods section.

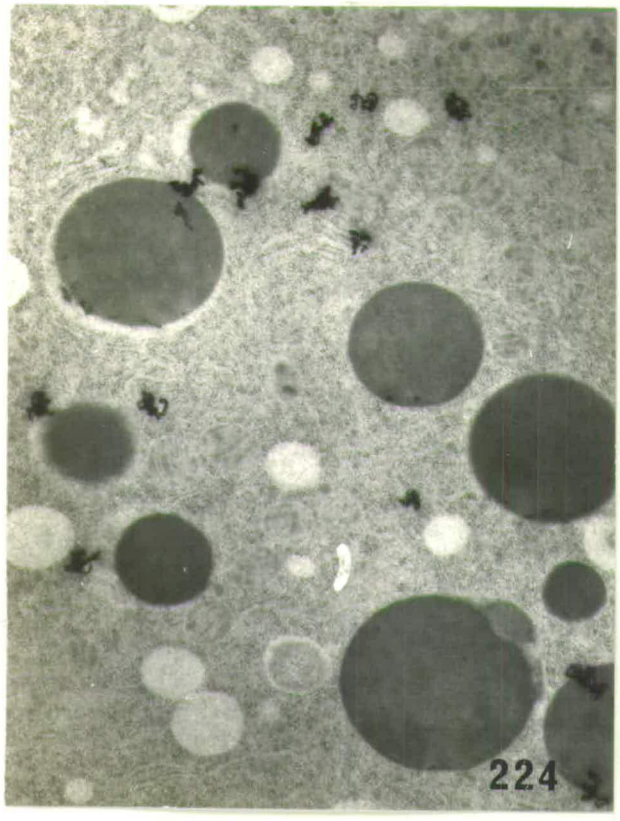
Twelve major protein fractions were resolved in the extracts of larvae haemolymph. The electrophoretic pattern obtained in different runs was reproducible and gels from two different runs are shown in Fig. 228. Preferential staining of the larval haemolymph proteins in the gels with benzidine revealed the existence of only one haemoglobin band with an Rf value of 0.14. Using appropriate stains, the lipoprotein and the glycoprotein bands were identified among the twelve

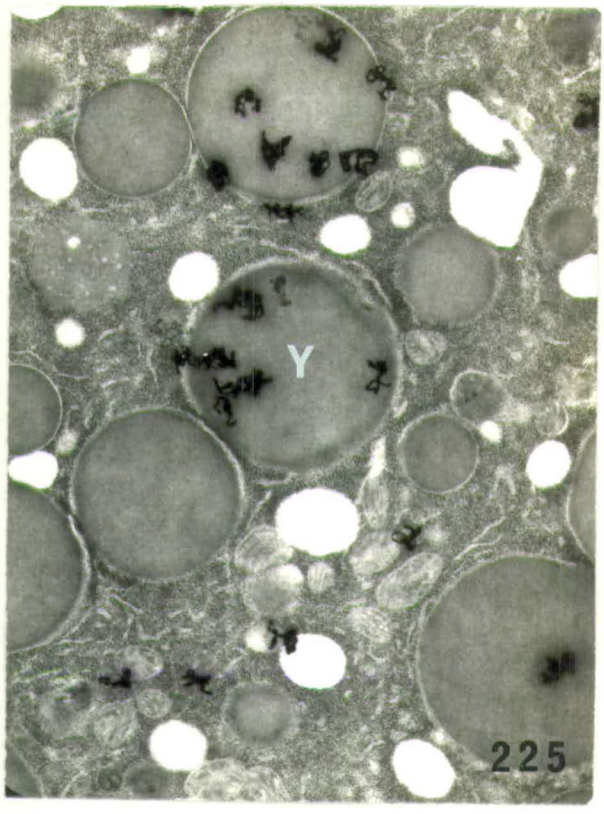
Fig. 224. An EM autoradiograph showing part of the central ooplasm of a stage 10 ovarian chamber taken from an ovary fixed 1 hour after injection of tritiated lysine. Note that only few and randomly distributed silver grains are visible in this preparation. The autoradiographic exposure was 7 days. x 12,000.

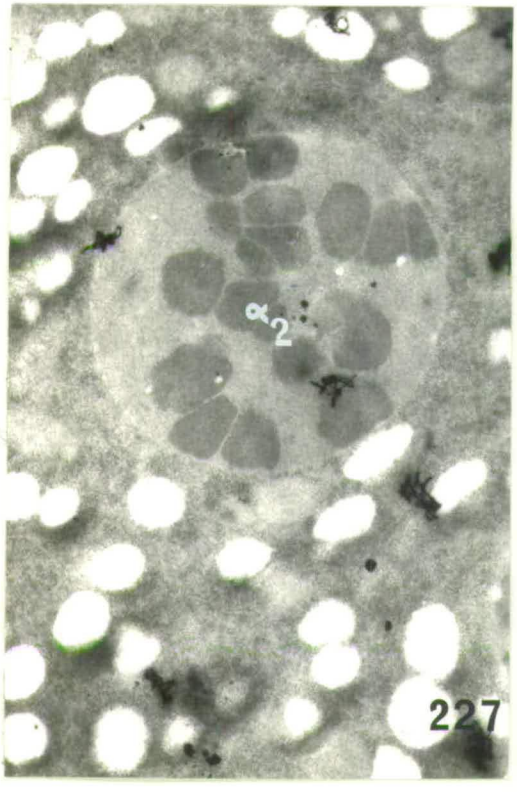
Fig. 225. Another EM autoradiograph of the central ooplasm of a stage 10 ovarian chamber taken from an ovary which was fixed 6 hours after the injection of tritiated lysine. Note that amidst two heavily labelled yolk platelets, there are also present several yolk platelets which are unlabelled. Silver grains are also seen in the yolk-free ooplasm. The autoradiographic exposure was 7 days. x 12,000.

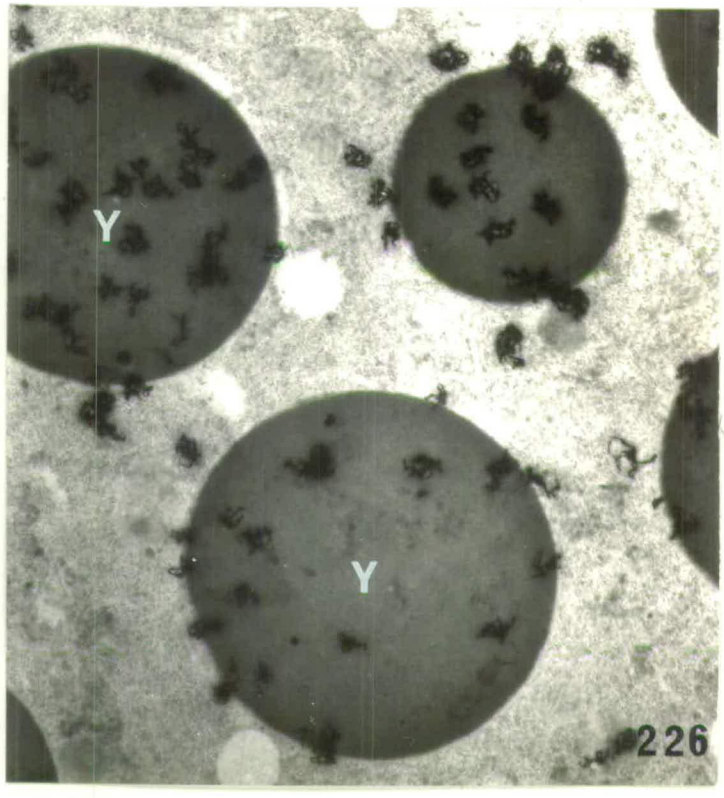
Fig. 226. An EM autoradiographic preparation of the central ooplasm of a stage 10 ovarian chamber taken from an ovary fixed 20 hours after the injection of tritiated lysine. Note that all yolk platelets are heavily labelled. The autoradiographic exposure was 7 days. x 12,000.

Fig. 227. An EM autoradiograph of a stage 14 oocyte showing an alpha-2 yolk platelet. The oocyte was taken from an ovary fixed 20 hours after the injection of tritiated lysine. Compare with Fig. 226; note that, although these two preparations are taken from ovaries both fixed 20 hours after exposure to the tracer, the alpha-2 yolk platelet contain fewer silver grains than the alpha-1 yolk platelets shown in Fig. 226. x 12,000.


224


Y
225


α2
227


Y
Y
226

bands visualized (see Fig. 228, Li and Gly). One major lipoprotein with an Rf value of 0.10 is present in the larval haemolymph. A second, very faintly stained band, is also present. These two bands also stain with the PAS technique for glycoproteins. In addition, three other bands appear stained by the PAS procedure, thus giving a total of 5 glycoprotein bands in the larval haemolymph.

When haemolymph from adult female flies was analyzed electrophoretically, a pattern essentially similar to that already described in the larvae was evident; differences were, however, apparent in the relative staining intensities of certain bands. In particular, the band exhibiting an Rf value of 0.37 which is stained slightly (with Coomassie blue) in the larval haemolymph is intensely stained in the haemolymph of the adult female. On the other hand, the two major bands of the larval haemolymph, with mean Rf values of 0.54 and 0.57 respectively are faintly stained in the adult female. Differences in the staining intensity of these bands may be taken to indicate differences in the relative concentration of these proteins in the haemolymph of larvae and adults. Although the protein concentrations of the various samples of haemolymph in the two instances were not determined by chemical methods, the samples were also prepared in an identical manner, and the volume loaded on each gel was always the same. The pattern of the differences in the staining intensity of the three bands mentioned also makes it unlikely that the differences are due to different concentration of proteins used for electrophoresis.

Of the twelve major fractions observed in the haemolymph of the adult female, only four fractions are seen in the electropherogram of the ovary. Of special interest is the band with an Rf value of 0.37. It was mentioned above that the staining intensity of this band is considerably higher in the haemolymph of the adult female compared with that in the larva; the same band appears even denser in the electropherogram of the ovary. On the othe hand, the bands with mean Rf values of 0.54 and 0.57 are less densely stained in the ovary. Another noticeable observation in the electropherogram of the ovary is that the band with an Rf value of 0.9 appears as a very thin one compared to that in the adult haemolymph. It would therefore seem that 8 bands seen in the adult haemolymph are absent in the ovary and of the 4 that are present in both tissues only one is very prominent.

Electrophoretic analysis of the fat bodies of adult female reveal only three bands with mobilities similar to those of the ovary. These three bands are also identifiable in the haemolymph of the adult female. Two other bands in the extracts of fat bodies, i.e. those with Rf values of about 0.20 and 0.80 do not seem to be present in the ovary but are present in the adult haemolymph. In the region of the Rf values 0.50 and 0.59, three bands are apparent in the extracts of fat bodies whereas, in the other tissues examined only two bands are visible. It is therefore possible that the extra band in this region of the gel represents a protein exclusive to the fat body.

(c) Analysis by isoelectrofocusing of *Drosophila* tissues exposed *in vivo* to ^{14}C-labelled amino acids.

