HISTOCHEMISTRY AND ULTRASTRUCTURE OF OOGENESIS IN *IBLA QUADRIVALVIS* AND *TETRACLITA ROSEA*, AND THE BIOCHEMICAL COMPOSITION OF DEVELOPING EMBRYOS OF *IBLA QUADRIVALVIS* (CRUSTACEA, CIRRIPEDIA).

by

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CONTENTS

SUMMARY

1. Oogenesis in I. quadrivalvis and T. rosea was divided by light microscopy into 7 stages. The stages were examined histologically and histochemically for nucleic acids, proteins, carbohydrates and lipids.

2. The 7 stages of oogenesis of I. quadrivalvis were examined by electron microscopy.

3. Oogenesis in both species is identical, except that I. quadrivalvis produces an egg about twice the linear dimensions of T. rosea.

4. The ovary in both species is a hollow tubular organ, branching into many ovarioles. Each ovariole is lined externally by a thick basement lamina, and lined internally by epithelial cells.

5. The epithelial cells of the ovary wall become mitotically active, forming germinal epithelial cells. The germinal epithelial cells undergo several mitotic divisions to eventually form clusters of 16 oogonia.

6. Some epithelial cells become involved in the resorption of ovarian tissue.

7. During previtellogenesis there are increases in the volumes of the nucleus, nucleolus and the cytoplasm.

8. The cytoplasm of the previtellogenetic oocyte becomes increasingly basophilic as numerous vesicles of the smooth endoplasmic reticulum derived from the outer nuclear membrane develop in the cytoplasm.

9. The presence of free ribosomes, lamellae of the rough and smooth endoplasmic reticulum, and Golgi bodies contributes to the increased cytoplasmic RNA and basic protein detected during previtellogenesis. The synthetic machinery of vitellogenesis is laid down during this stage.
10. The cytoplasm of the vitellogenetic oocyte becomes increasingly acidophilic as yolk accumulation begins.
11. The extrusion of nucleolar material through the pores of the nuclear membrane into the cytoplasm is discussed.
12. The lamellar form of the endoplasmic reticulum is no longer present in the cytoplasm. The vesicular form of the smooth and rough endoplasmic reticulum becomes a prominent organelle in the cytoplasm of vitellogenetic oocytes.
13. The Golgi complexes increase in size and number during vitellogenesis. Small membrane-bound secretory granules, 40-60 mμ in diameter are produced by the Golgi complex.
14. The granules become incorporated into vesicles of the endoplasmic reticulum to form small precursor yolk vesicles, staining positively for carbohydrate and basic protein.
15. Mitochondria increase in number and size during vitellogenesis. Microtubules increase in numbers, particularly in the peripheral regions of vitellogenetic oocytes.
16. At about the middle of vitellogenesis, lipid droplets are formed 'de novo' in the cytoplasm.
17. In addition, material is incorporated into the oocyte via micropinocytosis, and eventually gives rise to additional protein-carbohydrate yolk.

18. Towards the end of vitellogenesis, the precursor yolk vesicles undergo a process of maturation to form distinct protein-carbohydrate yolk bodies.

19. Towards the end of vitellogenesis, the surface of the oocyte develops microvilli and secretes a vitelline membrane.

20. Oogenesis in I. quadrivalvis and T. rosea is discussed in relationship to oogenesis in other crustaceans and in other animals.

21. The embryos of I. quadrivalvis were examined biochemically to determine total water, carbohydrate, glycogen, protein and lipid content.

22. The dry weight, and total glycogen and lipid contents remained constant throughout embryonic development. The water content increases considerably during embryonic development. There is a net decrease in total carbohydrate content, protein content shows a net increase, but the protein content fluctuates during embryonic development.

23. The changes in the above biochemical constituents during embryonic development of I. quadrivalvis are discussed in relationship to the changes in the biochemical composition of other cirripede embryos and other crustacean embryos.

24. A preliminary analysis of the pigments of ripe ovary and mature embryos of I. quadrivalvis shows that both astaxanthin and a chromolipid are present. The bulk of the pigment present in the ripe ovaries, still persists in the mature embryos.

25. This result is discussed in relation to pigment content of other cirripede embryos and other crustacean embryos.

INTRODUCTION

1. Oogenesis in Ibla quadrivalvis and Tetraclita rosea

In the early light-microscope studies on oogenesis in the Crustacea, attention was focussed mainly on the mode of synthesis of yolk inclusions, and the broad spectrum of oogenesis was generally ignored. Several different views were expressed concerning yolk formation in crustacean oogenesis. For example, protein yolk was considered to arise directly under the influence of the mitochondria in Oniscus (King, 1926), Cambarus (Kater, 1928) and Palaemon lamerrei (Bhatia and Nath, 1931). In contrast protein yolk in Carcinus maenas (Harvey, 1929) and in Paratalphusa spingera (Bhatia and Nath, 1931) was considered to develop in the cytoplasm directly from nucleolar extrusions.

Since the advent of electron microscopy, more exact studies have been possible on yolk synthesis in the oocytes of a wide variety of animals. Concomitantly, an extensive study of the organelles involved in general cellular synthesis, such as the Golgi and the endoplasmic reticulum, coupled with the firm evidence for the involvement of ribosomes in the synthesis of proteins, has given a clearer understanding of the synthesis of protein-rich secretions in cells of many diverse organisms (Brachet, 1960).

The means by which yolk is formed varies amongst different groups of animals. In crustacean oocytes, the protein and lipid yolk is synthesized within the oocyte (Beams and Kessel, 1962, 1963; Kessel, 1968a). The best evidence that synthesis occurs within the oocyte has been provided by Beams and Kessel (1962, 1963) in studies on the crayfish oocyte. In this oocyte, protein yolk is elaborated

within a well developed, interconnecting system of rough endoplasmic reticulum. Other studies on crustacean oocytes (Kessel, 1968a) have also shown that the synthesis of protein yolk granules is associated with the endoplasmic reticulum.

In the above examples, no evidence was obtained that the Golgi complex is involved in the protein yolk synthesis. However, numerous reports of the Golgi being actively involved in protein yolk synthesis have been presented for a wide variety of invertebrates (Kessel, 1966a, b; 1968b; 1968d; Dumond and Anderson, 1967; Beams and Sekhon, 1966). In a recent study of protein yolk synthesis in the spider crab Libinia emarginata, Hinsch and Cone (1969) have suggested that the Golgi complex may be involved in protein yolk synthesis to a greater extent than in other marine decapods.

No previous account of oogenesis has been given for the Cirripedia. The present study has been carried out primarily in order to compare the processes of yolk synthesis in the Malacostraca with those in a structurally simpler crustacean egg. In addition, the general course of cirripede oogenesis has been followed by histochemical and ultrastructural means, in order to elucidate the origins of cellular organelles and their interrelationships within the oocyte, and to gain a clearer understanding of the major cytoplasmic activities during crustacean oogenesis.

2. Biochemical Composition of embryos of I. quadrivalvis

One of the most conspicuous activities of crustacean oocytes is the synthesis and storage of protein and lipid yolk reserves. A study of the utilization of the yolk reserves during embryogenesis

and larval development may give an insight into the role of the various biochemical constituents synthesized and stored during oogenesis.

Studies on the rate and efficiency of transformation of matter and energy in the developing embryos of crustaceans have received increased attention in recent times (Urbani, 1959, 1962; Barnes, 1965; Green, 1965; Paffenhoff, 1967; Pandian, 1967; Pandian and Schumann, 1967). Studies on the biochemical composition of the embryos of Crangon crangon (Pandian, 1967) and Eupagurus bernhardus (Pandian and Schumann, 1967) for example, have shown that the bulk of the lipid yolk reserves are utilized during embryonic development. Recent studies on the biochemical composition of the embryos of the cirripedes Balanus balanoides and B. balanus (Barnes, 1965), have shown that all the yolk reserves are metabolized to some extent during embryonic development, but that in these cirripede embryos, which hatch into active feeding planktonic larvea, the lipid reserves are in part retained into the cyprid stages. I. quadrivalvis produces an egg which is larger than that produced by most cirripedes (Anderson, 1965) and has a greater yolk content. The nauplius larvae of I. quadrivalvis are active but non-feeding and the yolk reserves laid down in oogenesis are important not only during embryogenesis, but also in sustaining the planktonic larvae. The relative utilization of protein and lipid yolk in this sequence of development therefore becomes a matter of some interest.

MATERIALS AND METHODS

1. Histology of the ovary and oogenesis of I. quadrivalvis and T. rosea.

I. quadrivalvis is found in groups amongst the tube worm Galeolaria caespitosa Lamarck (Dakin, 1952). It occurs intertidally on the New South Wales coast and the material for the present study was collected from Bradley's Head on the north shore of Sydney Harbour.

T. rosea occurs in the warmer seas of the east coast of New South Wales and Queensland (Dakin, 1952). Specimens of T. rosea were collected from the intertidal regions of the rock platforms of the northern beaches of Sydney, chiefly Harbord and Balmoral.

Mature ovaries were found in both species throughout the year, but the best material for histological study was obtained in February-April, during the height of the breeding season. I. quadrivalvis breeds from August-May (Wisely and Blick, 1964). In T. rosea, egg production decreases during winter and increases towards spring-summer, to enter the height of the breeding season during February-April (Wisley and Blick, 1964). In both species, ripe ovaries were selected for all the histological work.

Freshly dissected ovaries were examined in sea water using a binocular microscope. As both species produce yolky eggs, the ovaries need to be treated with great care to avoid damaging the oocytes and disrupting the yolk. The ovary of I. quadrivalvis was not dissected out, but was fixed still attached to the peduncle. In T. rosea, the entire ovary was lifted out from the basal region of the carapace. The ovaries were fixed in Smith's formol bichromate and the procedure

outlined by Steedman (1960) was followed. The fixed preparations were dehydrated in a series of alcohols (30 minutes in each). While the ovaries were in 70% alcohol they were dissected into blocks ready for embedding. The ovaries were then passed through methyl benzoate and benzene, and embedded in paraffin (M.P. 56°C). Sections were cut at 8μ, and stained with Delafield's haematoxylin and eosin.

2. Histochemistry of the ovary and oogenesis of I. quadrivalvis and T. rosea

The following procedures were used to localize DNA, RNA, protein, carbohydrates and lipids.

(a) Nucleic Acids

Ovaries were fixed in Carnoy's fixative for no longer than 60 minutes at room temperature. They were then transferred to 70% alcohol, followed by 95% alcohol, methyl benzoate and benzene, and then embedded in paraffin (M.P. 56°C). Sections to be stained for DNA were cut at 8μ. Sections to be stained for RNA were cut at 5μ. Fresh material was used for the acridine orange method, as described in Pearse (1960). Small areas from the distal portion of the ovarioles were stained and gently squashed on the slide before examination by fluorescence microscopy using blue light.

Sections were stained as follows:-

DNA

1. The Feulgen reaction, as outlined by Pearse (1960) was employed. The sections were hydrolyzed in 1N HCl at 60°C for 8 minutes, and stained for 1 hour in Tomasi Schiff's reagent. Control sections were treated with crystalline DNase (1mgm/ml) at 38°C for 24 hours before staining, as outlined by Pearse (1960).

2. The acridine orange method (Pearse, 1960) modified for Carnoy fixed tissues was used on material sectioned at 5μ. Sections were stained for 3 minutes in acridine orange, then examined by fluorescence microscopy using blue light.

3. The pyronin/methyl green method described by Merritt and Rowlatt (1959) was used to demonstrate both DNA and RNA. Control sections were treated with DNase for 1 hour at 37°C, and then with RNase for 1 hour at 37°C before staining, as outlined by Pearse (1960).

RNA

Ribonucleic acid was localized by the two previously described methods: (1) acridine orange (Pearse, 1960) using both fresh ovaries and Carnoy fixed materials; (2) the pyronin/methyl green method of Merritt and Rowlatt (1959). Control sections were incubated with crystalline RNase for 1 hour at 37°C prior to staining.

(b) Proteins

A satisfactory cytoplasmic fixative was required to demonstrate proteins, and for both species Smith's formol bichromate (Steedman, 1960) was used as outlined above. As a general stain, the mercury bromphenol blue technique, as described by Pearse (1960), was used to demonstrate the presence of proteins. Before control sections were stained, they were treated with pespin for 1 hour at 37°C followed by trypsin at 37°C for 1 hour (Pearse, 1960).

Basic proteins

The presence of basic proteins was best demonstrated by the aqueous bromphenol blue method of Mazia, Brewer and Alfert (1953). The alkaline fast green F.C.F. technique of Alfert and Geschwind (1953)

as outlined by Pearse (1960) was also used to localize basic proteins. Control sections were incubated with trypsin for 1 hour at 37°C (Pearse, 1960) before staining.

Arginine

The Sakaguchi reaction for arginine (Baker's 1947 modification) was used as outlined by Pearse (1960). Control sections were incubated in trypsin for 1 hour at 37°C prior to staining, as described by Pearse (1960). Sections must be examined immediately after staining, as the dye fades rapidly.

Tryptophan

Estimation of tryptophan was carried out by the DMAB nitrite method for tryptophan (Adams, 1957)as described by Pearse (1960). Control sections were incubated with pepsin for 1 hour at 37°C (Pearse 1960) before staining.

(c) Carbohydrates

Various fixatives were used to demonstrate different kinds of carbohydrates, and again Smith's formol bichromate was used in addition to the recommended fixatives, as good cytoplasmic fixation was required.

Glycogen

Ovaries were fixed in cold Lison's "Gendre Fluid" (Pearse, 1960) for 24 hours at 0'C. The fixed tissue was passed through graded alcohols, 30 minutes in each, then through methyl benzoate and benzene,

and finally embedded in paraffin wax (M.P. 56°C). Sections were cut at 8μ. Best's carmine method (Pearse, 1960) was used to localize glycogen. The sections were stained for 20 minutes and rinsed in the differentiating solution for 10 seconds. Control sections were treated with undiluted saliva before staining, for at least 3 hours at 37°C. In addition, sections were stained by the periodic acid-Schiff reaction, as described by Hotchkiss (Pearse, 1960), using Barger and de Lamater Schiff reagent. Control sections were treated with saliva as described above. PAS-positive material that is removed in these sections is glycogen, and these sections were compared with the test sections for accurate localization of glycogen.

Polyasccharides other than Glycogen

Only high molecular weight polysaccharides can be localized histochemically. Other than glycogen, they are the neutral mucopolysaccharides, acid mucopolysaccharides, muco- and glycoproteins. Ovaries were fixed in Lillie's alcoholic lead nitrate (Pearse, 1960) for 24 hours at room temperature, dehydrated in a series of alcohols, then methyl benzoate and benzene and embedded in paraffin wax (M.P. 56°C).

Sections were stained as follows:-

(1) PAS-positive material

The sections were stained with PAS reagent as described above. Control sections were subjected to the acetylation-prevention of the PAS reaction (Lillie, 1954), as outlined by Pearse (1960).

(2) Acid mucopolysaccharides

Several methods were used to localize acid mucopolysaccharides since no adequate control can be established easily for these compounds.

1. Sections were stained with Alcian blue (1% solution) at various pH's as described by Lev and Spicer (1964).

2. Sections were stained with Colloidal Iron solution following the method of Hale, as outlined by Pearse (1960).

3. Sections were stained with mucihematein as outlined by Laskey (1950).

(d) Lipids

For the localization of lipids, the ovaries were fixed in Baker's formol calcium (Pantin, 1948) for 48 hours, and then transferred to Baker's formol-cadmium-calcium for storage (Pantin, 1948). The fixed tissue was washed for 4 hours before being embedded in a series of graded gelatin solutions; 10% gelatin for 1 hour at 37°C; followed by 1 hour in 15% gelatin, then 1 hour in 25% gelatin. The tissues were embedded in 25% gelatin and allowed to set in the cold. Once the gelatin had set, small blocks were cut and fixed in Baker's Cd-Ca formol for at least 24 hours. The blocks were washed for 4 hours before cutting at 15μ on a freezing microtome.

The controls for the following tests were fixed in a weak Bouin solution, followed by Baker's pyridine extraction test, as outlined in Pearse (1960). The material was then embedded in 25% gelatin solution as described above.

The following staining procedures were used to localize lipids:-

Neutral Fats

The Oil Red O method as outlined by Pearse (1960) was used to demonstrate the presence of neutral fats.

Phospholipids

The Copper Phthalocyanin method, as outlined by Pearse (1960) was used for the detection of phospholipids. Sections were stained for 18 hours at 60°C and counterstained in Mayer's Carmalum for 10 minutes.

3. Electron microscopy of oogenesis in I. quadrivalvis

Cytoplasmic organelles may vary in appearance according to the fixative used. Ovaries were fixed in 3 different buffer combinations in order to assist in the minimisation of fixation artifacts. Ripe ovaries were dissected free of the peduncle and immediately immersed into one of the following fixatives: (1) 2.5% glutaraldehyde in sea water; (2) 2.5% glutaraldehyde in .067M phosphate buffer saturated with 1.0M $CaCl_2$, with 0.44% NaCl added (pH, 7.45); (3) 2.5% glutaraldehyde in .067M phosphate buffer saturated with 1.0M $CaCl_2$, with 10% sucrose added (pH,7.45).

The ovaries were dissected into 1mm blocks which were left in one of the above fixatives for 3 hours at 0°C. The tissue was washed in 2 changes of cold buffer, and left in buffer overnight at 0°C. The material was then post-fixed in a 2% osmium tetroxide solution in the appropriate buffer for 3 hours at 0°C.

The blocks were rinsed in 0.01% acetic acid for 2-3 minutes at room temperature and stained with 1% aqueous uranium acetate for 1 hour at room temperature. The tissue was rapidly dehydrated by passing through a series of graded alcohols, rinsed twice in propylene oxide and left to infiltrate in a 50% mixture of propylene oxide and araldite (using the usual mixture) for 48 hours at room temperature. The blocks were then embedded in the usual araldite mixture at 60°C for 48 hours.

Sections of 600-900 A^{o} thickness were cut on a L.K.B. ultramicrotome using a glass knife, floated out on 10% acetone and picked up on a nitrocellulose-coated copper grid which had been previously stabilized with a carbon film. The sections were then stained in Karnovsky's (1959) lead stain for 10 minutes, washed in a stream of distilled water for 3 minutes, and dried with filter paper. A Siemens Elmiskop 1 (80KV,30μ or 50μ objective aperture) and a Philips electron microscope 200 (60 KV, 30μ objective aperture) with a cold stage to reduce contamination were used for viewing.

One micron thick sections were also cut from the araldite block with glass knives. The sections were peroxidated with 1 drop at 15% solution of H_2O_2, and the slide was left on a hot plate for about 5 minutes, until the peroxide has evaporated. One drop of alcoholic toludine blue was added, and the slide left for 30 seconds before rinsing in distilled water. A coverslip was mounted with immersion oil, and the slides were examined with the light microscope.

4. Embryonic Development of Cirripede Eggs

As repeated reference will be made to the different developmental stages of the embryos of I. quadrivalvis, a brief summary of the staging used is given below. A complete description of the developmental stages of I. quadrivalvis was given by Anderson (1965). The stages were numbered according to the system proposed by Crisp (1954) for Balanus embryos. Some of the stages were grouped together, as shown below, either because they last only for a short time, or because they cannot be distinguished from one another in the specialized embryos of I. quadrivalvis on the basis of external features.

The stages are:-

Stage 0: Mature ovary: Only ripe ovaries were used in all experiments. Care was taken to remove any muscle and connective tissue adhering to the ovary wall.

Stage 1: Freshly laid ovum: Fertilized or unfertilized ova were used. The yolk is uniformly distributed through the ovum, but after fertilization, segregation of the whitish, yolk-free cytoplasm occurs at the anterior pole.

Stage 2: Cleavage: About two hours after fertilization, the first cleavage furrow appears. Cleavage continues as described by Anderson (1965) until the blastula is formed.

Stage 4-6: Gastrulation: The gastrula can be distinguished from the blastula by the fact that it is covered with regular small micromeres which contrast sharply with the much larger macromeres of the blastula. A blastopore can be detected early in this stage.

Stage 7-8: Limb-bud formation: Three paired posterolateral thickenings appear on either side of a median posterior thickening. These are the rudiments of the naupliar limbs and the caudal papilla.

Stage 9: Development of appendages: The naupliar limb-buds are clearly visible as short bifid swellings on either side in front of the caudal papilla. The labrum and caudal papilla begin to elongate. The naupliar eye appears as a pair of minute red pigment spots in the ventral midline in front of the labrum.

Stage 10: Further development of appendages: The naupliar limbs elongate and become free from the embryo. The pigment in the naupliar eye turns brown.

Stage 11: Formation of the naupliar cuticle: The naupliar cuticle is secreted, and the naupliar limbs are covered with setae.

Stage 12: Mature embryo: The pigment in the embryo changes from a creamy yellow to a yellowish brown. Mature embryos can be distinguished by this alone. A carapace fold is developed around the periphery of the embryo. Limb movements begin as an irregular activity, which gradually becomes prolonged into regular continuous beating just prior to hatching.

These nine divisions were used in the previously described biochemical experiments.

Rate of Development

A time sequence for the developmental processes is required in order to relate the changes in gross biochemical material determined at each stage. The rate of development of embryos of Ibla quadrivalvis has been investigated by Anderson (1965). A brief summary of this data is set out in Table 1.

TABLE 1 RATE OF DEVELOPMENT OF EMBRYOS OF IBLA QUADRIVALVIS at 23°C (From Anderson, 1965).

Stage	Age at commencement of stage	Duration
1	0	2 hours
2-3	2 hours	3½ days
4-6	3½ days	1½ days
7	5 days	1½ days
8	6½ days	1½ days
9	8 days	3 days
10	11 days	1 day
11	12 days	1 day
12	13 days	4 days

5. Biochemical composition of mature ovaries and embryos of *I. quadrivalvis*

(a) Estimation of Wet weight

Egg masses containing developing embryos were removed from the mantle cavity of ovigerous females and placed in a clean petri dish containing freshly filtered sea water. A small piece of the egg mass was dissected free with fine dissecting needles. The embryos were then examined under a monocular microscope and the stage estimated. From this examination it was confirmed that synchronous development of the eggs was occurring throughout each egg mass. Early in the investigation, it was realized that the embryos in many egg masses were dead when removed from the adult. It was difficult in the younger stages to determine if the embryos were alive, but in egg masses containing dead eggs, yolk appeared to be extruded from the eggs and was observed floating within the egg mass. In every case, the dead eggs were infected with protozoans and nematodes. It was essential for the biochemical tests to only use live material.

After the embryos had been staged, the small piece of egg mass was rinsed three times in distilled water, gently blotted dry with filter paper and weighed. It was then returned to sea water, and the numbers of eggs within it counted under a dissecting microscope. This procedure was repeated until consistent results (within the limits of 0.5 micrograms) of wet weight/egg were obtained for each stage. Once a series of wet weights/egg for each stage was established, the numbers of eggs in the whole egg mass from any stage could be estimated by weighing.

All samples of ovary and egg masses used in biochemical

estimations were rinsed three times in glass-distilled water to remove any sea water adhering to the surface of the tissue prior to blotting with filter paper and weighing. The method of washing was that described for marine eggs by Fluchter and Pandian (1968). After evaporation, there would be a quantity of salts left from any sea water and this factor would vary with each set of egg masses or ovarian sample. As the distilled water has a detrimental effect on the embryos, the procedure was carried out as quickly as possible. It was observed that hatched nauplii from stage 12 were immobilized after about 1 minute in distilled water.

In order to be able to compare the results obtained in this study with results obtained for other cirripede embryos by Barnes (1965) the data of total carbohydrates, glycogen, protein and lipid composition are expressed as concentration per 10^6 eggs. As each pair of egg masses contains between 100-300 eggs (Anderson, 1965), it was necessary to pool egg masses from several individuals. Certain biochemical studies, particularly those which require over 100 mgm wet weight of tissue, could only be carried out on the ripe ovary and fully developed embryos (stage 12), as the latter has the longest duration of any stage and thus can be obtained from more individuals. It was very difficult to find large quantities of freshly laid ova (stage 1), as these enter cleavage 2 hours after fertilization. The intermediate stages could be incubated in sea water as described by Anderson (1965).

Each of the following biochemical experiments was repeated until consistant results, varying within the limits of 5 micrograms, were

obtained for each stage. Generally this involved repeating each experiment for each stage four times. Only gross changes in composition were considered in the present study. Once a set of data for each component was completed, the results were analysed using the Chi square test to determine if there is any significant difference, at a significance level of 5%, between measurements obtained for each stage of development.

(b) Determination of Water Content

After weighting, pooled egg masses were allowed to dry in a dessicator over solid calcium chloride. They were subsequently weighed to a constant weight. Egg masses were also dried in an oven at 60°C until a constant weight was obtained.

(c) Determination of Total Glycogen

Total glycogen was determined by the methods of Hassid and Abraham (1957) and Giese (1967). Not less than 25 mgm wet weight of tissue was homogenized in a teflon homogenizer in 1.0 ml of 30% KOH. Glucose and glycogen (analytical grade) were used as standards; aliquots were examined in a Hilger and Watts Uvispek photoelectric spectrophotometer.

(d) Determination of Total Carbohydrates

Approximately 25 mgm wet weight of tissue was homogenized in 5.0 mls of 5% trichloracetic acid (TCA). The homogenate was digested in the TCA solution for 1 hour in a boiling water bath. The method of Kemp et al (1954) was followed for the remainder of the procedure. Glucose (analytical grade) was used for the standard.

(e) Determination of Total Protein

The total protein content was determined by the Lowry method (Lowry et al, 1951) using bovine serum albumen (analytical grade) as the standard.

(f) Determination of Total Lipids

In the Lowry method above, the supernatants collected during the extraction of lipid for protein determinations were pooled and used to estimate the total lipid content. In addition to this method, pooled egg masses were homogenized in 2.0 mls of chloroform: methanol (2:1) solvent for about three minutes. The homogenate was centrifuged at $0^{o}C$ at 8,000 RPM for 15 minutes. The supernatant was collected, retained, and further extractions were carried out on the residue; the supernatants representing the total lipid content were pooled.

All the samples from both methods were stored at $-15^{o}C$. Total lipid was determined by transferring the samples to a weighed glass vial (2" x 1") and evaporating to dryness in an oven at $50^{o}C$ under nitrogen, which was pumped through constantly.

(g) Pigment Analysis

The method of Barnes (1965) was followed. A large quantity of tissue is required in order to get sufficient concentration of pigment to determine absorption spectra. Consequently absorption spectral studies were carried out only on ovarian tissue and mature embryos (stage 12). Equal wet weights (200 mgms) of both these stages were homogenized in chloroform:methanol (2:1) solvent for about three

minutes, and filtered through No. 42 Whatman filter paper under illuminating gas. The resulting pigment solution was made up to 5.0 mls with solvent. The absorption spectrum of both stages was determined in the spectrophotometer. Readings were made from 400 mμ to 600 mμ at 10 mμ intervals; at the maximum absorption range, readings were taken at 5 mμ intervals. This procedure enables a comparison of the absorption spectra of both these stages to be made.

Small aliquots of the pigment solution were also subjected to a series of chemical tests to further characterize the pigment, as described by Fox (1953).

RESULTS

1. DESCRIPTION OF THE OVARY

(a) Ibla quadrivalvis

In I. quadrivalvis a complemental male, about 3 mm long, is always found lodged in the mantle cavity of the full-sized female of hermaphroditic specimen (Dakin, 1952). The ovaries are found about half-way down the peduncle of large specimens. They are embedded in, and attached by connective tissue to the longitudinal muscles of the peduncle. The free surface of the ovary projects into the haemocoel of the peduncle and extends as numerous branching ovarioles. The ovarioles become packed with ova, which can be seen clearly when the ovary is ripe. As the oocytes mature, they then fill the lumen of the ovariole and the terminal region is almost always packed with mature oocytes. Immature and spent ovaries have few ovarioles, are smaller and contain more connective tissue than ripe ovaries.

The colour of the ovary changes several times as the ovary approaches maturity. Immature ovarian tissue is creamy-white, becoming yellow when most of the oocytes are in vitellogenesis, and finally deepening to orange when the ovary is ripe. At this stage the ovarioles fill the haemocoel of the peduncle and white strands of connective tissue can be seen distributed throughout the ovarioles, maintaining the ovary as a compact body. Although the majority of adults have developing or ripe ovaries throughout the year, spent ovaries are common only during the breeding season. In spent ovaries, ovarian tissue is resorbed and later replaced by connective tissue.

(b) Tetraclita rosea

In T. rosea the paired ovaries lie in the haemocoel, embedded in a layer of connective tissue between the mantle and the basement membrane. Each ovary consists of a mass of branched hollow ovarioles packed with oocytes. The paired ovaries do not meet on the undersurface of the animal, but are separated by a region of connective tissue. This region is always white in colour, and contrasts sharply with the cream colour of the mature ovary. The colour changes in T. rosea follow a similar trend to those observed in I. quadrivalvis.

2. OOGENESIS IN I. QUADRIVALVIS AND T. ROSEA

In the two species, the oocytes are formed in the wall of the ovarioles, pass through a previtellogenesis growth phase, and then enter a vitellogenesis period during which the mass of yolk and lipid material is deposited and the egg undergoes further massive growth (Table 3). The sequence of oogenesis in I. quadrivalvis and T. rosea is summarized diagramatically in Figures 2-4 and illustrated in Figures 5-10. Mature oocytes pass through the paired oviducts, which open on the bases of the first pair of thoracic limbs (Walley, 1965). Ripe oocytes are squeezed through the oviductal glands which form the egg masses and the two egg masses are held in the mantle cavity.

I. quadrivalvis produces a large, yolky, isolecithal egg about 400μ long and 275μ wide. This is about twice the linear dimensions of the egg produced by T. rosea (Table 4). The eggs of both species appear to be histologically similar, except for the size difference. There is no follicle layer around the oocyte. Oogenesis in both species can be divided into seven stages using the following criteria:-

(1) structure of the nucleus

(2) changes in the cytoplasmic contents

This tabulation is shown in Table 2.

In both species the ovaries are embedded in a mass of connective tissue composed of flattened, irregularly shaped cells. The wall of each ovariole consists of a thick lamina, folded on its outer surface and lined internally by flattened epithelial cells. When the epithelial cells of the wall become mitotically active, they round up and the nucleus increases in size. These cells are the germinal epithelial cells.

The nuclei of the germinal epithelial cells are identical with the nuclei of the surrounding somatic cells. In both, the nucleus contains dark compact chromosomes and is surrounded by a thin layer of hyaline cytoplasm. The germinal epithelial cells migrate from the ovary wall into the lumen of the ovariole. Here they undergo further mitotic divisions to form primary oogonia.

Primary oogonia are found near the wall and at all levels in the lumen of the ovariole, including the centre, where they are usually surrounded by mature oocytes. Each primary oogonium probably undergoes three mitotic divisions to form secondary oogonia, as these are usually found in clusters of eight. The chromosomes can be seen in the primary oogonia.

In the secondary oogonia, the nucleus is about the same size as the primary oogonia (Table 4), and the chromosomes are now compact dark bodies. The cessation of mitotic activity occurs concurrently with an increase in nuclear size (Table 3,4). This distinguishes

TABLE 2: Stages of oogenesis in I. quadrivalvis and T. rosea.

Major characteristics of each stage are summarized.

Stage	Nucleus	Cytoplasm
1 germinal epithelium	spherical nucleus; chromosomes compact bodies	thin layer of hyaline cytoplasm surrounds nucleus
2 primary oogonium	distinct chromosomes mitosis	as above
3 secondary oogonium	chromosomes indistinct compact bodies; mitosis	as above
4 previtellogenetic oocyte	formation of a nucleolus; chromosomes diffuse; germinal vesicle develops	thin layer of basophilic cytoplasm surrounds nucleus
5 vitellogenetic oocyte	as above, but greatly increased in size	acidophilic, protein-carbohydrate yolk deposited
6 vitellogenetic oocyte	germinal vesicle expands, gradually becomes basophilic	protein-carbohydrate yolk deposition continues; lipid yolk is deposited
7 vitellogenetic oocyte	complete break down of the germinal vesicle nucleus strongly basophilic	large granules of yolk and lipid dispersed throughout the cytoplasm; secretion of the vitelline membrane

TABLE 3: Stages in the Development of the Oocytes of Ibla quadrivalvis and Tetraclita rosea.
Ratio of the volumes of the components of the oocyte to each other.

