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CYTOLOGY AND ULTRASTRUCTURE OF DIFFERENTIATING
INTERSTITIAL CELLS IN SPERMATOGENESIS
OF HYDRA FUSCA

by
Anthony L. Schincariol

A Thesis
Submitted to the Faculty of Graduate Studies Through the
Department of Biology in Partial Fulfillment of the
Requirements for the Degree of
Master of Science at the
University of Windsor

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ABSTRACT

The cytology, cytochemistry and ultrastructure of interstitial cells differentiating into spermatozoa is described. Interstitial cells of Hydra fusca are induced to transform into spermatozoa rather than somatic cells by reducing the culture temperature from 20°C to 8°C. Within 30 days at 8°C, several testes containing spermatozoa appear as epidermal swellings. Spermatogenesis begins with the enlargement of the interstitial cell (diameter, 6.2 μ ; volume, 128 μ^3), which lacks endoplasmic reticulum and has few small organelles. Subsequently, the transforming interstitial cell passes through three successive phases: mitosis, meiosis, spermiogenesis. The spermatogonium (6.2-12.5 μ ; 123-1024 μ^3) in the mitotic phase is characterized by a more intense basophilia due to an abundance of ribosomes, absence of endoplasmic reticulum and the accumulation of DNA along the nuclear boundary and about the enlarged nucleolus. In the formation of the primary spermatocyte (6.1 μ ; 122 μ^3), the cytoplasmic basophilia diminishes, the nucleolus disappears and the nuclear DNA becomes more coarsely granular. After an extended prophase I, meiosis I is completed and the secondary spermatocyte (4.8 μ ; 60 μ^3) is formed. The second meiotic division ensues immediately. During this meiotic phase the chromosome number is reduced from 30 to 15. Finally.

in spermiogenesis of the spermatid (3.9μ ; $31\mu^3$), the nuclear volume is reduced and four large mitochondria are aligned at the point where the flagellum is elaborated. Electron microscopic studies show up to four spermatids connected by cytoplasmic bridges and each spermatozoan ($4 \times 1.5\mu$) surrounded by an electron dense layer.

Histochemical studies reveal that at the end of 30 days at 8°C the distribution of food materials in the gastrodermis of sexual hydra is altered as compared to that of non-sexual animals. Furthermore, some glycogen taken up by the gastrodermis may be passed through the mesoglea to the epidermal testes.

The events of spermatogenesis and the effect of reduced temperature on the differentiating cells is also discussed.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Hydra has been studied extensively as an experimental model for organismal control and cellular differentiation. (Burnett, 1961, 1966; Lentz, 1966). Consequently, the significance of the small interstitial cells in the maintenance of the animal by differentiating into specialized somatic cells has been well documented by several investigators (Brien and Reniers-Decoen, 1955; Burnett, 1961, 1966; Diehl and Burnett, 1964a, b, c; Lentz, 1966). However, germinal differentiation of the interstitial cell in Hydra has not been sufficiently researched. This thesis describes the cytology, histochemistry, and ultrastructure of differentiating interstitial cells in the spermatogenesis of Hydra fusca.

Classification, Morphology and Life Cycle of Hydra fusca

Classification -- The several species of Hydra Linneaus are fresh water organisms of the phylum Coelenterata or Cnidaria, the class Hydrozoa and the order Hydroida, (Hyman, 1959). H. fusca Linneaus 1767 is considered equivalent to H. oligactis Pallas 1766 (Hyman, 1930; Ewer, 1948). Other synonyms are given by Ewer (1948). Although Pallas' names have priority (Hyman, 1930), investigators of Hydra

have retained the term H. fusca in reference to stocks isolated in Europe.

The clone employed in this study, obtained from Dr. A.L. Burnett, (Developmental Biology Centre, Cleveland, Ohio), was originally derived from a European stock. We confirmed that this species is H. fusca (oligactis) by using Hyman's (1959) classification. In this key, the sizes, shape and coiling of the thread inside the nematocysts are important taxonomic characteristics. Of the four types of nematocysts (Fig. 1), the large glutinants (Fig. 1B) are diagnostic of this species; inside, the thread is coiled longitudinally, while in all other known species this thread is coiled transversely (Hyman, 1959; Ewer, 1948).

Fig. 1. The four types of nematocysts in H. fusca.
 A. Penetrants (stenotheles).
 B. Large glutinants (holotrichous isorhizas).
 C. Small glutinants (atrichous isorhizas).
 D. Volvents (desmonemes). The nematocysts were drawn from squash preparations of living animals.

Morphology -- H. fusca is from three to five mm long and can be divided into six regions anatomically, histologically and physiologically. (Burnett, 1959, 1961). These regions shown in Fig. 2A are the tentacles, hypostome, gastric region, budding region, peduncle, and basal disk. In cross section, hydra is a two layered tube of cells enclosing the digestive cavity. The outer layer or epidermis is separated from the inner layer or gastrodermis by a non-cellular mesoglea over which the two cell layers move (Fig. 2B; Shostak et al., 1965).

Fig. 2A. The six body regions of *Hydra*.

Fig. 2B. A longitudinal section of *H. fusca* at 20°C showing the three body layers. Dc, digestive cavity; E, epidermis; G, gastrodermis; Me, mesoglea. Karpechenko, 4μ, Flemming's triple stain, X50.

Six cell types distributed in definite tissue patterns within the six anatomical regions, comprise these two cell layers (Burnett, 1966; Lentz, 1966). Four such tissue patterns are seen in Fig. 3. Epitheliomuscular cells make up the bulk of the epidermis (Fig. 3A, B, C, D;e). In the basal disk these cells have transformed into gland cells containing a secretion which serves as an adhesive to hold the animal to a substrate (Fig. 3D;e).

Small interstitial cells, rich in ribonucleic acid (Cowden and Glocker, 1960), are usually found in clusters of 6-14 cells between the epitheliomuscular cells (Fig. 3B;i). They are known to differentiate into any of the other cell types (Lentz, 1965, 1966; Burnett, 1966). These interstitial cells occur mainly in the epidermis of the gastric and budding regions; some are also found in the adjacent gastrodermis.

Cnidoblasts elaborate one of the four types of nematocysts (Fig. 3B;c). Large numbers are seen in the gastric, hypostomal and budding regions of the epidermis. The nematocysts function in feeding and locomotion (Ewer, 1947).

Gastrodermal digestive cells are the largest cells in hydra. Burnett (1959) states that in the gastric region these cells contain numerous food vacuoles, lipid and protein droplets, glycogen, and other inclusions (Fig. 3B;d). In the peduncle, the digestive cells are highly vacuolated with few food inclusions (Fig. 3C;d). In the basal disk these gastrodermal cells are small non-vacuolated and rich in food materials (Fig. 3D;d).

Fig. 3. A diagram of four tissue patterns observed in *H. fusca*. E, epidermis; G, gastrodermis; Me, mesoglea; c, cnidoblast; d, digestive cell; e, epitheliomuscular cell; g, gland cell; mu, mucous cell.

According to Burnett (1959), the gastrodermis also contains mucous gland cells in the hypostome (Fig. 3A; mu) and zymogenic cells in the gastric region (Fig. 3B; g). These gland cells are interspersed among the digestive cells.

Epidermal ganglion, sensory and neurosecretory cells, have also been described by several investigators (Lentz and Barnett, 1965; Burnett and Diehl, 1964a; Lentz, 1966). The neurosecretory cells have been implicated as possible control factors of cellular differentiation in hydra (Burnett and Diehl, 1964b, c; Burnett, 1966).

Fig. 4

Fig. 4. The two modes of reproduction in the life cycle of *H. fusca*. Data concerning the sexual mode obtained from Brien (1965).

Life Cycle -- H. fusca reproduces mainly asexually by budding (Fig. 4). Animals in natural populations usually enter sexual reproduction in the winter (Fig. 4; Hyman, 1930). In H. fusca, spermatozoa and eggs are produced on separate animals and a zygote is formed. This zygote develops into a cyst from which a young hydra hatches in the spring.

The two modes of reproduction in H. fusca also occur in the laboratory. This species proliferates rapidly by budding at 20°C and both male and female clones can be induced to form spermatozoa and eggs respectively, at any time, by reducing the temperature to 8°C.

Review of Literature

Kleinenberg in 1872, cited by Downing (1905), was the first to point out the existence of the interstitial cells and to demonstrate that they differentiate into spermatozoa through a series of transformations. Subsequently an historical account and light microscopic description of cytological events during spermatogenesis was presented by Downing (1905) in several hydra species, by Tannreuther (1909) in H. viridis, by Brien and Reniers-Decoen in H. viridis (1950), in H. attenuata (1951) and by Brien in H. pirardi (1961), and briefly in H. fusca (1961). These authors were also concerned with the question of whether separate germinal and somatic interstitial cell lines exist. Brien (1961) and later Burnett and Diehl (1964c), and Burnett et al. (1966) presented evidence contradicting this dual interstitial cell line concept. Preliminary ultra-structural descriptions of

spermatogenesis have been presented by Burnett et al., (1966), in H. viridis, and Schincariol et al., (1967a, b), in H. fusca. Histochemical studies of spermatogenesis have been limited mainly to the distribution of food materials in the gastrodermis of sexual hydra (Burnett and Ferrier, 1963; Burnett and Diehl, 1964c; Schincariol et al., 1967b); some cytochemical changes of the transforming interstitial cells were recently reported by Schincariol et al., (1967a, b).

CHAPTER II

MATERIAL AND METHODS

Methods for Culturing Hydra fusca.

Four to five hundred animals were cultured at 19-20°C in 4½ inch diameter finger bowls and kept in a standard incubator. The culture medium, containing versene, calcium and bicarbonate, is a modified solution originally developed by Loomis and Lenhoff (1956). Hydra are fed daily with an excess of brine shrimp (Artemia salina), washed in tap water and returned to fresh culture medium. These techniques proved successful to routinely raise large numbers of animals and are fully described in Appendix I.

Induction of Spermatogenesis and Criteria for Selection of Animals

Spermatogenesis in our clone of male H. fusca is induced by reducing the temperature of the culture medium to 8°C. Preliminary experiments showed that well fed hydra usually failed to develop testes when subjected to 8°C. However, all animals formed testes if they were fed moderately for several days prior to incubation at 8°C. This precautionary procedure was adopted as a standard in all experiments.

After 30 days at 8°C, the testes, ranging from two to thirty per animal, contained motile spermatozoa or had released their spermatozoa into the culture medium. Consequently, animals were fixed and processed for microscopic examination

at various time intervals during this period of thirty days to study spermatogenesis. For the experiments large non-budding animals, occasionally with one bud, were chosen. Always thirty hydra, 24 hours after their last feeding at 20°C, were placed into small dishes (2½" diameter; 1" height), containing 30 ml of culture solution at 8°C and maintained in an incubator of the same temperature. These hydra were fed every second day from the time of their incubation.

Fixing, Embedding and Staining Procedures for Light Microscopy

Previous experiments indicated that significant cellular changes occur within 10 days at 8°C. Therefore, five animals per day were fixed for the first 10 days, then only every second day for another 20 days, always 24 hours after their last feeding, in each of Karpechenko's, Allen's PFA 3, and Maximov's fluids (Appendix II). The animals were then dehydrated in alcohol and embedded in tissuemat (Appendix II). To determine the changes in distribution of food materials in the animal with time, hydra at 20°C and after 4 and 19 days at 8°C were fixed 1, 3, 6, 18, 24 hours and 5 days post-feeding and treated as previously described. Serial sections were cut at 4μ with a Leitz microtome and from 3-5 sections were mounted serially on each slide. In this way 7-10 slides could be obtained per animal. Each slide from a series was then subjected to a different staining procedure or histochemical test so that all procedures employed in this study were performed on every animal.

For cytological examination, sections were stained with iron hematoxylin (Appendix III), and Flemming's triple stain (Appendix III). Living specimens were examined as squash preparations or macerated in Bela Haller's fixative (Appendix II) and examined in a Nikon Model M phase contrast microscope.

Histochemical Tests

Several histochemical methods were used to locate various chemical components in cells undergoing germinal differentiation and in the gastrodermal digestive cells.

Deoxyribonucleic Acid (DNA) -- The Feulgen technique was employed for the demonstration of DNA (Appendix III).

Ribonucleic Acid (RNA) -- The toluidine blue O method was used to demonstrate RNA and DNA. The histochemical confirmation of RNA by removal with ribonuclease or N-HCl(3h, 37°C) failed to give positive results after fixation in Karpechenko's Maximov's and Allen's PFA 3 fluids. It is speculated that these fixatives interfere in the removal of RNA by the enzyme or acid, as also observed by Brachet (1953). When animals were fixed in Carnoy's II solution, as recommended by Pearse (1960) and Brachet (1953), and subjected to the toluidine blue O or methyl green-pyronin methods, RNA was removed by ribonuclease treatment. Therefore, this procedure was used to demonstrate the sites of RNA in the differentiating cells (Appendix II, III).

Periodic Acid- Schiff Reactive Compounds (PAS) --

For the demonstration of polysaccharides (glycogen), neutral and mucopolysaccharides, muco- and glycoproteins, glycolipids, unsaturated lipids and phospholipids, the periodic-Schiff reaction was used (Appendix III). Hotchkiss, (1948), states that a PAS-positive reaction will occur with substances containing the 1, 2-glycol groupings, or the amino or alkyl amino derivatives of 1, 2-glycols, or the oxidation product CHOH-CO . Therefore, the chemical nature of the PAS-positive material was characterized further.

Glycogen -- To locate glycogen, sections were treated by Best's carmine and the PAS methods before and after digestion of glycogen with diastase (Appendix III, IV).

Proteins -- Proteins were demonstrated by the mercury-bromphenol blue procedure. Further confirmation of the proteinaceous material was obtained by subjecting several representative sections to trypsin treatment prior to staining (Appendix III, IV).

