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MOTILITY AND REVERSIBLE INACTIVATION OF HONEYBEE SPERMATOZOA *IN VIVO* AND *IN VITRO*

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SOMMAIRE

Les spermatozoïdes de la spermathèque ne peuvent se mouvoir. On les active en ajoutant la sécrétion d'une glande à mucus, des solutions tampons ayant un pH compris entre 3,5 et 9,0 ou de l'eau distillée. Les spermatozoïdes obtenus par éjaculation ou pris dans les vésicules séminales sont motiles. Le schéma de la motilité et la forme des spermatozoïdes dépendent de la densité et de la tonicité du diluant et non de leur provenance. Le pH du liquide de la spermathèque et des glandes à mucus était respectivement de 9 et 7. Les spermatozoïdes de l'abeille résistent à une ample gamme de températures (2° à 45°C).

INTRODUCTION

The fact that the fertilizing capacity of the spermatozoa of drones is preserved in the queen bee's spermatheca for years stimulates the question whether the spermatozoa are stored in a motile or immotile state. In the first case, a sufficient supply of energy-yielding substances and adequate conditions would have to be provided for high metabolic activity. whereas in the second case a depressed, energy-saving metabolism could be assumed. The state of sperm motility in the spermatheca is, therefore, indicative of factors relating to their longterm survival, such as their metabolism.

FLANDERS (1939), who discussed various aspects of sperm transport and motility in the queen bee's reproductive tract, postulated that the spermatozoa are preserved in the immotile state in the spermathecal fluid. In contrast to this assumption, much earlier, LEYDIG (1867) and BRESSLAU (1905) observed that spermatozoa are

in vigorous motion in the queen bee's spermatheca. Also DAVEY (1965) reported that spermatozoa are motile while stored in the spermatheca of insects. These observations were offered without any detailed description of the techniques employed. Since the data on the state of sperm motility in the queen bee's spermatheca appear to be inconclusive, the present study was undertaken to elucidate the problem.

Different patterns of motility as well as various forms of drone spermatozoa have been described (BISHOP, 1920 ; SMIRNOV, 1953 ; KURENNOI, 1954 ; ROTHSCHILD, 1955 ; DADE, 1962). Since temperature, dilution, pH, ionic composition and tonicity of the diluent are known to be the most important extraneous factors influencing sperm motility *in vitro* (MANN, 1964), it was proposed to study the effects of these factors on the sperm motility of drones. Such studies should permit the determination of optimal conditions for observation of spermatozoa derived from both male and female organs, and allow comparison and elucidation under what circumstances the forms described hither to appear.

MATERIALS AND METHODS

Spermathecae were obtained from one-year-old fertile *Italian* queens (*Apis mellifica* L. var. *ligustica* SPIN.). They were detached from the sperm-duct after removing the last three abdominal segments with watchmaker forceps.

Mature *Italian* drones served as semen donors. The semen was obtained either by exerting a gentle pressure on the drone's abdomen (BISHOP, 1920 ; MACKENSEN and ROBERTS, 1948) thus causing an ejaculation, or from the vesiculae seminales dissected from the drone's reproductive tract. The latter procedure was chosen when it was desired to eliminate admixture of the contents of the mucus glands to the semen, as occurs during ejaculation.

To dilute the semen, usually, five pairs of vesiculae were immersed in 0.5 ml of diluent and the contents dispersed by gentle stirring. Attempts to entirely remove the tissue from the spermatozoa by centrifugation were unsuccessful, and resulted in a considerable decrease in sperm motility. Therefore, the sperm suspension was not always homogenous, and clusters of spermatozoa attached to tissues of the vesiculae seminales were present. These masses of spermatozoa showed more vigorous motion than individually dispersed spermatozoa.

The following solutions were use for dilution of the semen :

a) Modified Ringer solution, buffered with 0.15 M phosphate buffer at pH 7.2.

b) Phosphate buffer isotonic to larval haemolymph, the freezing point depression of which is 0.81°C according to BISHOP (1923). The buffer was composed from two stock aqueous solutions of $Na_2HPO_4.12H_2O$ (65.5 g/l) and KH_4PO_2 (35.0 g/l), mixed in the requisite proportions to give the desired pH.

c) Veronal, glycine-NaOH, citrate and bicarbonate buffers (GOMORI, 1955) ; these were made isotonic to haemolymph by the addition of NaCl solution.

For observation of motility, the sperm suspension was sealed between slide and cover glass with liquid paraffin or was kept in a glass observation cell (SCHINDLER and NEVO, 1962). Both methods prevented the drying of the suspension and permitted prolonged observation of a single sample.

In order to determine the effect of temperature on sperm motility during the exposure period, high temperatures were maintained by an electrical heating stage ; lower ones, by means of pumping cold water through a microscopical stage. The temperature on the microscope slide was measured by means of a thermocouple. In other experiments, observation cells were submerged in a thermostatically controlled water bath or kept in a refrigerator, and motility observations were carried out subsequently at room temperature (20-25°C).

Motility was rated in six grades from 0 to 5 according to the percentage of motile cells, grade 5 indicating 80 to 100 per 100 motility and grade zero — no motility.

RESULTS

A. — *Motility of spermatozoa obtained from spermathecae*

In order to assess the state of motility of the spermatozoa in the spermathecal fluid and the rôle of the spermathecal glands on the initiation of motility, the following series of experiments were carried out.

1. *Motility of spermatozoa in crushed spermathecae.*

Spermathecae were crushed with or without the spermathecal glands between slide and cover glass. In the latter case, the glands were removed from the spermathecae with fine watchmaker forceps under a stereoscopic microscope.

In the case of spermathecae crushed with the spermathecal glands, a slow sperm motion was observed immediately, either at the margins of the preparation, spreading in toward the center of motionless spermatozoa, which were densely packed in bundles, and or at certain foci inside the preparation spreading out in wave formation (pl. 1, fig. 1 and 2). After a few minutes, the entire contents of the spermatheca were found to be in rapid motion (pl. 1, fig. 3).

In contrast, in spermathecae crushed after the removal of the spermathecal glands, dense masses of spermatozoa were observed in motionless bundles. In general, they remained immotile for periods of up to 11 minutes, at which point the diluent was added. However, in a few cases, a slight motion of the spermatozoa was observed, probably due to the fact that small fragments of the spermathecal glands were still attached to the spermatheca.

