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Oögenesis in the sea urchin Strongylocentrotus purpuratus was studied by histological methods and by histochemical techniques for polysaccharides, lipids, and nucleic acids. Urchins were collected at Yaquina Head, Oregon at regular intervals between April 1966 and March 1967. An attempt was made to correlate seasonal variations in the coastal water temperature with the gonadal cycle.

The ovaries of the sea urchin are large rebranched sacs covered with a flagellated peritoneal epithelium. Inside the peritoneum is a wall of collagenous connective tissue and smooth muscle. In the central portion of each saccule or acinus of the ovary are two main cell types: the sex cells, which develop into mature ova, and the accessory cells or nutritive phagocytes which apparently provide nutriment for the sex cells.

Oögonia can be found through out the year in small groups

scattered along the walls of the ovary, but are most numerous in the late spring and early summer when the ovary is spent. The oocytes start growing in the late summer and early fall when the accessory cells, which were depleted of nutriments in the spent ovary, start filling with lipid and polysaccharide globules. At this time the accessory cells are also found to have inclusions that appear to be degenerate sex cells. In the late fall and early winter, the oocytes continue to grow and their cytoplasm fills with lipid and polysaccharides. As the ova mature they move from the walls to the central portion of the acinus where they displace the accessory cells that had formerly been there.

The ova that have been shed or are about to be shed contain pyranophilic RNA which is not found in the oocytes. However, both ova and oocytes have RNA that is stainable with azure B. The pyranophilic RNA is also found in accessory cells.

Since all the oocytes do not mature at the same time, a sea urchin is able to shed many times during the breeding season which lasts from late winter to early spring. During this period the accessory cells progressively lose their globules. When the accessory cells are finally depleted of their lipid and polysaccharide, the oocytes no longer grow and the ovaries are spent.

A Histochemical Study of Oogenesis in the Sea Urchin, Strongylocentrotus purpuratus

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I. INTRODUCTION

The sea urchin has long been used for studies of fertilization and early development. The first description of the process of fertilization was made by Fol in 1879 using the sea urchin, and subsequently the eggs have been used not only to examine fertilization but also in the classic studies of embryogenesis and mitosis by many early workers. These studies are excellently compiled and reviewed by Harvey (1956) and Wilson (1925) with extensive bibliographies. Sea urchin material is particularly favorable for chemical and physiological studies of development, for large numbers of eggs can be obtained and when fertilized, they develop quite synchronously.

Although a great many studies have been carried out on the fertilized sea urchin egg and its early development, oögenesis, on which the early development of the embryo is ultimately dependent, has not received as much attention. As in most egg cells, the sea urchin ovum possesses a large store of yolk and other nutrients to carry the embryo through the early stages of development. Also stored in the egg is information in the form of messenger ribonucleic acid (RNA) to direct early protein synthesis, large numbers of

free ribosomes, lipids, cytoplasmic deoxyribonucleic acid (DNA) and polysaccharides derived from the mother which greatly influence early development. This subject has been extensively reviewed by Brachet (1960), Grant (1965), Raven (1961), and Williams (1965).

One of the first attempts to follow oögenesis in the sea urchin was that of Tennent and his co-workers (Miller and Smith, 1931; Tennent, Gardiner and Smith, 1931) in the Japanese urchin Echinometra lucunter. Attempts were made to identify the chemical composition of the ovary and immature eggs. The nutritive cells were found to be made up principally of fats and fatty acids, and it was believed by these authors that the nutritive cells broke down and their contents were taken up into the cytoplasm of the oöcytes. Morphological and cytological studies were also carried out on Mespilia globulus (Tennent and Ito, 1941), starting with the oögonia through the maturation of the ova. The work is very well illustrated, and while most points are clarified, the authors are uncertain about the origin of the germ cells. They suggest the germ cells arise from the peritoneal epithelium.

The primary oöcytes of the sea urchin, as most other primary gametocytes, go through a prolonged prophase during which the homologous chromosomes pair and become extremely condensed in preparation for the maturation divisions. During the diakinesis stage of the meiotic prophase the egg enters a growth phase which

Tennent and Ito (1941) refer to as the diffusion and resting nucleus stage while other workers have referred to it as the dictyotene or metabolic stage (Holland and Giese, 1965; Wilson, 1925). The nucleus of the oöcyte becomes enlarged and is referred to as the germinal vesicle, the chromosomes become very fine and elongate and a large dense nucleolus appears. Soon afterward, the cytoplasm begins to increase in volume until the oöcyte has reached the size of the mature egg. After the egg stops growing, the chromosomes recondense, the nucleolus disappears, and the germinal vesicle breaks down. The maturation spindles are formed near the egg membrane, with the result that the egg receives most of the cytoplasm and the polar bodies hardly any. The nucleus of the ovum is very small compared to the germinal vesicle, and it contains no nucleolus.

Most of the recent studies concerning sea urchin oögenesis have dealt with specific aspects of the problem. For example, many fine structure studies have been carried out on the cortical layer because of its importance in fertilization (Afzelius, 1956, 1957; Endo, 1952, 1961a, 1961b; Lönnig, 1963, 1964; Mercer and Wolpert, 1962; Runnstrom, 1966; and Wolpert and Mercer, 1961).

Other studies have concentrated on the synthesis of RNA, protein, and polysaccharides in the nucleolus and the transfer of these materials to the cytoplasm (Cowden, 1962; Esper, 1965;

Ficq, 1962, 1964; Ficq, Aiello and Scarono, 1963; Gross, Malkin and Hubbard, 1965; Piatogorsky, Ozaki and Tyler, 1967). Polyribosomes that appear to be derived from the nucleolus are extremely numerous in the cytoplasm and in addition, large amounts of messenger and transfer RNA are present in the egg. As the oöcyte matures, the amount of protein and polysaccharide in the cytoplasm increases, and the amount of cytoplasmic RNA becomes steadily diluted. Autoradiographic studies show that once the egg has matured into an ovum it no longer synthesizes RNA, but continues to produce protein (Ficq, 1964; Piatogosky, Ozaki and Tyler, 1967). Furthermore, these studies reveal the presence of a store of an inactive form of stable messenger RNA.

While these studies add greatly to our knowledge of sea urchin oögenesis they deal only with specific aspects of the growth or dictyotene phase of the oöcytes and do not take into consideration the possible role of the accessory cells or the seasonal variations in the reproductive cycle. In contrast, Fuji (1960) and Holland and Giese (1965) have based their studies on periodic observations made over the course of the annual reproductive cycle. Depending on the time of the year, great differences were found in the maturity of the egg cells and the state of the accessory cells. On the basis of these differences Fuji divided the yearly cycle into five stages: (I) "recovering spent", a few small primary oöcytes along the walls of

the ovary; (II) "growing", larger oöcytes line the walls; (III) "premature", large oöcytes along the walls and a few mature ova in the central lumen (IV) "mature", fertile with many mature ova; (V) "spent". In the spent stage the degenerating unshed ova are resorbed by phagocytes, and the number of oögonia and oöcytes increases until the ovary returns to stage one. Not only the gametes, but also the nutritive phagocytes, as Holland and Giese (1965) refer to the accessory cells, are found to undergo seasonal changes (Dawydoff, 1948; Holland and Giese, 1965). During the time that is equivalent to Fuji's first three stages these cells are filled with dense globules, but during the rest of the year they are relatively empty.

Holland and Giese (1965) studied the incorporation of DNA precursors into various types of ovarian cells over the course of the annual reproductive cycle. He found no evidence that the oögonia were derived from the visceral epithelium as suggested by Tennent and Ito (1941), that no DNA synthesis occurred in oöcytes after they had begun synapsis, and that no cytoplasmic DNA was labeled. His studies also showed that the nutritive phagocytes were derived by mitosis from previously existing ones and not from oögonia (Miller and Smith, 1931) or from hematocytes (Cowden, 1962).

Recently, Verhey and Moyer (1967a) have carried out an electron microscope examination of oögenesis in Arbacia punctulata, Lytechinus variegatus, and L. pictus. This was primarily a

morphological work and no mention was made of seasonal variations.

The present study was undertaken to correlate the morphological and biochemical changes in the egg cells and nutritive phagocytes with relation to the annual reproductive cycle, by histological and histochemical techniques.

II. MATERIALS AND METHODS

Strongylocentrotus purpuratus were collected at two week intervals from October 1966 to March 1967 in the tide pools of the mean low tide zone of Yaquina Head, Oregon (latitude 44° 40' 40"; longitude 124° 4' 40"). This is the period in which the greatest proliferation of the gonad and the spawning of eggs takes place. In addition samples of spent ovaries were sampled the proceeding April and May. Although no animals were obtained from June through September the May and October specimens overlapped sufficiently to present a continuous spectrum.

The sea urchins were sacrificed on the same day as collected or the morning immediately following a night collection. The wet weights of the animals were taken, they were then injected with 2 cc of 0.5 M KCl, and were allowed to stand for half an hour to see if they would spawn. Ovaries and gonads that could not be sexed by macroscopic inspection were removed from the urchins and fixed in Bouin's, Carnoy's, or calcium formal fixatives. The fixed material was then imbedded in gelatin for frozen sectioning or in paraffin. The gelatin imbedded tissues were quick frozen and sectioned on a microtome in a Model CTD International Harris Cryostat. Ten micron sections were made of both frozen and paraffin sections.

The hematoxylin and eosin method was used for standard

histological examination. Oil red O counterstained with Mayer's hematoxylin was used for neutral lipids; Feulgen reaction, for deoxyribonucleic acid (DNA); methyl green, pyronin (MGP) method was used for ribonucleic acid (RNA) in combination with mild acid hydrolysis of parallel sections, 1N HCl at 60°C for five minutes, as a control. The periodic acid Schiff (PAS) technique was used for the demonstration of polysaccharides with diastase digestion of parallel sections to indicate the presence of glycogen in the tissue. All histochemical techniques used followed the schedules as outlined in Barka and Anderson (1963). In addition, the presence of RNA was also determined by the azure B technique of Flax and Himes (1952) as modified by Szollosi (1965). Mild acid hydrolysis of parallel sections was again used to verify the presence of RNA.

Photomicrographs were made on a Leitz Wetzlar microscope with an Olympia 35 mm camera.

III. RESULTS

General Anatomy

The sea urchin has five ovaries. Each has its own gonaduct with a pore emptying on the aboral side near the anus. The gonad is a large rebranched sac and all saccules end in blind acini (Plate 20c). The thickness of the ovarian wall varies with the size of the ovary. When the ovary is gravid, the wall is stretched and the germinal layer appears to lie against the peritoneal epithelium. The outermost layer of the ovary consists of peritoneal cells and is three to six microns in width. These cells are small, flagellated and have large nuclei. In the gravid ovary they are more widely spaced. Underlying the peritoneal cells is a layer of connective tissue made up mainly of collagen fibers. In the spent ovary, it is three to six microns in width but can be stretched to one micron in gravid ovaries. Inside the collagen layer are bands of smooth muscle cells about four microns in width lying at right angles to the collagen fibers; the muscle layer also is much thinner in gravid ovaries. Beneath the smooth muscle layer there is another layer of collagenous connective tissue which is similar in width to the outer layer of connective tissue and is also at right angles to the smooth muscle. Finally there is the germinal layer of developing sex cells and nutritive phagocytes. There are two structures, 0.25-0.5 microns in width,

similar to basement membranes in the ovarian wall. The first lies between the peritoneal epithelium and the outer layer of connective tissue and the second between the inner layer of connective tissue and the germinal layer.

Annual Reproductive Cycle

In discussing the yearly reproductive cycle of Strongylocentrotus purpuratus the five stages of Fuji (1960) outlined above will be used. The yearly gross and histological changes as well as temperature and salinity data are summarized in Table 1.

Recovering Spent Stage

In urchins found along the central Oregon coast, this stage occurs from late summer to early fall, although there is a great deal of individual variation in this and other stages. The ovary at this time is small but firm and is slightly larger and more substantial than ovaries that have just been spent. Its color will vary from tan to orange depending on the number of dark brown degenerating bodies (discussed below) in the ovary and the amount and size of the immature eggs.

In histological section the accessory cells which are also referred to as nutritive phagocytes completely fill the entire ovary obscuring the lumina except for the small oöcytes that abut against the

wall of the ovary. These phagocytes contain many eosinophilic droplets (Plate 1), but the droplets are not as densely packed nor as numerous as in later stages. These globules stain very intensely with the periodic acid Schiff method, indicating large amounts of polysaccharides (Plate 8). Glycogen extraction only reduces the intensity of the stain evenly over the entire ovary, indicating that while glycogen may be present in large amounts other polysaccharides make up the greater percentage of these globules. These cells also contain many lipid inclusions (Plate 11A), and their nuclei are Feulgen positive.

Among the phagocytes many sex cells can be seen with an irregular or indistinct outline. Also, dark brown granules (Plate 1) and numerous acidophilic inclusions (Plate 17A circles) are present, although the granules and inclusions are somewhat more common in later stages (Plates 2A and 17A circles). The brown granules are Feulgen positive, indicating they contain DNA and are probably breakdown products of nutritive phagocytes, developing eggs of perhaps both (Plate 20). There are often other acidophilic granules in or between the nutritive phagocyes that contain concentrations of RNA and occasionally others which contain both RNA and DNA (Plates 14, 15A, and 18). Acidophilic phagocytes are often found to enclose a space, and sometimes the acidophilia will extend from the phagocytes into the space itself (Plate 17A).

Table I. Ovarian Cycle

	Apr.-Aug.	Aug.-Oct.	Oct.-Nov.	Nov.-Jan.	Jan.-Apr.
Nutritive Phagocytes	empty & refilling	lightly globulated	heavily globulated	same	emptying
Oogonia	very numerous	very few	few	more numerous	numerous
Dictyotene Oocytes	none growing 5-10μ	10-20μ	20-30μ	up to 60μ all sizes	same
Ova	degenerating	none	none	few	many
External Appearance	dark brown	dark brown	brown to orange	brown to orange	orange
	flaccid small	firm small	firm medium	"fluffy" full size	ripe full size
Surf Temperature °C Newport 1966*	8-16	10-15	10-14	9-12	8.5-11
Surf Temperature °C Depoe Bay 1967*	11-18	13-18	10-13	10-12	9-11
Salinity ‰ Newport 1966*	28-33	32-34	29-33	22-32	27-33
Salinity ‰ Depoe Bay 1967*	28-34	28-34	32-34	32-34	29-32

* Temperature and salinity data from Wyatt and Gilbert, 1967 and Gilbert and Wyatt, 1968.

Oögonia are very difficult to find in the recovering spent ovary; they occur singly or in very small groups scattered along the wall of the ovary. These oögonia, whose diameter is an average of five microns, have a scanty cytoplasm that does not stain specifically. Generally two small dense nucleoli distinguish them from primary oöcytes of the same size. The nuclei of the oögonia are slightly Feulgen positive. At this stage no primary oöcytes undergoing the early stages of the first meiotic prophase can be seen.

However, many small primary oöcytes just beginning their growth or dictyotic phase, resting or diffusion stage of Tennent and Ito (1941), can be seen along the wall of the ovary (Plate 1). There is generally an interval of about 20 microns between each one and they are slightly elongated in their axis parallel to the ovarian wall with their narrowest width ranging from 10-20 microns. The cytoplasm of these small oocytes shows a great concentration of azure B positive material which does not stain with pyronin (Plate 17A). Although some polysaccharide is present in the cytoplasm at this stage, it is very scant compared with the dense concentration in the adjacent phagocytes. No lipid appears in the oöcytes at this time and no Feulgen stain can be seen in the germinal vesicle.

The most prominent feature of the young oöcytes is the nucleolus, which is three to five microns in size. At this stage it often appears to be more eosinophilic than the cytoplasm, for it contains

not only material stainable with both pyronin (Plate 14), and azure B (Plate 17), but also non-diastate extractable polysaccharides (Plate 9). Despite this the nucleolus appears very dense and homogenous during this stage.