While it is possible that bands with the same electrophoretic mobility may be due to similar proteins, it would be quite incorrect to assume that identically located bands are necessarily identical proteins. The experiments described in the previous section, therefore, have to be interpreted in the light of these limitations. Similar electrophoretic patterns among different tissues constitute a strong indication of the identity of their protein components, but they do not necessarily prove it. The identity of proteins could only be established by using more sophisticated analytical and immunological techniques. Immunological analysis has been tried by a number of authors (Telfer, 1954; and Bell, 1970) in large insects where sufficient quantities of aliquots could be obtained easily. In view of the small size of *Drosophila*, another approach was employed, namely isoelectrofocusing to analyze the composition of ovarian proteins. In addition, it was hoped to follow the uptake of radioactively labelled proteins into the ovary and the haemolymph following injection of ^{14}C-labelled amino acids into adult flies.

When ovarian extracts were analyzed by isoelectrofocusing according to the procedure described in the Material and Methods section, numerous sharp bands (at least 26) could be resolved within the pH range of 3.5-10.0 (Fig. 229). On the other hand, when haemolymph from adult female was analyzed according to the same

procedure, there were fewer bands than in the ovary. The situation thus appears to be the opposite of that reported in the previous section, where the number of bands was higher in the electropherogram of the haemolymph. This difference may be explained as follows: electrophoresis of the adult haemolymph and ovary demonstrated only the water soluble proteins, whereas in isoelectrofocusing, when a solubilizing solution was used to prepare extracts of these tissues, all the proteins were rendered soluble (see Material and Methods). In view of the structural and chemical complexity of the ovary, a higher number of proteins may perhaps be expected in this tissue than in the haemolymph. Although the number of bands obtained by isoelectrofocusing are different in the two tissues examined (haemolymph and ovary) as mentioned above, the pattern of distribution of the major bands are quite similar (Fig. 229). The pattern of bands obtained in isoelectrofocusing was further examined by preparing autoradiograms of dried gels made from ovaries and haemolymph exposed *in vivo* for 24 hours to a mixture of radioactive aminoacids. In such preparations, it was found that all major bands common to both tissues were intensely labelled (Fig. 229).

The observations indicate that fractions of similar pI values exist in the extracts of both the ovary and the haemolymph. Only further experiments with progressively shorter periods of incubation *in vivo* can clarify the kinetics of uptake of the labelled proteins by the ovary from the haemolymph.

Fig. 228. Electrophoretic analyses of several tissues from larvae and adults of Drosophila. (Hb) haemoglobins, (Li) lipoproteins, (Gly) glycoproteins in the larval haemolymph. Details of the staining procedures for staining these substances are given in the Material and Methods section. (LHm) larval haemolymph, general staining for proteins with Coomassie blue; (AHm) adult haemolymph, general staining for proteins with Coomassie blue; (Ov) ovary, general staining for proteins with Coomassie blue; (FB) fat bodies, general staining for proteins with Coomassie blue. (Rf) ratio between the distance calculated for each major band from the origin of the gel, and the distance of the track dye band (bromophenol) from the origin of the gel. Note that LHm and AHm exhibit patterns essentially similar; some differences, however, are apparent in the relative staining intensity of the bands with the Rf value of 0.37, 0.50-0.58 and 0.55-0.59. The above mentioned bands are also visible in the electropherogram of the ovary and fat bodies.

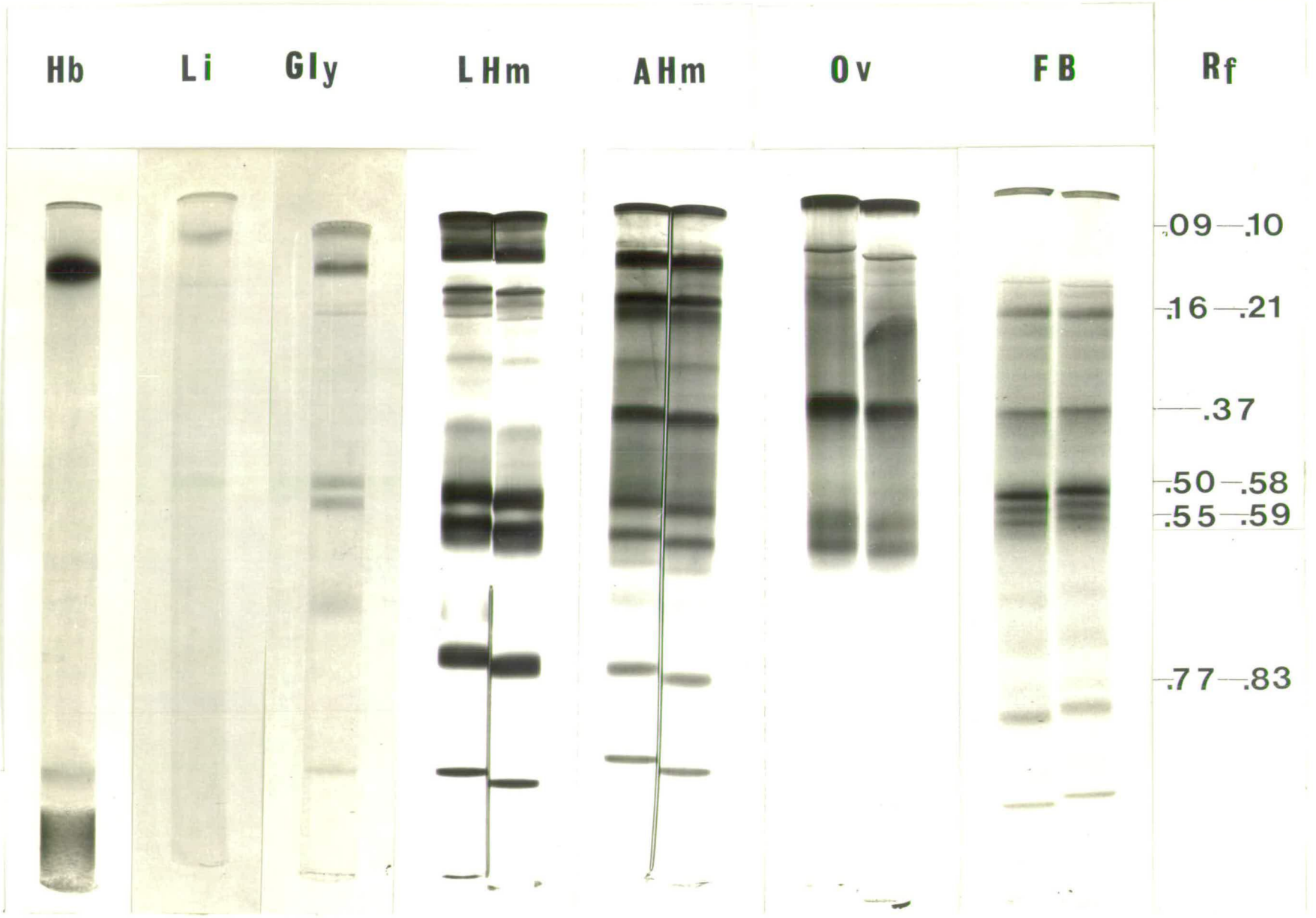
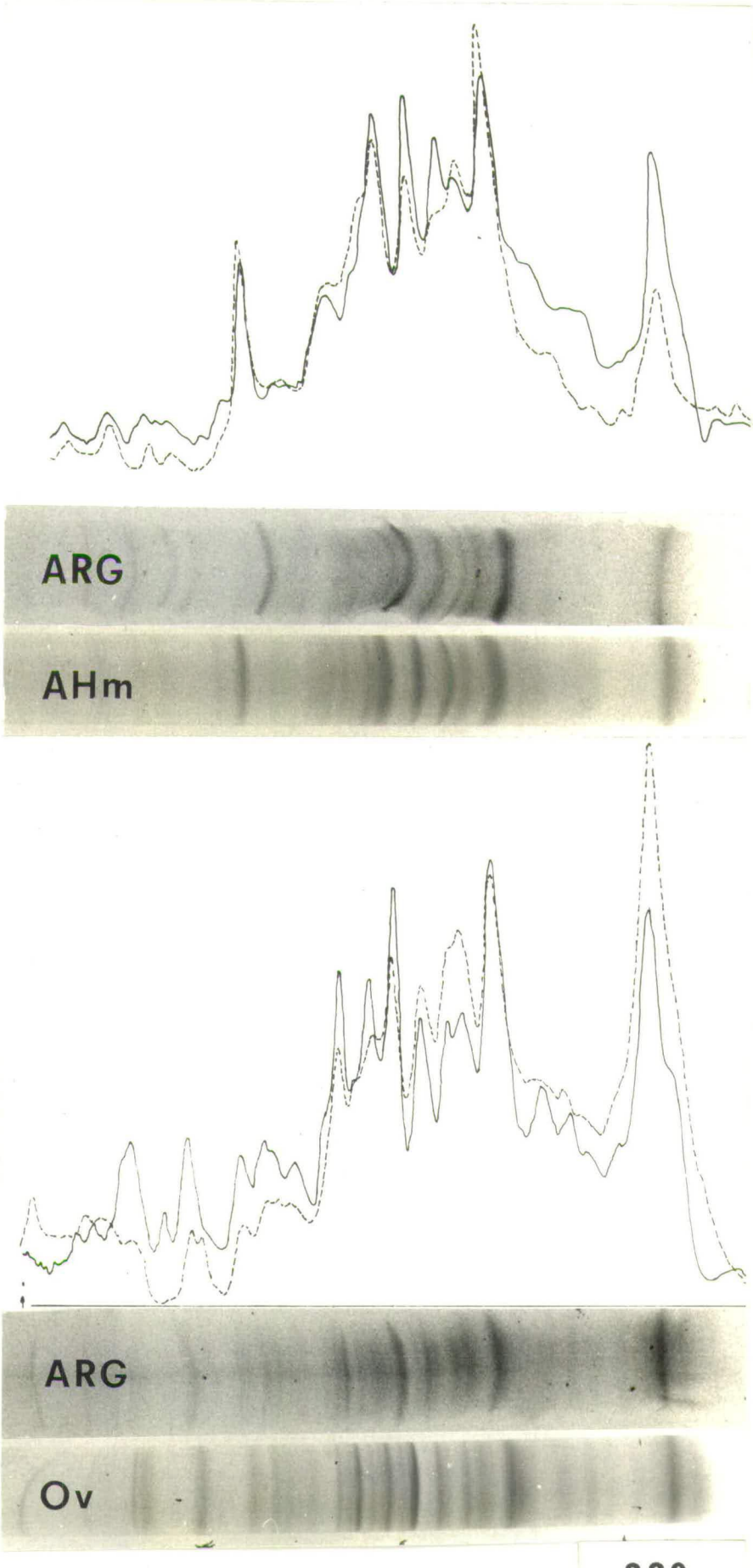

Hb
Li
Gly
LHm
AHm
Ov
FB
Rf
.09
.10
.16
.21
.37
.50
.58
.55
.59
.77
.83

Fig. 229. Isoelectrofocusing analyses of the haemolymph (AHm) and ovary (Ov) extracted from adults of Drosophila 24 hours after the injection of a mixture of 14-C-labelled aminoacids. (ARG), autoradiography of the dried gels. Note that numerous sharp bands (at least 26) can be resolved within the pH range of 3.5-10.0 of the ovary extracts, and that the pattern of distribution of several bands of the ovary extracts is quite similar to that of the haemolymph. The bands which are common to both tissues also appear heavily labelled in the autoradiograms. Staining procedure of the gels and autoradiographic technique are described in details in the Material and Methods section.


