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Studies on gametogenesis in the Echiuroid worm, Urechis caupo

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STUDIES ON GAMETOGENESIS IN THE ECHIUROID WORM, URECHIS CAUPO

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by

James Charles Hanson

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ACKNOWLEDGEMENT

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INTRODUCTION

During the spring of 1955, while attending classes at the Pacific Marine Station, under the direction of Dr. Alden E. Noble, Chairman of the Department of Zoology, College of the Pacific, Urechis caupo was introduced as a representative of the fauna of the mud flats of Tomales Bay. The unusual manner in which this animal lives would stimulate the curiosity of any student interested in biology, and especially those associated with marine life. The fact that the animal burrows its own home in a U-shaped tunnel and dwells there with three permanent commensals (Hesperonoë adventor, Skogsberg, a polynoid annelid; and 2 pinnotherid crabs, Scleroplax granulata, Rathbun, and Pinnixa franciscana, Rathbun) is a unique characteristic. The goby fish, Clevelandia ios (Jordon and Gilbert), may also be considered a commensal, but it uses the burrow as a retreat rather than a permanent residence (Fisher, 1946).

Studies on the early embryology of Urechis stimulated the writer to further study of the reproductive cycle. The puzzling descriptions and information pertaining to the immature gametes that are found in the coelomic fluid, coupled with the fact that no permanent gonad has been

observed or indeed, that it may be wholly lacking, supplied a challenge that he could not ignore. The primary purpose of this investigation is to attempt to evaluate the reproductive cycle and to attempt to locate some type of germinal epithelium.

URECHIS CAUPO

Historical Review

According to Fisher (1946), the first specimens of Urechis caupo were collected in 1903 by C. S. Thompson, at Morro Bay, California. These individuals were brought to Stanford University where they are apparently in a good state of preservation. In 1920, Fisher found an individual in Elkhorn Slough, Monterey Bay, California. A few years later, M. E. Johnson (1927), collected some specimens from which the figures in "Seashore Animals of the Pacific Coast" were drawn. In 1923, a Urechis was brought in by flounder fishermen from the sea bottom off the Hopkins Marine Station. However, it was not until 1926 and 1927 that these particular animals were studied intensively. G. E. MacGinitie, working in connection with an ecological exploration of Elkhorn Slough, sponsored by the Hopkins Marine Station, found them in quantity. Through collaboration with W. K. Fisher, he has discovered nearly every ecological factor pertaining to Urechis caupo known at the present time. By the year 1931, the blood pigments of Urechis had been investigated by J. P. Baumberger and Leonor Michaelis; the muscular activity and oxygen consumption by V. E. Hall; and the respiratory function of the blood by

A. C. Redfield and Marcel Florkin. In the early 1930's, W. W. Newby began his thorough study of the embryology of Urechis and in 1940 produced one of the more outstanding works on the embryology of an invertebrate. G. E. MacGinitie (1935), published two unique works concerning the normal functioning and experimental behavior of the sperm and egg collectors in Urechis, and the rearing of the larvae of Urechis caupo within the blood cavity of the adult animal. Later, in the early 1940's, both MacGinitie and Newby worked respectively on the size of the mesh openings in the mucous feeding net and the development and structure of the slime-net glands of Urechis. Since 1947, investigations concerning the reactions of Urechis eggs to particular stimuli, such as ultra-violet light, have been performed, Also, a great deal of work has been done concerning the respiration and cell division of the mature ova (Erik Zuthen, University of Copenhagen, Denmark).

Description

Urechis caupo is an unsegmented, bilateral, fusiform or sacculiform animal with an anterior mouth situated within a proboscis, and a posterior anus. The average length is 150-180 mm. (MacGinitie), and the width varies from 20-55 mm., depending upon the state of contraction. Externally, 8

setae are situated about 3-5 mm. behind the groove leading to the mouth and 10 or 11 are encircled around the anus. However, there is no mid-ventral seta in the anal group. Three pairs of nephrostomes are present; these are the external ostia for the nephridia and are situated in two rows behind the anterior setae. The body is covered with a rough, rugose-type cuticle. Internally, a long divided intestine is present, with a hind-gut adapted for respiratory functions. The intestine is supported by muscular mesenteries which are attached to the body wall. Two anal vesicles, which are retroperitoneal, are situated in the posterior part of the animal, and communicate with the cloaca.

Three pairs of nephridia, with their collecting organs, hang freely in the coelomic cavity by attachment of the nephrostome, and may extend two-thirds the length of the animal. The coelom is lined with an epithelial peritoneum and, within the coelomic cavity, there will be found some 15-40 ml. of coelomic fluid, bright red to almost black in color. In very immature individuals, the coelomic fluid may not be so pigmented, but may be a light grey or yellow in color. This was observed in an animal about 33 mm. in length. This fluid represents the circulating blood, for the animal possesses no blood vessels. The coelomic fluid

is composed of a plasma comprising approximately 70% of the total volume and the various cells (blood cells, amoeboid cell, and reproductive cells) about 30%. The erythrocytes occupy from 18-40% of the total volume. A grey or pink layer of rather variable volume containing reproductive and other cells, separates between the red cells and plasma on settling, or by centrifuging. The plasma does not clot and is straw colored. The erythrocytes are either reddish or brown, each with a small nucleus, and may be as large as 35 microns in diameter. They are irregular in shape (stained preparations), from circular to almost oval and have been described as being subcircular (Fisher and MacGinitie, 1928). They contain hemoglobin (Redfield and Florkin, 1931), and large refractive granules which may be related to hematin (Baumberger and Michaelis, 1931). There are numerous types of cells which may be fusiform, spindle shaped, or oval to circular, which have been classified as amoeboid. These are yellow when aggregated (Fisher and MacGinitie, 1928). The nervous system is composed of a nerve ring which surrounds the pharynx, and continues posteriorly as a single ventral nerve.