1. Ibla quadrivalvis

Stage	Cytoplasm:nucleus	Cytoplasm:nucleolus	Nucleus:nucleolus
1	2:1	-	-
2	2:1	-	-
3	2:1	35:1	15:1
4	8:1	350:1	40:1
5	40:1	2,500:1	65:1
6	175:1	16,000:1	90:1
7	2,500:1	80,000:1	30:1

2. Tetraclita rosea

1	10:1	-	-
2	10:1	-	-
3	10:1	350:1	30:1
4	35:1	950:1	30:1
5	45:1	3,000:1	65:1
6	90:1	6,000:1	65:1
7	700:1	10,000:1	15:1

Figure 1. Mature adult of Ibla quadrivalvis

Figure 2 CROSS SECTION THROUGH A PORTION OF AN OVARIOLE OF I. quadrivalvis SHOWING STAGES OF OOGENESIS

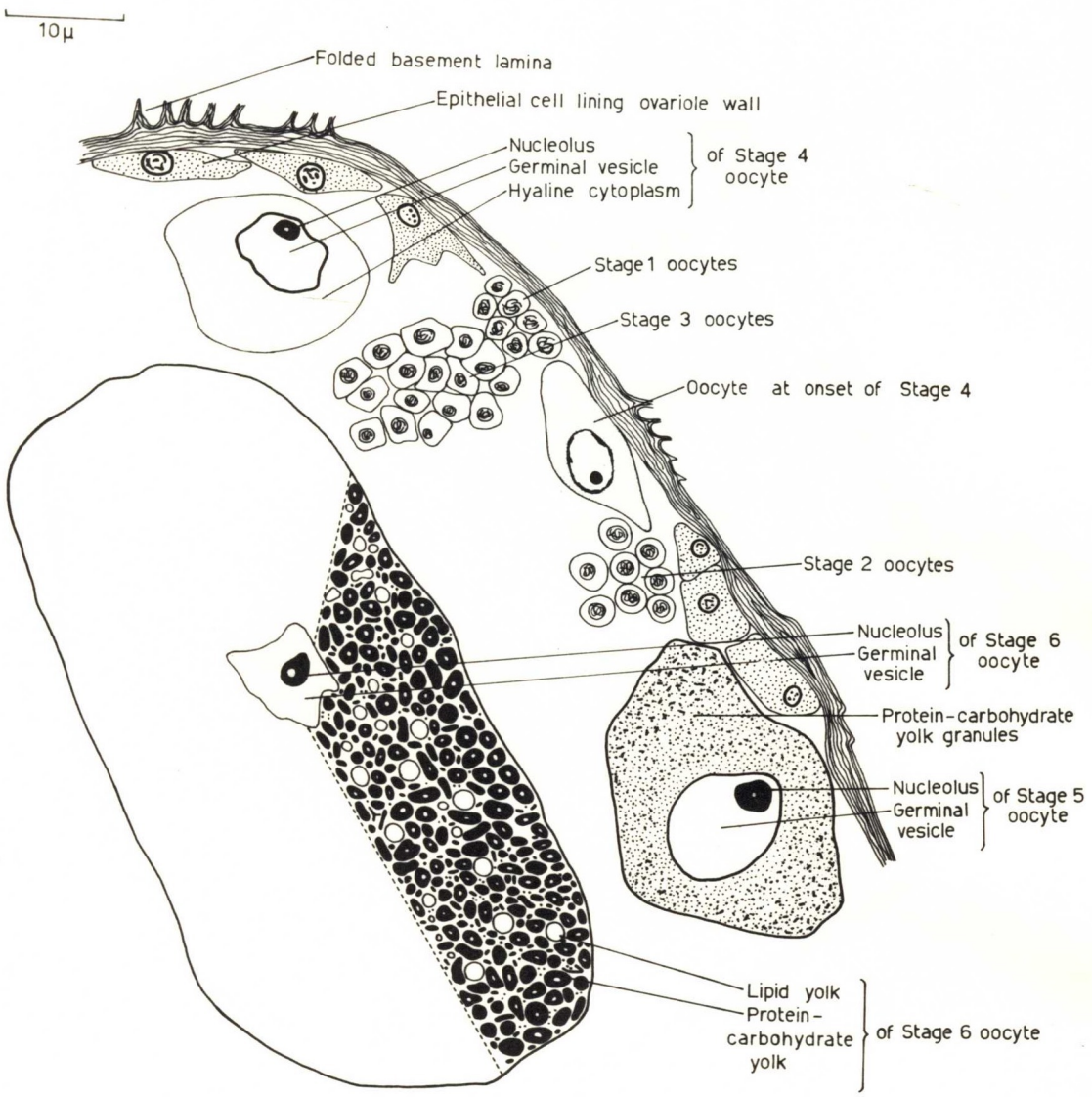


Figure 3 CROSS SECTION OF A STAGE 7 OOCYTE OF I. quadrivalvis

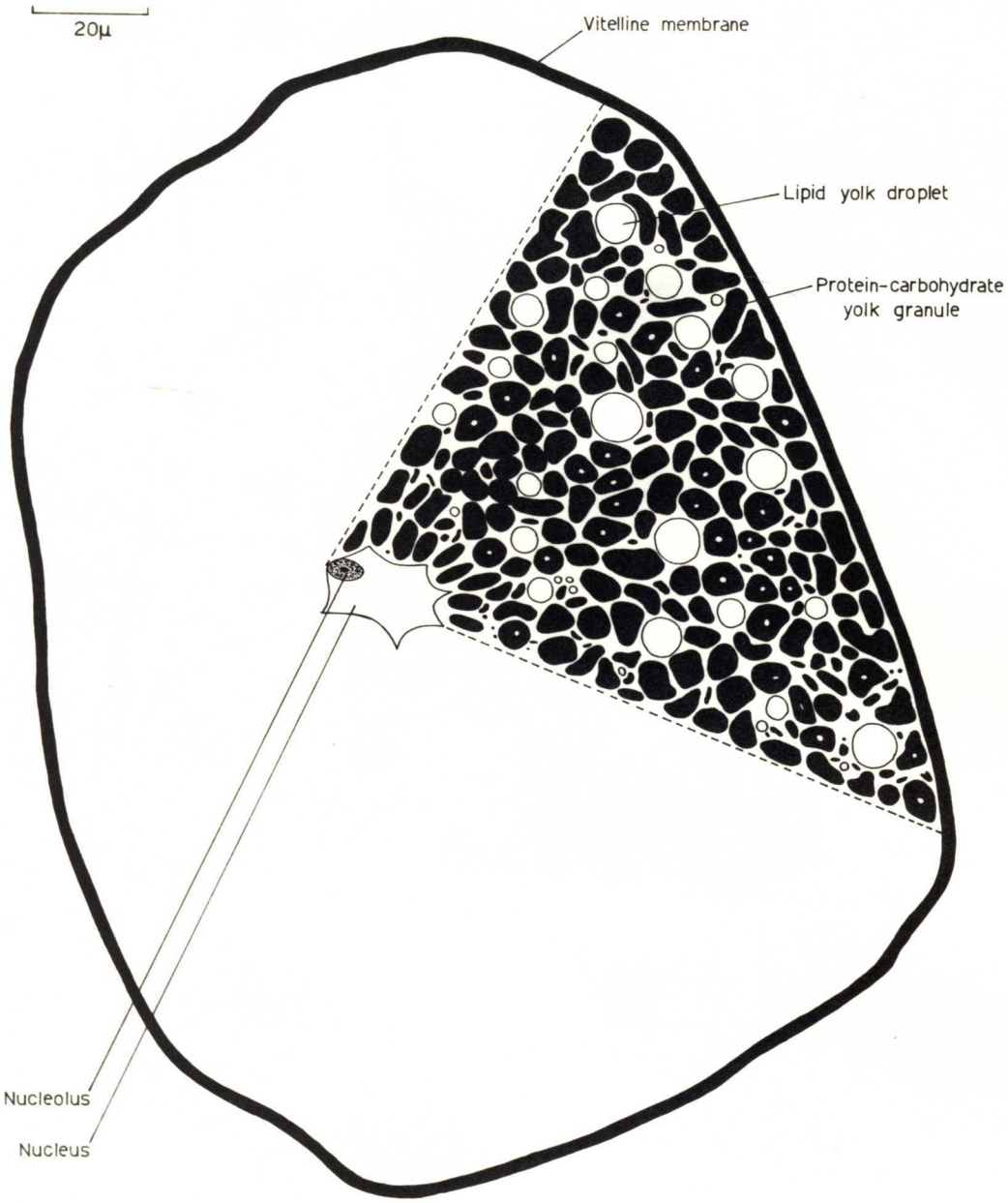


Figure 4 CROSS SECTION THROUGH A PORTION OF AN OVARIOLE OF T. rosea SHOWING STAGES OF OOGENESIS

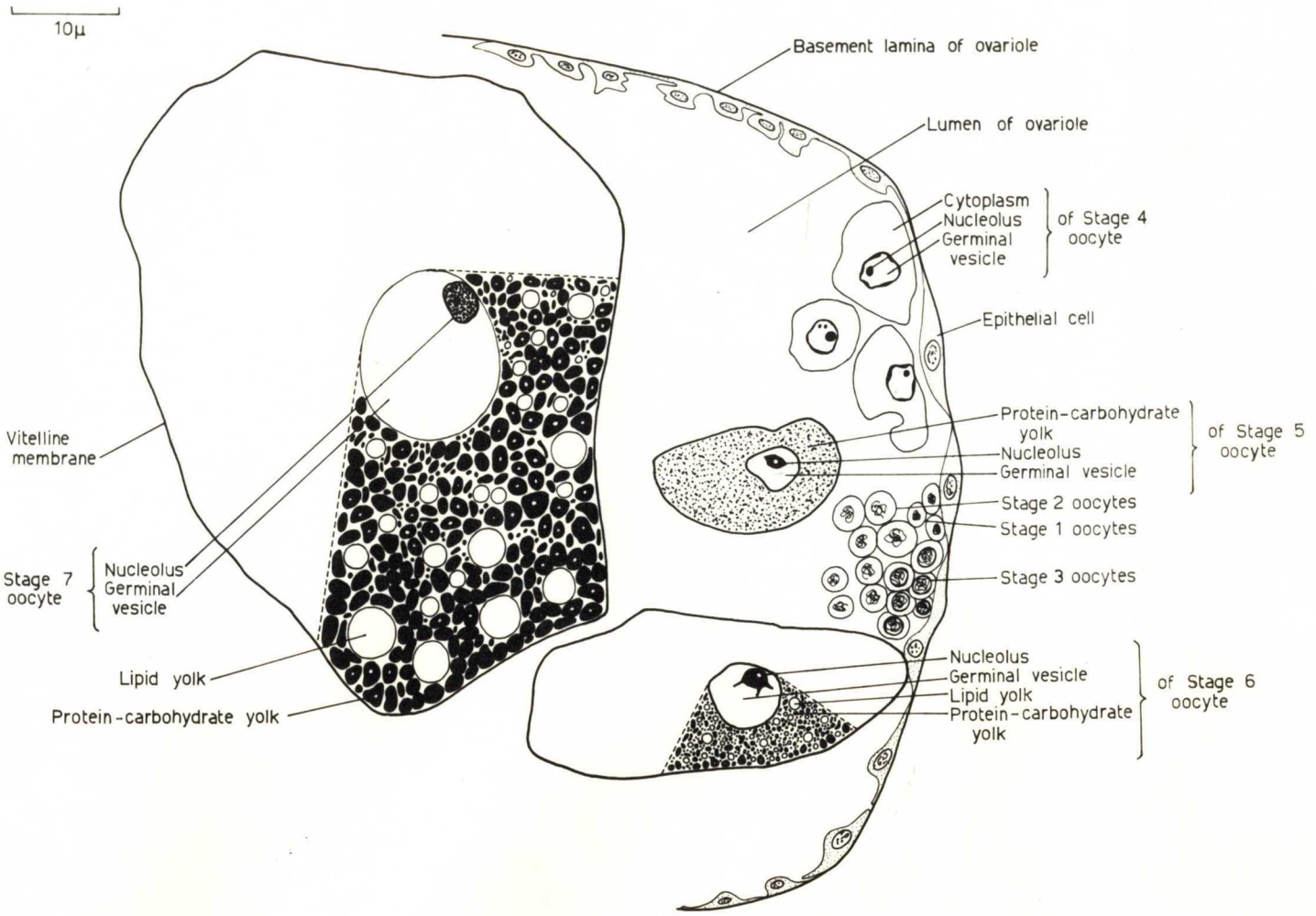


Figure 5. Transverse section through contiguous ovarioles from ovary of I. quadrivalvis, showing germinal epithelial cells, previtellogenetic oocytes and ripe oocytes.
Delafields heamatoxylin stain.

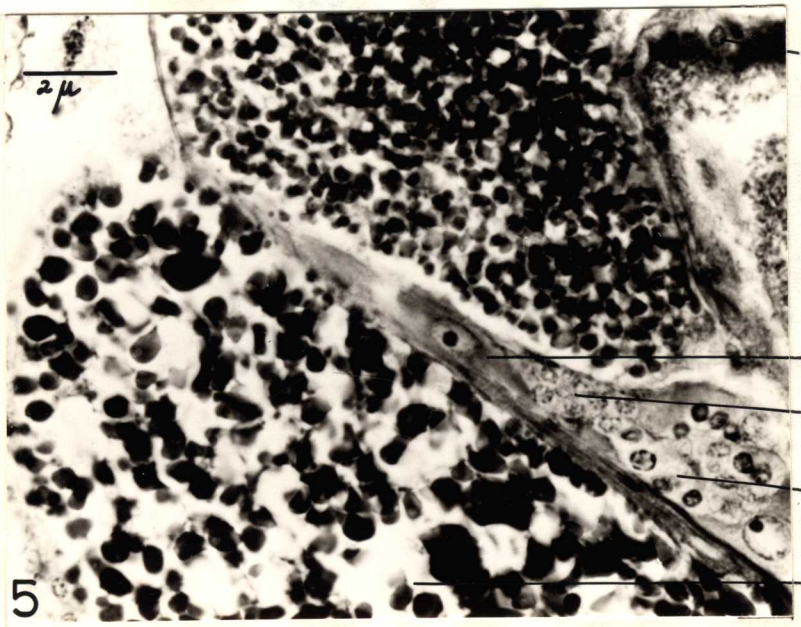


Figure 6. Transverse section of a stage 4 oocyte surrounded by stage 6 and stage 7 oocytes of I. quadrivalvis.
Delafields haematoxylin stain

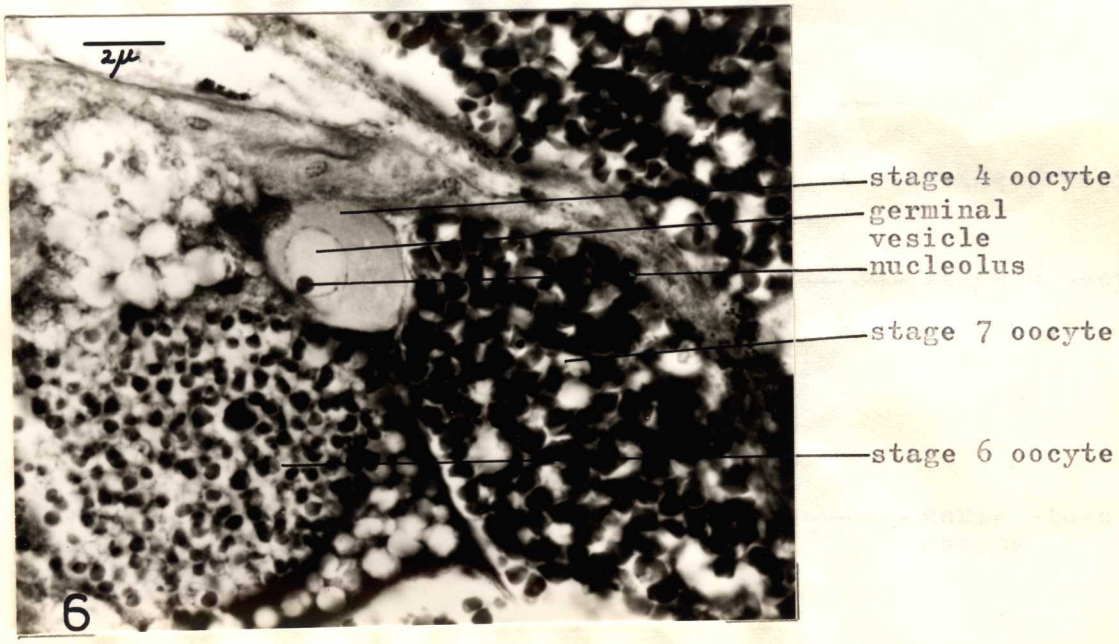
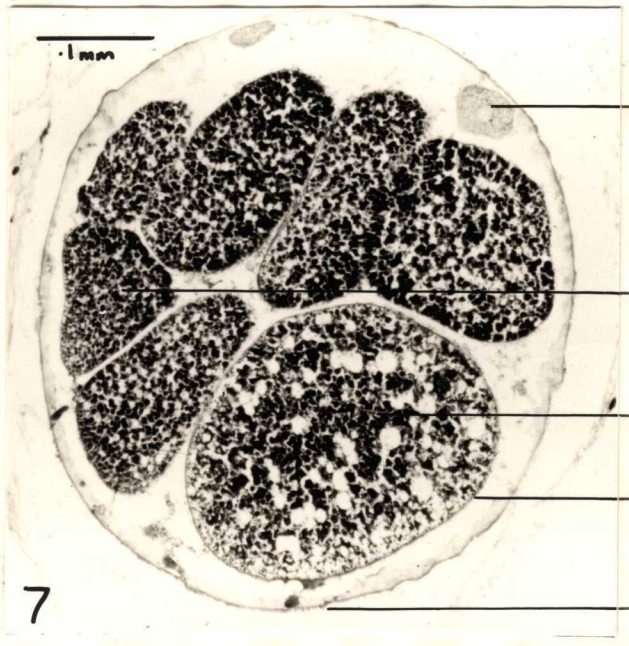


Figure 7. Cross section of an ovariole of I.quadrivalvis, showing all the stages of vitellogenesis.

Delafields haematoxylin stain



stage 5 oocyte

stage 6 oocyte

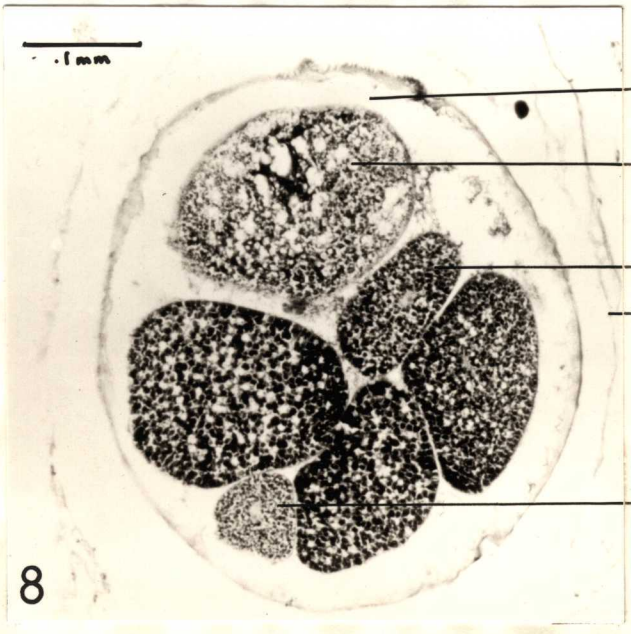
stage 7 oocyte

vitelline membrane

folded basement lamina

Figure 8. Cross section of an ovariole of I. quadrivalvis, showing various stages of vitellogenesis.

Delafields haematoxylin stain



lumen of ovariole

stage 7 oocyte

stage 6 oocyte

connective tissue

early stage 6 oocyte

Figure 9. Cross section of several ovarioles of T. rosea, showing various stages of oogenesis.

Delafields heamatoxylin stain.

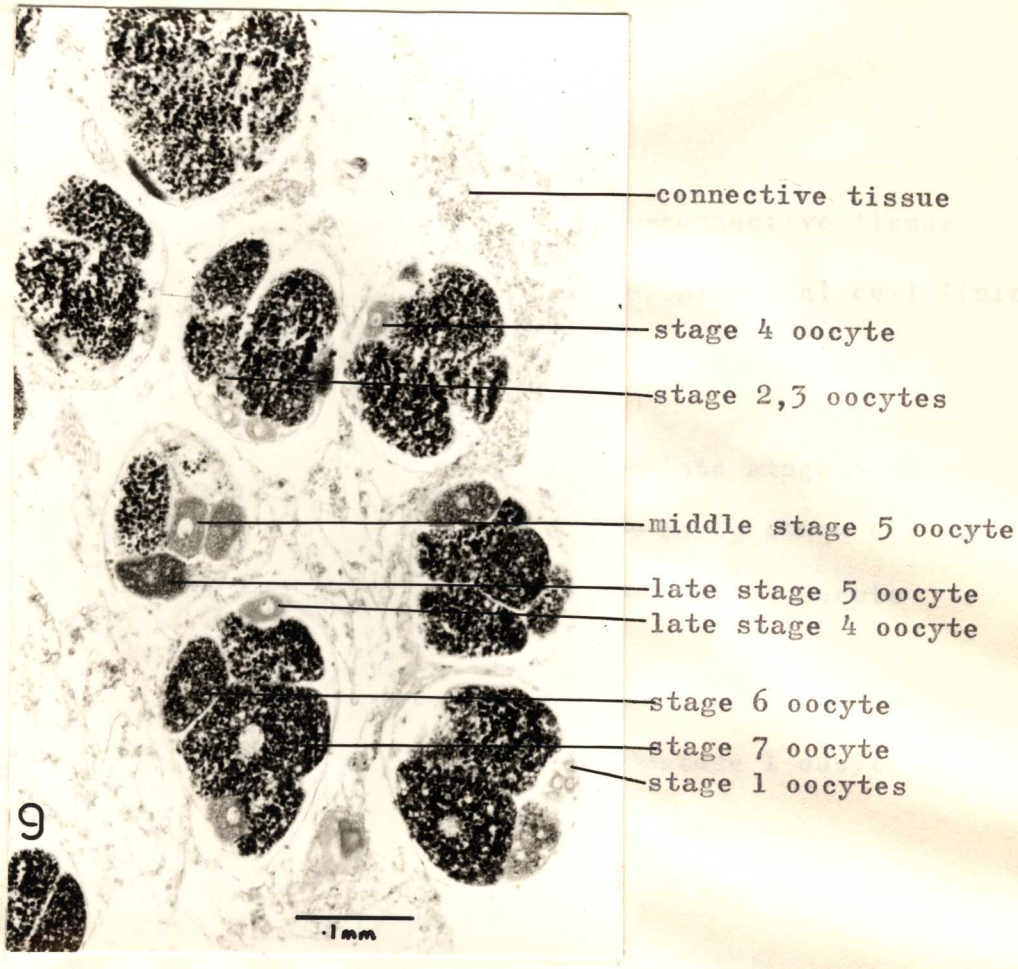
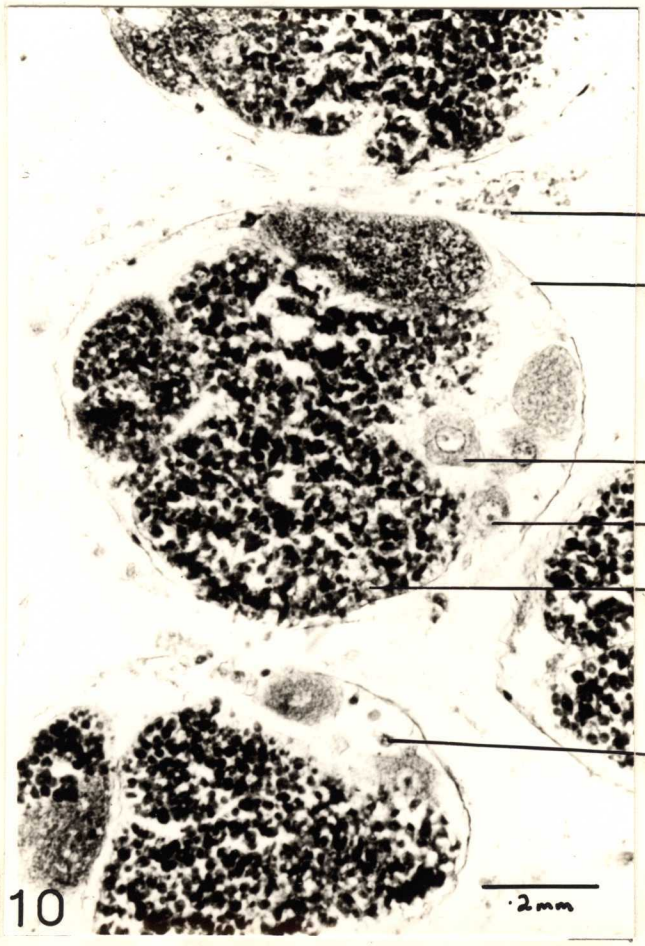


Figure 10. Cross section of an ovariole of T. rosea, showing various stages of oogenesis.

Delafields haematoxylin stain.



previtellogenetic oocytes (stage 4) from secondary oogonia (stage 3). The compact chromosomes of the secondary oogonia become diffuse and thread-like when the nucleus becomes arrested in premeiotic prophase. The nucleus of the secondary oogonium occupies most of the cell space and is surrounded by only a thin layer of hyaline cytoplasm (Table 3).

Once the eight oogonia of a group have passed synchronously through the stages of premeiotic prophase, they enter the previtellogenetic growth phase of oogenesis (stage 4). A nucleolus develops and the cytoplasm is no longer hyaline. Basophilic granules become apparent. During previtellogenesis, the nucleus swells to form a germinal vesicle and the thread-like chromosomes become diffuse. At the same time, most of the nucleoplasm becomes hyaline. As the germinal vesicle increases in size, the dense layer of chromatin that lies against the nuclear membrane, early stage 4 oocytes, disappears. A vacuole appears in the nucleolus. The growing cytoplasm becomes increasingly granular and more strongly basophilic, but develops no histologically distinct structures. The basophilia of the oocyte is related to the enlargement of the cytoplasm, not to yolk formation, as the cytoplasm becomes acidophilic before the yolk appears.

The previtellogenetic growth period is followed by the onset of vitellogenesis in stage 5. Initially the cytoplasm becomes less basophilic. Small granules, less than 1μ in diameter (Table 5) of protein-carbohydrate yolk are deposited in the perinuclear region. Once the oocyte has reached approximately 20μ in diameter at stage 6 (Table 4), the deposition of lipid yolk commences in the perinuclear region.

Much of the protein-carbohydrate yolk and lipid yolk is deposited during stage 6. Histologically, the lipid and protein-carbohydrate yolk granules do not appear to arise from any special cellular components. The deposition of the yolk extends outwards from the nuclear region until the entire volume of the oocyte is evenly packed with protein-carbohydrate yolk granules and lipid droplets. In fresh material the components of the yolk are approximately spherical in shape. The structure of the yolk varies in the histological sections, according to the fixative used, but generally it is similar to that of the fresh material. With the advancement of vitellogenesis in stage 7, larger protein-carbohydrate and lipid yolk granules are laid down in the more peripheral regions of the cytoplasm. Both these forms of yolk granules are approximately 5-6μ in diameter in the mature oocyte of I. quadrivalvis (Table 5). Yolk granules in T. rosea are about half the linear dimensions of those of I. quadrivalvis (Table 5). At the end of vitellogenesis, the protein-carbohydrate and lipid yolk granules are dispersed homogeneously throughout the cytoplasm of the oocyte in both species.

During the final stages of vitellogenesis (stages 6 and 7), a vitelline membrane is secreted by the oocyte.

The germinal vesicle begins to breakdown during stage 6 and gradually becomes basophilic. When this breakdown is completed in stage 7, the nucleus is small (Table 3) and strongly basophilic. The nucleolus does not increase in size during vitellogenesis (Table 4). The vacuole formed in the nucleolus at stage 4 persists throughout vitellogenesis, becoming larger. On completion of vitellogenesis, the nucleolus does not diminish in size, but the

TABLE: 4 Stages in the development of the oocyte.

Mean dimensions of the components of the oocytes in microns (based on measurements of 10 oocytes at each stage).

I. quadrivalvis

Stage	Diameter of the nucleus	Diameter of the nucleolus	Diameter of the cytoplasm
1	3.25	-	8
2	6	-	8
3	6	-	8
4	8.5	2.5	17.4
5	10	2.5	34
6	11.25	2.5	62.5
7	10	3.1	170

T.rosea

1	3.5	-	10
2	5	-	17.6
3	5	-	17.6
4	7.4	2.5	17.6
5	7.4	2.5	24.5
6	10	2.5	45

TABLE: 5 Mean dimensions of protein-carbohydrate yolk and lipid yolk granules at each stage of vitellogenesis in microns. (Based on measurements of 10 oocytes at the same stage).

Stage	Protein-carbohydrate yolk granules		Lipid yolk granules	
	I.quadrivalvis	T. rosea	I. quadrivalvis	T. rosea
5	less than 1	less than 1	-	-
6	2	2	1.3	1
7	5.2	2.5	6.5	4

cont. from page 29

vacuole disappears and is replaced by densely staining granules.

In many eggs during vitellogenesis, the nucleolus is surrounded by diffuse chromatin, which is closely applied to both the nucleolus and the nuclear membrane. Infrequently, nuclear material, other than the nucleolus shows strong pyroninophilia, suggesting that the nuclear material is composed of RNA. This material is invariable diffuse, and may be identical with nucleolar extrusion material observed by other authors.

3. HISTOCHEMISTRY OF OOGENESIS

(a) Distribution of DNA during oogenesis

The nuclei of the oogonia of stages 2 and 3 contain Feulgen positive chromatin. Nuclei of stages 3 oocytes show deep blue-green chromosomal material as well as a bright red nucleolus with the pyronin/methyl green technique. As soon as the nucleus begins to enlarge to form a germinal vesicle, however, the chromosomes are no longer Feulgen-positive. There is no further positive staining of the chromosomes by the Feulgen reaction or by the pyronin/methyl green technique for the remaining growth period of the oocyte, including stage 7. No cytoplasmic DNA was detected. This was not unexpected, as any cytoplasmic DNA would be in too small a concentration to be detected by the staining methods employed.

(b) Distribution of RNA during oogenesis

Nucleolus:-

A small, weakly pyroninophilic nucleolus is observed in oocytes at the onset of previtellogenesis at stage 4. With stains other than pyronin, the nucleolus of stage 4 is indistinguishable from the surrounding chromatin of the nucleus. During stage 4, the nucleolus undergoes several changes which result in its eventual vacuolation. Dense strands and granules of pyronin-positive material form within the nucleolus and around these strands and granules, weakly pyroninophilic areas develop. These dispersed areas converge early in stage 5 and form a central vacuole which is pyronin-negative. The outer edge of the nucleolus is strongly pyroninophilic and remains so throughout oogenesis.

During stage 6 the vacuole moves away from the centre until it adheres to the outer edge of the nucleolus. The vacuole is now weakly pyroninophilic. The vacuole becomes slightly larger during stage 6, and disappears when the oocyte reaches stage 7. The nucleolus of the ripe oocyte is strongly pyroninophilic and contains some flecks of densely staining material.

Cytoplasm:-

The cytoplasm of the epithelial cells which line the ovary wall is strongly pyroninophilic, indicating a high concentration of RNA. The cytoplasm of the oogonia of stages 2 and 3 show only weak pyroninophilia. Once the nucleolus is fully formed, the cytoplasm of the oocytes at stage 4 once again becomes strongly pyroninophilic. The RNA of these oocytes is uniformly distributed throughout the cytoplasm. As the yolk begins to form, the cytoplasmic RNA becomes more dispersed, but there is always some present in the cytoplasm during stage 5 and stage 6. Once the yolk is present in large quantities, the oocytes in both species no longer exhibit cytoplasmic pyroninophilia. Only the nucleolus continues to stain positively for RNA during stage 7.

Nucleoplasm and the nuclear membrane:-

Occasionally scattered strands of pyronin-positive material are found in the nucleoplasm closely associated with the nucleolus as mentioned previously. Other than these strands, the nucleoplasm shows no pyroninophilia during any stage of oogenesis. The nuclear membrane shows slight pyroninophilia during all stages, and this increases slightly when the oocytes have reached stage 7.

(c) Distribution of PAS-positive material during oogenesis

The epithelial cells of the ovary wall contain small granules of PAS-positive material. The cells of the connective tissue also contain small amounts of PAS-positive material. Oogonia and oocytes in the previtellogenetic stage of oogenesis are PAS-negative. With the onset of vitellogenesis, small PAS-positive granules appear in the cytoplasm. During vitellogenesis the amount of PAS-positive material increases greatly, and is found concentrated in the protein-carbohydrate yolk granules.

Glycogen was not detected in the cytoplasm at any stage of oogenesis.

The vitelline membrane, secreted by the oocyte during stage 7 is composed mainly of PAS-positive material.

No PAS-positive material was detected in the nucleus of the oocyte at any stage of oogenesis.

(d) Distribution of Acid mucopolysaccharides

No positive reaction was obtained with the techniques used to localize acid mucopolysaccharides.

(e) Distribution of Protein and Amino acids during oogenesis

The cytoplasm of the previtellogenetic oocytes stain a pale blue with mercury bromphenol blue. Positive results are also obtained with aqueous bromphenol blue and alkaline fast green F.C.F., indicating that most of the protein in the cytoplasm is basic. The protein-carbohydrate yolk granules stain intensely with mercury bromphenol blue and the basic protein stains, demonstrating the high proteineacous composition of the yolk granules, with an emphasis on basic proteins.

Basic proteins of the histone type contain high concentrations of arginine and tryptophan. The yolk granules stain intensely for both these amino acids, indicating that the basic protein moiety is of the histone type. Some degree of differentiation occurs in the mature yolk granules, as a central core develops which stains less intensely for basic proteins than the surrounding outer core.

The chromatin of the nuclei of the germinal epithelial cells and oogonia stains intensely for basic proteins. The diffuse chromosomes of the germinal vesicle of stage 4 oocytes stain positively for basic protein, arginine and tryptophan, demonstrating their high histone content. The nuclei of all stages of oogenesis also react intensely in the arginine and tryptophan tests.

(f) Distribution of Lipid during oogenesis

Small red-staining droplets of neutral fat appear in the cytoplasm of oocytes at stage 6. The size and number of droplets increases as the oocyte matures. In ripe oocytes of both species, the neutral fats appear as evenly-sized spherical droplets. The lipid droplets, about 5-6μ in diameter (Table 5), are generally larger than the protein-carbohydrate yolk granules. The bulk of the fat in the oocyte is neutral lipid.

There is no lipid material in the cytoplasm of the epithelial cells lining the ovary wall, nor in the nuclei of all stages of oogenesis.

Phospholipids are concentrated in the nucleolus, which stains an intense blue colour with copper phthalocyanin. Fine granules of phospholipids are found amongst the yolk in the mature oocytes of stage 7.

Summarising the histochemistry of oogenesis in I. quadrivalvis and T. rosea:-

In both species DNA is detected only in the nuclei of cells and oogonia not involved in active synthesis. The DNA is concentrated into clumps of Feulgen-positive heterochromatin. Once the oocytes approach the synthetic stage 4 of previtellogenesis, DNA can no longer be detected with histochemical stains. The heterochromatin clumps become dispersed, the amount of euchromatin increases as the germinal vesicle develops. Throughout the remaining stages of oogenesis, no DNA is detected in the germinal vesicle of the oocyte, which indicates that the oocyte continues to be involved in active synthesis.

The nucleolus, formed at stage 4 during previtellogenesis, has a high RNA and basic protein content. A central vacuole, of reduced RNA and basic protein content, develops at the onset of vitellogenesis (stage 5) and persists throughout vitellogenesis. The periphery of the nucleolus has a high RNA content throughout vitellogenesis. Arising from the nucleolus are strands of RNA-positive material, probably of nucleolar origin, which are attached to the nuclear membrane. The strands of RNA-positive material are probably involved in nucleolar extrusion, although no direct histochemical evidence of extrusion was obtained. Hence, the nucleolus becomes active immediately after its formation and perhaps remains a source of cytoplasmic RNA throughout oogenesis.

As soon as a nucleolus appears in the oocyte nucleus, the concentration of cytoplasmic RNA increases greatly. The concentration of RNA in the cytoplasm then decreases gradually during vitellogenesis

(stages 5-7). When vitellogenesis is completed at stage 7, no cytoplasmic RNA can be detected by histochemical means. Thus, as the synthetic activity of the oogonia is completed, the amount of RNA in the cytoplasm gradually becomes relatively depleted.

Histochemical evidence shows that only two types of yolk bodies are produced: protein-carbohydrate yolk and lipid yolk. The protein-carbohydrate yolk is synthesized before the lipid yolk. The former is composed of a carbohydrate and basic protein moiety which combine to form granules. The carbohydrate portion begins to be synthesized first, as PAS-positive granules arise in the perinuclear region of the cytoplasm at the onset of vitellogenesis (stage 5). Once the small granules of PAS-positive material have begun to be formed, they become associated with basic proteins and form the protein-carbohydrate yolk granules. When the oocyte reaches stage 7 of oogenesis, an inner core differentiates within each yolk granule. The inner core has a different histochemical composition from the remainder of the yolk granule, as it stains less intensely for PAS and basic protein. There is a complete absence of glycogen in any stage of oogenesis, and neutral polysaccharides appear to be the main form of carbohydrate stored in the oocytes.

Neutral fats are the dominant lipid material in oocytes of both species. Histochemical results do not indicate the degree of unsaturation amongst the fatty acids which make up the neutral fats. Phospholipids are found in the nucleolus, which may suggest a method of synthesis of the lipid material, as all the lipid material

appears to arise "de novo" in the cytoplasm of stage 6 and 7 oocytes.

A more penetrating analysis of the origin and synthesis of the components of the growing oocyte can be attained through an investigation of changes in ultrastructure. In the following section, the electron microscopy of oogenesis in I. quadrivalvis is described in detail.

4. ELECTRON MICROSCOPY OF OOGENESIS IN IBLA QUADRIVALVIS

Oogenesis in I. quadrivalvis has been divided above into seven histologically distinct stages. The ultrastructure of these stages, summarized in Figures 11,12,13 and 14, and illustrated in Plates 1-45, will now be discussed.

All the electron micrographs shown in the following plates 1-45 are of specimens fixed in glutaraldehyde/osmium in phosphate buffer with 0.44% NaCl added, unless otherwise mentioned. All the electron micrographs were taken on the Siemens electron microscope unless otherwise mentioned.

Legend of Letters used in Figures 11-14, and in Plates 1-45.

B. basement lamina

C. collagen fibres

CH. channel

CMS. complex membranous structures

CN. centriole

CT. connective tissue

DL. dense layer of chromatin

EC. euchromatin

EPC. epithelial cell

ET. electron transparent area of the basement lamina

ETA. electron transparent area of mature yolk bodies

F. filaments

FG. forming face of the Golgi complex

FV. fibrous material

G. Golgi complex

HC. heterochromatin

L. lumen of the ovariole

LD. lipid droplet

M. mitochondrion

MC. muscle cell

MV. microvillus

ML. myelin bodies

MPP. micropinocytotic pit.

MPV micropinocytotic vesicle.