ARG
AHm
ARG
Ov

VI. DISCUSSION AND CONCLUSIONS

1. The oocyte nucleus

The oocyte nucleus in stage 1 chamber of the ovary is easily distinguished from the nurse nuclei by the presence of synaptonemal complexes as well as by its posteriormost location within the cell cluster. Further, it contains a compact nucleolus with both fibrillar and granular components and rather dispersed chromatin. By stage 2, however, the peripheral granular part of the oocyte nucleolus begins to show a loose texture, and from stage 4 onwards, the granular constituents are no longer discernible. The central fibrillar part of the nucleolus persists as the endobody for the rest of the oogenesis. These observations are consistent with those of Mahowald and Tiefert (1970) who reported that during oogenesis in Drosophila the nucleolus of the oocyte becomes transformed into a fibrillar body. It is also clear that the nucleolar body consisting of only fibrils corresponds to the endobody described by Bier et al. (1967) in the oocyte nucleus of both meroistic and panoistic ovaries of insects.

During this study it was shown that when the nucleolus becomes transformed in the so-called endobody, the chromatin material gradually condenses to form a compact ring-shaped body. This body corresponds to what has been described by King et al. (1956, 1970) as the karyosome of Drosophila melanogaster. These authors, however, failed to notice that in ovarian chambers of late stages (stages 9-10) the compact structure of the karyosome is lost. Instead, it appears as a loosely

organized entity and at the same time two classes of granules appear in close association with it. When the granules appear in the oocyte nucleus in stages 9-10, the nuclear envelope shows several blebs. As described earlier, both membranes of the envelope are involved in the formation of the blebs. Material similar to that of the rest of the nucleus occurs inside the blebs. As far as one can judge from electron micrographs, there seems to be little doubt that they give rise to free vesicles when they detach from the nuclear envelope; vesicles of similar morphology, however, could not be detected in deeper regions of the ooplasm. It may well be that the vesicles that do occur in the deeper ooplasm are derivatives of the vesicles mentioned above, but have become morphologically altered as they moved away from the perinuclear cytoplasm.

Light microscope autoradiographs prepared during the course of this study showed that there was no incorporation of ^{3}H-uridine into the oocyte nucleus during stages 1 to 8. Some incorporation of the label was, however, noticed during stages 9-10 and this observation agrees with the autoradiographic evidence presented by Mahowald and Tiefert (1970) in their study of Drosophila ovary. Since the true nature of the endobody in the oocyte nucleus was not well established at that time, these authors suggested that RNA synthesized during stages 9-10 was not ribosomal. The present study has shown that the endobody is derived from the nucleolus of earlier stages, but the absence of particulate components in the endobody makes it unlikely that this body is involved in synthetic activity. It is, therefore,

clear that whatever RNA is synthesized by the oocyte nucleus is synthesized in association with the karyosome. Morphological evidence, namely the presence of two classes of granules closely associated with the karyosome, lend further support to this view. On the whole, therefore, it can be said that the chromatin of the developing oocyte makes only a small or negligible contribution to the RNA of the enlarging ooplasm. Further, in the light of our present knowledge of the function of the nucleolus it can be said with some certainty that in Drosophila, the developing oocyte does not derive much ribosomal RNA from its own nucleolus.

2. Previtellogenic ooplasm

The electron microscope observations made in the present study show that a number of cytoplasmic inclusions are present in the ooplasm of developing ovarian chambers with the earliest stage examined, i.e. stage 1. Apart from mitochondria, Golgi apparatus and fuzzy bodies, the other organelles present are a variety of lysosomal derivatives which range from typical multivesicular bodies to cytolysosomes. Their lysosomal nature has been confirmed in the present study by the localization of acid phosphatase in them. Nurse cells of ovarian chambers in previtellogenic stages also contain cytoplasmic inclusions similar to those of the ooplasm, and in numerous instances during the course of this study, such inclusions have been seen within the ring canals which connect the nurse cells with the oocyte. It, therefore, seems very likely that cytoplasmic inclusions from the nurse cells are transported to the

ooplasm and it is conceivable that this transport is caused by the rhythmical pulsations of the peritoneal sheath of the ovary.

My ultrastructural study has further clarified that several of the inclusions present in the ooplasm of stage 7-8 chambers exhibit intermediate characteristics between lysosomal derivatives and yolk platelets. It may be recalled how some of the inclusions were made up of, among other things, various electron dense regions bearing a close resemblance to yolk. In a recent study on Drosophila ovary, Mahowald (1972a)has found that some of the multivesicular bodies present in the ooplasm of previtellogenic stages exhibit an internal structure which makes them indistinguishable from fully grown yolk platelets. Similar observations have also been reported in the oocyte of Dytiscus marginalis by Steinert and Urbani (1969). The bodies they referred to as dense bodies are apparently lysosomal derivatives and were regarded by them as representing precursors of yolk platelets. Further, in previtellogenic oocytes of the European newt Triturus vulgaris, Spornitz and Kress (1973) have noted that multivesicular bodies exhibit a progressive condensation of their constituents so as to acquire a yolk-like appearance; accordingly they were termed primary yolk. My observations together with those of others cited above indicate that oocytes of several animals may come to contain yolk-like material prior to the period of initiation of what is generally regarded as active vitellogenesis.

In the case of the newt, the formation

of yolk-like material in the oocyte prior to active vitellogenesis can be considered as an autosynthetic activity in the oocyte itself as stated by Spornitz and Kress (1973). Such a view is untenable in the case of the meroistic ovaries such as that of Drosophila because the material in question is clearly derived from the nurse cells. In the light of my findings, it is difficult to reconcile with the view of Mahowald (1972a), who also worked with Drosophila melanogaster, that the multivesicular bodies of previtellogenic oocytes represent yolk proteins synthesized by the oocyte itself.

As described elsewhere, several of the lysosomal derivatives in the ooplasm as well as the nurse cell cytoplasm contain recognizable cytoplasmic organelles. The implication of this and the observations stated earlier is that there is focal degradation of cytoplasmic organelles in the nurse cells even during the previtellogenic stages. Evidence in support of this view is also available from some earlier investigations (King and Mills, 1962; Steinert and Urbani, 1969) in Drosophila and Dytiscus.

There are a number of examples in nature where somatic cells have been shown to acquire nutrients by means of enzymatic hydrolysis of sequestered material. The sequestration may be by autophagic or heterophagic activities (Swift and Hruban, 1964; Locke and Collins, 1965), and as pointed out by De Duve and Wattiaux (1966) there is sometimes no distinction between autophagic and heterophagic activities because material from the two sources may come to be digested within the same

cellular compartment. My observations from previtellogenic oocytes and oocytes in stages 7-8 suggest that autophagic activity in nurse cell cytoplasm makes a contribution to yolk platelets formation.

It is worth mentioning at this juncture that, when unicellular organisms enter the stationary phase; their mitochondria undergo an autophagic destruction (Eliott and Bak, 1964); the mitochondrial degeneration in these organisms appears to be a device by which a cell gets rid of its excess mitochondria and utilizes the degradation products. Indeed, autophagy appears to be a mechanism of widespread occurrence whereby cells operate a turnover of certain organelles and simultaneously reutilize their chemical components (Ericsson, 1969).

These studies suggest that, as far as the ability to undergo focal degradation of cytoplasmic organelles is concerned, the nurse cells in the ovarian chambers of Drosophila bear noticeable functional similarities with somatic cells.

3. Vitellogenic ooplasm

(a) Ultrastructural aspects of pinocytotic uptake. The ultrastructural study of the cortical region of the oocyte has shown that the beginning of vitellogenesis (stage 8) is marked by the appearance of several structural entities. These are the pits of the oolemma and the coated vesicles, tubules and yolk platelets of various sizes in the ooplasm.

It has been shown in the present study

that at the beginning of stage 8 the oocyte surface is thrown into microvilli-like folds and at the base of these there are present numerous depressions known as the pits. The pits have a three-layered structure; the outer most layer is the fuzzy layer, the middle or intermediate layer is continuous with the oolemma, and the inner layer facing the ooplasm has a characteristic spiked appearance. In other words, the pit region of the oolemma is characterized by the presence of a fuzzy layer and a spiked layer. It was explained earlier that the coated vesicles and the tubules, found in the deeper regions of the cortical ooplasm, exhibit ultrastructural features similar to those of the pits. On the basis of these similarities, it seems most probable that the coated vesicles represent internalized pits which have lost contact with the oolemma. The ultrastructural observations also lead to the assumption that the tubules in the cortical ooplasm have originated in turn by fusion of several coated vesicles. Indications are that the continued fusion of coated vesicles and tubules results in the formation of small yolk platelets in the cortical ooplasm. Actual images suggestive of fusion between coated vesicles and tubules with small yolk platelets have in fact been encountered.

The various entities described above, i.e. the pits, the coated vesicles and the tubules, have been reported in the cortical ooplasm of a variety of insects and have been interpreted as part of the mechanism for uptake of proteins from the haemolymph (Roth and Porter, 1964; Stay, 1965; Anderson, 1969; Anderson and Spielman

1971; Mahowald, 1972b).This mechanism envisages that proteins from the haemolymph gain access to the oocyte surface by passing through the intercellular spaces of the follicle layer and bind to the fuzzy layer of the pits. The proteins so bound are then taken in by pinocytosis and then ultimately sequestered into the growing yolk platelets. However, quite a different opinion has been expressed by Cummings and King (1970) in their study of the origin of yolk platelets in Drosophila. These workers reject the view that proteins as such are taken in from the haemolymph and incorporated into yolk platelets. Instead, they maintain that only "raw material" (precursors of biological macromolecules ?) are taken in by pinocytotic activity at the oocyte surface and that this material is then utilized for synthesis of yolk proteins by ribosomes in the oocyte attached to the endoplasmic reticulum. My work has helped to resolve this controversy and I have come to conclude that the view of Cummings and King (1970) is untenable for the following reasons. Firstly, the present work as well as that of Anderson and Spielman (1971) show that the pits of the oolemma are specialized regions for uptake of exogenous proteins (see observations from peroxidase and concanavalin experiments - pages 108 and 132 respectively, and also the discussion - pages 174-82), and that the so-called coated vesicles and tubules containing exogenous proteins make contact with small yolk platelets. These observations reinforce the view that the pits and their derivatives are part of the pinocytotic mechanism for the uptake of exogenous proteins from the haemolymph. Cummings and King's (1970)

view is also at variance with current knowledge of membrane transfer and dynamic exchanges between various compartments of the cell. According to De Duve (1969) there is no actual continuity, either static or dynamic, between the exoplasmic space and the endoplasmic reticulum, and membrane fusion does not occur between components of these compartments except through components derived from the Golgi apparatus. In their study, Cummings and King (1970) used osmium tetroxide as sole fixative and this may have contributed to some extent to their interpretation, because this fixative does not seem to permit a clear visualization of the fuzzy layer of the tubules and vesicles which fuse with themselves and with the small or forming yolk platelets. I noted during this study that double fixation, that is sequential fixation with glutaraldehyde and osmium is essential to reveal the presence of the fuzzy layer. Different lines of evidence in the present study point to the fact that the endoplasmic reticulum of the ooplasm makes no contact with either the growing yolk platelets or the incoming coated vesicles and tubules. Further, I have never during the course of this study come across a layer of endoplasmic reticulum in the cortical ooplasm of the developing oocyte as described by Cummings and King (1970).

