INTRAVITAM STUDIES ON GERM CELLS

by

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INTRAVITAM STUDIES ON

SEX CELLS

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THE STRUCTURE OF THE TESTIS AND MOVEMENT OF SPERMS IN CHORTOPHAGA VIRIDIFASCIATA AS DEMONSTRATED BY INTRAVITAM TECHNIQUE[1]

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THREE TEXT FIGURES AND TWO PLATES (TWENTY FIGURES)

AUTHOR'S ABSTRACT

By means of the 'intravitam technic' developed by Baumgartner and Payne ('31), the mature or maturing sperm of Chortophaga viridifasciata have been traced from the follicle of the testes in the male to the locus of fertilization in the female. The sperm aggregated into bundles, and held tight by a hyaline cytoplasmic cap, spiral up the follicle, turn and spiral back to the vas deferens by means of a periodic lashing and writhing of the sperm tails.

The genital tract is described briefly. In the vasa deferentia and storage tubules, the sperm bundles are usually in a quiescent state, having been inactivated most probably by secretions from the tubules.

Peristalsis and currents in the fluid contents of the tubules move the inactivated sperm from the vesicles of the male to the seminal receptacle of the female, where the cytoplasmic caps gradually disintegrate. This permits individual sperm to pass down the seminal duct and fertilize the ovum just before oviposition.

Single photomicrographs and a series of photomicrographs show the sperm in various parts of the genital system and making actual progress up a follicle. A stained preparation was used for only one of the photos. The other nineteen are from living unstained tissue. The intravitam observations are, most probably, more 'vital' than any heretofore recorded.

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I wish to express sincere gratitude to my major professor, Dr. W. J. Baumgartner, for his continual assistance and efficient direction during the development of this work. His helpful encouragement and invaluable suggestions, as well as the kindly interest manifested by other members of the faculty, will keep me ever a debtor to the University of Kansas.

INTRODUCTION AND GENERAL METHODS OF OBSERVATION

The interests of cytologists and of biologists generally center more and more in the living cell. Studies in vitro, manipulations with the ultraviolet microscope and intravitam observations prove, 1) that cells and tissues may be seen in a probably normal state, 2) that it is possible to distinguish cellular elements, and, 3) that function and movements of these elements as well as certain activities of the cell as a whole, may be carefully observed and examined.

Lewis and Robertson, '16; Strangeways and Canti, '22; Lewis, '23; Belar, '29; Koehring, '30; Nath, '30; Johnson, '31; and others, using the well-known 'hanging-drop' method, have cultured the cells and tissues of a host of vertebrates and invertebrates. Ectodermal cells, mesodermal cells, and entodermal cells behave in an orthodox manner, and, although occasionally an explanation or apology is tendered for the unusual behavior of certain cells, e.g., ameboid spermatocytes or binucleated neurons, etc., on the whole, studies of cells in vitro have accelerated the interest of the biologist and have fructified in many valuable contributions to the literature. Particularly valuable are the cytoplasmic analyses made possible through the addition of vital stains (Janus green, neutral reds, etc.) to the nutrient media in which the tissues are suspended, and although authors do not, at present, agree as regards the origin, structure, function, and fate of such intensively studied elements, as vacuomes, terminal vesicles, Centralkörper, etc., nevertheless, the multiple pertinent observations and discussions have aroused the interest of even the most lethargic, and directed attention to the cell.

Recent experimentation (Lucas and Stark, '31), has resulted in the development of a successful technic for optically sectioning living cells by means of the ultraviolet microscope, so that "an unstained living cell which would appear practically structureless under a visible-light microscope, appears much as though it had been stained when photographed by the ultraviolet microscope" (p. 92).

While the advantages of the ultraviolet microscope are striking, there are also certain disadvantages. All organic matter does not transmit ultraviolet light and the technique requires a high degree of mechanical skill. Not only the apparatus, but the individual operator as well, "must be tuned to a high state of perfection."

Besides, if the objective is intravitam study, a technique which subjects delicate reproductive cells, to ultraviolet rays less than 290 μμ arouses no slight degree of skepticism regarding the validity of the results obtained. Elles and Wells ('16, p. 271) state that "the lethal effect is exhibited by the rays of wave lengths shorter than 290 μμ (2900 Å).

In a previous paper (Lucas, '30) the wave length adopted for the analysis of the architecture of the living cell is 275 μμ. He asserts that "ultraviolet light of the intensity and wave length used in conjunction with the ultraviolet microscope appears to have little if *any,* harmful effects, so far as *some* types of living cells are concerned" (p. 606) (italics mine).

Other workers in the field do not hold that ultraviolet irradiation has little or no effect upon the living cell. Experiments on starfish eggs (Lillie and Baskerville, '22) demonstrate that brief exposures (2 to 7 min.) result in incomplete activation of the egg, while longer radiation causes local cytolytic effects, swellings, etc. (p. 71). They also show that ultraviolet irradiation decreases the motility and destroys the fertilizing power of spermatozoa. "The rate of injury as shown by loss of fertilizing power is much more rapid with these cells than with the egg."

Proteins are easily coagulated by ultraviolet irradiations (Bovie, '13). Spiegel-Adolf ('31), describes the effect of irradiation on a 1 per cent solution of albumen at 76.2 cm. The first sign of flocculation appears after 10 minutes exposure.

Burge ('15) says that ultraviolet kills living cells and tissues—the effective region of the spectrum in changing the living material of the cell or protoplasm to a coagulum lies between 254 μμ and 302 μμ (p. 344). It is to be noted that rays 275 μμ fall within these limits.

It may be possible to expose delicate cells to ultraviolet rays, without coagulating the protoplasm or inhibiting vital activities, but there is no perceptible change of the cellular elements in the series of photomicrographs by Lucas, ('30 and '31). The technic is undoubtedly good, and photomicrographs of the metamorphosis of a spermatid or of a spermatocyte passing through division stages, would banish all skepticism as regards harmless nature of the technic.

There is no need, however, to use ultraviolet light for the observation of living sperm cells of grasshoppers. It has been comparatively easy, through the use of intravitam technic, previously described in detail (Baumgartner and Payne, '30 and '31) to study the maturing sperm cells of Chortophaga viridifasciata and to see their structures within the unstained living specimen with a visible-light microscope. The unstained living cell is far from 'practically structureless,' as Lucas and Stark assert (p. 92).

In a later paper it is my intention to discuss early spermatocytes and their division stages. In this present work, with some slight modifications of our technic, I purpose to trace the path of the sperm bundles from the mature follicle of the male to the receptaculum seminis of the female.

McNabb ('28) has traced the fate of the sperm after its entrance into the egg. She says that the exact time that the sperm enters the egg is unknown, "although it would seem that it must be near the time of oviposition." My observations confirm this assumption.

MATERIALS AND TECHNIC

Following the previously described method, intravitam preparations were set up for observations upon the sperm within the follicle. If the testes were drawn far out from the body cavity, the taut vasa deferentia were exposed to view. Observations were made on the follicles and ducts with a Bausch & Lomb binocular microscope, 12.5 compensating oculars and either a 2 mm. water immersion lens or a 16 mm. lens. Spiraling movements of the sperm bundles were readily detected with a good dissecting microscope.

The testes, vasa deferentia, seminal vesicles, accessory glands, and the ductus ejaculatorius were removed from the grasshopper and placed in a nutrient medium. After gross examination, successive parts of the genital system were separated, placed on a glass slide in a drop of nutrient medium, and covered with a thin cover slip. The edges of the cover slip were sealed with melted paraffin to prevent convection currents, evaporation, etc. The mounts were studied with low and high power objectives and photomicrographs taken of the same preparations. Preparations of the female oviduct and seminal receptacle were similarly prepared after the thick coat of connective tissue and the aerating tubules which surround them were removed.

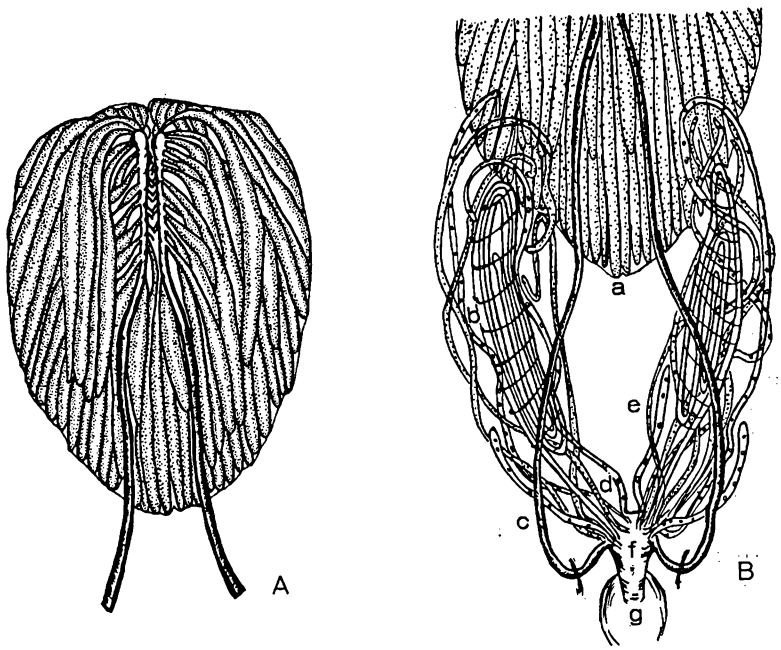
With two exceptions, no vital stains or other stains or fixatives were used in any of the preparations. Mounts of the testes, which were fixed in Allen's fluid and stained with methyl green and acid fuchsin, were examined for the purpose of determining the number of sperm in a bundle. Secondly, cross and longitudinal sections of vas deferens, accessory tubules, receptaculum seminis, etc., were examined, in order to determine the histological structure of the various parts of the genital systems. The latter tissues were fixed in Bouin's fluid and stained in hemotoxylin-eosin.

GENITAL SYSTEM OF CHORTOPHAGA

The two large testes, closely approximated, lie in the body cavity dorsal to the digestive tract and extend from the second to the fifth or sixth abdominal segment. Fatty, orange-colored connective tissues containing many simple and dilated tracheae are closely wrapped about the organs. Tracheal tubes pass from the encasing tissue into the testes, and each tubular gland has one or more air tubes adjacent to it.

From the dorsal side the testes appear as a single mass of tubules, but a ventral examination shows two vasa deferentia leading from the extreme anterior ends of the testes caudad (text fig. A). The testicular mass is held in place by delicate bands of hyaline tissue which extend from the

fatty covering of the testes to the body wall. Delicate cords of hyaline tissue extend from the ampullae or enlarged initial portions of the vasa deferentia (text fig. A) up to the septum which divides the thoracic and abdominal cavities. A common



Text fig. A A) Schematic drawing of the testes of Chortophaga viridifasciata. About thirty-seven follicles attached to each of the closely aligned vasa deferentia. The testicular mass is enclosed in a fatty connective-tissue covering. B) Schema of ejaculatory duct with vasa deferentia, seminal vesicles, and accessory tubules attached. Seminal vesicles are also enclosed in fatty connective tissue. *a*, testes; *b*, coiled seminal vesicle covered with orange-colored protective tissue; *c*, vas deferens; *d*, clear accessory tubule; *e*, milky tubule; *f*, ductus ejaculatorius; *g*, copulatory apparatus

band of tissue holds the ampullae of the vasa deferentia together, but the remaining parts of the ducts are independent of each other. The tubular follicles, 5 × 0.34 mm. are attached laterally and dorsally to the ampullae and the first 2 mm. of the ducts. The average count to a testis is 36, the extremes for 16 specimens being 32 and 43. One testis may have

34 tubules attached to it, while the adjacent one has 39 or 40. About 2.4 mm. from the cephalic end of the testicular mass the vasa deferentia emerge from the fatty tissue covering. Curving slightly, they continue caudad somewhat enlarged. Each tube is covered with the orange-colored connective tissue. The vasa deferentia pass under the mass of accessory glands, vesicula seminalis, etc., and empty into the large triangular lumen of the ductus ejaculatorius on the lateral ventral side (text fig. B).

The testes, in situ, average 7 by 5 mm. The size of the organs varies, of course, with the size of the grasshopper, the adult male Chortophaga varying in body length from 19.5 mm. to 23 mm. It varies also with the stage of development of the cells and sperm in the tubules and with the amount of expulsion of sperm.

The ventral face of the tip of the heavy walled muscular ductus ejaculatorius (photo 19) opens into the bulbous copulatory apparatus. The seminal vesicles and two kinds of accessory glands as well as the vasa deferentia, open into the upper corners of the ductus ejaculatorius (photo 20).

The two long convoluted masses of seminal vesicles and accessory tubules, measuring 10 by 3 mm. extend up on either side from the horns of the ductus ejaculatorius. They lie lateral and somewhat ventral to the digestive tract, the upper fourth of the mass curves up and lies just under the posterior part of the large testes (text fig. A, *B*).

The prominent seminal vesicle lies in the center of each mass. It is coiled 9 or 10 times, like the loops of a stiff rope and the coils are wrapped with the orange-colored connective tissue, similar to the tissue which encloses the testes. When uncoiled, the tube measures 37 by 0.35 mm. and terminates in a small ampulla (photo 11).

The accessory glands have no protective covering. They are of two types. First, long milky white tubules, somewhat coiled, located at the periphery of the mass of tissue. They measure 10 or 12 by 0.31 mm. when uncoiled. The milky white granular content runs out rapidly if the delicate walls

are punctured and the granules diffuse rapidly. They stain a deep orange in sudan III and turn black in 1 per cent osmic acid. There are four of the milky tubules on either side (photo 15).

The second type of accessory tubules are clear hyaline glands filled with a watery fluid in which many large, highly refractive granules are suspended. These granules do not stain with sudan III. The tubules twine in and out among the others, some measuring less than 8 mm. in length, others more than 20 mm. There are about ten tubules attached to each side, the long tubules being twice as numerous as the short ones (photo 13).

SPERM BUNDLES

In a follicle just below the cysts of young spermatids, which still have small globular heads and long tails loaded with droplets of cytoplasm, the rigid rod-shaped heads of the more mature sperm can be seen jutting into the upper cyst wall. Each head at first is separate, an individual sperm, but an aggregation of the sperm into a tight comet-like bundle soon occurs. The heads at the edges of the cyst wall are drawn toward a central point by a series of contractile waves, and a clear, cytoplasmic gelatinous cap, apparently homogeneous in structure, forms over the extreme tips of the heads which were embedded in the cyst wall. The heads then form a long blunt cone which measures 0.182×0.019 mm. The cap (photo 5), previously called a compound acrosome (Baumgartner and Payne, '31, p. 375), has been carefully examined with both intravitam and stain technic. It measures 0.011 by 0.019 mm. and resembles a clear flattened mushroom. It is hyaline and persistent, holding the sperm together from the time of aggregation until the final dissolution in the female receptacle. Bundles obtained from the follicles, the ducts, and the seminal vesicle could not be separated by pressure, jarring, etc., although the lower part of the sperm heads often spread widely apart. When stained with acid fuchsin and methyl green, the cap takes the acid fuchsin, while the

heads stain a brilliant green. No structural details can be ascertained even in the stained material, but functionally it leads in all movement of the sperm bundle.

After aggregation into bundles, a maturing period passes, during which the sperm are perfectly inactive (photo 1). Then periodically the tails vibrate; the entire bundle of sperm travels in a spiral pathway, left to right, just under the follicle wall, up and around the follicle (photo 4), to the secondary spermatocyte region where the direction of spiraling is changed to a downward course and the sperm bundle comes back, cap first (photo 5), down the follicle and out into the vas deferens.

Movement of sperm within the follicle is always the result of the activity of the sperm themselves. The lashing and whipping of the sperm tails, together with the slight boring action of the aggregated rod-like heads, result in the progress of the bundle, as a unit, the heads moving evenly and rapidly forward when sufficient energy has accumulated.