Electron Microscopic Methods

Animals after 10, 20, and 30 days at 8°C were fixed in 4% or 6.5% glutaraldehyde (Sabatini et al., 1963), postfixes in 1% osmium tetroxide containing 0.13 M sucrose (Caufield, 1957), both buffered in 0.1 M phosphate buffer (pH 7), dehydrated rapidly in alcohol and embedded in Epon 812 or Durcupam ACM (Pearse, 1964). Sections were cut on a Huxley ultramicrotome, mounted on carbon-coated Formvar-filmed grids, stained with

uranyl acetate (Watson, 1958) or lead citrate (Reynolds, 1963), and examined in an RCA EMU 3H electron microscope.

Determination of the Diameter and
Volume of Cells in Spermatogenesis

Cells in spermatogenesis undergo noticeable changes in size. The diameter of these cells could not be measured accurately due to their irregular shapes. To evaluate and correlate cellular size with the morphology of the consecutive cell stages, a method was devised whereby the cellular diameter and volume was calculated using weight as a criterion. In this procedure, cells were drawn using a camera lucida and further enlarged with a projector on graph paper. This final drawing was cut out with scissors and weighed on a Mettler Balance. The diameter (d) of the cells could now be determined from the relation $d = 15.4 \cdot x^{\frac{1}{2}}$ and the cellular volume (v) from the relation $v = 1,930 \cdot x^{\frac{2}{3}}$ where x is the weight of the final drawing in grams. Derivation of these formulae and the data are recorded in Appendix V.

CHAPTER III

RESULTS

H. fusca at 20°C reproduce asexually by budding (Fig. 5). However, animals after 10 days at 8°C cease budding and begin sexual reproduction. At this time, thickenings appear along the gastric portion of the epidermis. By 30 days the thickenings develop into as many as 30 testes per hydra (Fig. 6).

Microscopic observations of hydra at 20°C show several nests of basophilic interstitial cells and many cnidoblasts distributed throughout the gastric region of the epidermis (Fig. 3B; Fig. 7). This tissue pattern of the gastric region of the epidermis of animals at 20°C is altered progressively as sexuality at 8°C advances. After 10 days at this temperature, portions of the epidermis are thickened due to an increase in number of the small interstitial cells in these areas (Fig. 8). By 20 days these thickenings have developed into conical elevations containing increased numbers and different types of cells (Fig. 9). At the end of 30 days, testes have formed enclosing a large number of cells of varying morphology and spermatozoa, or appear empty due to the release of spermatozoa into the culture medium (Fig. 10). The cells of varying morphology represent the consecutive cellular stages of differentiation of the interstitial cell to the spermatozoan through a series of

Fig. 5. A living male H. fusca growing at 200C and exhibiting asexual reproduction by budding. Note the four buds (b), X20.

Fig. 6. A living male H. fusca after 30 days at 80C exhibiting sexual reproduction. Note the many conical testes (t), X25.

Fig. 7. A cross section through the gastric region of *H. fusca* growing at 20°C and showing nests of cnidoblasts (cn) and interstitial cells (i) in the epidermis (E). G, gastrodermis; Me, mesoglea. Karpechenko, 4μ, iron hematoxylin, X800.

Fig. 8. A cross section through the gastric region of *H. fusca* after 10 days at 8°C. showing a thickened epidermis (E) due to an increase in small, basophilic cells. G, gastrodermis; Me, mesoglea. Karpechenko, 4μ, iron hematoxylin, X300.

Fig. 9. A cross section through the gastric region of H. fusca after 20 days at 8°C, showing an epidermal elevation containing increased numbers of different types of cells. E, epidermis; G, gastrodermis; Me, mesoglea. Karpechenko, 4μ, iron hematoxylin, X800.

Fig. 10. A cross section through a portion of an epidermal testis of H. fusca, showing a large number of cells of varying morphology. E, epidermis, Karpechenko, 4μ, iron hematoxylin, X800.

characteristic cellular morphologies as illustrated in Fig. 11. To describe the stages of germinal differentiation in H. fusca, the same terminology is applied as was used by Brien (1961) in H. pirardi and is similar to other animal systems.

In analyzing the differentiation of interstitial cells into spermatozoa three phases were delineated: mitosis, meiosis and spermiogenesis (Fig. 11). Prior to the initiation of the first or mitotic phase, the interstitial cell which at 20°C differentiates into any of the somatic cells described earlier, enters germinal differentiation at 8°C. The interstitial cell becomes more basophilic and enlarges to form the primary spermatogonium. This enlarged cell (Fig. 11B), or primary spermatogonium, undergoes intense proliferation in the first or mitotic phase eventually forming the secondary spermatogonium (Fig. 11B-I). The secondary spermatogonium transforms into the primary spermatocyte through a series of nuclear changes, entering the second or meiotic phase. This primary spermatocyte undergoes meiosis I to form the secondary spermatocyte (Fig. 11J-O), which in turn enters meiosis II, resulting in the spermatid (Fig. 11O-Q). Finally, in the third phase or spermiogenesis, the spermatid transforms into the motile spermatozoan (Fig. 11Q-T).

Four criteria were employed to unravel this sequence of transformation stages from the interstitial cell to the spermatozoan (Fig. 11A-T): 1. the cytology and cytochemistry

Fig. 11 The Consecutive Stages of Interstitial Cells Differentiating Into Spermatozoa

of the differentiating cells; 2. the volume of the various cell stages; 3. the time of earliest occurrence of the various cell types; and 4. the spatial distribution of the cells in the testis.

Cytology and Cytochemistry
of the Interstitial Cell

Interstitial cells in budding hydra at 20°C are situated in groups of four to sixteen between epitheliomuscular cells (Fig. 12). These interstitial cells are small, round or oval and about 5-6 μ in diameter (Fig. 11A). The centrally located nucleus is about half the volume of the cell and shows small Feulgen-positive granules scattered throughout the nucleus and along the nuclear boundary. The small compact nucleolus stains darkly with iron hematoxylin and methods for RNA. In electron micrographs the nucleus contains dispersed granules of several sizes and densities

Fig. 12. A group of six interstitial cells (i) situated between the epitheliomuscular cells (e) in the gastric region of *H. fusca* grown at 20°C. N, nucleus; Nu, nucleolus. Karpechenko, 4 μ , iron hematoxylin, X2,000.

and the nucleolus is mainly composed of small granules similar to the cytoplasmic ribosomes (Lentz, 1965). The cytoplasm of the interstitial cells stains light grey with iron hematoxylin and strongly for RNA by the toluidine blue O method; it is filled with free ribosomes, has few smooth, membrane-bounded vesicles, some small mitochondria with few cristae and no endoplasmic reticulum (Lentz, 1965).

The interstitial cells stained by the mercury-bromphenol blue method resemble those stained with the toluidine blue O; all the regions containing nucleic acids give a strong reaction for protein. The nucleus, nucleolus and cytoplasm react negatively to the PAS and Best's carmine tests, indicating little or no glycogen or PAS-reactive compounds.

Mitotic figures are often observed either singly or in groups of two to six among interstitial cells. During division the chromatin stains more strongly for DNA and protein.

In a budding animal at 20°C, the interstitial cells continually differentiate into one of the several somatic cells; 99% of the differentiations are to the cnidoblast (Burnett, 1961). However, by placing an animal at 8°C the interstitial cell ceases to differentiate into a somatic cell and enlarges to become a primary spermatogonium.

Cytology and Cytochemistry of the Primary Spermatogonium

The primary spermatogonium, the first cell type in the mitotic phase, can be up to eight times the volume of the interstitial cell. These cells adopt a variety of shapes

and are closely packed in large groups between the epithelio-muscular cells (Fig. 13; Fig. 14). The round nucleus which occupies about half the volume of the cell is considerably enlarged and exhibits a fine meshwork of DNA accumulating along the nuclear boundary (Fig. 11B) and also encircling the nucleolus (Fig. 15). The latter, often doubled, is also enlarged and stains intensely with iron hematoxylin and toluidine blue O due to the accumulation of RNA (Fig. 16). The nucleoli do not stain with toluidine blue O or pyronin Y if the slides were previously treated with ribonuclease, thus confirming the RNA nature of the nucleolus. In the largest primary spermatogonium, small clear areas or vacuoles are seen

Fig. 13. A section through the epidermis of *H. fusca*, after 5 days at 8°C, showing numerous large primary spermatogonia (PS) between the epithelio-muscular cells (e). Note the dark cytoplasmic granules (cg) just outside the nuclear boundary. N, nucleus; Nu, nucleolus. Maximov, 4μ, iron hematoxylin, X2,000.

Fig. 14. A low magnification electron micrograph showing a group of primary spermatogonia in H. fusca after 20 days at 8°C. Note cell in division (M). Glutaraldehyde, post fixed in osmium tetroxide, uranyl acetate, X5,950.

Fig. 15. A high magnification photomicrograph of a group of primary spermatogonial nuclei showing the localization of DNA. Note the ring of DNA around the nucleolus (arrow). G, gastrodermis; Me, mesoglea; e, nucleus of epitheliomuscular cells. Karpechenko, 4 μ , Feulgen technique, X2,000.

Fig. 16. A single primary spermatogonium after 10 days at 80°C stained for RNA and DNA. Note the intense staining of the nucleolus (nu) and the cytoplasm due to RNA. E, epidermis; G, gastrodermis; Me, mesoglea. Carnoy II, 4 μ , toluidine blue O at pH 4, X2,000.

in the nucleolus (Fig. 17). In electron micrographs, the nucleus contains dispersed granules of several sizes and densities probably composed of DNA and associated proteins, and the nucleolus contains small granules similar to cytoplasmic ribosomes (Fig. 17).

The cytoplasm of the primary spermatogonia stains a darker grey with iron hematoxylin (Fig. 13) and is more intensely basophilic with toluidine blue O as compared to the interstitial cell. When sections are treated with ribonuclease, the cytoplasm does not stain with toluidine blue O. If the primary spermatogonia are fixed in Maximov's fluid and stained with iron hematoxylin, numerous darkly stained bodies of unknown nature are observed just outside the nuclear membrane (Fig. 13). Electron microscopic studies indicate that the cytoplasm contains an abundance of free ribosomes and few mitochondria with more cristae than in the interstitial cell. The endoplasmic reticulum is absent and occasionally an electron dense body is noted (Fig. 17), the significance of which is unknown.

In the primary spermatogonia all the regions containing RNA or DNA give a strong reaction for protein by the mercury-bromphenol blue method. The intensity of this reaction in regions of RNA is stronger than for the interstitial cell. The nucleus, nucleolus and cytoplasm also react negatively with PAS and Best's carmine tests.

Numerous mitotic figures (Fig. 11C-H; Fig. 14; Fig. 18) are observed among the primary spermatogonia resulting in a

Fig. 17. Electron micrograph of several primary spermatogonia in H. fusca, after 20 days at 8°C. Note the clear area (arrow) in the nucleolus (Nu) and numerous cytoplasmic ribosomes. N, nucleus; mi, mitochondria; edb, electron dense body. Glutaraldehyde, postfix in osmium tetroxide, uranyl acetate, X24,400.

Fig. 18. An electron micrograph of a group of primary spermatogonia showing several mitotic figures (M). Note distinct chromosomes (arrow). Glutaraldehyde, postfixed in osmium tetroxide, X5,950.

large increase in the number of these cells. The mitotic divisions occur singly but more commonly in groups. The prophase stage of mitosis begins with the condensation of nuclear DNA into long thin strands (Fig. 11C; Fig. 19) that border, along their longitudinal axis, on the nuclear boundary. These strands then thicken and shorten while the nucleolus disappears (Fig. 11D; Fig. 20). By the end of prophase these DNA-containing strands thicken and shorten further into about 30 chromosomes, and the nuclear boundary disappears (Fig. 11E; Fig. 21). At metaphase, the chromosomes have aligned at the metaphase plate and a spindle forms, converging at two small centrioles which stain strongly with iron hematoxylin (Fig. 11F; Fig. 22); no astral rays are noticeable. At anaphase the chromosomes move towards the two centrioles (Fig. 11G; Fig. 24); at telophase, the chromosomes are no longer distinct but have formed into DNA-containing bodies (Fig. 11H; Fig. 25). Finally cytokinesis occurs by furrowing (Fig. 11H; Fig. 25) and in the two daughter cells a nuclear boundary is reformed; the chromosomes disperse and the nucleolus reappears so that a cell similar to the original primary spermatogonium (Fig. 11B) is formed.

All of the mitotic chromosomes stain more intensely than the chromatin of the interphase nucleus with the Feulgen and toluidine blue O methods for DNA. Also, the mitotic chromosomes stain strongly for protein with the mercury-bromphenol blue technique.

In electron micrographs, the chromosomes are composed of aggregates of finely granular dense material. Ribosomes are scattered throughout the dividing cell whereas mitochondria,

Fig. 19. Two primary spermatogonia (arrow) in the epidermis of *H. fusca*, after 10 days at 8°C, at the early prophase stage of the mitotic phase of spermatogenesis. Karpechenko, 4μ, iron hematoxylin, X2,000.

Fig. 20. Two primary spermatogonia (arrow) in the epidermis of *H. fusca*, after 10 days at 8°C, at the middle prophase stage of the mitotic phase of spermatogenesis. Karpechenko, 4μ, iron hematoxylin, X2,000.

Fig. 21. A primary spermatogonium (arrow) in the epidermis of H. fusca, after 10 days at 8°C, at the late prophase stage of the mitotic phase of spermatogenesis. Karpechenko, 4 μ , iron hematoxylin, X2,000.

Fig. 22. A primary spermatogonium (arrow) in the epidermis of H. fusca, after 10 days at 8°C, at the metaphase stage of the mitotic phase of spermatogenesis. Note the centriol (ce), and spindle. Karpechenko, 4 μ , iron hematoxylin, X2,000.

Fig. 23. Four primary spermatogonia in the epidermis of *H. fusca*, after 10 days at 8°C at the metaphase stage of the mitotic phase of spermatogenesis. Note the several distinct chromosomes (arrow). This figure is a plane of cut perpendicular to that in Fig. 22. Karpechenko, 4μ, iron hematoxylin, X2,000.