The inclusion of gametes with blood cells in a circulating medium is a unique situation. The origin of these gametes and their entry into the coelom poses a

problem which it is hoped this investigation may help solve.

Collection of the Specimens

The animals used for this investigation were obtained from the mud flats of Tomales Bay, California, where they are abundantly found on the east shore line across from Pelican Point. All were collected between -0.1 to -0.5 ft. tides during the fall and spring of 1956 and the spring of 1957. The animals are located by their characteristic burrow apertures with fecal castings usually surrounding them. When collecting, the operator can either dig out the burrow by following it to the location of the animal, or a rubber hose at least $\frac{1}{4}$ in. in diameter can be used. When using the hose, the operator forces it into the burrow, at the same time blowing through it, until it cannot be forced any further. By digging carefully to the end of the hose, the animal can usually be found there, with its body expanded against the burrow wall.

MATERIALS AND METHODS

For preliminary observation of the coelomic fluid, Giemsa's Stain was used to differentiate the different types of cells. Thick smears were fixed in Schaudinn's Fluid for 15 minutes. The slides were then immersed in a 70% alcohol-Lugol's iodine solution for 15 minutes, followed by a 15 minute soaking in tap water. They were then stained in dilute Giemsa for 20-30 minutes, and then dehydrated in a graded series of acetone-xylol solutions. Clearing was done in xylol for 5 minutes, and the mounting in balsam.

Wright's Stain was used to substantiate the data obtained with Giemsa's Stain, using a dry smear instead of a wet-fixed smear. A smear was air dried and then flooded with thoroughly ripened Wright's Stain for 45 seconds. An equal amount of distilled water was added to the stain for 2 minutes. The smear was then washed thoroughly in running tap water, and allowed to dry.

Wet Heidenhain's Iron Haematoxylin Stain was used to differentiate the structure of the nucleus and nuclear inclusions. Two drops of coelomic fluid was mixed with a like quantity of Meyer's Albumin and then fixed in

Schaudinn's Fluid for 5 minutes. The Meyer's Albumin was used to aid the fixation of the smear on the slide, as the smear was often washed off the slide during differentiation in iron alum. The preparations were then transferred into a 70% alcohol-Lugol's iodine solution for 10 minutes. The smears were then hydrated in 50% alcohol for 6 minutes and washed in running tap water for 5 minutes. The slides were transferred into a 2% iron alum solution for 15-20 minutes and washed again in tap water for 5 minutes. The preparations were stained for 20-40 minutes in thoroughly ripened Heidenhain's and washed in running water for 5 minutes. A 2% iron alum solution was used to differentiate or destain the Heidenhain's (use a different iron alum solution for differentiation then was used as a mordant). The differentiated smears were then washed in running tap water for 15 minutes and dehydrated with alcohol solutions. Clearing was done in xylol for 5 minutes, and the mounting in balsam.

For preliminary observation of the epithelial lining of the coelom, scrapings were made by using an ordinary glass slide. The animal was incised from anterior to posterior on the mid-ventral line. The coelomic fluid was washed out thoroughly with sterile or boiled sea water. Using the longer edge of a glass slide, 2 or 3 scrapings were made from mid-dorsal to mid-ventral on the inner

lining of the coelom to remove most of the remaining coelomic fluid. With another slide abrasive scrapings were made in a localized area near the posterior of the animal, and the material was smeared on the slide. The scraped material was fixed in Schaudinn's Fluid, using Meyer's Albumin to maintain the smear on the slide. The slides were continued into the same process as used in the Wet Heidenhain's Stain.

For further study of the epithelial lining of the coelom, a one inch square section of body wall was removed and fixed in F.A.A. for 12 hours. The inner lining was removed with the aid of a scalpel and stained with Heidenhain's Iron Haematoxylin. The only difference is that the tissue was handled grossly instead of being fixed on a slide. A remaining portion of this tissue which was not stripped of its epithelium was prepared by sectioning and stained with Heidenhain's. Transverse and longitudinal sections at 15 microns were made.

Using a male animal, a section was removed from the posterior-ventral body wall. The tissue was fixed in the same manner as in the previous paragraph and frontal sections were made at 10 microns.

DESCRIPTION

Mature Ova from the Nephridia

The mature, unfertilized ova, as obtained from the nephridia (Plate I), are spherical in shape with concave depressions which may or may not be related to the polarity of the cell, Tyler (1931; 1932a), Taylor (1931). According to Taylor (1931), the average diameter of the egg is 128.3 (123-144) microns. However, other normal ova may be even smaller. The egg is completely surrounded by a vitelline membrane which is separated from the cytoplasmic portion by a perivitelline space. According to Tyler (1932c), the vitelline membrane is 0.3 microns thick and the perivitelline space is about 1.0 micron thick. The germinal vesicle may be spherical, ovoid, or amoeboid in shape and averages 55 (40-70) microns in diameter. The nucleolus (Plate IV, Figure 1), is spherical to ovoid and averages 10 (8-12) microns in diameter. Within the periphery of the nucleolus is a large, spheroid, nucleolar inclusion, 4.8 to 5.4 microns in diameter; this inclusion has a reticular chromatin pattern, and a spherical concentration of chromatin material. Throughout the cytoplasm of the unfertilized egg, relatively large, red, pigment granules are irregularly distributed. According to Baumberger and Michaelis (1931), these red

granules are masses of hematin and are probably related chemically to the respiratory pigment of the erythrocytes.