The following is a record of one of many similar observations:

A mature specimen was set up at 11.05. At 11.55 a bundle of sperm was seen at right angles to the edge of the follicle. The tails trailed off down the follicle. Suddenly that portion of the tails just behind the compact heads began a sinuous whipping. Power accumulated, and the bundle started to move. It passed straight across the cyst, turned down at the edge of the follicle, and moved on rapidly toward the open end between the bundles of maturing sperm and the follicle wall. It took just a few seconds for the bundle to traverse the follicle and pass down toward the open end. After traveling for a short space between the follicle wall and the cysts of sperm tails, the bundle spiraled on and disappeared with its long own tails, still writhing and whipping.

In another set-up, a bundle of heads had been stubbornly inactive for several hours. Then the tails commenced to vibrate, and, after several minutes, a sharp whipping action was in progress. The heads were tightly lodged between the

follicle wall and a turgid cyst of young spermatids. It seemed that no progress could be made. The tails separated into two portions and rose in rhythmic waves on either side of the head mass. The whipping brought the tail loops nearly to the cytoplasmic cap that cements the heads; but all in vain, the obstruction was an effective one and, after 10 minutes of active whipping, the tails gradually became quiescent.

Rarely are bundles seen in the act of passing down the follicle, while several hundred can, without any effort, be counted in the process of passing up. Yet, there is, what might be called a continuous sperm pathway just under the follicle wall, and outside of the mass of elongating tails and cyst walls. This pathway results from the decrease in width and increase in length of the cysts, due to the elongation of the sperm heads and the sloughing of superfluous cytoplasm down over the growing tail filaments. From the early spermatid region up to the region of the spermatogonia, the cyst walls are tightly appressed to the follicle wall, but from the spermatid region down to the open end a narrow open cylinder separates the follicle wall from the mass of tails (text fig. B). This space, probably filled with a nutritive or activating fluid, provides a pathway through which the sperm can spiral up, turn and spiral back down to the open end of the follicle.

Since the sperm bundles use the same pathway in spiraling up, about, and down through the follicle, why is it that so many bundles of sperm may be counted ascending the follicle and few or none seen descending? Several reasons present themselves. First, a physiological cause suggests itself, one associated with the probable state of maturity of the sperm itself. Movement of the sperm in Chortophaga is periodic. A bundle of heads may move 0.20 or 0.30 mm. up a cyst, then pause; another fraction of a millimeter is traversed, and another pause ensues; a third advance may be made, but the next period, after several advances, is apt to be prolonged—one of several hours rather than one of several minutes.

A series of photomicrographs was obtained showing the periodic movements and pauses. Photomicrographs 8, 9, and 10 are pictures of the same follicle taken at 3 and 3½ minute intervals. Two bundles of sperm are in the focal field. The upper bundle remains stationary, but the lower bundle advances and the mass of heads passes completely beyond the heads of the upper bundle. In photomicrograph 8, the heads of the lower bundle of sperm are just slightly in advance of the heads of the upper bundle. In no. 9 the lower bundle has moved about 0.09 mm. across the follicle and the tails of the head mass are in a slightly different configuration. In photomicrograph 10 a second advance of 0.06 mm. is recorded for the lower bundle. The upper bundle remains

Text fig. B Diagram of single follicle attached to vas deferens. Sperm pathway in the lower part of the follicle just under the follicular wall. Bundles of sperm ascending, crossing, and descending the follicle. *a*, sperm pathway; *b*, junction of follicle to vas deferens; *c*, follicle and cyst walls closely appressed; *d*, vas deferens.

quiescent. The tails of the moving bundle were writhing and changing position during the entire hour or more that the bundle was under observation, but no further progress was made, and eventually the tails became quiet.

Both bundles in the field are apparently in an identical stage of development and the loci are comparable, but one bundle advanced across the follicle while the neighboring bundle remained inactive. It seems that a difference in the physiological development would account for this variation in activity. When a bundle starts down the follicle, it has probably reached the stage of development necessary for continued or more rapid movement and so it passes on down the follicle and out into the vas deferens. Hence bundles are seldom quiescent, when passing down the follicle.

Again, when the sperm bundle travels down the follicle, it probably encounters less pressure, for the shrinking of the tails is greater toward the lower part of the cyst. Sperm bundles, in traveling up a follicle, are going against the force of gravity. This, though slight, is probably important.

The mass of debris at the bottom of the follicle apparently offers no resistance to mature sperm, for the vasa deferentia are crowded with bundles of sperm. In our previous work (Baumgartner and Payne, '31) we suggested that peristaltic contractions at the base of the follicle might aid this movement, for contractile waves were seen moving down the follicular wall.

POST-TESTICULAR MOVEMENTS

The sperm bundles in the narrow upper portion of the vas deferens are arranged in single file. The tail mass of one bundle, crowded and coiled, is followed immediately by another bundle of heads (photo 12). The lumen in the lower part of the duct is somewhat dilated, but even here the sperm are in close single file. The tails of the sperm in fourteen specimens examined were inactive in the vasa deferentia. When the ducts were torn and the bundles forced out into the nutrient medium, they remained in a passive state. Bundles of sperm do not flow out of the vas deferens readily.

Thousands of sperm bundles in the long seminal vesicles, apparently perfectly mature, but inactive, distend the bulbous ampulla which terminates the vesicle and fill loop after loop of the tubule, making it look dark or clouded (photo 11). A watery fluid, in which the sperm are crowded, flows out rapidly when the vesicles are ruptured, but there are apparently no granules or suspensions or inclusions, except the sperm bundles.

With 2 or 3 exceptions, the sperm bundles were passive in the seminal vesicles, and remained so when liberated into the nutrient medium. Here the caps of the bundles were very pronounced (photo 14). In one case all the tails of the sperm

in the ampullae and the first two loops of the ducts were actively writhing, the movement beginning in the ampullae and developing successively in the loops of the ducts. The sperm in the rest of the coils remained passive.

In three other cases individual bundles of sperm were seen forcing their way rapidly past masses of inactive sperm, along through an entire coil or two of the tubule. Then movement ceased. More than fifty preparations have been examined.

The accessory tubules, which are filled with the milk-white secretion, were macerated and added to sperm bundles obtained from the seminal vesicle. The bundles were not activated. Longitudinal and cross sections of these tubules, stained in Delafield's and eosin, sudan III, and osmic acid, give evidence that the tubular contents are cytoplasmic in origin and fatty in nature. Although the tubular secretion does not activate the sperm, it is found to form part of the fluid content passed from the male to the receptacle of the female. Hence it is most probably a nutritive fluid.

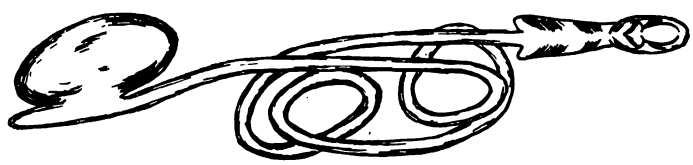
The contents of the hyaline glands were also added to the sperm bundles with like result. There was no activation. The large granular droplets suspended in the clear fluid of the hyaline tubules stain less heavily with cytoplasmic stains and do not react to tests for fat. They average 0.009 mm. in diameter.

In the 40 or 50 specimens examined, sperm bundles were not found in the short muscular ductus ejaculatorius (photos 19, 20). A careful study of the histological structure of the glandular tissue and a thorough analysis of the tubular contents of all the tubules must be made before an opinion as to the origin, structure, and use of the contents can be advanced. In the meantime I can say with certainty, from examinations of stained preparations, that secretions from the milky tubules and hyaline tubules are passed together with the bundles of sperm from the male to the female.

SPERM AFTER COPULATION

The seminal receptacle of the female is an oval pouch, measuring in large specimens as much as 2 by 1 mm. It folds back on the seminal duct and has a short spur-like process extending up from the point of junction. The coiled duct measures 17 mm. and ends in a bulbous muscular portion which attaches directly by a chitinous aperture to the ovipositor apparatus. The vagina opens into the same orifice above the aperture of the seminal duct. Hence fertilization most probably takes place just before oviposition.

In most mature females the seminal pouch is distended with sperm. At first glance, they seem to be in bundles (photo 18), but a close examination shows that the caps have been completely dissolved, although the sperm tend to remain in



Text fig. C Seminal receptacle and duct. Lower muscular portion is attached directly to the ovipositor apparatus (♀).

pseudobundles (photo 17). In some specimens the caps were found in various stages of dissolution. Hence a digestive enzyme, selective for the cytoplasmic cap, must be present in the female receptacle.

In the seminal receptacle the sperm are likewise inactive. At no time have the individual sperm been seen to move, either in the receptacle or when liberated into the nutrient medium (photo 17).

Several large eggs from a female Chortophaga were macerated and 1 cc. of the material mixed with 5 cc. of nutrient medium. Then some sperm from the female receptacle were added to the medium. They were not activated.

Definite peristaltic waves were observed running down the lower third of the tube adjacent to the seminal receptacle and on through the muscular terminal portion which is attached to the ovipositor apparatus (text fig. A, *b*).

DISCUSSION

The sperm of Chortophaga viridifasciata differ markedly from the short active sperm of closely related insects studied by other students in this laboratory. The peculiar 'hatted' sperm of Microcentrum rhombifolium, one of the long-horned grasshoppers, lash frantically either in or out of the follicle. In the follicle a pseudo-aggregation occurs, but they tend to separate when the follicle is broken. Blatta has short, blunt-headed active sperm. They are not aggregated into bundles, and spiral through the medium with a rapid corkscrew motion. The sperm of the cricket, Gryllus assimilis pennsylvanicus are more similar in form to those of Chortophaga, having the long rod-shaped heads and very long writhing tails, but they are not aggregated into tight bundles. The temporary or pseudo-aggregation occurs in the testes, but the sperm mature as individuals and are passed as such, in the complicated spermatophore, to the female. All of these sperm when liberated into the nutrient medium writhe and lash for a long period of time.

How does the sperm of Chortophaga get to the ovum? Does it make periodic progress, after maturing in the follicle, movement being dependent on physiological status? Is it forced through the vas deferens, gradually becoming more mature as it goes? Do currents in the fluid contents of the tubules carry it along, the fluid contents inactivating the mature bundles? Do two or more of these forces operate in enabling the sperm to eventually attain its destination?

As regards the mammals, the opinion has long been prevalent that sperm cells reach maturity during their long passage through the epididymis (Van der Stricht, 1893) and that the secretions of the epithelial cells strengthen the spermatozoa. Young ('29), who examined the genital tracts of at least four species of mammals, finds no evidence that sperm are strengthened as a result of retention or passage through the epididymis. He holds that the tubule serves as a sort of storage vessel, until the time of ejaculation, and that an aging process, as well as a maturing process (if development

was not complete when the sperm left the testis), goes on in the duct.

Braus and Redenz ('24) and others hold that the vitality of the sperm is retained by an inhibition of activity in the epididymis, due to high carbon dioxide tension, low oxygen content, or reduction of electrolytes in the fluid, whether carried to it from the testes or secreted by epididymal cells.

Young ('29) asserts that "the evidence for the movement of sperm out of the testis by means of their own flagellation is at variance with previously reported observations on the behavior of sperm from different levels of the epididymis and the testes (p. 478). The references quoted on which he bases his objections are studies on mammalian tissue and probably may be 'too conjectural,' but repeated intravitam observations on the follicle of Chortophaga show that the sperm spiral up the follicle, turn and spiral back down by means of their own activity.

Whether or not the inactive state, in the long coiled seminal receptacle, which might be compared to the epididymis of mammals, is for the purpose of conserving energy must at present remain a conjecture.

The view that the sperm of Chortophaga become mature in the testes, actively pass out into the tubules, and pass through the tubules in an inactive stage, the period of inactivity preserving the vitality of the sperm, for a longer period than would otherwise be possible, is in accord with that of Braus and Redenz ('24), etc.

The sperm may age in the tubules, for any living histozoan cell eventually becomes senescent, in spite of optimal environment, but optimal conditions prolong and maintain the period of vitality and normality considerably. The seminal vesicle in Chortophaga may not function "essentially to preserve the vitality of the sperm contained in it" (Young, p. 490), but thousands of bundles of sperms may be preserved in it for a period of 5 or 6 weeks in an apparently passive state.

If a certain state of physiological development is the *sine qua non* for progress through the ducts as well as up and around the follicle, then tubular secretions must either nourish or reinactivate the bundles and enable them to reach that state or condition necessary for active movement on their part, because they are inactivated most of the time that they are in the tubules and receptacles.

Probably post-testicular movement of sperm is generally due to peristaltic contractions which initiate streaming in the tubular contents. This does not preclude the possibility of an over-mature bundle of sperm due to its physiological state, initiating contractions which will carry it on toward its destination. Such an argument accounts for the 2 or 3 individual bundles of sperm that were seen in an active condition in the midst of the thousands of others that were perfectly inactive.

SUMMARY

1. Intravitam technique with ordinary transmitted light has certain advantages over other 'vital methods.' In vitro methods remove cells from probable environmental controls, maintained by the organ. Vital stains may or may not be vital. Ultraviolet light, in wave lengths less than 290 μμ, is harmful to protoplasm. It is difficult to use, and the apparatus is not always available.

2. By means of intravitam technique, the sperm of Chortophaga viridifasciata have been followed from the follicle of the male to the ovipositor apparatus of the female.

3. The sperm aggregate into bundles in the follicles of the testes, spiral up from left to right, turn in the early spermatid region, and spiral back down the follicle to the vas deferens.

4. The sperm travel in a space between the follicle and cyst walls, formed by the centrifugal shrinking or complete disappearance of the mature cyst walls in the lower part of the follicle.

5. The sperm bundles, held together by the cytoplasmic cap, pass out into the vas deferens and are carried on to the seminal vesicle.

6. Thousands of bundles of sperm are stored up in a passive state in the long coiled seminal vesicles.

7. The activity of the mature sperm, which passes from the follicle by means of its own activity, is probably inhibited by secretions during the period of retention in the seminal vesicle. Peristalsis, movements of fluids, etc., transmit the sperm.

8. In copula, the sperm, still in bundles, are passed to the seminal receptacle of the female where the caps gradually disintegrate.

9. The single sperm passes down the long coiled seminal duct and by contraction of the terminal muscular portion, are ejected on the egg, just before oviposition.

10. Movement throughout the male genital system and in the female receptacle and duct, as well as various types of movement, lashing of tails, forward movement of bundles, peristalsis, etc., is periodic.

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PLATES

All pictures, except figure 10, were taken with an arc illuminant on negative print paper. The tissues were taken from the testes or other parts of the genital tract, mounted in nutrient fluid, and immediately photographed. No stains or fixatives were used and the photographs are unretouched.

PLATE 1

EXPLANATION OF FIGURES

1 Bundles of sperm traveling up the follicles. The tails of one bundle of sperm in the middle follicle were writhing and whipping. × 30.

2 The same follicle 3 minutes later, showing the tails of the active bundle in different formation. The heads have not advanced. × 30.

3 A concentric spiral in the tails of an ascending bundle. × 120.

4 Two bundles of sperm crowding up between the follicle and cyst wall. The more distant bundle is not in sharp focus. × 100.

5 A bundle of sperm spiraling down the follicle. The cap which holds the heads together is in sharp focus. × 103.

6 A bundle of sperm traveling down the follicle, forcing its way between cyst and follicle wall. × 100.

7 Photomicrographs 7, 8, and 9 form a series taken at 3-minute intervals, demonstrating movement of sperm in a follicle. In no. 7, the head of the lower bundle is a little in advance of the upper or higher bundle. The writhing tails form a large imperfect circle under the follicle wall.

8 The same follicle focused 3 minutes later; the lower bundle of sperm has moved 0.09 mm., while the upper bundle remained stationary. × 92.