MT. microtubule

N. nucleus

NB. nuclear membrane blebs

NC. nucleolus

NM. nuclear membrane

NP. nuclear pore

PM. plasma membrane

PS. perivitelline space

PYV. precursor yolk vesicle

Pin.YV. pinocytotic yolk vesicle

R. free ribosomes

RER. rough endoplasmic reticulum

REV. rough endoplasmic vesicles

RO. ripe oocyte

S. septum of the nuclear pores

SER. smooth endoplasmic reticulum

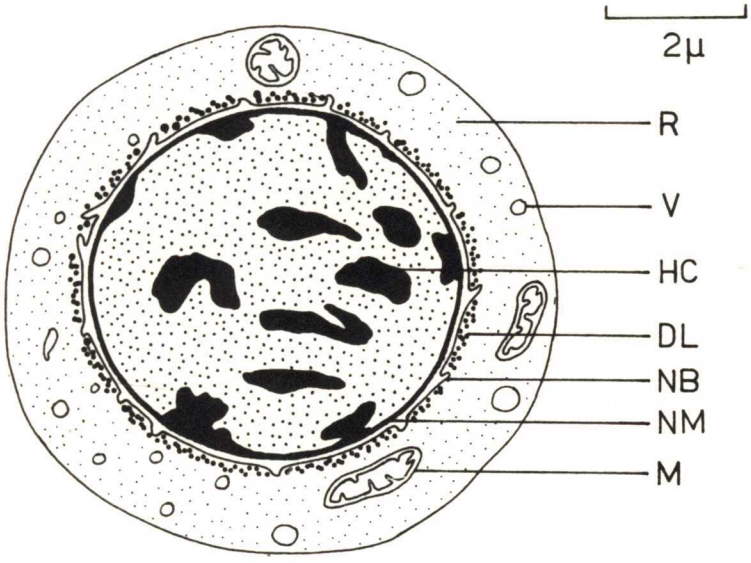
SEV. smooth endoplasmic vesicles

V. vesicles of the endoplasmic reticulum

VM. vitelline membrane

Figure 11

a) STAGE 1 OOCYTE



b) STAGE 2,3 OOCYTE

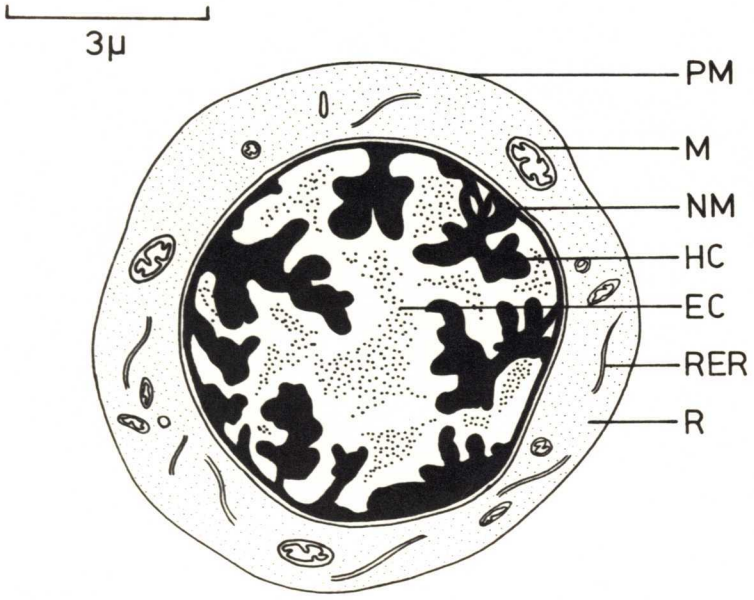
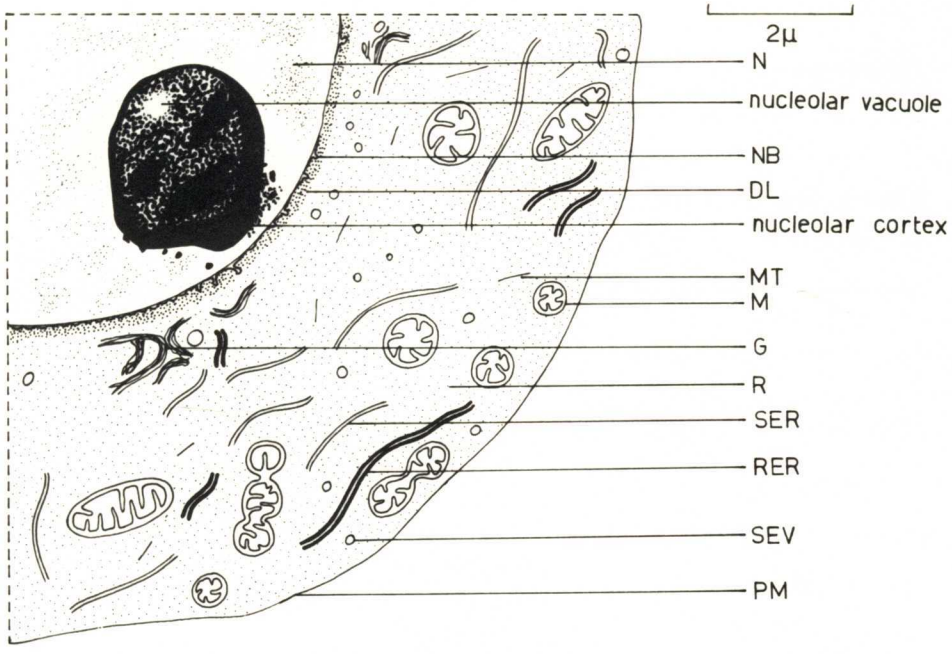


Figure 12

a) STAGE 4 OOCYTE



b) STAGE 5 OOCYTE

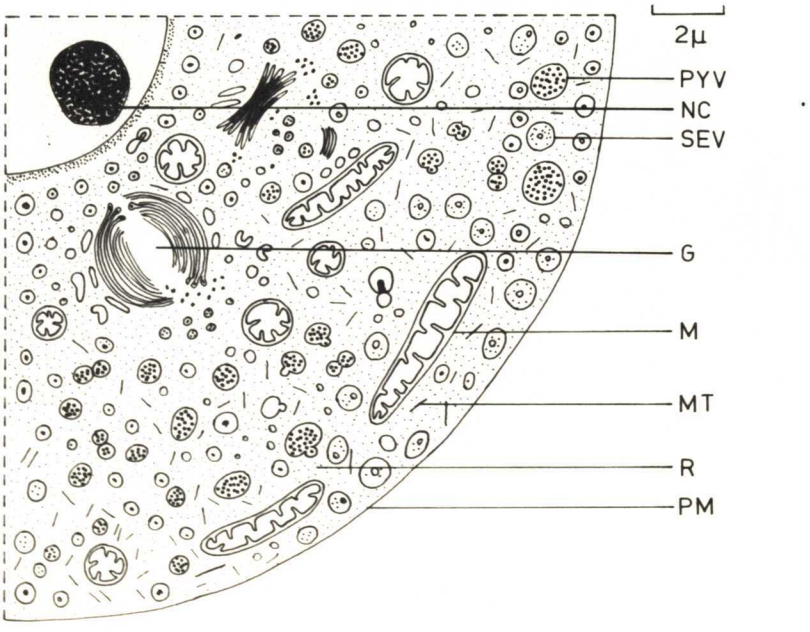


Figure 13

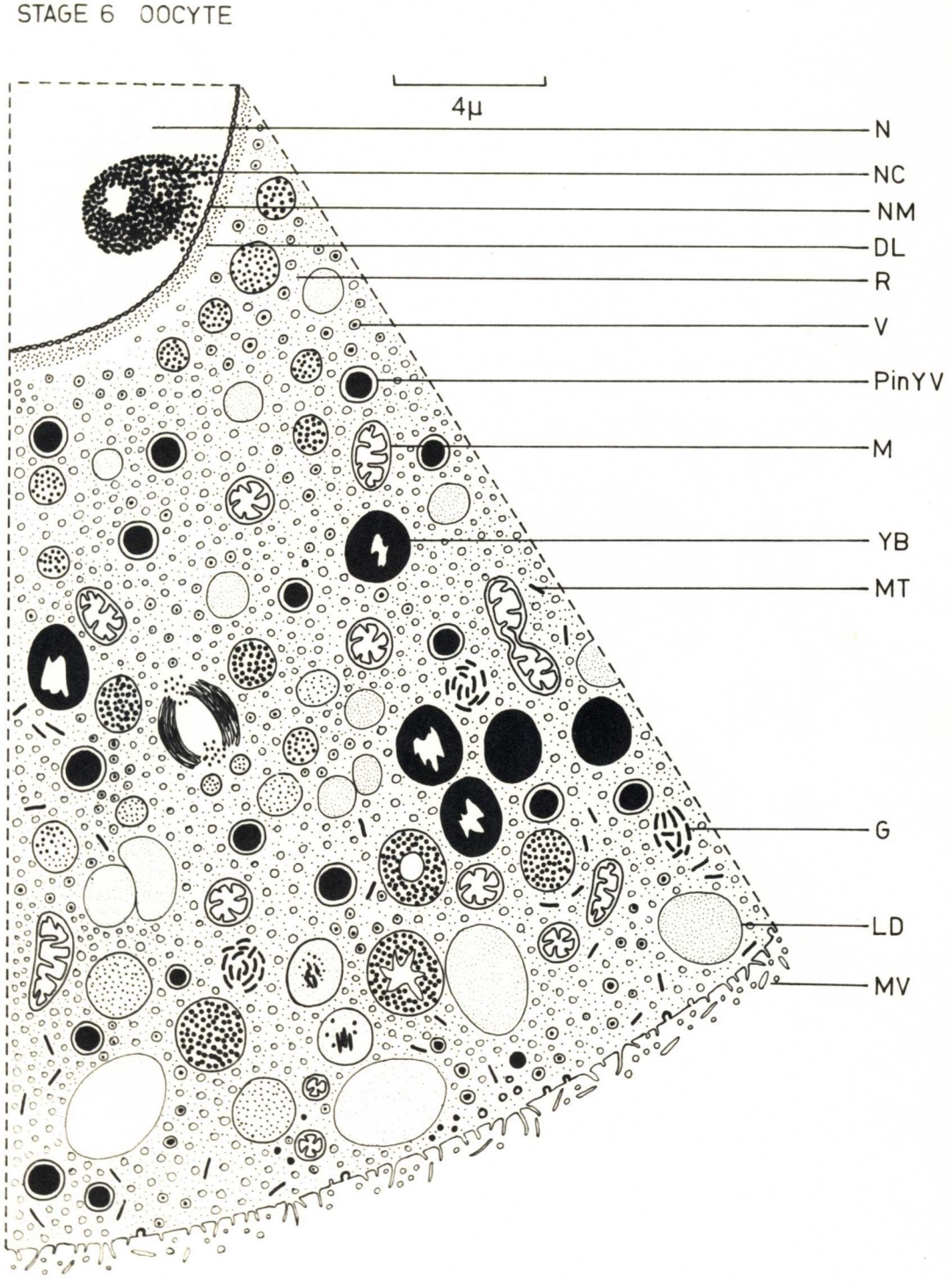
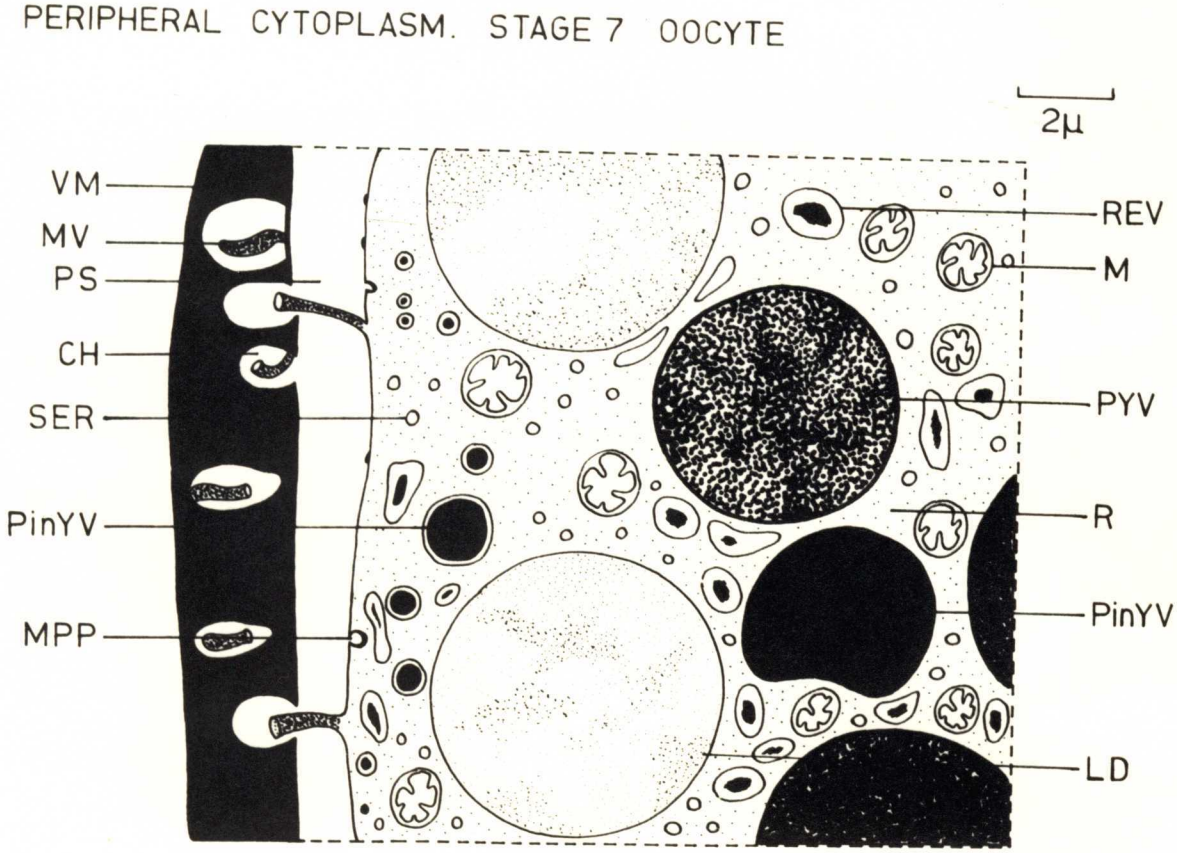


Figure 14


PERIPHERAL CYTOPLASM. STAGE 7 OOCYTE
2μ
VM
MV
PS
CH
SER
PinYV
MPP
REV
M
PYV
R
PinYV
LD

YB. mature protein-carbohydrate yolk bodies.

(a) Stage 1

Ovary wall:-

As shown by light microscopy, a thick basement lamina (Plate 1) forms the acellular external wall of the ovariole. The lamina usually exhibits some degree of folding in electron microscope preparations (Plate 2). The basement lamina is composed of a homogeneous, fibrous, osmophilic substance, which is layered. Electron transparent areas are visible between the layers. This phenomenon is particularly clear in Plate 2.

The thickness of the basement lamina varies from 1μ to 2μ, depending on the degree of folding that has occurred (Plate 2). The folding of the basement lamina is also observed in the light microscope study of fixed material. In some sections the basement lamina is flat (Plate 1), its average width being approximately 1μ. The acellular wall poses a thick barrier to the fixatives used in electron microscopy, which normally have low penetrating powers. Only oocytes close to the basement lamina were fixed adequately.

Epithelial Cells:-

Epithelial cells lie immediately beneath the basement lamina and form the cellular wall of the ovariole. The epithelial cells typically show an oblong nucleus, which lacks a nucleolus (Plates 1, 2, and 3). Several large heterochromatin clumps are dispersed throughout the nucleus. There is also a concentration of heterochromatin into a layer disposed around the periphery of the nucleus. The more central heterochromatin clumps are continuous with the dense peripheral layer (Plate 2). The nucleus has a typical double nuclear

membrane. Some sections show the outer nuclear membrane forming small blebs (Plate 2). These vesicles are presumably set free into the cytoplasm, and may account for the origin of the vesicular form of the rough and smooth endoplasmic reticulum seen in the epithelial cells as well as in developing oocytes.

The cytoplasm of epithelial cells consists mainly of mitochondria, endoplasmic reticulum, free ribosomes and microtubules. A layer, about 70 mμ thick, of free ribosomes surrounds the outer nuclear membrane. In many instances ribosomes were observed to be attached to the outer layer of the nuclear membrane (Plate 3).

The mitochondria are ovoid in shape and are usually smaller than those found in developing oocytes. They possess few cristae, but these exhibit the typical double membrane structure (Plate 1). The mitochondria do not appear to be associated with any other cellular organelles.

The rough and smooth endoplasmic reticulum only occurs in the vesicular form. As mentioned above, the vesicles may be derived from blebs of the outer nuclear membrane. The vesicles vary greatly in shape.

Several microtubules are visible in the cytoplasm of the epithelial cells. They are always few in number, and appear to have no discernable spatial orientation (Plate 1).

The epithelial cells lining the wall of the ovariole retain this pattern of differentiation (Plates 1,2,3,). However, as mentioned previously, some of the epithelial cells differentiate into oogonia, while others become involved in the resorption of ovarian tissues (Plates 4,5).

Epithelial cells which exhibit resorbing activities are much more varied in form than the typical epithelial cells described above. The cytoplasm increases greatly in size and rounds up into a more spherical shape. The nucleus may take various forms (Plate 4). In addition to the organelles already present in epithelial cells, complex membranous structures are found in the cytoplasm of cells involved in resorption (Plate 5). These complex membranous structures are bound by a single unit membrane, which surrounds many membrane-bound vesicles. In Plate 5 (arrow) a connection can be seen between the outer membrane of the complex and that of a vesicle of the endoplasmic reticulum. Microtubules, organized into bundles, are also found in the cytoplasm. Myelin bodies are found either singly in the cytoplasm or within the complex membranous structures (Plates 5,6).

Occasionally, an epithelial cell is found embedded in connective tissue and muscle cells (Plate 7). Large bundles of parallel microtubules are observed in the cytoplasm of these cells (Plate 7). Associated strands of collagen fibres are found outside the plasma membrane adjacent to the microtubules. It seems probable that this cell type is involved in the replacement of ovarian tissue by connective tissue. In spent ovaries, thick bundles of filaments are observed amongst epithelial cells which contain many complex membranous structures and myelin bodies (Plate 6).

(b) Oogonia (Stages 2 and 3)

Oogonia can be distinguished from epithelial cells by their characteristic, densely staining chromosomes. The nuclei of the oogonia (Plate 8) are approximately spherical in shape, having an average diameter of 5μ. The nucleus contains densely staining

heterochromatin in numerous clumps dispersed amongst finely granular euchromatin. A typical nuclear membrane is still present. The nucleus is surrounded by a thin layer of unspecialized cytoplasm (Plate 8). Except for a number of mitochondria, the cytoplasm is composed of free ribosomes, and a few lamellae of rough endoplasmic reticulum (Plate 8). No microtubules were observed in the cytoplasm of the oogonia.

(c) Previtellogenesis (Stage 4):-

The oocytes are approximately 9μ in diameter when they enter prophase of the first meiotic division. In one section, a centriole was observed lying adjacent to the interphase nucleus. The centriole is composed of several microtubules embedded in a dense amorphous matrix (Plate 9). The nucleus contains densely staining heterochromatin of a granular composition, interspersed with very fine wispy euchromatin. The nucleus has a typical double layered nuclear membrane, perforated by nuclear pores approximately 660A° in diameter, separated by a distance of 1,300 A° (Plate 9). The nuclear pores appear to be closed by a septum that extends across the middle of the pore. The septum is slightly thicker than either the inner or outer membranes of the nuclear envelope. In later previtellogenesis, the heterochromatin in the nucleus becomes dispersed, and is replaced by euchromatin; the layer of heterochromatin adjacent to the nuclear membrane disappears.

Ultrastructural studies revealed no information on the formation of the nucleolus. A definite nucleolus, more electron dense than the euchromatin, becomes apparent in oocytes when the nucleus has attained

a diameter of approximately 7μ. The youngest nucleolus observed had a diameter of approximately 1.5μ (Plate 10). By this stage, it has already undergone some differentiation. An excentric area, less osmiophilic than the surrounding area is now present, and appears to be composed of finely filamentous material. This area contrasts sharply with the remainder of the nucleolus, which is composed of densely granular material. As the nucleus swells to form a germinal vesicle, the nucleolus moves towards the nuclear membrane and becomes closely applied to it (Plate 10).

The cytoplasm of stage 4 oocytes contains several Golgi complexes. At this stage, the Golgi are found adjacent to the nuclear membrane. In fact, in Plate 12, a close association can be observed between the smooth endoplasmic reticulum of the forming face of the Golgi apparatus and the outer nuclear membrane. Continuity between the outer nuclear membrane and the lamellae of this smooth endoplasmic reticulum can also be observed (Plate 12). The Golgi apparatus, composed of 4-6 flattened saccules, has the usual associated vesicles and vacuoles (Plates 12 and 13). The remaining cytoplasm is filled with free ribosomes, lamellae of rough and smooth endoplasmic reticulum and a few mitochondria (Plates 11 and 14). During previtellogenesis the numbers of mitochondria begin to increase.

Scattered vesicles of smooth endoplasmic reticulum are also seen in the cytoplasm. The method of formation of these vesicles was not detected. The vesicles may be derived from blebs of the nuclear membrane as described previously (Plates 12,14). Plate 14 shows a typical portion of the oocyte cytoplasm in previtellogenesis, containing lamellae and vesicles of the endoplasmic reticulum, free ribosomes, microtubules and mitochondria.

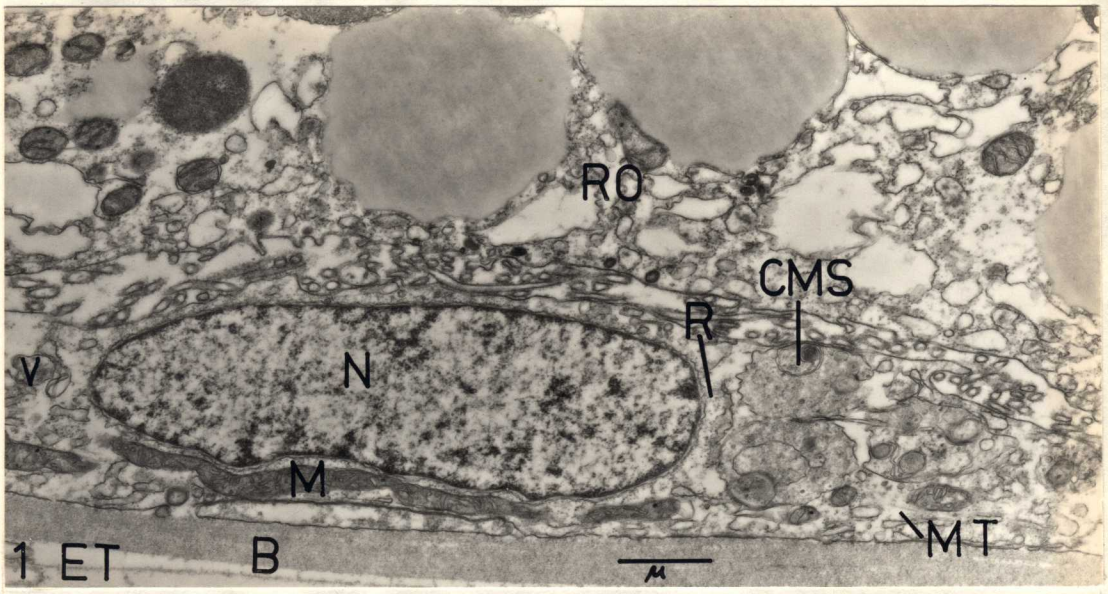


PLATE 1

Section through an epithelial cell which lies between a ripe oocyte (RO) and the basement lamina (B). Electron transparent area (ET) are present between the layers of the basement lamina. An oblong nucleus (N) is surrounded by a thin layer of unspecialized cytoplasm. Elongate mitochondria (M), vesicles of the endoplasmic reticulum (V), free ribosomes (R), microtubules (MT) are found in the cytoplasm. Complex membraneous structures (CMS), thought to be involved in the resorption of ovarian tissue are developing in the cytoplasm.

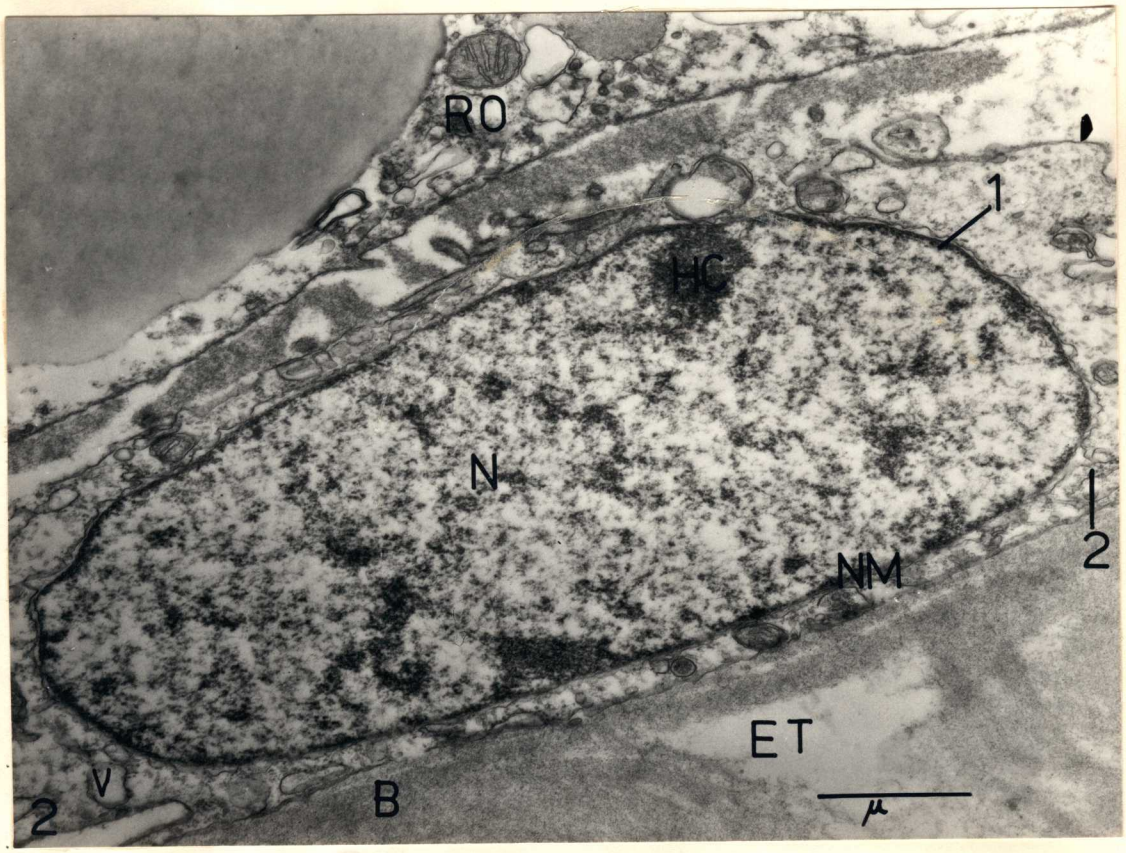


PLATE 2

High power view of an epithelial cell lying between the folded basement lamina (B) and a ripe oocyte (RO). Electron transparent areas (ET) are prominent in the basement lamina. The cell typically possess an oblong nucleus (N), which contains several clumps of heterochromatin (HC) contiguous with the dense layer of chromatin material disposed around the nuclear membrane (NM, arrow 1). The outer layer of the nuclear envelope forms blebs (arrow 2) which pinch off and lie free in the cytoplasm, forming vesicles of the rough and smooth endoplasmic reticulum (V). glutaraldehyde and osmium in phosphate buffer + 10% sucrose.

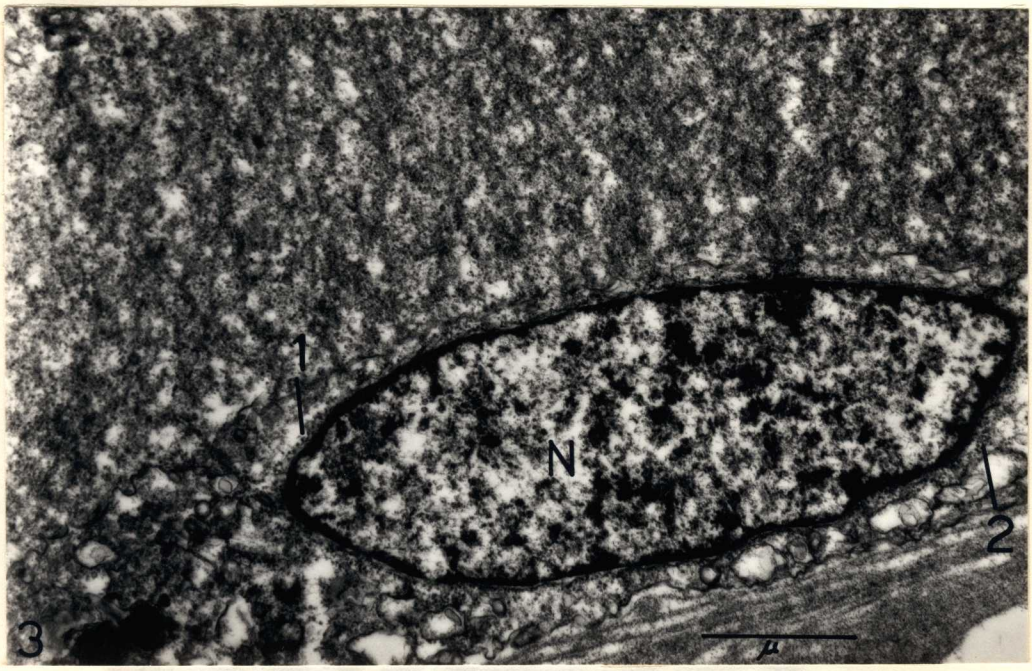


PLATE 3

Section through the nucleus (N) of an epithelial cell. Arrow 1 indicates the dense layer of free ribosomes around the nucleus, also showing some ribosomes attached to the outer nuclear membrane (NM). Arrow 2 shows a group of free ribosomes in the cytoplasm.

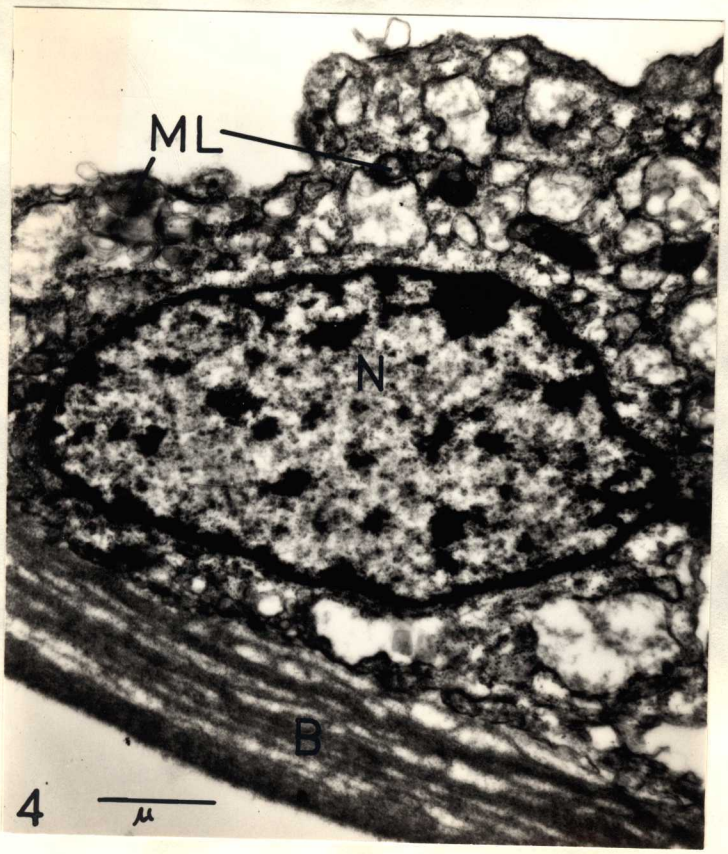


PLATE 4

Section through an epithelial cell which is involved in the resorption of ovarian tissue. This cell lacks any complex membraneous structures, but exhibits many myelin bodies (ML). On the outside of the cell, a thick layer of the basement lamina (B) is visible.

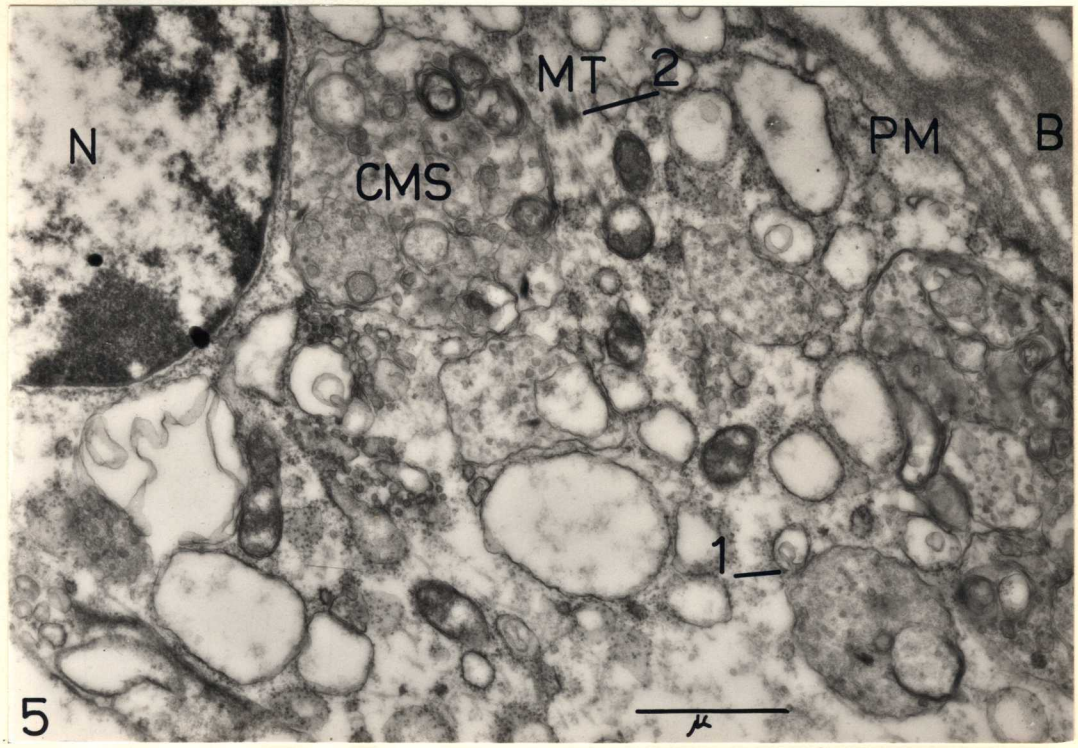


PLATE 5

Section through the perinuclear region of an epithelial cell showing resorbing activities. Many complex membraneous structures (CMS) are prominent in the cytoplasm. Arrow 1 shows the connection between the outer membrane of the complex membraneous structure and a vesicle of the rough endoplasmic reticulum (REV). Microtubules (MT) are organized into bundles (arrow 2), as well as scattered throughout the cytoplasm.

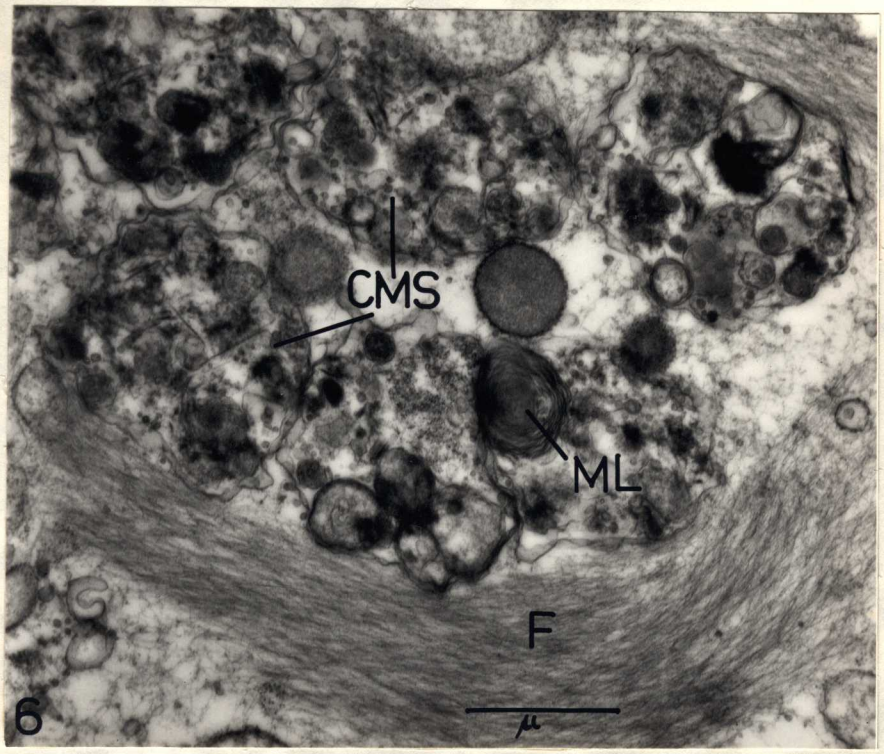


PLATE 6

Portion of an epithelial cell which is exhibiting resorbing activities. Large bundles of filaments (F) are present in the cytoplasm, as well as many complex membraneous structures (CMS). Myelin bodies (ML) are found either singly or within the complex membraneous structures.

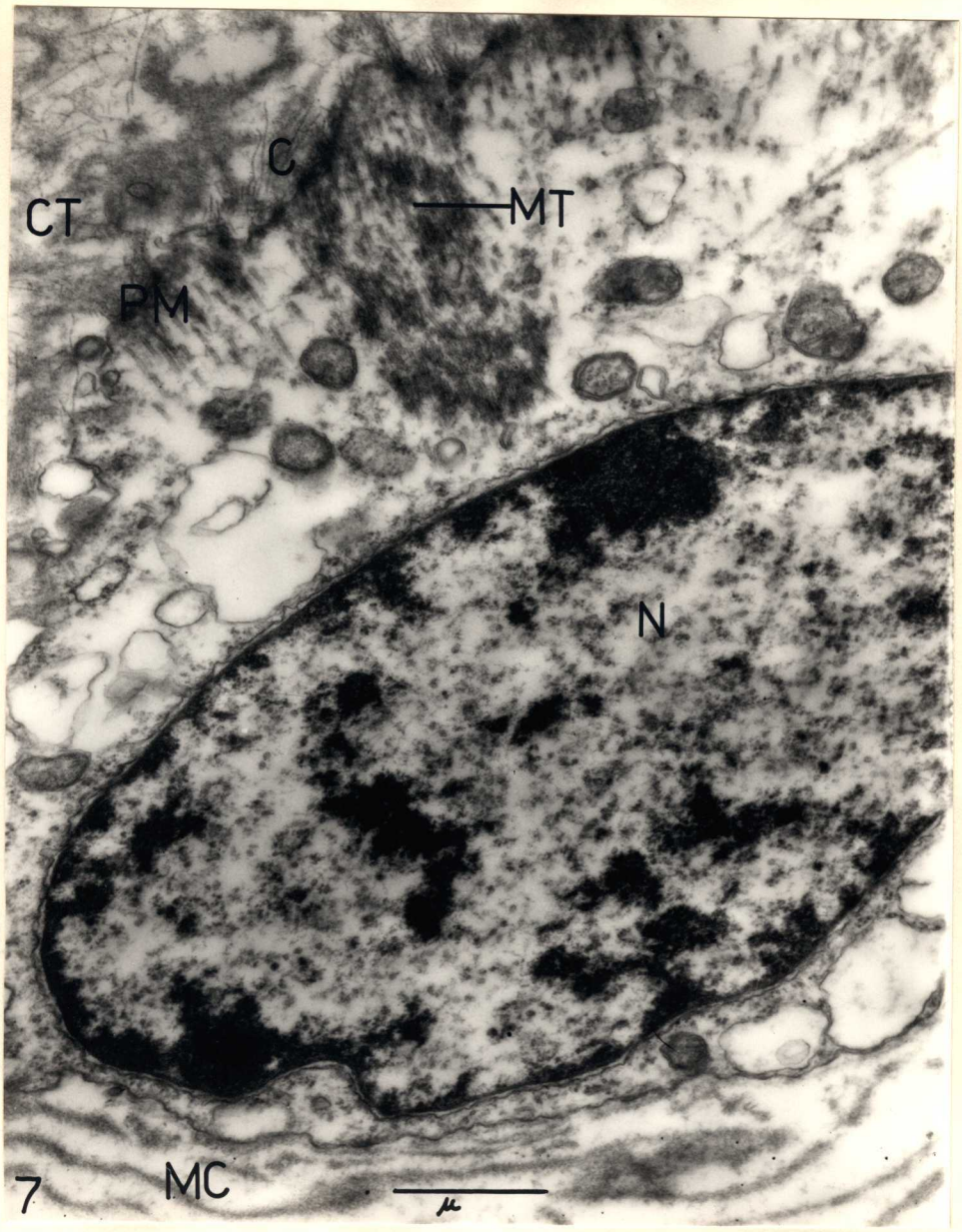


PLATE 7

Section through an epithelial cell which is embedded in connective tissue (CT) and muscle cells (MC). Large bundles of parallel microtubules (MT) are observed in the cytoplasm. They extend almost from the nucleus (N) to the plasma membrane (PM). Strands of collagen fibre (C) lie immediately outside the cell, amongst the connective tissue.