Growing Stage

In the late fall the ovaries enter the growing stage during which time they increase greatly in size. They have a mealy texture and vary in color from a cream to the golden orange color of mature eggs. At the cellular level, the nutritive phagocytes are now densely filled with polysaccharide and lipid globules (Plates 8 and 11B), and a large vacuole is present in many of these cells (Plate 2B). The accessory cells have attained their maximum size of 15-30 microns in diameter, and are irregular in shape due to crowding. Their cytoplasm is pyranophilic at this stage (Plate 11D and 14A) but only a barely perceptable bluing occurs with azure B (Plate 17B). The brown degenerative bodies are more numerous than in the previous stage. Many oöcytes seem to have indistinct borders where they abut with phagocytes, although this is difficult to ascertain exactly with the light microscope.

The oöcytes have attained a size of 20-30 microns in diameter; most are slightly elongate in the axis perpendicular to the acinar wall, while some are spherical. The cytoplasm of these oöcytes is

similar to that described in the previous stage, but the azure B stain is not as intense.

On the other hand, a slight change has occured in the nucleolus, it is more intensely pyranophilic than in the previous stage (Plate 11D and 14B) and often possesses one or two small vacuoles (Plate 19B). It has not kept pace with the growth of the rest of the cell and appears to be about the same size as it was in the previous stage. Scattered particles appear in the germinal vesicle that are as acidophilic as the nucleolus but stain neither as RNA or polysaccharides.

Premature Stage

In this stage which occurs in early winter the ovaries are golden orange and take on the outward appearance of mature ovaries. Although there are a few ripe eggs present in the ovary, they will not be shed even with the stimulus of KCl injections. The nutritive phagocytes remain much as described in the previous stage, but those in the more central portions of the ovary have lost their pyranophilia. Nests of oögonia and primary oöcytes in the pre-dictyotic phases are now quite frequent (Plate 3B).

The uniformity in the size of the growing oöcytes is lost, for all sizes can be found from the smallest to the largest which are about 55-65 microns in diameter, the size of the mature ovum. The

smaller oöcytes are usually found in the more peripheral acini. The cytoplasm of these small oöcytes stains much more intensely with azure B than that of the larger oöcytes (Plate 11A) while the larger oöcytes contain much more polysaccharide (Plate 10B arrows), and lipid appears in their cytoplasm for the first time (Plates 11C and 12). Although these two constituents are also found in the globules of nutritive phagocytes, they occur in the cytoplasm of the oöcyte as much finer grains. In the cytoplasm of a very few of the largest oöcytes there occasionally appears an irregular pyranophilic area for which there is no corresponding area marked by azure B. The nucleolar vacuoles become larger and more numerous and make up a large portion of the nucleolar area in the biggest oöcytes. Usually these vacuolated nucleoli have lost their pyranophilia, but still show visible staining with azure B and the PAS technique.

At the time of the maturation divisions the germinal vesicle becomes ill-defined as its membrane breaks down and the nucleolus diappears. The oöcyte either moves or is pushed toward the center of the acinus where meiosis takes place. This movement displaces the nutritive phagocytes that had formerly been in the center, but no patent lumen is formed. The spindle of the maturation divisions forms near the plasma membrane on one side of the egg, thus producing small polar bodies (Plate 4).

As the season progresses the ovary becomes studded with

the small pyknotic nuclei of the polar bodies, which can be identified most readily with the Feulgen stain (Plate 20A arrows). Few eggs can be found undergoing maturation divisions at any one time and it is often necessary to hunt carefully in serial sections to find any at all. The nucleus of the mature ovum is very small, seven to ten microns in diameter, as compared to the germinal vesicle of the oöcyte which is 20-25 microns in diameter. The chromosomes, which are now concentrated enough to be visibly stained by the Feulgen method, can be seen as small droplet-like areas adhering to the nuclear membrane.

Mature Stage

The ovaries usually become mature about mid-winter and the breeding season commonly lasts well into the spring. The ovaries are colored a golden orange by the mass of mature ova which they contain. They will shed after almost any mild shock and with KCl they will shed almost immediately.

There is often a large pyranophilic area in ova that are about to be shed and those that have already been shed. While still within the ovary this area is irregularly shaped, as are the ova themselves due to crowding, but once the ova are released both they and the pyranophilic area within them assume their normal spherical shape (Plate 16).

The mature ova within the ovary have displaced the nutritive phagocytes to the outside of the acinus where they form a single layer containing the oöcytes that have not yet matured (Plate 5A). The more peripheral acini retain an aspect very similar to that found in the premature ovaries, except that there tends to be a great variation in the sizes of the oöcytes (Plate 5B). Usually the smallest oöcytes, ten microns in diameter, occur in clusters in association with one or more nests of oögonia and pre-dictyotic oöcytes, all of which become more frequent as the season progresses (Plates 5B and 6B circles).

Due to the presence of continually growing oöcytes the urchin is able to shed several times during the breeding season. A definite, progressive change can be noted in the phagocytes over this time, which starts with the cells in the more central portion of the ovary and proceeds toward the more peripheral acini over the course of the season. The lipid and polysaccharide droplets within these cells become fewer in number (Plates 6 and 10) and eventually are entirely depleted in the spent stage (Plates 7 and 13). At this point many of the phagocytes disappear but some do however remain, identifiable only by their plasma membrane and small nucleus.

Toward the end of the breeding season the size of the oöcytes becomes progressively smaller as less cytoplasm is produced. They are elongate as opposed to the spherical or elliptical shape common

earlier in the season (Plate 6B). Usually these oöcytes are not shed even if they do mature and the ovary is now termed spent.

Spent Stage

At this time the external aspect of the ovary looks very much reduced, flabby, and has a dark brown color due to the presence of brown degenerating bodies. The nutritive phagocytes are empty, giving the inside of the ovary an open mesh appearance. Quite a few deteriorating, unshed ova can be seen in the now open lumina (Plates 6B and 7).

One very interesting and important event occurs at this time: the oögonia, pre-dictyotene oöcytes, and very small dictyotene oöcytes that have been slowly increasing in numbers over the course of the year are now quite numerous (Plate 7A). Together these various types of germ cells form a layer two or three cells thick just under the wall of the acini. As fall approaches the number of oögonia and oöcytes decreases, but some of the small oöcytes remain and begin growing along the wall of the ovary (Plate 1). The nutritive phagocytes begin refilling with globules as the ovary once again enters the recovering spent stage (Plate 1).

IV. DISCUSSION

A study of the annual reproductive cycle of the sea urchin shows that there is no period of the year when the ovary can be considered dormant. The cycle can roughly be divided into two parts, the first when the phagocytes are filling with globules and the majority of the sex cells are in the dictyotene phase, and the second when the phagocytes are being depleted of their globules and sex cells are in stages earlier than the dictyotene phase are numerous. Since the large growing oöcytes are most plentiful at that time of the year when the phagocytes are full of globules it seems that the globules are necessary for oöcyte growth, especially since polysaccharide and lipid appear in the phagocytes before they are seen in the cytoplasm of the oöcytes.

Although indistinct borders do occur between phagocytes and growing oöcytes as seen with the light microscope (Plate 19B arrow), it is unclear whether this represents an engulfment of the oöcyte by the phagocyte or active transport in the reverse direction. While Miller and Smith (1931) report incorporation of degenerating accessory cells by the oöcytes in Echinometra lucunter, the present author concurs with Holland and Giese (1965) in that no phagocytosis of the nutritive cells by the oöcytes was observed. Verhey and Moyer (1967a) report that no pinocytotic vesicles occur in the plasma

membrane of the oöcytes and therefore RNA and proteins elaborated in the nutritive phagocytes are not taken up by the oöcytes; however, these authors did not study the oöcytes over the course of the year, and it may be that pinocytosis occurs at a stage during the annual reproductive cycle other than the one they investigated. The same authors (1967b) also found none of the polysaccharide in the accessory cells of the sea urchins they examined was glycogen. On the other hand Strongylocentrotus purpuratus has large amounts of both glycogen and non-glycogen polysaccharides in its accessory cells (Plates 8, 9, and 10).

While it is not clear if the sex cells obtain nutriment directly from the phagocytes, there is evidence that the phagocytes engulf sex cells. Fuji (1960) reports absorption of unshed degenerating ova. Many phagocytes in the recovering spent phase contain RNA and DNA positive inclusions that under close inspection with the light microscope appear to be degenerate pre-dictyotene and early dictyotene sex cells (Plate 2A). Certainly this mechanism would account for the drastic reduction from the large number of immature sex cells seen along the walls of the spent ovary to the very few that are found in the late recovering spent ovary or in the early growing stage. This raises the question of whether the phagocytes exercise some control on the number of oöcytes that can develop.

Another question concerning the phagocytes is what causes the

cyclical appearance and disappearance of the globules contained within them. If the oöcytes do indeed derive nutriment from the phagocytes, it is easy enough to explain their deglobulation. However, it is unlikely that the phagocytes are completely refilled by incorporating degenerating ova or developing oöcytes for there are not enough of these to account for the abundance of polysaccharide and lipid that appears within them during the globulated stage. It is more reasonable to suppose that this variation is due to greater availability of food in the animals' habitat during the time when the phagocytes are filling and its relative scarcity when they are becoming depleted. Fluctuations in the food supply may likewise explain the great variability in gonad size and fertility from year to year (Boolootian, 1966).

It has also been suggested (Boolootian, 1966) that temperature and salinity may have some influence on the timing of the reproductive cycle. From the data available (see Table I) there seems to be a correlation between the filling of the phagocytes and temperature, but none with salinity. The water temperatures and salinities recorded are those of the open surf and would be correct for subtidal urchins; however, the intertidal ones caught in the tide pools would probably be exposed to much higher temperatures and increased salinity due to evaporation.

The seasonal variation in the amount of oögonia and predictyotene oöcytes raises the question of where the additional oögonia

come from. Tennent and Ito (1941) suggested that they were derived from the peritoneal epithelium, but they were unable to present any proof. Verher and Moyer's (1967a) work could have shed considerable light on this matter, but they adopted Tennent and Ito's theory without studying the matter further. Although the incorporation of DNA precursors was followed only over the course of one year (Holland and Giese, 1965), it does not give any support to Tennent and Ito's theory and in fact indicates that oögonia are derived from previously existing ones by mitosis. The cells of the visceral epithelium are highly differentiated, flagellated cells of the type described by Lyons, Bishop, and Bacon (1968) and occur commonly in other parts of the visceral epithelium as well. Furthermore, the muscle and collagen layer between the peritoneum and the inside of the ovary is very dense (Wilson, 1940) and would seem to preclude any migration through it.

No positive Feulgen reaction is seen in germinal vesicles of the dictyotene oöcytes because the DNA is presumably too dilute to be detected by this method. In the mature egg a Feulgen positive reaction can again be seen for the chromosomes have recondensed as small droplet-like areas adhering to the nuclear membrane. This configuration is apparently characteristic of sea urchins for it has also been reported by Burgos (1955) in Arbacia punctulata and by Agrell (1958, 1959) in Paracentrotus lividus, Arbacia lixula, and

Spantangas pareus.

The discrepencies between the results obtained with the pyronin and azure B methods for staining RNA can be explained if it is understood that they are staining different species of RNA. Immers (1961) stained unfertilized sea urchin eggs with pyronin and stated that if the phosphate groups of RNA are combined with protein as they are when the RNA is actively synthetic, the RNA will not take up the stain. On the other hand Flax and Himes (1952) state that azure B competes with protein for the phosphate groups of RNA and therefore is apparently able to stain RNA that would normally be combined with protein by replacing the protein. If this is true, then the azure B stains the RNA that is more highly saturated with protein and therefore presumably synthetically active while the pyronin stains the relatively inactive RNA that is not highly saturated with protein. On this basis the growing oöcytes contain actively synthetic RNA while the RNA found in the nutritive phagocytes is mainly inactive. Alternatively, this staining phenomenon may have something to do with the fact that some of the RNA is blocked with a protein inhibitor while the rest is not. The appearance of pyranophilia in mature ova (Plate 16) may correspond to the inactive form of messenger RNA said to be present in unfertilized sea urchin eggs (see reviews by Gross, 1967; Spirin, 1966). The position and time of appearance of the pyranophilia may correspond to the appearance of the heavy bodies

(Afzelius, 1957; Harris, 1967) for it rarely occurs in the oöcytes before they have undergone the maturation divisions and likewise heavy bodies do not appear until this time (Verhey and Moyer, 1967a). Unfortunately, the captions of the pictures (Figures 19 and 20) in their report, Verhey and Moyer (1967a) refer to ova as mature oöcytes which makes interpretation of their electron micrographs quite confusing.

Recently, Scheutz and Biggers (1967) have shown that there is a hormone that can be extracted from the radial nerve of the starfish, Asterias forbesi, that induces germinal vesicle breakdown and subsequent maturation division. It is possible that a similar mechanism may be at work in sea urchins and it may also cause the oöcytes to migrate from the ovarian wall to the central lumen. Another possibility is that the larger oöcytes may be capable of limited amoeboid movement that is largely supressed by the crowding of the nutritive phagocytes. When the phagocytes are smaller and deglobulated at the end of the breeding season, they no longer suppress this amoeboid movement and the oöcytes take on a very elongate shape (Plate 6B).

V. SUMMARY

The reproductive cycle of the ovaries of the sea urchin Strongylocentrotus purpuratus collected at Yaquina Head, Oregon was studied by using histological methods as well as histochemical techniques for the demonstration of polysaccharide, lipid, and nucleic acids.

The sea urchin has five separate ovaries; each covered by a flagellated peritoneal epithelium. The ovaries are large rebranched sacs and each saccule ends in a blind acinus. The wall of the ovary under the peritoneum is made up of collagenous connective tissue and smooth muscle. In the central portion of each acinus are two main cell types: the sex cells, which mature into ova, and the accessory cells or nutritive phagocytes, which apparently provide nutriment for the sex cells.

In the summer the nutritive phagocytes begin filling with polysaccharide and lipid globules. Dark brown granules that contain DNA and inclusions that look like degenerate sex cells are also found in the phagocytes. The sex cells are small and inconspicuous at this time and are found along the walls of the ovary.

As fall approaches the number of sex cells decreases but the ones remaining grow larger, and the accessory cells continue to fill with globules. The smaller dictyotene oöcytes stain more intensely

with azure B than do the larger ones, but the cytoplasm of the larger ones shows a higher concentration of lipids and polysaccharides. The nucleolus of the small oöcytes is very large and dense and contains polysacharides and RNA that stains with both pyronin and azure B. The nucleoli of the larger oöcytes contain the same components but they become less dense and vacuoles appear in them.

With the coming of winter some of the oöcytes start to mature into ova. The mature eggs move from the walls of the ovary to the central portion of the ovary displacing the accessory cells that had been there. The cytoplasm of ova that have been shed or are about to be shed contains pyranophilic RNA as well as RNA that stains with azure B. Oöcytes contain only the type of RNA that stains with azure B. The number of pre-dictyotene sex cells along the wall of the ovary begins to increase at this time. Nutritive phagocytes stop filling with globules and pyranophilic RNA appears within them.

The ovaries are mature from mid-winter to early spring and an urchin can shed eggs several times during this period since all the oöcytes mature at one time. The nutritive phagocytes are progressively depleted of their globules over the course of the breeding season. When all these globules are gone, the oöcytes no longer grow or mature and the ovaries are spent. In the spent ovary there are large numbers of pre-dictyotene and small early dictyotene oöcytes along the walls of the ovary. During the summer the

accessory cells once again begin filling with globules and the cycle starts again.