9 The third of the series, showing the sperm bundle moving up the follicle. Photograph 9 was taken 3 minutes after photograph 8. The total configuration has changed definitely and the heads of the lower moving bundle are completely past the heads of upper stationary bundle. × 92.

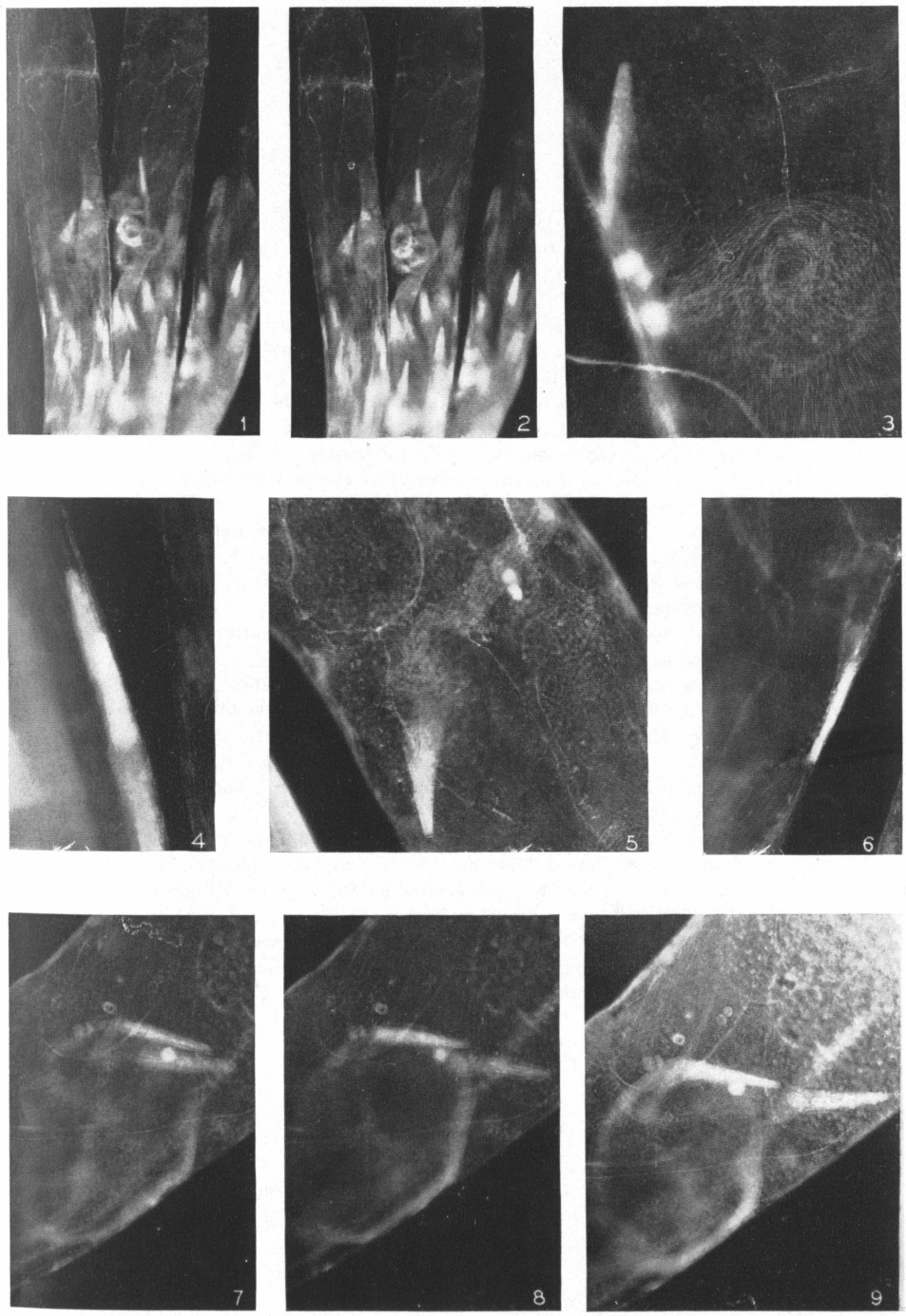
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PLATE 2

EXPLANATION OF FIGURES

10 Cross section of a follicle cut at right angles through the head. Counts of this and other bundles with a camera lucida gave an average of 267 sperm to a bundle. (Allen's fixation, methyl green and acid fuchsin.) × 650.

11 Ampulla and portion of seminal vesicle. Clouds of sperm can be seen through the wall. Bundles of sperm which flowed out from the ruptured end of the tube are in masses above and below the vesicle. × 35.

12 Portion of the vas deferens. Bundles of sperm traveling along in single file, from the testis to the seminal vesicle. × 42.

13 Portion of clear accessory tubule attached to the ductus ejaculatorius. Note large refractive granules suspended in the clear fluid. × 35.

14 Enlarged view of sperm from seminal vesicle. Head cap still holding sperm in a bundle. × 93.

15 Part of one of the milky tubules which are also attached to the upper portion of the ductus ejaculatorius. × 35.

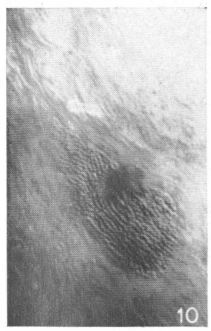
16 Ampulla and first 2 mm. of vas deferens to which about thirty-six follicles are attached. × 35. (Whitish areas are mostly remnants of the fatty connective-tissue covering, with which the entire testes, vasa deferentia, and seminal vesicles are sheathed.)

17 Enlarged view of lower middle third of figure 18. Note that bundles of sperm have lost the cytoplasmic caps. × 90.

18 Seminal receptacle from body of female Chortophaga viridifasciata. The masses of sperm were forced from the vesicle into the nutrient medium. × 15.

19 Ventral view of ductus ejaculatorius in focal plane of lumen of ductus ejaculatorius. (See diagram, text fig. A, *b*.) × 50.

20 Dorsal view of ductus ejaculatorius. Clear accessory glands, milky accessory glands, seminal vesicles, and vasa deferentia attached to upper portion. Numerous tracheae apparent. See diagram, text fig. A, *b*. × 50.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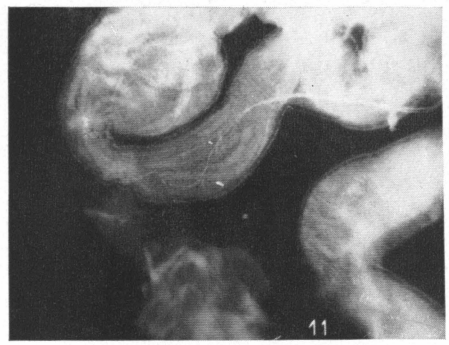
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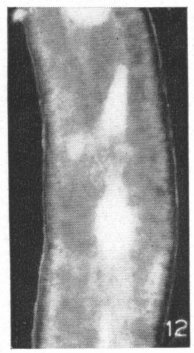
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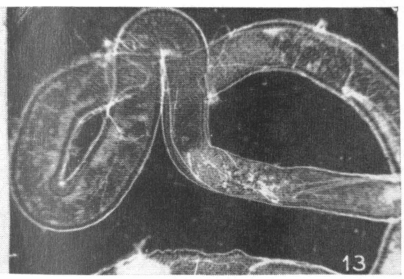
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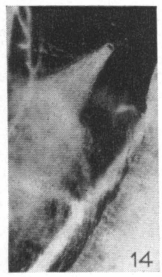
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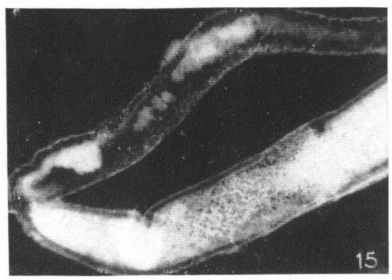
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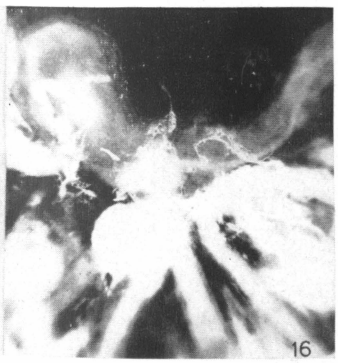
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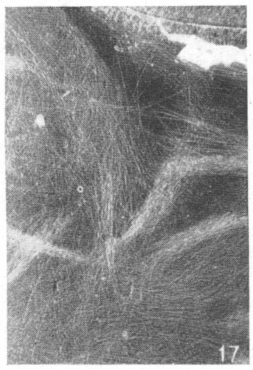
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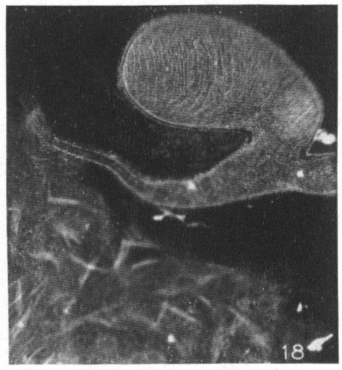
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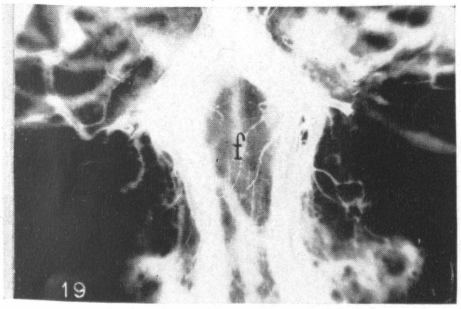
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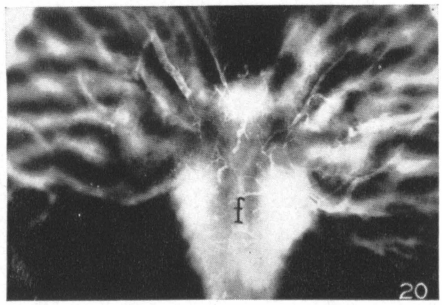
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II. Intravitam Studies on the Hemipteron, Leptocoris trivitatus. A Description of the Male Reproductive Organs and the Aggregation and Turning of the Sperms.

1) Introduction and method.

2) Description of the testes, ducts and accessory glands in the adult bug.

3) Aggregation and turning of the sperms.

4) Discussion.

5) Summary.

6) Literature cited.

7) Photomicrographs.

INTRODUCTION AND METHOD

Much cytological work has been done on the origin, growth and differentiation of the male germ cells in the Hemipteran bugs. (Paulmier, Wilson, Bowen, Montgomery, et al.) Nearly all of this work has been done on stained and fixed tissue for the reproductive cells of the Hemiptera, when compared with those of the Orthoptera, are exceedingly small and consequently do not lend themselves readily to intravitam studies. Besides the reproductive organs of many species of bugs are closely invested with a fatty pigmented connective tissue covering which is exceedingly hard to remove.

When this tissue has been successfully removed, without rupturing the follicle or cyst walls of the testes, it is found that light rays from an ordinary sub-stage lamp will not penetrate the protoplasm of the cells themselves, as easily as similar light rays pass through the cells of Orthoptera.

Hence in the bugs studies of spireme development, miotic phases and early details of spermatid transformation are made more readily on stained tissue.

However, it is exceedingly difficult to study certain phases of the growth and development of germ cells in fixed tissue for relationships, very evident in intravitam preparations, are not preserved in sectioned tissue. This is true of the aggregation and turning of the sperms within the testicular follicle. When only a part of a sperm bundle is present in a microscopic field, together with parts of spermatid cysts and portions of sperm tails from other sperm bundles, it is virtually impossible to realize that aggregation, turning and migration processes are under way (photomicrograph 7).

In intravitam preparations the problem of analysis is much simplified for the entire follicle, containing about 50 or 60 maturing and mature cysts can be readily examined under

low magnification. Later details in 3 dimensions can be studied with higher magnification and the evident relationship of cysts at various angles and in unexpected loci in the follicle become apparent. The cysts are spiraling across the follicle, just under the follicle wall, or the mature sperm are traveling down the follicle, heads first, toward the open end.

The method of aggregation and turning of the sperms in one of the Coreidae, Leptocoris trivitatus, is described in this paper. Aggregation and turning is very similar in several other bugs which have been observed in this laboratory and it is very probable that all sperms in the Hemipteran group aggregate in a manner very similar to that described for Leptocoris.

The methods developed and employed previously for the studies of the structure of the testis and movement of sperms in Chortophaga viridifasciata (Payne, '33) have been used for these analyses. The connective tissue covering had to be removed from the

follicles very carefully for both cysts and follicle walls are delicate. With very sharp slender needles the covering could be torn and pulled away in pieces, thus freeing the entire follicle for examination.

The nutrient medium used by Belar for his studies on the spermatogenesis of Chorthippus and which seemed perfectly satisfactory for my own analysis of the germ cells of Chortophaga, is not suitable for studies on Leptocoris. In this medium the sperms which have escaped from ruptured follicles have the tips of their tails coiled into rings while the bundles of mature sperm within the follicles, are inactivated. The spermatocytes and young spermatids, however, appear normal. These facts further indicate that the normal pressure maintained within the follicle by follicular and cystic fluids is a factor that must be considered when trying to establish normal environmental conditions for delicate germ cells. (Baumgartner and Payne. '31, p. 359).

Looke's nutrient medium seemed more suitable and was used during the first part of this study, but it is slightly hypertonic for sperms are abnormally activated by it and cyst walls and follicle walls rupture easily.

Finally Belar's medium was modified in the following manner so that it appeared to be isotonic for L. trivitatus. 1 cc. of a 9% NaCl solution; 1 cc. of a 1% $CaCl_2$; 1 cc. of a 1% KCl solution plus 5 cc. of a 10% dextrose solution were added to 10 cc. of Belar's nutrient medium, made up according to the original formula ('29). Then the whole was made up to 100 cc. by adding distilled HOH.

Difficulties encountered in attempts to balance an isotonic medium for L. trivitatus make an easy and accurate method for the determination of the pH of insect blood very desirable, for intravitam observations show that the limits of variation in hydrogen-ion which can be tolerated by maturing sex cells of most species of insects are very narrow. Furthermore a solution which is relatively isotonic for one family of insects may be hyper- or hypotonic for closely related families.

DESCRIPTION OF THE TESTES, DUCTS AND ACCESSORY GLANDS IN THE ADULT BUG.

Before describing the method of aggregation and turning of the sperms in this Coreid bug a detailed description of the testes, ducts and accessory glands is essential.

The two fan-shaped testes, lie in the body cavity in the second and third abdominal segments, under the edge of the dorsal wall and appressed to the lateral walls. Distally they are connected to the body-wall by delicate cords of connective tissue fibers. Proximally the follicles taper and open into the ampullae-like ends of the vasa deferentia. The average testicular mass is 1.65 mm. long and about 1.1 mm. wide at the upper end of the testis. It tapers to 0.7 mm. at the lower end. Often the testes from an apparently mature male are much shorter than this but the stage of development within the follicles closely approximates that in follicles of a testis of average size. (photomicrograph 1).

There are seven short plump follicles in each testis, which measure 1.65 mm. in length and 0.291 mm. in breadth at the distal end. Three of the follicles fit into the depressions left between the other four linearly arranged tubular follicles. The three finger-like follicles form the inner side of the testis, and lie against the lateral wall of the gastro-intestinal tract. The opposite side of the testis fits into the concavity of the lateral body-wall and is abundantly supplied with tracheae which come from the adjacent spiracle. All seven follicles are closely invested with a deep orange-colored fatty connective tissue coat which adheres tightly to each follicle and covers the testis as a whole. The connective tissue ends, however, just above the proximal ends of the follicles. Photomicrograph 9 shows a testis from which the connective tissue has been removed, permitting the follicles to spread out in the nutrient lake. A tiny fragment of connective tissue adheres to the first follicle which is ruptured and shows the escaping

sperms. The other six follicles are about half filled with mature sperms.