Cummings and King's (1970) criticism of the possibility of the formation of yolk in Drosophila through pinocytotic uptake of proteins lies in the observation that this process, because of the relative surface-to-volume ratios between vesicles and yolk platelets, would actually result in the transfer of

more membrane than protein content. These workers have estimated that if all yolk platelets were made through pinocytotic uptake of proteins as such, then ten thousand times more vesicles would be internalized in the oocyte than would suffice to provide the limiting membrane of the mature yolk platelets. Accordingly they argue that there should be a surplus of oolemma-derived membranes in the ooplasm.

In the present study there was no obvious accumulation of membranes at the site where pinocytotic vesicles (coated vesicles and tubules) fuse to form yolk platelets. It may be that the excess membrane is broken down to its molecular constituents or subunits and made available at another cellular site to be recycled back as new membranous fragments. Present knowledge of the molecular turnover of membranes makes it likely that large scale movements of membrane components occur at a level not detectable microscopically (Graham et al., 1973). Regulatory systems are also known to occur to maintain the balance of the synthesis and recycling of membranes both in detectable and non-detectable forms; among these are the Golgi apparatus which function in the assembly and differentiation of visible membranes and the lysosomal system which functions in the breakdown of membranes (Whaley et al., 1971).

The various considerations discussed above make it clear that there is no evidence at the present to support the view expressed by Cummings and King (1970) on the origin of the yolk platelets in Drosophila.

The existence of mechanisms involved in the breaking down of membranous components in the yolk platelets of Drosophila oocytes (see later) is made likely by the finding that lysosomal enzymes are present in them. In their study of the fat bodies of an insect, Locke and Collins (1968) came to the conclusion that an organelle which contains hydrolytic enzymes, such as for instance the multivesicular bodies, may be concerned with the breaking down of both the content and the limiting membrane of any exoplasmic vesicles which fuse with it.

It is worthwhile noting that pinocytotic activity, as a method to internalize large molecular aggregates prior to their final assimilation into the cell, is a rather widespread mechanism which occurs practically in every absorbing tissue (Friend and Farquhar, 1967). These considerations again emphasize that the problem relating to the ultimate fate of the invaginated membranes cannot per se be used as an argument against the extraovarian origin of yolk proteins in Drosophila melanogaster.

Another consequence of the pinocytotic uptake that has not so far received much attention is that short stretches of the oolemma (the pits) are continuously taken into the cell to form coated vesicles. On the basis of current knowledge of surface membrane turnover (Warren, 1969), a rapid rate of replacement of the oolemma may thus be predicted in oocytes during the period of active vitellogenesis. I have evidence in the present study which confirm this prediction. The

evidence come from the experiments with osmium tetroxide zinc iodide complex (OZI). In preparations of vitellogenic oocytes treated with the OZI complex there were, as described earlier (page 130), a number of vesicles containing OZI deposits in the region of the cortical ooplasm. In view of the known specificity of the OZI technique for elements of the Golgi apparatus (Niebauer et al., 1969), the above mentioned vesicles in the cortical ooplasm, may reasonably be assumed to be derived from the Golgi apparatus, and on their way to fuse with the oolemma. However, my observations so far have not revealed OZI deposits at the pits themselves as may be expected. Several reasons may be adduced to account for this but the most probable one is that the Golgi derived membranes undergo some modifications and lose their specificity for the OZI reaction as soon as they reach the surface and become integrated with the oolemma. There is evidence in the literature for membrane modification within the Golgi complex as well as subsequent to leaving the Golgi area (Northcote, 1972; Franke and Herth,1974).

A shift in the distribution of vesicles from the Golgi apparatus to the apical cytoplasm has been observed in the absorbing cells of the vas deferens in rat (Friend and Farquhar, 1967). A similar observation has also been reported in an unicellular alga, Ochromonas danica by Cole and Wynne (1973). More recently, Franke and Herth (1974) have presented convincing morphological evidence that the plasma membrane in plant cells may be formed de novo from from vesicles derived from the Golgi apparatus.

That, in vitellogenic oocytes of Drosophila, the Golgi apparatus in engaged in the synthesis and assembly of plasma membrane precursors is also suggested by my finding (see page 134) that acyltransferasæs are localized in this organelle. In every tissue where such enzymes have been studied cytochemically, their localization in the Golgi apparatus has been related to a high demand for plasma membrane replacements (Levine et al., 1972). Furthermore, the presence of acyltransferases in the Golgi apparatus has been consistently recorded in association with the appearance of a number of vesicles in the cytoplasmic region between the Golgi apparatus and the cell surface (Levine et al., 1972). In conclusion then, it may be stated that all available evidence in the present study point to the occurrence of a continuous flow of vesicles from the Golgi apparatus towards the oolemma, presumably to meet a high demand for membrane components related to pinocytotic activity.

(b) Ultrastructure and cytochemistry of yolk platelets. The observations on the ultrastructure of the yolk platelets have revealed for the first time several new features. Previously King and co-workers (1970) described the alpha-1 yolk platelets as consisting of a homogeneous membrane bounded body. The new features observed in alpha-1 yolk platelets are an asymmetrical superficial layer of low density within which a number of small empty looking spherules and a rounded associated body can be made out. Not all yolk platelets in any one section show the associated body;

this, however, is a consequence of the plane of sectioning.

My electron microscope observations on the ooplasm of ovarian chambers in stages later than 12 indicate that the above mentioned structural entities in the alpha-1 yolk platelets become rare with the growth of the yolk platelets. Furthermore, as previously described by King et al. (1966), a new type of yolk platelets becomes visible in the ooplasm of late ovarian chambers. My observations agree with the description of this structure given by King et al. (1966) who referred to it as the alpha-2 yolk platelets. When this type of yolk platelets appears in the ooplasm of stage 13 chambers, another inclusion is evident more or less concomitantly. The latter was previously described as being composed of subunits enriched in PAS-positive material and referred to as the beta-spheres by King et al. (1966). My observations on the ultrastructure of these inclusions agree with those provided by the earlier workers but my cytochemical experiments show that the PAS material in question is glycogen. Indeed, glycogen was already assumed to be present in the beta-spheres, but only on ultrastructural considerations (Mahowald, 1972b).

Previously, Cohn and Brown (1968) also observed that the number of alpha-1 yolk platelets present in the ooplasm of stage 13-14 chambers decreases progressively as the oocyte nears maturation. This seems to be accompanied by a concomitant increase in the number of alpha-2 yolk platelets. Cohn and Brown (1968) suggested a direct transformation of alpha-1

yolk platelets into alpha-2, and that this change should coincide with the release of PAS-positive material from the yolk platelets to the ooplasm.

During the course of this study, stages of possible transition of alpha-1 into alpha-2 yolk platelets were frequently observed and this therefore tends to substantiate Cohn and Brown's (1968) assumption.

Evidence from ^{3}H-lysine incorporation experiments demonstrated that alpha-2 yolk platelets in the ooplasm of stage 14 ovarian chambers have a lower density of autoradiographic grains than alpha-1 yolk platelets of stage 10 chambers exposed to the radioactive tracer for the same length of time. If alpha-1 yolk platelets are to be considered the forerunners of the alpha-2 yolk platelets, then the autoradiographic data indicate that the transformation involves some loss of material from alpha-1 yolk platelets to the ooplasm. Furthermore, since the PAS material to which they refer is now identified as glycogen then it seems likely that the appearance of glycogen-containing beta-spheres is somehow linked with the transformation of the alpha-1 yolk platelets into alpha-2. I have in fact demonstrated that glycogen is formed in intimate association with the alpha-1 yolk platelets.

Several evidence suggest that in oocytes approaching maturation glycogen is likely to be formed by a process of gluconeogenesis (Yurowitzky and Milman, 1972). The presence of microperoxisomes in the ooplasm of stage 14 ovarian chambers tends to confirm the occurrence of gluconeogenesis. It may then be

assumed that the aminoacids utilized for the gluconeogenic synthesis of glycogen are those derived from the degradation of certain proteins of the alpha-1 yolk platelets as they become transformed into alpha-2 platelets.

4. The role of the nurse cells in oogenesis.

The present study confirms and extends previous observations by other authors on the ultrastructure and function of nurse cells in meroistic ovaries of insects. Throughout the period of oogenesis, the nuclei of nurse cells undergo striking changes in size and shape which are accompanied by changes in the structural organization of their constituents. Nurse cells in previtellogenic chambers up to stage 4 exhibit fairly compact nucleoli, while during the same period the chromatin material appears in the form of four diffuse masses corresponding to the four pairs of chromosomes. In the succeeding stages, the nucleoli tend to increase in size and in sections they appear to consist of separate masses. Dapples and King (1970), however, have shown that the nucleolar masses are all interconnected. By the end of stage 10, the nucleolus especially that of the posterior nurse cells, becomes so large as to occupy most of the volume of the nucleus. At this stage, the nucleolus is like a thick network in the form of spherical shell and is situated close to the inner surface of the nuclear envelope.

From stage 4 onwards, the chromatin material appears as several aggregates scattered throughout the

nucleus and closely associated with the nucleolar mass. The ultrastructural features of nurse nuclei have been previously described by Dapples and King(1970), and the present observations confirm their account to a large extent. However, several new features have been noted now. By using the preferential staining method of Bernhard (1969) for ribonucleoproteins, it has been possible to demonstrate the occurrence of large interchromatin granules in nurse nuclei of the developing ovarian chambers. As stated earlier, there are two sizes of granules; one of these (400 A in diameter) is present in the nurse nuclei of chambers stages 1-10, and the other (about 600 A in size) is found in the nurse nuclei of stage 11-13 chambers. Interchromatin granules have been described in various vertebrate cells by Fakan and Bernhard (1973) and are believed to contain a slowly labelled RNA.

Much information have been gained in the present study about the nature and significance of amorphous material in the perinuclear cytoplasm of nurse cells; this is discussed later.

In all previous studies so far, the ultrastructure of nurse cells had been examined only up to stage 10. I have now extended this to stage 13 and shown by the cytochemical method of Bernhard (1969) that chromatin material can hardly be detected in the nurse nuclei of stage 13 chambers. All the nurse nuclei in stages 11-13 show ultrastructural features which are indicative of degeneration. The nuclear envelope appears ruptured in several places and the chromatin material have apparently passed into the cytoplasm and degraded.