Oöcytes from the Coelomic Fluid

The developing oöcytes found within the coelomic fluid (Plate II; Plate III) are distributed singly, and in relatively small numbers as related to the erythrocytes. The youngest (Plate III, Figure 3) are about 10 microns in diameter and can be distinguished from the red cells by their larger and more vesicular nuclei (stained preparations). When Giemsa's Stain is used, the oöcytes are stained blue and, on occasion, blue-green, which readily distinguishes them from the amber stained erythrocytes. The erythrocytes contain large refractive granules, which also aid in distinguishing them from the oöcytes. Oöcytes larger than 50 microns (fixed material) (Plate II), possess conspicuous vitelline membranes which are either absent or difficult to see in smaller cells. The nucleus possesses a rigid nuclear membrane, upon which may be found packets of chromatin material; such concentrations of chromatin are very conspicuous in cells under 30 microns in diameter. Within the nucleoplasm of the larger cells (diameter more than 90 microns), there are several inclusions which appear to possess chromatin granules (Plate II). A nucleolus is

present within, and there is an inclusion with a thread-like chromatin pattern (Plate IV, Figure 2). The cytoplasmic portions of the youngest oöcytes are either lacking pigment granules or the granules are not conspicuous. However, the medium and larger ones contain numerous granules. With Giemsa's Stain, these granules appear bright red as related to the bluish stained cytoplasm.

In the coelomic fluid, very few ova are found which possess the concave depressions. According to MacGinitie (1935a), the functional collecting organs are selective in extracting only those ova which possess such depressions.

Oöcytes from Coelomic Wall Scrapings

Scrapings of the ventral portion of the coelomic wall revealed the presence of several clusters of oöcytes which were more concentrated near the posterior end. These clusters of cells are represented by groupings of from 12 to 20 oocytes. The smaller cells are 5 to 6 microns in diameter, while the larger ones measure 8 to 9 microns. One particular cell had a nucleus and nucleolus of 6.4 and 2.4 microns in diameter, respectively. These cells are grey as compared to the more amber stained erythrocytes and possess larger, more vesicular nuclei. An erythrocyte

with a diameter of 24 microns possesses a nucleus with a diameter of 4.8 microns, which eliminates the possibility of these cells being immature red cells.

Male Gametes in the Coelomic Fluid

The gametes of the male Urechia are strikingly different from those of the female. Instead of being distributed singly throughout the coelomic fluid, the developing spermatocytes are packed into irregular aggregates of from some 20-100 cells. These clusters appear to be transitional stages of spermatogenesis. There are three principal types of clusters: the first, which possesses, in most cases, the primary spermatocytes; the second, which possesses the transitional secondary spermatocytes; and the third, which contains the developing spermatids and spermatozoa. In many cases, however, all of these stages are found to be in one particular aggregate.

Within the clusters of transitional secondary spermatocytes and spermatids there are relatively large, fine, granular-type cells, which may perhaps be considered nutritive cells. These might be the remains of the discarded cytoplasmic portions of the cells in their process of meiosis. This interpretation was made by observations of stained

material obtained from the coelom of these animals.

Iron Hematoxylin was used to observe the nuclear structure. The progressive decrease in the size of the nucleus correlated with progressively increased density aided the writer in his designations of cells as specific stages of spermatogenesis. In fresh, unstained coelomic fluid the mature spermatozoa not yet extracted by the collecting organs (MacGinitie, 1935a), can be seen moving in a jerky motion throughout the medium.

The mature spermatozoa, as obtained from the nephridia (Plate VIII), measure approximately 2 microns across the head and about 2.4 microns through the longer axis of the head. According to Newby (1940), the concave depression is about 0.4 microns in depth (a fifth of the length of the head). The flagella vary in length, but apparently reach no more than 10 microns (Newby, 1940). At the base of the head there is a middle piece, which seems to support the flagellum; it is about 1.5 microns in length.

The heads of the immature or developing spermatozoa (Plate VII, Figures 2, 3, and 4), are about 3.5 microns through the longer axis and about 2.8 microns across, with concave depressions at the anterior end. The cytoplasm is almost completely absent and, according to Newby (1940),

different stages in the development of the flagella are conspicuous. The spermatids (Plate VII, Figure 1), average 4.2 (3.6-4.8) microns in diameter, with a nucleus which averages about 3.2 microns. The cytoplasmic portions are reduced but are still conspicuous, and a definite oval shape is characteristic of this stage. The nucleus is densely packed with chromatin and might be best described as a mass of granular chromatin material.

The secondary spermatocytes (Plate VI, Figures 1 and 2) average 5.6 (4.8-6.4) microns in diameter with a nucleus averaging 4 microns. Less cytoplasm is present, and the nucleus possesses a finer, more dense, reticular-type of chromatin pattern than noted in primary spermatocytes. Most of the secondary spermatocytes appear to be in a stage of meiotic division.

The primary spermatocytes (Plate V, Figures 1 and 2), average 10.5 (9.7-11.3) microns through the longest axis, with a nucleus which averages 8 (7.2-8.8) microns. The cytoplasm is finely granular and within the nucleus the chromatin is dispersed with broad ribbons; conspicuous loosely packed, knot-like areas are present.

DISCUSSION

Mature ova and spermatozoa were taken from the nephridia (storage organs) of dissected individuals in an attempt to determine the normal nuclear structures and characters of the cytoplasm. Most of the ova obtained possessed conspicuous concave depressions; not infrequently, however, an almost spherical egg could be observed. The nucleus was nearly always half the diameter of the cell and possessed a conspicuous nucleolus. Within the nucleolus, inclusions were present. The cytoplasm contained characteristic refractive granules. Each cell was surrounded by a vitelline membrane which was separated from the cell by a perivitelline space.

The oöcytes found in the coelomic fluid range in size from that of mature ova (120 microns) to the youngest cells which were about 10 microns in diameter. The larger oöcytes usually appear singly; the younger cells (10-15 microns in diameter), infrequently appear in groups of four or five cells. Probably the younger cells increase in size by addition of cytoplasmic and nuclear materials presumably obtained from the coelomic fluid.