The vasa deferentia extend from the base of the testes to the median ventral line in the 4th body segment. At the beginning of the 5th segment they meet to form the ejaculatory ducts. There is an ampulla-like enlargement at the upper end of the vas deferens. In mature bugs this part of the duct is filled with sperm which are apparently perfectly mature but generally in a passive or quiescent state. They fill the lumen and extend down the duct to the point at which the contents of the accessory gland pours into the vas deferens (photo 6). In several preparations sperm in the ampullae of the vasa deferentia have been moving or contracting slightly but they have not been found in the ducts past that point at which the accessory glands open into the vasa deferentia in any of the hundred or more preparations which have been studied.

In many of the insects the ejaculatory apparatus consists of a common or single tube

which leads from the point of junction of the vasa deferentia to the exterior, but in Leptocoris there are two long tubes, closely approximated and wrapped in a common connective tissue covering. Each is a continuation of the right or left vas deferens respectively. These ducts are composed of narrow, hard chitinous-like rings which are fitted one to the other (photomicrograph 2). The ducts are longer than the vasa deferentia for they extend from the fifth body segment to the tip of the abdomen. At the very posterior part of the abdomen they fuse and continue as the short slender copulatory organ, the penis, which lies between the two heavy chitinous retractile claspers (photomicrograph 3).

Henneguy notes that the accessory glands of the insects vary in different species as regards the numbers and loci.

"Ces glandes peuvent être au nombre d'une, deux ou trois paires; ce cont des tubes allonges, souvent enroules sur eux-mêmes, ou des groupes de nombreux caecums. Elles viennent déboucher soit dans les conduits déférents, soit dans le canal éjaculateur, soit le plus souvent au point de réunion de ces canaux." (p. 175).

This statement about accessory glands in insects does not allow for the 12 or 14 pairs of glands that are attached to the ductus ejaculatorius in Chortophaga viridifaciata (Payne, '33), but Leptocoris has only one pair for a single large saccular gland is attached to each vas deferens, laterad and midway between the testis and the point of origin of the ejaculatory duct (photomicrographs 14, 18). This gland is usually distended with a finely granular milky secretion which fills the lumen (photomicrograph 5). The secretion is not homogeneous nor entirely fatty, although many of the droplets will stain a bright orange with sudan III. There is a pore or outlet at the base of the gland by which the secretion may escape into the vas deferens. Photomicrograph 18 shows the contents of a gland at the mouth of the funnel-like opening which leads into the duct.

The wall of the gland consists of a delicate serosa and an outer epithelial layer; a thin muscularis and a secreting mucosa. The serosa is

composed of a single outer layer of flat thin-walled cells and several irregular rows of cylindrical epithelial-like cells, from three to five cell-layers deep. These cells are massed in small bundles or trabeculae due to the fact that large tracheae run through the serosa and into the epithelial tissue, where they give off many delicate branches which ramify between groups of the stratified cells (photomicrograph 9). The entire gland is well aerated. The main tracheal tube which supplies many of the cells of the glandular wall as well as part of the adjacent vas deferens is shown in photomicrograph 18. A large nerve which runs from the ventral nerve cord into the wall of the vas deferens just above the point where the vasa deferentia meet, is also pictured.

The muscularis consists of a layer of loosely woven striated fibers. Most of the fibers run longitudinally but a few are at right angles to the longitudinal ones while several others cross from fiber to fiber and anastomose, forming small muscle bundles. The wall of the vas deferens also contains scattered

striped muscle fibers between the single row of epithelial cells and the relatively thin inner mucosa which lines the duct. There is a common wall, however, on that side of the duct to which the gland attaches, for the muscularis of the wall of the gland and duct have fused (photomicrograph 16).

The mucosal layer is composed of rows of small cylindrical cells, that vary in number. At the upper and lower end of the gland the cells may be only two or three layers deep but the walls composing the middle part of the lumen may be seven or eight cells in depth. The walls of the inner cells are very ragged and indefinite or the cell structure has completely disappeared and only remnants of the cytoplasm remain. Photomicrograph 5 is a cross section through a duct and adjacent gland. There a mass of mucosal cells can be seen projecting far into the lumen of the gland. The structure of the cells at the edge of this mass is very indefinite.

Several times when the entire reproductive system was removed intact and suspended in a

nutrient solution, a series of contractile waves ran down the wall of the gland to the region at which the pore from the lumen opens into the vas deferens. These waves commended in the upper third of the wall and traveled rapidly to the basal portion of the gland. A second wave commenced in the upper part of the gland before the first one had reached the base. They continued to follow one another for ten or twelve minutes and were quite pronounced. Similar contractions have been observed in glands before the testes and adjacent organs were removed from the abdominal cavity.

AGGREGATION AND TURNING OF THE SPERMS

The arrangement of the sex cells in the tubular follicles of Leptocoris is comparable to that found in Chortophaga. The distal end of the follicle is filled with small round cysts of spermatogonia, which are mostly in the resting stage. Next are older spermatogonia and very young spermatocytes in which the nuclear material is clumped in a mass in

the center of the nucleus and surrounded by a hyaline ring of clear nucleoplasm. This tight spireme of nuclear material is densely refractive and easily seen although the cells themselves are very small. Older cysts of cells are in division stages and the 13 chromosomes eventually give rise to the six tetrads and one dyad characteristic of the primary spermatocyte (Yocom, '23). These short plump chromosomes are easily counted in the living tissue. In secondary spermatocytes the six large chromosomes form a circle about a small chromosome which lies in the center. It is hard to distinguish any details of structure in young spermatids, other than the small nucleus and the refractive nebenkern. When the axial filaments have grown out slightly, however, a peculiar arrangement of the spermatids within the cyst becomes evident. There is a tendency for the filaments or tails to grow toward the center of the cyst and for the cell bodies, at the same time, to become arranged peripherally, just under the cyst wall. As the axial filaments grow, the heads become smaller

and smaller. Both the heads of the spermatids and the entire cyst of cells are perfectly spherical at this stage. Photomicrograph 13 shows several cysts of early spermatids that have flowed out from a ruptured follicle into the nutrient lake while in photomicrograph 12 two cysts of cells at this stage of development may be seen at the usual locus within the intact follicle. An optical section through one of these cysts looks like a compact wheel. The short filaments, sheathed by the double elongating nebenkern suggest the spokes of a wheel while the cell bodies, lying peripherially, wall to wall, form a perfect rim. The growth of the axial filaments continues centripetally until they meet in the center of the cyst where they commence to bend and coil, for their length becomes greater than the radii of the cyst. Then the entire mass of tails tends to push out in one direction and this line of growth causes the heads to move around to one side of the cyst which soon loses its spherical shape and becomes ovoid. Later a more or less

regular "spindle" is formed. The spindle gradually becomes more and more pointed, either due to the continued growth of the tails or to their straightening out. Eventually the heads have become slender rods and the entire mass is held in a tight clump by a small cap of cytoplasm which covers the tips of the heads. The tails, in the meantime, have elongated greatly, broken the cyst wall at the posterior end of the spindle and trail down from the head mass in characteristic fashion.

A typical "spindle cyst" may be seen crossing the follicle in photomicrograph 12. In this cyst the spermatid heads, fairly well elongated, are crowded in one end of the spindle while the tips of the tails are massed in the opposite end. The central part of the spindle bulges with cystic fluid containing many small globules of cytoplasm and numerous blebs aggregated on the middle part of the tails of the spermatids.

After the tails begin to push out from the center of the cyst, causing it to become more and more ovoid in shape, the entire cyst

gradually moves out under the follicle wall and then travels up the follicle for a short distance, crosses over, turns and spirals back down toward the open end of the follicle, tracing a pathway very similar to that followed by a bundle of grasshopper sperms. In the grasshopper, however, the mature sperms have aggregated into bundles and the long writhing tails have grown far beyond the confines of the ruptured cyst walls before the bundles commence to move in the follicles. In Leptocoris, the young cysts themselves, containing very immature spermatids, commence to move and turn in the follicle long before the sperm bundles have aggregated.

The method of turning and crossing is a spiraling process and commences when the tails push out from the central area of the rounded cyst. They gradually grow longer and longer, extending out toward the proximal end of the follicle. At the same time the head end is pointed up and across the follicle. Photomicrograph 15 shows a bundle of sperm in an early stage of migration. The head mass is

pointed up the follicle and is just about to cross over a cyst of spermatocytes. Other stages during the process of spiraling and turning are shown in photos 10, 11 and 12.

After cysts have spiraled across the follicle, the anterior or head end of the cyst always points to the open end of the follicle. A cyst of relatively mature spermatids with the head end pointing down the follicle may be seen just under the follicle wall on the right hand side of the follicle pictured in photomicrograph 11. After the cysts of spermatids have turned and commence to spiral down the follicle, the spermatids soon become sperms. The tails greatly elongate and the cyst walls disintegrate so that the lower two-thirds or more of the follicles in older bugs may be filled with mature sperm (photomicrograph 9, 10). Bundles of these mature sperm pass from the open ends of the follicles into the ampulla of the vas deferens and fill it down to the point previously described. In the ampullae the sperm are generally inactive but if a follicle is torn in a slightly hyper-

tonic solution the bundles of sperm commence to spiral rapidly through the medium. The bundles move clock-wise, spiraling across the microscopic field (16 mm. lens x 12.5 compensating oculars) in 10 seconds. (photomicrograph 17)

DISCUSSION

In most of the insects the sperm are aggregated in some manner before they are transferred to the female. Either a packet of sperms is massed together as in some of the Tettigoniidae, or a delicate, intricate spermatophore is constructed as in the Gryllidae (Baumgartner, '11) or the tips of the sperms are welded together with a cytoplasmic cap as in the Locustidae and Coreidae.

It seems plausible that this method of aggregating sperms into characteristic masses is for the purpose of insuring their safe transfer to the seminal receptacle of the female. Most female insects are prolific, laying hundreds of eggs, and a good supply of

sperm should be at hand to insure the fertilization of each ovum before ovi-position. The effectiveness with which nature accomplishes insemination is evidenced every time a mass of mantid eggs or a spider's egg case is hatched in the laboratory, for the nymphs pour forth for several days or young spiderlings appear in legions.

Although nature is prolific and hundreds of thousands of sperms are found in a single testis of Leptocoris, it appears, that up to the latter part of March, it is impossible for the sperm to pass beyond a certain point in the duct. Attention has been called to photomicrograph 6 in which the sperm are shown at that level in the duct to which they usually descend.

Female Leptocoris were examined periodically during this study and it was found that up to the same time the eggs were very immature. Neither could a seminal receptable containing sperms or sperm bundles be located in the female so it is probable that mating had not

occurred. This immaturity of the female sex cells would probably account for the fact that the sperm do not pass down into the ejaculatory ducts of the male earlier.

What is the function of the fluid in the accessory gland and does it activate or nourish the sperm? Some pertinent research has been carried on in this line by Young, Braus and Redenz, and others, but their observations have been made chiefly on mammals and the effect of epididymal retention and secretions. Hence their conclusions are not directly applicable to the insects. Nevertheless some light is thrown on the probable cause of passivity in the mature sperm by their studies.

The fluid contents of the single gland that is attached to the vas deferens cannot be forced through the opening pore at this stage of maturity. When pressure is brought to bear on the gland the wall ruptures and the contents flow out into the nutrient medium. Normal peristaltic waves and relaxation of the sphincter fibers at the mouth of the pore seem to be necessary for the passage of the glandular contents into the vas deferens. Neither

pressure stimulii nor mechanical stimulation cause the sphincter muscle at the mouth of the pore to relax.

If this glandular substance functions as an activator it must first be modified or activated itself, for sperm taken from the vas deferens and suspended together with it in a nutrient medium do not become motile. It is possible that a third substance, a catalyzer or enzyme-like secretion, is added to the sperm plus the glandular material when, upon proper stimulation, they are passed to the seminal receptacle of the female.

So many physiological reactions are based on the linkage of two chemical compounds to a third, e.g.,the prothrombin-calcium union which eventually acts on fibrinogen and brings about blood coagulation, and the more nearly related egg-fertilizin-sperm reaction described by Lillie ('19),that it is altogether plausible that the addition of another substance is necessary before the final activation of the sperm of Leptocoris. Since the ova of the females are not yet ready for fertilization the vitality

of the sperm can undoubtedly be maintained for a longer period of time in the male ducts, if they are retained in a passive state (Braus and Redenz '24).

It does not seem plausible that the contents of the accessory gland serve a purely nutritive function for the apparently mature sperm are in the ampulla of the vas deferens for several weeks and during this period none of the glandular contents pass into the duct.

In Leptocoris the cysts of young spermatids commence to turn in the follicle at a time when the tails are barely developed and certainly not motile. Their method of growth, however, seems to be the directive force in the turning process. The tails push out from the center of the cyst and down toward the open end of the follicle and at the same time the head ends of the sperms are forced up to the opposite end of the cyst and the entire upper end of the cyst moves out against the follicle wall. Growth and change of position occurs, of course, within the unbroken cyst wall. Cysts have been observed in which the tails of the

young spermatids had not yet elongated toward the open end of the follicle. Their tails had spiraled and coiled upon themselves in the center of the cyst and due to the packed condition, must have been under sufficient tension to give the anterior part of the cyst quite a forward push if they suddenly straightened out. In many of the more mature cysts, the spermatid tails are bent at quite an angle and appear to be under tension. Hence it is very probable that the main activating force, by which the cysts of spermatids turn and move in the follicle, is the elongation of the spermatid tails.

After the cyst walls have ruptured and the sperm have developed, the tails may writhe and move the sperm bundle along, but after the sperm bundles reach the vas deferens, peristalsis and translocation through the movement of fluids, are sufficient forces to account for the transference of the sperm to the copulatory organ.

SUMMARY

1. Analysis of certain phases of the development of germ cells is simplified through the use of intravitam technic, for with it mature and maturing cysts of cells can easily be examined and relationships of the cysts to the follicle, and of the germ cells to the cyst become evident. By means of intravitam technic the method of aggregation and turning of sperms in one of the Coreidae, Leptocoris trivitatus have been studied and described.

2. The genital system of Leptocoris consists of a pair of fan-shaped testes which lie between the lateral body wall and the gastro-intestinal tract. Two ducts or vasa deferentia lead from the testes and form a pseudo-fusion in the median line of the abdominal cavity. Two adjacent chitinous-like ejaculatory ducts continue from the point of fusion of the vasa deferentia to the posterior part of the abdomen where they join onto the short copulatory organ, and lastly, there is a

single pair of accessory glands attached to the vasa deferentia midway between the testes and the ejaculatory ducts. These large saccular glands are filled with a granular milky secretion. A pore opens from the inner basal wall of each gland into the adjacent vas deferens.

3. The wall of the gland consists of a serosa, a muscularis and a mucosal layer. The serosa is reenforced by several layers of epithelial-like cells. The muscularis is formed of loosely woven striped fibers and the inner row of the mucosal layer of cells seem to gradually degenerate and slough forming part of the secretory contents of the gland.

4. Peristaltic waves have been observed in the wall of the accessory gland. They start in the upper and outer wall of the gland and run down toward the base to the region in which the opening pore is situated.

5. The development of spermatogonia and spermatocytes proceeds in the usual way but

the aggregation and turning of the sperm differ from that found in some other insects. The young spermatids are at first arranged centripetally and the cyst is perfectly spherical. Then the elongating tails push out toward the open end of the follicle while the head pieces are forced up in the direction of the closed end. First an ovoid and later a spindle-shaped cyst is formed. The growing tails probably supply the force which moves the cyst across the follicle and finally turns the head end down toward the vas deferens.

6. The method of turning and crossing is a spiraling process. After a cyst starts down the follicle the cyst wall ruptures and disintegrates, leaving the sperm in characteristic bundles.

7. The sperm with the delicate tips of the heads held together by a clear cytoplasmic droplet pass on into the upper part of the vas deferens where they remain for some time in a passive state.