Fig. 24. A primary spermatogonium in the epidermis of *H. fusca*, after 10 days at 8°C, at the anaphase stage of the mitotic phase of spermatogenesis (arrow). Note the spindle and centriole. Karpechenko, 4μ, iron hematoxylin, X2,000.

membranous cisternae and occasionally electron dense bodies are located peripherally.

As the primary spermatogonia proliferate, the epitheliomuscular cells are continually forced away from the mesoglea. As a result, the testes are always covered by a layer of epitheliomuscular cells which often retain an attachment to the mesoglea by fine cytoplasmic extensions.

Fig. 25. A primary spermatogonium in the epidermis of H. fusca after 10 days at 8°C at the telophase stage of the mitotic phase (arrow). Note the spindle. Karpechenko, 4μ, iron hematoxylin, X2,000.

Cytology and Cytochemistry of the Secondary Spermatogonium

The final result after numerous primary spermatogonial divisions within the mitotic phase of spermatogenesis is the secondary spermatogonium. These cells occur in groups adjacent to the primary spermatogonia; they are round to oval and about the size of the original interstitial cell (Fig. 11I; Fig. 26). The centrally located nucleus is about half the volume of the cell and shows small granules of DNA scattered throughout the nucleus and along the nuclear boundary. The nucleolus is smaller and stains lightly with toluidine blue O and iron hematoxylin (Fig. 26). The cytoplasm reacts less intensely than the primary spermatogonium with iron hematoxylin and toluidine blue O (Fig. 26). In electron micrographs, the nucleus, nucleolus and cytoplasm appear similar to the interstitial cell.

Fig. 26. Several secondary spermatogonia in H. fusca after 15 days at 8°C. The nucleolus (Nu) is small and the cytoplasm is less basophilic than the primary spermatogonium. Karpechenko, 4 μ , iron hematoxylin, X2,000.

The nucleolus, DNA granules and the cytoplasm which react positively for nucleic acids also give a positive test for proteins and react negatively with the PAS and Best's carmine methods.

Mitotic figures are not observed among the secondary spermatogonia. Instead, these cells transform directly into the primary spermatocyte and initiate the second phase in spermatogenesis or meiosis.

Cytology and Cytochemistry of the Primary Spermatocyte

Primary spermatocytes occur in groups of various sizes adjacent to the secondary spermatogonia. In the transformation of the secondary spermatogonia to the primary spermatocytes (Fig. 11J), a series of nuclear changes are noted without alteration of cellular size. In the centrally located nucleus, the chromatin bodies which stain intensely with iron hematoxylin and the Feulgen procedure become more coarsely granular and the nucleolus is not apparent (Fig. 11J; Fig. 27). In electron micrographs, the diffusely scattered, dense nuclear granules aggregate into chromatin clumps. The cytoplasm appears less basophilic than the spermatogonia after iron hematoxylin and toluidine blue O. The large iron hematoxylin stained granules just outside the nuclear boundary of the primary spermatogonium are not visible in the primary spermatocyte. However, the cytoplasm still contains many free ribosomes and a paucity of other cellular organelles.

In the primary spermatocytes, the nucleolus, DNA-containing granules and the cytoplasm react positively for proteins, and negatively to the PAS and Best's carmine methods.

The primary spermatocytes undergo meiosis I (Fig. 11J-O) in small localized groups adjacent to non-dividing primary spermatocytes. Prophase I is characterized by a large irregular mass of chromatin in the nucleus (Fig. 11K; Fig. 27) enclosed by the nuclear membrane. The DNA material consisting of long thin intertwined strands probably represent the synapsed chromosomes. The cytoplasm is organized as a fine lightly basophilic ring around the nucleus (Fig. 11K; Fig. 27). At metaphase, the chromosomes become shorter, thicker and align at the metaphase plate (Fig. 28). A spindle, but no asters, converging at two dark centrioles also appears (Fig. 11L; Fig. 28). A cross section through the chromosomes at the metaphase plate perpendicular to the plane of cut in Fig. 28 shows about 15 distinct chromosomes which are much thicker than the metaphase chromosomes of mitosis (Fig. 11I; Fig. 29). During anaphase, the chromosomes become indistinct, move towards the two centrioles (Fig. 11M) and finally merge at telophase into two bean-shaped structures (Fig. 11N; Fig. 30) of the secondary spermatocyte (Fig. 11 O).

The chromosomes of meiosis I stain more intensely than the chromatin of the interphase nucleus with methods for DNA and protein, but negatively with the PAS and Best's carmine technique.

Fig. 27. A photomicrograph showing a large group of primary spermatocytes (large arrow) and a large group of primary spermatocytes at prophase I of meiosis (small arrow), in H. fusca, after 20 days at 8°C. Maximov, 4μ, iron hematoxylin, X2,000.

Fig. 28. A primary spermatocyte at metaphase I of meiosis in H. fusca after 20 days at 8°C. Note the spindle and two centrioles (arrow). Karpechenko, 4μ, iron hematoxylin, X2,000.

Fig. 29. A group of primary spermatocytes at metaphase I of meiosis in *H. fusca*, after 20 days at 8°C showing about 15 thick chromosomes (arrow). This section was cut at a plane perpendicular to that in Fig. 28. Karpechenko, 4 μ , iron hematoxylin, X2,000.

Fig. 30. A group of primary spermatocytes during meiosis in *H. fusca*, after 20 days at 8°C, showing several cells at telophase I (large arrow) and II (small arrow). Maximov, 4 μ , iron hematoxylin, X2,000.

In electron micrographs it has not been possible to distinguish meiosis I from meiosis II due to their similarities and an incomplete investigation of this phase in this study. The chromosomes are composed of finely granular dense material (Fig. 31); ribosomes are scattered throughout the dividing cell, whereas mitochondria are located peripherally.

Fig. 31. A low magnification electron micrograph of a portion of a testis of *H. fusca*, after 22 days at 8°C showing several cells undergoing division by meiosis (arrows). Glutaraldehyde, postfixed in osmium tetroxide, X5,950.

Cytology and Cytochemistry of
the Secondary Spermatocyte

The secondary spermatocytes are seen rarely among groups of cells in meiosis. These cells are round and one half the size of the primary spermatocyte. The nuclear region consists of a mass of DNA without a noticeable nuclear boundary. The cytoplasm surrounding the nucleus stains lightly with iron hematoxylin and toluidine blue 0 indicating a weak basophilic affinity. It is difficult to find the secondary spermatocyte in preparations for the light and electron microscopes, since this cellular stage is short lived (Fig. 32); the secondary spermatocyte enters meiosis II (Fig. 11 O-Q) immediately after the

Fig. 32. A portion of a testis of H. fusca, after 22 days at 8°C, showing a secondary spermatocyte (large arrow) and numerous spermatids (small arrows). Karpechenko, 4μ, iron hematoxylin, X2,000.

completion of meiosis I. In this process a spindle converging at two centrioles is formed and the central chromatin mass splits in half, each portion moving towards a centriole (Fig. 11P; Fig. 30) and the nucleus of the daughter cell is reformed. The daughter cells often remain joined by a fine cytoplasmic connection through which the spindle fibres seem to persist (Fig. 33).

The chromosomes during meiosis II stain more intensely than the chromatin of the interphase nucleus but show a similar intensity after the Feulgen and mercury-bromphenol blue procedures as the chromosomes during meiosis I. Also, the regions of the nucleus, nucleolus and cytoplasm, containing nucleic acids, stain strongly for proteins.

Fig. 33. A portion of a testis of H. fusca, after 22 days at 8°C, showing the two daughter cells, the result of meiosis II, connected by an intercellular bridge (arrow). Karpechenko, 4 μ iron hematoxylin, X2,000.

Cytology and Cytochemistry of the Spermatid

The small round spermatids (Fig. 11Q) are tightly packed in groups adjacent to the primary spermatocytes (Fig. 32). These cells are about one quarter the volume of the primary spermatocytes. Dense irregular masses of DNA are scattered throughout the nucleus and along the nuclear membrane (Fig. 11Q; Fig. 32); from electron micrographs these masses appear to be composed of chromatin granules which are scattered throughout the nucleus and along the nuclear membrane. A nucleolus is absent in the spermatid. A small ring of cytoplasm around the nucleus stains very lightly with iron hematoxylin and toluidine blue O; however, this perinuclear region still contains many free ribosomes and few other cell organelles (Fig. 34). All regions containing nucleic acids react positively with the mercury-bromphenol blue method and the spermatids react negatively to the PAS and Best's carmine procedure. The spermatid now enters the third phase of spermatogenesis or spermiogenesis.

Cytology and Cytochemistry of the Spermatozoan

Motile spermatozoa are found in those regions of the testis outermost from the mesoglea, bathed in a fluid that does not stain with any of the procedures employed in this study. In phase contrast microscopy, the living spermatozoan consists of a dense head ($1.5\mu \times 3.5\mu$), a lighter middle piece ($1.0\mu \times 1.5\mu$) and a long flagellum (about 30μ) (Fig. 35).

Fig. 34. An electron micrograph of a portion of a testis in *H. fusca*, after 22 days at 8°C, showing several spermatids (large arrow), many transforming spermatids at the diffuse stage of nuclear condensation (S1) and several at the dense stage of nuclear condensation (S2). F, flagellum; IC, intercellular bridge; mi, mitochondrion. Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X5,950.

In fixed microscopic preparations, the head stains intensely with iron hematoxylin, the Feulgen technique and toluidine blue O, indicating its DNA composition; the mitochondria and flagellum are not noticeable in stained preparations.

Fig. 35. A phase contrast photomicrograph of living motile spermatozoa from a squash preparation of *H. fusca*, after 30 days at 8°C, showing the head (N), middle piece (mi) and flagellum (F). X2,000.

An electron micrograph of the spermatozoan shows a homogeneous, electron dense head, a middle piece consisting of four large spherical mitochondria and a flagellum (Fig. 36). In addition, there is a thick electron dense layer surrounding the head and middle piece. Occasionally some cytoplasm surrounding the head is noted in electron micrographs.

The spermatozoan is formed through a series of transformations from the spermatid (Fig. 11Q-T). The cellular

Fig. 36. Spermatozoan of *H. fusca* after 28 days at 8°C, demonstrating the head (N), middle piece consisting of four mitochondria (two shown here) (mi), a flagellum (F) and an outer electron dense layer (L). Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X19,100.

size becomes reduced by elimination of most of the cytoplasm and a condensation of the nuclear chromatin into the spermatozoan head; four mitochondria are oriented at the middle piece; and a flagellum is elaborated. These light and electron microscopic findings are described below.

Nuclear and cytoplasmic changes in forming the Spermatozoan -- Condensation of the nuclear chromatin

begins with the accumulation of DNA along the nuclear boundary of the spermatid (Fig. 11R). Subsequently, two dense accumulations of DNA appear at the opposite ends of the

nucleus (Fig. 11S; Fig. 37). Finally, the nucleus becomes pyriform in shape and stains intensely and homogeneously with iron hematoxylin, toluidine blue O and using the Feulgen technique (Fig. 11T; Fig. 37). Electron microscopic studies of these nuclear transformations show a progressive increase in the concentration of the chromatin granules. The aggregated granules of chromatin in the spermatid pass from a diffuse and homogeneous appearance (Fig. 38) to a densely homogeneous stage (Fig. 34). The nuclear membrane recedes to surround the condensed nucleus as the nuclear chromatin condenses (Fig. 39). The cytoplasm of the spermatid stains progressively lighter with iron hematoxylin and toluidine blue O as the nucleus condenses. In electron

Fig. 37. A portion of a testis of *H. fusca*, after 22 days at 8°C, showing several spermatids in the phase of spermiogenesis (arrow). C, cytoplasm; N, nucleus. Karpechenko, 4μ, iron hematoxylin, X2,000.

Fig. 38. Two transforming spermatids at the diffuse stage of nuclear condensation, connected by an intercellular bridge. *H. fusca* after 22 days at 80°C. bb, basal body; mi, mitochondrion; r, cytoplasmic ribosomes; F, flagellum; N, nucleus. Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X12,800.

micrographs numerous ribosomes in the cytoplasm persist during spermiogenesis (Fig. 38: Fig. 40). Eventually the cytoplasm becomes much reduced in volume and only a few ribosomes are observed (Fig. 41). The cytoplasmic membrane recedes to surround the condensed nucleus and the middle piece as spermiogenesis is completed (Fig. 42).

Cytology of the mitochondria in the middle piece --

With the embedding procedures employed in this study for light microscopy, mitochondria are not preserved. However, when living squash preparations are examined in the phase

Fig. 39. A transforming spermatid at the dense stage of nuclear condensation showing the double-layered nuclear membrane (NM) receding about the nucleus (N). *H. fusca* after 28 days at 8°C. mi, mitochondrion; CM, cell membrane. Glutaraldehyde, postfixes in osmium tetroxide, lead citrate, X24,000.

Fig. 40. A transforming spermatid at the dense stage of nuclear condensation in *H. fusca*, after 28 days at 8°C. Note the cytoplasmic ribosomes (r) and the star-shaped filaments in the emerging flagellum (arrow). mi, mitochondrion; N, nucleus; NM, nuclear membrane ; CM, cytoplasmic membrane. Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X26,800.

contrast microscope, four refractile bodies are noted close to the flagellum and sperm head in transforming spermatids (Fig. 43A; Fig. 43B); these bodies correspond in location to the position of the mitochondria in electron micrographs (compare Fig. 43A and Figs. 38-42). Only two mitochondria are seen in longitudinal sections of spermatozoa (Fig. 38-42), whereas in cross sections through the middle piece there appears to be four mitochondria. The small mitochondria of the spermatids seem to enlarge as the nucleus condenses to the spermatozoan head and the number of cristae are increased within these large mitochondria (Fig. 34; Fig. 39-42).

Fig. 41. A transforming spermatid at a late dense stage of nuclear condensation showing the extent of reduction in the volume of the cytoplasm. *H. fusca* after 28 days at 80C. mi, mitochondrion; CM, cytoplasmic membrane; F, flagellum; N, nucleus. Glutaraldehyde postfixed in osmium tetroxide, uranyl acetate, X21,500.