The developing male gametes found in the coelomic fluid, range from primary spermatocytes to mature spermatozoa. The developing male gametes are grouped into three distinct aggregated types: the primary spermatocytes, the transitional secondary spermatocytes, and the spermatids and spermatozoa. Most of the cells in the primary clusters appear to be resting stage primary spermatocytes, but infrequently a cell will be observed in meiotic division. In one particular cluster, a cell with two nuclei was observed, which might represent the telophase stage of meiosis and the origin of secondary spermatocytes. The transitional secondary aggregates are composed of dividing secondary spermatocytes and their transition into spermatids. The spermatids and developing spermatozoa are grouped into a third type of cluster. Spermatozoa are also observed freely moving throughout the coelomic fluid.

It should be understood that the male gametes demonstrate a successive pattern of spermatogenesis and, commonly, primary and secondary spermatocytes together with spermatids and spermatozoa are observed within a single colony. From this observation, there is a possibility that the primary spermatocytes are proliferated continually into the coelom as colonies and develop into mature spermatozoa within the same colony. Investigations on seventeen males during both

the spring and fall of 1956 and the spring of 1957 showed that all stages of development were present in each animal. The relative ratio of types of colonies was approximately the same in each individual, which might indicate proliferation of primary groups during all seasons of the year.

According to Newby (1940), a statement concerning the temporary appearance of a gonad was made. If a temporary gonad were to appear and disappear seasonally, or perhaps only once, it would seem that, within a single individual, nearly all the same stages of development would appear at once. Since this was not observed in this investigation, there is possibility that the gonad or germinal epithelium proliferates immature gametes throughout the entire year.

Assuming that proliferation of these cells might occur within the body wall of the individual, scrapings of the epithelium were made in localized areas. It was found that an area on the interior-posterior-lateral portion of the body wall yielded four aggregates of what appear to be immature oöcytes. The relative size and characteristics of the nuclei indicated that they were

oöcytes. The diameters of the nuclei were larger in oöcytes than were these structures in the mature erythrocytes. No other cells found in the coelomic fluid have nuclei resembling those of the oocytes. The grey color, which is a unique character of these cells when stained with Heidenhain's, is also an obvious character.

The latero-posterior portion of the body wall was excised and removed from a single female whose maximum contracted length was not more than 50 mm. Approximately one-fourth of this area was stripped of its epithelial lining and stained with Heidenhain's Iron Haematoxylin. Many cells were found resembling immature oöcytes; their diameters ranged from 4 to 6 microns. The nuclei of these cells were without any appreciable cytoplasm and the nuclear structures were difficult to differentiate. Small, budded bodies were observed upon a strand of what appeared to be muscle tissue, but no nuclei could be distinguished.

Observations of the sectioned material from the latero-posterior portion of the body wall in a single female revealed no identifiable bodies which were reproductive in origin.

Observations of the sectioned material from the posterior-ventral body wall in a mature male also revealed no identifiable bodies which were reproductive in origin.

SUMMARY

1. The nephridia of Urechis caupo contain mature gametes. They have been selectively extracted by the collecting organs from the coelomic fluid.

2. In male individuals, three types of reproductive colonies are evident; these demonstrate gametogenesis from primary spermatocytes into spermatozoa.

3. In females, oöcytes ranging from 10 microns to mature ova of about 120 microns are found.

4. Immature oöcytes ranging from 5 to 8 microns were observed by scrapings made from the epithelial lining of the coelom.

5. No reproductive cells were found in sections of body wall in the posterior-lateral position of either female or male individuals.

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EXPLANATION OF THE PLATES

All of the figures and plates are composite drawings made by observations through a 97X oil immersion objective and a 10X ocular.

Abbreviations:

Ch.----------Chromatin
C. D.----------Concave depression
C. P.----------Connecting piece
Cyto.----------Cytoplasm
Fl.----------Flagellum
G. V.----------Germinal vesicle
H.----------Head
N.----------Nucleus
N. I.----------Nuclear inclusion
N. M.----------Nuclear membrane
Nucleol.----------Nucleolus
Nuc. I.----------Nucleolar inclusion
P. G.----------Pigment granule
Pv. S.----------Perivitelline space
V. M.----------Vitelline membrane

Plate I.

Diagrammatic hemisected mature ovum as obtained from the nephridia, x 1000. The concave depression and relative distribution of pigment granules are demonstrated.

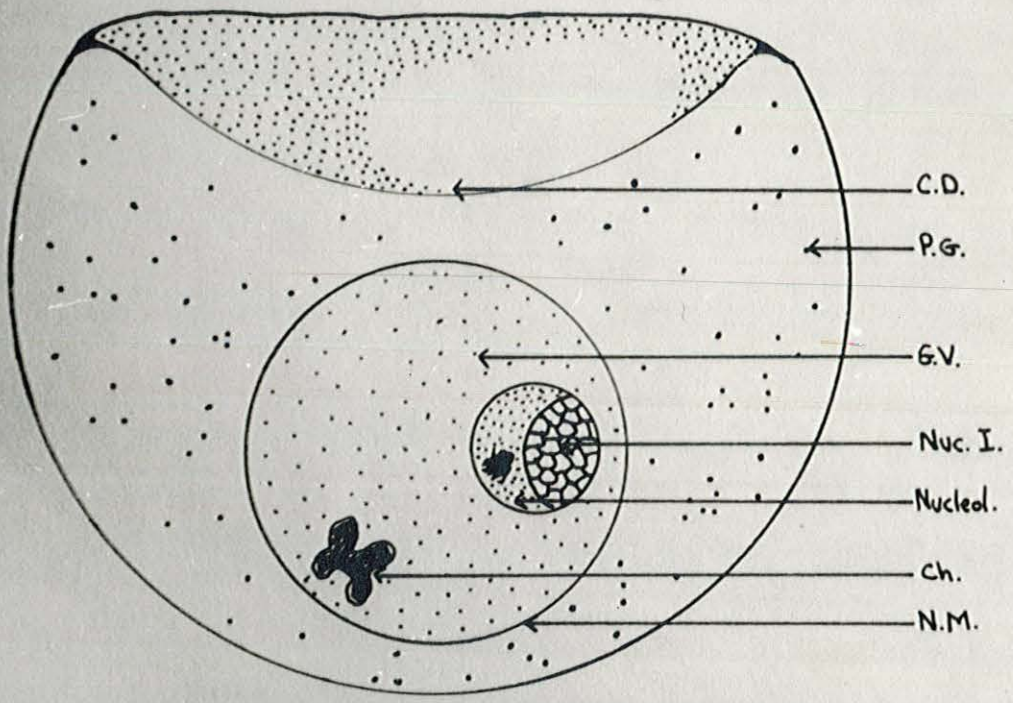

PLATE I

Plate II.