2. *Motility of spermatozoa in the fluid obtained from the spermatheca.*

Part of the spermathecal fluid was extracted by using a fine, hair-tapered glass capillary in order to avoid contamination of the spermathecal fluid with traces of secretion from the spermathecal glands. The capillary was inserted through the spermathecal wall and the fluid collected was transferred to microscope slides for immediate observation. In all samples obtained by this technique, the dense masses of spermatozoa in the fluid were found to be immotile. When the same spermathecae with the spermathecal glands still attached were crushed, the spermatozoa which had remained in the spermatheca and now came into contact with the secretion from the spermathecal glands became motile. The activating effect of the spermathecal glands on spermatozoa was demonstrated in further experiments. Fluid with spermatozoa was extracted from spermathecae with a glass capillary, and the immotility of the spermatozoa was confirmed ; on addition, however, of drops of spermathecal fluid to crushed spermathecal glands which had been dissected from the same spermathecae, the spermatozoa became motile (pl. 1, fig. 4).

It would seem evident that the spermatozoa are stored in the spermatheca in an immotile state and are activated when they come into contact with the secretion from the spermathecal glands.

3. *Activating effect of various diluents.*

In order to determine whether the activating effect of the spermathecal gland secretion is specifically a function of its composition, the effect of buffer solutions of various compositions and pH values on the motility of spermathecal spermatozoa was investigated.

Phosphate buffer (pH 7.1) was added to motionless spermatozoa obtained from crushed spermathecae and deposited on microscopic slides. Immediate vigorous motion of the dense masses of spermatozoa was observed. The same effect was found when the phosphate buffer was added to spermathecal fluid obtained by puncture of the spermatheca.

In order to test whether the activating effect was due to a specific action of the phosphate ion, buffers of different composition were tested. Veronal (pH 8.6), glycine-NaOH (pH 9.0), citrate (pH 4.3) and bicarbonate (pH 8.7) immediately activated the spermatozoa suspensed in the spermathecal fluid.

The experiments with buffers of pH varying from 4.3 to 9.0 demonstrated that the activating effect is independent of pH within this range. This observation was confirmed by tests with phosphate buffer at pH's of 4.4, 8.0 and 9.1. Using the same buffer system, the activating effect was immediate at all pH values tested.

Since activation of the spermatozoa appeared to be independent both of pH and ion composition of the buffer solution, it was suspected that it might be merely a function of dilution *per se*. Therefore, immotile spermatozoa withdrawn from spermathecae by means of a capillary were diluted with distilled water. A sudden outburst of sperm motility followed ; however, the activity ceased after a few minutes and no reactivation of the spermatozoa was obtained on addition of isotonic phosphate buffer.

It appears from these results that the activation of the immotile spermatozoa can be achieved by mere dilution, regardless of the pH or ionic composition of the diluent, and even by distilled water.

B. — *Patterns of motility and shape of spermatozoa in undiluted and diluted specimens*

Microscopic examinations of undiluted ejaculated semen revealed densely packed spermatozoa, embedded in mucus gland secretion and exhibiting vigorous motion. Dense masses of vigorously moving spermatozoa were also observed in seminal vesicles which had been crushed on microscope slides. The mass movement in these samples was the same as that observed in slightly diluted spermathecal fluid (pl. 2, fig. 1-3). The spermatozoa were arranged parallel to each other in independently moving bundles, thus creating a swirling motion. Each bundle exhibited a wave-like motion ressembling an S-form which apparently resulted, according to ROTHSCHILD (1955), from synchronous movements of the tails.

In diluted samples of semen obtained either from the drone or from the spermatheca, individual, circular-shaped spermatozoa were found outside the masses, in less crowded areas (pl. 3, fig. 1), exhibiting an undulatory movement of the tail perpendicular to their own axis. These undulatory movements acting on the circular-shaped cells often induced a rotation motion of the whole cell. Two or three undulating cells lying on each other presented the image of a rotating spiral.

In addition to individual cells appearing as rotating circles, snake-like forms were observed (pl. 2, fig. 2), apparently the same as those described by SMIRNOV (1953). Their motility, however, was usually less than that of the circular forms from which they had evolved. They represented cells which had somewhat lost vitality. This observation was supported by the fact that spermatozoa which detached themselves from the dense masses were always of circular form, while the snake-like forms appeared in areas more distant from the main sperm concentrations.

It was noticed that in all the motility patterns observed, the drone spermatozoa showed an undulation of the tail which resulted in stationary but not locomotory motion, except when the spermatozoa were diluted, in which case they tended to move from the densely packed and probably more viscous areas to those containing the diluent. It was impossible to determine whether the movement was active or passive.

When the spermatozoa were suspended in an isotonic solution the head and tail were of almost identical with and could hardly be distinguished from each other, as pointed out by SMIRNOV (1953). However, when observed with phase contrast, the head is darker than the tail. A spike-like processus (the acrosome, according to ROTHSCHILD, 1955), was visible at the anterior end of the sperm head (pl. 3, fig. 3). The end of the tail is tapered and can easily be distinguished from the anterior part of the sperm cell. When photographed after drying of the preparation, the head appeared to be slightly swollen and wider than the tail, exhibiting a rectangular capsule-like image (pl. 3, fig. 4). It resembled the form which had been drawn by DADE (1962) from dried, stained preparations.

When spermathecal fluid, obtained by means of a capillary, was suspended in distilled water, the activated spermatozoa showed the following sequence of changes in their appearance within a few minutes. As soon as the individual spermatozoa detached themselves from the undulating bundles, loops were formed at the extreme end of the tail (pl. 4, fig. 1), as observed by KURENNOI (1954) in a hypotonic solution. The whole cell then assumed a coiled form, while continuing to rotate (pl. 4, fig. 2). In the next phase, the cells uncoiled, stretched out and became motionless. The occurrence of death of the cell at the end of the sequence was established, since no reactivation took place on addition of isotonic buffer solution.

In spermathecal fluid obtained from queen bees which died three hours prior to the dissection of the spermathecae, the spermatozoa were found to be motionless and could not be activated by the addition of buffer. The spermatozoa were stretched out and loops were present at the extreme end of the tails (pl. 4, fig. 3).

C. — *Hydrogen ion concentration in fluids from the reproductive organs of the queen bee and drone*

Since the activating effect of the accessory glands on the spermatozoa has been attributed to its neutralizing action on the acid spermathecal fluid (FLANDERS, 1939, 1950), pH measurements of the fluids from both organs were carried out. Because of the extremely small amounts of fluid available, indicator paper (pH range : 8.2-10.0 [1]) and pH range, 8.4-10.0) [2]) was used as the test system, the reliability of

[1]) E. Merck, A. G., Darmstadt, Germany.
[2]) Johnsons of Hendon Ltd., Hendon, London, England.

which was checked with standard buffers. Five samples of spermathecal fluid obtained with a capillary had pH values of about 9. The same value was obtained for six spermathecae crushed separately without the spermathecal glands on the indicator paper. Two pairs of accessory glands crushed on the indicator paper had a pH of about 7.