In stage 13, the nuclei seem to contain only RNA material, presumably the remnants of the nucleolus, some of which, as described earlier, pass out of the nucleus.

King and co-workers (1970) have put forward the interpretation that the spatial organization of the nucleolar material in nurse nuclei results from the process of "polyploidization" of the chromosomes followed by separation of the individual chromatids. The work of Jacob and Sirlin (1960) has amply demonstrated that the development of nurse nuclei involves a number of endomitotic doublings. The separation of the chromatids following polytenization causes the dispersal of the nucleolar organizer regions and this can be expected to result in the dispersal of the nucleolar masses (Painter and Biesele, 1966).

Molecular hybridization experiments with labelled ribosomal RNA and DNA from Drosophila stocks containing different doses of nucleolar organizers have shown (Ritossa and Spiegelmann, 1965) that the proportion of the DNA complementary to RNA in the different stocks corresponds to the number of organizers present. This and subsequent experiments by Wallace and Birnstiel (1966) have firmly established that the nucleolar organizer region is the site of the cistrons which code for ribosomal RNA. It may hence by assumed that the endomitotic doublings of nurse nuclei in Drosophila lead to an increase in the amount of ribosomal DNA. The ribosomal cistrons of Drosophila melanogaster are said to be located in the alpha heterochromatin of the X and Y chromosomes (Cooper, 1959; Ritossa and

Spiegelmann, 1965) and this raises some important questions concerning the replication of ribosomal cistrons during polytenization. In his classical studies on heterochromatin, Heitz (1934) suggested that the alpha-heterochromatin portion of the genome like the whole of the Y-chromosome and some proximal segments of the X and autosomes do not "grow" during polytenization with the result that polytene nuclei come to possess a very small amount of heterochromatin. This question of disproportionately lower amount of heterochromatin in polytene chromosomes of Drosophila has been examined by several workers using different techniques. The recent work of Rudkin (1972) using Feulgen cytophotometry suggests that with increasing polyteny salivary gland nuclei in Drosophila melanogaster exhibit a lower proportion of DNA in the centric heterochromatin. A combined autoradiographic and cytophotometric study of polytene nuclei in the brain ganglion cells of D. hydei (Berendes and Keyl, 1967) has also indicated that there is relatively less heterochromatin in the polytene nuclei of the brain compared with the diploid nuclei. The most recent work of Lakhotia (1974) using electron microscope autoradiography has revealed that the alpha-heterochromatin does not replicate at all from after hatching till late third instar larvae, while the beta-heterochromatin replicates as much as the euchromatin. It has been previously suggested that while the alpha-heterochromatin remains unreplicated in polytene nuclei, the rDNA cistrons located within this region replicate (Rodman, 1969; Pardue et al., 1970) perhaps not to the same level of polyteny as the

euchromatin (Henning and Meer, 1971; Spear and Gall,1973). If this is the case, then the rDNA cistrons of the alpha-heterochromatin must be assumed to be under independent control for their replication in polytene nuclei (Spear, 1974). With regard to the polytene nuclei of the nurse cells in ovarian chambers of Drosophila, it is not known, as far as I am aware, whether there is an underreplication of the ribosomal cistrons or not during polytenization. In view of the fact that these cells are involved in oogenesis, it may well be that the independent control mechanism of rDNA replication envisaged by Spear (1974) may operate to ensure that the rDNA replicates at least to the same extent as the euchromatin. The question, however, remains to be worked out.

The present study has shown, as in the previous work of other authors, that as ovarian chambers develop there is a concomitant increase in the size of nurse nuclei as well as of nurse nucleoli. An analysis of EM autoradiographs (Text-Fig. 2) of ^{3}H-uridine labelled material in the present study clearly shows that the nucleolar areas are intensely labelled. The data also show that the grain density over the nucleoli increases steadily from early stages up to about stage 10. This suggests that there is a steady increase in the rate of transcription of nucleolar RNA as the chamber develops. At any given stage, however, the grain density over the nucleoli in nurse cells is comparable with that in follicle cells. The larger size of the nucleoli in the nurse cells can, however, be expected to ensure a far

greater output of ribosomal RNA, since the RNA produced must be a function of both the rate of transcription and the amount of template available for transcription.

In my light microscope autoradiographic experiments with ^{3}H-uridine *in vivo* (see Figs. 111-115), the pattern of grains distribution at different intervals of time in the nurse cells and in the ooplasm in ovarian chambers of different stages is clearly indicative of a transfer of RNA, presumably as ribosomes, from nurse cells to the ooplasm. The route of transport of the ribosomes would of course be the ring canals. Evidence from the present study (already discussed) is also indicative of the fact that the contribution of the oocyte nucleus to the population of ribosomes in the ooplasm is negligible.

From early previtellogenic stages, some amorphous material is present in the perinuclear cytoplasm adjoining the nuclear envelope of all nurse nuclei examined. Earlier in this thesis, I have provided a detailed description of this material and what appears to be its transformation into fuzzy bodies. A stage-by-stage analysis of this material, especially in the posterior nurse nuclei in developing chambers, also indicates that the number of fuzzy bodies in the deeper regions of the nurse cytoplasm increases from stage 5 onwards. The increasing frequency with which fuzzy bodies are encountered within or in the vicinity of ring canals and in the ooplasm as development progresses may be regarded as evidence of a continuous flow of these bodies from the nurse cytoplasm to the ooplasm. However, during

stages 7-8, the fuzzy bodies tend to become rare or at any rate are not so frequently encountered as in the early stages of oogenesis. At the same time a narrow but continuous layer of extremely fine fibrils makes its appearance on the cytoplasmic side of the nuclear envelope of nurse cells. The significance of this layer of fibrils is unknown, but the timing of its appearance is interesting. It may reflect a large scale movement or transport of a particular kind of macromolecular constituents from the nucleus to the cytoplasm. In suitably designed experiments, it may be possible to examine in future, whether there is any change in the rate of transport of material across the nuclear envelope in nurse cells which possess the narrow band of fibrils close to the nuclear envelope. At any rate, the above mentioned observations indicate that the presence of the fuzzy bodies in the nurse cytoplasm is restricted to a limited period of time during oogenesis.

It was pointed out before that a consistent topographical relationship is observable between the fuzzy bodies and clusters of mitochondria in the nurse cytoplasm. Ultrastructural observations pointing to a close association between fibrillo-granular material (presumably derived from the nucleus) and mitochondria have been reported in several amphibians (Balinsky and Devis, 1963; Kessel, 1969; De Silva, 1973); in one instance (De Silva, 1973), it was suggested that the material contain RNA. In amphibian oogenesis it is known that mitochondria play a role, more directly than in insects, in the formation of yolk. Although

there is a morphological similarity in the association between mitochondria and elements derived from the nucleus in these instances, it is not possible to attribute any definite function to this association.

My autoradiographic studies using tritium labelled uridine indicate that the amorphous material, which most probably subsequently transform into fuzzy bodies, contain RNA. This suggests that the material in question is indeed coming out of the nucleus and is not something transported in the opposite direction; the difficulty of deciding the direction of movement of material observed in the static pictures in electron microscopy is a well known one. It is, however, surprising that the fuzzy bodies themselves appeared unlabelled in nurse cells of ovaries incubated *in vivo* in ^{3}H-uridine for as long as 12 hours. This question is considered further in the following paragraph.

Evidence from the autoradiographic studies using tritiated lysine indicate that the amorphous material in nurse nuclei during stages 4 to 7 contain proteins. The presence of proteins in similar material of *Rana* oocytes has been previously demonstrated by Clerot (1968) and also by Eddy and Ito (1971) by enzyme digestion and autoradiographic methods. The view generally accepted at the present time is that all nuclear proteins are made in the cytoplasm and transferred to the nucleus (Robbins and Borun, 1967; Goldstein, 1970). Craig and Perry (1971) have provided convincing evidence that the ribosomal proteins are predominantly, if not entirely, synthesized on cytoplasmic ribosomes. In my autoradiographic preparations made

after an hour long incubation in radioactively labelled lysine it is impossible to determine the site of synthesis of proteins, but in view of the earlier mentioned possibility that the amorphous material contain RNA, it is conceivable that the autoradiographic evidence following the use of lysine indicates proteins complexed with RNA moving out into the cytoplasm.

The results of the RNase extraction studies on the amorphous material were described earlier. It was pointed out, how RNase had the effect of increasing the contrast of this material. The failure of the enzyme to extract material, needsnot necessarily to be taken to mean that it contains no RNA, because it is possible that the nature of the RNA-protein complex is such as to protect the RNA from direct attack by the enzyme. The increase in electron density after RNase recalls the observations of Yotsuyanagi (1960) who suggested that this may, in fact, be indicative of the presence of DNA in the material. He suggested that Rnase, being a basic protein may bind to acidic DNA and the complex so formed may stain densely with uranyl acetate. The increase in electron density after RNase treatment was even more pronounced in the case of the fuzzy bodies. The results of the attempts to distinguish between ribonucleoproteins and deoxyribonucleoproteins by the Bernhard's (1969) procedure provide further indication that chromatin-like material is present in the fuzzy bodies and probably in the amorphous material as well. These observations taken together with the results of uridine labelling (viz. that the amorphous material is labelled with uridine, but the fuzzy bodies are not)

suggest that, as the amorphous material transforms into fuzzy bodies, it somehow loses RNA into the surrounding cytoplasm. As a result, it may be assumed that the fuzzy bodies come to contain predominantly deoxyribo-nucleo-proteins.

In their autoradiographic study of Drosophila oocytes, Muckenthaler and Mahowald (1966) found that a considerable portion of the total DNA which is present in the ooplasm is not associated with mitochondria; they expressed the opinion that nurse nuclei of stages later than 10 are a probable source of this ooplasmic DNA. In the light of the information now available on the chemical nature of the amorphous material and fuzzy bodies, one may venture to suggest that those entities provide morphological basis for the hypothesis put forward by Muckenthaler and Mahowald (1966). This, however, awaits confirmation by more direct means such as DNase extraction in formaldehyde fixed sections and autoradiography using labelled thymidine. If the amorphous material and the fuzzy bodies are the carriers of some DNA from the nurse cells to the ooplasm, then, the present study shows that this transfer occurs much earlier than originally suspected by Muckenthaler and Mahowald (1966).

5. The role of follicle cells in oogenesis

I have made a detailed study of the ultrastructural and functional aspects of the follicle cells in various stages of development of the ovarian chambers. Of special interest are the observations relating to the formation of the egg coverings.

It has been generally recognized that the vitelline membrane precursors are synthesized in the follicle cells. In an early account of the ovarian follicle cells in Drosophila, using light microscope, King and Koch (1963) described the presence of small sudanophil bodies, the "epithelial bodies" which they assumed to be the site of formation of the vitelline membrane precursors. Later, Beams and Kessel (1969) reported that in the ovary of dragonfly, the vitelline membrane precursors are made of vesicles derived from the cisternae of the Golgi apparatus of the follicle cells. More recently, Mahowald (1972a)also came to the conclusion that the Golgi apparatus are involved in the process of vitelline membrane formation in Drosophila melanogaster and commented on the inadequacy of the nomenclature adopted by King and Koch (1963). The latter authors still seem to hold the view (see King, 1970) that the epithelial bodies are not related to the Golgi apparatus. In their LM autoradiographic study, Cummings et al. (1971) also failed to associate the vitelline precursors with Golgi-derived vesicles.