Maturing oöcyte as obtained within the coelomic fluid, x 1000. The vitelline membrane, perivitelline space, and nuclear inclusions are demonstrated.

Measurements:

Diameter of cell	105	microns
Diameter of nucleus	47	microns
Diameter of nucleolus	8.5	microns
Diameter of nucleolar inclusion	6.4	microns

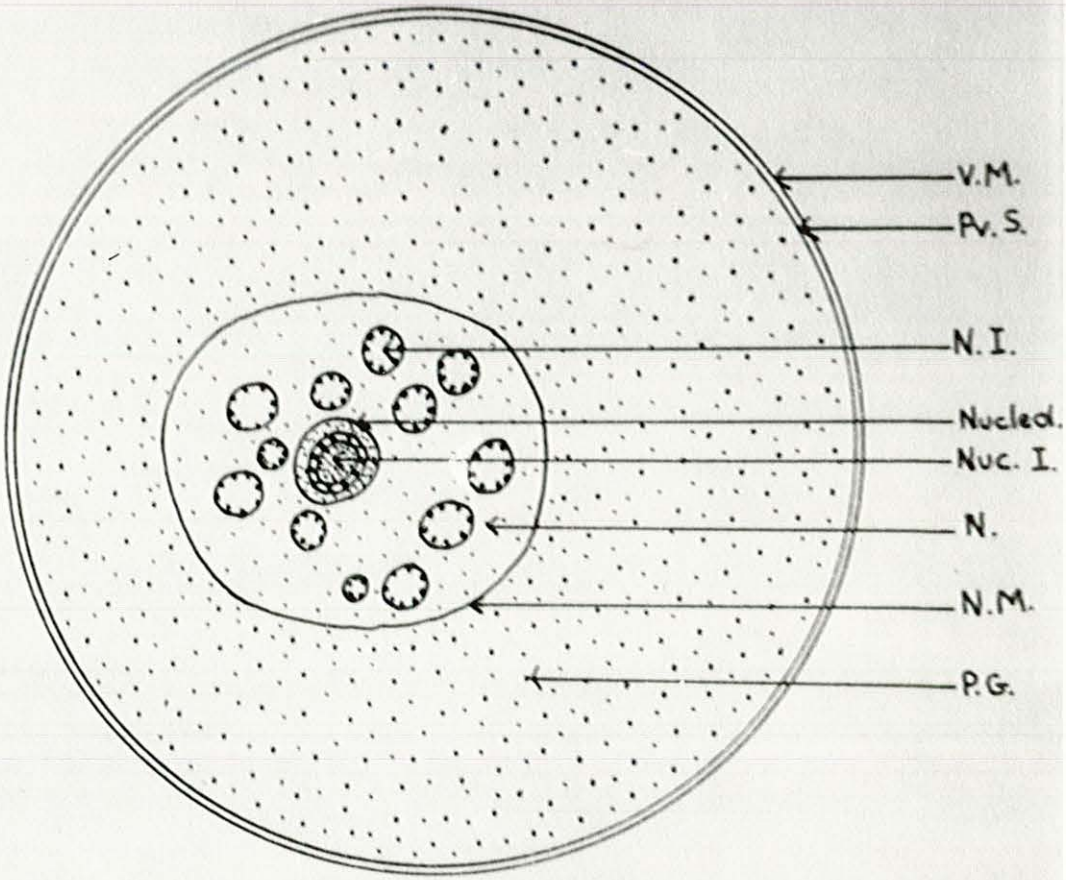

PLATE II

Plate III.

Figure 1.

Medium sized oöcyte as observed within the coelomic fluid, x 1000. The vitelline membrane, perivitelline space, and nucleolus are demonstrated.

Measurements:

Diameter of cell	65	microns
Diameter of nucleus	26	microns
Diameter of nucleolus	8.1	microns
Diameter of nucleolar inclusion	5.3	microns

Figure 2.

Young oöcyte as observed within the coelomic fluid, x 1000. The thick chromatin packets mounted on the nuclear membrane are demonstrated.

Measurements:

Diameter of cell	22	microns
Diameter of nucleus	13	microns
Diameter of nucleolus	5.6	microns
Diameter of nucleolar inclusion	4.8	microns

Figure 3.

Youngest oöcyte observed within the coelomic fluid, x 2000. The characteristic chromatin pattern of the

nucleus is demonstrated.

Measurements:

Diameter of cell----------9.7 microns

Diameter of nucleus----------4.9 microns

Diameter of nucleolus----------2.4 microns

Figure 4.

Oöcyte as obtained from body wall scraping, x 2000. The large nucleus with chromatin packets which are mounted on the nuclear membrane is demonstrated.