Fig. 42. A transforming spermatid at a late dense stage of nuclear condensation showing the cytoplasmic membrane (arrow), receding about the nucleus (N) and middle piece. *H. fusca* after 28 days at 8°C. Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X38,200.

Fig. 43 A, B. Phase contrast photomicrographs of transforming spermatids, demonstrating the appearance of refractive bodies (arrows). Note the two spermatids in a single vesicle in Fig. 43A. From a squash preparation of *H. fusca* after 24 days at 8°C. X2,000.

Cytology of the flagellum -- The flagellum is either not preserved or lost in the preparations fixed and stained for light microscopy. However, the flagellum is clearly visible in phase contrast microscopy of living spermatids (Fig. 43A). In electron micrographs, it is already formed by the time the nuclear granules have condensed to the diffuse stage (Fig. 38). The flagellum emerges from a basal body located between the mitochondria and appear to be composed of the usual two central and nine outer double filaments (the axial filament complex) (Fig. 38; Fig. 41).

Two to four transforming spermatids or spermatozoa are often contained within a single vesicle (Fig. 43A). In electron micrographs, up to four transforming spermatids also appear interconnected by cytoplasmic bridges (Fig. 34; Fig. 38), and up to four spermatozoa are found enclosed in a single unit (Fig. 44). Four cells enclosed in this manner probably correspond to four spermatids, resulting from two meiotic divisions of one primary spermatocyte, which have not separated from each other due to incomplete cytokinesis. An indication of this hypothesis is presented in Fig. 33 where a cytoplasmic bridge persists between the two spermatids.

Cellular Volumes of the Consecutive Transformation Stages

The measurement of cellular diameters and volumes has been useful in conjunction with the cytology of the differentiating interstitial cell as described and illustrated in Fig. 11. The results presented in Table 1, correspond to the changes expected of the interstitial cell in spermatogenesis. To

Fig. 44. A cross section of four spermatozoa (N) of H. fusca after 28 days at 8°C, enclosed within a vesicle. Glutaraldehyde, postfixed in osmium tetroxide, uranyl acetate, X26,800.

compare volumetrically the various cellular types in Fig. 11, the prophase stage of the primary spermatocyte (cell K) was chosen as the standard reference cell. This cell is observed in large numbers in most testes and is easy to measure because of its consistent spherical shape.

The interstitial is of a similar diameter and volume as the prophase stage of the primary spermatocyte. In transforming to the primary spermatogonium, the interstitial cell enlarges greatly (Table 1, column B). Actual determinations of the volume of the primary spermatogonia lie within the limits of two to eight times the volume of the interstitial cell (Appendix V, Table 4). This suggests that if an interstitial cell enlarges to its maximum volume (eight times or $1024\mu^3$; Table 1), then a maximum of three mitoses must occur for its volume to be reduced to that of the secondary spermatogonium. As a consequence, there should be three families of primary spermatogonia of volumes 1024, 512, $255\mu^3$ corresponding to 1, 2, and 3 mitoses. This assumes that each primary spermatogonial division results in two daughter cells of equal volume. Due to continued growth and division, cellular volumes between the above limits are also found (Appendix V, Table 4). Thus, it now appears possible that an interstitial cell will grow to eight times its original volume and then divide three times to form the secondary spermatogonium.

The secondary spermatogonium is half the volume of the smallest primary spermatogonium and of the same volume as the primary spermatocyte and the reference cell. These

TABLE 1

THE VOLUME, DIAMETER, AND TIME OF FIRST APPEARANCE
OF THE CONSECUTIVE TRANSFORMATION STAGES FROM
THE INTERSTITIAL CELL TO THE SPERMATOZOAN*

A	B	C	D
TYPE OF CELL	VOLUME (μ^3)	DIAMETER (μ)	DAY OF FIRST APPEARANCE AT 8°C
Interstitial	128	6.2	
Primary Spermatogonium**	1024 512 256	12.5 9.9 7.9	
Secondary Spermatogonium	123	6.2	14
Primary Spermatocyte	122	6.1	16
Prophase stage of the Primary Spermatocyte	123	6.2	16
Secondary Spermatocyte	60	4.8	18
Spermatid	31	3.9	18
Spermatid in the phase of Spermiogenesis	21	3.4	18
Spermatozoan		4 x 1.5 μ	20

* The complete data are reported in Appendix V, Table 4 and 5.

** See text (P. 53) for an explanation of the values for the primary spermatogonium.

values would be expected if the secondary spermatogonium was the result of the last primary spermatogonial mitosis and if it transformed directly into the primary spermatocyte without division.

The primary spermatocyte is the same volume as the prophase stage of meiosis I signifying that it transforms into the prophase stage without growth or division.

The secondary spermatocyte is about half the volume of the primary spermatocyte, and the spermatid is about half the volume of the secondary spermatocyte. This corresponds to the change in volume expected from meiosis I and II respectively.

Finally, the spermatids transforming into spermatozoa are smaller than the spermatids. This is expected if the cellular material of the spermatid is being condensed or eliminated during the phase of spermiogenesis.

The Time of First Appearance and
the Spatial Distribution of the
Differentiating Cells in the Testis

The cells depicted in Fig. 11 (from stages A-T) occur consecutively. The time at which each cellular stage first appears within the 30 days of examination is presented in Table 1, column D. Initially, the interstitial cells cease to differentiate into somatic cells and begin germinal differentiation when the culture temperature is reduced to 8°C. This change in the differentiation pathway of the interstitial cell occurs most likely within the first few days at 8°C. To evaluate

this hypothesis, the number of primary spermatogonia and cnidoblasts from the gastric region of the epidermis was counted in relation to the number of days at 8°C. The results are reported in Table 2, and illustrated in Graph 1. The number of primary spermatogonia increases steadily in the first 12 days at 8°C, whereas the number of cnidoblasts or somatic cell differentiation of the interstitial cells decreases. After 30 days at 8°C the number of cnidoblasts is drastically reduced in the tentacles and gastric region.

The primary spermatogonium is the only cell type in the testis for the first 14 days at 8°C. These cells increase greatly in number during these 14 days within the mitotic phase of spermatogenesis. To evaluate this increase with time a count was made of the number of spermatogonial mitoses between 1-30 days at 8°C and the results are indicated in Table 3 and illustrated in Graph 2. Cellular proliferation increases slowly for the initial five days, then rapidly to a peak at about 16 days; by the 28th day, the mitotic activity has declined to a level below the first day. A similar pattern is demonstrated in Table 2 and Graph 1. The number of spermatogonia increases slowly for the first 5 days and then more intensively from 5-12 days. It appears that spermatogonial proliferation begins with the initiation of sexuality and continues to the end of the 30 day period at 8°C.

The secondary spermatogonia are first noticed after about 14 days in groups adjacent to the primary spermatogonia

TABLE 2

THE NUMBER OF PRIMARY SPERMATOGONIA AND CNIDOBLASTS
IN THE GASTRIC REGION OF THE EPIDERMIS IN RELATION TO
THE NUMBER OF DAYS AT 8°C.*

(Compare with Graph 1)

A Number of days at 8°C*	B Primary Spermatogonia		C Cnidoblasts	
	Mean Number	Observed Range	Mean Number	Observed Range
0**	24	12-47	49	28-71
3	37	16-65	41	26-53
4	37	15-59	43	22-75
5	42	30-63	52	29-74
7	51	30-72	38	15-69
9	71	36-101	35	23-88
12	181	147-249	40	14-56

* The complete data is given in Table 6, Appendix VI.

** These values are for a budding animal at 20°C.

GRAPH 1

THE NUMBER OF PRIMARY SPERMATOGONIA AND CNIDOBLASTS
IN THE GASTRIC REGION OF THE EPIDERMIS IN RELATION TO
THE NUMBER OF DAYS AT 8°C

(COMPARE WITH TABLE 2)

TABLE 3

THE NUMBER OF PRIMARY SPERMATOGONIAL
MITOSES IN HISTOLOGICAL PREPARATIONS
IN RELATION TO THE NUMBER OF DAYS AT 8°C*

(Compare with Graph 2)

Number of days at 8°C	Mean Number of Mitoses	Number of days at 8°C	Mean Number of Mitoses
0**	10	12	116
1	12	14	143
2	14	16	155
3	12	18	152
4	14	20	134
5	16	22	105
7	22	24	72
8	30	26	16
9	54	28	6
10	56	30	3

* The complete data is given in Table 7, Appendix VI.

** This value is for budding animals at 20°C.

GRAPH 2

THE NUMBER OF PRIMARY SPERMATOGONIAL
MITOSES IN HISTOLOGICAL PREPARATIONS
IN RELATION TO THE NUMBER OF DAYS AT 8°C

(COMPARE WITH TABLE 3)

and outermost from the mesoglea (Table 1, column D).

The primary spermatocyte and prophase stage of meiosis I, first appear after 16 days at 8°C in groups adjacent to the secondary spermatogonia and outermost from the mesoglea (Table 1, column D). Therefore, within 2 days the secondary spermatogonium has transformed to the prophase stage of meiosis I and the second phase of spermatogenesis has begun.

The secondary spermatocytes and spermatids first appear after 18 days adjacent to the primary spermatocyte (Table 1, column D). Thus, the second or meiotic phase begins after about 15-16 days and requires about 48-72 hours for completion. However, the meiotic phase does not cease after 18 days but continues up to and beyond 30 days at 8°C.

The first spermatozoa are noticed after 20 days at 8°C. Therefore, the process of spermiogenesis also requires about 48 hours and like the meiotic phase, it continues up to and beyond 30 days at 8°C.

A diagrammatic representation of a typical testis after about 22 days at 8°C shows that the consecutive cell stages are distributed in the testis in an orderly manner (Fig.45). The differentiating cells are found usually in a gradient with the primary spermatozoa nearest the mesoglea, the motile spermatozoa outermost and the other cellular stages between these limits. In any one testis this gradient is not always as ideal as diagrammed. The order of the cells can be more random and all stages might not be present in a single testis.

Fig. 45. A diagram demonstrating the spatial distribution of the differentiating cells within a testis of *H. fusca*, after about 22 days at 80C. Note the occasional extension of the epitheliomuscular cells into the testis. PS, primary spermatogonium; SS, secondary spermatogonium; P Spc, primary spermatocyte; S Spc, secondary spermatocyte; Spt, spermatid; Sp, spermatids in spermiogenesis; Sz, spermatozoan; Ep, epitheliomuscular cell. X1,300.

The continued formation of spermatozoa from 20-30 days at 8°C expands the testis to the conical shape. At 30 days all testes contain spermatozoa but most have released them and appear empty with a large reduction in number of all the cell stages described. Beyond 30 days at 8°C the remaining spermatogonia, spermatocytes and spermatids continue to transform to spermatozoa. Eventually, the remnants of the testes after moving down the body column, slough off at the basal disk and the animal again begins asexual reproduction by budding at 8°C.

Each successive cell stage always appears first in those testes nearest the hypostome; within each testis they usually appear at a point farthest from the mesoglea but occasionally a group of cells will appear out of order. Furthermore, neither all testes in the same animal nor all animals after equal times at 8°C will have progressed to the same stage of differentiation.

The Effect of The Reduced Temperature On the Differentiating Cells

As described earlier, the interstitial cells differentiate continuously into somatic cells in animals kept at 20°C. By reducing the temperature to 8°C, the interstitial cells begin germinal differentiation and enlarge to form the primary spermatogonia. In an effort to determine if the reduced temperature is always necessary for sexual development to continue or acted solely as the original stimulus, four groups of fifteen animals each were placed at 8°C and then returned to 20°C after 5, 10, 15, and 20 days at the

low temperature. Those returned after 5 and 10 days did not develop testes, although definite epidermal thickenings are observed in hydra exposed for 10 days to 8°C. When animals are returned after 15 days, 3 of the 15 hydra at 20°C did continue to form testes. Although these testes were not as large as testes on animals kept continuously at 8°C, spermatozoa are formed. All hydra in the group after 20 days at 8°C formed testes at 20°C, although these testes did not appear as large as those at 8°C. Control cultures at 8°C formed testes and those at 20°C failed to do so. It appears that until 15 days incubation primary spermatogonia are not completely determined to form spermatozoa. This time period of 15 days corresponds approximately to the time meiosis begins (Table 1, column D).

When male and female clones of H. fusca are maintained at 8°C spermatozoa and eggs are formed respectively. Eventually small hydra hatch from some of the eggs. Thus it can be concluded that the use of reduced temperature to initiate spermatogenesis results in a functional spermatozoan.

The Distribution of Food Materials In Sexual Animals

The quantity of glycogen, proteins and PAS-reactive materials in the gastrodermis of sexual hydra differs from non-sexual animals. Histochemical studies reveal that at the end of 30 days at 8°C, the gastrodermis of sexual hydra is much depleted in these food materials. The digestive cells are more extensively vacuolated, except in those regions adjacent to the epidermal testes, and contain larger

accumulations of protein, glycogen and PAS-positive material. This difference between sexual and non-sexual animals is illustrated in Fig. 7 and Fig. 9. From these illustrations it is obvious that the gastrodermis of sexual hydra contains a greater amount of proteinaceous and PAS-positive material localized in cytoplasmic vacuoles. These vacuoles also contain RNA and DNA in varying proportions. Glycogen is found as small cytoplasmic granules near the periphery of the cell or adjacent to the mesoglea.

Studies concerning the changes in distribution of food materials with time in the cellular layers of hydra indicate that within 3 hours postfeeding, glycogen is concentrated along the enteric border of gastrodermal cells, and particularly in those digestive cells adjacent to the testes. After 6 hours the mesoglea is much thickened by accumulations of glycogen (Fig. 46); by 24 hours the mesoglea has returned to normal and small accumulations of glycogen are noted between the differentiating cells in the testis (Fig. 47). Based on these observations it appears that some glycogen taken up the gastrodermis is passed to the testis. Proteins and PAS-positive materials are not distributed in the same manner as glycogen.