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APPENDIX

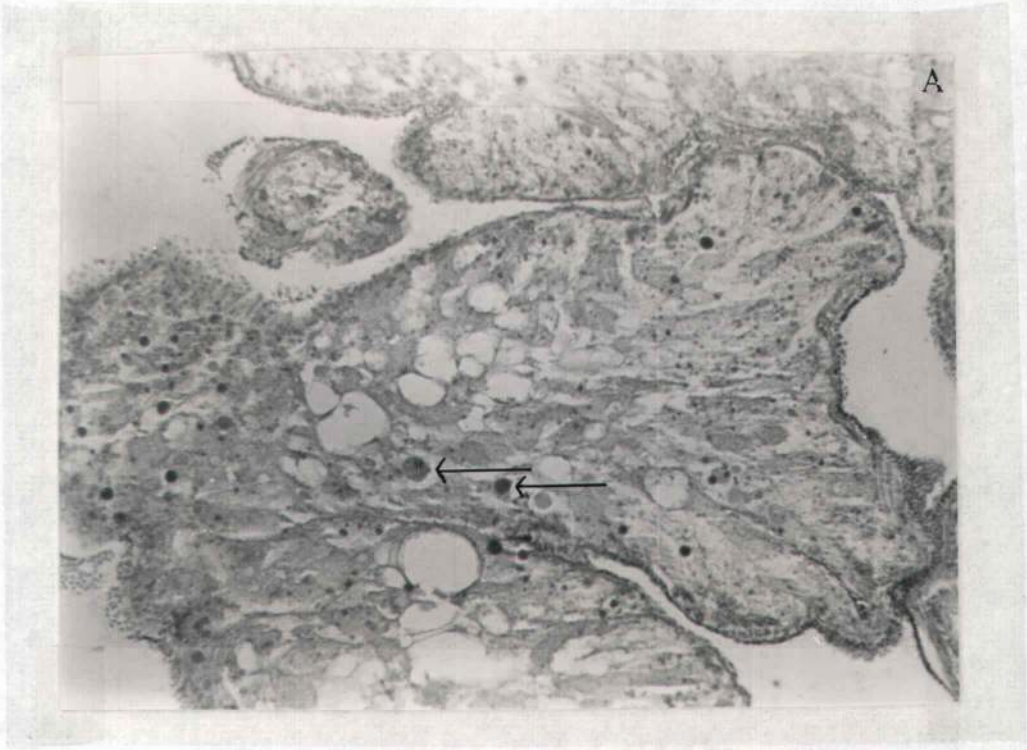
PLATE 1

A. An early recovering spent ovary with some full accessory cells (nutritive phagocytes) and many empty ones. There are many small sex cells along the ovarian wall. Note dark granular inclusions (arrows).

hematoxylin and eosin; 350 X

B. A late recovering spent ovary with accessory cells full of globules. Also note dark granular inclusions in accessory cells (arrows). See Plate 2A for an enlargement of inclusions. Small oöcytes along ovarian wall.

hematoxylin and eosin; 350 X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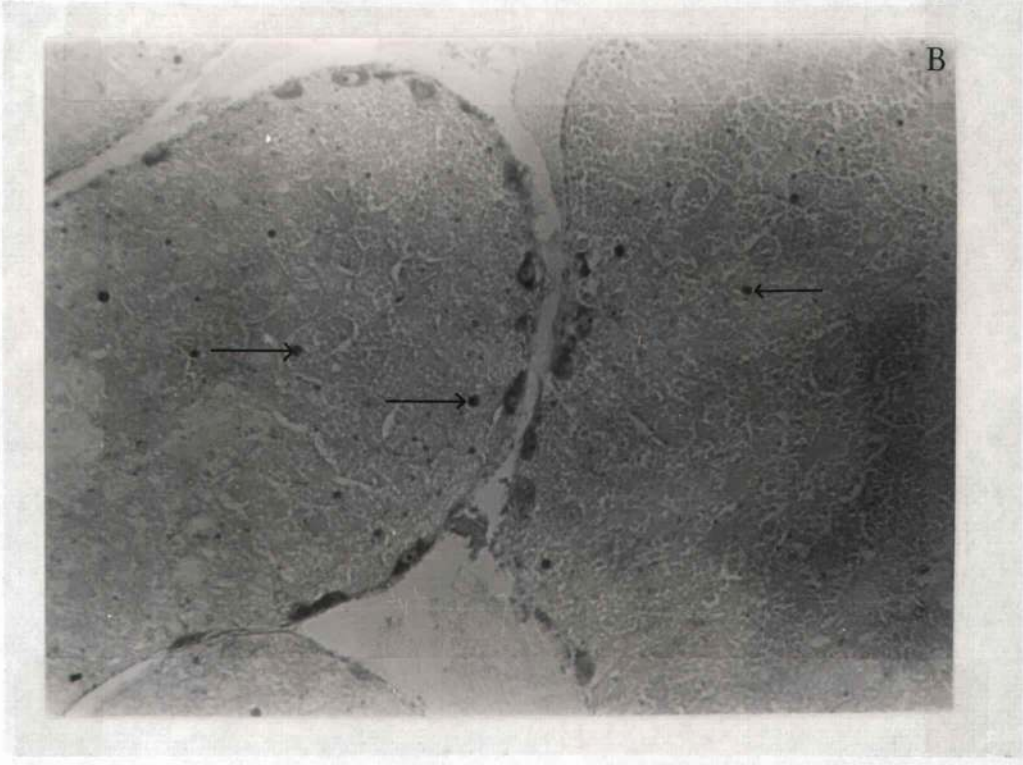
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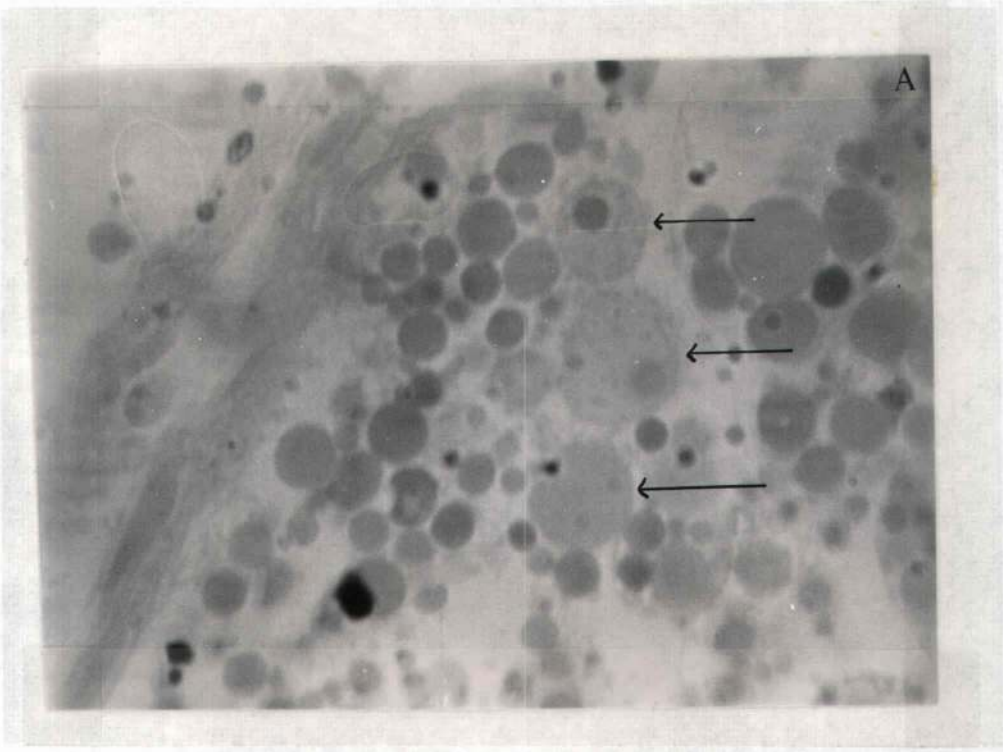
PLATE 2

A. Late recovering spent ovary, showing globular inclusions of the accessory cells and inclusions that look like nuclei of former sex cells (arrows).

gluteraldehyde fixed; osmium tetroxide post-fixed; araldite imbedded; methylene blue stained; 3395 X

B. Growing ovary with full phagocytes. Many of the phagocytes contain vacuoles (v). Size of the oöcytes is uniform.

hematoxylin and eosin; 350 X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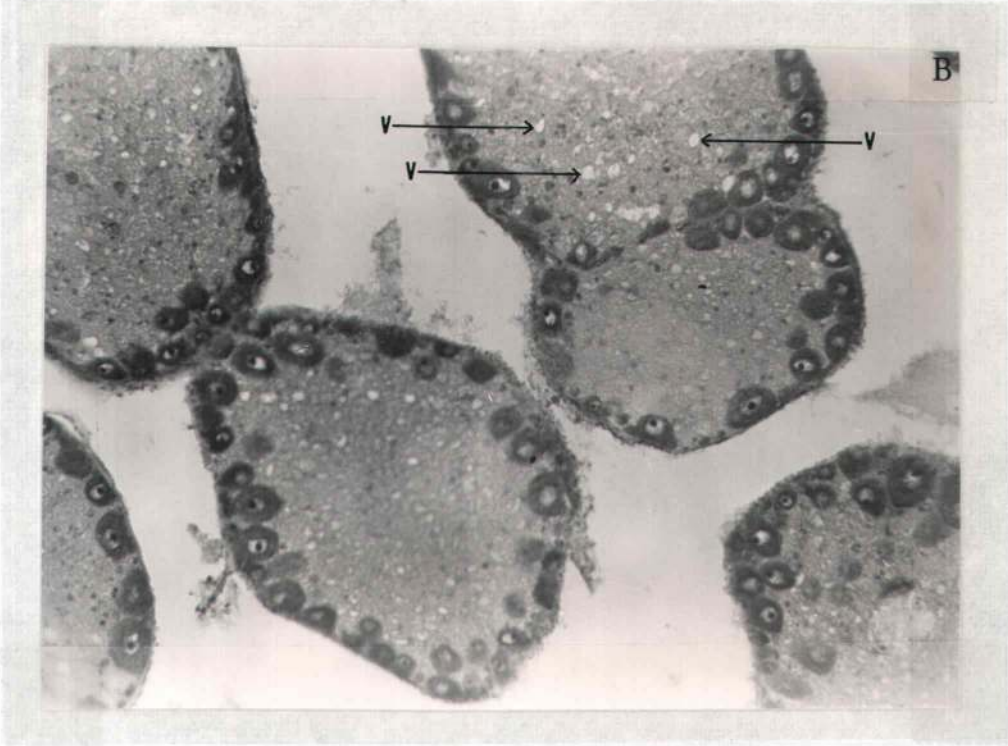
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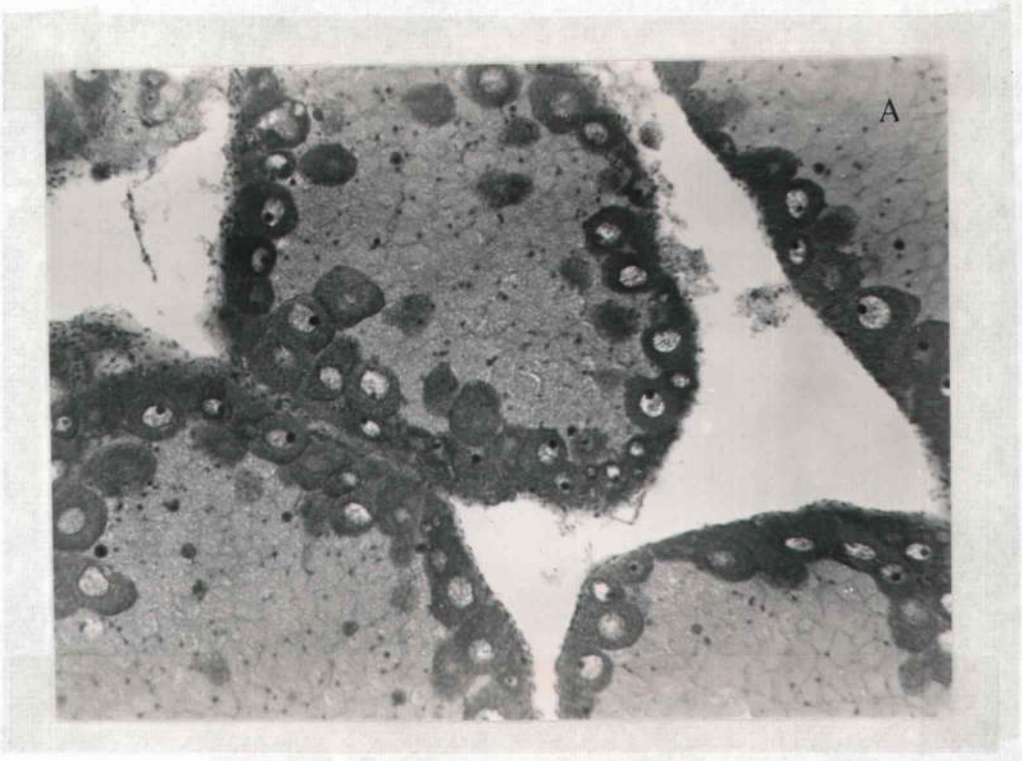
PLATE 3

A. & B. Premature ovaries have sex cells of all types, oöcytes have lost uniformity of size; many pre-dictyotene sex cells are present (circles).

hematoxylin and eosin; 350 X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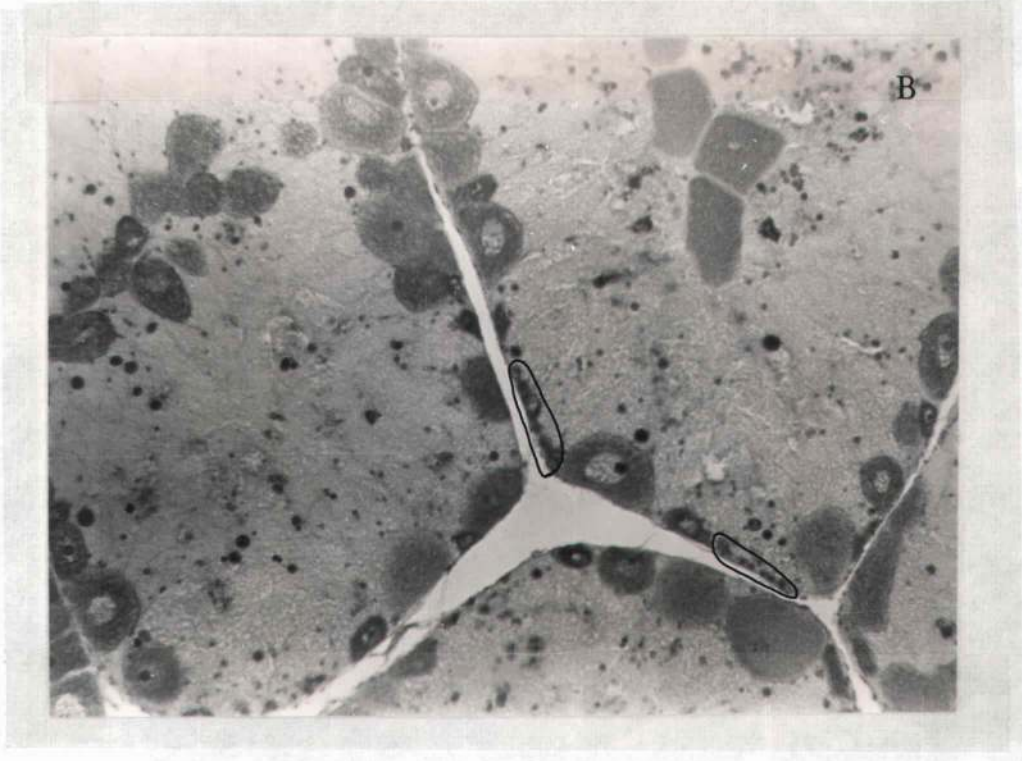
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PLATE 4