Measurements:

Diameter of cell----------8.1 microns

Diameter of nucleus----------6.4 microns

Diameter of nucleolus----------2.4 microns

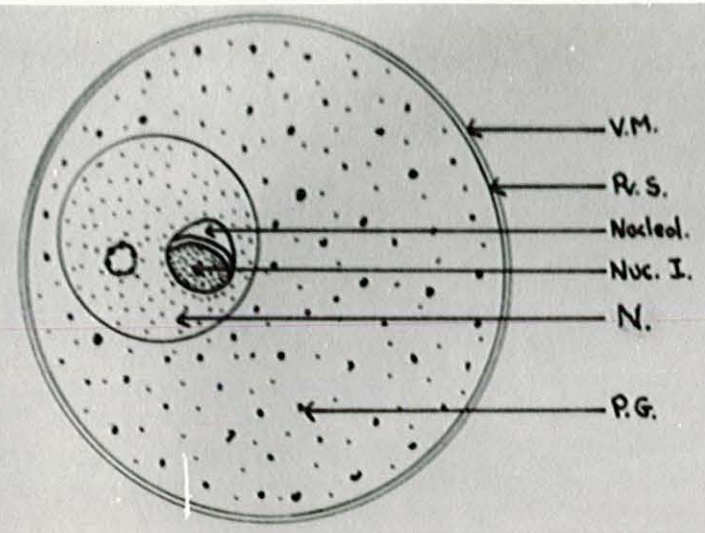

FIG. 1

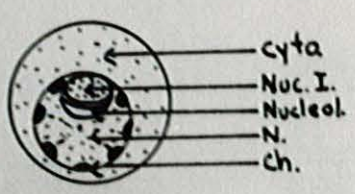

FIG. 2

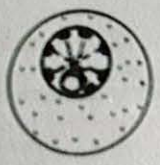

FIG. 3

FIG. 4

PLATE III

Plate IV.

Figure 1.

Nucleolus, as observed within a mature ovum. A nucleolar inclusion possessing a reticular type chromatin pattern is demonstrated.

Figure 2.

Nucleolus, as observed within a larger oöcyte, as obtained from the coelomic fluid. A nucleolar inclusion possessing string-like bands of chromatin material and a second nucleolar inclusion are demonstrated.

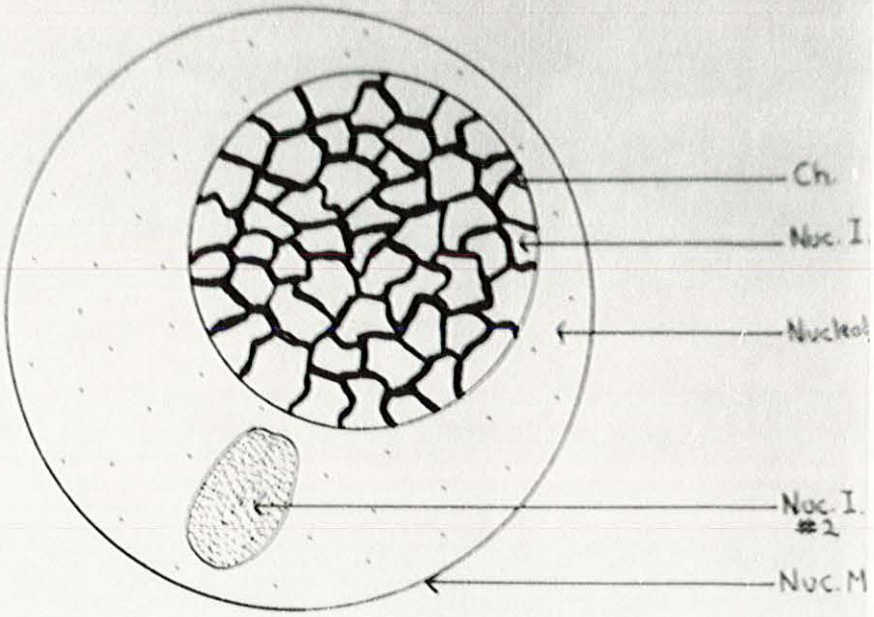

FIG. 1

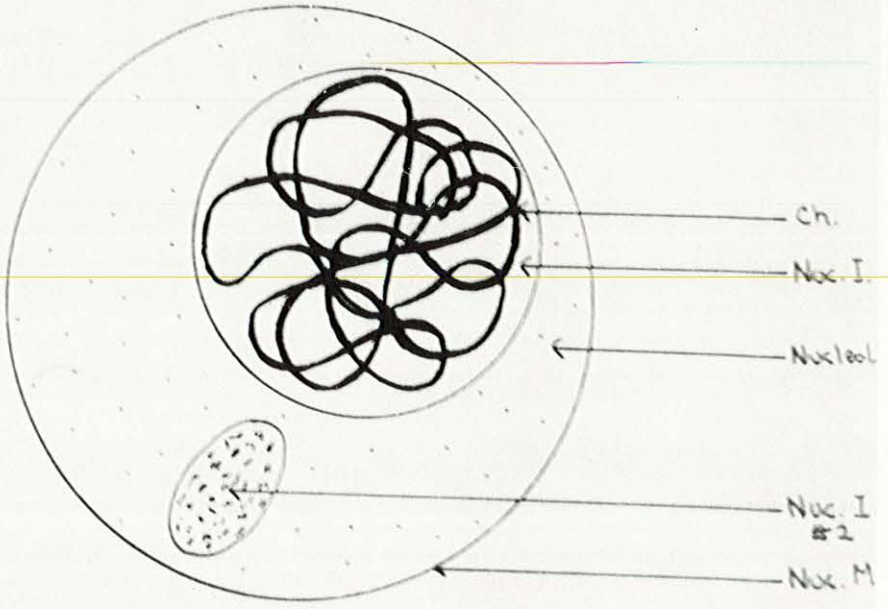

FIG. 2

PLATE IV

Plate V.

Figure 1.

Primary spermatocyte, as observed on the periphery of the primary colonies in the coelomic fluid, x 10,000. The characteristic broad chromatin bands with knoted areas are demonstrated within the nucleus.

Figure 2.

Primary spermatocyte, as observed within the median area of the primary colonies in the coelomic fluid, x 10,000.