8. The process of massing of the insect sperms into bundles insures effective trans-

location to the seminal vesicles of the female which are usually extremely prolific and hence need a good supply of sperm in order to insure insemination at the time of oviposition.

9. The glandular secretion is not purely nutritive but most probably functions as an activator. However a third substance must be added to modify or stimulate the secretion itself for sperm are not activated by this secretion alone. It is probable that the third substance is added by the female after copulation.

10. After the sperm have aggregated in the cyst and the cyst walls have ruptured, the sperm tails may writhe and whip and so move the massed heads out of the lower part of the follicle into the vas deferens but here peristalsis and movement of tubular fluids are sufficiently pronounced to account for the translocation of the sperm to the copulatory organ.

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PLATE I

EXPLANATION OF FIGURES

Photomicrographs are pictures of unfixed unstained tissue (nos. 5, 7, 8, and 16 excepted). The tissue was removed from the organism, suspended in nutrient medium and immediately photographed. Belar's nutrient medium or a modified Belar's was used in preparing the tissue mounts for photographing, except nos. 13 and 17, which were suspended in Locke's nutrient medium.

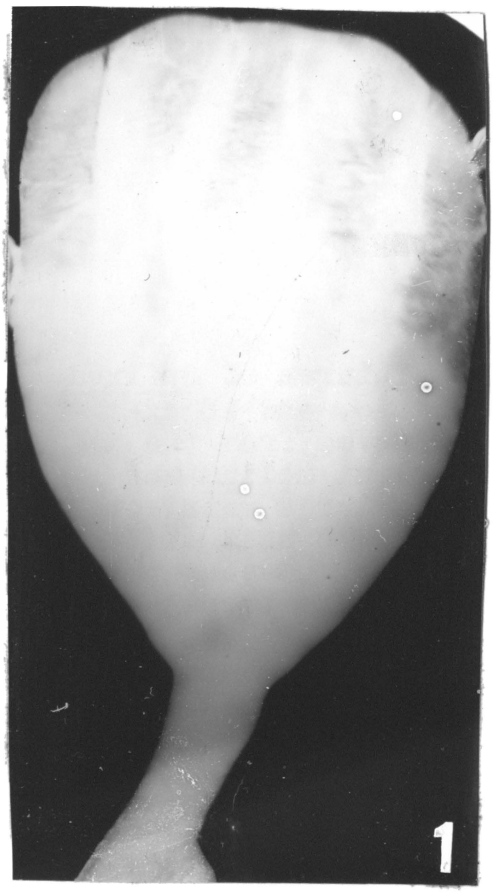
1. Fan-shaped testes, covered with the fatty yellow connective tissue. X 110.

2. Portion of the ejaculatory ducts, showing the two tubes, the chitinous-like rings of which they are constructed and the loose connective-tissue wrapping which covers them. X 138.

3. The short copulatory organ to which the ejaculatory ducts join. It lies between the heavy chitinous partly-retractile claspers. X 138.

4. The delicate cords that attach the testes to the abdominal wall. X 180.

5. Cross section through vas deferens and attached accessory gland. The duct is filled with sections of sperms; the gland, with a milky secretion. A mass of mucosal cells from the upper wall of the gland projects deeply into the lumen and is readily distinguished from the more opaque secretion (stained tissue). X 240.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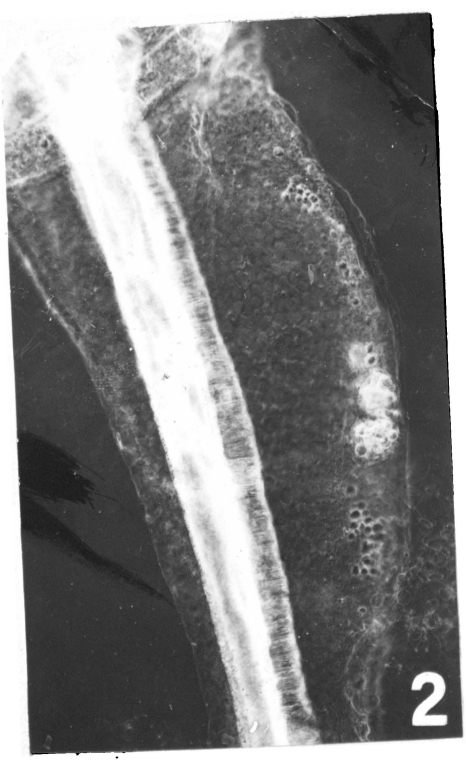
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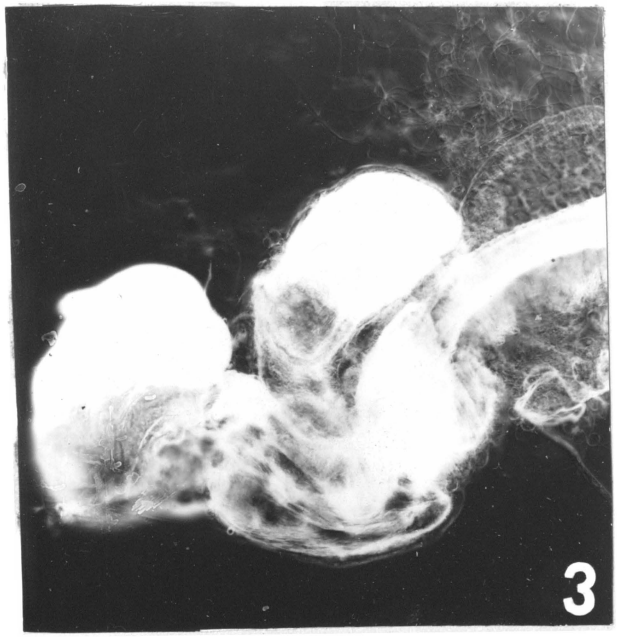
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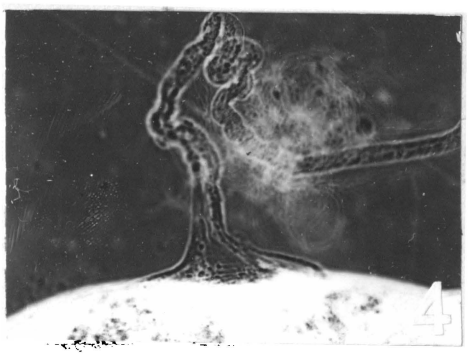
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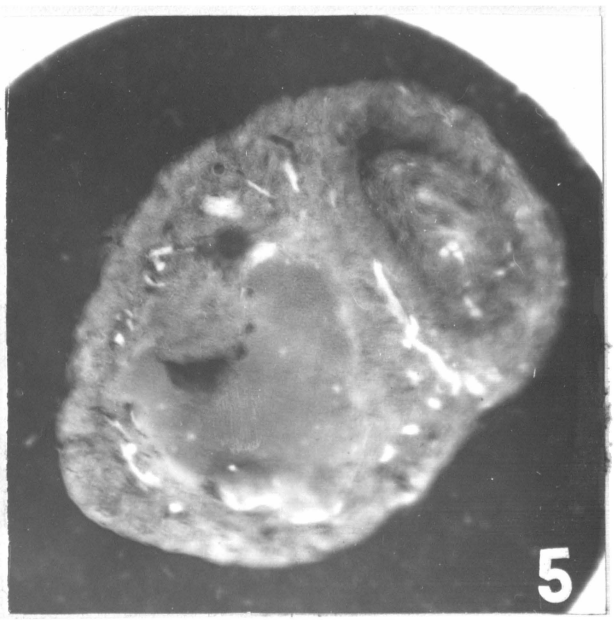
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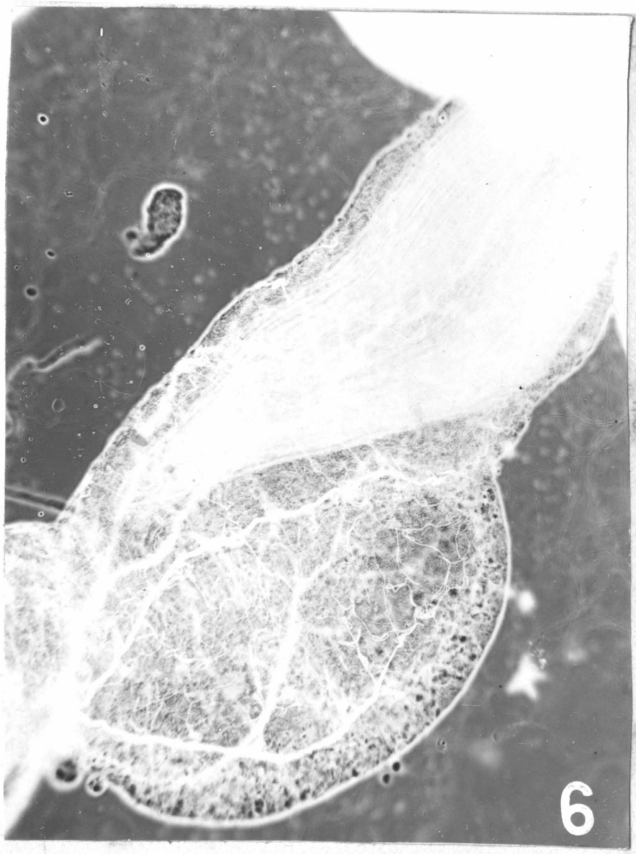
PLATE II.

6. A mass of sperms in the vas deferens. They have descended to the level at which the accessory gland opens into the duct. The ramifications of several larger tracheal tubes can be seen in the wall of the gland and duct. X 165.

7. Cross section of prepared tissue (Bouins fixation; hemotoxylin and light green stain). The head mass and attached tails of one bundle of sperms are shown in one follicle, together with parts of detached head masses of two other bundles. The adjacent follicle contains only sections through tails of other bundles of sperms. X 270.

8. Cross-section through the wall of the accessory gland, showing loosely woven striped muscle fibers and in the upper right-hand wall of the gland, portions of several tracheal tubes. (stained tissue). X 310.

9. A testis from which most of the connective tissue covering has been successfully removed. A narrow band and fragment of the covering remain at the base of the follicles. Each testis is composed of seven follicles, arranged in two linear groups of three and four, respectively. The three follicles that lie against the outer abdominal wall, fit in between the angles formed by the four tubular inner follicles. In this photograph the seven follicles have spread apart and lie in one plane. The wall of the first follicle has ruptured. X 60.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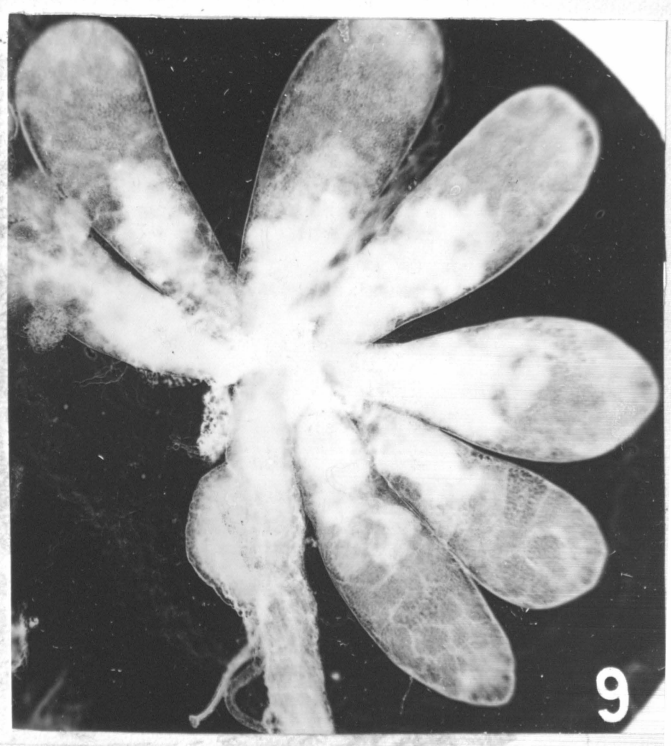
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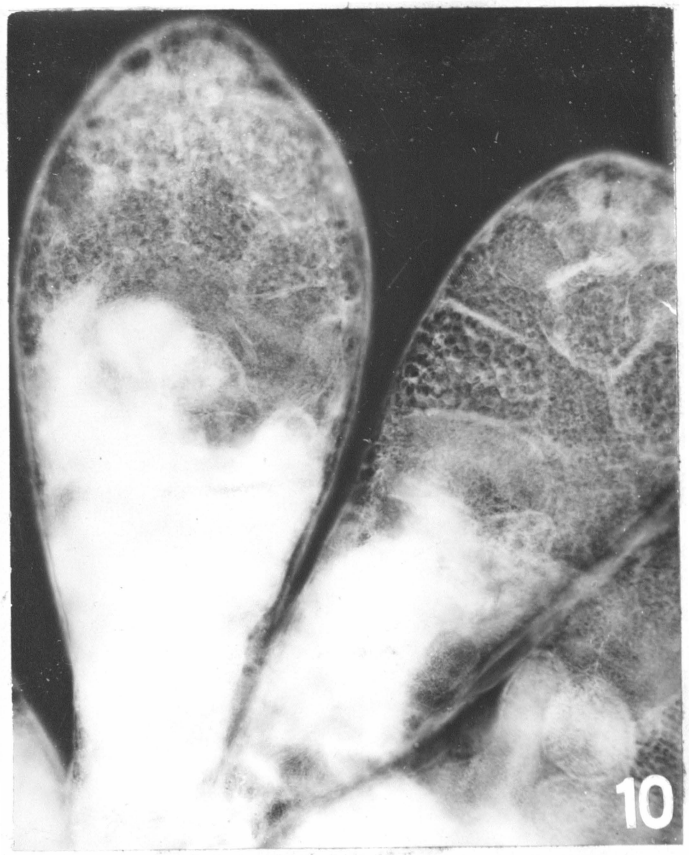
PLATE III

10. Follicles containing cysts of spermatogonia and spermatocytes in different stages of development. Cysts of large primary spermatocytes may be seen just above the spermatid region. X 130.

11. A follicle nearly filled with turning and maturing cysts of spermatids. Under the wall on the right side of the follicle, two cysts which are in the process of ascending the follicle may be distinguished. On the left hand side a single cyst is going down the follicle. The cysts turn and migrate in the "sperm pathway", a narrow space between the follicle wall and the central mass of maturing sperms. X 140.