Not only extreme alkaline but also extreme acid pH values were found in the queen bee. The pH of the queen bee venom was found by us to be about 4.5. This value is similar to that established for bee worker venom (pH 5.2) by FISCHER and NEUMANN (1953).

The pH of the ejaculate, also determined using indicator paper, was found to be 7.2. This value is similar to that reported by SMIRNOV (1953).

The pH of freshly dissected seminal vesicles and mucus glands was determined using a Cambridge Bench Type pH Meter, a soft tissue glass electrode and a KCl agar bridge being employed in addition to the calomel reference electrode. For comparison, the pH of drone haemolymph, collected by inserting a sharply pointed glass capillary into the dorsal vessel, between the 3rd and 4th abdominal segments (LENSKY, 1964 *a*), was also measured. Average pH values were : mucus glands — 6.8 ; seminal vesicles — 6.6 ; haemolymph — 6.9.

D. — *Effect of temperature upon the motility and survival of spermatozoa*

Spermatozoa were obtained from seminal vesicles and suspended in phosphate-buffered Ringer's solution.

Sperm suspensions held between microscope slides and cover glasses sealed with liquid paraffin were observed during exposures to temperatures of 19°, 29° end 36°C. It was found that at 19° and at 29°C a slight decrease in sperm motility occurred at the end of the second hour of exposure, whereas at 36°C a considerable decrease in motility was evident at the end of the first hour of exposure.

In order to further elucidate the effects of elevated temperatures on sperm motility the following experiments were carried out. Sperm suspensions kept on a heating stage at 37°C were observed continuously under the microscope. When after about 1 hour motility considerably decreased, the suspension was transferred to a cold stage at 19°C, where upon motility again became vigorous. Repeated transfers from hot to cold gave the same reversible changes each time.

Sperm suspensions in observation cells were exposed to temperature ranging from — 10° to 50°C for periods of up to one hour and their motility was observed at room temperature (25°C) immediately after their exposure to the experimental temperatures. Figure 1 shows that temperatures of — 10°, 2° and 50°C completely immobilized the spermatozoa after an exposure of 15 to 30 min. At 16°C a decrease in sperm motility was evident, while sperm motility remained almost unchanged after exposure of 1 hour to temperatures of 24°, 30°, 37°, 40° and 45°C. The depressing effect of high temperatures on motility was not evident in these experiments, because the spermatozoa were examined at room temperature rather than during the high temperature exposure, and had evidently become re-activated.

In order to determine whether there were any permanent after-effects resulting

from exposure to the different temperatures, the spermatozoa were continuously observed at room temperature for 1 hour. Spermatozoa which had been exposed to — 10°C during 5, 15 and 30 minutes took about 30 minutes to recover maximum motility at room temperature. Exposure to — 10°C for 1 hour immobilized the spermatozoa irreversibly. In contrast to sperm suspensions, spermatozoa kept inside the spermathecae of 2 months-old fertilized queens at — 10°C for 2 days were found to be motile when trought to room temperature.

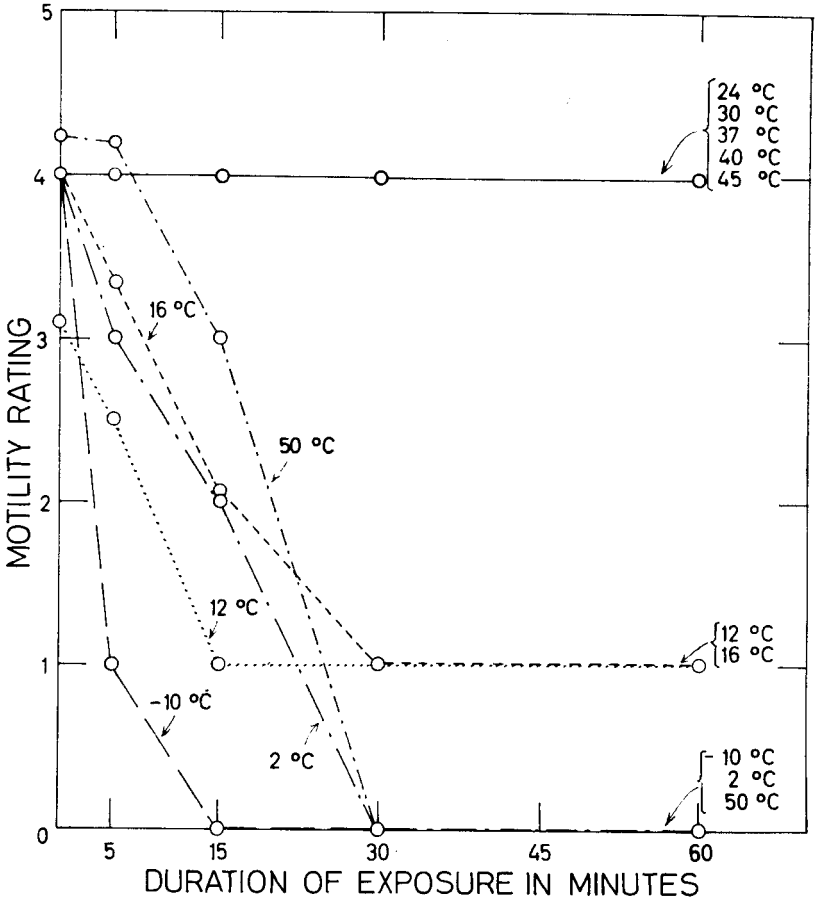

FIG. 1 — *Effects of different exposures to various temperatures upon the motility of spermatozoa examined in observation cells immediately after the experimental treatment*

(grade 5 indicating 80 to 100 % motility and grade zero — no motility)

Spermatozoa which had been kept at 2°C even for prolonged periods regained maximum motility after about 15 minutes at room temperature.

Samples exposed to 7°, 13° and 37°C for 15, 30, 45 and 60 minutes and subsequently observed for 6 hours at room temperature (25°C) were found to be unaffected by these prior exposures as regards their duration of motility.

Exposure to 50°C did not affect subsequent motility at room temperature, as long as the exposure time did not exceed 15 minutes. Longer exposure times resulted in irreversible immobilization of the spermatozoa.

DISCUSSION

Contradictory opinions have been expressed regarding the state of sperm motility in the spermatheca. LEYDIG (1867) [1] stated that the spermatozoa are motile inside the spermatheca, and this observation was confirmed by BRESSLAU (1905). No description of the techniques are given by these workers. If we assume, as may be concluded from page 15 of LEYDIG'S paper, that be examined one spermatheca only, from a queen bee preserved during one year in alcohol, his statement on the motility of the spermatozoa inside the spermatheca is apparently an interpretation of findings from fixed material. Histological sections of spermatheca indeed show an arrangement of the sperm bundles as if their wavelike motion had suddenly been interrupted by the fixation process (pl. 4, fig. 4). Though LEYDIG did not mention that he observed live spermatozoa, there exists also the possibility that he obtained spermatozoa from crushed spermatheca. In this case the spermatozoa were activated either by the secretion of the spermathecal glands or by the fluid which served for the suspension of the spermatozoa.