The starvation of both sexual and non-sexual hydra for 5 days lowers drastically the amount of protein droplets in the gastrodermis and reduces considerably the amount of glycogen and PAS-positive vacuoles. The gastrodermis of sexual animals contains less glycogen and PAS-positive material than the non-sexual form; those compounds present are more concentrated in the cells adjacent to the testes.

Fig. 46. A photomicrograph showing the thickened mesoglea that stains positively for glycogen (arrow). H. fusca after 19 days at 8°C and 6 hours post-feeding. Allen's PFA 3, 4 μ , Best's carmine. X1,760.

Fig. 47. A portion of a testis of H. fusca after 22 days at 8°C and 24 hours postfeeding, showing glycogen granules (arrows) among the spermatozoa. Allen's PFA 3, 4 μ , Best's carmine, X1,760.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

This study has further elucidated the cytology, cytochemistry and ultrastructure of the differentiating interstitial cell during spermatogenesis of H. fusca. The interstitial cell is induced to enter germinal differentiation by a reduction in temperature. This stimulus is sufficient in our clone of H. fusca to cause the epidermal interstitial cell to become spermatozoa, a transformation it does not normally undergo. As a result there is a progressive alteration of the macroscopic and microscopic morphologies of the animal. Macroscopically, asexual reproduction by budding ceases and testes are formed along the gastric region of the epidermis. Microscopically, the interstitial cell in spermatogenesis passes through a series of consecutive transformation stages resulting in functional spermatozoa.

Cytology and Cytochemistry of Differentiating Interstitial Cells in Spermatogenesis

Spermatogenesis of H. fusca is described in three consecutive phases of mitosis, meiosis and spermiogenesis. The interstitial cell undergoes characteristic morphological changes through each of these phases. Initially, the interstitial cell ceases to differentiate into a somatic cell (cnidoblast), and enlarges up to eight times its original

volume, becoming the primary spermatogonium. Cellular proliferation of the primary spermatogonium in the phase of mitosis serves to greatly increase the number of cells that will eventually be released as spermatozoa. Because of this proliferation, the phase of mitosis is a period of intense RNA synthesis which continues essentially throughout spermatogenesis with a peak after about 16 days at 8°C. In the primary spermatogonium, the localization of large amounts of RNA in the nucleolus and cytoplasm and the perinucleolar ring of DNA are characteristics of a cell actively synthesizing proteins for growth or multiplication (Casperson, 1950; Brachet, 1957; De Robertis et al., 1965). The ribosome-like granules in the nucleolus are probably precursors of the cytoplasmic ribosomes which are part of the cellular protein-synthesizing apparatus (Fawcett, 1966; Georgiev, 1967). In the subsequent phases of meiosis and spermiogenesis, the RNA content of the cytoplasm of the differentiating cell gradually decreases until little or none is observed in the spermatozoan.

After 16 days at 8°C, the spermatogonium situated outermost from the mesoglea is of the same volume as the interstitial cell and no longer grows and divides. Instead, this secondary spermatogonium transforms into the primary spermatocyte and begins the second phase of spermatogenesis or meiosis, which continues until the end of the thirty day period at 8°C. In other animals, a reduction of the chromosome number (reduction division) and also a halving of

the DNA content is the predominant feature of the meiotic phase of spermatogenesis (Brachet, 1957). Since quantitative measurements were not made in this study, it is not certain that the DNA content of the spermatid is half that of the primary spermatocyte as would be expected. The observation of 30 chromosomes in spermatogonial mitoses and 15 synapsed chromosomes at metaphase I of meiosis correlates with the number of chromosomes in epitheliomuscular cell mitoses (Niiyama, 1944). The characteristics of meiosis in H. fusca are similar to those in other animal systems, particularly in regard to the extended prophase I and the short prophase II (De Robertis et al., 1965). However, the numerous subdivisions of prophase I are not discernable in H. fusca.

The spermatid resulting from meiosis I and II is one fourth the volume of the primary spermatocyte as expected after the meiotic divisions. Our observations reveal that the spermatids have not completely separated from each other but remain joined by fine cytoplasmic bridges observable particularly in electron micrographs. Because of this incomplete separation, living spermatids transforming to spermatozoa are often seen in groups of four within a common vesicle in phase contrast microscopy. These four haploid spermatids probably are the result of a single primary spermatocyte undergoing meiosis I and II.

After 18 days at 8°C, the spermatid has entered the third phase of spermatogenesis or spermiogenesis and transforms into the motile spermatozoan. The events of

spermiogenesis in H. fusca are similar to those in other animal systems, in relation to the condensation of the nucleus to the pyriform shape, the elimination of cytoplasm and the elaboration of a flagellum from a centriole (De Robertis et al., 1965; Swanson, 1957; Balinsky, 1965). However, a nebenkern or mass of mitochondria which form a helical mitochondrion at the middle piece is not present. Instead, four spherical mitochondria comparatively larger than in previous cell types, orient in a plane at the middle piece. The larger number of cristae in the spermatozoan mitochondria may be an indication of their increased oxidative capacity required for flagellar movement (Fawcett, 1966). In addition, an acrosomal system present in mammals is not apparent in electron micrographs of the spermatozoan of H. fusca, although a Golgi complex has been noted occasionally in transforming spermatids. The Golgi complex was rarely observed in electron microscopic preparations of transforming spermatids probably because of the staining methods used (Pearse, 1964).

Comparison of germinal and somatic cell differentiation of the interstitial cell reveals similarities and differences. In somatic cell differentiation, one or more cytoplasmic organelles become prominent (Lentz, 1965), whereas in spermatogenesis nuclear changes and some cytoplasmic changes are predominant. In both types of differentiation (somatic and germinal), the nucleolus initially enlarges, ribosomes become less numerous, mitochondria enlarge and contain more

cristae, and all cells acquire a Golgi apparatus.

Our findings of spermatogenesis in H. fusca reveal similar stages of a differentiating interstitial cell to those reported by earlier authors in other species. However, groups of differentiating interstitial cells are not contained within a cyst as Tannreuther (1909) reported for H. viridis, but are delimited into columns by cytoplasmic extension of the epitheliomuscular cells. This is in agreement with the studies of Brien and Reniers-Decoen in H. viridis, (1950). In addition, only Tannreuther (1909) describes a vesicle-like structure containing four spermatids transforming into four spermatozoa. Other authors failed to observe this vesicle because they did not study living preparations which we find of major importance in observing these vesicles. Downing (1905) stated that the first testis to contain spermatozoa were those nearest the peduncle, whereas in H. fusca they appear first in those testes nearest the hypostome. We are also unable to confirm the extensive detail described by Downing. At the ultrastructural level, differentiation in H. fusca appears similar to that in H. viridis described briefly by Burnett et al., (1966), particularly with regard to cytoplasmic bridges between cells, condensation of nuclear material, and the thick outer electron-dense layer of spermatozoa. However, Burnett et al., (1966), observed only three mitochondria in spermatozoa, whereas four are present in H. fusca.

Volume Changes of the Differentiating Cells

The daughter cells resulting from each mitotic or meiotic division are one-half the volume of the parent cell. The volumes of the consecutive cellular stages of spermatogenesis agree with the changes expected of an interstitial cell differentiating to spermatozoa. In determining these cell volumes we assumed the cells to be spherical. Most of the cells measured were round to oval. Furthermore, it has been demonstrated that characterization of cell populations by measuring cell diameters proves to be an adequate method in following processes of change in cell populations (Simon, 1967). In such populations cells are present with volumes related in the ratio 1:2:4 and so on as a result of cellular division (Simon, 1967).

Distribution of Food Materials in Sexual Animals

Food distribution is altered in the gastrodermis as the processes of spermatogenesis occur in the epidermis. The concentration of food materials in digestive cells adjacent to the testes is probably the result of the need for food materials by differentiating cells. On the other hand, it is possible that the reduced temperature affects the food distribution and this in turn causes epidermal interstitial cells to become spermatozoa. A similar observation concerning the distribution of food materials in sexual animals has been previously reported by Downing (1905), and Burnett and Diehl (1964c). These authors however, did not observe the possible passage of glycogen

from the gastrodermis to the testis reported here.

The Effects of Reduced Temperature on
the Induction of Sexuality

There has been considerable interest concerning the factors responsible for the induction of sexuality. Various agents have been proposed including temperature change, PCO_2 , pO_2 , pH, starvation, rich feeding, day length, redistribution of food reserves and cessation of neurosecretion. (Burnett and Diehl, 1964c; Park et al., 1965) Of all these stimuli, temperature change has been the most consistent inducer for all Hydra species (Park et al., 1965). Consequently, Park et al., (1965), have presented the hypothesis accounting for sexual differentiation in terms of a temperature effect on a chemical substance which initiates sexual differentiation. Burnett (1966), on the other hand, maintains that interstitial cells differentiate into gametes whenever hypostomal neurosecretion ceases, which can be caused by a change in temperature. Evidence is presented in this thesis suggesting that reduced temperature is necessary to induce spermatogenesis, but as soon as the differentiating cell has entered the meiotic phase (after about 16 days) it continues to form the spermatozoan regardless of the temperature. Thus, it may be that any of the inducing agents postulated in the theories mentioned allow the interstitial cell to grow and divide. Consequently, on removal of animals from the cold, the primary spermatogonium would cease to grow and divide and would enter somatic cell differentiation. This would explain the smaller testes on

animals returned to 20°C after 15 and 20 days at 8°C, because cellular proliferation would not have been as extensive. It has been shown that the primary spermatogonia remaining in the testes after 30 days, become cnidoblasts rather than spermatozoa if the temperature is returned to 20°C (Burnett and Diehl, 1964c). Furthermore, the chemical agent(s) should attain a maximum change in its concentration after about 16 days at 8°C when the number of mitoses is maximum and the meiotic phase of spermatogenesis can first begin. This hypothesis could be tested initially by mitotic counts of primary spermatogonia in animals returned to 20°C after various times at 8°C. Evaluation of the chemical agent(s) must await isolation and/or characterization. Recent evidence suggests that this chemical control agent might be a hypostomal neurosecretory product which is reduced in quantity after 30 days at the reduced temperature (Lesh and Burnett, 1964; Burnett, 1966).

CHAPTER V

SUMMARY

1. The cytology, cytochemistry and ultrastructure of interstitial cells differentiating into spermatozoa of H. fusca was studied. Spermatogenesis can be induced by lowering the culture temperature from 20°C to 8°C. Within 30 days asexual reproduction by budding ceases and epidermal testes containing motile spermatozoa are formed.

2. The consecutive stages of interstitial cells differentiating into spermatozoa can be divided into three phases: mitosis, meiosis and spermiogenesis. Initially, the interstitial cells cease to form cnidoblasts and enlarge to become the primary spermatogonia.

3. The mitotic phase is a period of intense RNA synthesis due to primary spermatogonial growth and multiplication. These cells are characterized by a large nucleolus and a cytoplasm with numerous ribosomes. Cells at metaphase of mitosis have a spindle converging at two centrioles but no astral rays.

4. During the meiotic phase, the chromosome number is reduced from 30 to 15. The primary spermatocyte lacks a nucleolus and shows many cytoplasmic ribosomes. Subdivisions are not distinguishable in the extended prophase I. At metaphase I, synapsed chromosomes align at the metaphase plate and a spindle converging at two centrioles is formed.

In the secondary spermatocyte, the nucleus does not reform but the cell enters immediately into meiosis II. The resulting spermatid reforms a nucleus but not a nucleolus.

5. During spermiogenesis the spermatid is transformed into the motile spermatozoan by a condensation of nuclear chromatin, elimination of cytoplasm, enlargement and orientation of four spherical mitochondria at the middle piece and elaboration of a flagellum. As many as four spermatids, probably daughter cells of a single primary spermatocyte after meiosis I and II may remain joined together by fine cytoplasmic extensions.

6. The spermatozoan consists of a dense pyriform head ($1.5\mu \times 3.5\mu$), a middle piece containing four mitochondria ($1.0\mu \times 1.5\mu$), a flagellum (about 30μ), and an outer electron dense layer.

7. The diameter and volume of the consecutive cellular stages was determined. The interstitial cell (diameter 6.2μ) grows up to eight times its original volume ($128\mu^3$) to form the primary spermatogonium (diameter 12.5μ ; volume, $1024\mu^3$). Three families of primary spermatogonia can be distinguished (diameters, 7.9, 9.9, 12.5μ ; volumes 256, 512, $1024\mu^3$). The secondary spermatogonium and primary spermatocyte are the same size as the interstitial cell (diameter, 6.2μ ; volume, $123\mu^3$). The secondary spermatocyte is half the volume of the primary spermatocyte (diameter, 4.8μ ; volume $60\mu^3$) and the spermatid is one fourth the volume of the primary spermatocyte (diameter, 3.9μ ; volume $31\mu^3$).

8. From mitotic counts, it is concluded that the mitotic phase begins during the first day and continues throughout the 30 day period at 8°C. The meiotic phase begins after 16 days and continues to the end of the 30 day period. Spermiogenesis begins after 18 days at the reduced temperature and continues to the end of the 30 day period. The first spermatozoa appear after 20 days. It is concluded from these figures that both meiosis and spermiogenesis require about 48-72 hours.

9. The consecutive transformation stages are distributed in the testis in a gradient with the primary spermatogonia nearest the mesoglea, the spermatozoa outermost, and the other stages in between these limits. Each successive stage appeared first at a level outermost from the mesoglea and in the testis nearest the hypostome.

10. Food distribution appears altered in the gastrodermis of sexual hydra compared to non-sexual hydra. The digestive cells adjacent to the epidermal testes contain large accumulations of PAS-positive and proteinaceous material. It seems that glycogen passes from the gastrodermis through the mesoglea to the epidermal testis. Starvation of sexual hydra for 5 days markedly reduces the amount of protein and glycogen in the gastrodermis.