My study is probably the first complete account of the structural and correlated functional aspects of the follicle cells at the level of the electron microscope. It was mentioned earlier that the Golgi apparatus in the follicle cells of early previtellogenic stages consist mainly of empty looking vesicles. On the other hand, as active vitellogenesis commences (stage 8), the Golgi apparatus exhibit a number of flattened cisternae and dense vesicles. The latter can be interpreted as the precursors of the

vitelline membrane material and two types of vesicles can be distinguished on the basis of their electron density. These observations agree with those already reported in Drosophila by Quattropani and Anderson (1969). It is of interest that the Golgi apparatus of the follicle cells is involved in the simultaneous elaboration of two distinct products both of which were shown to contribute ultimately to the vitelline membrane. Such a function of Golgi apparatus may not perhaps be uncommon. It may simply be that in the present material the two products are distinguishable by their electron density. The simultaneous production of two products has been reported in the Golgi apparatus of the secretory cells of the multifid gland of a snail by Ovtracht and Thiery (1972). The EM observations made in the present study leave little doubt that in Drosophila the precursors of the vitelline membrane are derived from the Golgi apparatus.

My autoradiographic experiments with tritium labelled lysine and the pattern of labelling obtained further confirm that the Golgi apparatus are involved in the elaboration of the vitelline precursors. In chambers of stages preceding the formation of the vitelline membrane there was no indication that the follicle cells contributed any products to the ooplasm. This observation is at variance from that described by Anderson adn Telfer (1969).

The ultrastructural and autoradiographic evidence that I have in the follicle cells are compatible with the generally accepted view of protein synthesis

on the ribosomes attached to the endoplasmic reticulum and their transport through the Golgi apparatus to the site of secretion in the interspaces between the oocyte and the follicle cells.

The present investigation has revealed that features essentially similar to those which characterize the follicle cell cytoplasm during the period of vitelline membrane formation (stages 9-10) are also present when (stage 11) the endochorion is being formed. On the other hand, the endochorion and the vitelline membrane are reported to exhibit clear cut chemical differences (King and Koch, 1963). This would imply that the Golgi apparatus in a given cell may elaborate products of different chemical composition at different times. A number of such situations have been recorded in the literature. For instance, Baiton and Farquhar (1966) have described two types of granules produced by the Golgi apparatus of polymorphonuclear leucocytes in two distinct periods of their life cycle.

The deposition of egg coverings ends with the apposition of the exochorion on the external side of the endochorion. The exochorion is formed when no secretory activity is apparent in the follicle layer. It has been suggested (Quattropani and Anderson, 1969) that the exochorion is formed by "wispy" material lifted from the plasma membrane of the follicle cells. Images suggestive of such a process have been seen occasionally during the course of the present study, but in the absence of further ultrastructural and experimental data it is not possible to comment on the

validity of this hypothesis. Quattropani and Anderson (1969) have also proposed that the exochorion could be regarded as equivalent to the insect epicuticle, while the endochorion and the vitelline membrane could be regarded as cuticle. Such a viewpoint has been criticized by Cummings et al. (1971) who proposed, on embryological considerations, that the formation of the egg coverings and cuticle in insects are separate phenomena. It was described earlier (page 117) that the endochorion as well as its precursors exhibit peroxidase activity. In insects, Locke (1969) has already demonstrated that peroxidasic oxidation occurs during the formation of the cuticle. On this basis, therefore, one may regard the endochorion of the egg and the cuticle of the adult as analogous structures, although they have different embryological derivation.

The presence of various cellular junctions in the follicle cells was mentioned earlier and have also been noted by other workers. It is of interest to note that the columnar follicle cells are joined by desmosomal junctions during the formation of the vitelline membrane, while later, as endochorion begins to be formed, these cells are joined by septate junctions. The appearance of the septate junctions also coincides more or less in time with the stretching of the follicle cell layer (stages 11-13), but the functional relationship between these two events is a matter of speculation.

Another structure which deserves mention is the intercellular bridge present between adjacent cells of the follicle layer. As stated earlier these

bridges are observable from stage 1, and it is therefore surprising that this structure was not identified by any of the investigators so far. In an electron micrograph published by Mahowald (1972a, Fig. 1), a structure present in the follicle layer of an ovarian chamber in Drosophila, is labelled by him as midbody; this structure, however, could more appropriately be interpreted as a tangential or oblique section of an intercellular bridge. In a previous work by Poodry and Schneiderman (1970), on the developing leg of Drosophila, intercellular bridges were seen connecting epithelial cells, and the authors speculated that the bridges are derived from midbodies formed during mitosis. From a limited study of serial sections of intercellular bridges in the present material, I am inclined to speculate that the bridges are derived by transformation of desmosomes. The origin of the intercellular bridges is therefore still a matter for conjecture, and the choice between the two alternatives mentioned must await more detailed studies.

6. The route of entry of substances into vitellogenic oocytes.

The present study has shown that tracers such as ruthenium red and lanthanum nitrate gain access to the oocyte by initially passing through the intercellular spaces of the follicle layer. Such a route for the entry of blood proteins was previously demonstrated in the oocyte of the cecropia moth by the use of fluorescein labelled antibodies (Telfer, 1961).

Since the tracers enter the interspaces between the follicle cells, these experiments can also be taken as demonstrating that the basement lamina - the outermost egg covering - is not an impermeable barrier for molecules of the size of the tracer employed. Evidence from experiments with thorotrast indicate that this colloidal tracer does not gain access to any site within the ovarian chambers, either in *in vivo* or *in vitro* conditions. On the other hand, I have shown that this tracer gains access to certain cells at the base of the ovary which are devoid of the basement lamina. It may thus be reasonably assumed that the basement lamina normally prevents diffusion of large sized particles such as those of the colloidal thorotrast. In their study of the vitellogenesis of *Aedes aegypti*, Anderson and Spielman (1971) noted that among the tracers they used, thorotrast was the one which penetrated more slowly into the ooplasm. The fact that in *Drosophila* ovary this tracer does not penetrate at all may be taken to indicate that the basement lamina in this species has different sieving properties from those of *Aedes aegypti*.

As stated earlier, although the various experiments with the above mentioned tracers have provided valuable information, they do not allow a study of the kinetics of the uptake in vitellogenic oocytes. The unavoidable necessity to use ruthenium red and lanthanum nitrate along with fixatives limits the usefulness of these tracers in that they do not permit a kinetic approach to absorption of substances into the egg. However, this limitation was overcome in

the present study by the use of peroxidase which can be supplied to the tissue in *in vivo* conditions.

Evidence from experiments with peroxidase demonstrate that at the beginning of vitellogenesis (stages 8-10) the tracer enters the intercellular spaces between the follicle cells and the space between the follicle cells and the oocyte. At about the same time the tracer can also be detected within the oocytes. On the other hand, in ovarian chambers which were at stage 10 when the experiment started it was not possible to detect peroxidase within the oocyte. It would thus seem that the vitelline membrane, once it is completed (at stage 10) is a barrier to the entry of substances into the oocyte.

The peroxidase detected within the oocyte is associated with the coated vesicles, tubules and small yolk platelets. The evidence that I have presented (see part II) may be interpreted as indicating that proteins from external sources (haemocoel) enter the oocyte at the region of the pits along the oolemma by pinocytosis, and that tubular profiles in the cortical ooplasm are derived by fusion of the coated vesicles which themselves are derived by pinocytosis from the region of the pits. There was never any indication of peroxidase in the cisternae of the endoplasmic reticulum of the oocytes when maximum pinocytotic uptake may be expected to occur. In view of this observation, the suggestion of Cummings and King (1970) that the tubules in the cortical ooplasm of *Drosophila* eggs are derived from the endoplasmic reticulum is untenable. Evidence

from the experiment with peroxidase also show that the number of labelled yolk platelets increases progressively with increasing durations of exposure to the tracer.

When peroxidase was supplied to the ovary in *in vitro* conditions, it was explained how the results were quite different from those observed in *in vivo* experiments. The presence of peroxidase in some coated vesicles and tubules of the cortical ooplasm indicates that pinocytotic uptake occurs in *in vitro* conditions, but the absence of the tracer from yolk platelets and morphological alterations in the cortical ooplasm are suggestive of some abnormality related to the later phases of pinocytotic uptake. It may be that pinocytotic activity occurs to some extent during the initial periods of culture *in vitro* but as time goes by the uptake process gradually comes to a stop. As explained in an earlier section of this thesis, the tubular aggregates that come to be formed in the cortical ooplasm during this experiment must be assumed to be derived from the first formed pinocytotic vesicles. In view of the similarities of the electron microscope images seen in *in vitro* cultured ovaries and in fibroblasts cultured *in vitro* with peroxidase (Steinman *et al.*, 1974), it may be assumed that factors other than those necessary to initiate the pinocytotic uptake are in fact required in vitellogenic oocytes to accumulate the proteins into yolk platelets.

Cohn and Benson (1965) have observed that the rate of pinocytotic uptake in *in vitro* cultures of phagocytes is greatly dependent upon the concentration of serum in the culture medium, and that no other factor

influences this function in physiological conditions. On the other hand, Bell (1969) has demonstrated that, if vitellogenin is restored to normal levels in the blood of previously allatectomized females of Periplaneta americana, there is no appreciable deposition of yolk in growing oocytes. However, if under these conditions farnesyl methyl ether - a substitute for juvenile hormone - is injected, it initiates vitellogenesis. More recently, it has been found that deposition of yolk could be restored in developing oocytes of starved females of Periplaneta americana either through injection of juvenile hormone or implantation of corpora allata but not through injection of oocyte homogenate (Bell and Barth, 1971). These experiments provide strong indication that vitellogenesis is a hormone-controlled process and that perhaps juvenile hormone is required for the formation of yolk platelets.

The appearance of atypical ultrastructural features such as the formation of tubular aggregates in the cortical ooplasm of ovaries cultured in vitro with peroxidase is best interpreted in terms of a functional alteration related to the lack of juvenile hormone. The ideal way to check the validity of this hypothesis would have been to add juvenile hormone to the culture medium containing peroxidase. Unfortunately, it was not possible to obtain the required juvenile hormone from any source. Further, it also needs to be checked if the atypical morphology of the cortical ooplasm is also related to a functional alteration resulting from the lack of specific proteins. This could be checked by adding extracted yolk proteins to the culture medium

containing peroxidase, but I have not so far attempted to extract and purify yolk proteins from eggs of Drosophila; this remains a problem for the future. In the mean time, it was hoped that conditions simulating this requirement could be obtained by adding extracts of fat bodies from adult females. It may be pointed out at this juncture that there is some evidence from my electrophoretic study that the protein content of fat bodies have electrophoretic mobilities similar to those found in the ovary. It may also be added that, in several insects, the fat bodies have been shown (Pan et al., 1969) to contain proteins immunologically identical to those of the ovary.