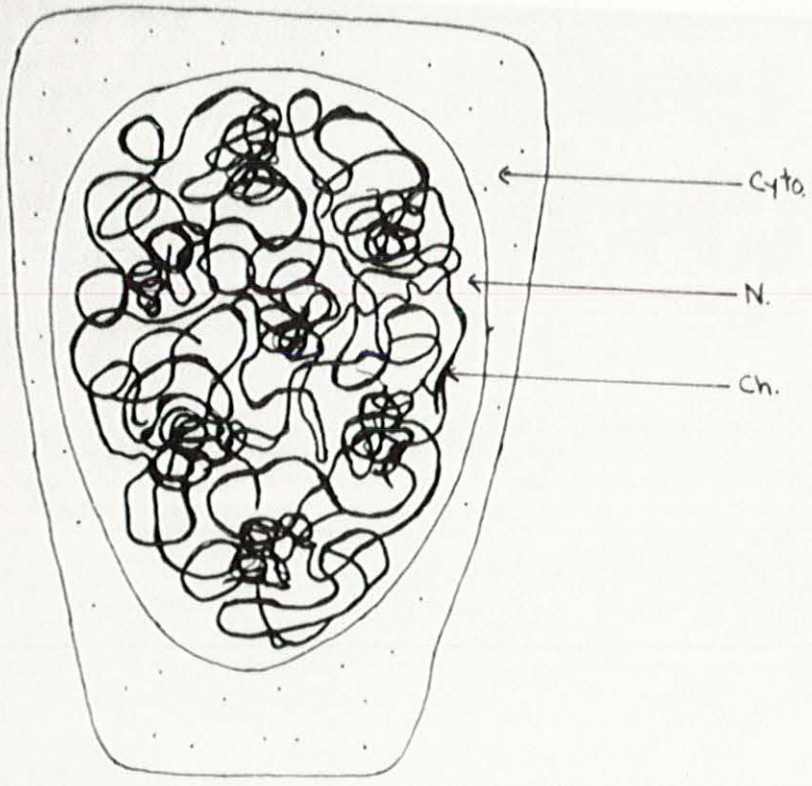

FIG. 1

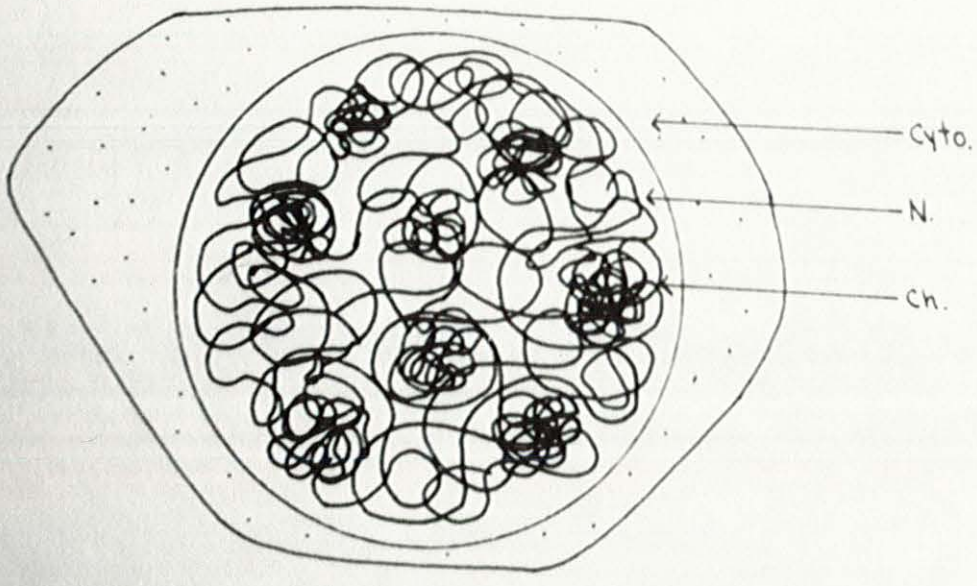

FIG. 2

PLATE V

Plate VI.

Figure 1.

Secondary spermatocyte in resting stage, as observed in a transitional colony in the coelomic fluid, x 10,000. The characteristic narrow bands of finely packed chromatin are demonstrated within the nucleus.

Figure 2.

Secondary spermatocyte in meiotic division, as observed in the transitional colonies in the coelomic fluid, x 10,000. The chromatin pattern within the nucleus has become more compact without any particular formation.

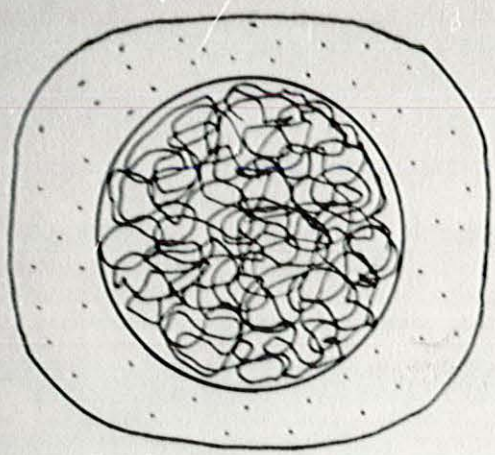

FIG 1

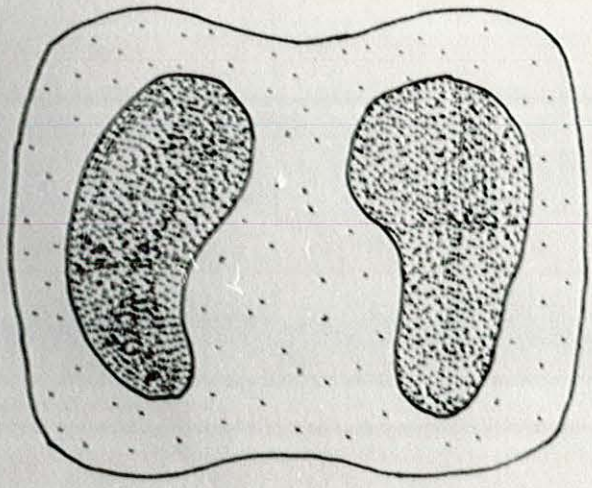

FIG. 2

PLATE VI

Plate VII.