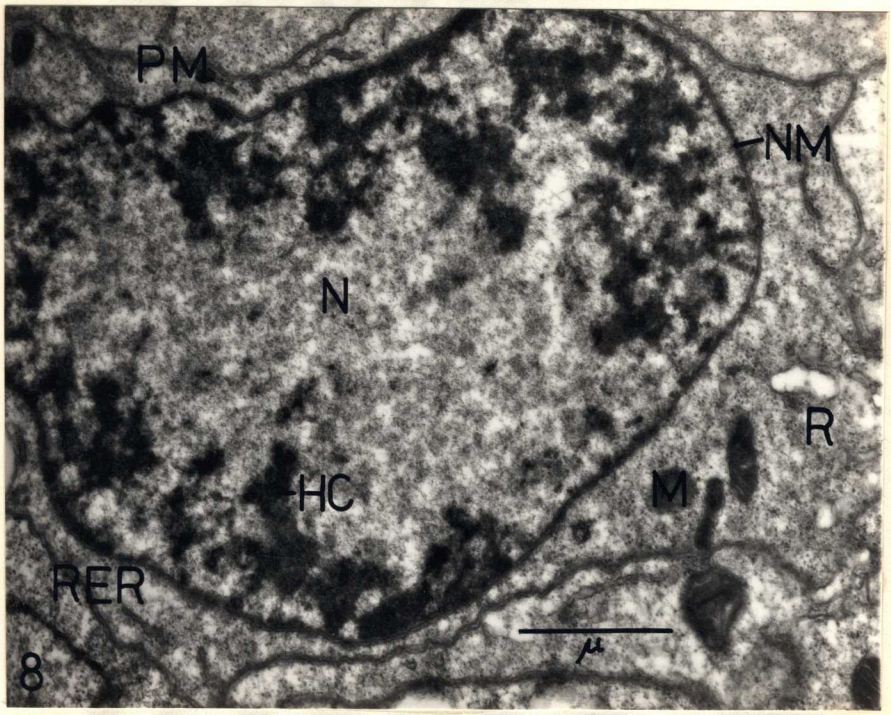


PLATE 8

High power view of an oogonium showing the unspecialized cytoplasm surrounding the nucleus. The nucleus (N) containing clumps of heterochromatin (HC), is bounded by a typical double-layered nuclear membrane (NM). The thin layer of cytoplasm contains a few mitochondria (M), many free ribosomes (R) and lamellae of rough endoplasmic reticulum (RER).

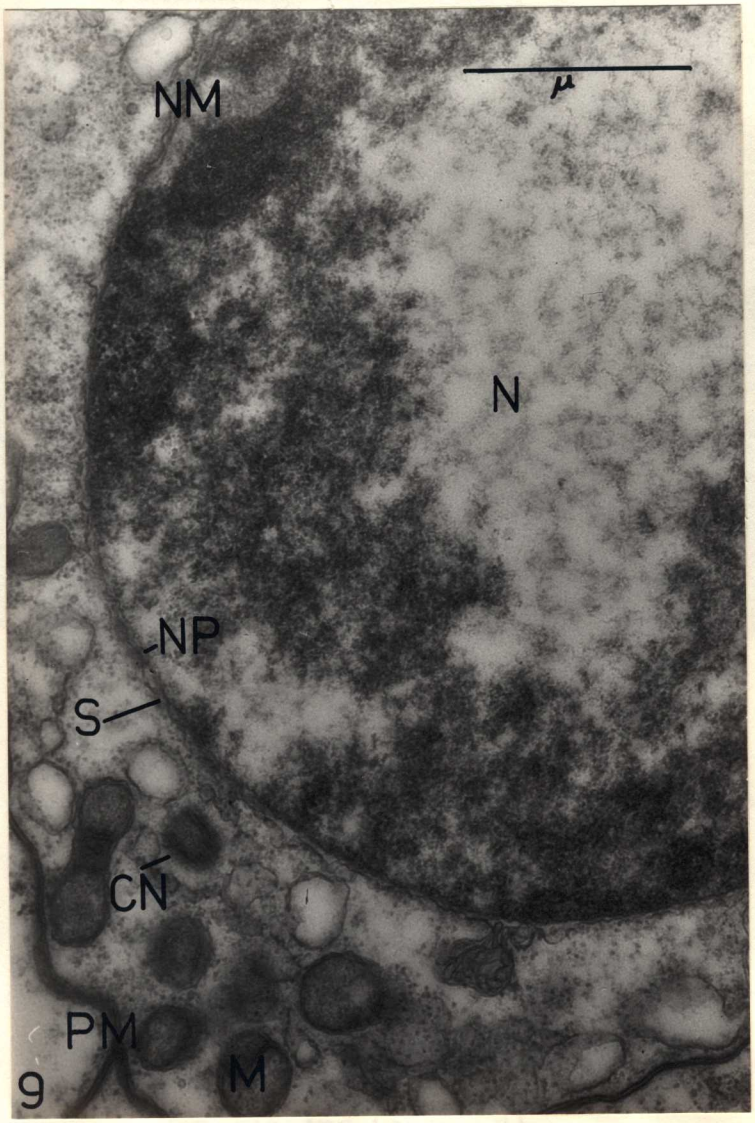
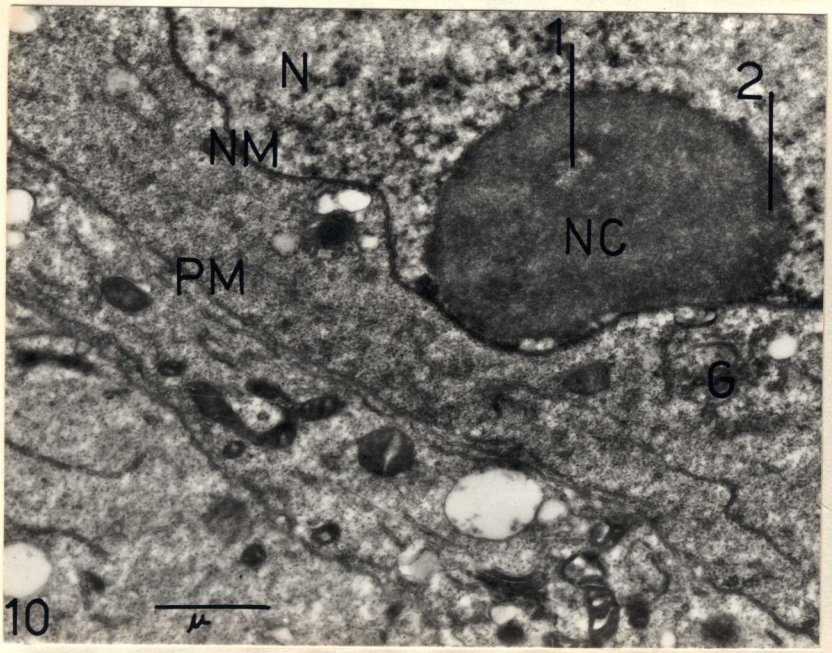


PLATE 9

A section through an oocyte at the onset of previtellogenesis (stage 4). The chromatin material is dispersed as the nucleus (N) enters interphase at the completion of the mitotic division. A centriole (CN) is found adjacent to the nucleus. The nuclear membrane (NM) is interrupted by nuclear pores (NP). The pores show the septum (S, arrow), which extends across the pore.



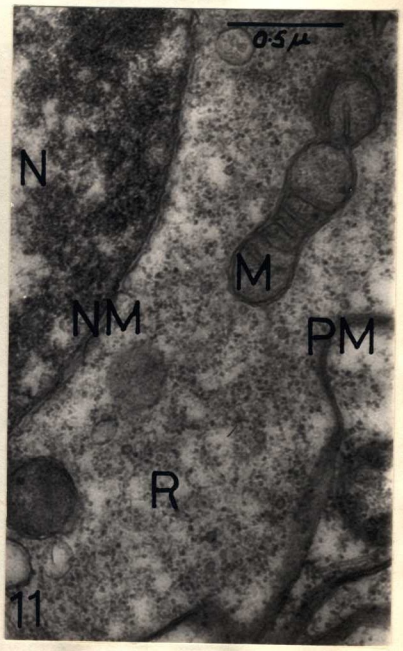


PLATE 10

Section through the nucleus (N) including the nucleolus (NC) of an oocyte in an early phase of previtellogenesis (stage 4). The nucleolus is closely applied to the nuclear membrane (NM). A less electron-dense excentric area (arrow 1) has developed within the nucleolus, and a more electron-dense area occupies the periphery of the nucleolus (arrow 2).

PLATE 11

A typical portion of the thin layer of cytoplasm surrounding the nucleus (N) of an oocyte in an early phase of previtellogenesis (stage 4). The cytoplasm is chiefly composed of free ribosomes (R) which extend from the nuclear membrane (NM) to the plasma membrane (PM) of the oocyte. Mitochondria (M) are found throughout the cytoplasm.

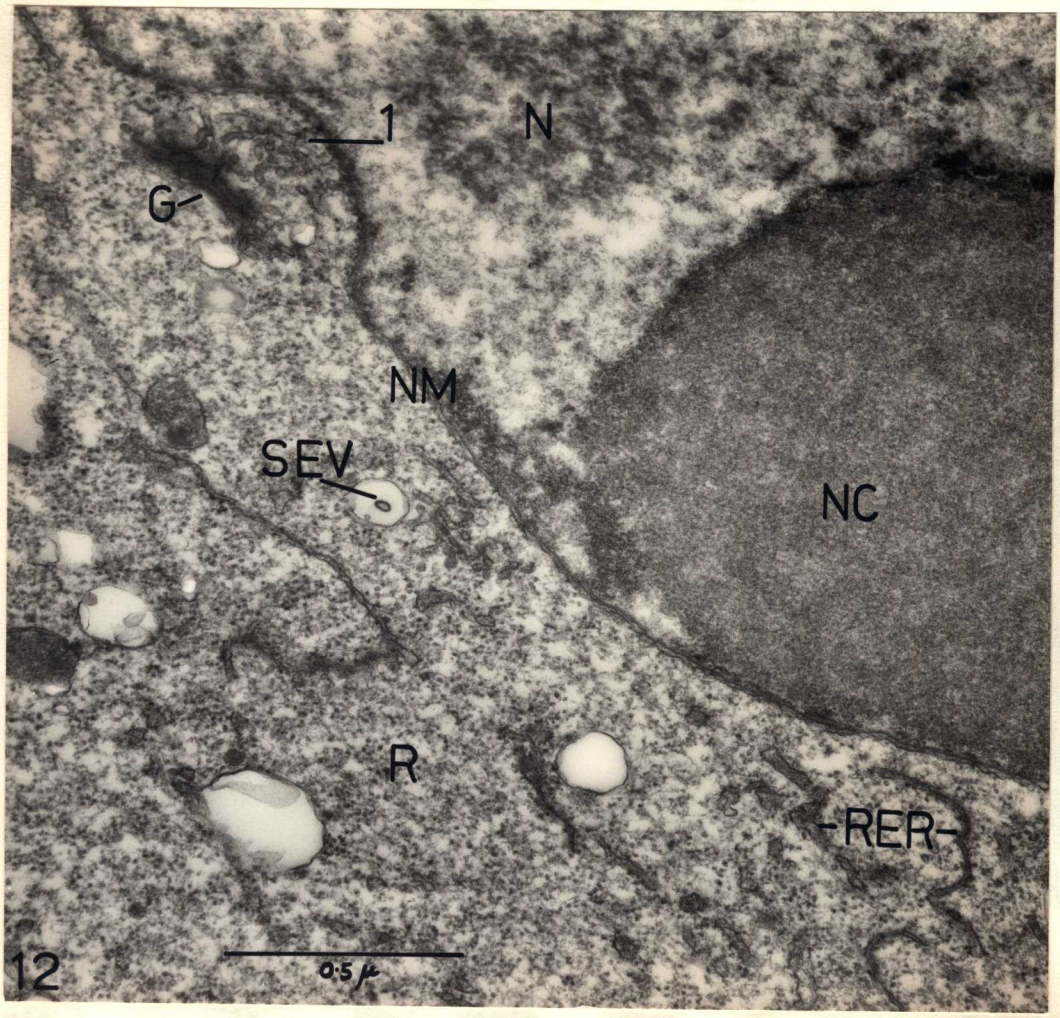


PLATE 12

Section through the nucleus (N) and adjacent cytoplasm of an oocyte in the middle of previtellogenesis (stage 4). A Golgi complex (G), composed of 5 flattened saccules is found with Golgi vesicles and lies adjacent to the nuclear membrane (NM). Arrow 1 indicates the continuation of the outer layer of the nuclear membrane and the lammelae of the smooth endoplasmic reticulum at the forming face of the Golgi apparatus. The cytoplasm is composed of free ribosomes (R), lamellae of endoplasmic reticulum (RER), vesicles of smooth endoplasmic reticulum (SEV).

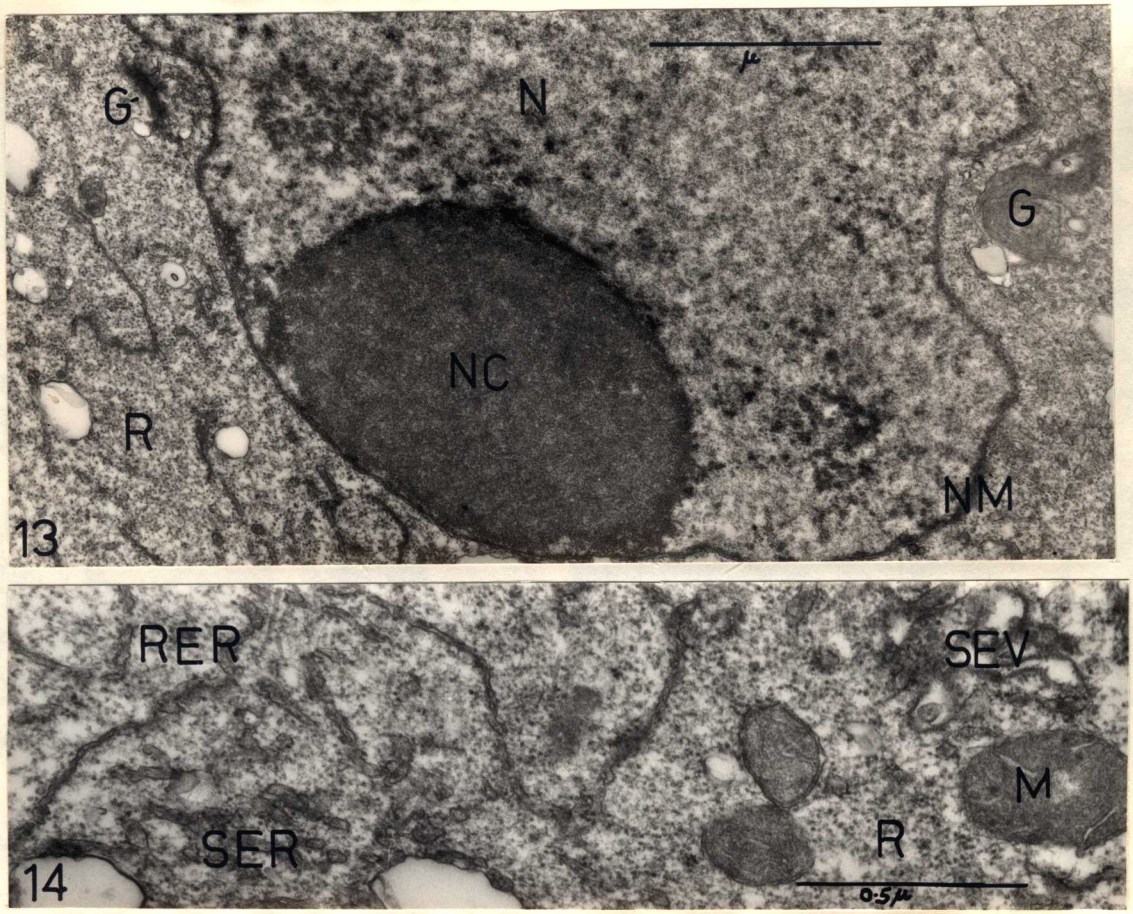
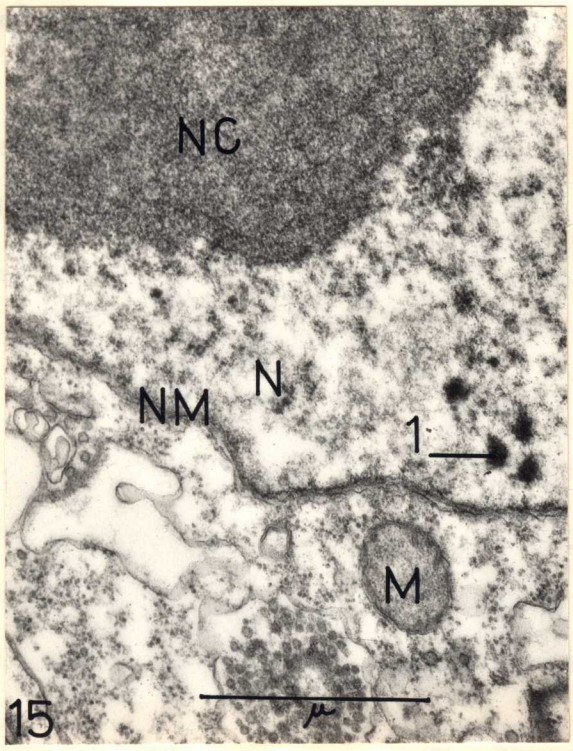


PLATE 13

Oocyte in a middle phase of previtellogenesis (stage 4). Two Golgi complexes (G) lie adjacent to the nuclear membrane (NM). Much of the cytoplasm of the oocyte is filled with free ribosomes (R).

PLATE 14

Typical portion of the cytoplasm of an oocyte in a late phase of previtellogenesis (stage 4). Lamellae of rough and smooth endoplasmic reticulum (RER, SER, respectively) are found amongst free ribosomes (R). Vesicles of smooth endoplasmic reticulum (SEV) are also seen in the cytoplasm.



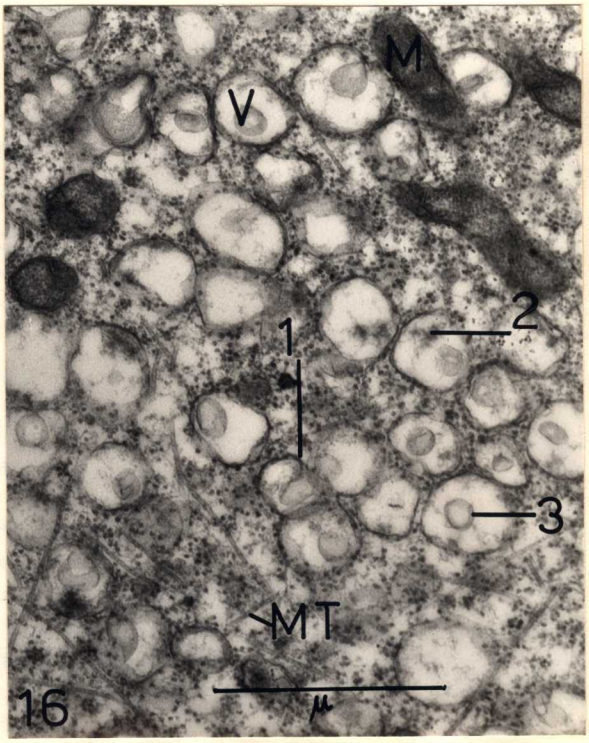


PLATE 15

Portion of the nucleus (N), nucleolus (NC), and perinuclear cytoplasm of an oocyte early in vitellogenesis (stage 5). Nucleolar material appears to have separated from the nucleolus and moved towards the nuclear membrane (arrow 1).

PLATE 16

Peripheral cytoplasm of an oocyte at about the middle of stage 5 of oogenesis. The cytoplasm is densely packed with vesicles of the endoplasmic reticulum (V), formed from blebs of the outer nuclear membrane of the nuclear envelope. Once these vesicles are free in the cytoplasm, they appear to invaginate (arrow 1) to form a "vesicle within a vesicle", arrow 3. The interior of the vesicle is mostly electron-transparent but partly contains some fine wispy material (arrow 2). Mitochondria (M) and microtubules (MT) are also present in the cytoplasm.

(d) Vitellogenesis (Stage 5):-

The youngest oocyte containing yolk (Plate 15) is only slightly larger than the largest previtellogenetic oocyte. The diameter of the germinal vesicle in the oldest previtellogenetic oocyte observed is approximately 9μ. This has increased to approximately 10μ in the youngest stage 5 oocyte.

The heterochromatin of earlier stages can no longer be seen. The nucleolus, approximately 2.5μ in diameter, is morphologically similar to that of the stage 4 oocyte. In Plate 15 some nucleolar material appears to have become detached from the nucleolus and is dispersed towards the nuclear membrane. Nevertheless no direct evidence of nuclear extrusion of this material was obtained. The typical double nuclear membrane persists, as in previous stages, with nuclear pores identical to those described for previtellogenetic oocytes (Plate 17).

Once the oocyte enters vitellogenesis, the cytoplasm undergoes several fundamental changes. However, it still retains the dense layer of free ribosomes around the nucleus (Plate 17). The most prominent organelle in the cytoplasm of a stage 5 oocyte is the vesicular form of the rough and smooth endoplasmic reticulum (Plate 16). These vesicles probably produced from blebs from the outer nuclear membrane, are dispersed throughout the cytoplasm, with no discernable spatial orientation. Initially the vesicles of the endoplasmic reticulum are about 160 mμ in diameter. In most instances, the outer surface of the vesicle has several to many ribosomes attached.

Once the vesicles are formed, they appear to undergo a process of invagination (Plate 16) to form a "vesicle within a vesicle". The inner vesicle has an approximate diameter of 60 mμ. The contents of the vesicles are mostly electron transparent and the vesicles appear to be mostly "empty". However, many do contain a few wisps of fine material. The vesicles appear to increase in size by fusion with one another (Plate 18) to attain an approximate diameter of 600 mμ. They do not appear to increase beyond this size in stage 5 oocytes.

There is a complete lack of the lamellar form of rough endoplasmic reticulum. Flattened cisternae of smooth endoplasmic reticulum are observed associated with the Golgi complex only.

Many free ribosomes are found in the cytoplasm of stage 5 oocytes, but their concentration is much less than that found in stage 4 oocytes (compare Plates 14, and 20).

Mitochondria increase in number during the course of vitellogenesis. Concomitantly, they increase in size by elongation. Plate 19 shows a mitochondrion which is approximately 7.5 μ long.

Many short microtubules are prominent in the cytoplasm of stage 5 oocytes. The microtubules range from about 250 mμ to about 1.5 μ in length, and show no specific spatial orientation. (Plate 21). The electron micrographs reveal that microtubules are concentrated mainly in the peripheral region of the oocyte. Their numbers are reduced towards the end of stage 5.

Several Golgi complexes are present in the perinuclear region of the cytoplasm of stage 5 oocytes (Plate 20). Golgi complexes are found both singly in the cytoplasm and in groups of 2 or 3 (Plates 17 and 22). As the Golgi become active, they move towards

the peripheral regions of the oocyte (Plate 29).

A close association between the Golgi complexes and the vesicles of the endoplasmic reticulum is observed. The ultrastructure of the oocyte indicates that the protein-carbohydrate yolk is synthesized initially by the incorporation of secretory granules, produced by the Golgi apparatus, into the endoplasmic vesicles formed early in stage 5. This process will now be discussed in detail.

The synthesis of Protein-carbohydrate yolk during stage 5:-

Two organelles appear to be directly involved in the synthesis of protein-carbohydrate yolk. These are the vesicular form of the endoplasmic reticulum and the Golgi complexes.

The smallest Golgi are approximately 160 mμ wide. As each Golgi becomes involved in secretory processes, its width increases to a maximum of 330 mμ. The active Golgi complexes reach a maximum length of about 2.5 μ. The most active Golgi are found in stage 5 and 6 oocytes, during early vitellogenesis (Plate 23).

An active Golgi complex consists of 8-10 flattened membranous saccules. The flattened saccules are piled one upon the other in close parallel array, and may be flattened or curved. Active Golgi complexes exhibit both a forming face and a maturing face. The forming face is characterized by the sometimes greater width of the saccules, and the presence of many flattened vesicles of smooth endoplasmic reticulum. The maturing face is characterized by the presence of dense homogeneous material within the innermost saccules. Clustered around the convex surface of the forming face are many vesicles of smooth endoplasmic reticulum (Plate 26), which appear to become flattened and form cisternae which then lie almost parallel

to the forming face of the Golgi (Plate 24). Dense homogeneous material is observed within the cisternae. This material appears to be identical in electron density with that found in the saccules at the maturing face of the Golgi (Plate 24). In addition to this material, granules of greater electron density, approximately 25 mμ in diameter, are located within the cisternae (Plate 24). Although smaller in size, these granules have a similar electron density to those produced by the saccules at the maturing face of the Golgi complex (Plate 26).

Two secretory products, directly derived from the Golgi apparatus are observed to accumulate adjacent to the maturing face of the Golgi complex. From the ends of the flattened saccules, discrete membrane-bound secretory granules, approximately 40-60 mμ in diameter are released free into the surrounding cytoplasm (Plates 22,23 and 24). These granules are incorporated into vesicles of the smooth endoplasmic reticulum (Plate 22, arrow) which then move away from the Golgi complex (Plate 26). In addition, the ends of the saccules swell to form Golgi vesicles which, in turn, contain many discrete secretory granules, about 40-60 mμ in diameter (Plates 25,26). In size and electron density, the two types of granules derived separately from the Golgi appear to be identical.

Both the smooth endoplasmic vesicles with their enclosed granules and the Golgi vesicles are considered to be precursory yolk vesicles. Each vesicle is approximately 200 mμ in diameter and contains only a small number of secretory granules (Plates 20,25, and 26). The precursor yolk vesicles move away from the Golgi region and begin the complex process of maturation into yolk bodies.

Maturation is initiated in stage 5 by an increase in size of the precursor yolk vesicles. Electron micrographs indicate that this occurs by a process of fusion of two or more small yolk-containing vesicles (Plate 27). Precursor yolk vesicles are also observed to fuse with other vesicles of the endoplasmic reticulum. The endoplasmic vesicles have ribosomes attached to their cytoplasmic surface, and contain fine wispy material, as described previously (Plate 30).

In stage 5, the largest precursor yolk vesicles observed are about 900 mμ in diameter. They still contain discrete granules between 40-60 mμ in diameter, the more central ones having lost their outer membrane. The granules appear to concentrate in the central regions of the vesicles and form a distinct inner core (Plate 28).

(e) Vitellogenesis (Stage 6):-

Most of the yolk present in the ripe oocyte is laid down during stage 6 of oogenesis. The formation of precursor yolk vesicles, initiated in stage 5, continues and the number of precursor yolk vesicles increases sharply. In addition, material is incorporated into the oocyte by micropinocytosis across the plasma membrane to form a second kind of protein-carbohydrate yolk body (see below). Simultaneously, lipid accumulation commences (Plate 29).

During stage 6, direct evidence of the extrusion of nucleolar material through the nuclear pores of the nuclear membrane was obtained. The nuclear pores still have the same structure and size as described in previous stages. The extruded material does not

appear to accumulate in the cytoplasm but becomes dispersed into electron dense particles resembling the ribosomes free in the cytoplasm. In Plate 30, a portion of the nucleolus is apparent and particles appear to come from this and pass through the nuclear pores. An enlargement of the nuclear membrane in Plate 31 shows this process clearly.

The synthesis of lipid droplets:-

The first lipid droplets are observed during the initial phases of stage 6. Ultrastructural studies reveal no information on the synthesis of lipid droplets. Initially small droplets appear "de novo" in the cytoplasm of stage 6 oocytes. The smallest droplets observed are about 1 u in diameter (Plate 29). The electron micrographs reveal a dense line around the periphery of the lipid bodies (Plate 30). However, this is not considered to be an indication of a limiting outer membrane. It has been shown (Fawcett, 1966) that an intense reduction of osmium tetroxide occurs at the interface between lipid and its surrounding cytoplasm. This phenomenon produces a dense line.

Lipid droplets occur initially in the perinuclear regions of the oocyte. As the oocyte grows, the lipid droplets fuse with one another to form increasingly larger droplets (Plate 32). At no time in the formation of the lipid are the droplets specifically associated with any cytoplasmic organelles. Judging by their reaction with osmium tetroxide staining, the lipid droplets contain a quantity of unsaturated fats.

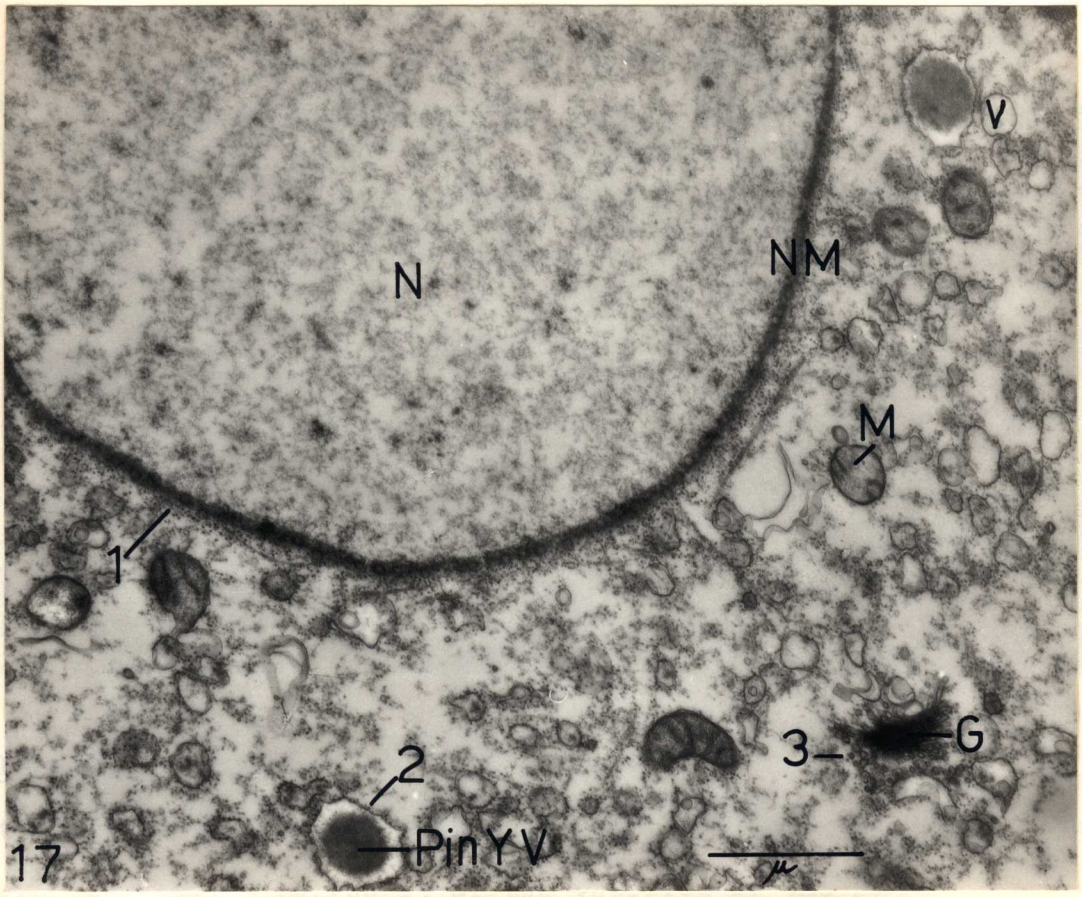
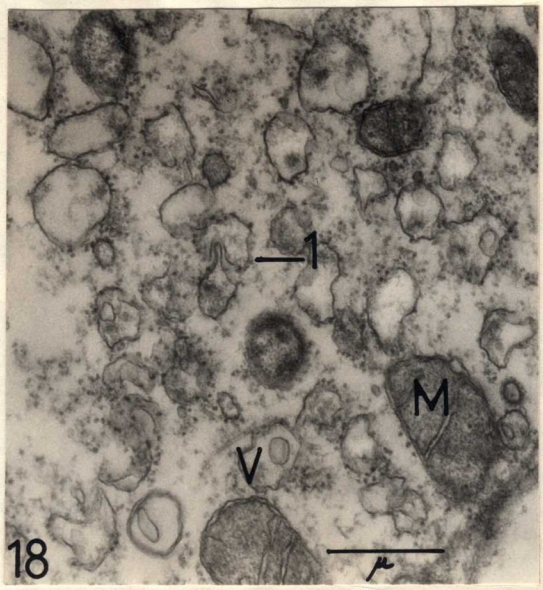


PLATE 17

Portion of the nucleus (N) and the cytoplasm of an oocyte at the onset of stage 6 of oogenesis. An active Golgi complex (G) is present at a distance from the nuclear membrane. Arrow 1 shows the layer of free ribosomes (R) surrounding the nucleus. Some pinocytotic yolk vesicles (Pin. YV) are present in the cytoplasm of this oocyte, indicating that the oocyte is just at the onset of stage 6 of oogenesis. Arrow 2 indicates ribosomes attached to the outer edge of the pinocytotic yolk vesicle. Arrow 3 indicates the many secretory granules, produced by the Golgi (G), which are released free into the cytoplasm.



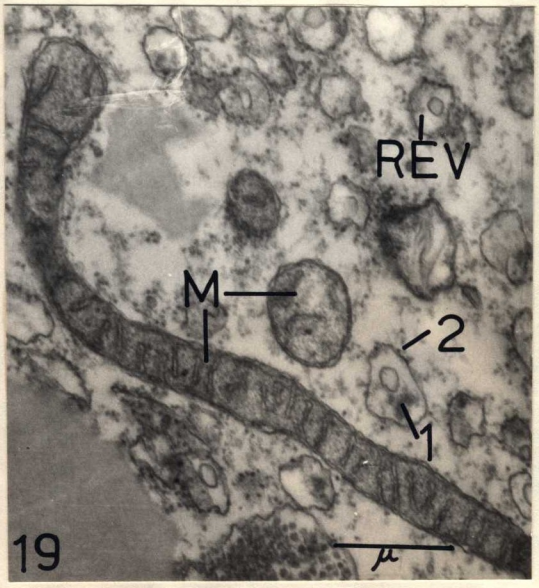


PLATE 18

Portion of the cytoplasm of an oocyte late in stage 5 of oogenesis showing the fusion (arrow 1) of two vesicles of the rough endoplasmic reticulum, to form larger vesicles of the endoplasmic reticulum (V).

PLATE 19

Portion of the cytoplasm of an oocyte at the onset of stage 6 of oogenesis showing an elongated mitochondrion, sectioned longitudinally. Other mitochondria (M) in the cytoplasm have been cut transversely. Vesicles of rough endoplasmic reticulum (REV) are also apparent in the cytoplasm. Arrow 1 shows the accumulation of fine wispy material within these vesicles; arrow 2 shows ribosomes attached to the cytoplasmic aspect of the vesicle.

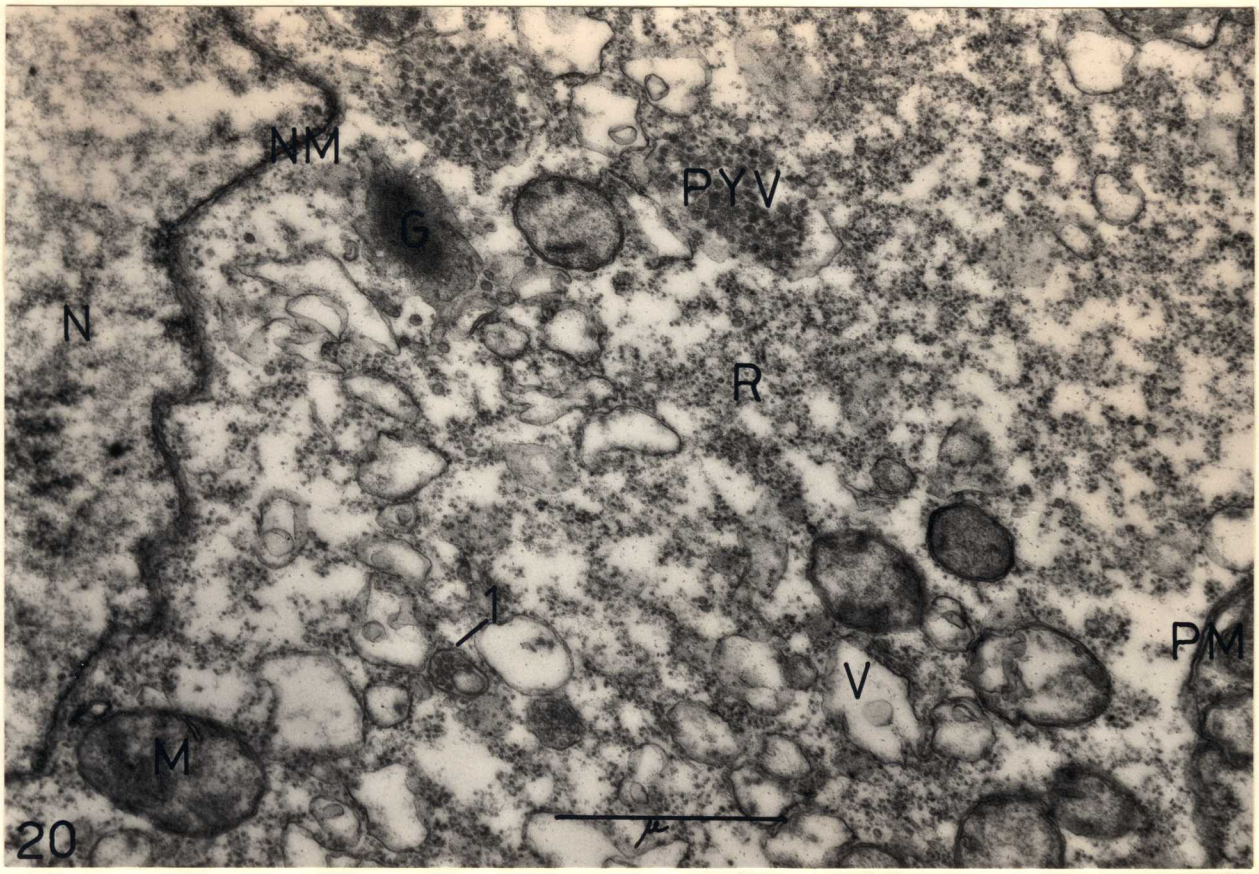


PLATE 20

The nucleus (N) and cytoplasm of an oocyte at the onset of vitellogenesis (stage 5). The cytoplasm is composed chiefly of vesicles of the endoplasmic reticulum (V), mitochondria (M), free ribosomes (R) and Golgi complexes (G). Precursor yolk vesicles (PYV), containing the secretory products of the Golgi complexes, are also present in the cytoplasm. Arrow 1 shows a small PYV containing only a small number of secretory granules.