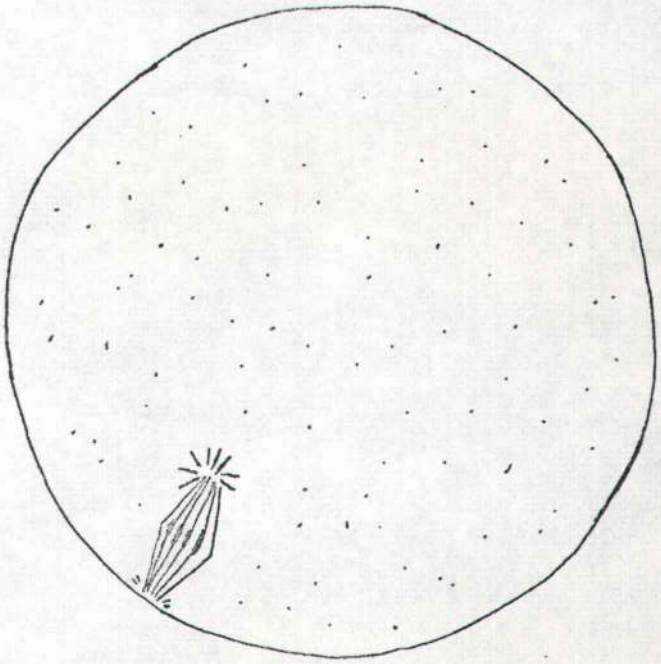
A. Diagram of meiotic metaphase.

about 2000 X

B. Meiotic metaphase. Tangential section cutting off part of egg with spindle. Note chromosomes.

hematoxylin and eosin; 3395 X

A



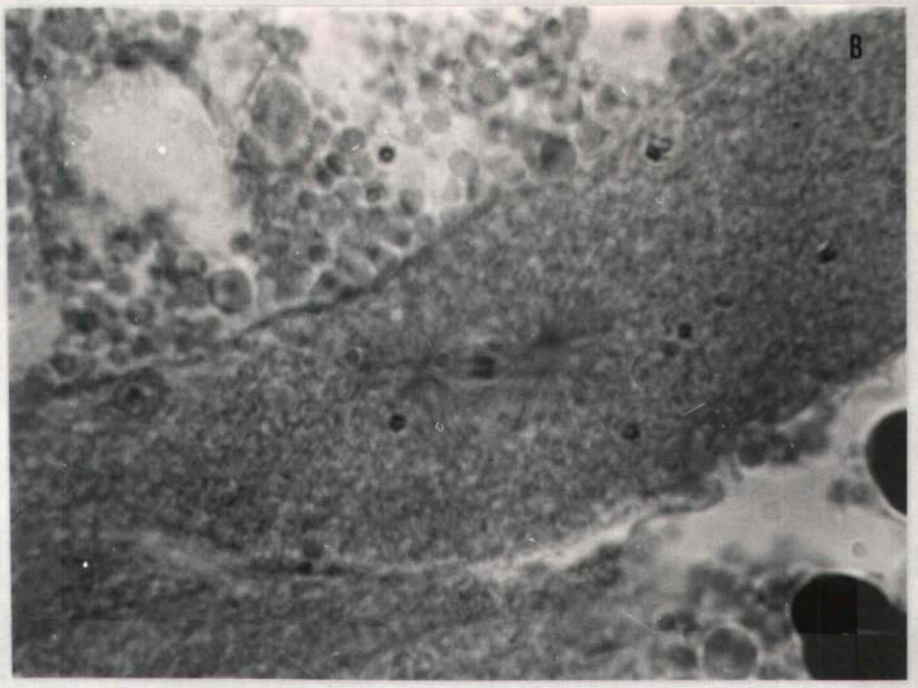


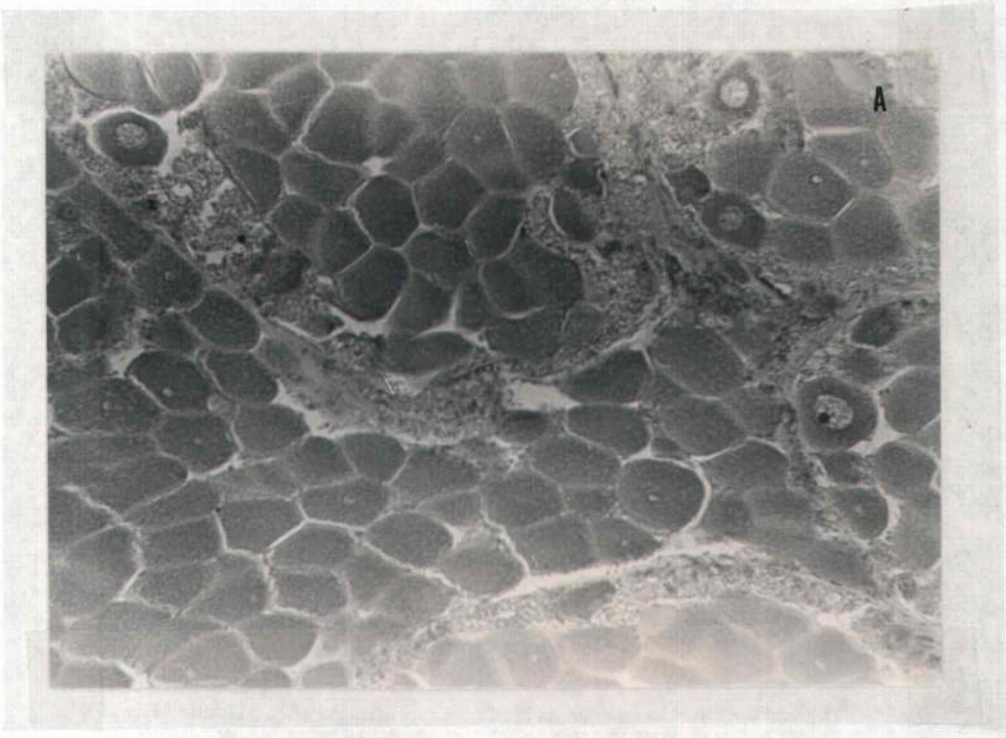
PLATE 5

A. Mature gravid ovary with layer of immature egg cells and accessory cells along acinar wall.

hematoxylin and eosin; 450 X

B. Mature ovary, peripheral acini; accessory cells still full of globules. Circles indicate nests of oögonia and small oöcytes.

hematoxylin and eosin; 350 X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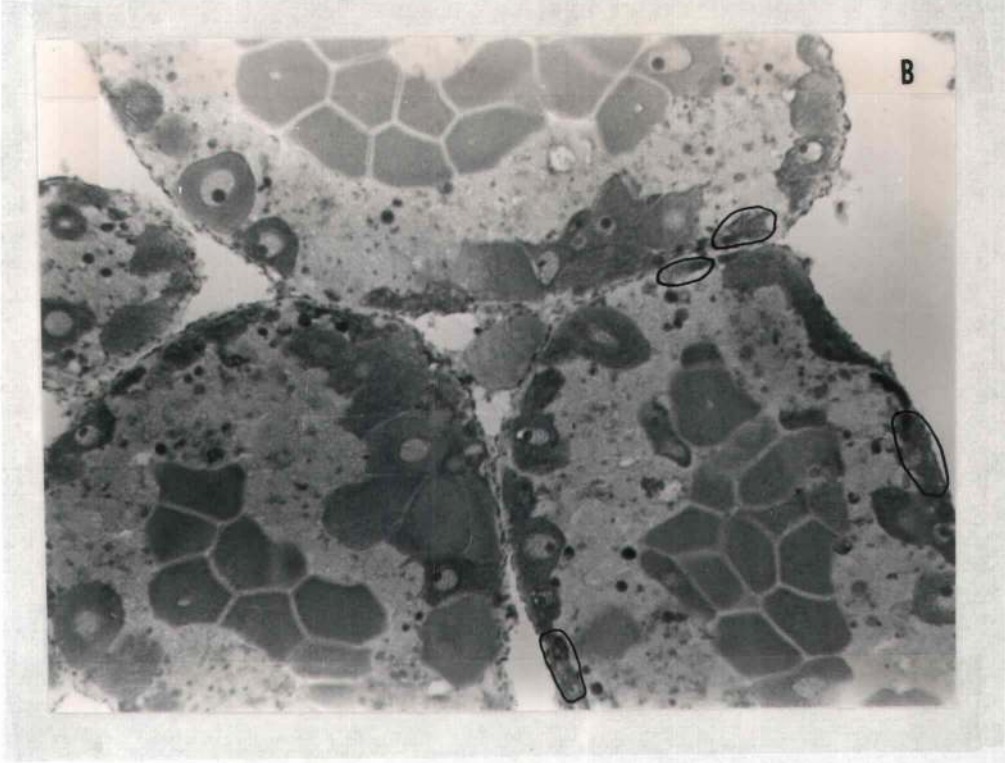
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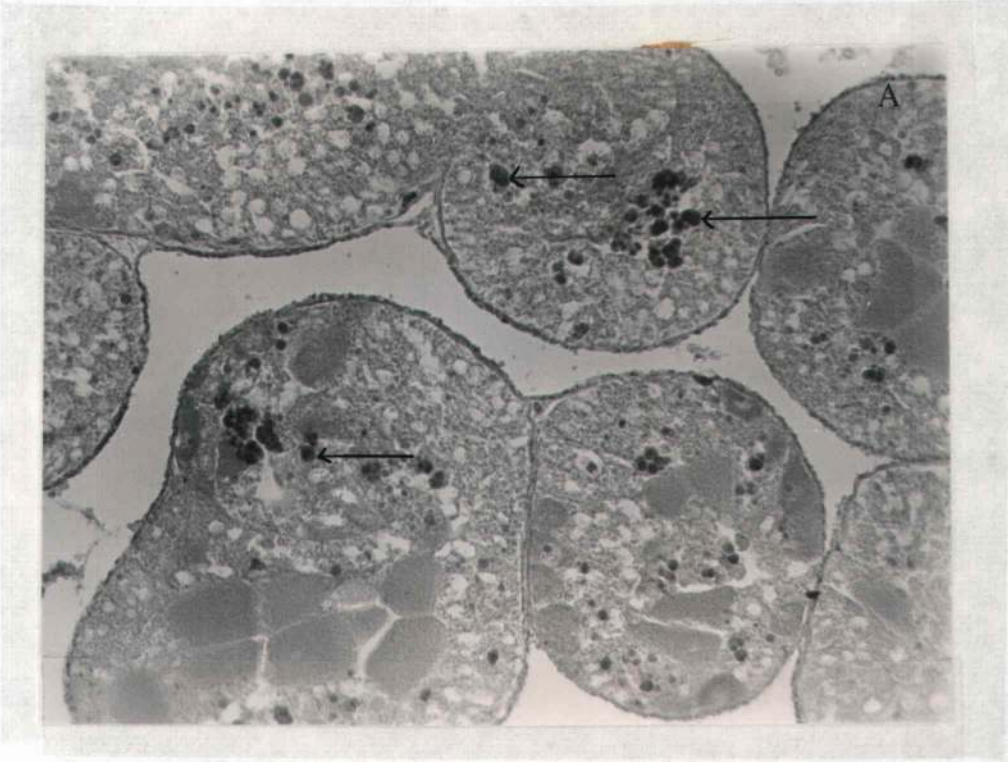
PLATE 6

A. Late mature ovary, peripheral acini; accessory cells emptying of globules. Note dark granular inclusions (arrows).

hematoxylin and eosin; 350 X

B. Spent ovary with elongate oöcytes; accessory cells empty. Circles indicate nests of oögonia and small oöcytes.

hematoxylin and eosin; 425 X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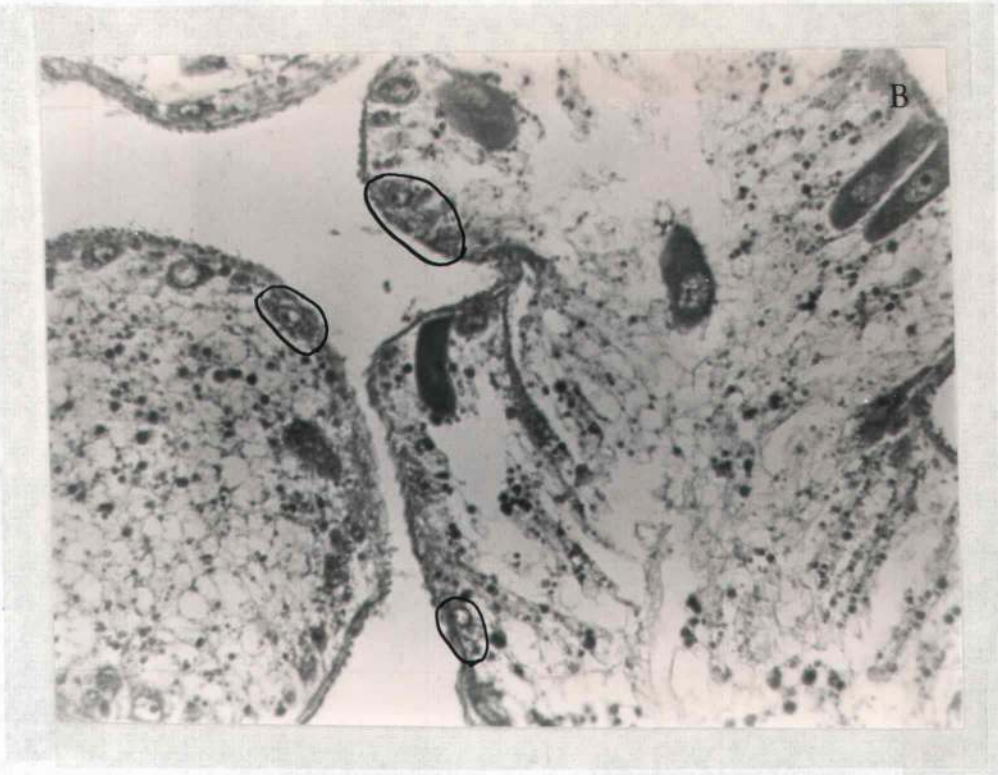
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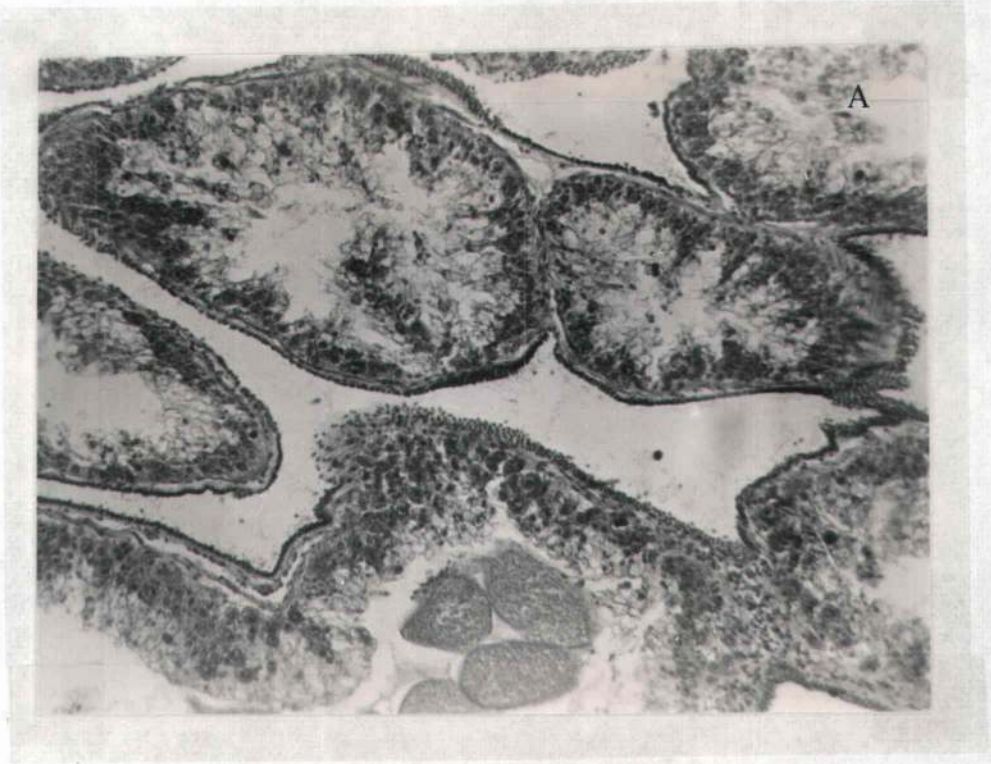
PLATE 7