FLANDERS (1939) has opined that the spermatozoa are immotile in the spermatheca. His assumption was based on the postulation of LILLIE (1919), that the immotility of sea urchin spermatozoa in dense aggregates is due to their own respiratory CO_2. Therefore FLANDERS, assuming the existence of a CO_2 gradient from the queen bee oviduct through the sperm duct to the spermatheca, caused by sperm respiration, concluded that the sperm would be immotile. This assumption has been widely cited ever since (SNODGRASS, 1956 ; RUTTNER, 1956, 1960 ; GOETZE, 1964). However, LILLIE'S (1919) and subsequently FLANDERS' (1939) assumptions on the causes of sperm quiescence are negated by the findings of ROTHSCHILD (1948), who showed that the lack of sperm movement in sperm aggregations in sea urchins is due to oxygen deficiency. The present experiments indicate that neither the accumulation of CO_2 nor oxygenen deficiency are likely to be the cause of sperm immobility in the spermatheca, since aeration of the spermathecal fluid did not activate the spermatozoa. Further evidence that the sperm quiescence is not due to an acid environment lies in the fact that the pH of the spermathecal fluid (without admixture of spermathecal gland secretion) is highly alkaline (pH 9). Our experimental evidence while confirming FLANDERS' assumption that the spermatozoa are immotile in the spermatheca, does not support his « CO_2 theory » of sperm quiescence.

The activation of quiescent spermatozoa leaving the spermatheca for fertilization has been discussed by FLANDERS (1939). While BRESSLAU (1905) believed that the secretion of the spermathecal glands supplies nutrients to the spermatozoa in the sper-

[1] p. 76 — « Allein es darf nicht unerwähnt gelassen werden, dass doch die wirbelnden Bewegungen der Zoospermien innerhalb der Samentasche keineswegs den Anblick gewähren, als ob die Fäden dem Lumen des Ausführungsganges zustrebten und nur durch die Contractionen des Samenganges abgehalten würden, in dessen Lichtung vor- und durchzudringen. »

matheca, and ADAM (1913) assumed that this secretion serves as a medium for the spermatozoa passing from the spermatheca to the site of fertilization, FLANDERS was apparently the first to postulate that this secretion serves to activate the spermathecal spermatozoa. Since he assumed that the quiescence of the spermatozoa in the spermathecal fluid is due to an acid environment, he precluded the possibility that the secretion of the spermathecal gland is other than alkaline. However, our measurements show that the pH of the spermathecal gland secretion is approximately neutral.

In the present work it has been shown that the activation of the immotile spermathecal spermatozoa can be achieved by the secretion of the spermathecal glands, as well as by addition of buffer solutions varying widely in their ionic composition and in pH, or even by distilled water. These facts indicate that the activation of spermatozoa is due to a dilution effect *per se* and not to a specific component of the diluent.

The question now arises as to what are the possible factors responsible for the inhibition of sperm motility in the spermatheca. Although the work presented here does not encompass the causes of immotility of spermathecal spermatozoa, the following suggestions may be made, based on the fact that dilution *per se* activates the spermatozoa : *a*) high viscosity of the spermathecal fluid ; *b*) partial dehydration of the spermatozoa due to hypertonicity of the spermathecal fluid ; *c*) the presence of an inhibitor in the spermathecal fluid the activity of which may be diminished by dilution ; *d*) deficiency of oxygen in the spermathecal fluid. This last proposed mechanism seems less likely than the former ones, because the procedures employed in the preparation of the specimen should supply a sufficiency of aeration, at any rate at the edges of the semen drop.

It appears that all three types of motility — undulating mass movement, circular movement and snake like movement of individual spermatozoa — were independent of the source from which the spermatozoa were derived, i.e. vesiculae seminales, ejaculate or spermatheca, and were dependent only upon the state of their density. Similar patterns of movement have been described by other authors. BISHOP (1920) and ROTHSCHILD (1955) described a wave-like movement of masses of spermatozoa, while SMIRNOV (1953) and KURENNOI (1953) working with diluted semen suspensions observed the snake-like movement. FYG (1963) considers the circular shape of the spermatozoa in the spermatheca to be the result of a disease of the queen bee. The forms which have been presented by FYG (1963) and called by him « circular » are not the same as those represented in the present work as circular (pl, 3, fig. 1). They rather resemble the sperm forms obtained in the present work following their suspension in water (Plate 4, fig. 1 and 2). Also the shapes of spermatozoa reported by DADE (1962) strongly resemble those obtained in the present work following suspension of spermatozoa in water, showing all the stages of coiling and uncoiling. Thus, they cannot be considered as natural occurring forms, but rather seem to be artefacts of a hypotonic medium.

It was shown that drone spermatozoa are reversibly inactivated by high and low temperature exposure but that their subsequent survival is not affected over a wide range of temperatures. Only at extreme temperatures of — 10° and +50 °C, irreversible damage was caused to the cells. In view of this high resistance of drone spermatozoa to a wide range of temperatures it is interesting to note that the honey-

bee may change its body temperature from 4° to 48°C under the influence of the environmental temperature (PIRSCH, 1923) and survive under a wide range of extreme temperature conditions (KALABUCHOV, 1934 ; LENSKY, 1964 *b*).

The possible recovery of sperm motility after inactivation at 0°C was mentioned by BISHOP (1920) and by SMIRNOV (1953). JAYCOX (1960) also described the inactivation phenomenon which occurs after a short period following ejaculation, and he reported that he was able to reactivate the spermatozoa by heat.

KURENNOI (1954), while observing semen drops on microscope slides at various temperatures, found that sperm motility lasted 140 minutes on the average at 20°C, and 5 minutes or less only at 10° and 44°C. In view of the present findings it appears that the apparent death of the spermatozoa observed by KURENNOI at low and high temperatures was an inactivation which probably could have been reversed if the spermatozoa had again been exposed to room temperature.

The fact that drone spermatozoa are reversibly inactivated by certain temperatures, has to be taken into consideration when motility observations are carried out. It appears that optimal temperatures for such observations lie between 19° and 30°C. When spermatozoa have been kept below or above this temperature, it is necessary to bring them back to room temperature in order to obtain maximum sperm motility.

The remarkable resistance of the honey bee spermatozoa to a wide range of conditions is shown also by the following phenomenon. It was found that the pH in the seminal vesicles where the spermatozoa are stored for at least 16 days until mating (KURENNOI, 1953) is close to 7. A similar pH was found for the ejaculate. However, after entrance into the spermatheca the spermatozoa are subjected to a medium with a pH of about 9 and are stored under these conditions for years.