11. The events of spermatogenesis and the effect of reduced temperature on the differentiating cells are discussed.

APPENDIX I

CULTURING TECHNIQUES

A. Culture Solution for HydraStock A:

Versene (ethylenediaminetetraacetic acid).....	10 g
NaHCO ₃	20 g
Distilled Water.....	1000 ml

Stock B:

CaCl ₂	40 g
Distilled Water.....	1000 ml

Stock A and B can be made up in large quantities and kept indefinitely. The culture solution is made up by adding 10 ml of stock A into 2000 ml of distilled water and mixing it thoroughly; finally, 10 ml of stock B are added and mixed thoroughly.

Versene removes toxic copper ions from the water (Loomis and Lenhoff, 1956) and must be added first when the culture solution is prepared. The bicarbonate serves to maintain the pH between 7.2-7.5. Stock B provides calcium ions which are necessary for growth of Hydra (Loomis and Lenhoff, 1956). The culture solution can be used immediately but routinely it is used 24 hours after preparation.

B. Hatching and Treatment of Shrimp

Hydra are fed daily or every second day with freshly hatched shrimp (Artemia salina). These shrimp are hatched and treated as described by Loomis and Lenhoff (1956) with some modifications.

1. 1000 ml of tap water are mixed with 30 ml of a stock salt solution (360 g of coarse Windsor salt per liter of tap water) in an erlenmeyer flask and poured into standard pyrex dishes (9" x 12" x 2").

2. $1\frac{1}{2}$ to 2 ml of shrimp eggs are sprinkled over this solution; at a temperature of 22°C most shrimp hatch in 48 hours. By placing a 60 watt light in front of the pyrex dishes, the shrimp gather at a point nearest the light and are collected with a baster.

3. The shrimp are poured into a bolting cloth (#20) held in a standard household strainer. When a desirable amount is collected the animals are thoroughly washed under running tap water (adjusted to 19-20°C) to remove adhering salt.

4. The washed shrimp are suspended in a beaker containing some tap water. The animals are added to the culture dishes containing the hydra with a medicine dropper.

C. Treatment of the Hydra Culture After Feeding

While the hydra are feeding, the dishes are left undisturbed for at least 1 hour. The excess shrimp are then removed as follows:

1. The contents of the dish are poured into bolting cloth (#1). The dish is then rinsed twice with tap water (at 20°C) which is also poured into the bolting cloth. Immediately the dish containing the hydra is filled with fresh culture solution.

2. The contents in the bolting cloth, usually with some hydra, are then washed in running tap water to remove

most of the excess shrimp and then suspended in a dish containing tap water. After swirling the water in the dish, the hydra accumulate in the center and are transferred with a medicine dropper into a second dish containing tap water. This swirling procedure is repeated until all of the excess shrimp are removed; finally, the hydra are returned to their culture dish with a medicine dropper.

D. Cleaning the Hydra Culture Dishes

Hydra secrete slime at the proximal portion of their bodies. To prevent excessive growth of microorganisms the culture dishes of hydra are cleaned once or twice a week as follows:

1. All hydra are gently loosened by hand, transferred into bolting cloth and rinsed under tap water (at 20°C).
2. The culture dish is then rubbed with the fingers under tap water until all the slime is removed from the glass.
3. The animals are then resuspended in the dish in fresh culture solution.

E. Incubation Conditions

The dishes containing the hydra are kept in a standard incubator (General Electric, Model 805) at 19-20°C. An 18" fluorescent light (Type 115, RSTP, Wiener Electronic, Toronto, Ontario) provides a continuous illumination for 24 hours a day.

F. Efficiency of the Culture Techniques for Raising Hydra

Over a period of two years no difficulties were encountered to raise H. fusca or other species. This is

illustrated in Graph 3, showing a logarithmic growth curve of H. fusca. Under optimal conditions of rich daily feedings and cleanliness of the culture dishes, H. fusca will double its number every 4-5 days, maintaining a characteristic morphology with up to six or seven buds (Fig. 5).

Graph 3. A typical logarithmic growth curve of H. fusca growing at 20°C.

APPENDIX II

FIXATIVES AND EMBEDDING PROCEDURE

A. Fixatives Used1. Karpechenko's Fixative:

Chromic acid.....	0.5 g
Acetic acid.....	5.0 ml
Formalin, concentrated.....	40.0 ml
Distilled water.....	54.5 ml

The chromic acid is dissolved in distilled water and kept as a stock solution. Immediately before use the glacial acetic acid and the formalin are added. Animals are fixed for $\frac{1}{2}$ hour in the dark, then washed for $\frac{1}{2}$ hour in distilled water. We have found that this solution penetrates rapidly and is a good fixative for nuclei, chromosomes, cytoplasm, and the transformation stages of spermatids. Iron hematoxylin and basic aniline dyes also give good results with this fixative (Galigher and Kozloff, 1964).

2. Allen's PFA 3 Fixative:

Picric acid (saturated aqueous solution)....	75 ml
Formalin, concentrated.....	15 ml
Glacial acetic acid.....	10 ml
Urea.....	1 g

Animals are fixed for $\frac{1}{2}$ hour and washed for $\frac{1}{2}$ hour in 70% alcohol. This solution is a good nuclear fixative (Galigher and Kozloff, 1964).

3. Maximov's Fluid:

Potassium dichromate.....	2.5g
Mercuric chloride.....	5 g
Sodium sulfate	1 g
Formalin, concentrated (add just before use)	10 ml
Distilled water	100 ml

A stock solution of all the components and the formalin is prepared and is added immediately before use. Animals are fixed for $\frac{1}{2}$ hour and washed for $\frac{1}{2}$ hour in distilled water. Hydra fixed in this solution are treated with Lugol's iodine (Iodine, 2g; Potassium iodide, 3g; 95% ethanol, 100 ml) to insure removal of mercuric chloride crystals (Galigher and Kozloff, 1964). This is done by adding Lugol's solution dropwise to the 70% alcohol of the dehydration series until a deep amber colour is obtained.

This fixative preserves nuclei, cytoplasm, connective tissue, mucin, zymogen granules and other cytoplasmic granules (Galigher and Kozloff, 1964).

4. Carnoy's II Fixative:

Ethanol.....	60 ml
Chloroform.....	30 ml
Glacial acetic acid.....	10 ml

Animals are fixed at room temperature for not more than 45-60 minutes (Galigher and Kozloff, 1964). Specimens are transferred to 95% ethanol and then 100% ethanol. Times in alcohol are kept short because the alcohol extracts the nucleic acids (Pearse, 1960). When this fixative was used, benzene was substituted for xylene in the embedding procedure.

5. Bela Haller's Fixative:

Glacial acetic acid.....50 ml
Glycerine.....100 ml

Animals are fixed for about 30 seconds, then the fixative is blotted off and a drop of water is added. A cover slip is placed on the specimen and examined (Gatenby and Beams, 1950).

B. Embedding and Sectioning:

Throughout the embedding procedure, the animals are handled in small glass rings (diameter, 1cm; height, 1cm) covered at one end with #1 bolting cloth. After fixation, animals are washed as required after each fixative. The specimens are dehydrated in 10% gradations of alcohol (2 min. each), cleared in xylene/alcohol mixtures in the ration of 1:4, 2:3, 3:2, 4:1 (2 min. each), followed by three changes of pure xylene (5 min. each). Finally, the hydra are embedded in tissuemat (melting point 56.5°C), after passing through xylene/paraffin mixtures in the ratio of 3:1, 2:2, 1:3 (2 min. each) and three changes of pure paraffin (2 min. each). All paraffin mixtures are maintained at 60°C.

In order to easily recognize the hydra in the tissuemat block, animals are lightly stained with eosin dissolved in the 90% alcohol of the dehydration series.

The resulting block containing the hydra is trimmed and sectioned at 4 μ using a Leitz microtome. The sections are stretched on a water bath, kept at 41°C and affixed to slides with Mayer's egg albumen.

APPENDIX III

STAINING PROCEDURES AND HISTOCHEMICAL TESTS

A. Iron Hematoxylin1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov.

2. Reagents:(a) Mordant:

Ferric Ammonium Sulfate
"iron alum", violet crystals..... 2 g
Distilled water.....100 ml
Filter before use.

(b) Staining Solution:

Hematoxylin (light crystals). A 0.5% solution in distilled water is made and allowed to ripen to a deep port wine colour (about 6 weeks). Three drops of a saturated LiCO_3 solution is added immediately before use to each 100 ml of ripened solution to make it slightly alkaline.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Pass slides into fresh mordant, 2-3 hours.
- (c) Rinse in distilled water, 15-30 seconds.
- (d) Rinse in tap water, 1-3 minutes.
- (e) Pass into stain solution, 2-3 hours.
- (f) Rinse in distilled water.
- (g) Pass slides into running tap water, 5 minutes.
- (h) Destain under the microscope with 2% mordant until the cytoplasm is light grey but the chromatin is still blue-black.
- (i) Wash in running tap water, 30 minutes.
- (j) Dehydrate in alcohol.
- (k) Clear in xylene and mount in permount.

4. Results:

Hematoxylin with iron as the mordant is considered the best cytological stain for nuclei and chromosomes which appear blue-black. It also stains the cytoplasm in various shades of grey and is valuable in studies of mitosis, spermatogenesis and development.

5. Reference:

Galigher and Kozloff, (1964).

B. Flemming's Triple Stain

1. Fixative:

Karpechenko

2. Procedure:

- (a) Bring slides from xylene to 1% safranin in 50 % ethanol, 6 hours or overnight.
- (b) Differentiate in 50 % ethanol until cytoplasm and spindles are pink.
- (c) Rinse in distilled water.
- (d) Pass slides into 1% aqueous crystal violet, 10-30 minutes.
- (e) Dip into distilled water several times.
- (f) Pass into 1% aqueous orange G, 30-60 seconds. Prolonged exposure may remove too much crystal violet.
- (g) Dip several times in 95% ethanol.
- (h) Pass into 100% ethanol, 5-15 seconds.
- (i) Flood slide with clove oil and observe.
- (j) Quickly immerse in xylene.
- (k) Clear in xylene and mount in permount.

3. Results:

The chromosomes at metaphase and anaphase stain bright red, the cytoplasm yellow and the mitotic spindle and nuclei are blue. This stain is useful in correlating the results of iron hematoxylin with the Feulgen procedure.

4. Reference:

Galigher and Kozloff, (1964).

C. The Feulgen Technique

1. Fixatives:

Karpechenko, Allen's PFA 3 and Maximov.

2. Reagents:

(a) Schiff's Reagent. One gram of basic fuchsin (National Aniline Certified Stain) is dissolved in 200 ml of boiling distilled water, then shook and cooled to exactly 50°C. This solution is filtered and 20 ml of N-HCl is added to the filtrate. After cooling to 25°C, 1 g of potassium metabisulphite is added ($\text{Na}_2\text{S}_2\text{O}_5$). The mixture is allowed to stand in a brown bottle for 12 hours, then 2 g of activated charcoal are added and the contents shook for one minute. After being filtered in the dark, the contents are stored in a brown bottle in the refrigerator. This reagent is used in the dark at room temperature after filtering.

(b) N-HCl.

(c) SO₂ water. A stock solution is made by dissolving 1 g of potassium metabisulphite in 200 ml of tap water. 10 ml of N-HCl are added immediately before use.

3. Times of Hydrolysis In N-HCl at 60°C:

(a) Karpechenko's fixative.....	15 minutes
(b) Allen's PFA 3 fixative.....	22 minutes
(c) Maximov's fixative.....	5 minutes

4. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Pass slides into N-HCl at room temperature, 1 minute.
- (c) Hydrolyze in N-HCl at 60°C for required time (see above).
- (d) Rinse briefly in cold N-HCl.
- (e) Rinse in two changes of distilled water.
- (f) Transfer to Schiff's solution, 2 hours in the dark.
- (g) Rinse in three changes of SO₂ water, 10 minutes each.
- (h) Wash in tap water, 5 minutes.
- (i) Dehydrate in alcohol.
- (j) Counterstain in 0.5 % fast green FCF in 90 % alcohol, 30-60 seconds.
- (k) Clear in xylene and mount in permount.

5. Results:

DNA appears bright pink or shades of reddish-purple. Control slides subjected to the procedure described above but kept in distilled water at 60°C instead of N-HCl, did not give a positive test.

6. Reference:

Pearse, (1960).

D. Toluidine Blue O for RNA and DNA

1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov, Carnoy's II.

2. Reagents:

(a) Staining Solution. A 0.05% toluidine blue O solution in 1/10 N McIlvaine's citric acid-disodium phosphate buffer at pH 4.0 is prepared.

(b) Buffer. Solution A = 28.4g Na_2HPO_4 in 1000 ml distilled water.

Solution B = 21.0g citric acid in 1000 ml distilled water.

77.1 ml of solution A is mixed with 122.9 ml of solution B for a working buffer at pH 4.0.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Stain in 0.05% toluidine blue O at 37°C, 40 minutes.
- (c) Rinse off excess stain in two changes of tertiary-butyl alcohol.
- (d) Differentiate in fresh tertiary-butyl alcohol, 18 hours.
- (e) Clear in xylene and mount in permount.

4. Results:

RNA and DNA are stained in shades of blue.

5. References:

Bonhag, (1956); Pearse, (1960).

E. Methyl Green-Pyronin Method for RNA and DNA

1. Fixative:

Carnoy's II

2. Reagents:

- (a) A 2% aqueous solution of Pyronin Y (G.T. Gurr) is made and extracted with chloroform by shaking in a separatory funnel until the chloroform layer becomes colourless. Spectrophotometric graphs of the pyronin solution before and after extraction were similar, indicating a high degree of purity of the original dye.
- (b) A 2% aqueous methyl green solution is made and extracted with chloroform as above, until the chloroform layer is no longer violet.
- (c) To make the final staining solution, 12.5 ml pyronin Y and 7.5 ml of methyl green are mixed with 30 ml of distilled water.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Stain in the methyl green-pyronin solution, 6 minutes.
- (c) Blot with filter paper.
- (d) Pass through two changes of n-butyl alcohol, 5 minutes each.
- (e) Pass into xylene, 5 minutes.
- (f) Pass into cedar oil, 5 minutes, and mount in permount.