A. Spent ovary with sex cells proliferating along ovarian wall and empty accessory cells. Note degenerating unshed ova in lumen.

hematoxylin and eosin; 350 X

B. Spent ovary. Number of sex cells along the wall has decreased. Note numerous dark granular inclusions (arrows).

hematoxylin and eosin; 425 X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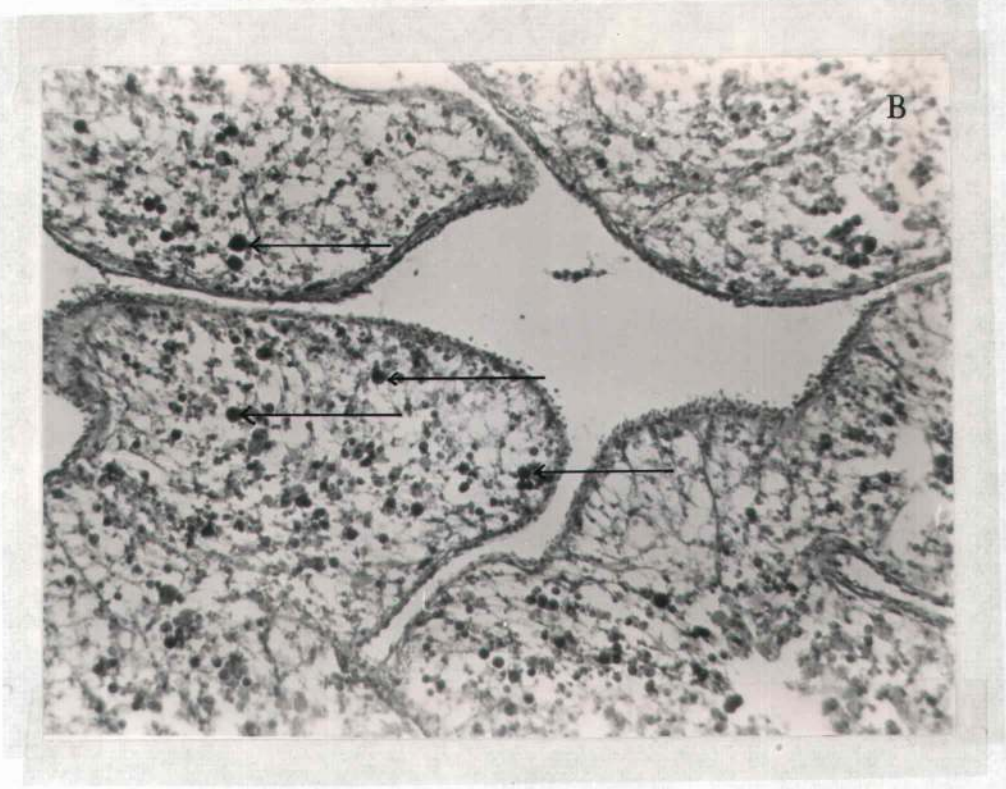
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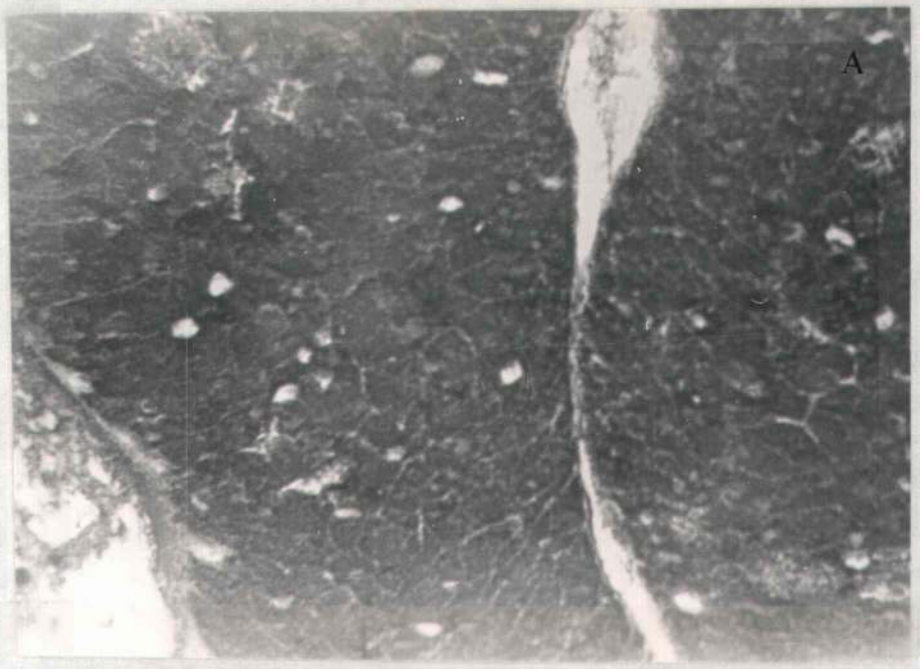
PLATE 8

A. Late recovering spent ovary with accessory cells showing intense PAS staining.

periodic acid Sciff (PAS); 425 X

B. Same as above with glycogen extracted.

diastase treated, PAS; 425 X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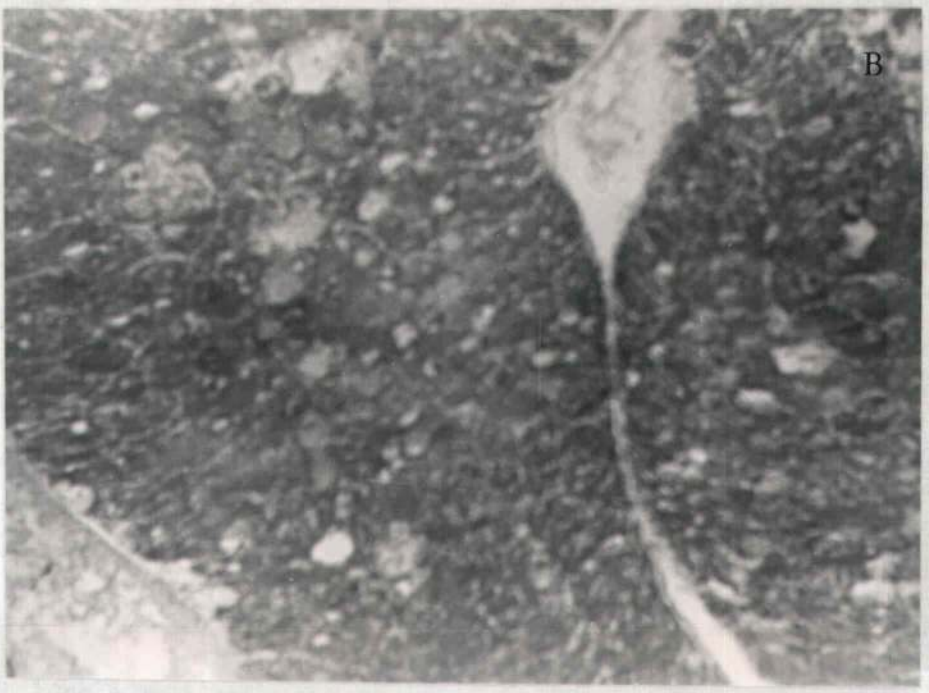
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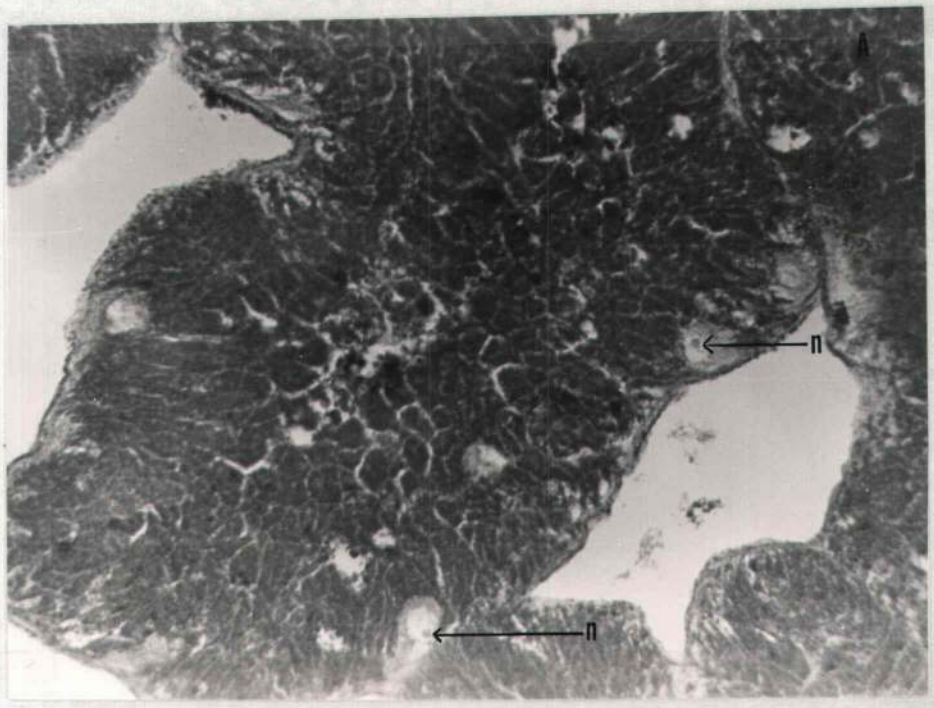
PLATE 9

A. Growing ovary. Oöcyte cytoplasm much lighter than accessory cells. Nucleolus (n) contains polysaccharides.

PAS; 425 X

B. Same as above with glycogen extracted. Nucleolus (n) still PAS positive.

diastase treated, PAS; 425 X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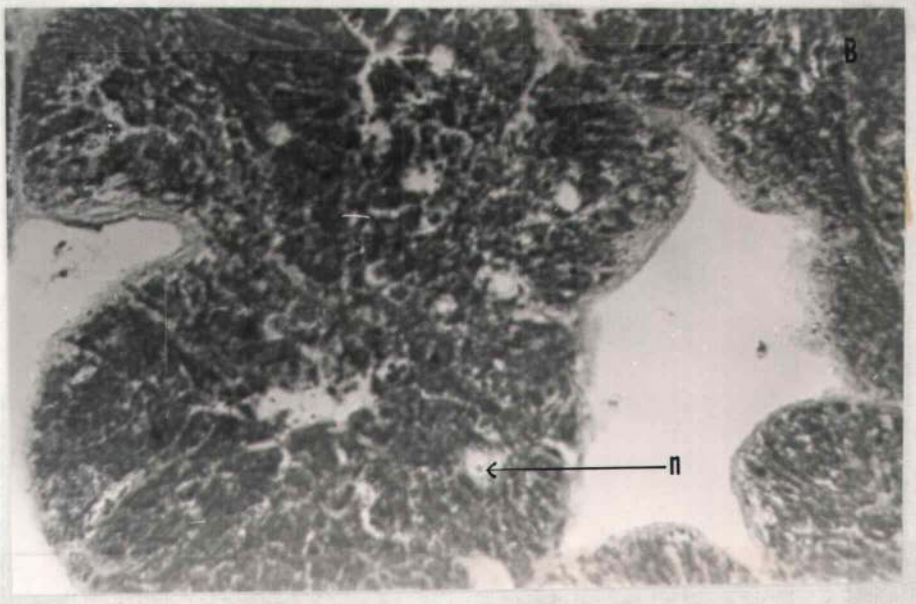
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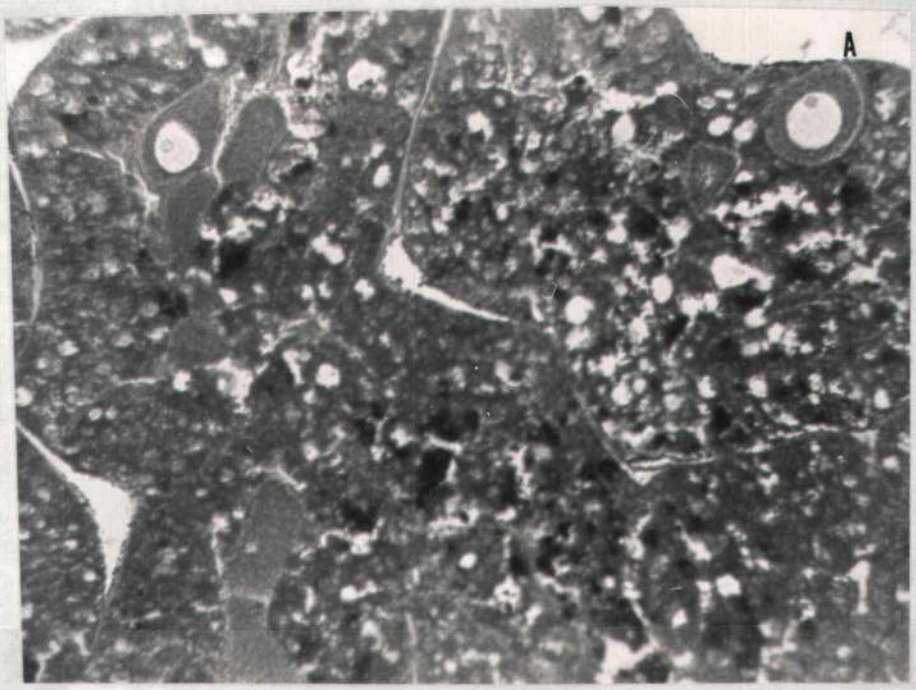
PLATE 10

A. Mature ovary, PAS stain not as intense as in accessory cells in Plate 9.

PAS; 425 X

B. Same as above with glycogen extracted. Growing oöcytes (arrows) have lighter staining cytoplasm than ova.

diastase treated, PAS; 425 X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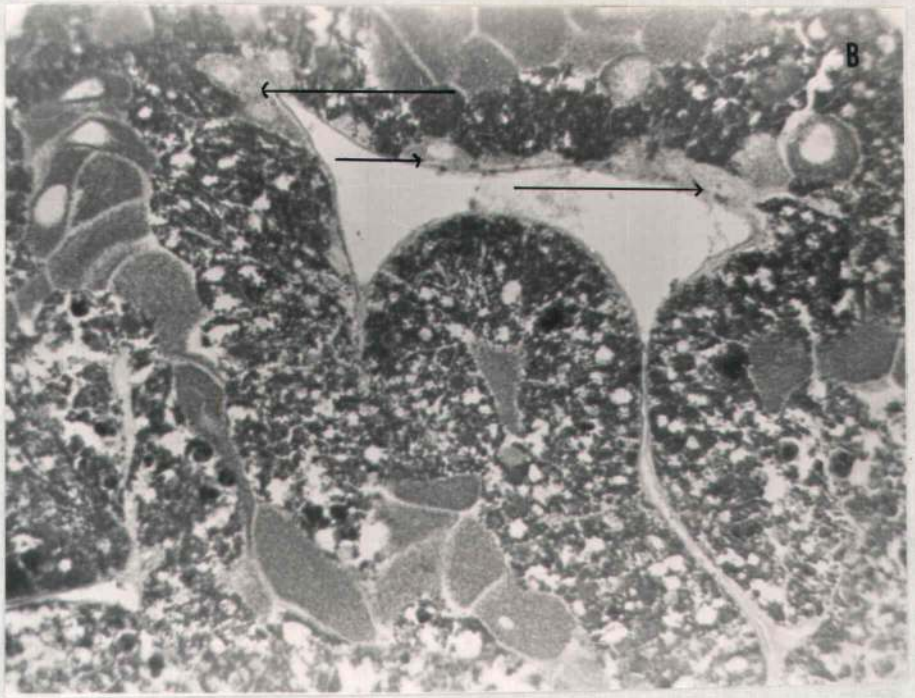
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PLATE 11