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SUMMARY

A study was made of the state of motility of spermatozoa obtained from the spermatheca of the queen bee and from the ejaculate and vesiculae seminales of drones. The effects of temperature, dilution, pH, ionic composition and tonicity of the diluent on bee spermatozoa were also investigated.

The spermatozoa were found to be immotile in the spermatheca ; they could be activated by the addition of spermathecal gland secretion, or various buffer solutions at pH ranging from 4.5 to 9.0 or distilled water. The activation of the spermatozoa was established to be due to dilution *per se*, and not to any specific component of the diluents tested.

Spermatozoa obtained from the ejaculates or from the vesiculae seminales were found to be motile In these samples, as well as in slightly diluted samples from the spermathecae, the spermatozoa were found to move in a swirling mass. In diluted samples from all sources, individual spermatozoa of circular and snake-like forms were observed. The pattern of motility and the shape of the spermatozoa were dependent upon the state of density and the tonicity of the diluent and not the organ from which they were taken.

The pH of the spermathecal fluid and of the spermathecal glands was found to be 9 and 7 respectively. This negates the supposition that the immobilisation of spermatozoa inside the spermatheca is due to the accumulation of respiratory CO_2, and that the spermathecal glands activate the spermatozoa by neutralisation of the acid medium in the spermatheca.

The honey bee spermatozoa were resistant to a wide range of temperatures (2°-45°C) ; at the low and high temperatures of this range they were inactivated, but could be reactivated on transfer to room temperature.

RÉSUMÉ

MOTILITÉ ET ACTIVATION RÉVERSIBLE DE SPERMATOZOIDES D'ABEILLES « IN VIVO » ET « IN VITRO »

Les auteurs étudient la motilité des spermatozoïdes prélevés dans la spermathèque de la reine d'abeille ou dans les vésicules séminales des mâles ou obtenus par éjaculation. Les recherches ont également concerné l'action de la température, de la dilution, du pH, de la composition ionique et de la tonicité du diluant sur les spermatozoïdes des abeilles.

Les spermatozoïdes de la spermathèque ne peuvent se mouvoir. On peut les activer par l'addition de la sécrétion d'une glande de la spermathèque, de plusieurs solutions tampons ayant un pH compris entre 4,5 et 9,0 ou d'eau distillée. On a constaté que l'activation des spermatozoïdes est due à une dilution *per se* et non à un quelconque composant des diluants testés.

Les spermatozoïdes obtenus par éjaculation ou pris dans les vésicules séminales se sont révélés mobiles. Dans ces échantillons et dans les échantillons, légèrement dilués, de la spermathèque, les spermatozoïdes se mouvaient dans une masse tourbillonnante. Dans les dilutions d'échantillons des trois provenances on a remarqué certains spermatozoïdes circulaires ou anguiformes. Le schéma de motilité et la forme des spermatozoïdes dépendaient de la densité et de la tonicité du diluant et non de leur provenance.

Le pH du liquide de la spermathèque et des glandes de la spermathèque était respectivement de 9 et 7. Ceci dément l'hypothèse selon laquelle l'immobilisation des spermatozoïdes à l'intérieur de la spermathèque est due à l'accumulation de CO_2 et que les glandes de la spermathèque activent les spermatozoïdes par neutralisation du milieu acide dans la spermathèque.

Les spermatozoïdes de l'abeille résistent à une ample gamme de températures (2° à 45°C). Aux basses et hautes températures de cette gamme, ils sont inactifs mais peuvent être réactivés par transfert à la température ambiante.

REFERENCES

ADAM A., 1913. Bau und Mechanismus des Receptaculum seminis bei den Bienen, Wespen und Ameisen. *Zool. Jahrb, Abt. Anat.* **35**, 1-74.

BISHOP G. H., 1920. Fertilization of the honeybee. 1. The male sexual organs. *J. exper. Zool.* **31**, 225-266.

BISHOP G. H., 1923. Body fluids of the honeybee larva. *J. biol. Chem.*, **58**, 543-565.

BRESSLAU E., 1905. Der Samenblasengang der Bienenkönigin. *Zool. Anz.*, **29**, 299-323.

DADE H. A., 1962. *Anatomy and Dissection of the Honeybee.* Bee Research Association, London.

DAVEY K. G., 1965. *Reproduction in the Insects.* Oliver and Boyd, Edinburgh and London.

FISCHER ,F. G., NEUMANN, W. P., 1953. Das Gift der Honigbiene. Trennung und chemische Charakterisierung der beiden Hauptfraktionen. *Biochem. Z.*, **324**, 447-464.

FLANDERS S. E., 1939. Environmental control of sex in Hymenopterous insects *Ann. entomol. Soc. Amer.* **32**, 11-26.

FLANDERS S. E., 1950. Control of sex in the honeybee. *Sci. Monthly*, **71**, 235-240.

FYG, W., 1963. Anomalies et maladies des reines d'abeilles. *Symp. Pathol. apicole de l'O. I. E.*, Bern.

GOETZE G., 1964. *Die Honigbiene in natürlicher Zuchtauslese. Teil I. Systematik, Zeugung und Vererbung.* Paul Parey, Hamburg and Berlin.

GOMORI G., 1955. Preparation of buffers for use in enzyme studies. In *Methods of Enzymology* (Ed. by Collowick, S. P. and Kaplan, N. O.) Vol. 1, p. 138-146. Academic Press Inc., New York.

JAYCOX E. R., 1960. The effect of drying and various diluents on spermatozoa of the honeybee (*Apis mellifera*, L.) *J. econ. Entomol.*, **53**, 266-269.

KALABUCHOV N. I., 1934. Beiträge zur Kenntnis der Kältestarre (Winterschlaf und Anabiose) bei der Biene (*Apis mellifica*). *Zool. J. Abt. Physiol.* **53**, 567-602.

KURRENOI N. M., 1953. The mating of the queen and drone. *Pchelovodstvo.* **30** (10), 19-25.

KURRENOI N. M., 1954. Structure and vitality of drone spermatozoa. *Pchelovodstvo*, **31** (10), 41-44.

LENSKY Y., 1964 *a*. Variations glycémiques du sang des abeilles soumises à une chaleur extrême. *J. Insect Physiol.*, **10**, 279-282.

LENSKY Y., 1964 *b*. Résistance des abeilles (*Apis mellifica* L. var., *ligustica*) à des températures élevées. *Insectes soc.*, **11**, 293-300.

LEYDIG F., 1867. Der Eierstock und die Samentasche der Insekten. *Novorum Actorum Academiae Caesareae Leopoldino-Carolinae germanicae Naturea Curiosorum*, **33**, 1-88.

LILLIE F. R., 1919. *Problems of Fertilization.* University of Chicago Scientific Series, University of Chicago, Chicago.