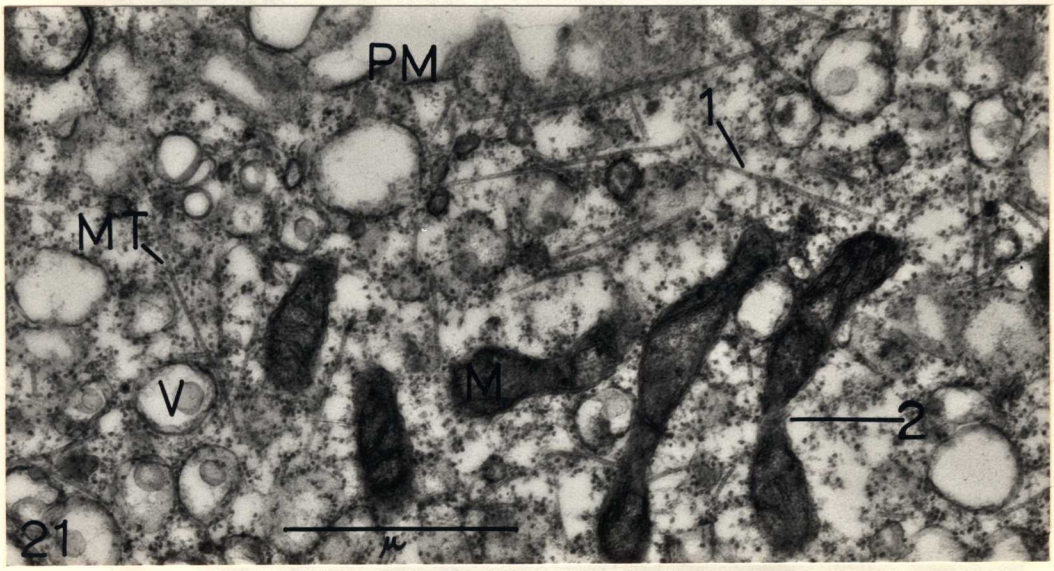


PLATE 21

Peripheral region of the cytoplasm of an oocyte in the middle of stage 5 of oogenesis. Many microtubules (arrow 1) (MT) are scattered throughout the cytoplasm without any discernable spatial orientation. Mitochondria (M) may be budding and dividing into two (arrow 2). The plasma membrane (PM) shows no micropinocytotic activity or microvilli at this stage.

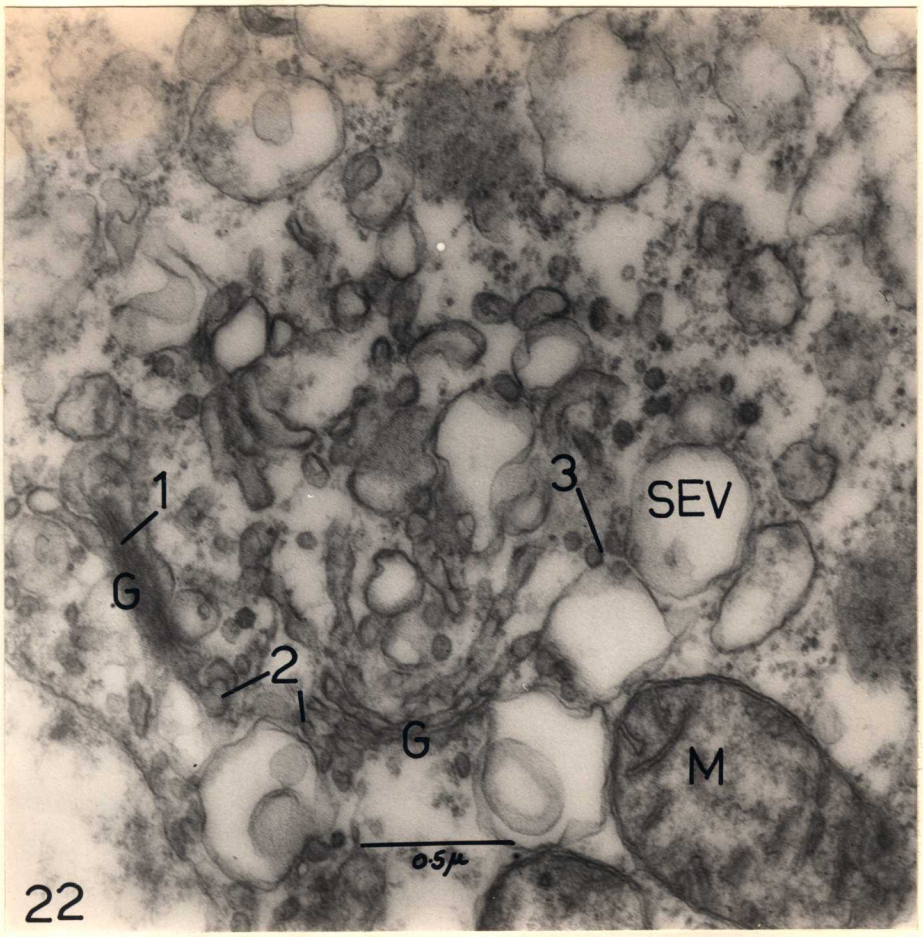


PLATE 22

Two Golgi complexes (G), in portion of the cytoplasm of a stage 5 oocyte, are in close association. Dense homogeneous material is evident in the saccules of the Golgi complex (arrow 1). From the ends of the saccules, many membrane-bound secretory granules (arrow 2), approximately 40-60mμ in diameter, are apparent at the maturing face of the Golgi. These secretory granules are then incorporated into adjacent vesicles of the smooth endoplasmic reticulum (SEV) arrow 3, to form precursor yolk vesicles.

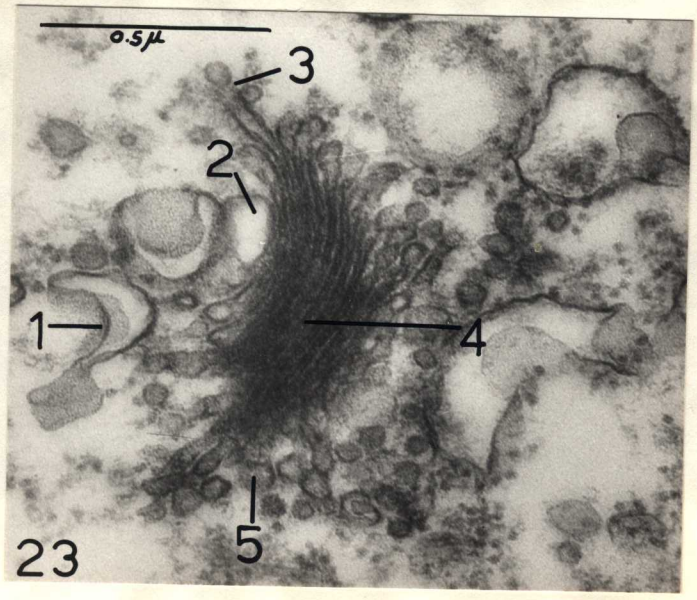


PLATE 23

A high power view of the Golgi complex from Plate 17. Vesicles of the smooth endoplasmic reticulum at the forming face (FG) of the Golgi appear to become flattened and lie adjacent to the saccules (arrow 1,2). Membrane-bound secretory granules, about 40-60 mμ in diameter, are formed at the ends of the saccules of the Golgi and then released into the surrounding cytoplasm (arrow 3). The innermost saccules at the maturing face of the Golgi contain dense homogeneous material (arrow 4). Many secretory granules can be seen at the maturing face of the Golgi, arrow 5.

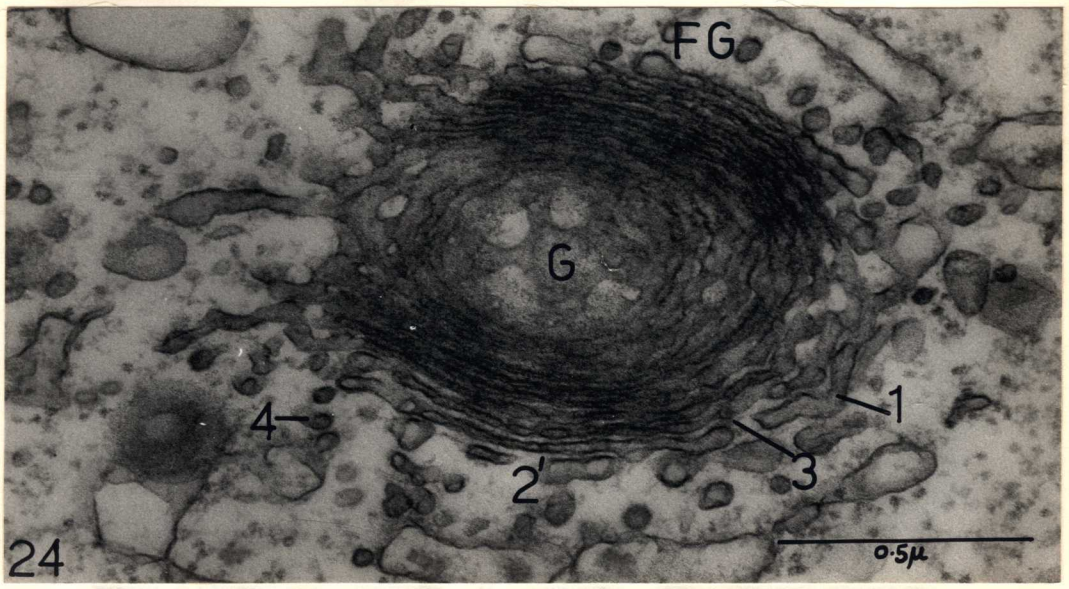


PLATE 24

Section through an active Golgi (G) from portion of the cytoplasm about halfway between the nucleus and the plasma membrane of an oocyte in stage 6 of oogenesis. The tubular cisternae of the smooth endoplasmic reticulum (arrow 1) are apparent at the forming face (FG) of the Golgi. Dense homogeneous material is apparent within the cisternae (arrow 2). In addition, smaller granules of greater electron density are obvious in the tubular cisternae of the smooth endoplasmic reticulum (arrow 3). Many membrane-bound secretory granules formed by the Golgi accumulate in the surrounding cytoplasm (arrow 4).

Philips 200

glutaraldehyde/osmium in phosphate buffer with 10% sucrose added.

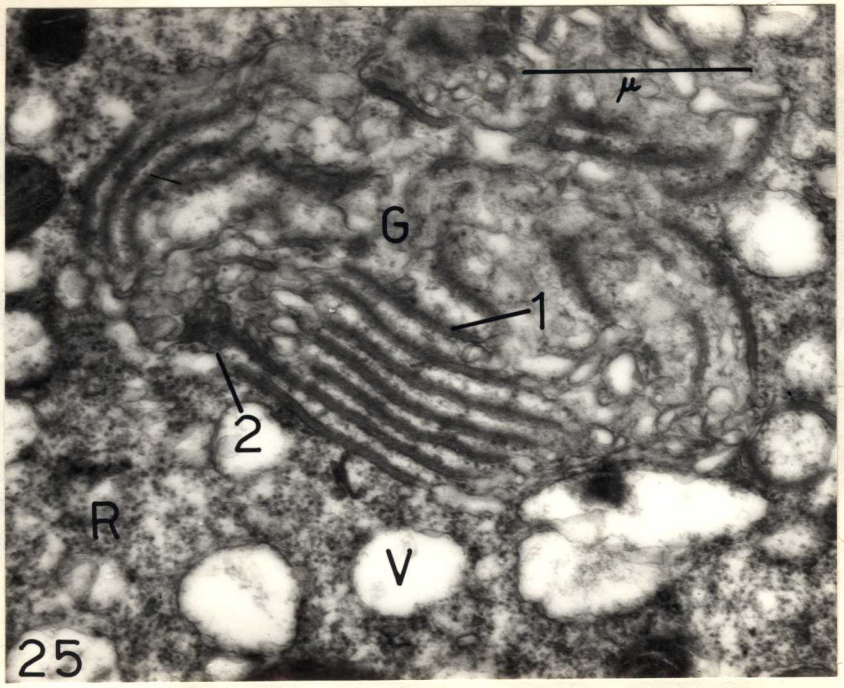


PLATE 25

Section through a complex group of Golgi bodies (G) from portion of the cytoplasm about halfway between the nucleus and the plasma membrane of a stage 6 oocyte. Dense homogeneous material is evident in the saccules of the Golgi complex (arrow 1). The ends of these saccules are seen to swell to form a Golgi vesicle (arrow 2). The Golgi vesicles contain several secretory granules produced by the Golgi itself. The Golgi vesicle, with its enclosed secretory granules, becomes detached from the Golgi, and lies free in the cytoplasm, forming the precursor yolk vesicles.

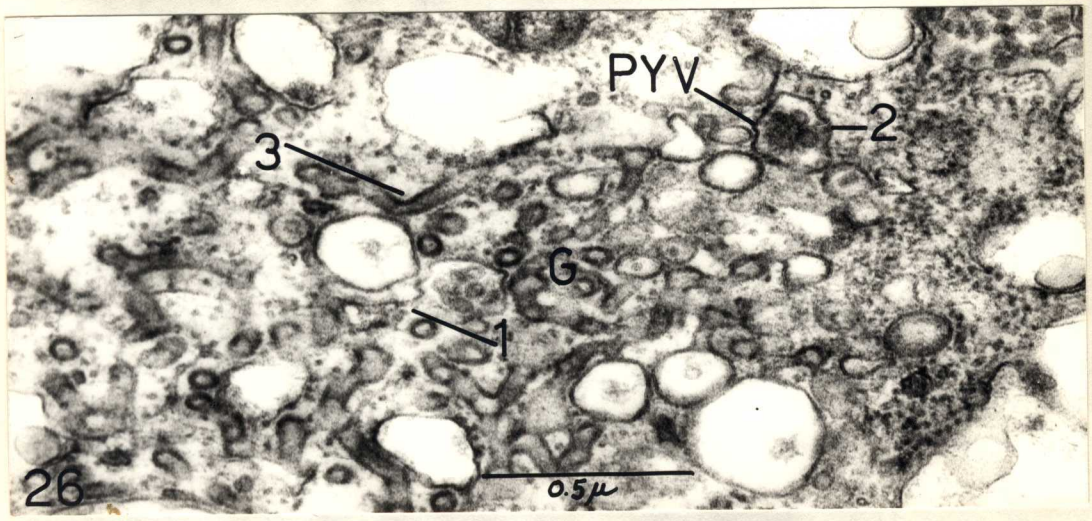


PLATE 26

A section through the peripheral region of the Golgi complex (G), showing a Golgi vesicle containing many secretory granules still connected to the Golgi saccules (arrow 1). The small precursor yolk vesicles (PYV) then move away from the Golgi (arrow 2) into the surrounding cytoplasm. Arrow 3 shows the tubular smooth endoplasmic reticulum which forms the peripheral regions of the Golgi complex.

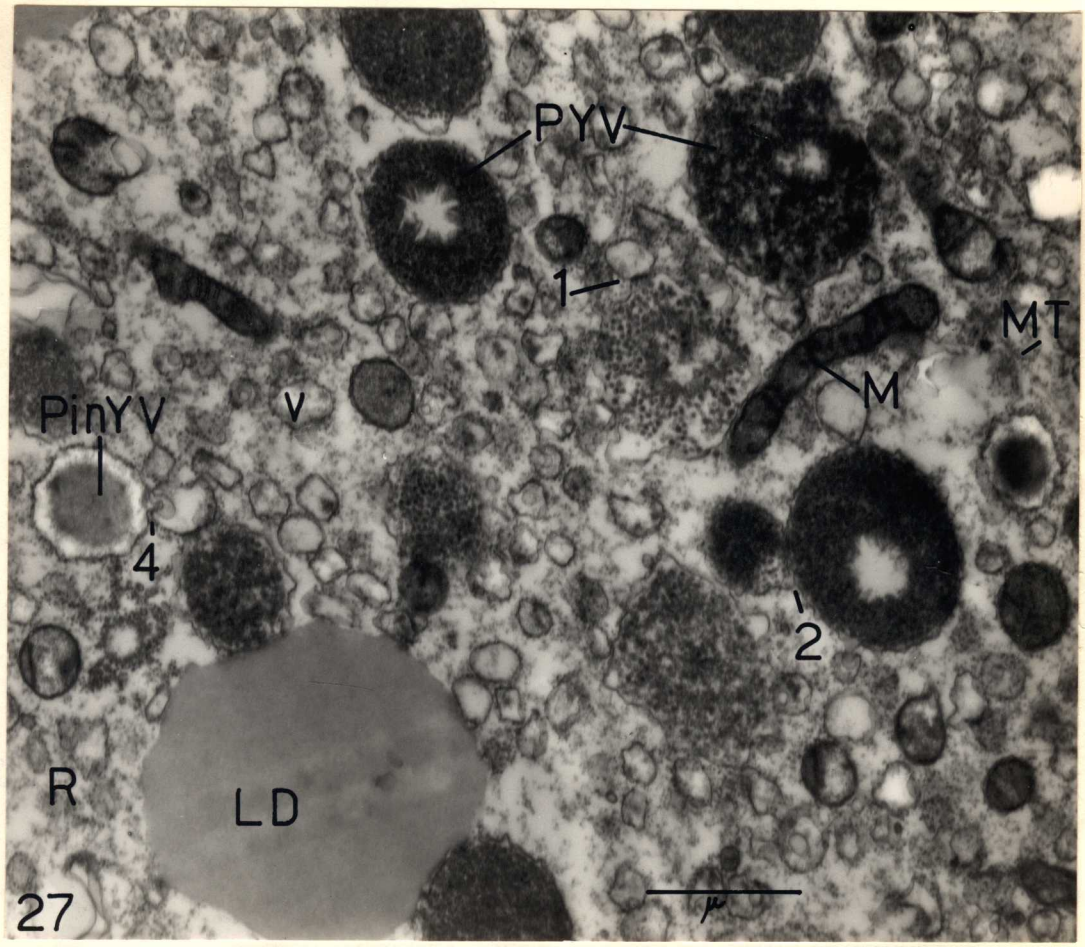


PLATE 27

A portion of the peripheral cytoplasm of an oocyte in stage 6 of oogenesis. Immature precursor yolk vesicles (PYV) are seen at various stages of development. They can be seen to fuse with the small vesicles of the rough endoplasmic reticulum (arrow 1) which contain fine wispy material, and smaller PYV (arrow 2). Pinocytotic yolk vesicles (see Plate 40) are evident in the cytoplasm (Pin YV) and can be seen fusing with small vesicles of the rough endoplasmic reticulum (arrow 4). Ribosomes are seen attached to the outer membrane of the pinocytotic yolk vesicle; but are lacking from the precursor yolk vesicles. Also present in the cytoplasm are lipid droplets, (LD), mitochondria (M), microtubules (MT), free ribosomes (R) and vesicles of the endoplasmic reticulum (V).

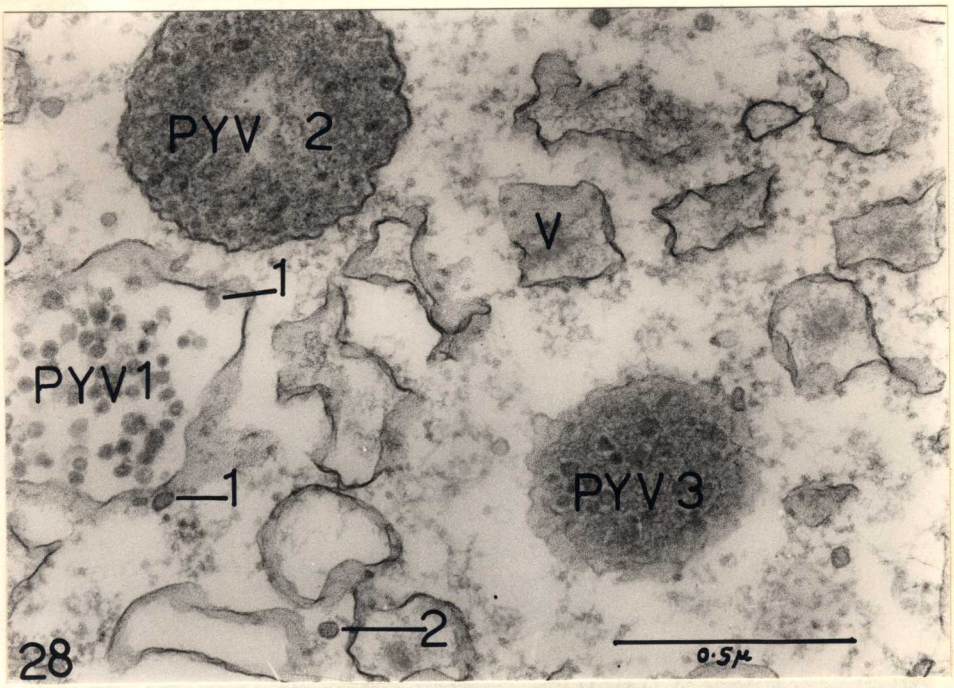


PLATE 28

A high power study of precursor yolk vesicles (PYV) in the cytoplasm near the Golgi region of an oocyte at the end of stage 5 of oogenesis. PYV 1 shows a vesicle composed of membrane-bound secretory granules only. Arrow 1 indicates granules that may be incorporated into the precursor yolk vesicles. PYV 2,3, are more mature yolk vesicles. Secretory granules can be seen towards the outer regions of the precursor yolk vesicles, the central areas being composed of finely granular material. This apparently forms when the secretory granules lose their outer membranes and the contents of the granules fuse together. Many vesicles (V) of the endoplasmic reticulum are present in the cytoplasm. Arrow 2 shows a secretory granule formed at the Golgi free in the cytoplasm.

Glutaraldehyde-osmium in phosphate buffer with 10% sucrose added.

Philips 200.

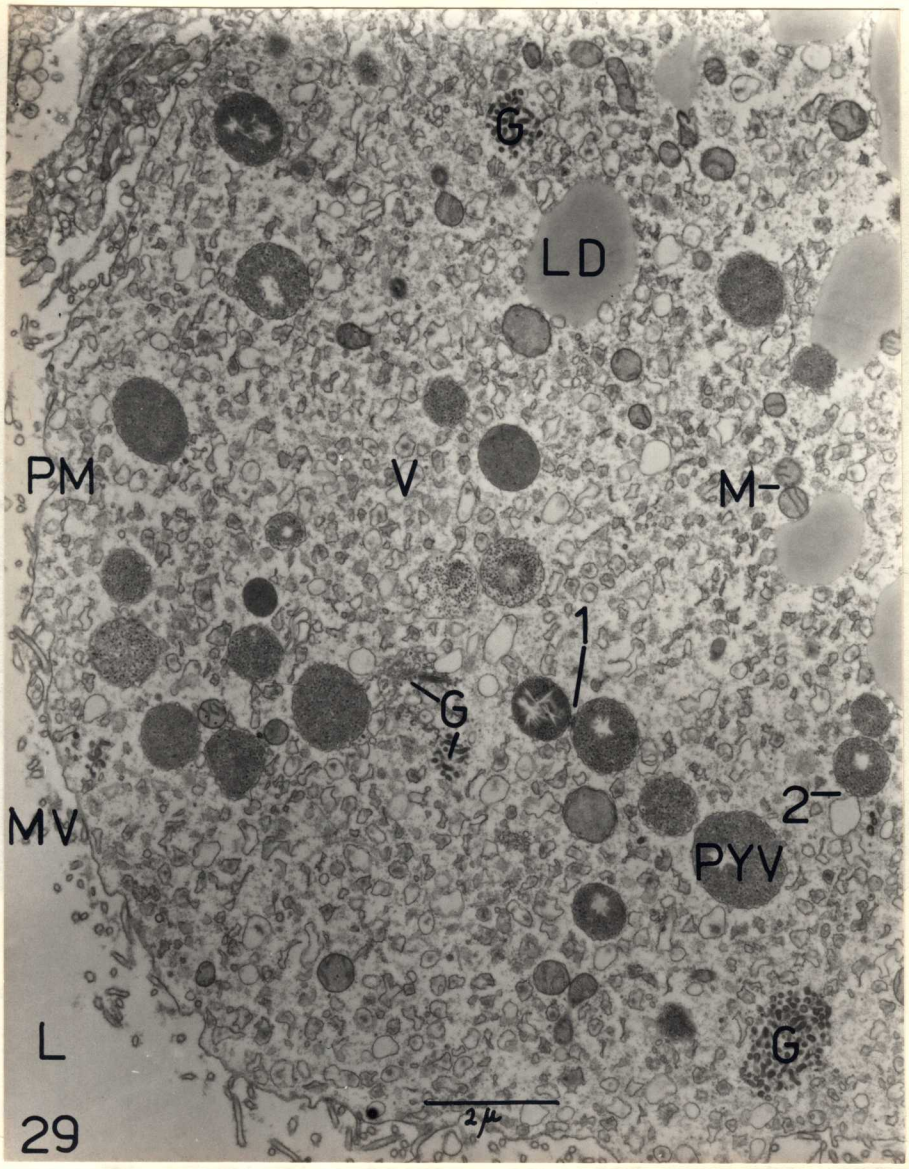


PLATE 29

Low power view of the peripheral cytoplasm of an oocyte in stage 6 of oogenesis, showing many stages of precursor yolk maturation, lipid synthesis and formation of microvilli. Arrow 1 indicates possible fusion between two mature yolk bodies. Arrow 2 shows fusion of precursor yolk vesicles (PYV) with the small vesicles of rough endoplasmic reticulum (V).

glutaraldehyde-osmium in phosphate buffer with 10% sucrose added.

Philips 200

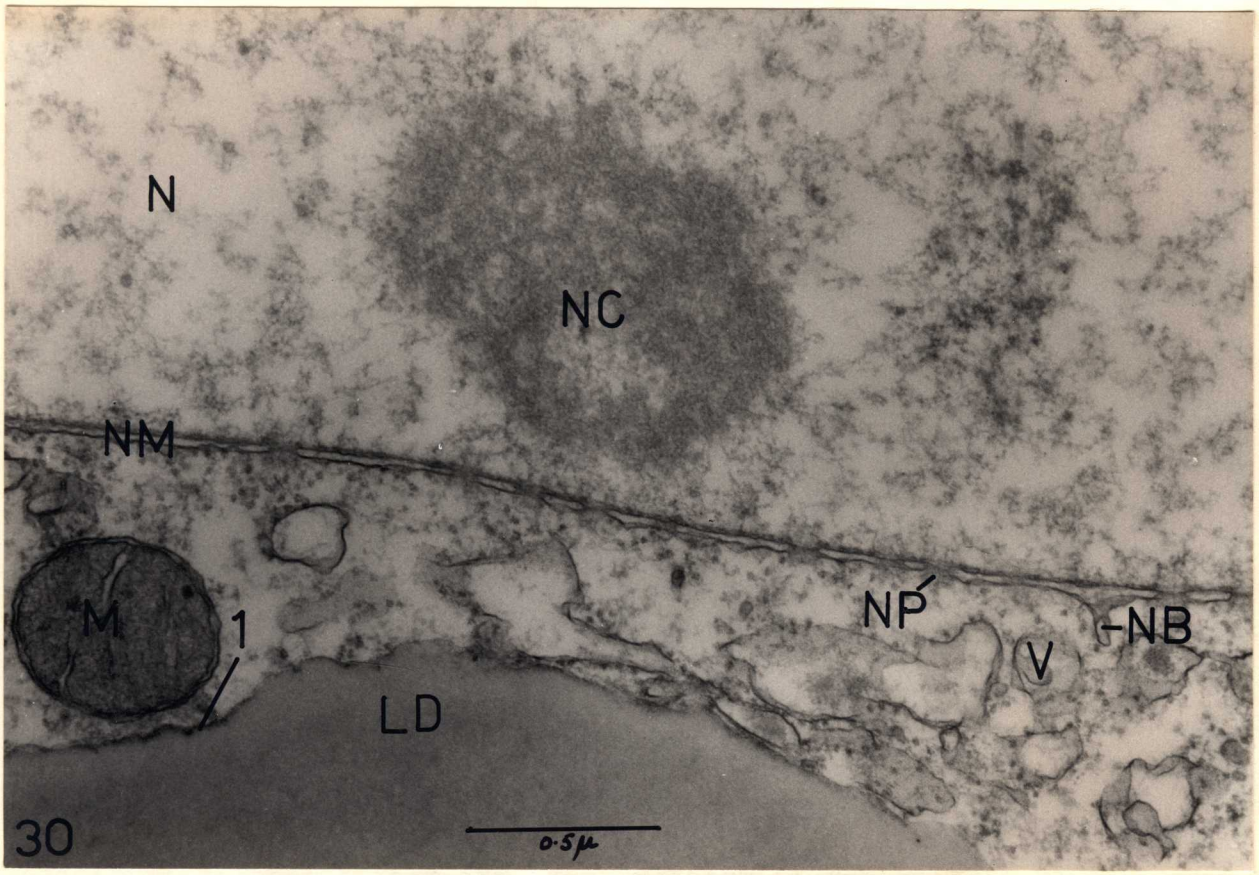


PLATE 30

A nucleus (N) from an oocyte at stage 6 of oogenesis. Part of the nucleolus (NC) is visible in the nucleus, close to the nuclear membrane (NM). Particles become separated from the nucleolus and move towards the nuclear membrane. The nuclear membrane is perforated with nuclear pores (NP). The septum of the pores does not show in this electron micrograph. A lipid droplet (LD) is apparent adjacent to the nucleus, showing the dense line (arrow 1) which gives the false impression of an outer limiting membrane.
Philips 200
glutaraldehye-osmium in phosphate buffer with 10% sucrose added.

Micropinocytosis:-

In addition to the formation of precursor yolk vesicles as described above, precursor yolk material is observed to be incorporated into the oocyte from the lumen of the ovariole. This process occurs by the formation of bristle-coated micropinocytotic vesicles at the oocyte surface. The plasma membrane at the site of invagination is coated on its outer aspect with a thin layer of granular material of appreciable density (Plate 33). The cytoplasmic surface of the plasma membrane shows a local condensation which forms the bristle coat, typical of alveolate vesicles. The micropinocytotic pits, containing dense granular material, project deeper into the cytoplasm, and eventually pinch off from the plasma membrane (Plate 34).

Once free in the peripheral cytoplasm of the oocyte, these micropinocytotic vesicles can be identified by the bristle coat surrounding their membrane (Plate 35). Their average shape is ovoid, with approximate dimensions of 150x80mμ. The micropinocytotic vesicles are observed to aggregate in the peripheral cytoplasm (Plate 35). Moreover, individual micropinocytotic vesicles become incorporated into rough endoplasmic vesicles (Plate 35). Once the micropinocytotic vesicles are included within the rough endoplasmic vesicles, they lose their bristle coats, and fuse to form a homogeneous material. Eventually all the micropinocytotic vesicles become incorporated into endoplasmic vesicles in this way. The contents of the micropinocytotic vesicles appear to have the same homogeneous finely granular appearance and electron density as the material in the rough endoplasmic vesicles. These structures are a second form of precursor yolk body, and will now be described as

pinocytotic yolk vesicles (Plate 36).

Ribosomes persist at the cytoplasmic surfaces of the pinocytotic yolk vesicles (Plates 17 and 36). In addition, the cytoplasm surrounding the vesicles abounds with free ribosomes. This may suggest that synthesized proteins become incorporated into the vesicles, which may in turn account for their increase in size. The contents of the pinocytotic yolk vesicles are loosely surrounded by the external membrane (Plate 36). Fine wispy material, identical in electron density with that found in the small rough endoplasmic vesicles, can be observed in some parts of the outer "empty" region. Electron micrographs indicate that the pinocytotic yolk vesicles may also increase in size by fusion with smaller rough endoplasmic vesicles (Plate 36); in this respect, they are similar to the precursor yolk vesicles described above as originating in association with the Golgi complexes.

As each pinocytotic yolk vesicle matures, the interior of the vesicle is gradually completely occupied by the finely granular material described previously (Plate 37). Once a diameter of about 900 mμ has been reached the pinocytotic yolk vesicle becomes morphologically indistinguishable from a mature precursor yolk vesicle (see stage 7).

(f) Vitellogenesis (Stage 7):-

During the final phase of oogenesis, the synthesis of lipid and protein-carbohydrate yolk is completed. Towards the end of stage 6, microvilli begin to project from the plasma membrane of the oocyte into the lumen of the ovariole. This is the commencement

of the formation of the vitelline membrane, which is completed in stage 7. There is little evidence of yolk synthesis during this stage. The main activity within the oocyte is the maturation of existing precursor yolk vesicles into the membrane-bound, finely granular yolk bodies. The process of maturation is initiated during stage 6 and continues into stage 7.

Maturation of precursor yolk vesicles:-

As the precursor yolk vesicles approach a diameter of about 1μ, the granules in the central region of each vesicle appear to lose their outer membrane and fuse to form "rod-like" structures (Plate 38). This material forms a definite central core within the precursor yolk vesicle, parts of which become electron-transparent. As the precursor yolk vesicle continues to mature, the electron-transparent areas either remain rod-like or becomes stellate in shape (Plate 38). This may be due to the plane of the section through the yolk bodies (Plate 39). Staining of 1μ thick sections with toludine blue reveals a lower uptake of the stain in this region, indicating a reduced protein content as compared with the periphery of the yolk body. No further information on the structure of the central region could be ascertained due to its electron transparency. However, it seems likely that the material of the central core is very hard, as this region often tore out of the mature yolk bodies when sections were cut with glass knives. This problem was solved by a more gradual embedding procedure.

Concomitant with the formation of an electron-transparent stellate core, the individual granules within the precursor yolk vesicles lose their outer membrane and fuse with one another to form a homogeneous

material (Plate 29). At this stage they become indistinguishable from the pinocytotic yolk vesicles. This process of fusion does not occur simultaneously throughout the contents of the precursor yolk vesicle. In some mature yolk vesicles, individual granules are seen randomly dispersed amongst the homogeneous contents. Mature yolk bodies may still fuse with smaller vesicles (Plates 29, and 40), but this process is not as common as in stage 6 of oogenesis. The mature yolk bodies retain their outer limiting membrane (Plate 40).

Vitelline membrane:-

During the latter part of stage 6, as mentioned above, microvilli project from the plasma membrane of the oocyte into the lumen of the ovariole (Plate 41). The microvilli measure approximately 760 mμ in length. Not all the microvilli project perpendicular to the plasma membrane, since most sections show the microvilli in cross section (Plate 41), suspended in the perivitelline space. The plasma membrane of the microvillus is more electron dense than the plasma membrane of the oocyte. This difference appears to be caused by local condensation of dense granular material on the outer membrane of the microvillus (Plate 41). Homogeneous material forms within the microvilli, and appears to be identical in electron density with the fibrous material being laid down between the microvilli (Plate 42). Presumably the fibrous material in the microvilli is passed in clumps into the perivitelline space (Plate 42).

As the oocyte matures, increasing quantities of the fibrous material are deposited in the perivitelline space. The fibrous material deposited by each microvillus fuses with that produced by

adjacent microvilli. In mature oocytes, a vitelline membrane, composed of a 2μ thick fibrous layer can be seen. This layer is interrupted by microvilli projecting from the plasma membrane through channels running into the fibrous material (Plates 43,44). The presence of the microvilli is a constant feature of the vitelline membrane of all mature oocytes. Although the connection between the plasma membrane and the microvilli is observed in most sections, a small perivitelline space, open to the channels around the microvilli, is always found between the plasma membrane and the fibrous material of the vitelline coat. The perivitelline space ranges in size from about 130mμ to 400mμ in mature oocytes (Plate 45). Extraoocytic material can still be seen being incorporated via micropinocytosis even in the ripe oocyte.

The completed vitelline membrane, 2μ thick forms a barrier to fixatives. Consequently, the contents of ripe oocytes are not well fixed. The cytoplasm of the ripe oocyte consists mostly of mature lipid and protein-carbohydrate yolk bodies. Small inactive Golgi are apparent in the peripheral regions of the cytoplasm of ripe oocytes, together with a few vesicles of the endoplasmic reticulum. The number of free ribosomes in the cytoplasm is greatly reduced during stage 7. Small mitochondria are evident, much reduced in size as compared with those found during the synthetic phases of vitellogenesis.

It was considered that the fixation of ripe oocytes was generally poor, and thus little ultrastructural information could be gleaned from the electron micrographs (Plates 1, 37 and 45).

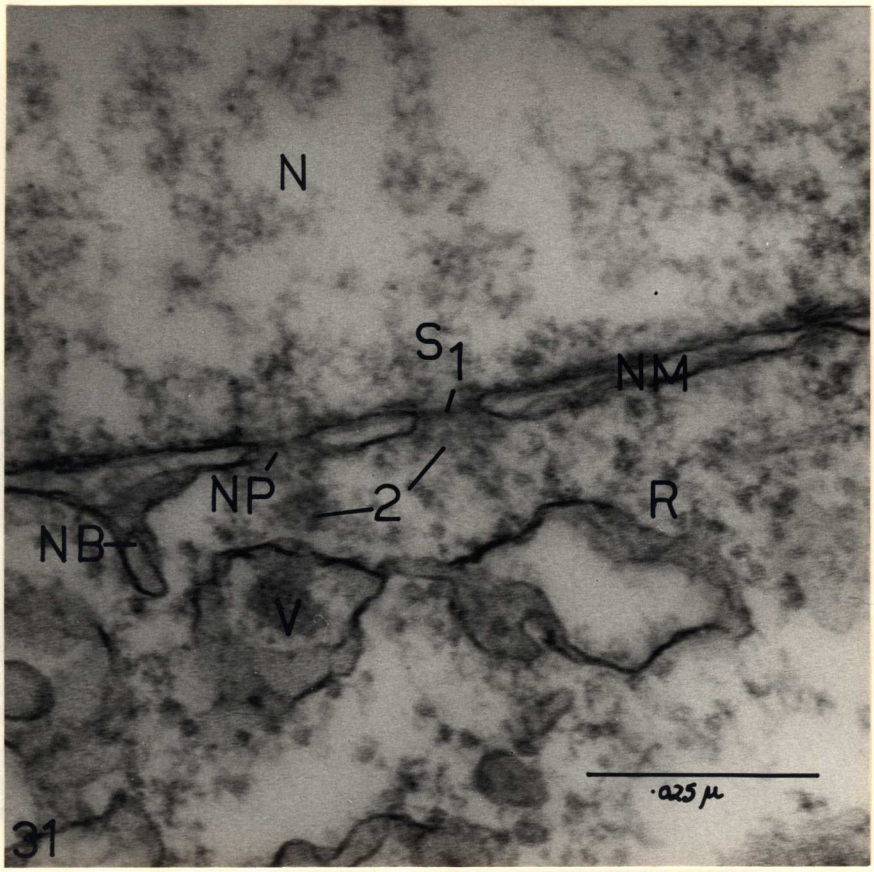


PLATE 31

High power study of the nuclear membrane (NM) of Plate 30. Nucleolar material can be seen passing through the nuclear pores (NP). The septun (S) is just visible in these pores (arrow 1). The extruded material (arrow 2) appears to disperse in the cytoplasm and is identical in size and electron density to the free ribosomes in the cytoplasm (R).

glutaraldehyde-osmium in phosphate buffer with 10% sucrose added.