A. Late recovering spent ovary. Nutritive phagocytes stain red with oil red O, an indication of the presence of neutral lipids. Oöcytes stain dark blue.

gelatin imbedded; oil red O and Mayer's hematoxylin; 200 X

B. Growing ovary. Nutritive phagocytes stain red indicating presence of lipid. Oöcytes acidophilic.

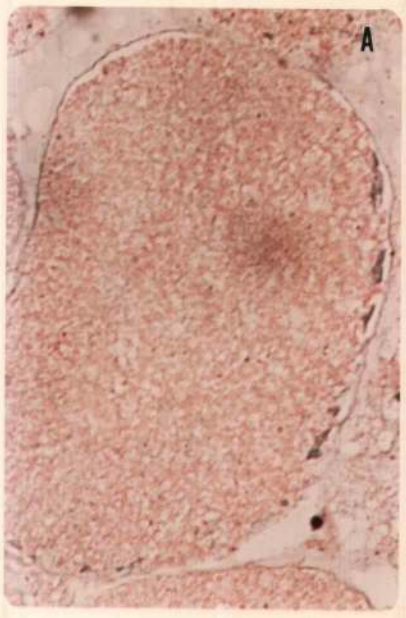
gelatin imbedded; oil red O and Mayer's hematoxylin; 200 X

C. Premature ovary. Grazing section through ovarian wall. Nucleoli of oöcytes are acidophilic and are stained light blue. Purple staining indicates the presence of both acidophilic and lipid containing substances.

gelatin imbedded, oil red O and Mayer's hematoxylin; 200 X

D. Growing ovary. Nutritive phagocytes pyranophilic. Nucleolus of oöcyte also pyranophilic (arrow), but cytoplasm of oöcyte is not.

methyl green and pyronin; 200 X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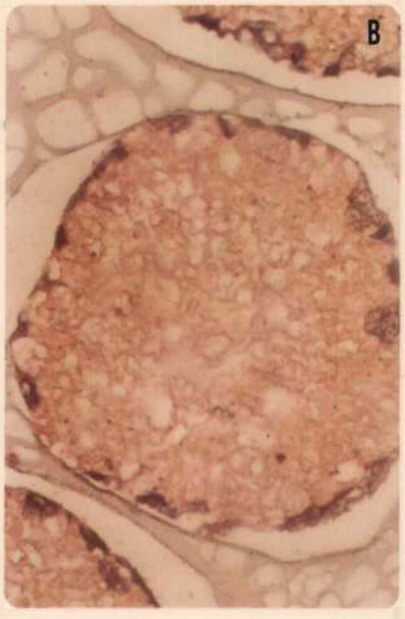
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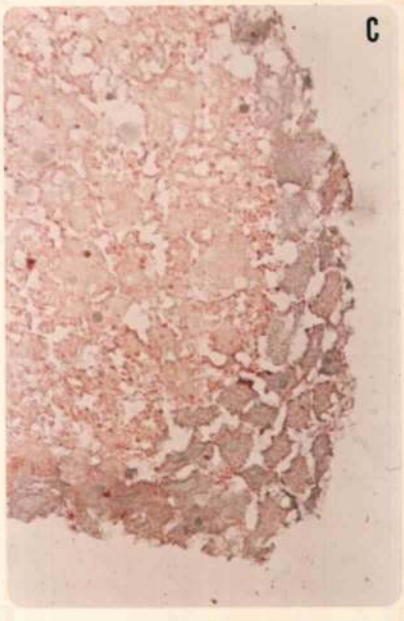
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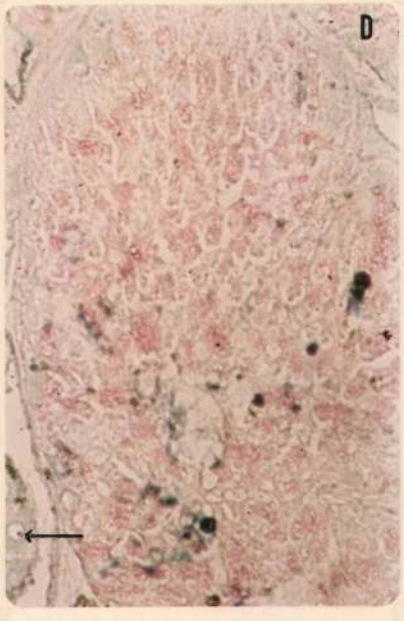
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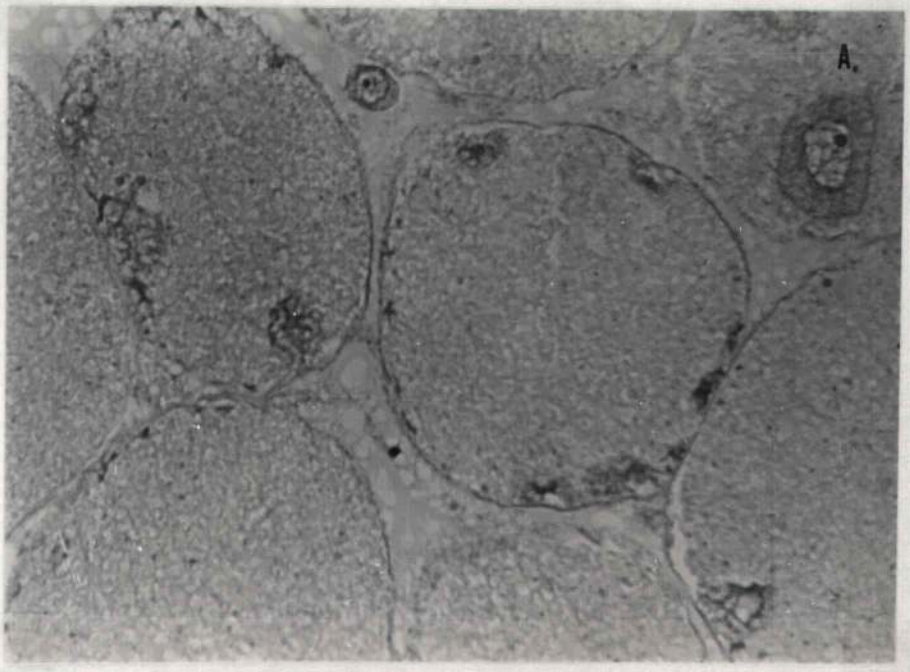
PLATE 12

A. & B. Premature ovary; phagocytes full of lipid globules; cytoplasm of large oöcytes lipid positive; small oöcytes stain only with hematoxylin. See also Plate 11 C.

gelatin imbedded; oil red O and Mayer's hematoxylin; 350 X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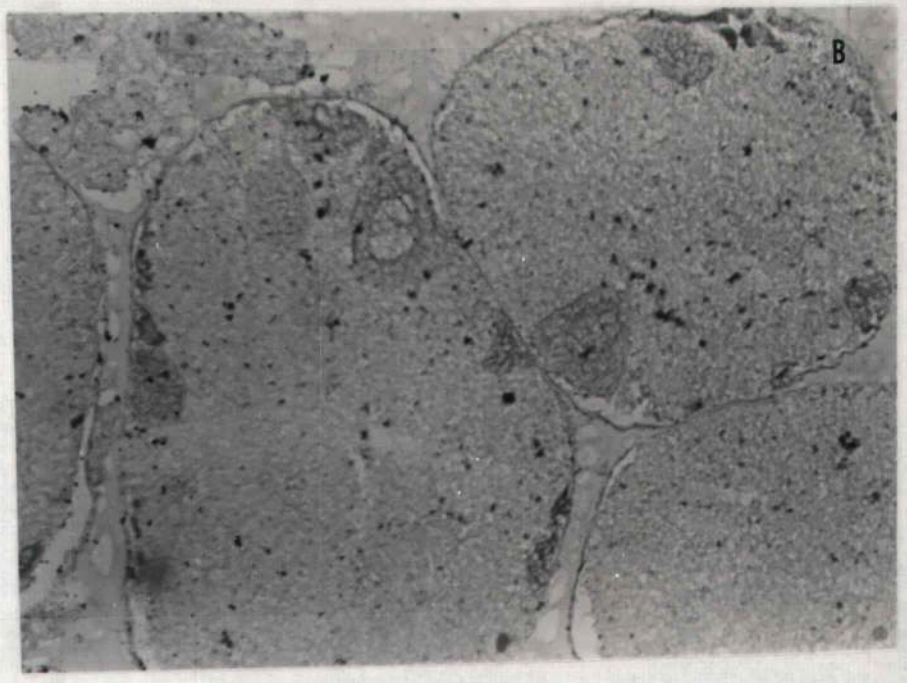
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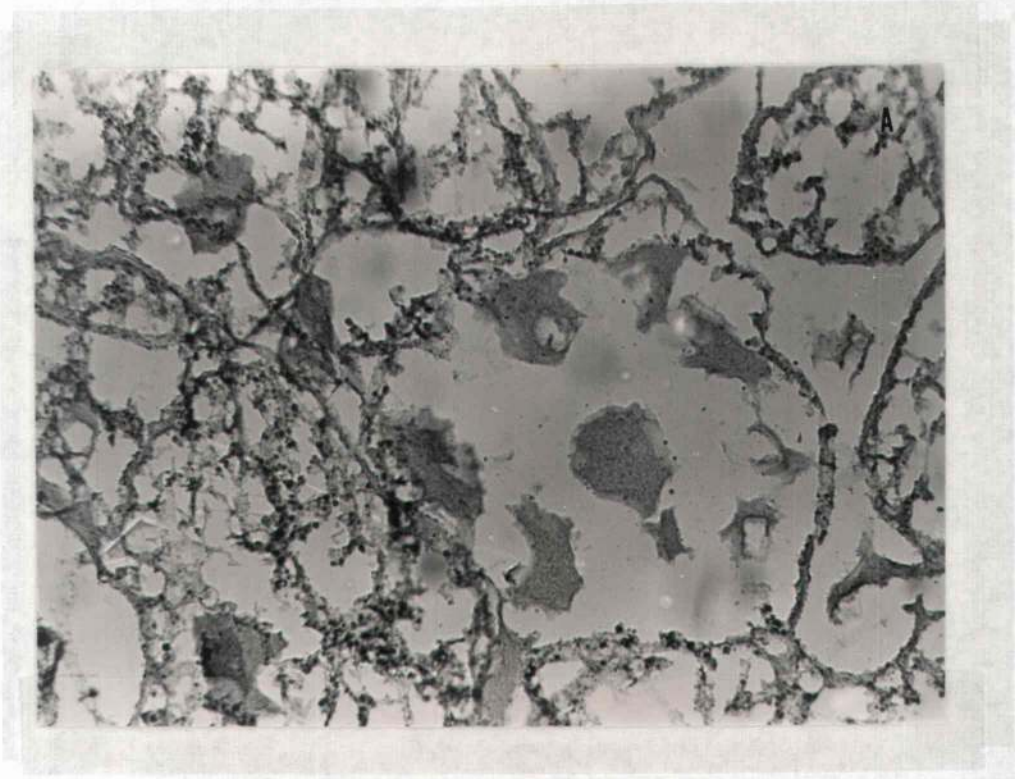
PLATE 13

A. & B. Spent ovary. Phagocytes devoid of lipid globules; ova stain only with lipid stain. No hematoxylin stain present. gelatin imbedded; oil red O and Mayer's hematoxylin; 350 X


A

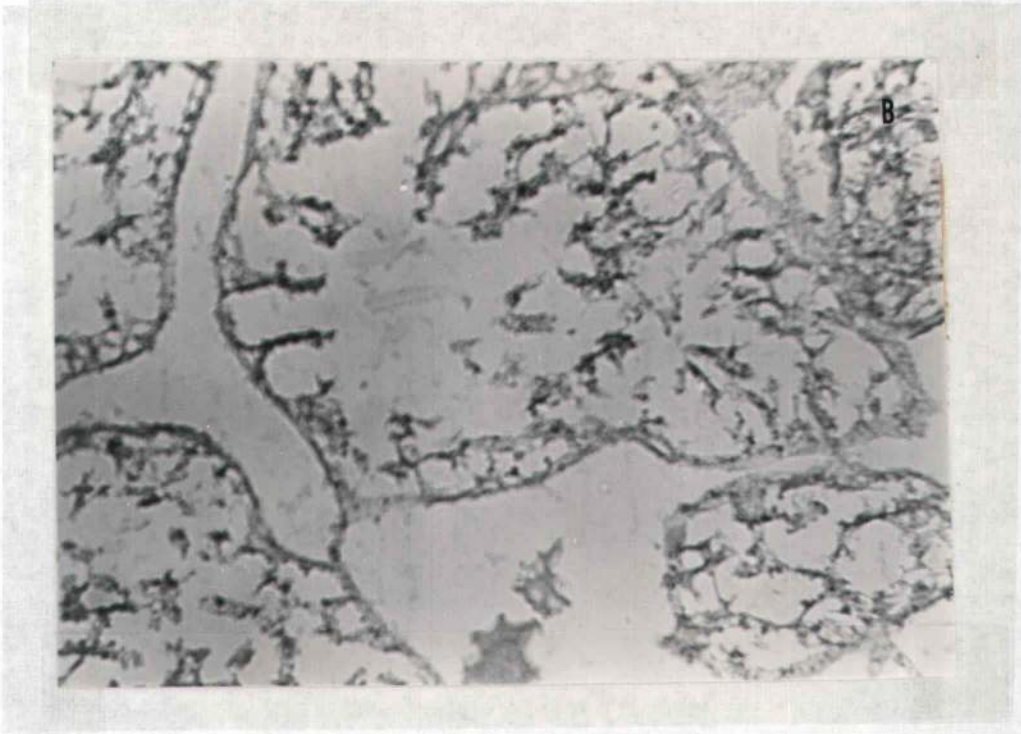

B

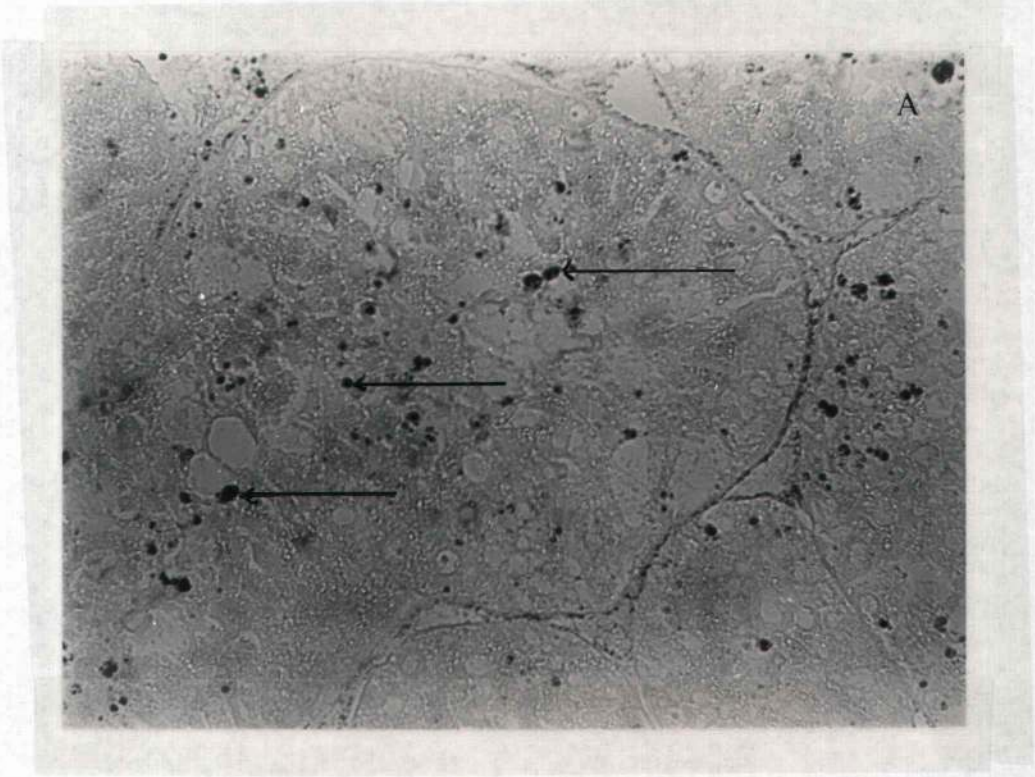
PLATE 14

A. Growing ovary; phagocytes pyranophilic; dark granular inclusions stained with methyl green (arrows). See also Plate 11D.