MACKENSEN O., ROBERTS, V. C., 1948. *A Manual for the Artificial Insemination of Queen Bees.* U. S. D. A. ET-250.

MANN T., 1964. *The Biochemistry of Semen and of the Male Reproductive Tract.* Methuen and Co., London.

PIRSCH G. B., 1923. Studies on the temperature of individual insects, with special reference to the honeybee. *J. agric. Res.*, **24**, 275-287.

ROTHSCHILD (Lord) 1948. The physiology of sea urchin spermatozoa. Lack of movement of semen. *J. exper. Biol.*, **25**, 344-352.

ROTHSCHILD (Lord) 1955. The spermatozoa of the honeybee. *Trans. r. entomol. Soc., London*, **107**, 289-294.

RUTTNER F., 1956. The mating of the honeybee. *Bee World*, **37**, 2-15 ; 23-24.

RUTTNER F., 1960. Fortpflanzung and Vererbung. In *Biene und Bienenzucht* (Ed. by Büdel A. and Herold, E.) p. 5-22. Ehrenwirth. München.

SCHINDLER H., NEVO A., 1962. Reversible inactivation and agglutination of fowl and bull spermatozoa under anaerobic conditions. *J. Reprod. Fert.* **4**, 251-265.

SMIRNOV I. V., 1953. New data on the sperm of drones. *Pchelovodstvo*, **2**, 23-25. *Apicultural Abstr.*, Vol. 5 in *Bee World*, **35**, 118.

SNODGRASS R. E., 1956. *Anatomy of the Honey Bee.* Comstock Publishing Associates, Ithaca, N. Y.

PLATE 1. — *Spermatozoa obtained from spermathecae of queen bees and photographed after activation*

Action of different temperature exposures on the motility of spermatozoa examined in the cells, immediately after experimental treatment

(degree 5 indicates 80 to 100 p. 100 motility and degree 0 absence of motility)

FIG. 1. — Spermatheca crushed without the accessory glands. The spermatozoa were activated by the addition of a buffer solution. They spread out from one center in S-like wave motion. Whorls resulting from independently moving bundles can also be seen (Phase contrast, 162 ×).

FIG. 2. — Spermatheca crushed with the accessory glands. In the blurred parts of the picture the spermatozoa were in motion at time of photography, whereas in the other parts motionless bundles of spermatozoa can be seen ; these became active later (Phase contrast, 432 ×).

FIG. 3. — Spermatheca crushed with the accessory glands. Swirling motion of sperm bundles can be seen (Phase contrast, 162 ×).

FIG. 4. — Spermathecal spermatozoa deposited near a punctured accessory gland (on the left hand side of the picture) and activated by its secretion (Phase contrast, 81 ×).

PLANCHE 1. — *Spermatozoïdes provenant de la spermathèque de reines d'abeilles et photographiés après l'activation*

Action de l'exposition à différentes températures sur la motilité des spermatozoïdes examinés dans les cellules immédiatement après le traitement expérimental

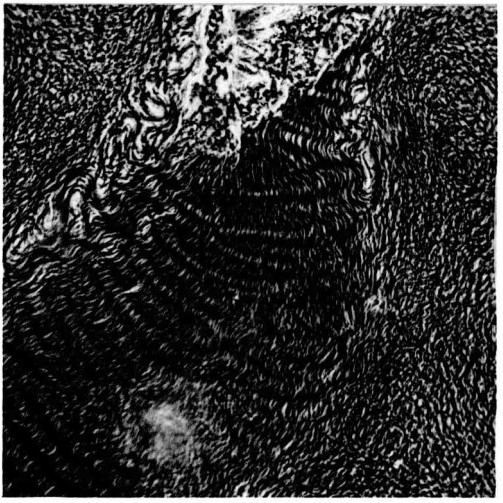
(le degré 5 indique 80 à 100 p. 100 de motilité et le degré 0 l'absence de motilité)

FIG. 1. — Spermathèque broyée avec les glandes à mucus. Les spermatozoïdes ont été activés par l'addition d'une solution tampon. Ils se répandent à partir d'un point unique avec un mouvement anguiforme. On peut voir aussi des circonvolutions résultant d'amas se mouvant indépendamment (contraste de phase, 162 ×).

FIG. 2. — Spermathèque broyée avec les glandes à mucus. Dans les parties floues de la photographie les spermatozoïdes étaient en mouvement au moment de la prise de vue, tandis qu'en d'autres points des amas immobiles de spermatozoïdes sont visibles ceux-ci sont devenus mobiles plus tard (contraste de phase, 324 ×).

FIG. 3. — Spermathèque broyée avec les glandes à mucus. On peut voir le mouvement en spirale des amas de sperme (contraste de phase, 162 ×).

FIG. 4. — Spermatozoïdes de la spermathèque déposés près d'une glande à mucus perforée (à gauche de la photo) et activés par sa sécrétion (contraste de phase, 81 ×).



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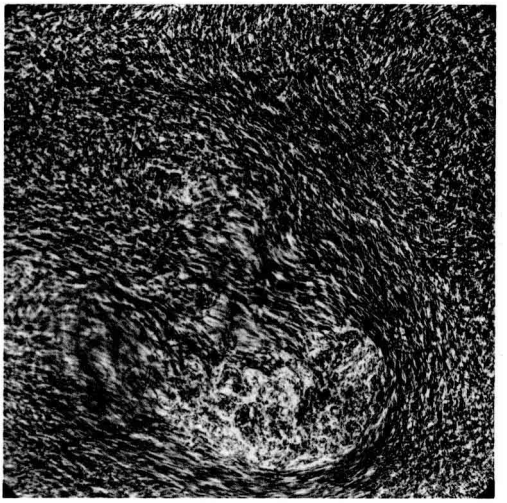

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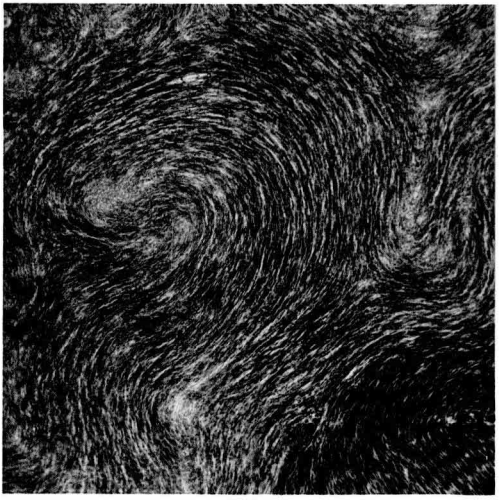

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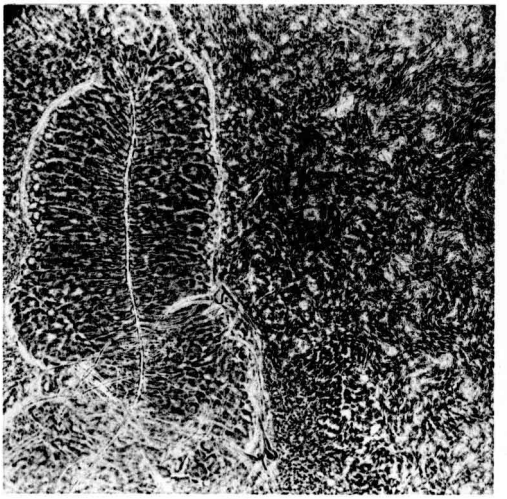


4 100 μ

PLATE 2

Vigorously moving bee spermatozoa obtained from three different sources : vesiculae seminales, ejaculate and spermatheca. The spermatozoa move in bundles which exhibit a wave-like motion resembling an S-form.