Figure 1.

Spermatid, as observed in the third type colonies in the coelomic fluid, x 10,000. The chromatin material in the nucleus is more concentrated as related to the secondary spermatocyte.

Figures 2, 3, and 4.

Developing spermatozoan as observed in the third type reproductive colonies in the coelomic fluid, x 10,000.

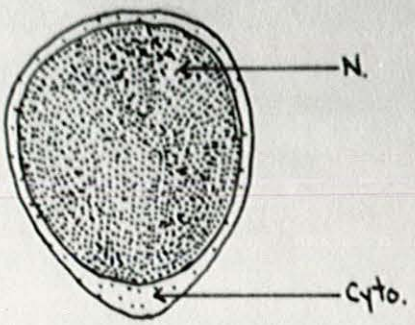

FIG. 1

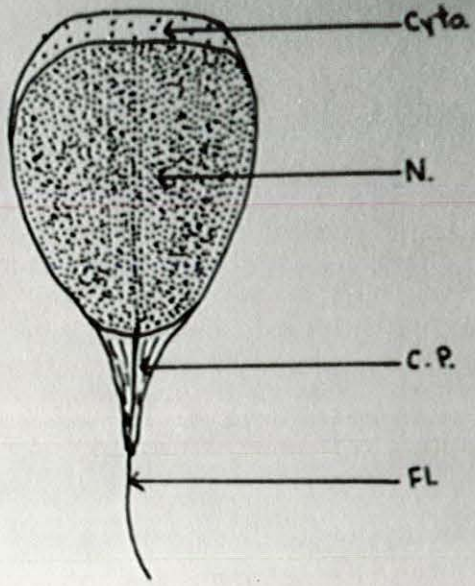

FIG. 2

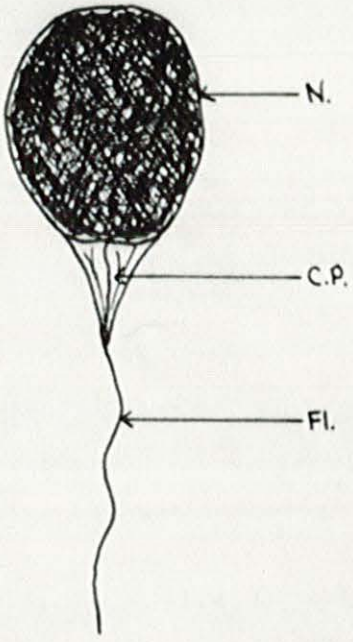

FIG. 3

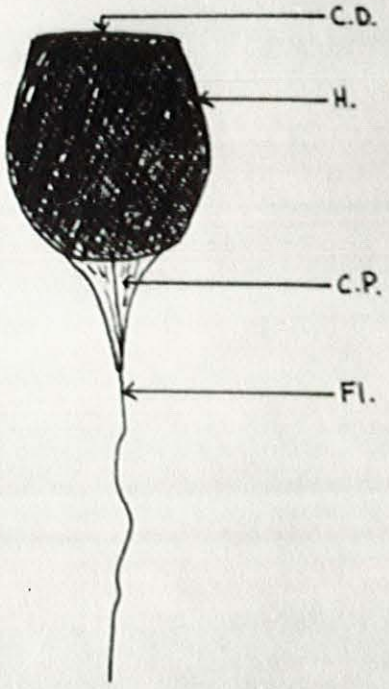

FIG. 4

PLATE VII

Plate VIII.

Mature spermatozoan, as obtained from the nephridia, x 10,000. The characteristic concave depression and connecting piece are demonstrated.

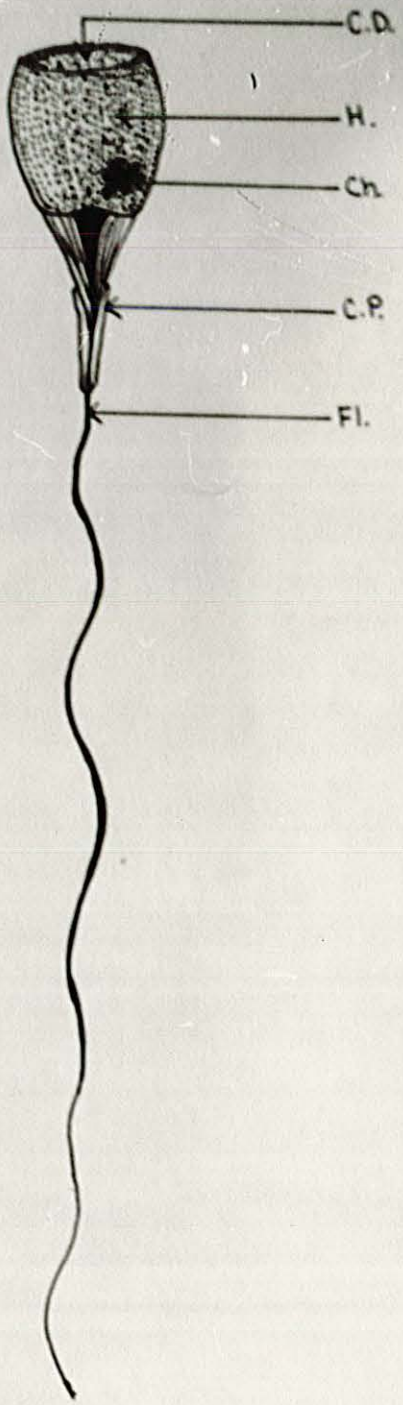

PLATE VIII