4. Results:

Chromatin is clear green; nucleolar and cytoplasmic RNA are bright red.

5. References:

Brachet, (1953); Kurnick, (1958); Pearse, (1960).

F. The Periodic Acid-Schiff Technique (PAS)

1. Fixatives:

Karpechenko, Allen's PFA 3 and Maximov.

2. Reagents:

- (a) Schiff reagent. See Feulgen technique.
- (b) 0.5% aqueous periodic acid.

3. Procedure:

- (a) Bring slides from xylene to distilled water. Mercury is removed after Maximov's fluid. (See Appendix II).
- (b) Oxidize in 0.5% periodic acid, 5 minutes.
- (c) Wash in distilled water, 5 minutes.
- (d) Pass into Schiff's reagent, 15 minutes.
- (e) Wash in running water, 10 minutes.
- (f) Dehydrate, clear in xylene and mount in permount.

4. Results:

Carbohydrate-containing proteins stain in various shades of reddish-purple. Glycogen stains strongly. Control slides subjected to the procedure described above, but kept in distilled water instead of 0.5% periodic acid, did not give a positive test.

5. References:

Hotchkiss, (1948); Pearse, (1960); Galigher and Kozloff, (1964).

G. Best's Carmine Stain for Glycogen

1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov.

2. Reagents:

- (a) Carmine Stock Solution. 2g carmine, 1g potassium carbonate and 5g potassium chloride are added to 60 ml of distilled water. This is boiled gently for 5 minutes, cooled and filtered. 20 ml of ammonium hydroxide are added to the filtrate. (Sp. G. 0.880). This solution lasts 3 months at 0-4°C.
- (b) Carmine Staining Solution. 15 ml of the stock solution is diluted with 12.5 ml ammonium hydroxide and 12.5 ml of methyl alcohol. This solution lasts for 2-3 weeks.
- (c) Best's Differentiator.

Absolute alcohol.....	8 ml
Methyl alcohol.....	4 ml
Distilled water.....	10 ml

3. Procedure:

- (a) Bring sections from xylene to absolute alcohol.
- (b) Place sections in 1% celloidin in absolute alcohol/ether (equal parts), 2 minutes.
- (c) Dry in air until the celloidin coat congeals.
- (d) With a steady motion, lower the slide into 70% ethanol, 2 minutes.
- (e) Pass through alcohols to water. Leave only a few seconds in water.
- (f) Stain nuclei in Ehrlich's hematoxylin, 5 minutes.
- (g) Rinse and differentiate rapidly in 1% acid alcohol.
- (h) Rinse in water.
- (i) Stain in Best's carmine solution, 15-30 minutes.
- (j) Differentiate in Best's differentiator without previous rinsing, 5-60 seconds or until stain ceases to diffuse from the sections.
- (k) Wash in 80% alcohol.
- (l) Dehydrate in absolute alcohol, clear in xylene and mount in permount.

4. Results:

Nuclei stain dark blue and glycogen red.

5. References:

Pearse, (1960); Galigher and Kozloff, (1964).

H. Mercury-Bromphenol Blue Method for Proteins

1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov and Carnoy's II.

2. Reagents:

1% HgCl_2 and 0.05% bromphenol blue in 2% aqueous acetic acid.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Pass into staining solution, 2 hours.
- (c) Rinse sections in 0.5% acetic acid, 5 minutes.
- (d) Transfer directly to tertiary-butyl alcohol. Change and leave for 12-18 hours at 37°C to differentiate. This step obviates the necessity for restoring pH to the neutral point in order to produce the blue form of the dye.
- (e) Clear in xylene and mount in permount.

4. Results:

Proteins stain deep clear blue or reddish.

5. References:

Mazia et al., (1953); Pearse, (1960).

APPENDIX IV

ENZYME EXTRACTION TECHNIQUES

A. Ribonuclease Extraction1. Fixative:

Carnoy's II.

2. Preparation of Stain:

4mg/ml of ribonuclease (ribonuclease - A from bovine pancreas. 5X crystallized. Type 1-A. 55 Kunitz units/mg. Sigma Chemical Co.) is dissolved in deionized water and heated to 80°C for 10 minutes to inactivate any contaminating deoxyribonuclease (Marmur, 1961). The final solution is diluted 20-fold with deionized water (0.2 mg/ml, final concentration).

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Place slides in the enzyme solution at 37°C for 1 hour. Control section are kept in deionized water at 37°C.
- (c) Wash in deionized water and continue with 3b of the Toluidine Blue O or 3b of the Methyl Green-Pyronin Method.

4. Result:

Nuclear and cytoplasmic RNA are removed and thus not stained in the test slide but are stained in the control slide.

5. References:

Brachet, (1953); Pearse, (1960).

B. Diastase and Saliva Extraction1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov, Carnoy's II.

2. Preparation of Enzyme Solution:

1g of malt diastase is dissolved in 100 ml of distilled water.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Place slides in the enzyme solution at 37°C for 1 hour or saliva at room temperature for 30 minutes. Control slides are placed in distilled water only.
- (c) Wash in distilled water and continue with 3b of the PAS or 3f of the Best's Carmine Procedure.

4. Results:

All glycogen is removed and therefore not stained in the test slide, but is stained in the control slide.

5. References:

Pearse, (1960).

C. Trypsin Treatment

1. Fixatives:

Karpechenko, Allen's PFA 3, Maximov, Carnoy's II.

2. Preparations of Enzyme Solution:

A 0.05 M borate buffer at pH 8.9 is made with deionized water. To make the working enzyme solution, 0.2mg/ml of trypsin are added to this buffer.

3. Procedure:

- (a) Bring slides from xylene to distilled water.
- (b) Incubate at 37°C in the enzyme solution, 15-60 minutes. Control sections are kept in buffer only.
- (c) Wash test and control slides in water.
- (d) Continue with 3b of the Mercury-Bromphenol Blue Method.

4. Results:

Histone and non-histone proteins are degraded and removed in the test slide but not in the control slide, by the subsequent washing.

APPENDIX V

DETERMINATION OF CELLULAR DIAMETER AND VOLUME

Due to the irregular shape of many differentiating cells and to obviate the necessity of averaging two or three measurements of the diameter of any particular cell, a technique was devised whereby the diameter and volume of the cell was calculated mathematically from a measurement of weight.

A. Theoretical Basis

The diameter of a sphere of unknown dimensions can easily be determined by measuring the diameter of a plane that passes through the centre of this sphere. If it is not possible to measure directly the diameter of this plane, then it can be found by calculating the area of the plane according to the relation, $\text{area} = \pi r^2$. To obtain the area, the image of the plane is drawn on paper, cut out with scissors and weighed. The weight of this circular piece of paper is directly proportional to the area of the image; if the weight of the paper per centimeter square is known, the actual area can now be calculated. From the area, the diameter and volume of the sphere can be calculated as shown below.

B. Procedure for Determining The Cellular Diameter and Volume

The cell chosen for measurement was magnified X1,300 and drawn with a Wild camera lucida (objective, X100;

ocular, X15), on white paper. This image was further enlarged X5.5 with a projector (Bausch and Lomb, Model LRM-18), on graph paper of known weight per centimeter square. This final image was cut out with scissors and weighed on a Mettler balance (Type H16). The diameter (d) was then calculated from the relation, $d = 15.4 \cdot x^{\frac{1}{2}}$ and the volume (v) from the relation, $v = 1,930 \cdot x^{\frac{2}{3}}$, where x is the weight of the final image in grams.

The total enlargement of the original cell, in this procedure is $1300 \times 5.5 = 7,150$. On this basis 1 cm of the final image is equivalent to 1.4μ on the microscope slide.

C. Derivation of the Formulae For the Diameter and Volume

In the following derivations it is assumed that the cells being measured are spherical. This is valid because most of the cells chosen for measurement were separated from neighbouring cells and had adopted a more spherical shape. To insure that the plane of the cell chosen passed through the diameter, adjacent serial sections were examined to find the section with the widest part of the cell. Since the thickness of the section was 4μ , even the largest cells would not occupy more than 2-3 sections.

The following steps were performed to derive the formulae:

1. The weight of 50 pages of graph paper	= 198.0 g
The weight of 1 page of graph paper	= 3.3 g
The area of 1 page of graph paper	= 622.1 cm^2
Therefore, the area of 1g of graph paper	= $186.8 \text{ cm}^2/\text{g}$
Therefore, the area of xg of graph paper	= $x \cdot 186.8 \text{ cm}^2$

$$\begin{aligned}
 2. \text{ The area of a circle} &= \pi r^2 \\
 \text{Therefore, the radius (r)} &= (\text{area}/\pi)^{\frac{1}{2}} \\
 &= (186.8 \cdot x / 4)^{\frac{1}{2}} \\
 &= 7.7 \cdot x^{\frac{1}{2}} \text{ cm} \\
 \text{Therefore, the diameter (d)} &= 15.4 \cdot x^{\frac{1}{2}} \text{ cm}
 \end{aligned}$$

This last relation gives a measure of the diameter of the cell assuming that the cell is spherical. The unknown value, x, is the weight of the final drawing in grams.

$$\begin{aligned}
 3. \text{ The volume (v) of a sphere} &= 4.25^3 \\
 \text{Therefore, the volume of a cell} &= 4.2(7.7 \cdot x^{\frac{1}{2}})^3 \text{ cm}^3 \\
 &= 1,930 \cdot x^{3/2} \text{ cm}^3
 \end{aligned}$$

This last relation gives a measure of the volume of the cell assuming that the cell is spherical. The unknown value, x is the weight of the final drawing in grams.

The values for the diameter (d) and the volume (v) can be converted to microns on the basis that 1cm of the final image = 1.4μ on the microscope.

TABLE 4

DATA PERTAINING TO THE DETERMINATION OF
THE VOLUME OF THE PRIMARY SPERMATOGONIUM

A	B	C	A	B	C
Serial Number	x* (g)	y** (μ^3)	Serial Number	x* (g)	y** (μ^3)
1	0.1689	370	46	0.1446	295
2	0.1465	299	47	0.2217	560
3	0.1372	268	48	0.3576	1140
4	0.1400	278	49	0.2750	770
5	0.1605	338	50	0.2525	680
6	0.1355	260	51	0.2486	660
7	0.1379	275	52	0.3127	930
8	0.1438	290	53	0.3202	965
9	0.1573	330	54	0.2962	855
10	0.1573	330	55	0.3543	1110
11	0.1502	308	56	0.3404	1050
12	0.1421	283	57	0.1655	355
13	0.1204	220	58	0.2953	845
14	0.2402	638	59	0.2618	770
15	0.1632	350	60	0.3493	1090
16	0.1652	360	61	0.2368	615
17	0.1442	290	62	0.2565	700
18	0.1432	285	63	0.2783	775
19	0.1385	275	64	0.3049	895
20	0.1140	200	65	0.1610	355
21	0.1475	299	66	0.1692	370
22	0.1471	299	67	0.1650	360
23	0.1658	360	68	0.1480	295
24	0.1149	200	69	0.1799	405
25	0.1100	190	70	0.1838	420
26	0.1575	330	71	0.1960	465
27	0.3350	1030	72	0.2999	875
28	0.2725	760	73	0.2913	830
29	0.2735	760	74	0.2091	505
30	0.2006	480	75	0.2407	630
31	0.2304	585	76	0.2524	670
32	0.2608	710	77	0.1744	380
33	0.3230	985	78	0.2735	765
34	0.2533	675	79	0.1480	300
35	0.1873	430	80	0.1902	435
36	0.2105	515	81	0.1267	240
37	0.2062	500	82	0.2926	840
38	0.1401	278	83	0.2601	705
39	0.2365	610	84	0.2856	815
40	0.2458	645	85	0.1461	775
41	0.1313	250	86	0.0995	165
42	0.1886	440	87	0.2009	480
43	0.3238	980	88	0.2251	565
44	0.2282	580	89	0.2679	740
45	0.2217	560	90	0.2404	630

TABLE 4 (cont'd)

A	B	C	A	B	C
Serial Number	x* (g)	v** (μ^3)	Serial Number	x* (g)	v** (μ^3)
91	0.2791	780	96	0.2206	550
92	0.3065	900	97	0.1929	505
93	0.2773	775	98	0.2026	495
94	0.2756	770	99	0.1615	350
95	0.3041	945	100	0.1204	220

* x = the weight of the final drawing in grams. All specimens were fixed in Maximov's fluid and stained with iron hematoxylin.

** v = the volume of the cell in microns cubed, calculated from the relation, $v = 1,930 \cdot x^{3/2} \text{ cm}^3$ and converted to microns on the basis that 1cm of the final drawing is equivalent to 1.4μ on the microscope.