FIG. 1. — Spermatozoa from vesiculae seminales (Phase contrast, 162x).

FIG. 2. — Ejaculated spermatozoa (Phase contrast, 162x).

FIG. 3. — Spermatozoa from a spermatheca (Bright field, 810x).

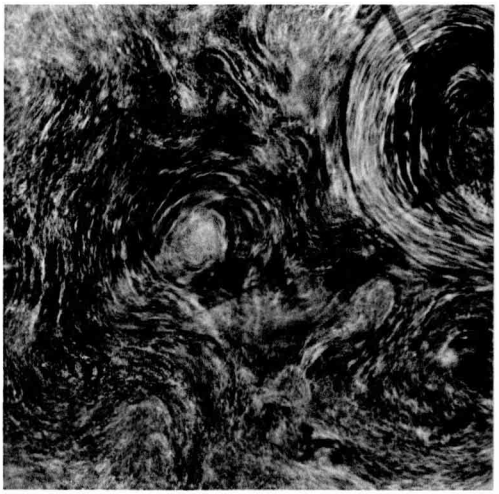
PLANCHE 2

Spermatozoïdes très mobiles provenant de trois sources différentes : vésicules séminales, éjaculation et spermathèque. Les spermatozoïdes se présentent en amas ayant un mouvement anguiforme.

FIG. 1. — Spermatozoïdes provenant des vésicules séminales (contraste de phase, 162x).

FIG. 2. — Spermatozoïdes provenant de l'éjaculation (contraste de phase, 162x).

FIG. 3. — Spermatozoïdes provenant de la spermathèque (champ brillant, 810x).



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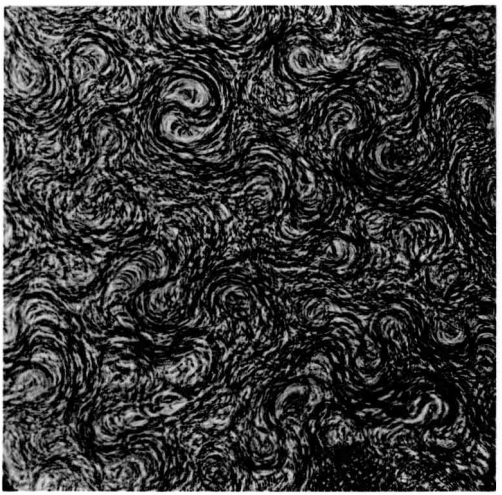

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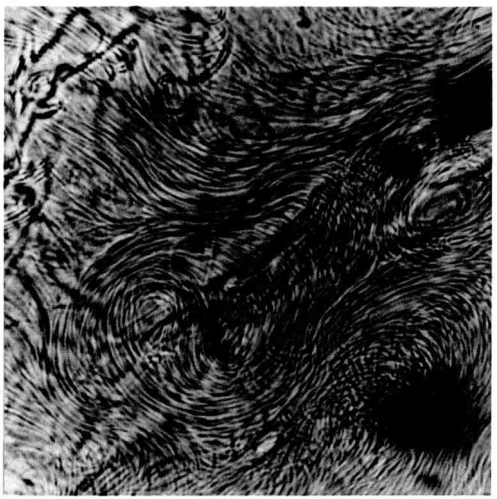


3 10 µ

PLATE 3

Different shapes of honey bee spermatozoa.

FIG. 1. — Circular shaped spermatozoa in a dilute suspension of an ejaculate. Rotating spirals as well as a few snake-like forms on the right hand side of the picture may also be seen (Phase contrast, 162x).

FIG. 2. — Snake-like pattern of spermatozoa in a dilute sperm suspension (Phase contrast, 162x).

FIG. 3. — Spermatozoa suspended in haemolymph. The head is distinguished from the tail by its darker appearance. A spike-like processus (the acrosome) may be seen (Phase contrast, 810x).

FIG. 4. — Spermatozoa photographed after drying of the preparation. The head appears to be wider than the tail and the acrosome (Bright field, 810x).

PLANCHE 3

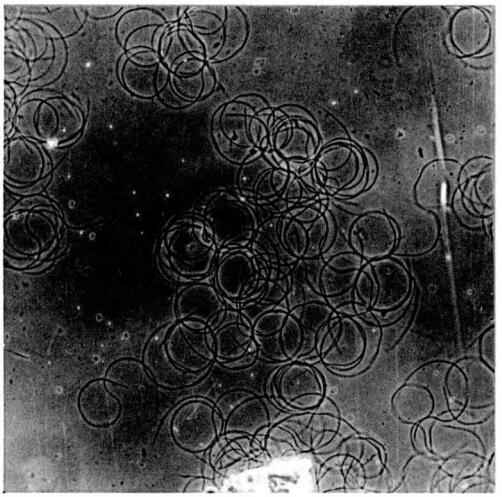
Différentes formes de spermatozoïdes d'abeille.

FIG. 1. — Spermatozoïdes de forme circulaire dans une dilution de la suspension éjaculée. On peut voir à droite de la photo des formes en spirales rotatives et d'autres anguiformes (contraste de phase, 162x).

FIG. 2. — Figure anguiforme de spermatozoïdes dans une suspension de sperme diluée (contraste de phase, 162x).

FIG. 3. — Spermatozoïdes en suspension dans l'hémolymphe. La tête se distingue de la queue par son aspect plus sombre. On peut voir une excroissance en forme de lance (l'acrosome) (contraste de phase, 810x).

FIG. 4. — Spermatozoïdes photographiés après séchage de la préparation. La tête semble plus large que la queue et l'acrosome (champ brillant, 810x).