12. Young cysts of spermatids, centripetally arranged, are visible in the upper part of the follicle. An older spindle-shaped cyst is migrating across the follicle. X 150.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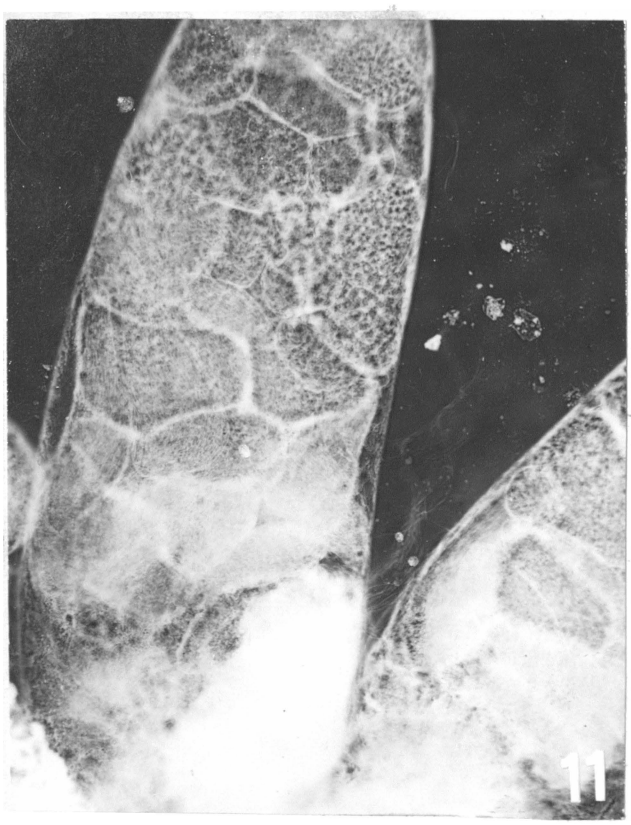
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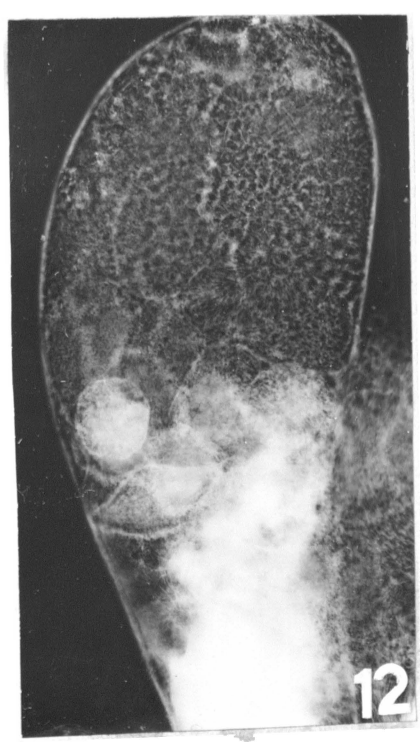
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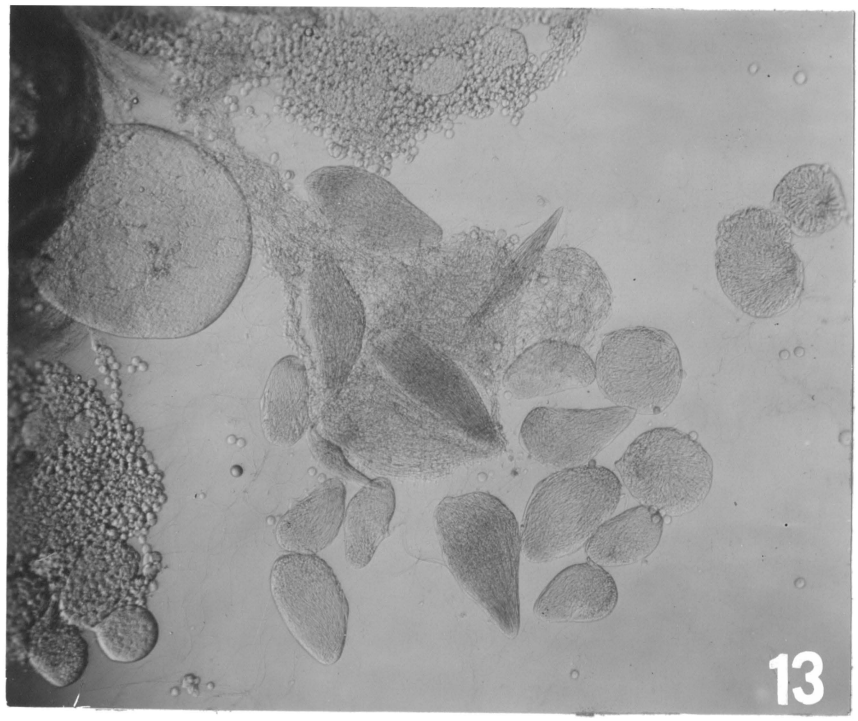
PLATE IV.

13. Bundles of spermatocytes in different stages of development. These flowed out into the nutrient lake from a ruptured follicle. Cysts of spermatogonia and spermatocytes, as well as free cells from ruptured cysts are also in the lake of nutrient medium. X 136.

14. The single saccular gland, firmly attached to the vas deferens. X 86.

15. Cyst of young spermatids starting to travel up and across the follicle. The cytoplasmic cap is forming at the anterior end and the tails are pushing out and downward. Cytoplasmic blebs can be seen in the mid-part of the cyst. X 420.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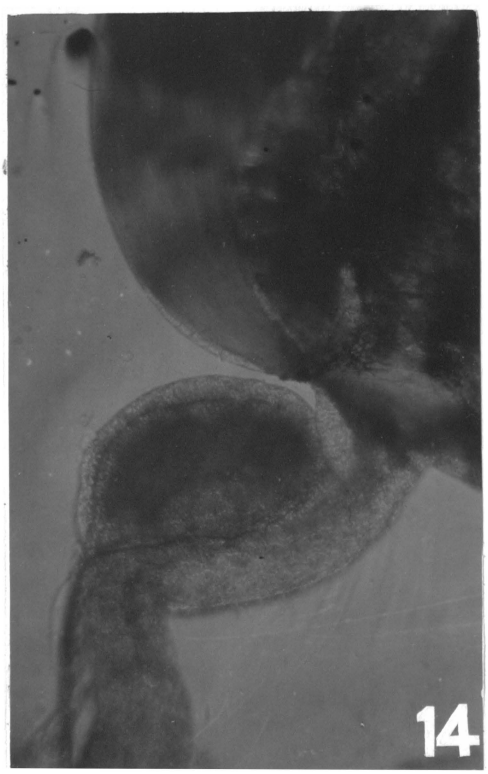
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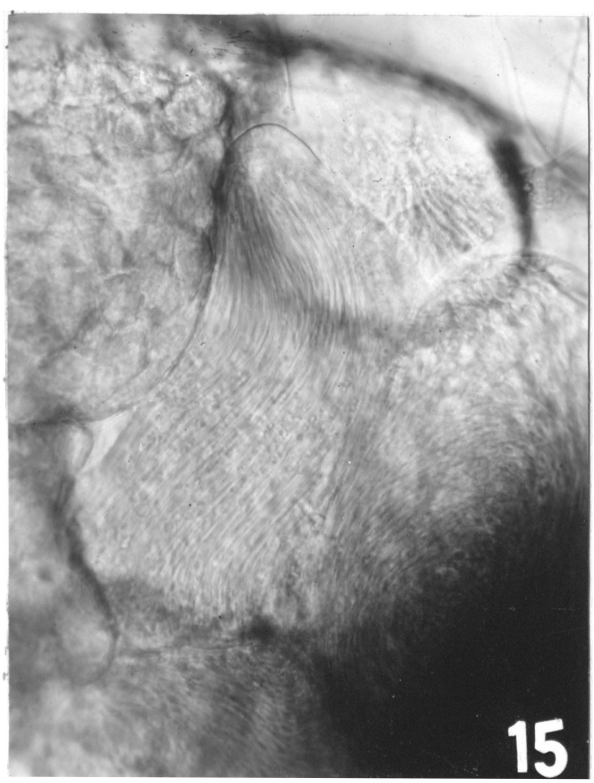
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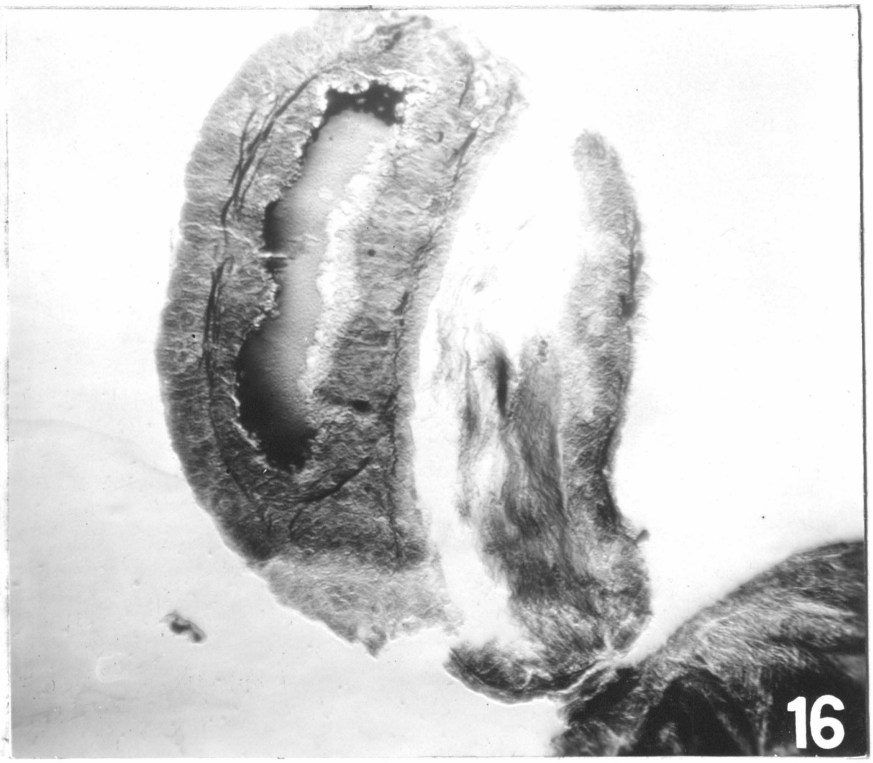
PLATE V.

16. A longitudinal section through the vas deferens and attached accessory gland. The muscle fibers may be seen in the outer wall of the duct and gland while the relatively thin inner wall of the duct has fused to the muscularis of the inner wall of the gland, all intervening tissues having disappeared. X 210.

17. Two bundles of sperm from a broken follicle out in the nutrient lake. The heads, which are held together by droplets of cytoplasm, are propelled through the water by the writhing tails. The bundles spiral from left to right and cross the field on the microscopic stage in a few seconds. Spermatogonia and spermatocytes from broken cysts are in the same field. X 210.

18. In this photomicrograph the milky secretion distends the gland and funnel-shaped opening near the base, through which its fluid contents pour into the vas deferens. The large tracheal tube which aerates the gland and the duct, as well as the small nerve which runs to the wall of duct from the ventral nerve cord, can be seen. X 110.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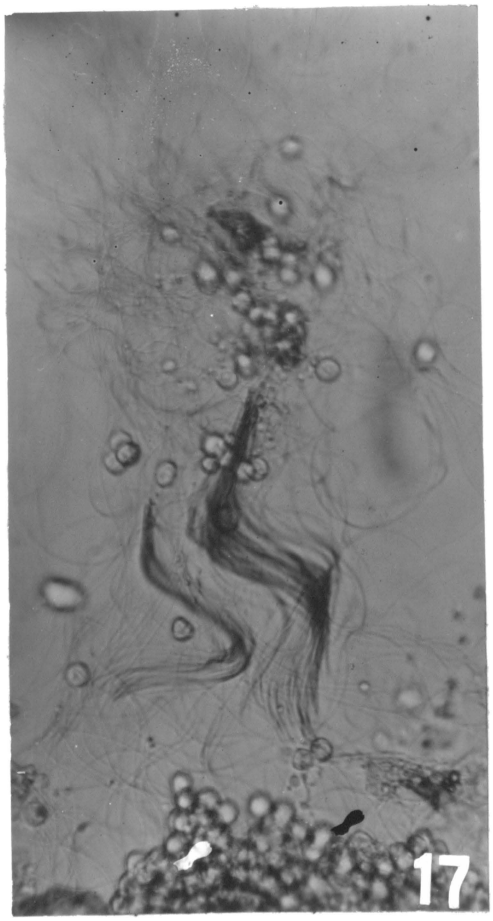
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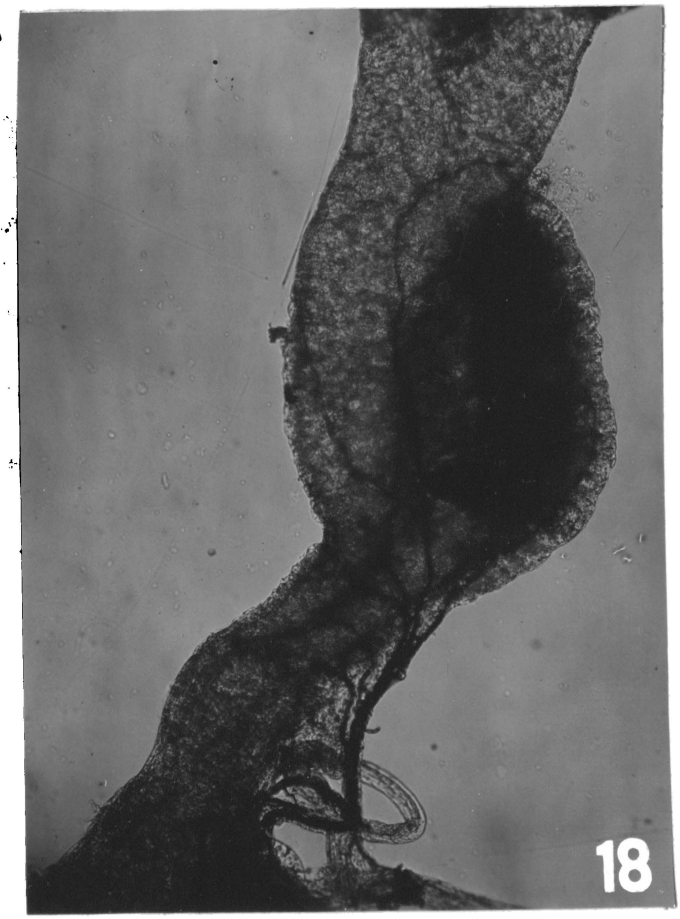
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III. ARE MITOCHONDRIA DYNAMIC CYTOPLASMIC STRUCTURES?

Introduction

Intravitam Observations

Discussion

1. Are mitochondria the causative factor in the origin of species?
2. Are normal mitochondria soluble?
3. Is there similarity in form, staining reaction and functional properties between mitochondria and bacteria?
4. What is the specific functional property of mitochondria in the axial filament?

Summary

Photomicrographs

ARE MITOCHONDRIA DYNAMIC CYTOPLASMIC STRUCTURES?

Most biologists or bacteriologists assume a skeptical attitude when asked if they do not think that mitochondria are symbiotic bacteria. The average cytologist will answer "Of course they are not. That can be easily proven."

However, a very cursory reading of Ivan E. Wallin's early papers and his later comprehensive treatise "Symbionticism and the Origin of Species" disillusions the neophyte as regards the ease with which this thesis can be shelved, for much evidence from many sources is advanced in support of the assertion that "Mitochondria are symbiotic bacteria in the cytoplasm of the cell of all higher organisms whose symbiotic existence had its inception at the dawn of phylogenetic evolution." (Wallin, '22 b. p. 466.)

The unqualified stand which the author takes justifies critical analysis of his "evidences" and deep respect for his persistent effort for there are few scientists in this 20th century who will speak with certitude as regards either the beginnings of phylogenetic evolution or the status of mitochondria.

Wallin's work on this subject has been extensive. His first paper appeared in '22 and a review and summation of this and later research was published in book form in '27. Recently Hurst and Strong ('32) at a meeting of the American

Society of Zoologists presented a paper entitled "The Culture of Mitochondria." The abstract appearing in the American Record (Vol. 54, No. 3) says:

"The writers seem to have been able to grow mitochondria in culture media ..... Each granule seemed to be enclosed in a lipoid membrane..... "

Such a report in the recent literature recalls Wallin's conclusions.

Wallin's theory is an elaboration of the hypothesis of Portier ('18) which caused a heated controversy among scientists in France. Portier, too, held that mitochondria were symbiotic bacteria. The Société de Biologie de Paris appointed a committee to examine his work. Portier's conclusions were found unsound. (C.R. Soc. Biol. LXXXIII, 654, '20.) His "Symbionts" when cultured outside of the cell in bacteriological media, withstood a temperature of 115° C.; 5% phenol for 50 hours; absolute alcohol for several hours; 20% formaldehyde, etc. These characteristics were too phenomenal even for a French Committee. They concluded that the media had been contaminated. In 1924 Wallin succeeded in cultivating "mitochondria" outside of the cells in other bacteriological media. He says:

"When grown independently in artificial culture media, they behave in all observed particulars like bacteria."