methyl green and pyronin (MGP); 350 X

B. Growing ovary; nucleoli (n) are pyranophilic; peritoneal cells and granular inclusions stain with methyl green.

MGP; 1470 X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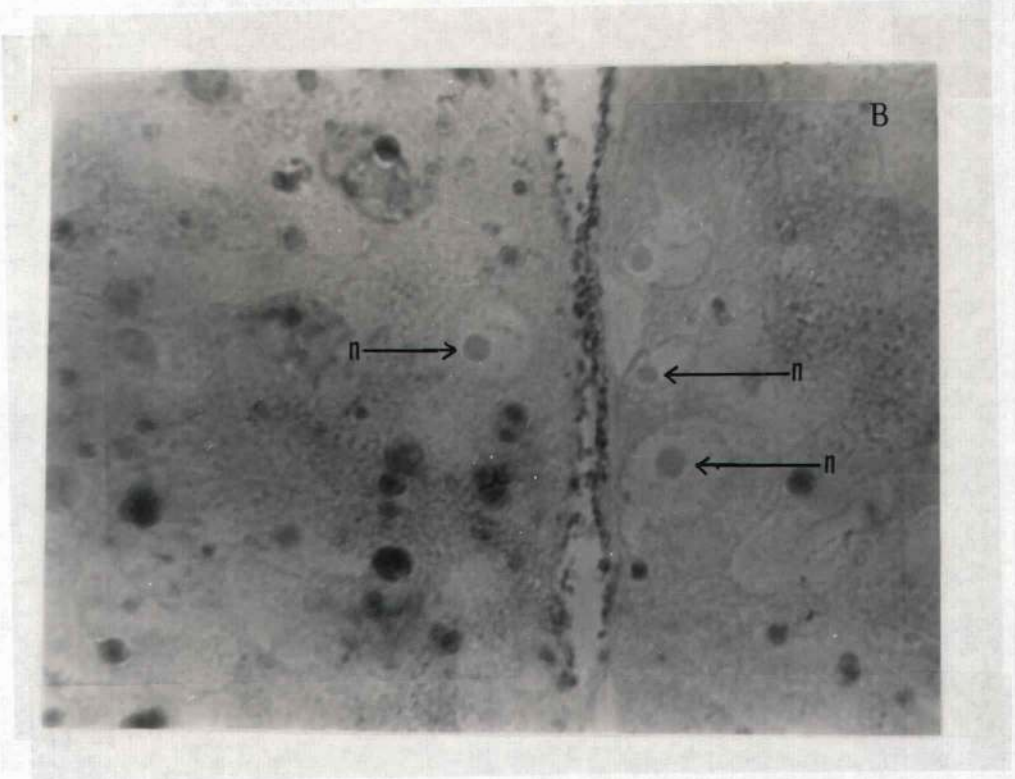

B

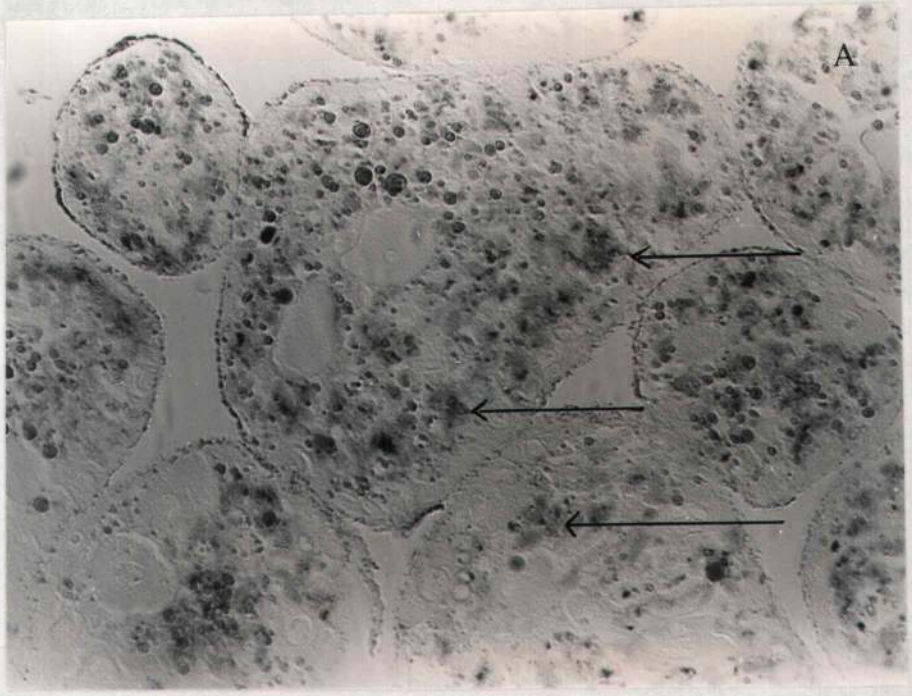
PLATE 15

A. Premature ovary; accessory cells pyranophilic (arrows); dark granular inclusions stain with methyl green.

MGP; 350 X

B. Same as above; RNA extracted with HCl and therefore no areas are stained with pyronin.

MGP; 350 X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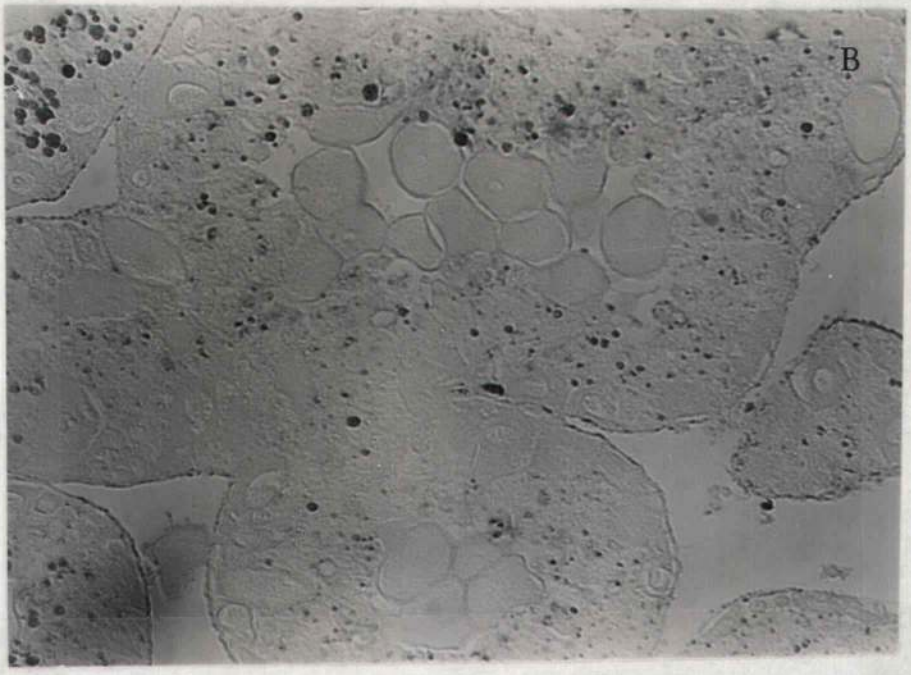

B

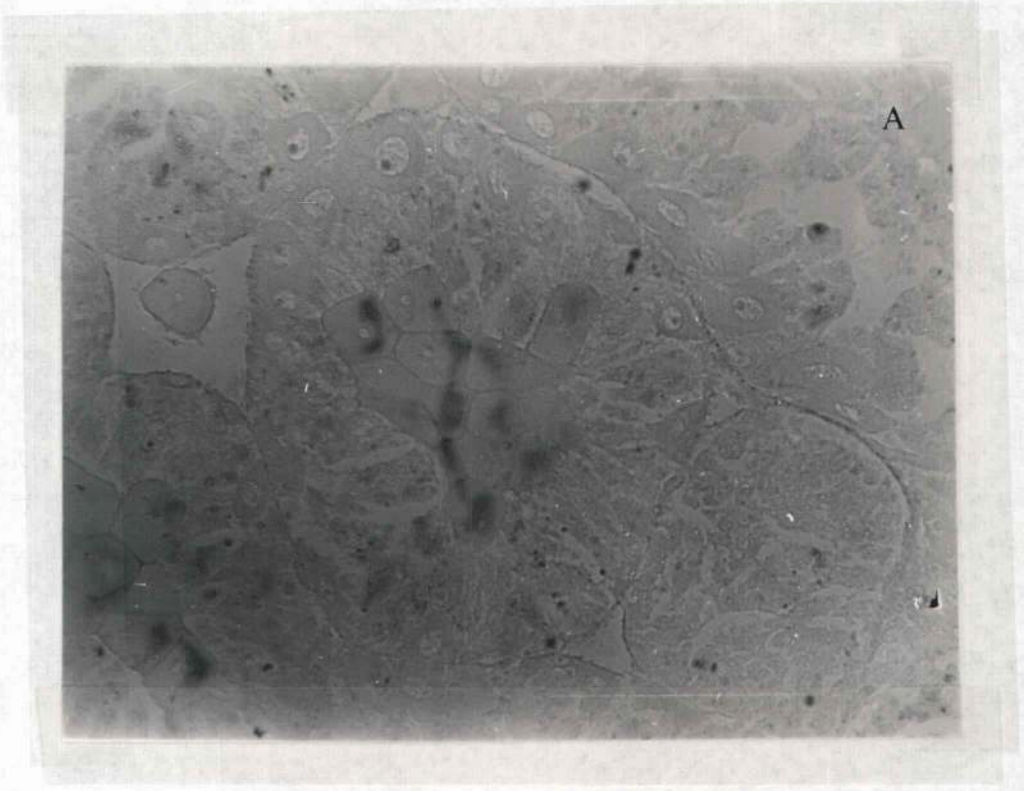
PLATE 16

A. Mature ovary with unshed pyranophilic ova.

MGP; 350 X

B. Mature ovary with three shed pyranophilic ova.

MGP; 450 X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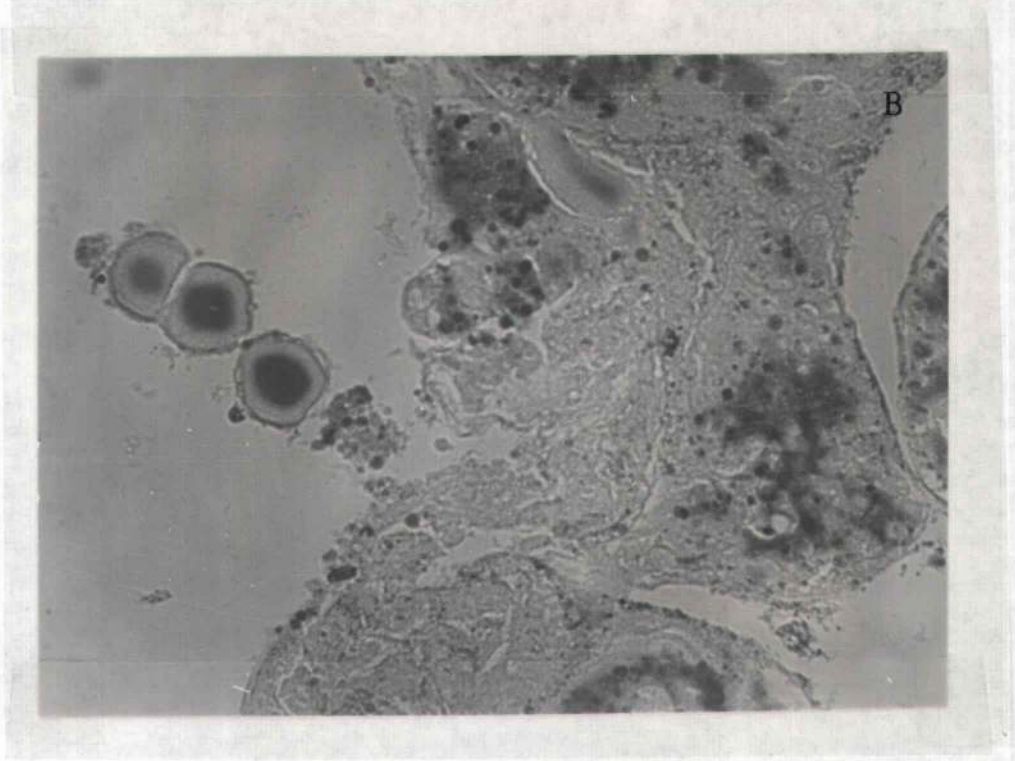

B

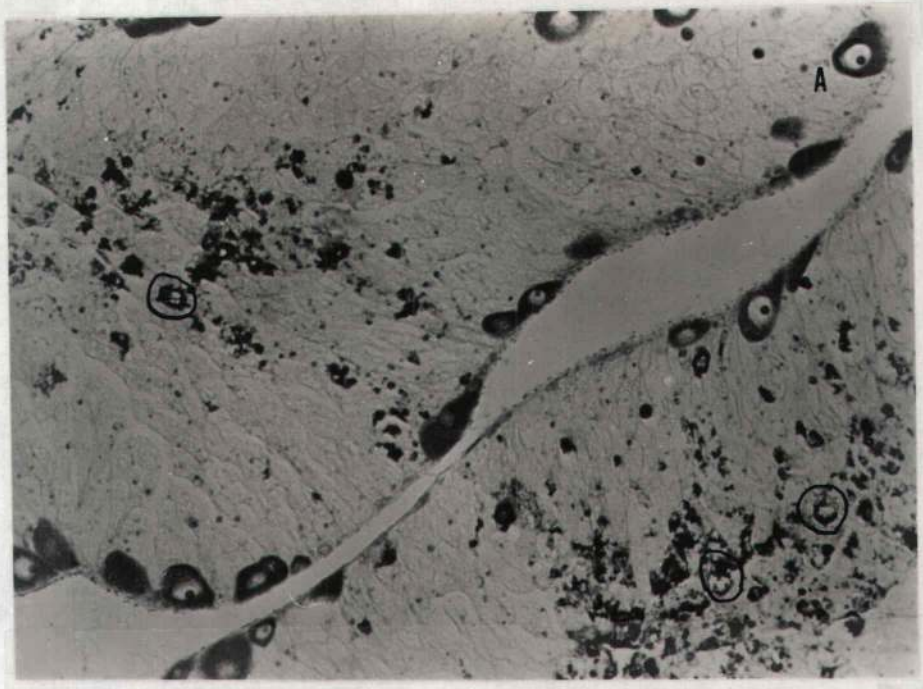
PLATE 17

A. Late recovering spent ovary; oöcytes stained dark blue; dark acidophilic areas in central acinus stain purple. Note some of these acidophilic areas surround an unstained space. (circles).