I have sought to obtain some clarification of the points discussed above by various in vitro culture experiments with haemolymph or fat bodies. The haemolymph of just-hatched females may be expected to contain both hormonal (juvenile hormone) factor and yolk proteins and, therefore, the addition of this to the culture medium may mimic the situation found in in vivo conditions in the female. The male haemolymph may be expected to contain the hormone only (Gilbert and Schneiderman , 1961), apart from other non-yolk proteins, and the addition of this to the culture medium may provide an experimental condition similar to the one described above but without the influence of the yolk. Finally, the addition of homogenate of fat bodies to the culture medium may be expected to provide an experimental condition in which the hormonal factor is absent but the yolk is present. The results of the experiments with these incubation media confirm the prediction described above that the

absence of peroxidase-labelled yolk platelets and the abnormal ultrastructural features of the cortical ooplasm of ovaries cultured *in vitro* are most probably due to the lack or deficiency of the juvenile hormone.

Two factors then appear to be involved in the mechanism of uptake in vitellogenic oocytes; blood proteins to fill the forming yolk platelets and juvenile hormone to control the cell surface modifications. The process of pinocytosis would therefore seem to be divisible into two distinct phases: (a) the adsorption of particles on the cell surface, and (b) the invagination of the loaded membrane (Jacques, 1969). It is of interest to note in this connection that the hormone oxytocin has been shown to induce pinocytotic uptake in the epithelial mucosa of the bladder in *Bufo marinus* (Masur *et al.*, 1969).

The experiments with peroxidase have shown that vitellogenic oocytes in *Drosophila* are capable of incorporating exogenous yolk proteins into the yolk platelets. A further clarification of the mechanism of uptake of yolk proteins was sought in autoradiographic experiments using tritiated lysine. The pattern of labelling was carefully analyzed by EM autoradiography and, as explained earlier, there is a distinctly heavy labelling of yolk platelets about 6 hours after injection of the tracer. In his work on protein synthesis in the ovary of *Musca domestica*, Bier (1963) also noted a considerable time-lag in the labelling of yolk platelets. Also in the honey bee ovary Engels (1972) showed that the yolk fraction was labelled

after a considerable delay following the labelling of other ovarian proteins.

The level of labelling in the yolk-free ooplasm during the time lag discussed above is very low by comparison, and this labelling could be attributed to protein containing material (ribonucleoproteins ?) transferred from the nurse cells through the ring canals. In view of the possibility of such a transfer, it is difficult to detect if there is a low level of synthetic activity in the ooplasm *in situ*. In contrast, the cytoplasm of the follicle cells shows heavy incorporation of the aminoacid even 1 hour after injection. The labelling patterns observed in the ovarian chambers demonstrate that, at any rate, there is no synthesis of proteins in the ooplasm even remotely comparable to that which occurs in the follicle cells. Further, the delayed appearance of label over the yolk platelets could conceivably be attributed to the time required for yolk proteins to be synthesized in an extraovarian tissue(s) and then transferred to the oocyte through the blood stream. The present findings together with the observations of other authors already mentioned (Bier, 1963; Engels, 1972) establish fairly well that in these insects the yolk proteins are synthesized in non-ovarian sites and that this accounts for the inability of the ovary to form yolk in *in vitro* conditions.

The similarities in the electrophoretic patterns of proteins extracted from the ovary and haemolymph, taken together with evidence from various other experiments in the present study provide reasonably conclusive evidence that the yolk proteins

are taken up by the oocyte from the haemolymph. Support for this view also comes from isoelectrofocusing experiments using solubilized proteins of ovary and haemolymph and autoradiography of such gels prepared after injection of radioactive aminoacids.

The next question that remains is the actual site of synthesis of the yolk proteins and this is a problem for the future. Some workers in this field (Pan et al., 1969) regard the fat bodies as the organ where the yolk proteins are synthesized. On the other hand, certain other workers (Roth and Porter, 1964) have assumed that the intestinal cells are the source of the yolk proteins. If this were the case, it could be that the yolk proteins which are synthesized in the intestinal cells pass into the haemolymph and then are taken up both by the fat bodies and the ovaries. It may be of interest to mention in this context that, during my autoradiographic study using labelled aminoacids, it was observed that the intestinal cells become extremely well labelled. This finding confirms earlier observations by Roth and Porter (1964) in the adult of Aedes aegypti. It remains, however, to be established whether any of the proteins synthesized in these cells are yolk proteins. A possible way to tackle this question in the future may be by immunological techniques in combination with autoradiography.

7. The activity of endogenous peroxidase in vitellogenic oocytes

By using the diaminobenzidine incubation medium under different conditions, it has

been possible to establish that two kinds of endogenous enzyme activities are present in Drosophila oocytes during stages 12-14. One is peroxidase activity which is restricted to the endoplasmic reticulum of both the nurse cells and the oocyte and which persists from stage 12 up to the completion of vitellogenesis. The other enzyme is catalase which is present in membrane-bounded organelles of only the ooplasm in stages 13-14.

Peroxidases are widely distributed in animal cells (Churg and Anderson, 1974) and a variety of functions have been attributed to these enzymes. For example, they have been credited with a bactericidal role and also implicated in the metabolism of halogens but no precise role has been so far established in relation to any definite physiological activity. The peroxidase present in the later oocytes of Drosophila is probably involved in some of the activities mentioned above, but as yet there is no evidence to substantiate this.

Several factors suggest that the catalase-containing organelles present in the ooplasm of stage 13-14 chambers in Drosophila are microperoxisomes. They are small in size and of variable shape; further, they do not exhibit an inner crystalloid core and appear to be in intimate contact with cisternae of the endoplasmic reticulum. In early EM studies of mammalian tissues (cf. Hruban and Rechcigl, Jr., 1969) certain organelles which contained a crystalloid core were referred to as microbodies. Subsequently, catalase activity was demonstrated cytochemically in these bodies (Fahimi, 1969), and on this basis they were identified as peroxisomes

that were first described in biochemical investigations (cf. De Duve and Baudhuin, 1966). The crystalloid core of the peroxisomes is assumed to be due to the presence of uricase (Hruban and Rechcigl, 1969). Further studies, however, showed that a class of peroxisomes does not possess the crystalloid core and may be intimately associated with the endoplasmic reticulum; these entities have come to be called microperoxisomes (Novikoff et al., 1972).

Peroxisomal enzymes, of which catalase, is normally taken to be the cytochemical marker (Novikoff and Novikoff, 1972), are recognized to have a definite functional significance in various animal tissues. De Duve and Baudhuin (1966), for instance, have demonstrated that peroxisomes may oxidise the reduced NAD by forming hydrogen peroxide. The excess hydrogen peroxide which is produced in this reaction, is subsequently reduced to water by means of a catalatic mechanism. In view of the participation of peroxisomal enzymes in this type of metabolic pathway, De Duve and Baudhuin (1966) suggested that peroxisomes could be involved in the process of gluconeogenesis. This interpretation is in fact consistent with the finding that all cell types from which peroxisomes have been extracted are known to synthesize glycogen by gluconeogenesis (Hand, 1973).

Evidence have been presented in the present study to show that the beta-spheres in the mature oocytes in Drosophila contain large amount of glycogen and that this glycogen is formed in intimate association with the alpha-1 yolk platelets (see page 158). Ovaries

of several insects are known to store glycogen in large amounts during advanced stages of oogenesis (Engels and Bier, 1967). There are also indications that the synthesis of glycogen during the later stages of oogenesis in several organisms occurs through gluconeogenesis (Yurowitzky and Milman, 1972). Therefore, on the basis of the information available, the catalase activity in the ooplasm of late ovarian chambers in Drosophila may be assumed to be functionally related to the synthesis of glycogen by gluconeogenesis.

8. Carbohydrates and yolk platelets

My investigations indicate that the superficial layer of alpha-1 yolk platelets (which are present in the ooplasm in stage 9-12 chambers) contains carbohydrates. This was demonstrated by the use of the silver methanamine method; this method, however, does not reveal any carbohydrates in the associated body embedded in the superficial layer of the yolk platelet. When the oocytes were treated according to other cytochemical procedures which are know to reveal carbohydrates (phosphotungstic acid and periodic acid - thiosemicarbazide - silver proteinate procedures) a reaction was evident only in the associated body and not in the rest of the superficial layer. It may therefore be concluded that the associated body and the rest of the superficial layer contain different types of carbohydrates. The periodic acid - thiosemicarbazide - silver proteinate procedure, as used in the present study, is known to reveal glycoproteins (Thiery, 1967).

My observations from several cytochemical experiments (PTA, PASM and ruthenium red) indicate the presence of carbohydrate material along the follicle-oocyte border in vitellogenic chambers. Following the above mentioned cytochemical procedures it is not possible to discern any ultrastructural elements in the follicle-oocyte border. A continuous amorphous cell surface coat has been reported in numerous instances (Rambourg, 1971). High resolution electron micrographs of the oocyte surface of Drosophila do not reveal such a coat; in this instance the amorphous coat (the fuzzy layer) is restricted to scattered regions (the pits) of the oolemma. The results obtained in the cytochemical experiments described above may therefore be taken to indicate the occurrence of some carbohydrate material (glycoproteins) which has reached the oocyte surface from the haemolymph, after passing through the intercellular spaces of the follicle layer.

I have clearly demonstrated the carbohydrate nature of the fuzzy layer at the pits of the oolemma by the use of concanavalin-peroxidase conjugate. With this technique, however, the glycoproteins indicated by the use of other cytochemical reactions such as PTA, PASM and ruthenium red are not revealed. It is thus possible to distinguish a carbohydrate component of the oolemma from the glycoproteins that occur in the interspaces between the oolemma and the follicle cell layer. Further, the fact that PTA stains the interspace between the oocyte and the follicle cells and also the inner layer of the coated vesicles in the cortical

ooplasm of vitellogenic oocytes makes it likely that the glycoproteins in the interspace are taken up into the oocyte.

Ultrastructural observations as well as the evidence derived from other experiments (peroxidase) point to the pit region of the oolemma as the sites of pinocytosis. It may now be suggested that the pinocytotic uptake of the glycoproteins occurs by their binding initially to the fuzzy layer. A similar suggestion was put forward by Favard-Sereno (1969) in vitellogenic oocytes of Gryllus. Glycoproteins which taken up by the oocyte are, as mentioned on several occasions before, derived most probably from the haemolymph.

In the in vivo experiments with concanavalin that were designed to show the kinetics of uptake of material by oocytes, the agglutinin appeared to be transported via the coated vesicles and tubules to the forming yolk platelets and segregated into the superficial layer. This shows that the carbohydrates of the fuzzy layer of the pits to which concanavalin is bound finally accumulate in the superficial layer of the yolk platelets. The PTA experiments, on the other hand, indicate that the glycoproteins taken up into the oocyte via the coated vesicles and tubules are stored in the associated body embedded in the superficial layer of the alpha-1 yolk platelets.

The specific staining reaction of the OZI with the Golgi apparatus has enabled me to identify Golgi elements in the superficial layer of the yolk platelets in chambers stages 8-12. This finding leaves

open the possibility that the Golgi-derived vesicles which are transferred to the yolk platelets may also be the carriers of some carbohydrate material.