Philips 200

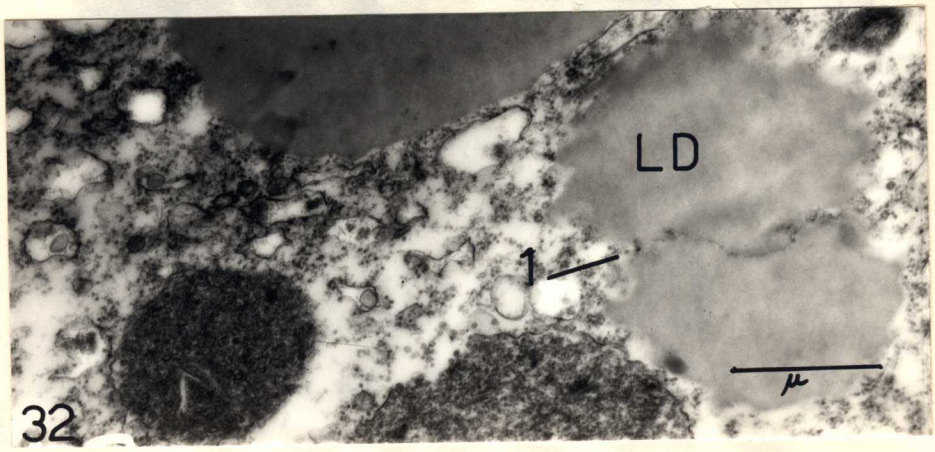
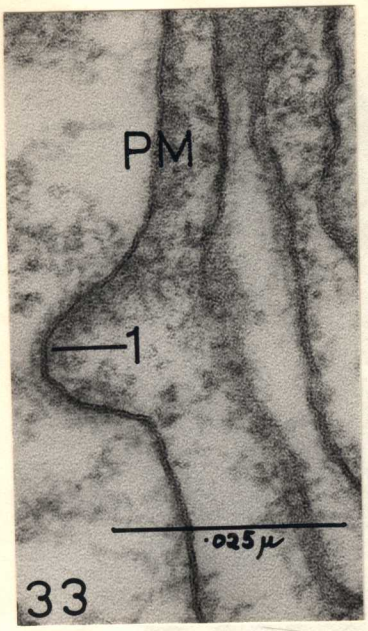


PLATE 32

Peripheral region of the cytoplasm of an oocyte early in stage 6 of oogenesis. Two small lipid droplets (LD) are apparently fusing (arrow 1) to form a larger lipid droplet.



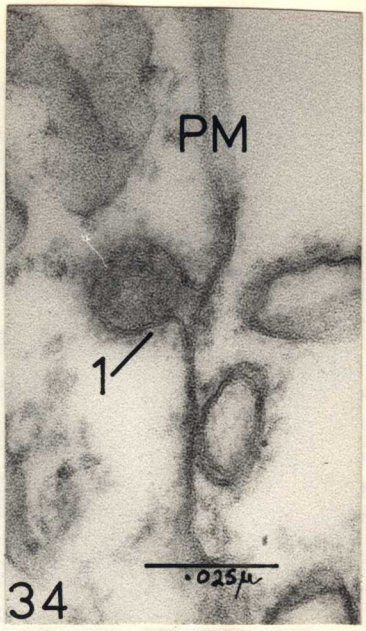


PLATE 33

High power view of the plasma membrane (PM) of an oocyte early in stage 6 of oogenesis. The invagination of the plasma membrane (arrow 1) indicates the commencement of a micropinocytotic pit. This is indicated by the local condensation of material on the outer surface of the plasma membrane. In addition, there is a deposition of fine material on the cytoplasmic surface of the micropinocytotic pit to form a bristle coat.

glutaraldehyde-osmium with 10% sucrose added. Philips 200

PLATE 34

High power view of the plasma membrane (PM) of an oocyte early in stage 6 of oogenesis. The micropinocytotic pit in this electron micrograph is about to pinch off from the plasma membrane (arrow 1). The micropinocytotic pit still retains its bristle coat.

glutaraldehyde-osmium in phosphate buffer with 10% sucrose added. Philips 200.

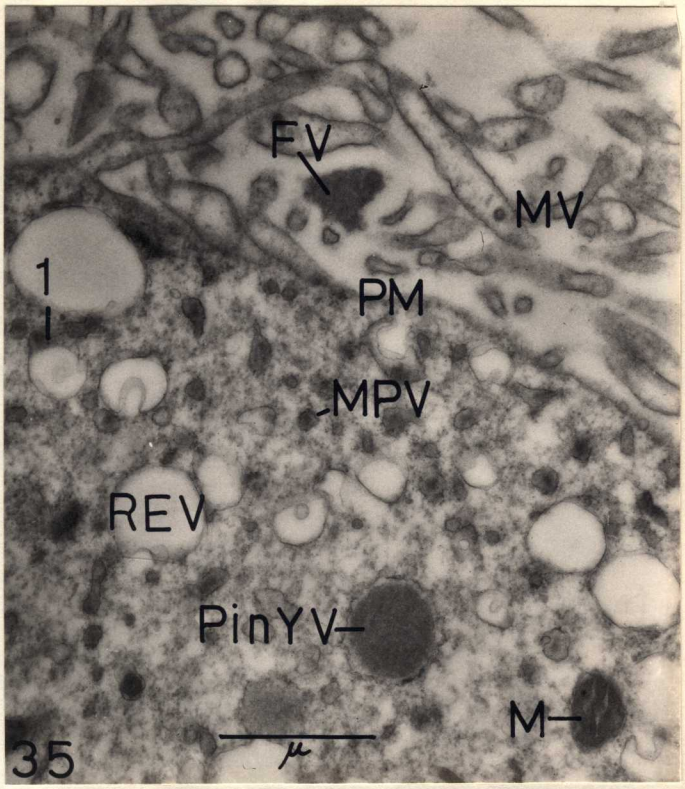


PLATE 35

Portion of the peripheral cytoplasm of an oocyte late in stage 6 of oogenesis. Immediately below the plasma membrane (PM) of this oocyte many micropinocytotic vesicles (MPV) are evident. Arrow 1 indicates the incorporation of a micropinocytotic vesicle into a vesicle of the rough endoplasmic reticulum (REV). The outer layer of the oocyte is enclosed in dense layers of microvilli, (MV) which are laying down fibrous material (FV) which forms the vitelline membrane.

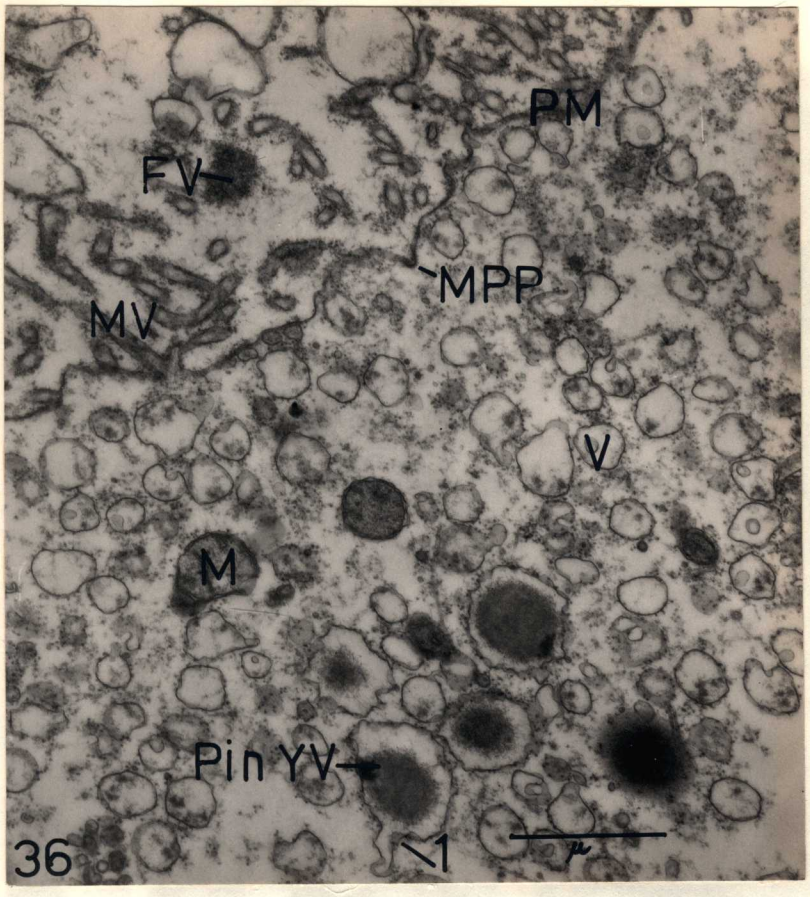


PLATE 36

Portion of the peripheral region of an oocyte in the middle of stage 6 of oogenesis. Pinocytotic yolk vesicles (Pin YV) arrow 1 are seen to fuse with the small vesicles of the rough endoplasmic reticulum (REV). Ribosomes are attached to the cytoplasmic surface of the pinocytotic yolk vesicles.

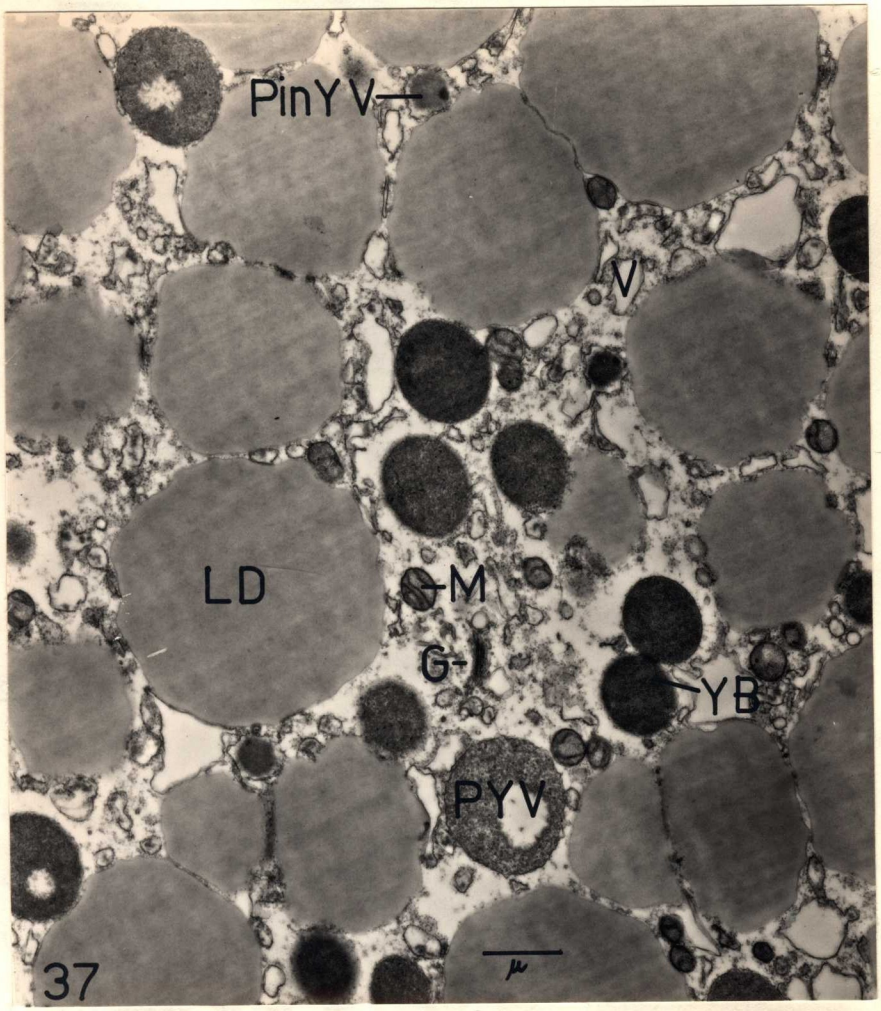


PLATE 37

Portion of the cytoplasm of an oocyte at the onset of stage 7 of oogenesis. Many large lipid droplets (LD) are prominent in the cytoplasm. Precursor yolk vesicles (PYV) and pinocytotic yolk vesicles (Pin YV) are evident, at various stages of maturation, in the cytoplasm. The cytoplasm between the yolk inclusions is less packed with vesicles of endoplasmic reticulum than in earlier stages of vitellogenesis (stages 5 and 6 of oogenesis). Small mitochondria (M), Golgi (G), vesicles of endoplasmic (V) are also present.

Philips 200

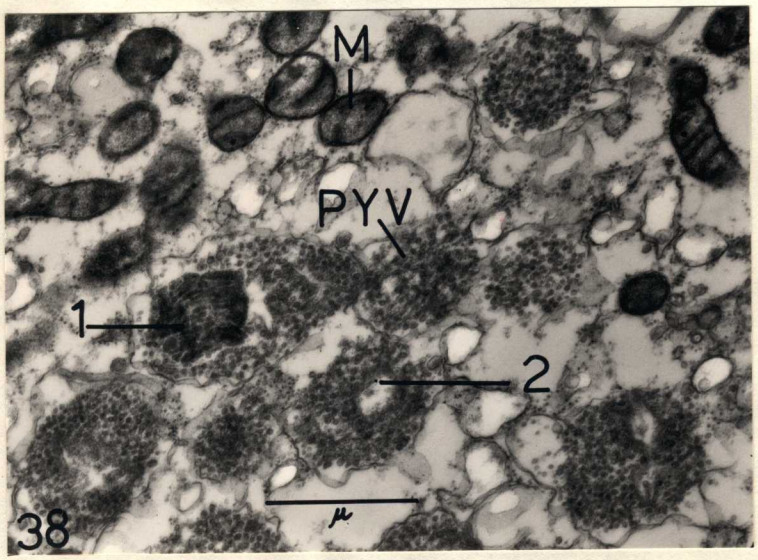


PLATE 38

Portion of the cytoplasm of an oocyte late in stage 6 of oogenesis. This section shows the early stages in the maturation of the precursor yolk vesicles (PYV). Arrow 1 shows the formation of "rod-like" structures which appear to form by fusion of the secretory granules after they have lost their outer membranes. Arrow 2 indicates an electron transparent area within another precursor yolk vesicle. In most instances, the electron transparent areas are stellate in shape.

glutaraldehyde-osmium in phosphate buffer with 4% NaCl added.

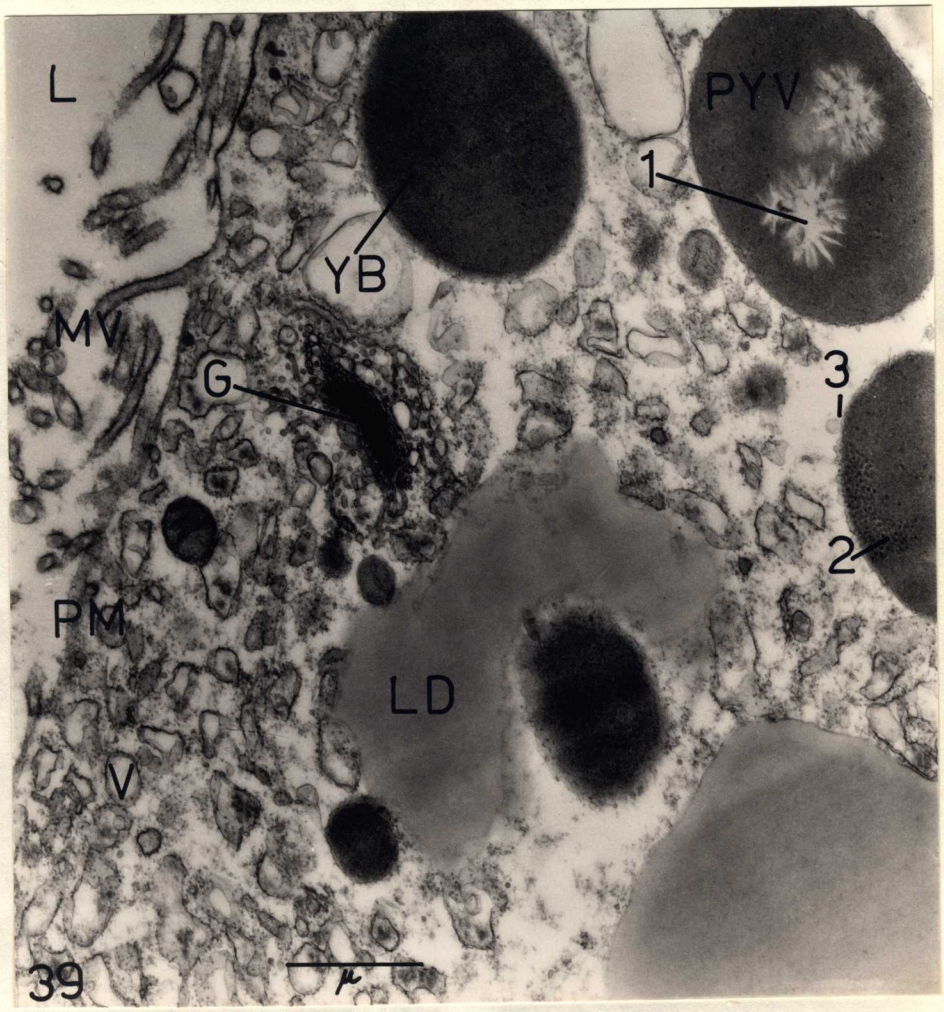


PLATE 39

Portion of the peripheral cytoplasm of an oocyte late in stage 6 of oogenesis. Mature precursor yolk vesicles (PYV) are prominent in the cytoplasm of the oocyte. Electron transparent areas (arrow 1), are in the form of a stellate structure. Small individual secretory granules (arrow 2) can still be seen amongst the fine granular material. These mature precursor yolk vesicles may still fuse with smaller precursor yolk vesicles (arrow 3), and grow further in size. Microvilli (MV) formed from the plasma membrane (PM) are being laid down in the lumen of the ovariole (L). A Golgi (G) is lying close to the plasma membrane (PM). Philips 200.

glutaraldehyde/osmium in phosphate buffer with 10% sucrose added.

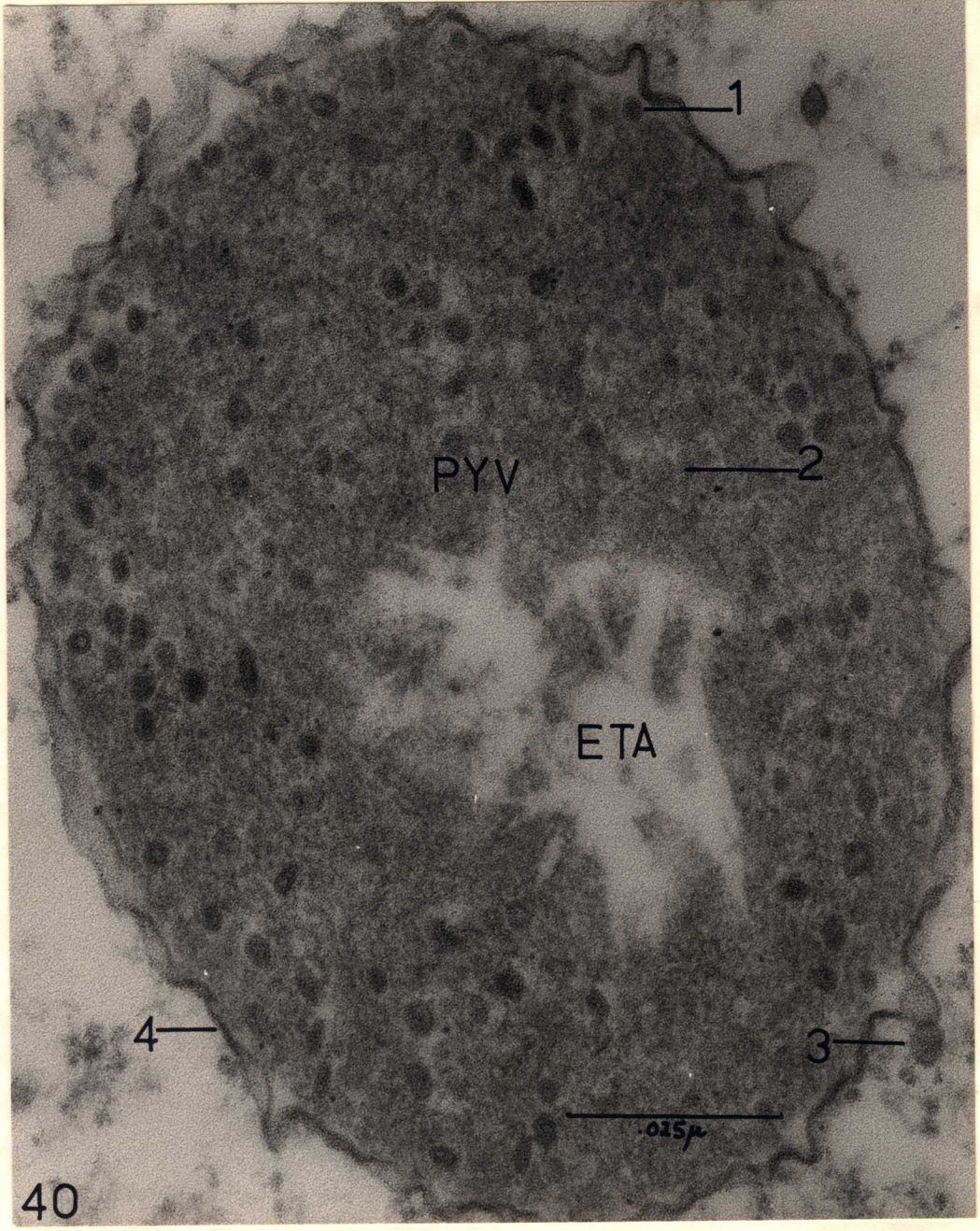


PLATE 40

High power view of a maturing precursor yolk vesicle (PYV). Electron transparent areas (ETA) are found in the central regions and are stellate in shape. The peripheral regions of the vesicle contain many individual membrane-bound secretory granules (arrow 1). As they move towards the central regions of the vesicle, they lose their outer membrane and fuse to form the finely granular central core (arrow 2). Arrow 3 indicates a secretory granule apparently about to be incorporated into the PYV. Arrow 4 indicates the outer limiting membrane of the PYV.

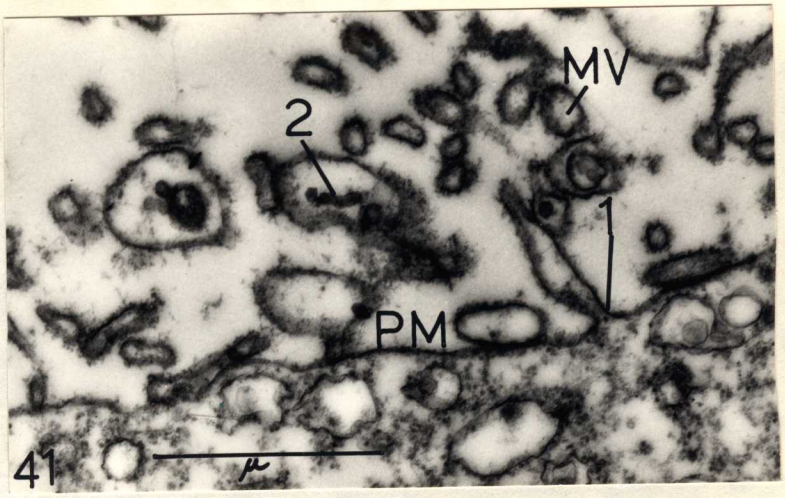


PLATE 41

Portion of the peripheral cytoplasm of an oocyte early in stage 6 of oogenesis. Microvilli (MV) are projected from the plasma membrane (PM), arrow 1. Fibrous material is conspicuous in the cavity of the microvillus, arrow 2, but as yet the fibrous material that forms the vitelline membrane has not been laid down by the microvilli.

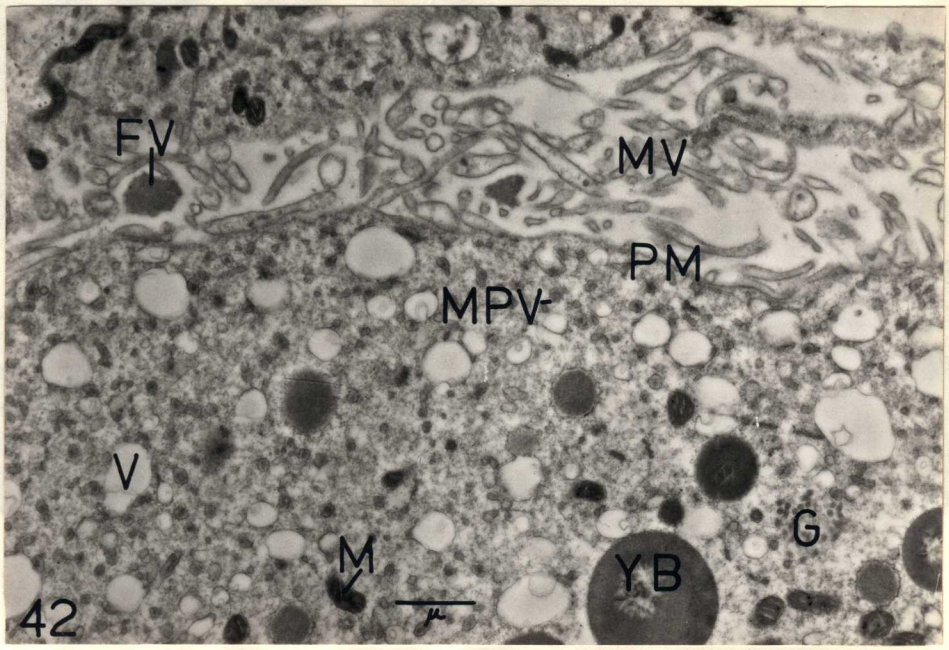


PLATE 42

Low power study of the peripheral portion of the oocyte at the onset of stage 7 of oogenesis. Many elongate microvilli (MV) are salient in the perivitelline space. Amongst these microvilli clumps of fibrous material (FV) presumably laid down by the microvilli are evident. Fusion of the clumps of fibrous material will eventually form the vitelline membrane. Many micropinocytotic vesicles, (MPV), are evident in the peripheral cytoplasm. Mature protein-carbohydrate yolk bodies (YB) are seen in the cytoplasm.

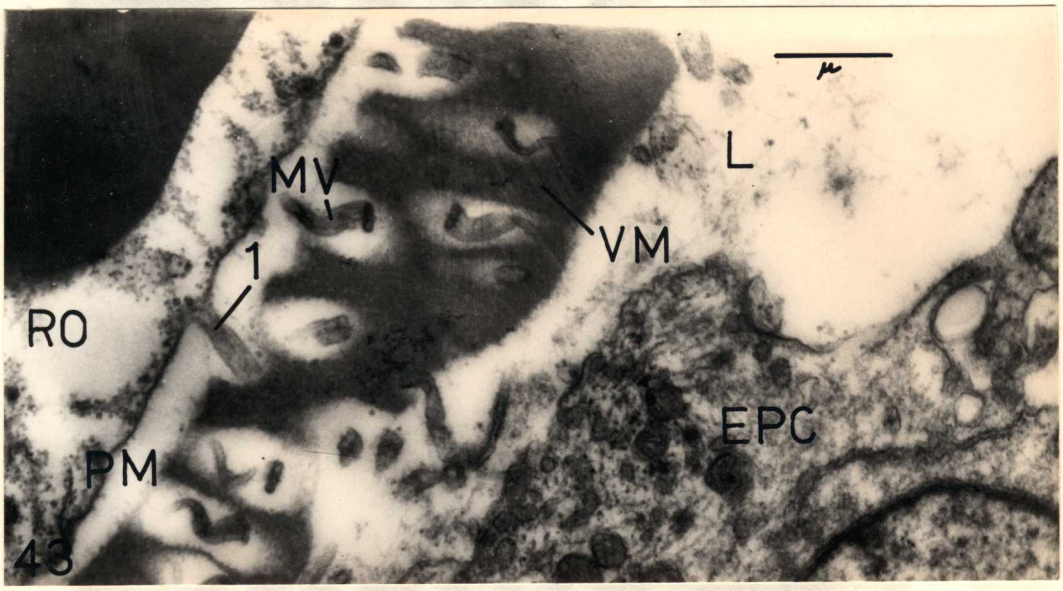


PLATE 43

Low power view of the completed vitelline coat (VM) which surrounds a ripe oocyte (RO). A microvillus, arrow 1, showing its continuation with the plasma membrane (PM) can be seen extending into the vitelline membrane. Other microvilli (MV) are lying in wide channels in the vitelline membrane.

glutaraldehyde-osmium in sea water.

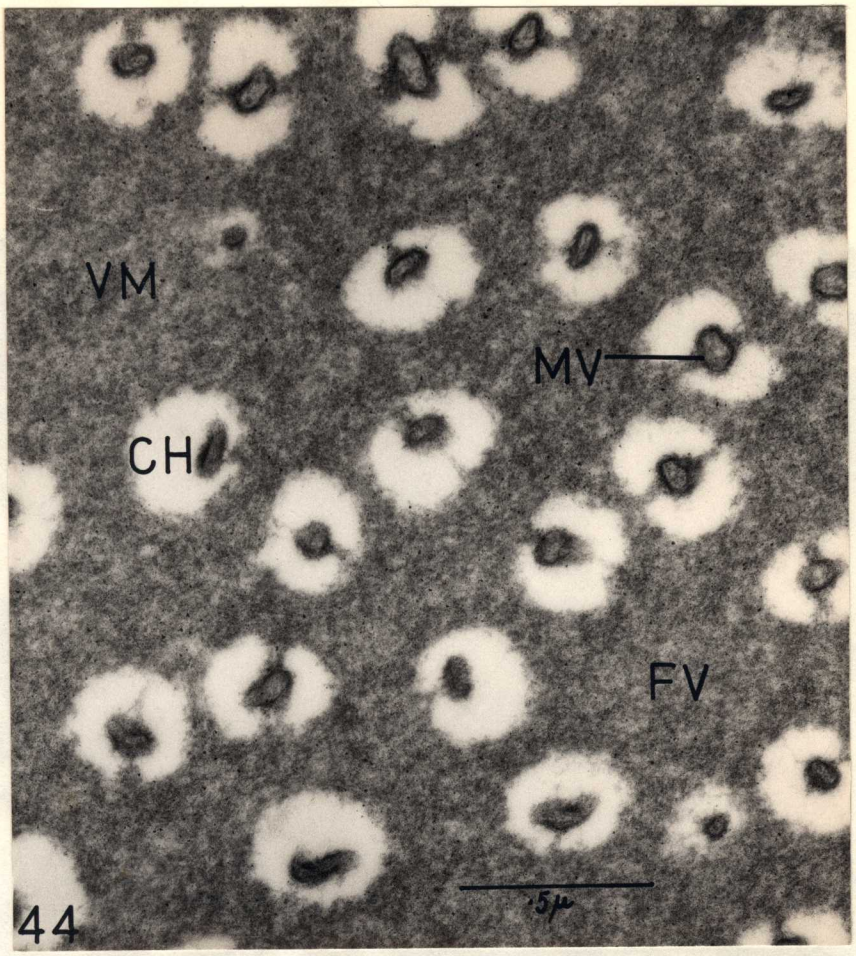


PLATE 44

A tangential section of the vitelline membrane (VM) of a ripe oocyte (stage 7 of oogenesis). The microvilli (MV) lie in the wide electron transparent channels (CH) which perforate the vitelline membrane. The vitelline membrane is composed of dense fibrous material (FV).

Philips 200.

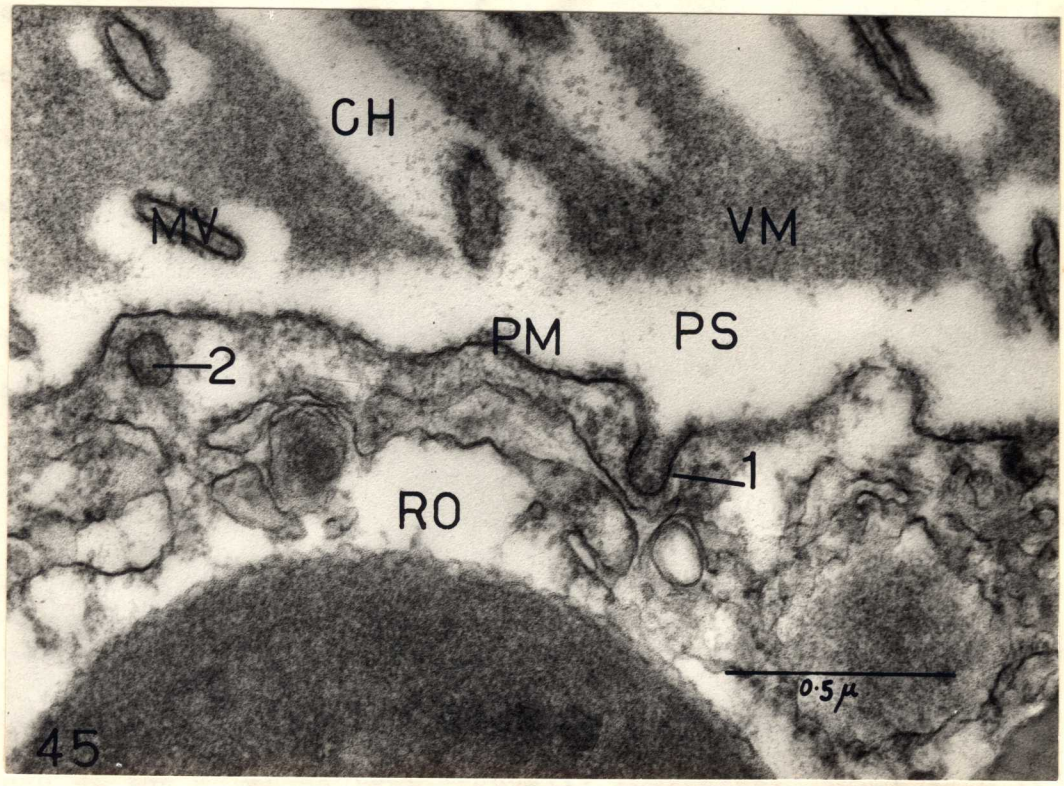


PLATE 45

Section of the plasma membrane (PM) and vitelline membrane (VM) of a ripe oocyte (RO) still undergoing micropinocytosis. Arrow 1 indicates a micropinocytotic pit formed at the plasma membrane. Arrow 2 indicates a micropinocytotic vesicle free in the cytoplasm. The continuity between the plasma membrane and the lumen of the ovariole can be seen via the perivitelline space (PS) and the channels (CH) in the vitelline membrane.

Philips 200.

RESULTS

5. BIOCHEMICAL COMPOSITION OF EMBRYOS OF I. QUADRIVALVIS

Histochemical and ultrastructural results have shown that the cytoplasm of the ripe oocyte of I. quadrivalvis is full of yolk. The yolk components consist of lipid droplets, composed chiefly of neutral triglycerides containing a quantity of unsaturated fatty acids, and protein-carbohydrate yolk bodies. In the following section, the total content of the following biochemical constituents, water, carbohydrate, glycogen, protein and lipid were estimated at each stage of embryonic development of I. quadrivalvis in order to gain an insight into yolk utilization.

(a) Changes in Total Water Content:-

There is a total net increase in wet weight of 120% throughout the development of the embryos from cleavage (stage 2) to hatching (stage 12)(Table 6). The wet weight of the embryos remains constant until gastrulation is completed (stage 4-6). From stage 4-6 to stage 9, the water content gradually increases. There is a total increase of 80% from cleavage to the completion of the limb-bud development at stage 10. The wet weight of the embryos then increases gradually again from stage 10 until hatching.

The dry weight does not show any total change from cleavage (stage 2-3) to hatching (stage 12), and this remains constant throughout the development of the embryos (Table 6).

The total water content during the development of the embryos is given in Table 6 and Figure 15. As expected from the measurements of wet weight and dry weight, there is a 215% increase in the total water content during development (Table 6). The water content

remains stable from cleavage until the end of gastrulation (stages 2-6). At the commencement of limb-bud formation (stages 7-8), the water content increases by 40%. This is followed by a further increase of 40% at stage 9. The water content remains stable from stage 9 to stage 11 (Table 6), and increases by a further 30% as the embryos approach hatching. The increase in water content at stage 11 occurs simultaneously with the increase in the volume of the developing haemocoel and the midgut cavities, as illustrated in Figure 16.