In brief, some of his experiments may be summarized as follows. Pieces of liver from foetal rabbits were cul-

tured in urea media. Deep clouded growths resulted around the liver-plants. the clouds were examined and proved to be cocci, diplocci and clumped or agglutinated cocci. The bug-a-boo of contamination had been ruled out. Hence he says,"These facts (the masses of pleomorphic cocci) apparently admit of no other interpretation than that mitochondria are living organisms, symbiotically combined with the cells of plants and animals." (Wallin '27, p. 38) The pleomorphism of the mitochondria of the several growths is readily accounted for by the author to his satisfaction. It is due to variation in the culture media! (The medium was adjusted from pH 6.4 to pH 8.36) In one of Wallin's liver cultures the medium shifted during the course of the experiment from an original pH 7.5 to pH 8.8 (idem, p. 51.)

In his book Wallin refers to and affirms statements in the literature to the effect that <u>normal</u> mitochondria are <u>soluble</u>. (pp. 12 and 102.) He says that his own observations on the mitochondria of lymphocytes confirm those of Cowdry. Just which observations of Cowdry's were confirmed is not definite but Cowdry is not <u>positive</u> in his statements as regards the activity of mitochondria. I have located one reference in which he says:

".....we have certain cells in which the mitochondria appear to go into solution." (Cowdry, '16 b. p. 41.)

He refers to the chromophile cells of the nervous system.

Cowdry favors the view that mitochondria arise "de novo" and shifts the burden of demonstrating their continuity to the geneticist, who, he holds should be vitally interested. In his discussion of the morphology of mitochondria he says:

"We should bear in mind the possibility that in all cells, individual mitochondria go into solution and gradually fade away and reappear again, even though the change escape our observation." (Cowdry, '18. p. 69.)

In the first quotation he says that they appear to go into solution. In the second he mentions the possibility.

Chambers, ('15, p. 292) in his microdissection studies on the germ cells, described mitochondria.

"At the end of cell division, each daughter cell contains a cluster of mitochondrial filaments which have already begun to be transformed into a granular network mass which gradually spreads around the nucleus. The mitochondria are not stable structures. Granules at one end may draw out into threads, or coalesce with their neighbors or go into solution, freshly formed granules replacing them."

Wallin says there is "no occasion to question Chambers' observation." However in the several hundred intravitam preparations which I have examined, I have not seen mitochondria take form or disappear, except in the conventional ways listed later. At least this is true of apparently normal mitochondria of grasshopper cells and the description from Chambers quoted above is based on studies made on similar cells.

When an investigator asserts that "up to the present time the evidence submitted for the bacterial nature of

mitochondria has neither been confirmed nor discredited." (Wallin, '28, p. 117) it seems proper that any pertinent piece of research, either confirming or discrediting the "evidence" should be presented. Hence the following observations and microphotographs of mitochondria in living grasshopper cells are submitted.

INTRAVITAM OBSERVATIONS

By means of intravitam methods, previously described, (Baumgartner and Payne, '31, p. 36) the mitochondria of the developing germ cells of several different species of grasshoppers have been carefully studied. The work is in part, similar to the investigation of Lewis and Robertson, '15, in in-vitro cultures and corroborates closely their succinct descriptions. The analyses, due to improved technic, however, have enabled me to add several observations to their findings and the photomicrographs of unstained, unfixed tissue illustrate that at least a few of the statements which have appeared in the literature need revision.

With good illumination,[1] the mitochondria in the spermatogonia appear as small granules evenly distributed

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1. A 40 watt glazed mazda lamp is placed directly under the microscope. This gives light rays which are effective in penetrating follicle, cyst and cell walls. In some instances and ordinary sub-stage lamp gives sufficient illumination.

through the cytoplasm. Later, in first spermatocytes the granules clump about the nuclear wall while the peripheral part of the cytoplasm is relatively clear and hyaline. (Microphoto 1.)

At times the granules in the spermatogonia and in young spermatocytes simulate Brownian movement. Whether this apparent movement is due to the state of gelation of the rest of the cytoplasm, or to the physiological state of the microsomes, or to some other cause, remains a problem. The fact has been observed, however, that cysts in other preparations, containing cells in the same stage of development, show no perceptible movement.

In older spermatocytes the mitochondria, still in the granular form, aggregate into a clump to one side of the slightly eccentric nucleus and opposite the large formed refractive element that is just under the nuclear wall. (Microphoto 2.)

In mitotic phases the mitochondria appear in rod-form aggregated outside of the spindle area. In metaphase they are "stave-shaped" and not very long. In anaphase, the long axis of the mitochondrial rod is parallel to the long axis of the spindle. (Microphoto 4, 5.) They elongate in late anaphase as the cell does; the elongation apparently being due to the different shape that the cell takes. The mitochondrial threads in late anaphase, up to the time of

interkinesis, are passive, but when flexion of the elongate cell commences, the mitochondrial threads seem to be at least one of the factors in flexion, for they themselves bend rapidly.

When a mitochondrial band in the stage of development shown in microphoto 6 is brought into focus from above, it appears that the ends of the band force down the clumped masses of chromosomes which they nearly touch. When the lesser angle of flexion between the halves of the mitochondrial band is about 100 degrees, the threads that form one end of the band spread out and cup under the nuclear mass; but the tips of the threads at the other end of the band seldom exhibit the same cupping action. This peculiar cupping position is held by the threads for a very short time. Then they contract, and the sheaf-formation, so common in stained preparations, follows. (Miscrophoto 7.) If transformation continues, the mitochondrial threads contract into a loose ball in about three hours.

In only about 10% of my preparations have the mitochondrial threads been active. Cellular activity in these was apparently normal and so undoubtedly worth reporting. Sufficient data to justify the compilation of a time table for nebenkern formation, however, has not been secured, but from onservations, it seems that it requires many hours or even days for the apparently homogeneous nebenkern to form. (Microphoto 8.) In preparations that were kept under

observation for ten hours, no appreciable change occurred.

Cysts are often found in which the single body has divided into halves. Microphoto 9 shows a cyst in which the halves of the nebenkern have elongated slightly. In microphoto 10 the mitochondria have slipped on down the tail. The two adjacent cysts of spermatids are in different stages of development. If the cyst containing the cells with the round nuclei is traced down, the tails are found to have the mitochondrial blebs. In the other cyst, the acrosme at one end of the nucleus and the centrosome at the opposite end are perfectly apparent in the unfixed unstained tissue although this microphoto, as well as all of the others, was taken through the follicle and the cyst walls.

DISCUSSION

A careful study of the varying morphology as well as the activity of the mitochondria in intravitam preparations during spermatid transformation suggests the following questions:

1. Are mitochondria the causative factor in the origin of species?
2. Are normal mitochondria soluble?
3. Is there similarity in form, staining reaction and functional properties between mitochondria and bacteria?
4. What is the specific functional property of mitochondria in the axial filament?

1. If the theory of Symbionticism is presented as the causative factor in the origin of species then a clear statement of the author's idea of species is very desirable for species differ from species in proportion to the limitation of the idea. Most definitions of species demand, at least, that the specific group of organisms have one or more striking characteristics in common and that they be able to interbreed easily. Now, it is conceivable that micro-invasion by a symbiont could bring about such a pronounced type of morphological and physiological modification in the invader as well as in the invaded organism, that difficulty might be encountered by a taxonomist in his attempt to classify the speciments. But whether these modifications, which generally result from the invasion of epidermal or endodermal cells, could proceed to that stage of finesse required for successful interbreeding, or could result in the loss of the capacity to mate, is problematic.

Wallin says that symbionticism is "a fundamental causative factor in the origin of species." (Wallin, '27, p. 114.) How does this fundamental causative factor operate? He discusses, in the light of microsymbiosis, the formation of the luminiferous organs of three cephalopods. His figures of diagrammatic sections through the light organs of Rondeletia minor, Sepiola intermedia and Pterygiotheuthis maculata show the organs to be comparable in structure. All have an epidermal layer over a greatly thickened lens. There is a striking resemblance between the organ that perceives light and the organ

that produces light. Wallin says that in the cephalopods the dedifferentiation of the nidamental gland, which later becomes the luminous element in the light organ is "essentially a response to bacterial influence, and the entire structure apparently is designed to utilize symbiotic bacteria." (Wallin, '27, p. 89.)

There are several different kinds of bacteria found in specific regions of the light organ. One of the symbionts, not luminiferous in the early stages of development, is the cause of the development of the luminous element in the light organs, whereas another species, a non-luminous one, causes the lens to form! (idem. p. 89.)

It is generally accepted that the light of a bacterium, or a bug or a cephalopod is due to the oxidation of a photogenic substance, luciferin or noctilucine, or some similar compound that has been elaborated by living cells. It is true that the "Bacterial Theory of Bio-luminescence" has gained ground recently, but in most of the species in which luminescence seems to be due to the bacterial symbionts, the bacteria, when cultured apart from the partner, have given forth no light. Such was the case when the symbionts of Anamalous and Photoblepharon were cultured. (Harvey, '22, p. 43.) Even before this, Pierantoni ('17, p. 30) cultured symbiotic organisms from certain light organs and found that these organisms were "collectively colored yellow and were opalescent." They did not give light. Later he worked on cephalopods.

Nevertheless Buchner in his work "Die Symbiose" ('21, p. 34) makes a sweeping generalization as regards the source of luminescence in fire-flies, fire-flames and cuttle-fish. The source of the light is luminous bacteria. Wallin adds the statement that the luminous organs themselves are essentially due to bacterial influence. Now result does not transcend the cause. There is good evidence for the conclusion that bioluminescence is the result of an energetic reaction between licuferin and O_2 in the presence of luciferase. (Duboid, '86 and Harvey, '20.) It is probable that the gland cells of the luminous organs of the cephalopods secrete an activator which not only accelerates the oxidation of the luciferin, if present, but also enables the symbionts, when in the organs of the molluscs, to glow. If this is the case, is it not as logical to say that the capacity for luminosity in the symbiont is a direct response to the presence of luciferace secreted by the gland cells of the cephalopod itself? There are so many possible causes to which response and organ formation may have been due that one is not inclined to accept symbiotic relations or luminous bacteria as probable causes.

Even if the light organs were the result of symbiosis, would minimal requisites to justify the establishment of another species be present?

2. That type of rod or granule which can be found time and again in intravitam set-ups where the environmental factors are constant is considered normal if the pH of the

medium in which the tissue is immersed approaches closely the pH of the blood of the insect under observation.

Recent research has shown that the limits of variability in the pH of the normal human blood stream are very narrow. The range is from pH 7.35 to pH 7.38. (Woodard, etc. '31, p. 705.)

The wide range of hydrogen ion concentration in Wallin's liver cultures has been noted previously. In one experiment the medium shifted from an original pH 7.5 to pH 8.8. Morphological changes as well as physiological reactions might be expected in delicate symbionts or in dependent protoplasmic constituents by such changes in hydrogen ion concentration. Mitochondria would be notably affected for it is common knowledge that anisotonic solutions affect living protoplasm. The literature has numerous accounts of swellings, abnormal vacuolizations, coagulations and solutions that have occurred as a result of anisotonicity of culture media. (Chambers, '14 and '24; Belar, '29; Lewis, '23, etc.)

That mitochondria cannot be "stable structures" in an abnormal environment is readily proven by the use of a drop of acetic acid. In intravitam preparations the effect of a slight hypo - or hypertonic medium is easily seen. In a slightly hypotonic medium the mitochondria swell and become very stable, insoluble. It seems, however, that slight hypertonicity of the medium has a fixing effect on delicate

cells and the cellular inclusions. A slight gelation of the protoplasm prevents further cellular activity, but there is no evident distortion of the cellular contents. A higher degree of hypertonicity brings about the "pseudopod" activity which has been so often described in cells outside of the follicle wall. (Lewis and Robertson,'15; Strangeways,'27; etc.) When the cells are subjected to normal pressures within the follicle, however, pseudopods never form. (Payne,'33.) The entire cell and all of its component parts remain very stable.

Several references or descriptions in the paper of Chambers to which Wallin refers raise a query in regard to the isotonicity of the medium in which these microdissections were made. Chambers mentions two spermatozoa that "corkscrewed their way between the meshes of the mitochondrial spindle. Whenever their tails touched the viscous material of the meshes,violent lashings were necessary to set themselves free." (Chambers, '15, p. 291.)

The mature sperms of the average grasshopper are tightly aggregated into bundles of 250 or more. It is these mature sperm bundles that are periodically motile, not as individuals, but as entire bundles. (Payne, '33, p. 331.)

In the same paper the tips of mitochondrial threads are described as "lumpy" after construction of the common wall of the forming daughter cells. Then in the summary it is said that mitochondria cannot be classified as persistent structures; they pass from a granular stage into strands; they reappear and must be merely changes in

physical states of the colloids which compose the cytoplasm.

Is it not doubtful whether these recorded changes would have appeared if a properly balanced isotonic medium had been used? Chambers used normal saline which at that time was considered a satisfactory medium for in-vitro studies, but at present is not used for careful cytological studies of living cells of cold-blooded animals.

Lewis and Robertson's ('15, p. 105.) account of their disappearing granules is conceivable in the light of the fact that they used Janus green B., for it is well known that this dye quickly undergoes chemical change in the cell with the liberation of potentially toxic compounds. These toxins would cause changes, even hydrolysis.

"After preparations (grasshopper sex cells) had been stained for a short time the granules coalesce into large globules and finally they disappear in the cytoplasm. Long thread-like mitochondria rapidly break into granules when stained with Janus green."

The conventional ways in which mitochondria are said to disappear are the following:

a. Solution, through the use of solvents, acetic acid, alcohols, etc.

b. Hydrolysis, if the surrounding fluids are anisotonic.

Cowdry ('18, p. 139.) says in his discussion of mitochondria in acidosis:

".....mitochondria respond to a wide range of noxious influences by swelling up before going into solution, which might well be due to the effect of increased H-ion concentration upon their protein fraction, causing it to become hygroscopic and to swell."

Microsomes and mitochondrial granules at times manifest a Brownian-like movement. If the movement sets up delicate currents in the sol-cytoplasm, it is possible that those microgranules, which are in the microscopic field, could be carried out of the focal plane and disappear. The disappearance is not due to solution but apparent solution through location for in intravitam preparations such disappearing mitochondria can be made to reappear with a partial turn of the micrometer screw.

It is possible that mitochondria may be dissolved and their substance be used again in cell economy, if they become too abundant. However, it seems that such a process would take place slowly as do most normal physiological functions. A sudden disappearance of mitochondria that can be followed under the microscope is not conceivable unless the disappearance occurs through the action of unsuitable culture media or protoplasmic solvents.

Mitochondria may appear by

a. Meristic division during mitotic activity or in preparation for mitosis.

b. Through fusion or agglutination.

Meristic division of mitochondrial elements to daughter cells in cell division has been described in so many forms that it seems universally applicable. Even in a mitochondrial syncitium, the characteristic nebenkern, a division into two equal parts eventually occurs.

There is no reason why ultramicroscopic units of mitochondria should not increase in size or number, through fusion or growth or agglutination as other cytoplasmic constituents do, but it is very probable that an observer could not <u>see</u> this process, because typical organic growth of a cell component or body is ordinarily not perceptible to the eye.

Hence any proof for the theory that "mitochondria are symbiotic bacteria" which rests on their appearance or on the "solution phenomenon" (Wallin, '27, p. 102.) seems inadequate.

3. In the preceding discussion the miscibility and fragility of normal mitochondria in <u>suitable</u> environment have been discussed. The same conditions, normality and suitable environmental medium are postulated for the following discussion of some of the points which appear in the General Résumé of Wallin's article, "On the Nature of Mitochondria." ('22 b. IV, p. 465) There he cites further facts and biological principles in support of the bacterial nature of mitochondria among which are the following:

Similarity in form
Similarity in staining reaction
Similarity in functional properties.