The next question that remains to be considered is the origin of the carbohydrates of the fuzzy layer of the pits. From a survey of the literature is seems universally recognized that the Golgi apparatus is the site of synthesis of carbohydrates (Rambourg et al., 1969 ; Neutra and Leblond, 1966) and that Golgi vesicles transport carbohydrates to the plasma membrane to form the cell coat (cf. Bennett et al., 1974). It may, therefore, be reasonably assumed that in the present material the carbohydrates of the pits are synthesized by the Golgi apparatus of the oocytes and transported to the surface in the form of vesicles. My attempts to follow this process by autoradiography following incubation of oocytes in a medium containing tritiated fucose were unsuccessful.

9. Lysosomes and yolk formation.

The Gomori method is a reliable method for detecting acid phosphatase and this enzyme is widely accepted as a marker for lysosomes. In Drosophila oocytes, the Gomori procedure has revealed that the forming yolk platelets contain acid phosphatase and that as the yolk platelets increase in size, it is progressively difficult to demonstrate the enzyme. The present study, thus, confirms earlier light microscopic observations of Cone and Eschenberg (1966) and Hopkins and King (1967) on the occurrence of lysosomes in the cortical region of insect oocytes

during the early phases of vitellogenesis.

It is current knowledge that fusion between lysosomes and heterophagosomes determine the initiation of the process of digestion of the ingested material in the resulting phagolysosomes (Strauss, 1964, 1967). As a result of the lytic activity of lysosomal enzymes, it has been shown by Mego and McQueen (1965) and Neely and Martinore(1974) that low molecular weight degradation products will rapidly diffuse out of the phagolysosome into the surrounding area of the cell cytoplasm. The presence of acid phosphatase in yolk platelets in the present material may similarly suggests that some hydrolytic digestion takes place in the yolk platelets.

In the process of pinocytotic uptake of proteins envisaged in the present instance, one may assume that only specific yolk proteins are bound to the fuzzy layer of the pits. When the coated vesicles are subsequently formed, it may be further assumed that the free content of these vesicles, i.e. material not adsorbed on the fuzzy layer, consists of the non-yolk proteins. Because of the very small size of the vesicles, they may be expected to have a larger content of yolk proteins compared with the non-yolk proteins trapped in the vesicles, (Telfer, 1965). To some extent, therefore, this would lead to a preferential uptake of yolk proteins but, as explained already, the uptake is by no means exclusive. This probably accounts for the finding of Telfer (1965) that every protein detected immunologically in the blood has an antigenic counterpart in the vitellogenic

oocytes of insects. It is in this context that a meaningful explanation can be suggested for the presence of acid phosphatase and presumably of other lysosomal enzymes, in the growing yolk platelets. The suggestion is that the lysosomal enzymes are required to degrade all the non-yolk proteins and thus make possible the accumulation of specific yolk proteins in the maturing yolk platelets. In fact, some sort of selective hydrolysis has been assumed by Roth and Porter (1964) to occur during the transition of a homogeneous looking yolk platelet of Aedes aegypti into one with a crystalline structure.

There are indications from my autoradiographic experiments that some degradation of proteins takes place in oocytes of Drosophila. In stage 9-10 chambers that were fixed after up to 10 hours of injection of tritiated lysine into the haemocoel, there was a steady increase in grain density over the yolk platelets. When, however, chambers of the same stage were fixed after exposure to the tracer for over 10 hours, the density of grains over the yolk platelets was much less. The higher grain density in the first formed yolk platelets and the loss of radioactivity during their further development is consistent with the interpretation that some of the labelled proteins found initially in the yolk platelets undergo degradation during maturation of the yolk.

Although a number of previous investigators have demonstrated in light microscopy studies the occurrence of acid phosphatase in several locations in the oocytes of many animals, the origin of this enzyme in yolk platelets is still a matter of

controversy. The present study provides an original contribution towards an understanding of how acid phosphatase and perhaps also other lysosomal enzymes are brought into the yolk platelets.

A large body of evidence, mainly from biochemical work, has established that lysosomal enzymes, like other enzymes and proteins, are synthesized in the rough endoplasmic reticulum, transferred to the Golgi apparatus and then released as vesicles (lysosomes) in the cytoplasm (De Duve and Wattiaux, 1966). Numerous cytochemical investigations have further substantiated this concept; for instance, Novikoff et al. (1964) regarded the vesicles derived from the Golgi region (which they referred to as GERL) as primary lysosomes or vesicles containing lytic enzymes not yet involved in digestive processes.

My ultrastructural and cytochemical studies have clearly shown that the oocytes acquire a number of Golgi apparatus from the nurse cells, through the ring canals. The use of the Gomori method has revealed that the Golgi apparatus in nurse cells and ooplasm contains large amount of acid phosphatase. The Golgi apparatus of the follicle cells also react positively for acid phosphatase, but there is no evidence at all of any transfer of this to the ooplasm. Therefore, the major part of the acid phosphatase present in the vitellogenic ooplasm appears to be derived from the Golgi apparatus of the nurse cells. Some contribution of acid phosphatase, however, may also be expected from the cytolysosomes, which have passed into the ooplasm from the nurse cells even prior to the initiation of

vitellogenesis.

Experiments to demonstrate thiamine pyrophosphatase activity by the Novikoff and Goldfischer's (1961) procedure have revealed clear cut differences between the lysosomes of the follicle cells on the one hand and the nurse cells and the oocyte on the other. This finding further substantiates the hypothesis that the follicle cells do not contribute to the enzymatic make up of the ooplasm.

The lack of ultrastructural clarity in preparations used for the localization of acid phosphatase does not allow an unambiguous demonstration of the method of transfer of the enzyme from the Golgi apparatus to the yolk platelets. Added to this is the difficulty posed by the fact that enzymatic activity is either lost or become cytochemically undetectable in Golgi vesicles as they move away from the Golgi apparatus (Friend and Farquhar, 1967). The value of the OZI method in this context is that it stains selectively the Golgi apparatus and the vesicles derived from it on a non-enzymatic basis. By using this method it was shown that Golgi derived vesicles may fuse with the yolk platelets. Furthermore, the OZI deposits are observable in the superficial layer of several yolk platelets. The simultaneous occurrence of acid phosphatase and OZI deposits in the forming yolk platelets provides strong evidence that the Golgi vesicles are the link between the Golgi apparatus and the yolk platelets. It must; however, be mentioned that no membrane-limited vesicles are visible in the superficial layer of the yolk platelets even in the preparations made for study of ultrastructure.

This suggests that the Golgi derived vesicles lose their membrane as they fuse with the yolk platelets.

The interpretation given above for the occurrence of acid phosphatase in the yolk platelets of the oocyte of *Drosophila* recalls the method of origin of hydrolytic enzymes described by Daems *et al*. (1969) in endocytotic vacuoles of several absorbing tissues. The mechanism of membrane fusion between lysosomal derivatives and endocytotic vacuoles (heterophagosomes) is in fact a well recognized process which enables enzymatic digestion of extracellular material to occur within an internalized membrane limited space, without damaging the cell cytoplasm (Hirsch and Cohn, 1964). Other ways in which lysosomal enzymes may be transferred from one cell compartment to another are also known. Gordon *et al*. (1965), for instance, have demonstrated that intact Golgi vesicles may be engulfed within a phagosome by a temporary interruption of its limiting membrane. However, incorporation of intact Golgi vesicles within a membrane-enclosed space has been assumed to be related to a type of delayed intracellular digestion which could be accomplished by controlling the permeability properties of the engulfed vesicles (De Duve and Wattiaux, 1966). In vitellogenic oocytes, acid phosphatase is present in the early stages of yolk formation and, as I have mentioned several times before, it seems to disappear from this region as the yolk platelets increase in size.

There is no evidence in the present material to suggest the occurrence of any mechanism for delaying the release of acid phosphatase as in the instance cited by Gordon *et al*. (1965) and De Duve and Wattiaux (1966).

In the smaller yolk platelets, the enzyme is more or less uniformly distributed, while it seems to become progressively restricted in distribution in the growing yolk platelets. In the larger yolk platelets, the enzyme appears to become localized to the associated body and finally, with further transformation of the alpha-1 into alpha-2 yolk platelets, the enzyme is no longer demonstrable by the Gomori procedure. The presence of acid phosphatase and presumably of other lysosomal enzymes in the main body of early yolk platelets and their later localization in the associated body of the yolk platelets raises a crucial question, that is, how the yolk proteins escape digestion. An attempt is made below to offer an explanation.

One may speculate that the hydrolytic enzymes in the young yolk platelets do not act on the specific yolk proteins brought into the yolk platelets via the fuzzy layer of the coated vesicles but instead act only on the remaining proteins (non-yolk proteins ?). In this context, it is relevant to refer to the hypothesis formulated by Brambell (1964, 1966) on the transfer of certain γ-globulins from mother to the young in pregnant mammals. According to this hypothesis, all plasma proteins are taken up by pinocytosis at the level of the placenta and transported to the opposite side through the lysosomal system. During the transport, the γ-globulins alone were assumed to escape degradation because they were bound to certain protecting substances on the wall of the pinocytotic vesicles. More recently Jacques (1969) has proposed that, in cells capable of pinocytotic uptake, the mucopolysaccharide coat of the cell surface could

very well function as the protecting substance postulated by Brambell (1966). Further, Snellman and Sylven (1974) have actually demonstrated that substances may be protected from hydrolytic enzymes by virtue of their binding to carbohydrate moiety. In the present material, one may attribute the carbohydrate of the fuzzy layer to serve as the substance protecting the yolk proteins from degradation by the hydrolytic enzymes in the yolk. In line with the assumption so far made, one may further speculate that in the later stages of yolk development, the yolk proteins dissociate from the carbohydrate moiety (which has so far provided protection from the lysosomal enzymes) and get packed in the main body of the yolk platelet, while the carbohydrate accumulates to form the associated body of the yolk platelet. A final speculation would be that the carbohydrates in question then bind to the residual hydrolytic activity thereby accounting for the presence of both acid phosphatase and PTA-stainable carbohydrates in the associated body. If this interpretation is correct, then the formation of the associated body may also be regarded as a device to inactivate residual lysosomal enzymes, at the end of the process of yolk formation.

It would, therefore, seem that the lysosomal enzymes present in forming yolk platelets serve initially to ensure the degradation of non-yolk proteins that are taken up together with specific yolk proteins during pinocytosis. The later sequestration of the hydrolytic enzymes - in an inactive form - in the so-called associated body, besides serving to prevent the degradation of the stored yolk proteins,

may have a role to play in yolk utilization in the embryo. Although the associated body has not been distinguished in the yolk platelets of mature eggs, it could be that the trapped enzymes are still present in the yolk and serve to initiate degradation of the yolk in early embryonic development.

A few years ago Bluemink (1969) suggested that the functional concept of lysosome may apply to the process of formation and utilization of yolk material. This may now indeed be deemed to be a likely proposition. The whole concept, however, rests on several speculations and each of the steps in the scheme outlined above requires clarification and experimental confirmation.

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