(b) Changes in Total Carbohydrates:-

The total carbohydrate content during development is given in Table 7 and Figure 17. There is a loss of 20% in total measurable carbohydrates during the development of the embryos. The carbohydrate content does not change significantly until the embryos reach stage 9. At this stage there is a net loss of 30% in total carbohydrates. From stage 9 to hatching at stage 12, the measurable carbohydrate content initially increases gradually and then remains more or less constant. Chitin is laid down at stage 10-12. The carbohydrate content of chitin is not measured by the method used. It is probable that free glucose would be used in the synthesis of chitin, so that the total increase in carbohydrates must be greater than that measured (Table 7).

(c) Changes in Total Glycogen:-

The total glycogen content during development is given in Table 7 and Figure 18. In contrast to the decrease in total carbohydrates, there is no significant net change in the total glycogen content from stage 1 to stage 12. The glycogen content

also remains constant at each stage throughout the development of the embryos, from freshly laid ovum (stage 1) to hatching (stage 12).

(d) Changes in Total Protein:-

The Folin-Coicalteu phenol-determining reagent used to determine protein in the Lowry method has several disadvantages which are summarized by Lowry et al (1951). The colour developed depends upon the quantity of tyrosine and tryptophan residues in the protein, and the Lowry determination rests on a presumed similarity in the level of these amino acids in the unknown and in the standard (Giese, 1967). However, it is an acceptable method for measuring mixed protein, particularly when absolute values are not required (Lowry, 1951). Only significant changes in the total content of protein are required in the present study, and these can be adequately detected by the Lowry method.

The total protein content during development is given in Table 7 and Figure 19. There is a net increase in total protein during development of about 70%. Total protein content remains stable until gastrulation is completed. There is then a 50% increase in total protein content between gastrulation (stage 4-6) and the development of limb-bud rudiments (stages 7-8). From cleavage (stage 2-3) to the formation of limb-buds at stage 7-8, there is a net increase of 100% in total protein content. As the embryos enter the more prolonged stage 9 (Table 1) there is a 100% decrease in the total protein content (Table 7). The protein content remains constant during stage 9 and stage 10, but increases again by 70% at stage 11, and remains more or less constant until hatching (stage 12).

(e) Changes in Total Lipid:-

The total lipid content during development is given in Table 7 and Figure 20. There is no significant net change in total lipid content during development. The total lipid content also remains constant at each stage throughout the development of the embryos from cleavage (stage 2-3) to hatching (stage 12)(Table 7).

The comparison of weights of constituents per 10^6 eggs, given in Table 6 and 7, shows that the total lipid content in the embryos at any stage is slightly greater than the amount of total protein; both the latter components greatly exceed the amounts of carbohydrates, and glycogen found in the embryos at any stage of development.

(f) Results of the Pigment Analysis:-

During the embryonic development of the eggs of Ibla quadrivalvis the pigment of the eggs and ovarian tissue changes gradually from a creamy yellow to the dark brown colour visible in the hatching embryos. This process first becomes evident at stage 11, when the pigment as seen in reflected light slowly becomes brown. The pigment extracted from stage 0 (ripe ovary) and stage 12 (mature embryos) has a similar bright yellow colour. The results of the absorption spectra are shown on Figure 21. The composition of the pigment from the ripe ovary and the mature embryo remains constant, and little pigment is utilized during development. Thus the change in the colour of the pigment that is observed during embryogenesis is caused by other factors, such as changes in tissue transparency. Maximum absorption bands are obtained at approximately 460 mμ, in both stages, indicating the presence of the pigment astaxanthin.

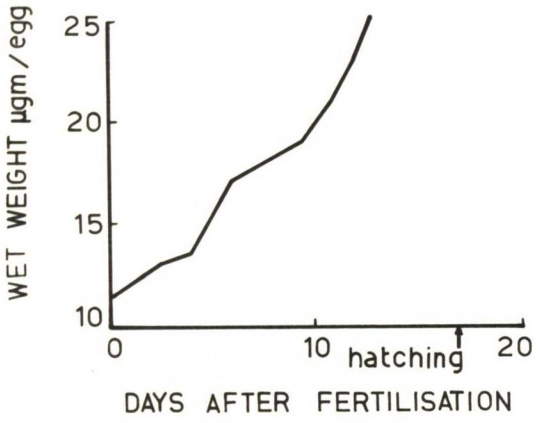
TABLE 6: Water Content of embryos of I. quadrivalvis

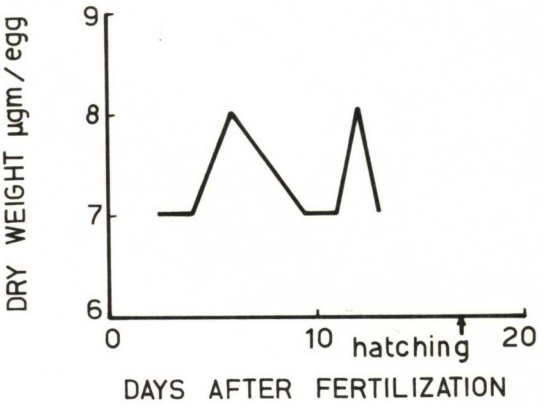
Stage	Wet Weight ugm/egg	Dry Weight ugm/egg	Total water content $x10^2$mgms/10^6 eggs
1	11.5	-	-
2-3	13	7	58
4-6	13.5	7	64
7-8	17	8	89
9	19	7	125
10	21	7	140
11	23	8	148
12	25	7	182

TABLE 7: Total Carbohydrate, Glycogen, Protein and Lipid content in embryos of Ibla quadrivalvis

Stage	Total Carbohydrate $x10^2$mgms/10^6 eggs	Total Glycogen content $x10^2$mgms/10^6 eggs	Total Protein content $x10^2$mgms/10^6 eggs	Total Lipid content $x10^2$mgms/10^6 eggs
1	-	.8	-	-
2-3	2.7	.8	31	75
4-6	2.7	.8	41	72
7-8	2.5	.8	60	68
9	2.1	.8	30	79
10	2.2	.95	33	81
11	2.2	.9	54	77
12	2.3	.9	52	68

Figure 15 TOTAL WATER CONTENT IN EMBRYOS OF I. quadrivalvis





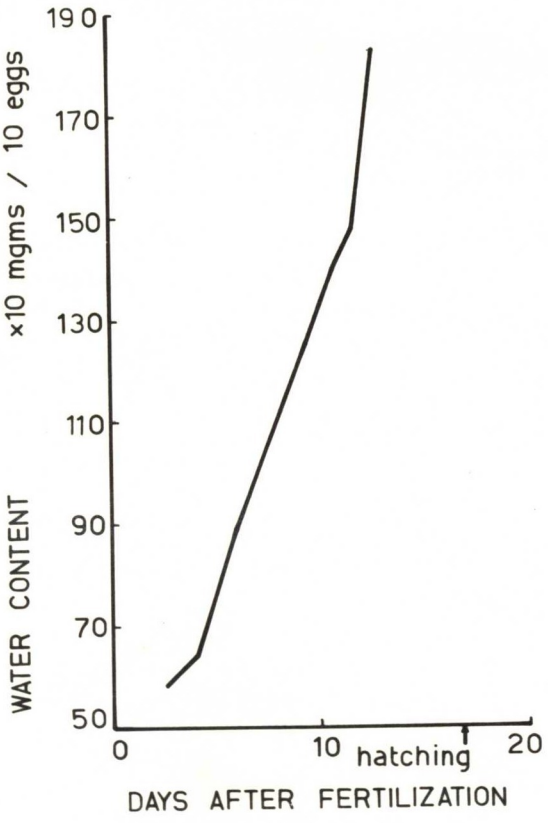


Figure 16

(a) LONGITUDINAL SECTION OF STAGE 9 EMBRYO OF I. quadrivalvis

(b) LONGITUDINAL SECTION OF STAGE 11 EMBRYO OF I. quadrivalvis

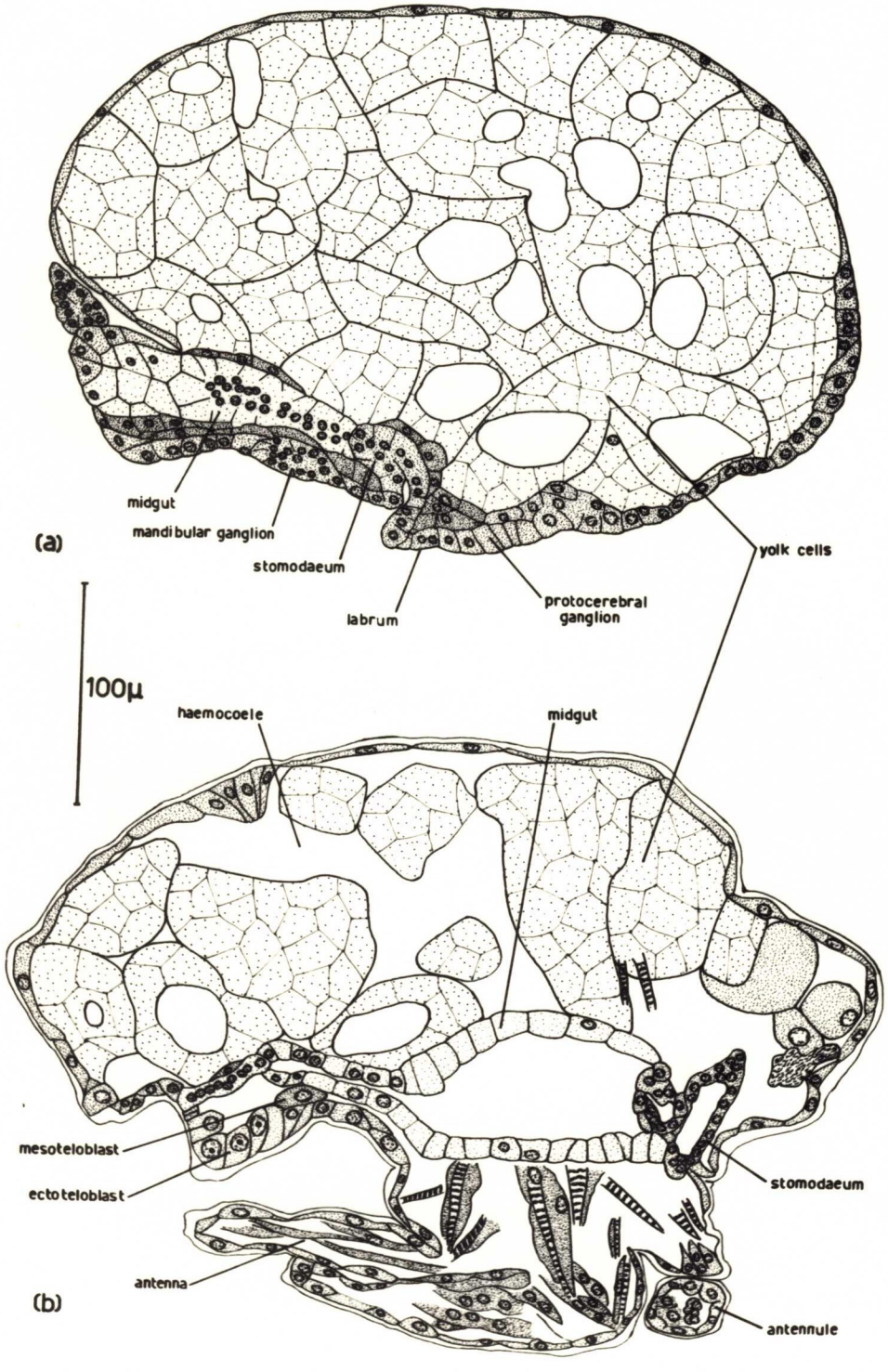


Figure 17 CHANGES IN TOTAL CARBOHYDRATE CONTENT IN EMBRYOS OF I. quadrivalvis

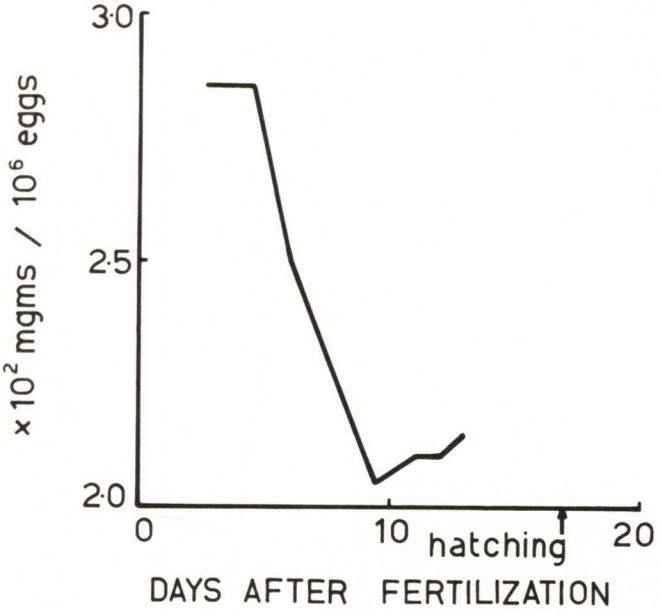


Figure 18 CHANGES IN TOTAL GLYCOGEN CONTENT IN EMBRYOS OF I. quadrivalvis

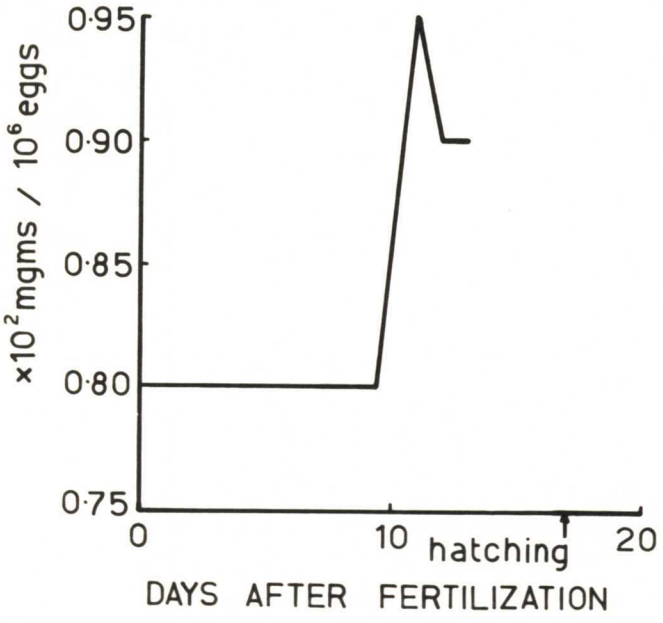


Figure 19 CHANGES IN TOTAL PROTEIN CONTENT IN EMBRYOS OF I. quadrivalvis

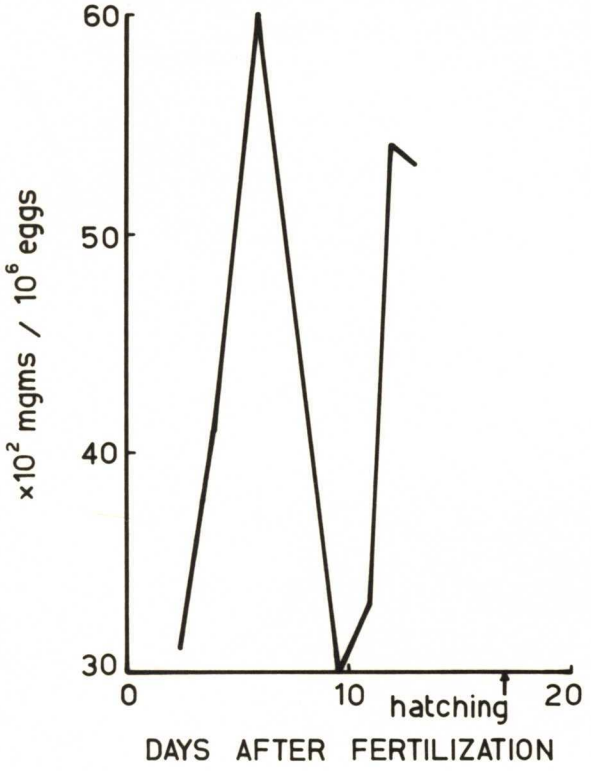


Figure 20 CHANGES IN TOTAL LIPID CONTENT IN EMBRYOS OF I. quadrivalvis

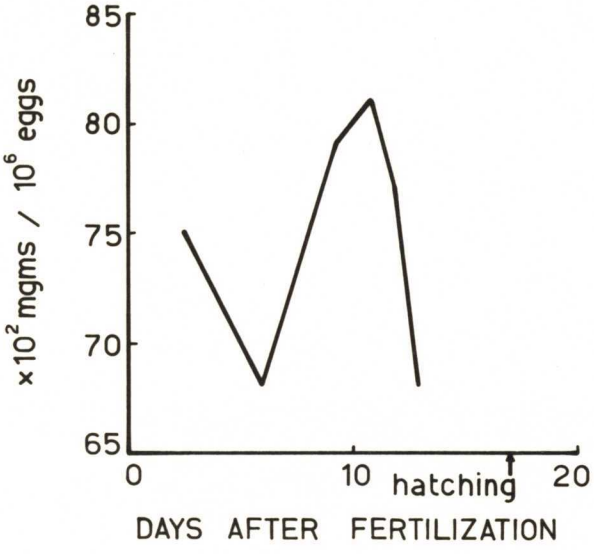
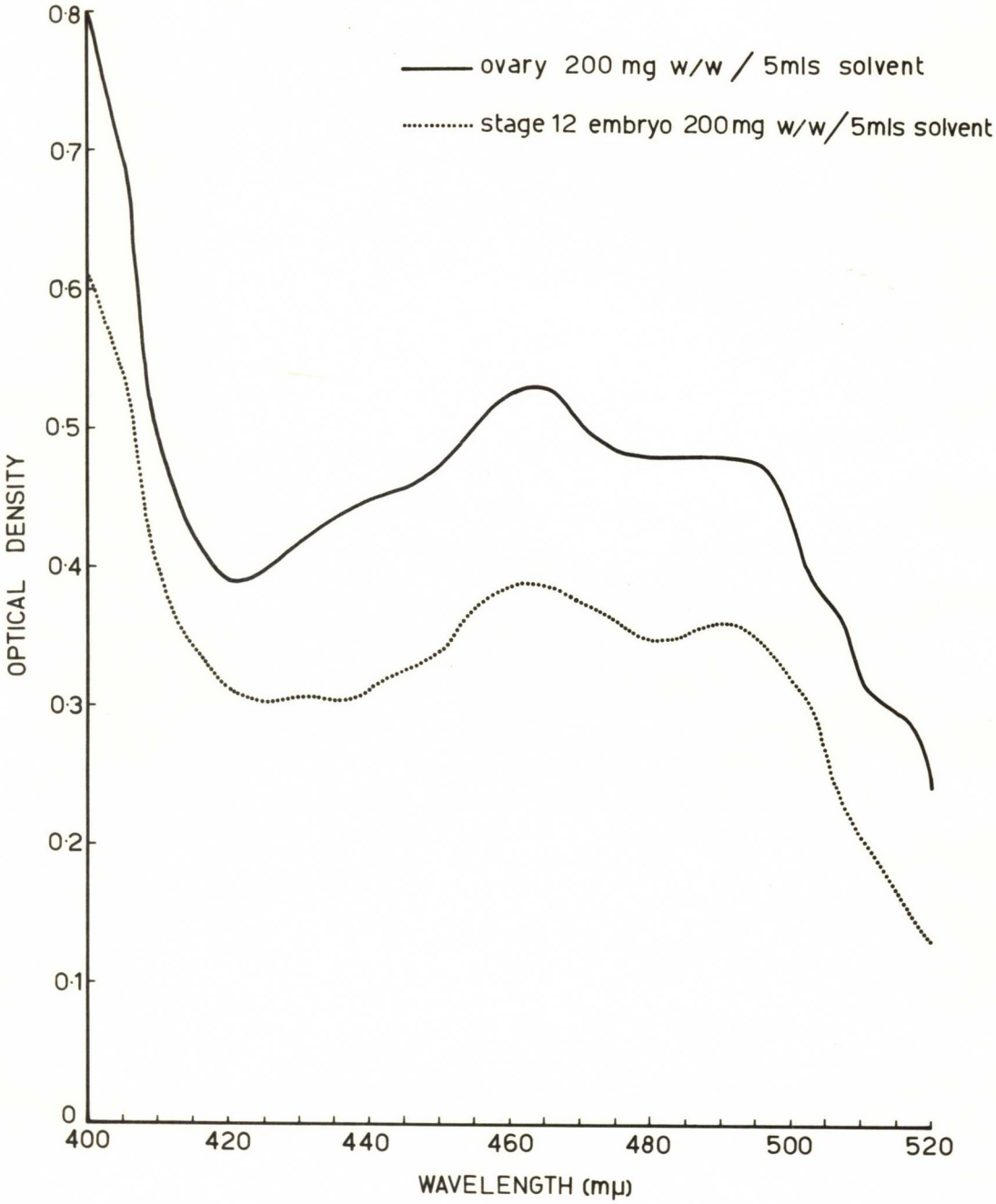


Figure 21 RELATIVE ABSORPTION CURVES OF RIPE OVARY AND MATURE EMBRYOS OF I. quadrivalvis IN CHLOROFORM : METHANOL (2:1) SOLVENT



cont. from page 60.

However, there is a stronger absorption band in the spectral range below 400 mμ, continued into the ultraviolet, which suggests the presence of a chromolipid.

The results of the chemical tests used to assist in characterizing the pigment extract are summarized below. When aliquots of pigment samples were added to a small quantity of concentrated H_2SO_4, the pigment became unstable and turned dark red in a few minutes. The pigment was stable in dilute mineral acids and in some oxidising agents such as iodine, but reacted with hydrogen peroxide, the latter turning the pigment from yellow to blue.

These results suggest that the pigment extracted is a mixture of at least astaxanthin and chromolipids, the latter appearing to be the more predominant pigment in the mixture. The astaxanthin is probably linked non-covalently with a protein moiety, and the chromolipid is dissolved in the lipid fraction.

DISCUSSION

1. The Ovary

The paired ovaries of I. quadrivalvis and T. rosea are hollow tubular organs lying in the haemocoel of the peduncle and branching into many hollow ovarioles. In this respect the ovaries of these two cirripedes are similar to those of insects (Raven, 1961). In other Crustaceans, particularly the Branchiopoda, Copepoda and the Malacostraca (King, 1948; Fautrez-Firlefyn, 1951; Longhurst, 1955; Linder, 1959; Cummings, 1961; Beams and Kessel, 1963; Kessel and Beams, 1963a; Park, 1966; Kessel, 1968a; Hinsch and Cone, 1969), the paired ovaries are usually hollow tubular organs with out-pocketings, if they occur, in one restricted area only.

The ovaries of I. quadrivalvis and T. rosea are embedded in connective tissue, as are the ovaries of the Branchiopoda and Malacostraca. Unlike the other crustaceans studied, however, strands of muscle are found associated with the connective tissue surrounding the cirripede ovary.

Blood vessels ramify throughout the connective tissue of the ovaries of the lobsters Homarus and Panulirus (Kessel, 1968a). **Blood vessels were not found in the connective tissue surrounding the ovary of either I. quadrivalvis or T. rosea.** Although the Cirripedia do not possess a true heart, Cannon (1947) postulated the presence of a blood pump which forces blood around the body of the pedunculate barnacle Lithotrya. This circulation presumably bathes the surface of the ovaries directly. Contraction of the longitudinal muscles of the peduncle provides an accessory pumping mechanism to circulate blood throughout the pedunculate haemocoel.

A similar system exists in most cirripedes (Cannon, 1947), and means that the ovaries of I. quadrivalvis and T. rosea are bathed in a constantly renewed haemolymph.

The ovary wall in both I. quadrivalvis and T. rosea is lined internally with unspecialized epithelial cells and externally by a thick acellular wall. Any of the epithelial cells of the ovary wall may give rise to germinal epithelial cells. The ovaries of the Branchiopoda, Copepoda and Malacostraca, in contrast, have a restricted area, the zone of proliferation, from which the germinal epithelial cells arise (King, 1948; Fautrez-Firelfyn, 1951; Linder, 1959; Park, 1966).

2. Oogenesis in I. quadrivalvis and T. rosea

In spite of the structural peculiarities of the cirripede ovary, oogenesis in I. quadrivalvis and T. rosea follows the general crustacean pattern (King, 1926; Kater, 1928; Harvey, 1929; Hilton, 1931; Witschi, 1935; Fautrez-Firlefyn, 1951; Hilgard, 1959; Linder, 1959; Beams and Kessel, 1963; Kessel and Beams, 1963a; Park, 1966; Cassidy and Beauregard, 1967; Kessel, 1968a; Hinsch and Cone, 1969). It is, however, simpler that the oogenesis of the Malacostraca and lacks the follicle and nurse cell complications of the Branchiopoda.

Oogenesis in both I. quadrivalvis and T. rosea can be divided, on basis of histological and histochemical evidence, into seven stages. The significance of this staging is emphasized more clearly in ultrastructural studies of oogenesis in I. quadrivalvis, as each stage represents a distinct phase of oocyte activity which directly affects the subsequent stage. As no follicle cells or nurse cells are associated with the oocytes in either I. quadrivalvis and T. rosea,

the oocyte itself is the source of the bulk of the material synthesized in previtellogenesis and vitellogenesis, and develops an elaborate synthesizing system in the cytoplasm and nucleus.

Oogenesis is similar in I. quadrivalvis and T. rosea, but I. quadrivalvis produces a much larger egg. The size difference is entirely a result of increased yolk content, and is reflected in subsequent development. The larvae of I. quadrivalvis do not feed after hatching (Anderson, 1965), whereas the planktonic larvae of T. rosea begin to feed after hatching, in the usual cirripede manner.

3. Ultrastructural Changes during oogenesis.

(a) The germinal epithelial cells (stage 1)

No previous ultrastructural studies have been made on germinal epithelial cells in crustaceans. Furthermore, in the many studies of crustacean oogenesis, only Harvey (1929) has described the histochemistry of the germinal epithelial cells. Harvey (1929) considered that the germinal epithelial cells of the lobster Carcinus maenas are in the form of a syncitium. Distinct cell boundaries can be seen in the germinal epithelial cells in I. quadrivalvis and T. rosea even with the light microscope.

The nucleus of the germinal epithelial cell in I. quadrivalvis and T. rosea is rich in DNA and basic protein, as in Carcinus maenas and other invertebrate oocytes (Raven, 1961). Electron micrographs reveal dense clumps of heterochromatin dispersed throughout the nucleus. The same material is disposed as a layer around the nuclear membrane. In Carcinus maenas, a nucleolus is located in the nucleus of germinal

epithelial cells (Harvey, 1929). A nucleolus is lacking in the nucleus of germinal epithelial cells in both I. quadrivalvis and R. rosea.

Histochemical studies reveal that the cytoplasm of the germinal epithelial cells is rich in RNA and basic proteins. The electron micrographs reveal that the cytoplasm is ultrastructurally unspecialized, consisting chiefly of many free ribosomes, vesicles of the endoplasmic reticulum and a few mitochondria. Some microtubules are present, but no Golgi complexes were detected. Ultrastructurally, the germinal epithelial cells are similar to those found in other invertebrate groups (Raven, 1961). It can reasonably be assumed that the cells are mainly involved in the rapid synthesis of DNA, as they are in the sea urchin Strongylocentrotus purpuratus (Holland and Giese, 1965).

The general epithelial cells which line the ovary wall are similar in their ultrastructure to those which form the clusters of germinal epithelial cells. However some epithelial cells have complex membranous structures in the cytoplasm, identical to those observed by Beams and Kessel, (1963) in the cytoplasm of crayfish oocytes. The complex membranous structures also resemble the residual bodies and myelin figures observed in somatic cells (Novikoff, Essner and Quinatana, 1964). The continuity observed between the vesicle of the smooth endoplasmic reticulum and the outer limiting membrane of the complex membranous structure is similar to that observed in somatic cells (Goldfischer et al, 1966). Goldfischer et al (1966) reported that serial sections of hepatocytes suggest that the smooth

endoplasmic reticulum, by enveloping an area of the cytoplasm, forms an autophage vacuole, a type of lysosome. The epithelial cells containing complex membranous structures in I. quadrivalvis are involved in the resorption of ovarian tissue.

(b) Oogonia, stages 2 and 3

No previous electron microscope studies have been made of oogonia in crustaceans, although extensive histological and histochemical studies have been carried out on oogonia in the Branchiopoda.

The oogonial nucleus in both I. quadrivalvis and T. rosea is similar to that of Artemia salina (Fautrez-Firlefyn, 1951) and other animals (Raven, 1961), in that it is mitotically active and has a high DNA content. A nucleolus was not detected, in contrast to oogonia of Artemia salina, which possess a nucleolus during the mitotic divisions, but lose this structure at the onset of meiotic prophase (Fautrez-Firelfyn, 1951).

The thin layer of cytoplasm surrounding the nucleus appears hyaline at the light microscope level and has a low RNA content. Electron micrographs reveal that the cytoplasm is more densely packed with free ribosomes than in the germinal epithelial cells. A few lamellae of rough endoplasmic reticulum are dispersed amongst the ribosomes. Several typical mitochondria are found in the cytoplasm. Fautrez-Firlefyn (1951), using histochemical methods found many granules of RNA in the cytoplasm of the oogonia of Artemia salina while a nucleolus was present in the nucleus. The concentration of cytoplasmic RNA then became reduced when the nucleolus disappeared. The lack of a nucleolus in the oogonia of I. quadrivalvis and T. rosea may account for the reduced concentration of cytoplasmic RNA. The

nucleolus is the major site of RNA synthesis in a wide variety of cell types, including developing oocytes (Raven, 1961). Further evidence on the synthetic role of nucleoli is given by Brown (1965) who demonstrated that the disappearance of nucleoli in the oocytes of Xenopus laevis during meiosis occurred simultaneously with the cessation of ribosomal RNA synthesis.

As in the germinal epithelial cells, the main activity of oogonia appears to be DNA synthesis. In autoradiographic investigations on the ovary of Strongylocentrotus purpuratus, Holland and Giese (1965) demonstrated that premeiotic oogonia show intense DNA synthesis. Once the oocyte enters premeiotic prophase no DNA synthesis can be detected.

(c) Previtellogenesis (stage 4).

The previtellogenetic oocytes of I. quadrivalvis and T. rosea are similar histologically to those described for other crustaceans (Fautrez-Firlefyn, 1951;Linder, 1959; Beams and Kessel, 1963; Kessel and Beams, 1963a; Park, 1966; Hinsch and Cone, 1969), and other invertebrates (Raven, 1961).

Previtellogenesis is characterized by an increase in nuclear size, the formation of a nucleolus and the commencement of its synthetic activity, and an increase in the volume of the cytoplasm (Raven, 1961; Beams, 1964). As in the previtellogenetic oocytes of other crustaceans (Beams and Kessel, 1963; Kessel and Beams, 1963a; Anteunis et al 1966a,b,c; Kessel, 1968a; Hinsch and Cone, 1969) the electron micrographs reveal the main activity of oocytes of

I. quadrivalvis at this stage is the formation of cytoplasmic constituents, particularly a membrane system (endoplasmic reticulum, Golgi bodies) that will function in yolk synthesis during vitellogenesis.

1. The nucleus (germinal vesicle).

The ultrastructure of the nucleus in the previtellogenetic oocyte of I. quadrivalvis is similar to that of Artemia salina (Anteunis, et al, 1966a,b,c), Cambarus and Orconectes (Beams and Kessel, 1963), Homarus (Kessel and Beams, 1963a), and Libinia emarginata (Hinsch and Cone, 1969). As the nucleus grows to form a germinal vesicle, the heterochromatin clumps become dispersed and the nucleoplasm is composed of finely granular euchromatin.

The formation of diffuse chromosomes corresponds to a reduction in DNA stainability and a probable cessation of DNA synthesis. Holland and Giese (1965) found no evidence of DNA synthesis in oocytes after the premeiotic stage in Strongylocentrotus purpuratus. Lampbrush chromosomes of many animals, as shown by autoradiographic experiments in the amphibian oocytes (Williams, 1965) do not synthesize DNA.

The nuclear envelope of the previtellogenetic oocyte of I. quadrivalvis is a double-layered porous structure, similar to that found in other crustaceans (Beams and Kessel, 1963; Kessel, 1968a; Hinsch and Cone, 1969), and other invertebrate oocytes (Raven, 1961), and in somatic cells (Fawcett, 1966). The nuclear pores are retained throughout oogenesis. As in other crustacean oocytes (Beams, 1964; Hinsch and Cone, 1969), and in somatic cells (Fawcett, 1966) the nuclear pores in the oocyte of I. quadrivalvis are closed by a septum which extends completely across the pore. Hinsch and Cone (1969)

found evidence of nuclear material passing through the nuclear pores in previtellogenetic oocytes of Libinia emarginata. Although extrusion of nuclear material through the nuclear membrane is common in many oocytes (Raven, 1961) it was not observed during previtellogenetis in I. quadrivalvis.

2. The nucleolus

A single nucleolus develops in the nucleus of the previtellogenetic oocyte of I. quadrivalvis and T. rosea as in the branchiopod Chirocephalopsis bundyi. More than one nucleolus, in contrast, is found in oocytes of Cambarus and Palaemon (Raven, 1961), Carcinus maenas (Harvey, 1929), Artemia salina Fautrez-Firlefyn, 1951; Anteunis et al, 1966 a,b,c; Cassidy and Beauregard, 1967), crayfish (Beams and Kessel, 1963) and calanoid copepod Epilabidocera amphitrites (Park, 1966). There is a typical high RNA content in the nucleoli of the previtellogenetic oocytes of I. quadrivalvis and T. rosea, as in all oocytes examined (Raven, 1961). Histochemical studies indicate that the bulk of the RNA in the nucleus is localized within the nucleolus. It seems likely that the nucleolus of stage 4 oocytes of I. quadrivalvis and T. rosea is the site of RNA synthesis. Allen (1967) has shown by autoradiographic investigations that the most rapid synthesis of RNA in oocytes of the syllid Autolyus edwarsi occurs in the nucleolus, from which the RNA migrates to the rest of the nucleus and then to the cytoplasm.

Once the nucleolus is established in I. quadrivalvis and T. rosea, a nucleolar vacuole of reduced RNA content develops. The ultrastructure of the nucleolar vacuole is similar to that seen in Artemia salina (Anteunis et al, 1966a,b,c), Epilabidocera amphitrites

(Park, 1966), and the trematode Gorgoderina attenuata (Koulish, 1965). Vacuolation of the nucleolus appears to be a constant feature in the developing oocytes of many invertebrates (Raven, 1961). The vacuole persists throughout oogenesis until the oocyte is mature (stage 7). The pattern of nucleolar vacuolation in I. quadrivalvis and T. rosea is identical with that found in oocytes of sea urchins (Esper, 1965; Verhey and Moyer, 1967; Millonig et al, 1968).

Esper (1965) summarizes evidence that the vacuole in the nucleolus is inactive in RNA and protein synthesis. It appears that the nucleolar vacuole serves primarily as a means of increasing the surface area of the nucleolar cortex. Nucleolar surface area is related to cellular synthetic activity and the greater the surface area, the greater the amount of total cellular RNA (Esper, 1965). In oocytes of Xenopus laevis, the synthesis of ribosomal RNA correlates precisely with the presence and size of nucleoli. However, the synthesis of DNA-like RNA and other forms of RNA involved in protein synthesis is not related to the presence or size of the nucleoli (Brown, 1965).

The nucleolus is usually found adjacent to the nuclear membrane during previtellogenetis in I. quadrivalvis and T. rosea. Raven (1961) summarizes evidence that a single nucleolus is usually found in the centre of the nucleus, or excentrically. In Cyclops the nucleolus is connected to the nuclear membrane by a funnel at the onset of vitellogenesis (Raven, 1961). Koulish (1965) found the single nucleolus of previtellogenetic oocytes of Gorgoderina attenuata to be close to, or in contact with the nuclear membrane. There was no evidence of nucleolar extrusion in the previtellogenetic oocytes of

I. quadrivalvis, although this phenomenon has been reported in many other invertebrate oocytes (Raven, 1961), and occurs during vitellogenesis in I. quadrivalvis (see below).

3. The cytoplasm

A centriole was found adjacent to the interphase nucleus in the oocyte of I. quadrivalvis at the onset of previtellogenesis (stage 4). The centriole has a similar ultrastructure to those found in the previtellogenetic oocytes of the hydrozoan jellyfish Trachylina (Kessel, 1968d), being composed of microtubules embedded in an amorphous matrix.

After an initial growth period, the oocyte cytoplasm begins to synthesize the membrane system found during vitellogenesis. Electron micrographs reveal that a great elaboration of the endoplasmic reticulum takes place. This is reflected histochemically in an increase in cytoplasmic RNA and basic protein. During the early stages of previtellogenesis, the majority of the endoplasmic reticulum is in the form of rough lamellae. Some smooth lamellae are located near the nuclear membrane. The density of free ribosomes is reduced during previtellogenesis, as many ribosomes become attached to the lamellae of the rough endoplasmic reticulum.

Several Golgi complexes are found adjacent to the nuclear membrane after about the middle of previtellogenesis. Hinsch and Cone (1969) found several well developed Golgi complexes adjacent to the nuclear membrane in previtellogenetic oocytes of Libinia emarginata. The Golgi complex is also a prominent organelle in previtellogenetic oocytes of the horseshoe crab Limulus polyphemus (Dumont, 1967; Dumont and Anderson, 1967) and of the human ovary (Hertig and Adams,

1967). The Golgi complex is not a prominent feature in the cytoplasm of other crustacean oocytes (Anteunis, et al, 1966 a,b,c; Beams and Kessel, 1962, 1963; Kessel and Beams, 1963a; Kessel, 1968a).

The Golgi complexes in the previtellogenetic oocytes of I. quadrivalvis show the same ultrastructure as in other oocytes (Raven, 1961), and in somatic cells (Fawcett, 1966). Each is composed of 4-6 flattened saccules. The Golgi are small and inactive during previtellogenesis. Each complex exhibits both a forming face and a maturing face. These are involved in replacement and removal of material from the Golgi, as summarized by Novikoff and Shin (1964).