One of Bowen's conclusions based on an exhaustive study of mitochondria in insect sex cells (stained tissue) is that all mitochondria are composed of two substances, a chromophobic substance, covered by an outer or chromophilic envelope. The mitochondrial granules of the young spermatocyte fuse to form the characteristic rods of the mature spermatocyte. Later in the transformation stages of the spermatid there is a complete fusion of the chromophobic and the chromophilic substances of the mitochondrial rods into the nebenkern. Analysis with stain technic shows that the chromophobic centers of the mitochondria have fused to form the central mass of the nebenkern while the balance of each mitochondrium, the chromophilic part, together with similar remnants of other mitochondria form the outer envelope. So constant and regular is the syncitium of the parts that Bowen described two fundamental types of nebenkern, the "spireme" type and the "plate-work" type.

No single species of bacterium known assumes during its life cycle such varied complex forms nor does there occur in any group of bacteria a similar disintergration and fusion of parts into a functional whole. Of course there is the "analogous" amorphous mass which Löhnis ('21, p. 195) has called a symplasm of bacteria, but here, after a longer or shorter period of union, the individual bacterium leaves the mass and pursues its way as a specific individual. Only a biological clumping or agglutination has occurred. The case is different with the army of individual mitochondria. With these there is a fusion or clumping of parts. The individual

mitochondria loses all semblance of specificity and individuality yet the heterogeneous nebenkern resulting continues its morphological differentiation. The mass divides into halves, the halves stretch out and sheathe the axial filament and eventually the entire mass is discarded by the living sperm unit. Where is the analogy?

In the case of Bacillus radicicola the authors' illustrations show that the limits of morphological variation are very narrow. He illustrates mature, senile and juvenile forms. Some of the mature ones in his figure 1. are identical with drawings in figure 2. (Wallin, '22 b. IV. p, 465.)

The drawings of forms from the stem of white clover, where the bacteria, judging by locus, should be mature, are comparable to the illustrations of bacteria from a young unfolded leaf, where they are probably juvenile. These limited variations cannot easily be homologized to the marked variations seen in any intravitam preparation of the metamorphosing germ cell.

4. Finally, I would mention a possible specific functional property of mitochondria in germ cells related to their sheathing of the axial filament. When interkinesis occurs the mitochondrial rods have been seen to writhe and turn, particularly at the extremities of the mitochondrial band, where the cupping of the nucleus occurs. The mature sperm tails, when completely sheathed in mitochondria, at times become extremely active, whipping and writhing, and forcing the sperm heads along to their destination. The

degree, as well as the constancy of the whipping movement, varies in different species; but the whipping tail is characteristic of most sperms. Now this mitochondrial substance which sheathes the axial filament in the sperm tail may be the contractile element that enables the sperm to make its way to the mature egg. A hygroscopic variation in its chemical composition or an activating substance from an accessory gland may be the condition necessary to bring on the whipping process. Sperms in which the sheath of mitochondria is not completed cannot be activated. At times it is hard to activate even apparently mature sperms. (Payne, '33, p. 332)

Hegner said in 1914 that "Mitochondria are cellular constituents which have only comparatively recently come into prominence, and ideas concerning their nature and functions are still in a very chaotic condition." (Hegner '14, p. 146) This statement remains partially true today. New theories, argumentations, laboratory data are at hand but chaos remains. If they will not fit into the kingdom of the bacteria they must still be classified as cellular constituents. It is hoped that a more extensive use of living tissue will eventually help to solve some of the mitochondrial problems satisfactorily.

SUMMARY

An examination is made of the "evidence" presented by Wallin in favor of the thesis "Mitochondria are symbiotic bacteria in the cytoplasm of the cell of all higher organisms whose symbiotic existence had its inception at the dawn of phylogenetic evolution."

Wallin's theory is an elaboration of Portier's hypothesis. A French Committee examined Portier's cultures and concluded that they had become contaminated. Wallin raises pleomorphic cocci from liver plants in culture media that ranges in pH from 6.4 to 8.36. He too says that the growths which appear and cloud the medium are masses of mitochondria.

Mitochondria are said to be soluble. In studying several hundred preparations of living germ cells I have not seen normal mitochondria go into solution. An account of these intravitamobservations and a series of microphotographs of mitochondria from intravitam preparations are presented.

In young germ cells the mitochondria are granular and quiescent. In older cells they simulate a Brownian-like movement. In telokinesis they have become a compact band of rods. The tips of the mitochondrial rods that form one end of the band cup under the nuclear mass. After flexion, the band of rods divides near the middle and two loose balls of mitochondrial threads form in both daughter cells. Later the characteristic nebenkern appears. It eventually elongates

and slips on down, sheathing the axial filament.

Intravitam observations raise several questions. Can mitochondria be functional causative factors in the formation of species? Are normal mitochondria soluble? Are they similar to bacteria and finally, what is their function?

A precise definition of the author's idea of species is deemed necessary before he can say that symbionticism is "a fundamental causative factor in the origin of species." Light organs are not necessarily a response to bacterial influence nor are bacteria the only causative agents which bring about photogenesis in different species. Even if the light organs of certain cephalopods, etc. were the result of the presence of mitochondria, the criterion of sterility and fertility has not been taken into consideration.

The confirmed methods by which mitochondria disappear are through the use of solvents and in hydrolysis. They appear or multiply in meristic division and by fusion. Hence any proof for the theory that mitochondria are "symbiotic bacteria" which rests on their appearances or on the "solution phenomenon" seems inadequate.

Similarity in form, staining reaction and functional properties between mitochondria of germ cells and bacteria, as demonstrated by intravitam studies and stain technic is to say the least, slight. There is a syncitium of parts in the mitochondrial nebenkern, a phenomenon that does not occur in the bacterial symplasm.

It is suggested that the mitochondrial substance which sheathes the axial filament in the sperm tail may be the contractile element that enables the sperm to make its way to the mature egg. Insect spermatids in which the sheath is not complete are inactive while the whipping tail, an axial filament sheathed with mitochondria, is characteristic of most mature insect sperms.

It is hoped that a wider use of intravitam technic will help solve some of the mitochondrial problems that still perplex the cytologist today.

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PLATE I.

EXPLANATION OF PHOTOMICROGRAPHS

(All photographs are of unstained unfixed tissue. Magnifications, 420 and 980.)

1. Spermatogonia showing granular mitochondria clumped about the nuclear wall. Formed x-element just under the nuclear wall.

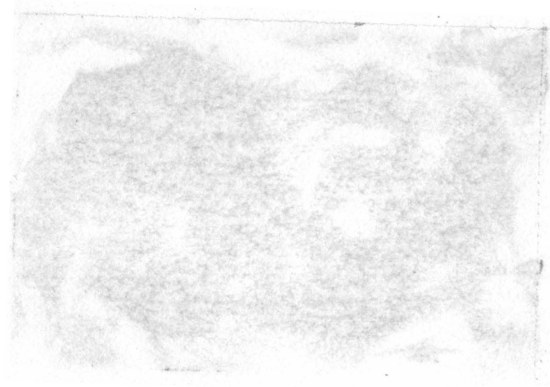
2. Young spermatocytes showing granular mitochondria clumped on one side of the nuclear wall, opposite the large formed x-element.

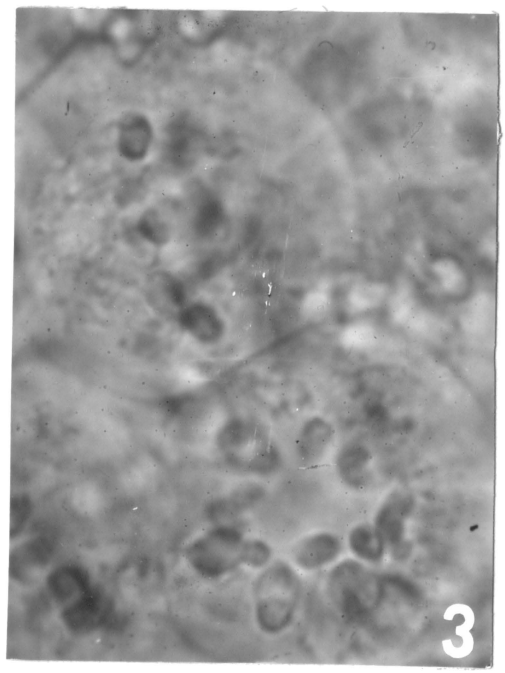
3. Polar and lateral views of spermatocytes in metaphase stage of cell division. The mitochondria are distributed outside of the ring of tetrads.

4. A focal section through cells in late anaphase, the mitochondrial threads aggregated into a heavy band, have lengthened with the elongating cell.

5. Earlier anaphase. The mitochondrial rods are stave-shaped.


1

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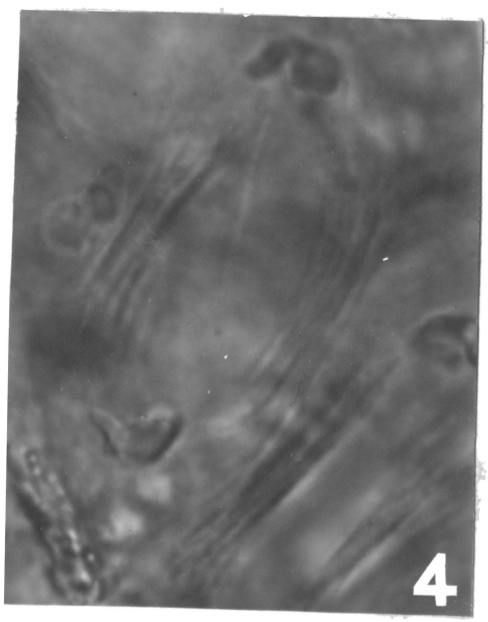
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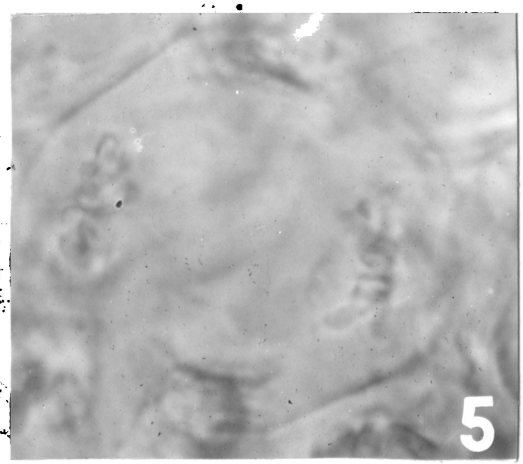
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PLATE 2

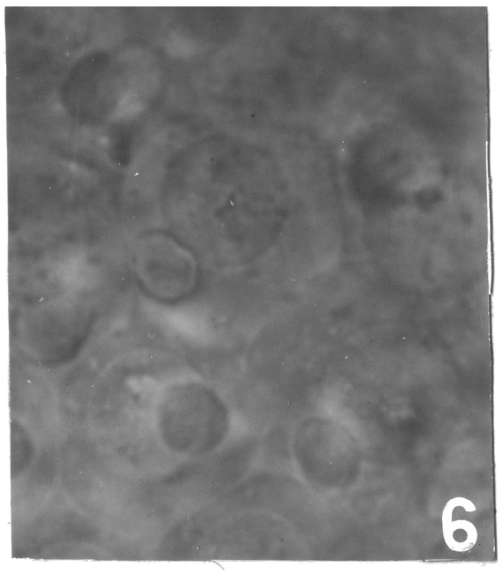
6. Interkinesis. Polar view of mitochondrial band.

7. Sheaf-shaped mitochondria. Initial step in the formation of the nebenkern.

8. Young spermatids. Nebenkern, a single body. The nebenkern in one cell is commencing to divide.

9. Cyst of maturing spermatids. The two halves of the nebenkern are elongating.

10. Two adjacent cysts of older spermatids. In one the nuclear masses are still rounded. The mitochondria sheathes the axial filaments, many blebs being present on each tail. In the adjacent cyst the nuclear masses have elongated. Acrosomes and centrosomes appear without stain or fixation.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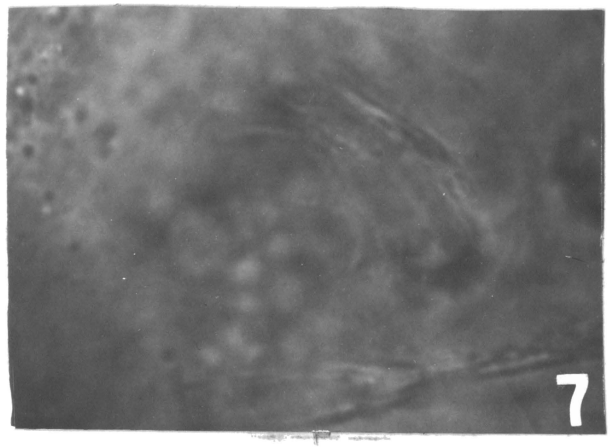
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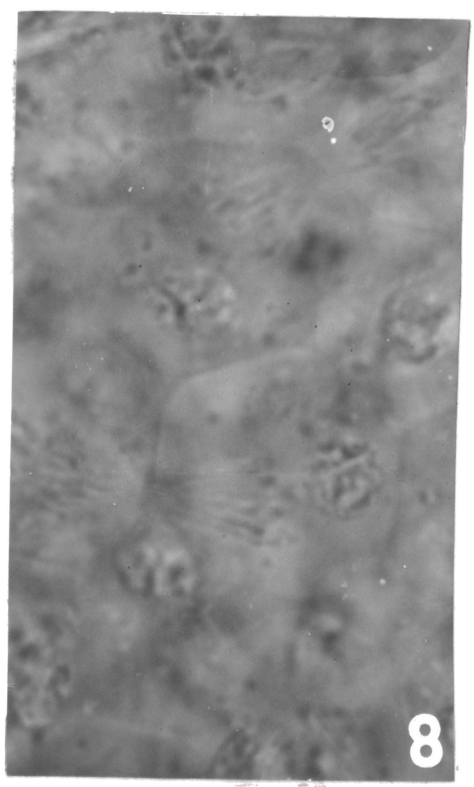
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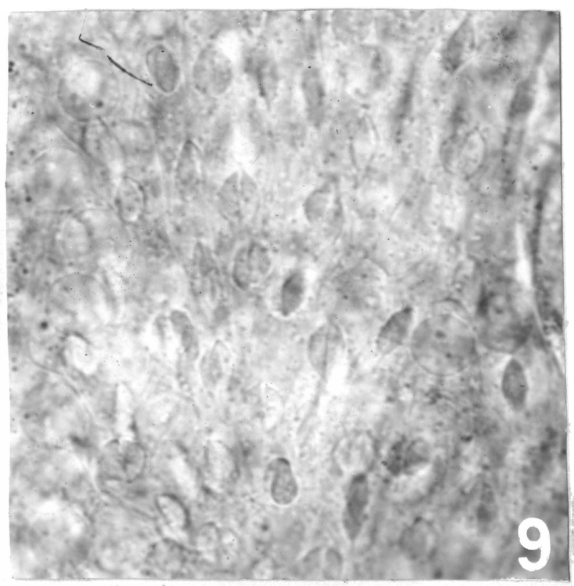
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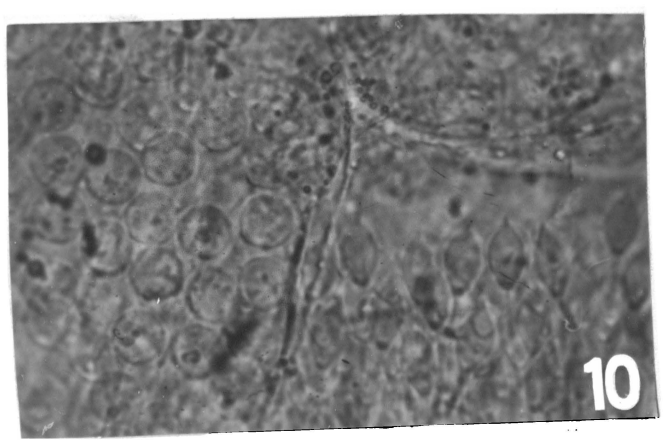
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