azure B; 350 X

B. Premature ovary. Acidophilic areas less numerous.

azure B; 450 X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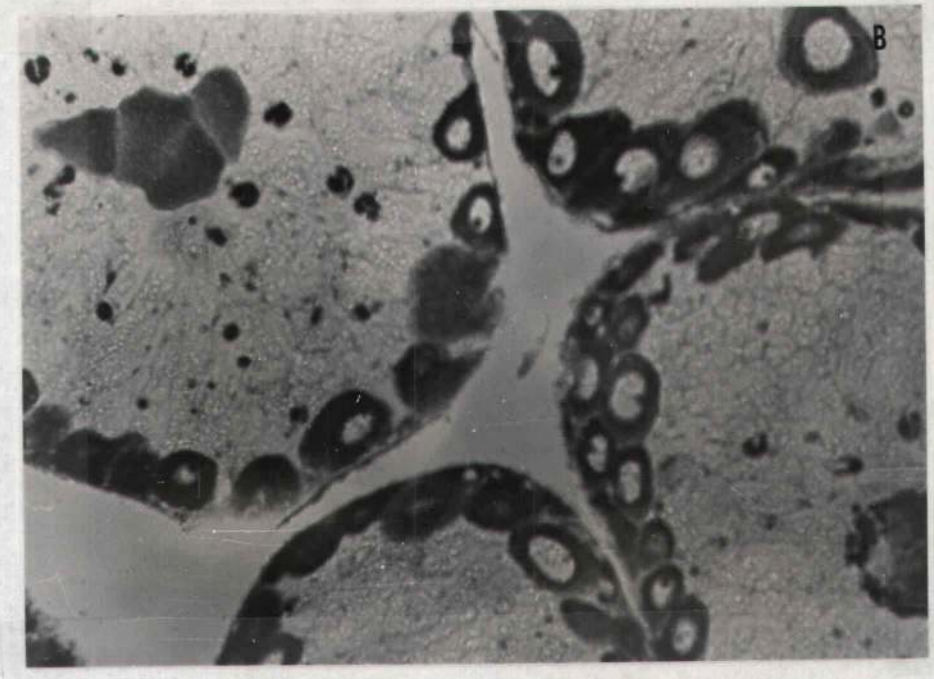

B

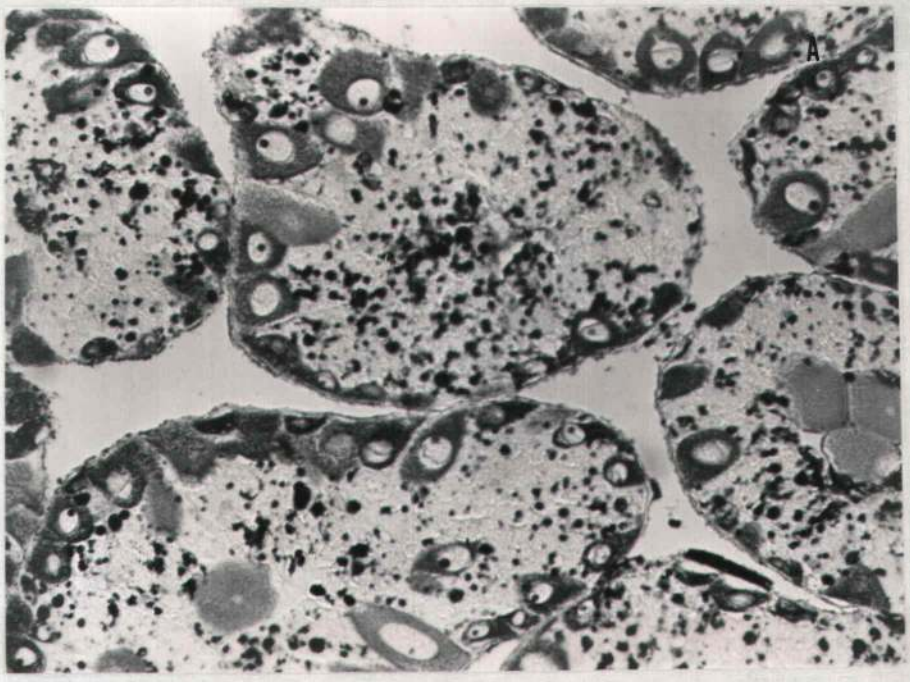
PLATE 18

A. Premature ovary. Note that larger oöcytes stain more lightly than small oöcytes.

azure B; 350 X

B. Same as above; RNA removed with HCl.

azure B; 350 X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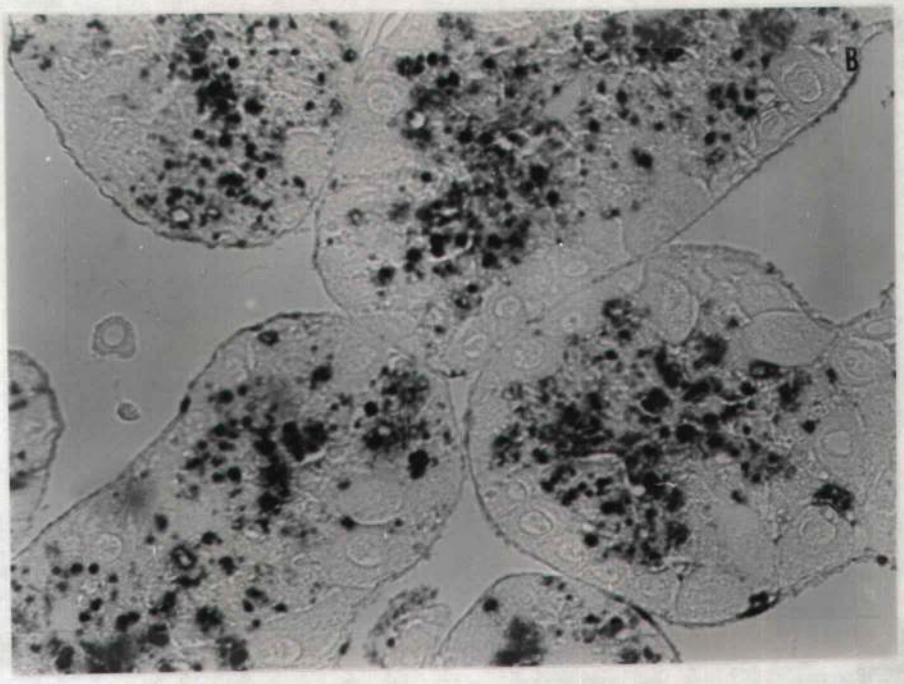

B

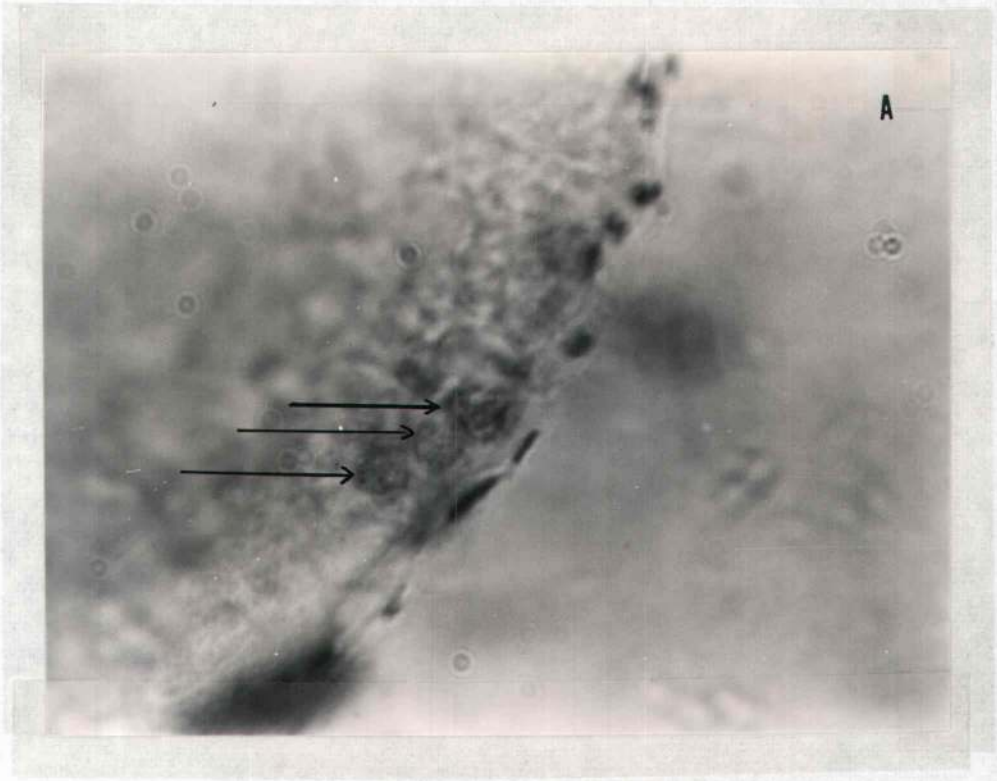
PLATE 19

A. Pre-dictyotene oöcytes (arrows).

hematoxylin and eosin; 3500 X

B. Dictyotene oöcytes. Note nuclear vacuole and indistinct border between oöcyte and phagocyte (arrow).

azure B; 3500 X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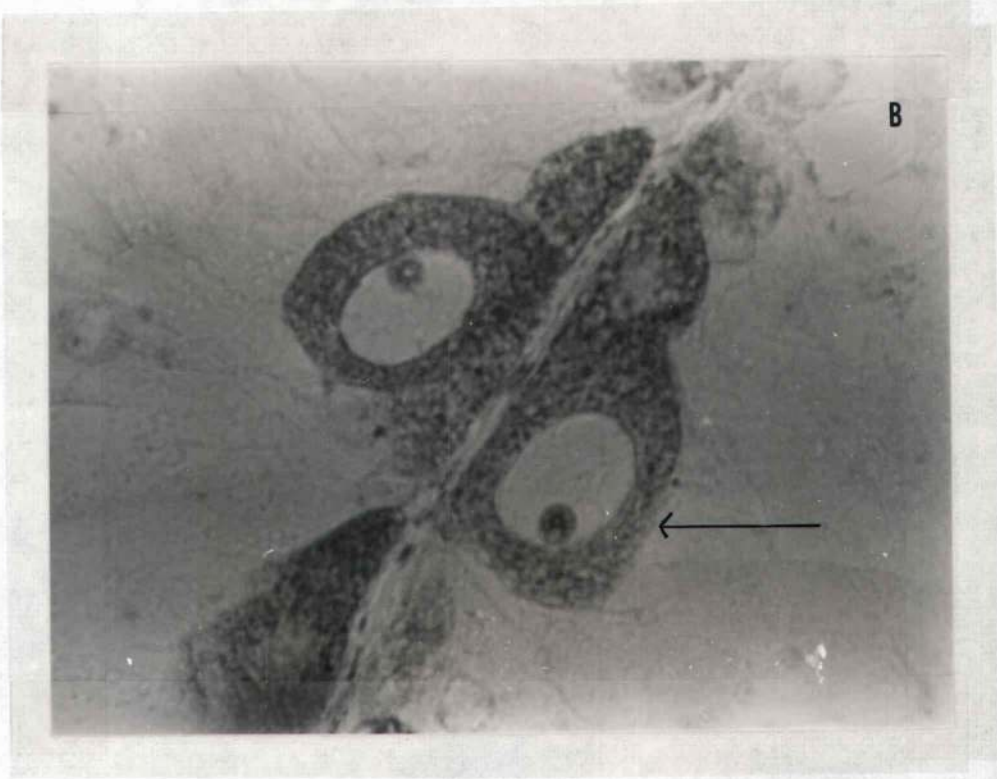

B

PLATE 20

A. Mature ovary; note DNA positive material in dark granular inclusions and in small nuclei of polar bodies (arrows).

Feulgen; 350 X

B. Pre-dictyotene oöcytes (arrows).

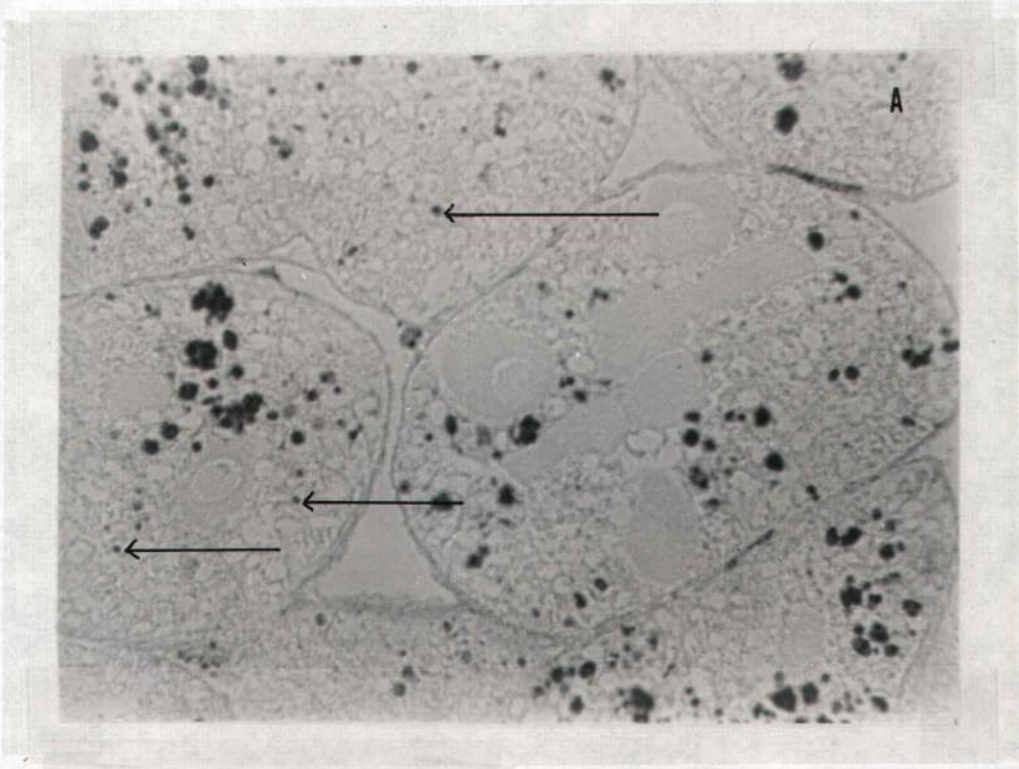
Feulgen; 1470 X

C. Diagram of ovary showing gross morphology.

a - acinus

gd - gonaduct

gp - gonapore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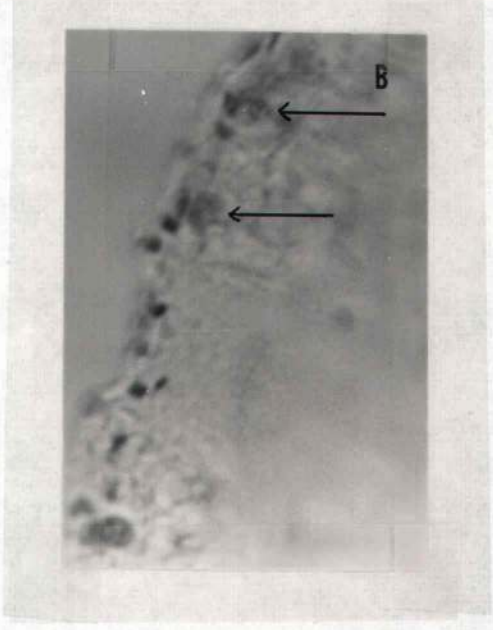
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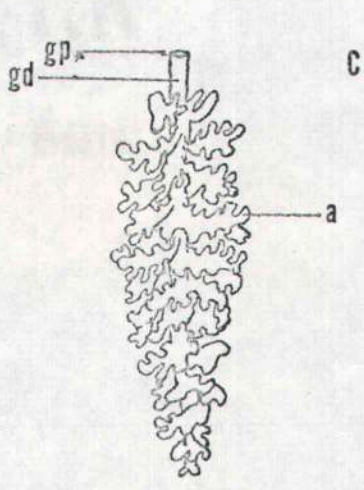
gp
gd
C
a