TABLE 5
DATA PERTAINING TO THE DETERMINATION OF THE
DIAMETER AND VOLUME OF CELLS IN SPERMATOGENESIS

Serial Number	Interstitial Cell x^* (g)	Secondary Spermatogonia x^* (g)	Primary Spermatocyte x^* (g)	Prohphase of Primary Spermatocyte x^* (g)	Secondary Spermatocyte x^* (g)	Spermatid x^* (g)	Cells in Spermiogenesis x^* (g)
1	0.0922	0.0888	0.0904	0.0828	0.0498	0.0375	0.0287
2	0.0989	0.0717	0.0864	0.0866	0.0543	0.0339	0.0223
3	0.0793	0.0795	0.0894	0.0868	0.0573	0.0410	0.0273
4	0.0854	0.0909	0.0810	0.0692	0.0438	0.0314	0.0237
5	0.1059	0.1068	0.0720	0.0929	0.0428	0.0273	0.0246
6	0.1060	0.0627	0.0796	0.0788	0.0398	0.0324	0.0257
7	0.1131	0.0852	0.0725	0.0900	0.0416	0.0309	0.0246
8	0.1199	0.0723	0.0941	0.0876	0.0433	0.0349	0.0310
9	0.0882	0.0816	0.0805	0.0799	0.0421	0.0285	0.0263
10	0.0770	0.0660	0.0732	0.0855	0.0441	0.0253	0.0271
11	0.0872	0.0658	0.0800	0.0740	0.0508	0.0368	0.0257
12	0.1002	0.0631	0.0791	0.0762	0.0475	0.0339	0.0286
13	0.0937	0.0914	0.0760	0.0786	0.0549	0.0237	0.0309
14	0.0674	0.0759	0.0760	0.0852	0.0572	0.0249	0.0267
15	0.0630	0.0844	0.0960	0.0775	0.0423	0.0300	0.0278
16	0.0928	0.0839	0.0691	0.0707	0.0556	0.0267	0.0294
17	0.0766	0.0775	0.0399	0.0850	0.0557	0.0309	0.0229
18	0.0853	0.0701	0.0773	0.0762	0.0570	0.0258	0.0275
19	0.0585	0.0950	0.0745	0.0828	0.0422	0.0272	0.0263
20	0.0941	0.0846	0.0964	0.0783	0.0625	0.0238	0.0250
21	0.0796	0.0727	0.0939	0.0804	0.0494	0.0329	0.0280
22	0.0618	0.0777	0.0755	0.0803	0.0543	0.0273	0.0248
23	0.0622	0.0658	0.0848	0.0707	0.0557	0.0359	0.0225
24	0.0623	0.0638	0.0696	0.0939	0.0554	0.0415	0.0251
25	0.0648	0.0993	0.0694	0.0999	0.0561	0.0294	0.0187

26	0.0844	0.0869	0.0647	0.0807	0.0454	0.0427	0.0224
27	0.0807	0.0894	0.0864	0.0802	0.0552	0.0351	0.0186
28	0.0698	0.0744	0.0860	0.0762	0.0449	0.0334	0.0190
29	0.0669	0.0924	0.0750	0.0798	0.0519	0.0395	0.0199
30	0.0602	0.1069	0.0752	0.0720	0.0504	0.0432	0.0206
TOTAL	2.4782	2.4265	2.4135	2.4387	1.5015	0.9743	0.7506
AVERAGE	0.0826	0.0810	0.0805	0.0813	0.0501	0.0325	0.0250
VOLUME**							
(cm ³)	46.4	44.4	44.2	44.8	21.8	11.6	7.6
(u ³)	125.2	122.8	121.5	122.8	59.2	31.2	21.0
Diameter***							
(cm)	4.46	4.40	4.38	4.40	3.46	2.78	2.44
(u)	6.24	6.16	6.14	6.16	4.84	3.90	3.42

* x = the weight of the final drawing in grams. All specimens were fixed in Maximov's fluid and stained with iron hematoxylin.

** Calculated from the relation, $v = 1.930 \cdot x^{3/2} \text{cm}^3$ and converted to microns on the basis that 1 cm of the final image = 1.4μ on the microscope slide.

*** Calculated from the relation, $d = 15.4 \cdot x^{1/2} \text{cm}$ and converted to microns as above**.

APPENDIX VI

COMPILATION OF THE DATA PERTAINING
TO MITOTIC AND CELL COUNTS

TABLE 6

THE NUMBER OF PRIMARY SPERMATOGONIA AND
CNIDOBLASTS IN HISTOLOGICAL PREPARATIONS
IN RELATION TO THE NUMBER OF DAYS AT 8°C

A	B	C	D
Number of Days at 8°C	Fixative*	Number of Primary Spermatogonia**	Number of Cnidoblasts**
0	K	12	28
		22	55
		30	62
		36	66
		47	69
		SUBTOTAL	147
			280
0	K	30	61
		16	35
		25	71
		27	31
		22	56
		SUBTOTAL	120
			254
0	K	18	41
		25	57
		13	26
		16	24
		19	45
		SUBTOTAL	91
		TOTAL	358
		AVERAGE	24
			49

<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
3	K	53	46
		16	53
		35	26
		43	32
		41	50
		SUBTOTAL 188	207
3	K	41	37
		30	40
		54	29
		26	41
		23	43
		SUBTOTAL 174	190
3	K	26	31
		65	49
		40	44
		37	49
		38	38
		SUBTOTAL 206	211
		TOTAL 568	608
		AVERAGE 37	41
4	K	48	75
		31	38
		33	31
		36	29
		35	38
		SUBTOTAL 183	211
4	A	28	56
		38	41
		52	22
		15	34
		35	41
		SUBTOTAL 168	194
4	M	25	54
		59	47
		29	37
		37	41
		50	60
		SUBTOTAL 200	239
		TOTAL 551	644
		AVERAGE 37	43

<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
5	K	63	67
		42	47
		32	29
		43	45
		35	34
		SUBTOTAL 215	222
5	M	52	54
		59	50
		37	49
		30	37
		37	49
		SUBTOTAL 215	239
5	A	42	74
		39	69
		37	29
		36	91
		44	60
		SUBTOTAL 198	323
		TOTAL 628	784
		AVERAGE 42	52
7	K	53	20
		36	30
		32	33
		30	15
		53	60
		SUBTOTAL 204	158
7	M	55	20
		55	40
		52	45
		39	35
		52	45
		SUBTOTAL 253	185
7	M	60	47
		60	69
		72	36
		58	45
		54	24
		SUBTOTAL 304	221
		TOTAL 761	564
		AVERAGE 51	38

<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
9	M	69	33
		72	29
		82	25
		56	23
		36	88
		SUBTOTAL 315	148
9	M	101	45
		60	44
		101	26
		93	44
		79	22
		SUBTOTAL 434	181
9	A	47	34
		65	63
		47	25
		77	37
		85	32
		SUBTOTAL 321	191
		TOTAL 1064	520
		AVERAGE 71	35
12	K	154	53
		147	56
		249	43
		166	14
		187	33
		TOTAL 903	199
		AVERAGE 181	40

* K = Karpechenko

A = Allen's PFA 3

M = Maximov

All sections were stained with iron hematoxylin.

** These values represent the number of cells for every ten epitheliomuscular cells per section in the gastric region of H. fusca. Each group of values represent a set of serial sections cut longitudinally.

TABLE 7

THE NUMBER OF PRIMARY SPERMATOGONIAL
MITOSES IN HISTOLOGICAL PREPARATIONS
IN RELATION TO THE NUMBER OF DAYS AT 8°C.

A	B	C	A	B	C
Number of Days at 8°C	Fixative*	Number of Mitoses**	Number of Days at 8°C	Fixative*	Number of Mitoses**
0	K	6 16 15 7 <u>44</u>	1	K	13 4 2 0 1 <u>20</u>
	SUBTOTAL average	11		SUBTOTAL average	4
0	K	10 10 5 7 3 8 <u>43</u>	1	M	8 4 3 1 9 <u>21</u>
	SUBTOTAL average	7		SUBTOTAL average	8
0	M	1 1 7 4 10 21 <u>44</u>	1	A	4 13 30 16 17 <u>80</u>
	SUBTOTAL average	7		SUBTOTAL average	16
0	K	11 11 27 18 8 12 <u>87</u>	1	K	18 24 31 11 <u>103</u>
	SUBTOTAL average	15		SUBTOTAL average	21
	TOTAL AVERAGE	218 10		TOTAL AVERAGE	249 12

<u>A</u>	<u>B</u>	<u>C</u>
2	K	4 4 9 <u>3</u> 20 5
	SUBTOTAL	20
	average	5
2	K	11 11 11 30 <u>11</u> 74 15
	SUBTOTAL	74
	average	15
2	M	17 14 22 27 <u>22</u> 102 20
	SUBTOTAL	102
	average	20
2	M	15 13 12 16 <u>22</u> 78 16
	SUBTOTAL	78
	average	16
	<u>TOTAL</u>	276
	<u>AVERAGE</u>	14
3	M	9 7 <u>11</u> 27 9
	SUBTOTAL	27
	average	9
3	A	14 13 <u>7</u> 34 11
	SUBTOTAL	34
	average	11

<u>A</u>	<u>B</u>	<u>C</u>
3	K	16 17 <u>17</u> 50 17
	SUBTOTAL	50
	average	17
3	M	7 5 9 15 <u>20</u> 56 11
	SUBTOTAL	56
	average	11
	<u>TOTAL</u>	167
	<u>AVERAGE</u>	12
4	K	5 16 16 18 <u>25</u> 80 16
	SUBTOTAL	80
	average	16
4	M	9 11 8 19 <u>14</u> 61 12
	SUBTOTAL	61
	average	12
4	A	19 17 10 10 10 <u>10</u> 66 13
	SUBTOTAL	66
	average	13
	<u>TOTAL</u>	207
	<u>AVERAGE</u>	14

<u>A</u>	<u>B</u>	<u>C</u>
5	K	13 20 10 15 25 22
	SUBTOTAL	105
	average	18
5	M	4 16 15 10 21
	SUBTOTAL	66
	average	13
5	A	19 17 26 17 12
	SUBTOTAL	91
	average	18
	TOTAL	262
	<u>AVERAGE</u>	16
7	A	7 13 14 14 9
	SUBTOTAL	57
	average	11
7	K	5 8 9 16 34
	SUBTOTAL	72
	average	14

<u>A</u>	<u>B</u>	<u>C</u>
7	M	51 48 48
	SUBTOTAL	147
	average	49
7	M	13 10 15 8 6 8
	SUBTOTAL	60
	average	10
7	A	23 24 21 13 17 18 18
	SUBTOTAL	134
	average	19
7	M	30 16 18 22 51
	SUBTOTAL	137
	average	27
	TOTAL	607
	<u>AVERAGE</u>	22
8	K	29 15 15 14 22
	SUBTOTAL	95
	average	19

A	B	C
8	K	12 19 21 SUBTOTAL 52 average 17
8	M	10 35 43 42 51 SUBTOTAL 181 average 36
8	A	62 51 55 79 42 SUBTOTAL 289 average 58
8	M	18 11 10 32 36 SUBTOTAL 107 average 21 TOTAL 724 AVERAGE 30
9	K	19 27 20 41 53 SUBTOTAL 160 average 32
9	M	78 58 53 83 67 SUBTOTAL 339 average 70

A	B	C
9	A	58 64 44 56 SUBTOTAL 222 average 56
9	M	79 56 68 40 SUBTOTAL 243 average 61
9	M	18 37 54 74 51 SUBTOTAL 234 average 47
9	A	62 42 72 65 SUBTOTAL 241 average 60 TOTAL 1,438 AVERAGE 54
10	K	52 40 43 37 38 SUBTOTAL 210 average 45
10	K	56 63 59 SUBTOTAL 178 average 59

<u>A</u>	<u>B</u>	<u>C</u>
10	A	40 31 52 82 <u>73</u> SUBTOTAL 278 average 56
10	M	49 58 32 28 <u>58</u> SUBTOTAL 225 average 45
10	M	85 69 76 59 <u>78</u> SUBTOTAL 367 average 74
10	K	55 55 62 SUBTOTAL 172 average 57
10	M	75 50 50 SUBTOTAL 175 average 58 TOTAL 1,605 <u>AVERAGE</u> 56
12	A	65 47 29 48 <u>41</u> SUBTOTAL 230 average 46

<u>A</u>	<u>B</u>	<u>C</u>
12	M	252 286 219 <u>285</u> SUBTOTAL 1042 average 260
12	K	31 57 39 <u>42</u> SUBTOTAL 169 average 42 TOTAL 1441 <u>AVERAGE</u> 116
14	K	159 175 <u>165</u> SUBTOTAL 499 average 166
14	M	110 107 83 100 <u>85</u> SUBTOTAL 485 average 97
14	A	103 107 <u>127</u> SUBTOTAL 337 average 112
14	M	193 196 <u>199</u> SUBTOTAL 588 average 196 TOTAL 1909 <u>AVERAGE</u> 143

<u>A</u>	<u>B</u>	<u>C</u>	<u>A</u>	<u>B</u>	<u>C</u>
16	A	150 176 145 159 143 <u>SUBTOTAL</u> 773 average 155	20	M	145 155 168 100 133 <u>SUBTOTAL</u> 701 average 140
16	A	112 167 149 173 176 <u>SUBTOTAL</u> 777 average 155 <u>TOTAL</u> 1550 <u>AVERAGE</u> 155	22	K	143 140 140 <u>SUBTOTAL</u> 423 average 141
18	K	164 153 <u>SUBTOTAL</u> 317 average 159	22	M	137 107 117 <u>SUBTOTAL</u> 361 average 120
18	M	158 175 146 145 <u>SUBTOTAL</u> 624 average 156	22	M	32 67 60 69 37 <u>SUBTOTAL</u> 265 average 53
18	K	140 148 131 140 153 <u>SUBTOTAL</u> 712 average 142 <u>TOTAL</u> 1653 <u>AVERAGE</u> 152	24	K	82 91 86 <u>SUBTOTAL</u> 259 average 86
20	K	160 111 129 113 <u>SUBTOTAL</u> 513 average 128	24	M	62 63 44 55 <u>SUBTOTAL</u> 224 average 56 <u>TOTAL</u> 483 <u>AVERAGE</u> 72

<u>A</u>	<u>B</u>	<u>C</u>
26	A	13 13 <u>16</u> 42
	SUBTOTAL average	14
26	K	23 19 <u>10</u> 52
	SUBTOTAL average	17
	<u>TOTAL</u> <u>AVERAGE</u>	94 16
28	M	4 3 <u>3</u> 10
	SUBTOTAL average	3
28	K	8 3 <u>12</u> 23
	SUBTOTAL average	8
	<u>TOTAL</u> <u>AVERAGE</u>	33 6
30	M	5 12 <u>0</u> 17
	SUBTOTAL average	6
30	K	3 0 <u>0</u> 3
	SUBTOTAL average	1
	<u>TOTAL</u> <u>AVERAGE</u>	20 3

* K = Karpechenko
A = Allen's PFA 3
M = Maximov

All sections were stained with iron hematoxylin or Flemming's triple stain. Section thickness was 4 μ .

** Each value represents the total number of epidermal mitoses in a single longitudinal section. Each group of numbers represents a set of serial sections. Sections were counted only if the plane of cut was through both the hypostome and the basal disk. Epitheliomuscular cells in mitosis were not counted.

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