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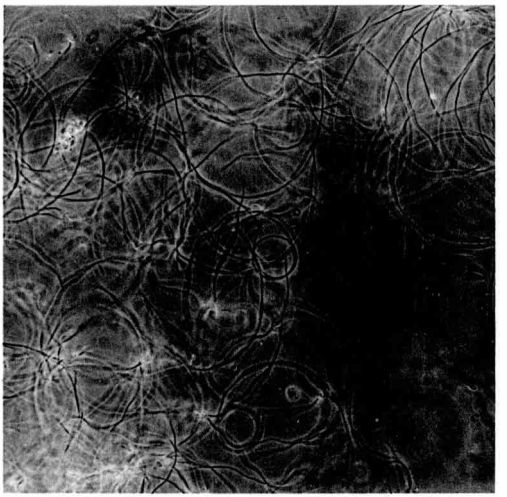

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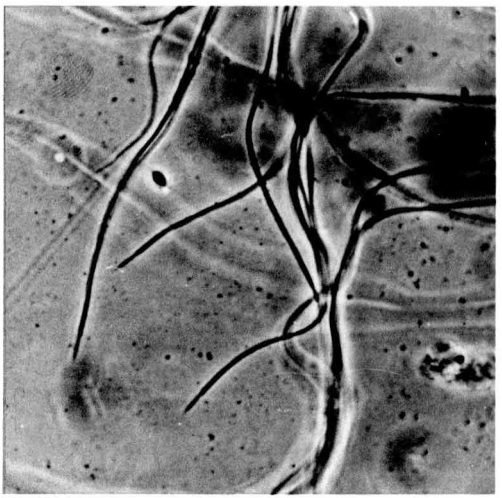

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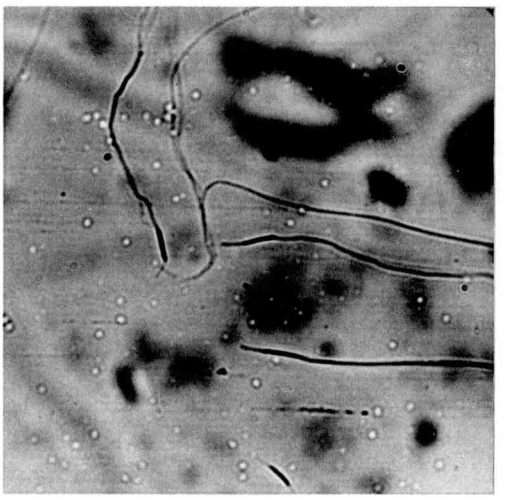

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PLATE 4

FIG. 1 and 2. — Coiled and uncoiled forms of spermatozoa from spermathecal fluid suspended in distilled water (Phase contrast, 324x).

FIG. 3. — Spermatozoa from a dead queen's spermatheca. The motionless cells are stretched, and have a loop at the end of their tail (Phase contrast, 324x).

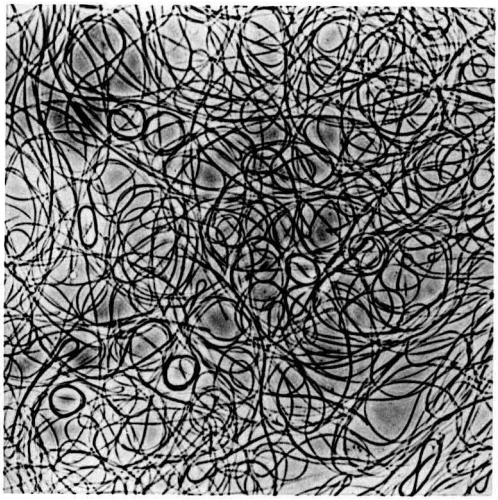
FIG. 4. — Histological section of a spermatheca showing the spermatozoa arranged in bundles. (Haematoxylin-Eosin, 81x) (from unpublished data on the histology and histochemistry of the spermatheca and accessory gland of the queen bee).

PLANCHE 4

FIG. 1 et 2 — Formes enroulées et déroulées de spermatozoïdes provenant de la spermathèque et en suspension dans l'eau distillée (contraste de phase, 324x).

FIG. 3. — Spermatozoïdes provenant d'une reine morte. Les cellules sont étirées et ont une boucle au bout de la queue (contraste de phase, 324x).

FIG. 4. — Section histologique d'une spermathèque montrant les spermatozoïdes rangés en tas. (Hématoxyline-Éosine, 81x) (Données non publiées sur l'histologie et l'histochimie de la spermathèque et la glande à mucus de la reine d'abeille).



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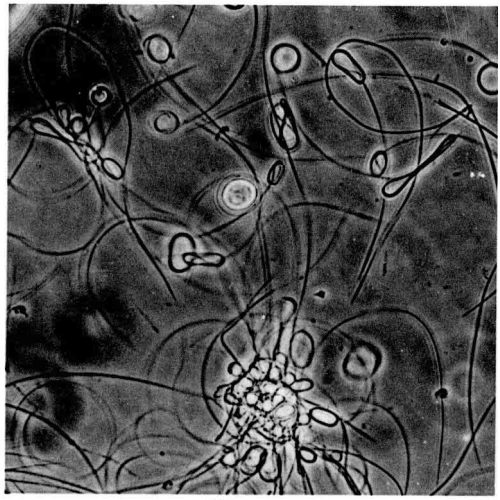

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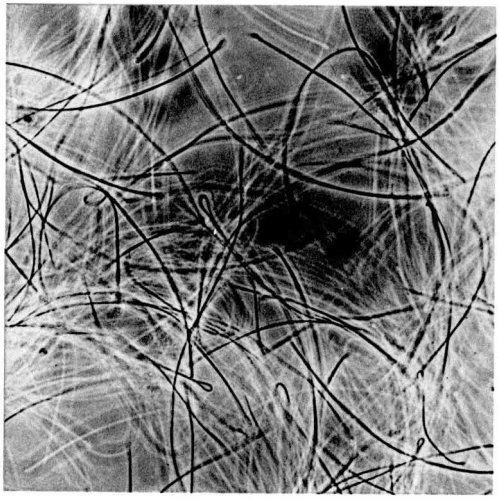

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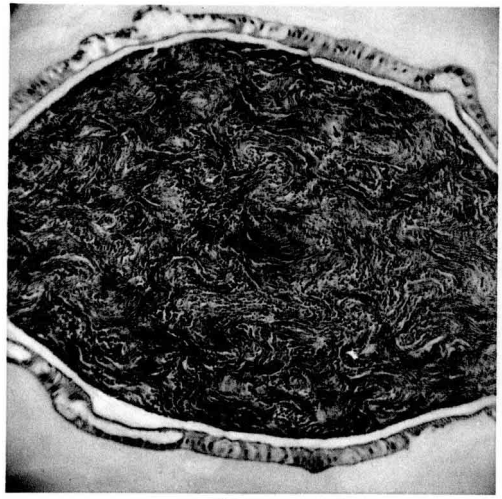

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