A close topographical relationship is observed between the outer membrane of the nuclear envelope, the lamellae of smooth endoplasmic reticulum and the forming face of the Golgi complex. This phenomenon has not previously been described in the oocytes of any animal. However, Essner and Novikoff (1962) described an identical situation in somatic cells. In rat pancreatic cells, the rough endoplasmic reticulum is formed from the nuclear membrane. There is a flow of smooth endoplasmic reticulum from the rough endoplasmic reticulum to the forming face of the Golgi complex. As material becomes detached from the maturing face of the Golgi, the complex is "remade" by addition of lamellae of smooth endoplasmic reticulum at the forming face of the Golgi complex.

Towards the end of previtellogenesis, the endoplasmic reticulum undergoes several changes. The lamellae of the rough endoplasmic reticulum becomes dispersed, and small vesicles of smooth endoplasmic reticulum appear adjacent to the nuclear membrane. The endoplasmic vesicles are formed by an out-pocketing and budding of vesicles from

the outer membrane of the nuclear envelope. The delamination of smooth endoplasmic vesicles from the outer nuclear membrane continues during the initial stages of vitellogenesis (stages 5 and 6). Simultaneously the lamellae of rough endoplasmic reticulum disappear. Beams (1964) described an identical formation of vesicles in the cytoplasm of oocytes of Cambarus and Orconectes, and Kessel (1968a) found an identical system in oocytes of Homarus, Panulirus and other marine decapods. Unlike the endoplasmic vesicles produced by crayfish oocytes, the vesicles of the smooth endoplasmic reticulum formed in the oocyte of I. quadrivalvis retain their vesicular form throughout oogenesis. To some extent the vesicular form of the endoplasmic reticulum is retained in oocytes of Homarus and Panulirus and other marine decapods (Kessel, 1968a). In crayfish oocytes, however, the endoplasmic vesicles flatten and become tubular, and arrange themselves into interconnecting stacks (Beams and Kessel, 1962, 1963).

As in the lobster oocyte (Kessel, 1968a) ribosomes may become attached to the outer surface of the smooth endoplasmic vesicles, and thus by definition transform them into vesicles of rough endoplasmic reticulum. Once these vesicles are formed, they appear to undergo a process of invagination to form "a vesicle within a vesicle". This structure has been reported in the oocytes of Homarus and Panulirus (Kessel, 1968a), but in the latter the inner vesicles in the smooth endoplasmic vesicles are said to be derived by the fusion of two endoplasmic vesicles.

Throughout previtellogenesis, the mitochondria in the oocytes of I. quadrivalvis retain the usual structure and arrangement of cristae

(Fawcett, 1966). Unlike those of the crayfish oocyte (Beams and Kessel, 1963) they are uniform in their ultrastructure. During the course of previtellogenesis, the number of mitochondria increases. Norrevang (1966) has shown that mitochondria increase in number by elongation and subsequent division or by a budding process. Mitochondria exhibiting a dumb-bell configuration, representing stages of duplication, were observed in oocytes of the polychaete Diopatra cuprea (Anderson and Huebner, 1968). Conclusive evidence of the division of mitochondria was not obtained in I. quadrivalvis.

There is no apparent spatial orientation of the oocyte mitochondria in I. quadrivalvis. In many invertebrate oocytes (Raven, 1961) the mitochondria accumulate in a dense group around the Golgi zone on one side of the nucleus. In the previtellogenetic oocytes of the polychaete Diopatra cuprea (Anderson and Huebner, 1968), and the ophuroid Ophioderma panaminsis (Kessel, 1968b), mitochondria preferentially align at the surface of the nucleus. Hertig and Adams (1967) observed a similar spatial arrangement of mitochondria in oocytes from the human ovary, in which the mitochondria were not only disposed around the nuclear membrane, but clustered at the Golgi zone.

Microtubules are present in the peripheral cytoplasm of the previtellogenetic oocyte of I. quadrivalvis. Microtubules in oocytes of Cambarus, Orconectes and Procambarus (Beams and Kessel, 1963) are aligned in elongate groups at the periphery of the cytoplasm. Microtubules are involved in the maintainance of cellular shape and may also function as an intracellular transport system (Anderson, Weissman and Ellis, 1966; Hertig and Adams, 1967; Gibbins et al, 1969; Tilney and Gibbins, 1969). However, those of I. quadrivalvis oocytes have no obvious spatial orientation.

(d) Vitellogenesis (stages 5,6 and 7).

2. The nucleus (germinal vesicle).

The germinal vesicle, formed during previtellogenesis (stage 4) remains morphologically unchanged and grows slightly larger during stages 6 and 7 of oogenesis in I. quadrivalvis and T. rosea. The nucleoplasm of I. quadrivalvis remains devoid of any resolvable ultrastructural components except those associated with the nucleolus. The nuclear pores observed during previtellogenesis (stage 4) persist throughout vitellogenesis, as in other oocytes (Hinsch and Cone, 1969). The outer layer of the nuclear membrane forms blebs which become free in the cytoplasm, forming vesicles of the endoplasmic reticulum. This sequence is identical with that described in other crustacean oocytes (Beams and Kessel, 1963; Beams, 1964; Kessel, 1968a; Hinsch and Cone, 1969).

As in other oocytes (Raven, 1961) the germinal vesicle breaks down prior to the final meiotic divisions (stage 7). Schuetz and Biggers (1967) have isolated a small molecular eight compound which causes the breakdown of the germinal vesicle of isolated immature starfish oocytes. It was also demonstrated by Schuetz and Biggers (1967) that the compound which regulates shedding of the ova in the starfish also regulates the breakdown of the germinal vesicle in mature oocytes.

In contrast to many other oocytes (Raven, 1961), the nuclear membrane does not disappear in either I. quadrivalvis or T. rosea, nor does the nucleoplasm become Feuglen positive at the end of stage 7. It therefore can be concluded that the final maturation stages in both species occurs in the oviduct prior to the releasing of the ova.

2. The nucleolus

The nucleolus remains approximately the same size throughout stages 5 and 6 of oogenesis in I. quadrivalvis and T. rosea, and enlarges at stage 7 in the former, in contrast to T. rosea, in which the nucleolus becomes reduced in size. The nucleolar vacuole formed during previtellogenesis (stage 4) persists throughout stages 5 and 6. Once the oocyte is ripe and the germinal vesicle breaks down (stage 7) the nucleolar vacuole disappears and is replaced by dense granules. Similar events have been described in other oocytes (Raven, 1961; Esper, 1965).

Electron micrographs of nucleoli in the vitellogenetic oocytes (stages 5 and 6) of I. quadrivalvis reveal that nucleolar material becomes separated from the main body of the nucleolus and moves towards the nuclear membrane. Kessel (1968b) observed a similar migration of nucleolar material in the vitellogenetic oocytes of the ophuroid Ophioderma panamensis.

As in other crustacean oocytes (Beams and Kessel, 1963; Kessel, 1968a; Hinsch and Cone, 1969) nucleolar material was observed to pass through the nuclear pores during vitellogensis in I. quadrivalvis. As in other oocytes (Kessel, 1966 a,b) the extruded nucleolar material appears to disintegrate into small particles which resemble ribosomes.

The nucleolus of stage 7 oocytes in both I. quadrivalvis and T. Rosea still retains a high concentration of RNA and basic protein.

(3) The cytoplasm

(a) The synthesis of Protein-carbohydrate yolk

During vitellogenesis in I. quadrivalvis and T. rosea, the bulk of the protein-carbohydrate yolk and lipid yolk is synthesized and stored within the oocyte. Numerous studies on vitellogenesis in other animals have suggested that the bulk of the yolk is synthesized within the oocyte (Raven, 1961). The yolk of many diverse groups, however, such as insects, molluscs, amphibians and mammals, is not synthesized within the oocyte. Yolk components are produced at a site external to the oocyte and transported in soluble form to the oocyte, where they are converted into yolk (Telfer, 1961, Kessel and Beams, 1963a; Roth and Porter, 1964; Anderson, 1964; Norrevang, 1966; Cone and Scalzi, 1967; Bodemer, 1968; Scheurer, 1969).

Recent ultrastructural studies on the vitellogenetic oocytes of crustaceans show that the bulk of the yolk is always synthesized within the oocyte (Beams and Kessel, 1962, 1963; Kessel and Beams, 1963a; Hinsch and Cone, 1969). The details of the synthetic machinery differ considerably amongst species (Beams, 1964; Kessel, 1968a) but generally proteinaceous yolk particles appear to arise directly within a well developed system of endoplasmic reticulum. Golgi complexes are involved in the synthesis of protein-carbohydrate yolk in I. quadrivalvis and to a lesser extent in Libinia emarginata (Hinsch and Cone, 1969). Micropinocytosis also plays a role in contributing to protein-carbohydrate yolk synthesis in I. quadrivalvis as in crayfish oocytes (Beams and Kessel, 1963), lobster oocytes (Kessel, 1968a) and in Libinia emarginata (Hinsch and Cone, 1969).

The method of yolk synthesis observed in I. quadrivalvis resembles that found in a diverse series of other invertebrates, especially in oocytes of the following animals; the hydrozoan jellyfish Trachylina (Kessel, 1968d); the polycheate Diopatra cuprea (Anderson and Huebner, 1969); the fresh water mussel Anodonata (Beams and Sekhon, 1966); the horseshoe crab Limulus polyphemus (Dumont and Anderson, 1967); the ophuroid Ophioderma panamensis (Kessel, 1968b); the holothuroid Thyone briareus (Kessel, 1966a) and the tunicate Ciona intestinalis (Kessel, 1966b).

As in other oocytes (Kessel, 1966a,b; Dumont and Anderson, 1967; Kessel, 1968b; Kessel, 1968d) the Golgi complexes increase in size and number at the onset of vitellogenesis (stage 5), and move away from the nuclear membrane. The Golgi complexes in I. quadrivalvis usually become associated with each other to form complex groups, as in other vitellogenetic oocytes in which the Golgi are prominent in the cytoplasm (Beams and Sekhon, 1966; Kessel, 1966a,b; Dumont and Anderson, 1967; Kessel, 1968b; Kessel, 1968d).

Vesicles of the smooth endoplasmic reticulum are found associated with both the forming face and the maturing face of the Golgi. During vitellogenesis, the forming face of the Golgi is characterized by the sometimes greater width of the saccules, and the presence of many flattened vesicles of smooth endoplasmic reticulum which are involved in the renewal of Golgi saccules (Essner and Novikoff, 1962; Novikoff and Shin, 1964; Kessel, 1968b). The maturing face of the Golgi is characterized by the presence of dense homogeneous material within the innermost saccules. The maturing face of the Golgi in I. quadrivalvis is identical with those found in other cells involved

in active synthesis (Novikoff and Shin, 1964; Novikoff and Essner and Quiatanus, 1964; Kessel, 1966 a,b).

Electron dense granules, 40-60mμ in diameter, are produced by the Golgi complexes. Granules of a similar diameter are produced within the cisternae of the rough endoplasmic reticulum in crayfish oocytes (Beams and Kessel, 1962, 1963). Droller and Roth observed that numerous 70mμ diameter vesicles apparently pinch off from the Golgi complex in vitellogenetic oocytes of Lebistes reticulatus. Ulrich (1969) observed many secretory vesicles, synthesized 'in situ' by the Golgi complex, around the Golgi complex of vitellogenetic oocytes of the teleost Branchydanio rerio. In I. quadrivalvis, as in the vitellogenetic oocytes of some echinoderms and tunicates and the horseshoe crab (Kessel, 1966 a,b; Dumont and Anderson, 1967), membrane-bound secretory granules are produced at the swollen ends of the saccules of the Golgi and released into the cytoplasm. Once free in the cytoplasm, the individual secretory granules are incorporated into vesicles of the smooth endoplasmic reticulum to form precursor yolk vesicles.

In addition, the ends of the saccules of the Golgi swell to form Golgi vesicles which also contain secretory granules. The Golgi vesicles become detached from the Golgi complex, and lie free in the cytoplasm, and are identical to the precursor yolk vesicles described above. The latter method of synthesis of precursor yolk vesicles is similar to that described in Trachylina by Kessel (1968d).

The precursor yolk vesicles of I. quadrivalvis have the same ultrastructure as those of Limulus polyphemus (Dumont and Anderson, 1966), Anodonata (Beams and Sekhon, 1966), and Trachylina (Kessel,

1968d). In addition, the precursor yolk vesicles of I. quadrivalvis are not unlike those in other crustacean oocytes (Beams and Kessel, 1962, 1963; Kessel, 1968a), although the latter are not produced by the Golgi. The vesicles are all membrane-bound, lack attached ribosomes and contain many secretory granules.

Histochemical evidence suggests that the products of the Golgi may be a polysaccharide, since PAS positive granules first appear in the perinuclear region of the stage 5 oocyte. Caro and Palade (1964) and Jamieson and Palade (1967a,b) have demonstrated in autoradiographic experiments on the synthesis of zymogen granules in rat pancreas, that proteins are synthesized on the rough endoplasmic reticulum and then transported to and condensed with the Golgi saccules, to be released from the Golgi complex as zymogen granules. Neutra and Leblond (1966 a,b) have demonstrated that the Golgi saccules can synthesize complex carbohydrates, which later become added to immigrant protein to form glycoprotein. Ulrich (1969) has shown that the mucopolysaccharide yolk in vitellogenetic oocytes of Brachydanio rerio is synthesized 'in situ' in the Golgi complex. The dense homogeneous material apparent in the saccules at the forming face of the Golgi in I. quadrivalvis may be the first stage in the production of the complex carbohydrate. Dense granules, smaller than the final secretory product, are evident in the peripheral regions of the Golgi. It appears that the peripheral regions of the Golgi may synthesize the polysaccharide, which is later packaged and released at the maturing face of the Golgi.

Dumont and Anderson (1967) postulated that the Golgi may synthesize a carbohydrate moiety which later combines with a protein moiety to form the complex carbohydrate-protein yolk found in the precursor yolk vesicles of Limulus polyphemus. This theory may account for the formation of the carbohydrate moiety in precursor yolk vesicles of I. quadrivalvis. The protein moiety may be supplied by the vesicles of the endoplasmic reticulum.

As in vitellogenetic oocytes of Homarus and other marine decapods (Kessel, 1968a), and of Lebistes reticulatus (Droller and Roth, 1966), the vesicular form of the endoplasmic reticulum is a prominent organelle in the cytoplasm of the vitellogenetic oocytes of I. quadrivalvis. At no time during vitellogenesis does the endoplasmic reticulum resume the lamellae form, or ever form large interconnecting cisternae which are characteristic of vitellogenetic oocytes in other crustaceans (Beams and Kessel, 1962, 1963).

The formation of the vesicular form of the endoplasmic reticulum commences at the end of stage 4 as discussed previously. The vesicles are evenly dispersed throughout the cytoplasm. As in oocytes of Libinia emarginata (Hinsch and Cone, 1969), and Lebistes reticulatus (Droller and Roth, 1966) the vesicles have ribosomes attached to the outer surface, at least temporarily. As mentioned in the previous section, the vesicles of the smooth endoplasmic reticulum are involved in the formation of precursor yolk vesicles. In addition, some vesicles of rough endoplasmic reticulum appear to synthesize a fine wispy material. These vesicles and their contents fuse with the small precursor yolk vesicles to form the histochemically distinct protein-carbohydrate yolk complex.

On the basis of morphological and histochemical evidence, it can be postulated that in I. quadrivalvis the protein moiety of the carbohydrate-protein complex is derived solely from the vesicular form of the endoplasmic reticulum, whereas the carbohydrate portion is derived from the Golgi complex. This precise sequence of synthesis has not been described in vitellogenetic oocytes of other animals, but as discussed above, a very close topographical relationship has been observed between the Golgi and the endoplasmic reticulum in the production of the protein-carbohydrate yolk, particularly in oocytes of Limulus polyphemus (Dumont and Anderson, 1967) and in Libinia emarginata (Hinsch and Cone, 1969).

(b) Maturation of Precursor Yolk Vesicles

The maturation of the precursor yolk vesicles observed in the vitellogenetic oocytes (stages 6 and 7) of I. quadrivalvis is similar to that described in other animals in which either the Golgi complex or endoplasmic reticulum or both are active in the synthesis of the protein-carbohydrate yolk (Beams and Kessel, 1963; Kessel, 1966 a,b; Beams and Sekhon, 1966; Dumont and Anderson, 1967; Kessel, 1968a; Kessel, 1968d ;Hinsch and Cone, 1969). However, maturation lacks the complications observed in tunicates (Kessel, 1966b), amphibians (Ward, 1962; Wischnitzer, 1964; Karasaki, 1963) or oocytes of the hen (Bellairs, 1967).

Initially the precursor yolk vesicles increase in size. As in other oocytes in which the Golgi complex is involved in the synthesis of protein-carbohydrate yolk (Beams and Sekhon, 1966; Droller and Roth, 1966; Dumont and Anderson, 1967; Anderson and Heubner, 1968; Kessel, 1968d)the enlargement occurs by the fusion of many small

precursor yolk vesicles. In addition, in I. quadrivalvis as in other oocytes (Droller and Roth, 1966), the precursor yolk vesicles also fuse with vesicles of the endoplasmic reticulum.

Once the precursor yolk vesicles have reached approximately 1μ in diameter, changes occur in the contents. Initially in I. quadrivalvis, as in Anodonta (Beams and Sekhon, 1966), small irregularly shaped elongate bodies appear within a central core. However, the cross striations in the central core of the precursor yolk vesicles observed by Beams and Sekhon (1966) in Anodonata were not found in I. quadrivalvis. As in other oocytes (Droller and Roth, 1966; Dumont and Anderson, 1967; Kessel, 1968a; Kessel, 1968d)the central core in I. quadrivalvis now disintegrates leaving membrane-bound granules restricted to the cortex. Dissolution of the granules continues in the cortex until the yolk vesicles are composed of a homogeneous finely granular material. Mature yolk bodies in I. quadrivalvis resemble those found in other crustaceans (Beams and Kessel, 1963; Kessel, 1968a; Hinsch and Cone, 1969) and other invertebrates (Dumont and Anderson, 1967).

The regular crystalline structures observed in yolk platelets of the amphibian oocyte and various invertebrate oocytes as summarized by Raven (1961) and Williams (1967) and in the oocyte of the hen (Bellairs, 1967) were not observed in the central core of I. quadrivalvis or in other crustaceans (Kessel, 1968a). However, an electron-transparent stellate structure of reduced protein and PAS-positive content, develops in mature yolk bodies during stage 7 of I. quadrivalvis. The occurrence of this structure has not been previously reported in other animal oocytes. "Empty patches" develop

in mature yolk bodies of other crustaceans (Kessel, 1968a), but these do not resemble the electron-transparent areas found in the mature yolk bodies of I. quadrivalvis.

(c) Micropinocytosis

Not all protein-carbohydrate yolk in crustaceans is packaged by the methods discussed above. The formation and development of micropinocytotic pits and subsequent pinocytotic vesicles during stages 6 and 7 of oogenesis in I. quadrivalvis follows the pattern described in other oocytes (Beams and Kessel, 1963; Kessel and Beams, 1963b; Anderson, 1964; Roth and Porter, 1964; Beams and Sekhon, 1966; Droller and Roth, 1966; Kessel, 1966 a,b; Kessel, 1968a; Hinsch and Cone, 1969) and shows a striking resemblance to the observations of Dumont and Anderson (1967) on the vitellogenetic oocytes of Limulus polyphemus.

Micropinocytosis plays a relatively minor role in protein-carbohydrate yolk formation in I. quadrivalvis, as other crustaceans (Beams and Kessel, 1963; Kessel, 1968a; Hinsch and Cone, 1969) and certain other invertebrates (Beams and Sekhon, 1966; Dumont and Anderson, 1967). As in Limulus polyphemus (Dumont and Anderson, 1967) and Libinia emarginata (Hinsch and Cone, 1969), micropinocytosis does not commence until much of the protein-carbohydrate yolk has been synthesized by the oocyte itself. In contrast, micropinocytosis plays a major role in the accumulation of yolk in insect oocytes (Telfer, 1961; Kessel and Beams, 1963b; Anderson, 1964; Roth and Porter, 1964).

As in other invertebrates (Kessel and Beams, 1963b) local condensations of material form on the external surface of the plasma membrane at stage 6. The surface coating on the plasma membrane may endow the plasma membrane with some degree of local

selective permeability (Fawcett, 1966; Anderson, 1964; Roth and Porter, 1964). A micropinocytotic pit develops the plasma membrane beneath each condensation, having a diameter similar to that described in other oocytes (Kessel and Beams, 1963b; Fawcett, 1966). The cytoplasmic surface of the micropinocytotic pit is also coated with dense material forming a bristle coat. The micropinocytotic pits eventually pinch off from the plasma membrane to lie free in the cytoplasm, and lose their bristle coats.

As in the oocytes of Limulus polyphemus (Dumont and Anderson, 1967) the micropinocytotic vesicles of I. quadrivalvis, once free in the cytoplasm, fuse with each other to form pinocytotic yolk vesicles. As in the lobster oocyte (Kessel, 1968a), the micropinocytotic vesicles of I. quadrivalvis also fuse with the vesicles of the rough endoplasmic reticulum to form pinocytotic yolk vesicles. The pinocytotic yolk vesicles are morphologically distinct from the precursor yolk vesicles.

The pinocytotic yolk vesicles grow by fusion with one another, as in the oocytes of Limulus polyphemus (Dumont and Anderson, 1967), and undergo a short period of maturation to eventually form protein-carbohydrate yolk bodies morphologically indistinct from those formed by maturation of the precursor yolk vesicles.

As in Limulus polyphemus (Dumont and Anderson, 1967), mature oocytes of I. quadrivalvis secrete a thick vitelline membrane. As micropinocytosis still continues in mature oocytes of I. quadrivalvis, as in oocytes of Limulus polyphemus, (Dumont and Anderson, 1967), the material incorporated is capable of reaching the surface of the oocyte through the channels which perforate the vitelline membrane.

(d) Lipid synthesis

The histochemical identification of lipid yolk can be confused by the simultaneous staining of the phospholipid membrane of the Golgi, mitochondria and endoplasmic reticulum (Raven, 1961). This problem has resulted in conflicting reports on the localization of lipid yolk, particularly before the advent of electron microscopy. In vitellogenetic oocytes of Carcinus maenas (Harvey, 1929) and Lygia (Steopoe, 1929) lipid droplets were observed to arise 'de novo' in the cytoplasm. However, Bhatia and Nath (1931) considered the Golgi complex to be the site of lipid synthesis in both the prawn Palaemon lamerri and the crab Paratalphase spinigna.

In stage 6 oocytes of I. quadrivalvis and T. rosea lipid droplets arise 'de novo' in the perinuclear regions of the cytoplasm. Formation and aggregation of lipid droplets continues throughout stages 6 and 7 of oogenesis, until the lipid forms the bulk of the yolk in the ripe oocytes. Recent ultrastructural studies on vitellogenetic oocytes of other crustaceans (Beams and Kessel, 1963; Kessel and Beams, 1963a; Kessel, 1968a; Hinsch and Cone, 1969) have shown that a formation of lipid droplets 'de novo' in the cytoplasm is characteristic of crustacean oocytes.

(e) Other cytoplasmic Organelles

Apart from the organelles associated with the synthesis of protein-carbohydrate and lipid yolk, the major components in the cytoplasm are the mitochondria and the microtubules.

The mitochondria, as in previtellogenetic oocytes (stage 4) do not show any unusual features. The number of mitochondria appears to increase. As discussed previously, this may occur by budding or dividing into two.

The microtubules, as in previtellogenetic oocytes (stage 4) have no spatial orientation. However, they become concentrated throughout the peripheral regions of the oocyte in stages 5 and 6. As suggested by Kessel (1968a), the microtubules might play a role in stabilizing the "cytoarchitecture" of the oocyte, particularly in stages of rapid growth.

During the final stages of oogenesis (stage 7) the cytoplasm between the yolk granules begins to disintegrate and disperse. Fixation of stage 7 oocytes was poor and little ultrastructural information could be ascertained. No specialized cortex could be found in the peripheral cytoplasm of ripe oocytes of I. quadrivalvis.

4. Membrane Formation

As in most oocytes (Raven, 1961), a vitelline membrane is secreted by the oocyte towards the end of vitellogenesis. In I. quadrivalvis the vitelline membrane is laid down during stage 7 of oogenesis. In Carcinus maenas (Harvey, 1929; Cheung, 1966), the vitelline membrane is secreted once the oocyte is free from the syncytial germinating zone. However, the formation of the vitelline membrane in I. quadrivalvis appears to be similar to that described for other invertebrate oocytes (Droller and Roth, 1966), especially the surf clam Spisula solidissima (Rebhun, 1962), and some vertebrates, as summarized by Anderson, (1966).

At the onset of stage 6, microvilli arise from the plasma membrane and project into the lumen of the ovariole. The formation of microvilli prior to the deposition of the vitelline membrane appears to be a constant feature in oocytes which secrete this membrane (Rebhun, 1962; Beams and Sekhon, 1966; Droller and Roth, 1966;

Anderson, 1967).

In I. quadrivalvis, the dense fibrous material of the vitelline membrane is deposited in clumps between adjacent microvilli. The origin of the vitelline material was not resolved.

Deposition of the vitelline secretion continues throughout stage 7, eventually giving rise to a homogeneous vitelline membrane about 2μ thick. Rebhun (1962) observed a similar deposition of vitelline secretion in Spisula solidissima but two distinct layers differentiate within the vitelline membrane of this species. The formation of several distinct layers occurs to a greater degree amongst vertebrate oocytes (Anderson, 1967).

As in other invertebrate oocytes (Rebhun, 1962; Dumont and Anderson, 1967) the completed vitelline membrane is perforated by wide channels in which the microvilli lie. These channels would allow movement of extraoocytic material from the ovariole lumen to the oocyte surface (Dumont and Anderson, 1967). As in other oocytes (Rebhun, 1962; Dumont and Anderson, 1967; Kessel, 1968c) there is always a thin perivitelline space between the plasma membrane, which would allow the circulation of extraoocytic material. Micropinocytosis continues in the ripe oocyte.

5. BIOCHEMICAL COMPOSITION OF EMBRYOS OF I. QUADRIVALVIS

Ultrastructural and histochemical studies on the oocytes of I. quadrivalvis have shown that the ripe oocyte is full of yolk. Biochemical studies have shown that the bulk of the yolk is retained throughout embryogenesis until hatching, which at 23°C occurs approximately 17 days after oviposition (Anderson, 1965). The

planktonic naupliar larvae of I. quadrivalvis, in contrast to most barnacle nauplii, do not feed. Thus the yolk reserves must sustain the nauplii of I. quadrivalvis throughout the final metamorphic stages, which occupy at least another 7 days (Anderson, 1965).

(a) Dry weight:-

Measurements of dry weight provides a simple means of studying changes in total yolk reserves during development. The dry weight of many crustacean embryos from marine environments remain stable (Green, 1965). Particularly in embryos of Artemia salina, in which the dry weight remains constant throughout embryonic development, this has been attributed to the uptake of salts (Dutrieu, 1960). The dry weight of embryos of I. quadrivalvis remains constant throughout embryonic development, presumably again due to the uptake of salts. In contrast, other marine embryos, such as the hermit crab Eupagurus bernhardus (Pandian and Schumann, 1967) and the shrimp Crangon crangon (Pandian, 1967) lose about 30% of their dry weight during embryonic development. As both these crustacean embryos incorporate water and salts from the environment (Pandian and Schumann, 1967; Pandian, 1967), the loss in dry weight reflects a reduction in total yolk reserves.

(b) Wet weight:-

The wet weight of embryos of I. quadrivalvis increases by 120% from cleavage to hatching. The increase in water content is correlated with an increase in the size of the nauplii. The increase in water content of 70% just prior to hatching reflects the enlargement of the haemocoel and midgut cavities. Zotin (1964) summarizes evidence that water uptake in many marine embryos is correlated with an increase in body cavity volume and a consequent increase in total volume.

Uptake of water seems to be a common feature of developing crustacean eggs. In Balanus balanoides and B. balanus (Barnes, 1965), Crangon crangon, Ligia oceanica, Homarus gammarus and Artermis salina (Pandian, 1967), and in Eupagurus bernhardus (Pandian and Schumann, 1967) as in I. quadrivalvis, the increase in water content is related to the increase in volume of the embryos.

(c) Carbohydrates:-

Total measurable carbohydrates only account for about 4% of the dry weight of embryos of I. quadrivalvis. This value is similar to that found by Barnes (1965) in embryos of the cirripedes Balanus balanoides and B.balanus. Thus, as in other crustacean embryos (Urbani, 1959; Green, 1965; Dawson and Barnes, 1966; Barnes and Evens, 1967; Williams, 1967) it seems on biochemical evidence that carbohydrates do not make up the bulk of the nutrients stored during embryonic development. However, histochemical studies of the ripe oocytes of I. quadrivalvis reveal that a major part of the yolk is stored as a protein-carbohydrate complex. It therefore seems likely that in biochemical analyses, the attached carbohydrate moiety of the protein-carbohydrate complex may not be measured. Giese (1967) summarizes evidence that all the polysaccharides, oligosaccharides and glycogen will be estimated by the method used, but the low carbohydrate value may only be a measure of carbohydrates previously released from the protein-carbohydrate complex.

During the embryonic development of I. quadrivalvis, there is a net loss of 20% in total measurable carbohydrates. In I. quadrivalvis as in the cirripedes Balanus balanoides and B.balanus (Barnes, 1965), a greater percentage of the carbohydrate fraction is utilized than of

either the protein or lipid fraction. There is an initial loss of measurable carbohydrates, as in Balanus balanoides and B.balanus (Barnes, 1965) between cleavage and the formation of limb-buds (stage 9). From stage 9 to hatching, the amount of measurable carbohydrates remains more or less constant, again as in Balanus balanoides and B.balanus (Barnes, 1965). Within this period, during the final development of the embryos, the remaining yolk is rapidly digested, and there is thus a greater release of carbohydrates. A slight temporary increase in total measurable carbohydrates occurs during stages 11-12. Concurrently, the secretion of chitin commences at stages 10-11, and the increase in measurable carbohydrates probably reflects in part a storage of glucose for the synthesis of chitin (Passano, 1960).

Glycogen was not detected in either histochemical or ultrastructural studies on the oocytes of I. quadrivalvis. Nevertheless, in more specific biochemical tests for glycogen, small amounts, less than 2% of the dry weight, were detected in the developing embryos. The glycogen fraction was not divided into an acid soluble and an acid insoluble form as described by Stetten and Stetten (1960) and Barnes (1965). Giese (1967) considers such a separation as artificial, as the form glycogen extracted depends on the homogenizing method used.

In small crustacean embryos with relatively little yolk, such as those of Simocephalus vetulus (Green, 1965), Balanus balanoides and B.balanus (Barnes, 1965), the glycogen reserves are rapidly consumed during development. In the embryos of I. quadrivalvis, which have a greater yolk content, the glycogen level remains constant. The reason for this is unknown. In the embryos of Artemia salina, which is a

special case of a diapausing egg, glycogen is synthesized during development from the carbohydrate reserve trehalose (Green, 1965). The non-dormant type of egg in Artemia salina, which is retained in the brood pouch of the female until the nauplius larvae emerges, shows a difference in carbohydrate metabolism from the egg which becomes encysted and dormant. In the latter, the glycogen content remains at a low level until development resumes. In contrast, the glycogen content of the non-dormant egg increases by 70% during embryonic development (Green, 1965). Presumably glycogen serves as an energy source during the subsequent rapid larval development of Artemia salina.

(d) Proteins:-

Proteins are one of the major storage forms of nutrients in embryos of I. quadrivalvis, as in other cirripedes (Balanus balanoides and B.balanus, Barnes, 1965; Barnes and Evens, 1967) and other crustacean embryos (Artemia salina, Williams, 1967). Histological and ultra-structural evidence shows that proteins are a major constituent stored in the unfertilized eggs of crustaceans (Kessel, 1968a).

There is an increase of 100% in the total protein content of the embryos of I. quadrivalvis between cleavage and the formation of limb-bud rudiments (stage 8). This increase in protein content corresponds to the decrease in carbohydrates, which suggests that the carbohydrates are not only utilized as energy reserves, but may also act as metabolic intermediates for protein synthesis. There is a rapid decrease of 100% in protein content at stage 9 in embryos of I. quadrivalvis, followed by an increase at stage 11. The rapid decrease at stage 9, which has a duration of three days at 23°C, suggests that protein may now provide an energy source in conjunction

with energy supplied by the carbohydrates. From stage 11 to hatching, the protein content of embryos of I. quadrivalvis remains more or less constant.

In contrast to the fluctuations in protein content detected in embryos of I. quadrivalvis, protein content remains stable in Artemia salina (Urbani, 1959; Green, 1965) and in Balanus balanoides (Barnes, 1965), so that protein is apparently not used to any significant extent as a respiratory substrate in these species. The protein content of Balanus balanus embryos remains stable until the final stages of development, during which there is an increase of 10% followed by a decrease of 7.5% (Barnes, 1965). However, a net increase of 10% in protein content occurs in the developing eggs of Eupagurus bernhardus and Crangon crangon (Pandian and Schumann, 1967; Pandian, 1967). These results also suggest that proteins are not utilized as respiratory substrates in these crustacean embryos.

(e) Lipids:-

Histological, ultrastructural and biochemical evidence shows that lipid is the second major nutrient stored in eggs and embryos of I. quadrivalvis. Lipids also form a major nutrient store in embryos of the cirripedes Balanus balanoides and B.balanus (Barnes, 1965).

The lipid content of I. quadrivalvis embryos remains more or less constant throughout embryonic development. There is a loss of 11.5% in the total lipid reserves in embryos of Balanus balanoides and a slightly higher loss of 17.3% in embryos of B.balanus (Barnes, 1965). In contrast, the eggs of the shrimp Crangon crangon (Pandian, 1967) lose about 55% of the lipid reserves between cleavage and hatching. Pandian (1967) suggests that the oxidation of fat is the main energy

source of Crangon crangon and for other crustacean embryos (Eupagurus bernhardus, Ligia oceanica, Homarus gammarus, Loligo vulgares and Crepidula fornicata).

Although some metabolism of lipids occurs in the cirripedes Balanus balanoides and B.balanus (Barnes, 1965), it is apparent that the bulk of the lipid is retained in cirripede embryos. In part, the lipid reserves of embryos of I. quadrivalvis, as in most cirripedes, are retained into the cyprid stages. Large reserves of oil are usually found in the anterior ends of the cirripede cypris (Crisp, 1967).

Lipid storage, giving a relatively low density in relation to mass, is considered to be an adaptation to motility and thus could be an important mechanism for survival in small planktonic larvae (White, Handler and Smith, 1964). The energy set free in oxidation of 1 gram of fat is over twice that obtained for the same weight of either carbohydrate or protein (Downes, 1962). Thus it would be reasonable to assume that the lipid reserves, laid down in oogenesis and retained during embryogenesis, are probably the main source during the active, non-feeding larval development of I. quadrivalvis.

(f) Pigments:-

The eggs of many crustaceans have been long noted for their intense and distinctive colouration, which ranges from pink in Triops, green in Daphnia pulex and blue in Lepas to red in Palinurus vulgaris (Cheesman, Lee and Zagalsky, 1967). During the course of embryonic development the pigment usually undergoes colour changes. The visual appearance of the embryos is not a reliable guide to the pigment content, the former being much under the influence of other factors, such as the transparency of tissues (Ball, 1944; Goodwin, 1951;

Barnes, 1965). The pigment responsible for the variable colouration is a carotenoid (Fox, 1953).

In I. quadrivalvis, as in most crustacean embryos, the carotenoid moiety is astaxanthin, which is usually non-covalently bonded to a lipid and/or a protein complex (Goodwin, 1960; Cheesman and Prebble, 1967; Wallance, Walker and Hauschka, 1967). The carotenoprotein or carotenolipid is usually a major component of crustacean yolk (Wallace, Walker and Hauschka, 1967).

The function of carotenoid pigments in crustacean embryos is not resolved. Although in Daphnia magna about half of the mother's carotenoid pigments are transferred to the eggs at each brood, little if any of this pigment is utilized by the embryos during development (Herring, 1968a). Herring (1968b) has further shown that the carotenoids in the eggs of Daphnia magna are not necessary for their normal development. Goodwin (1951) could not find any evidence of pigment metabolism in the embryos of Homarus. The carotenoid pigments may function in stabilizing the protein complex during early development (Lee and Zagalsky, 1966; Cheesman, Lee and Zagalsky, 1967). In the Cladocera (Green, 1965) breakdown of the pigment occurs towards the end of embryonic development, when the free carotenoid passes into fat droplets. Goodwin (1951) suggested that the carotenoid pigments of Homarus eggs may have hormonal properties, since the protein complex is disrupted and the free astaxanthin is liberated just before hatching. Carotenoproteins may function by absorbing light and protecting embryos from injury by solar radiation (Green, 1965; Cheesman, Lee and Zagalsky, 1967; Herring, 1968b).

In addition to astaxanthin, a large amount of chromolipid is found in the pigment fraction in both the ovary and mature embryos of I. quadrivalvis. Chromolipids are an ill-defined group of pigments, which are separated from the carotenoids by their chemical and spectraphotometrical properties (Fox, 1953). Chromolipids have been identified in the eggs of other cirripedes Balanus balanus and B.balanoides (Barnes, 1965), and are also found in the eggs of mammals and hens.

Chromolipids are closely linked with lipid metabolism (Fox, 1953). The role of chromolipids in the embryonic tissues of crustaceans is not resolved. They may function as a catalyst for the oxidation of unsaturated fatty acids (Fox, 1953). Lipids of marine animals are distinguished from those of most terrestrial animals by their high content of polyunsaturated fatty acids (Rodegker and Nevenzel, 1964; Dawson and Barnes, 1966). There is a slight reduction in total pigment content in the mature embryos of I. quadrivalvis, which suggests that some pigment may be metabolized during development. However, as in the cirripedes Balanus balanoides and B.balanus (Barnes, 1965) a large part of the pigment complex originally present, still persists on completion of embryonic development.

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