ASPECTS OF GAMETOGENESIS AND RADIATION PATHOLOGY IN THE ONION FLY, *HYLEMYA ANTIQUA* (MEIGEN). I. GAMETOGENESIS

J. A. B. M. THEUNISSEN

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Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te 's-Gravenhage op 10 april 1937, is goedgekeurd door de promotoren, Dr. J. de Wilde, hoogleraar in het dierkundig deel van de Plantenziektenkunde en mej. Dr. L. P. M. Timmermans, lector in de algemene dierkunde

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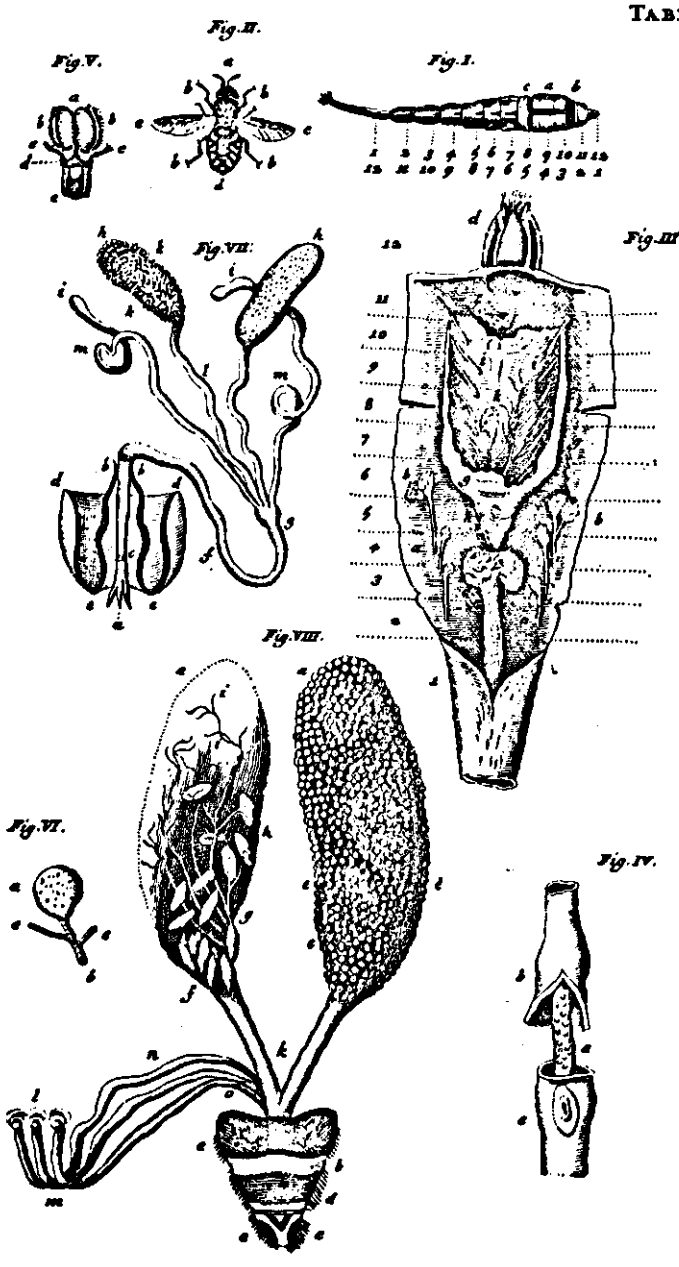
(with a summary in Dutch)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. IR. J. P. H. VAN DER WANT,
HOOGLERAAR IN DE VIROLOGIE,
IN HET OPENBAAR TE VERDEDIGEN OP
VRIJDAG 21 MEI 1976
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWHOGESCHOOL
TE WAGENINGEN

H. VEENMAN & ZONEN B.V. – WAGENINGEN – 1976



Male (fig. VII) and female (fig. VIII) reproductive organs of Tabanus, as has been drawn by the Dutch Entomologist Jan Swammerdam around 1669.
Source: Joannis Swammerdammie, Biblia Naturae sive Historia Insectorum, Leyden MDCCXXXVIII. (University Library, Wageningen).

STELLINGEN

I

De definities van de begrippen 'predefinitive', 'indefinitive' en 'definitive' spermatogoniën zoals ze worden geformuleerd door Hannah-Alava, leiden tot de conclusie dat de categorie van de 'indefinitive' spermatogoniën overbodig is.

Dit proefschrift.
HANNAH-ALAVA, A. 1965. Adv. in Genetics, **13**, 157–226.

II

De invoering van de term 'ovarioles adenomorphes' door Martoja ter aanduiding van het ovariooltype van *Steraspis speciosa* is overbodig en ongewenst.

MARTOJA, R. 1964. Bull. Soc. Zool. Fr., **89**, 614–641.

III

De conclusie van Causse, Feron en Pereau-Leroy dat in *Ceratitis capitata* het verdwijnen van de spermatiden binnen vier dagen na bestraling moet worden toegeschreven aan voortgezette differentiatie, wordt ten onrechte getrokken.

CAUSSE, R., FERON, M. en PEREAU-LEROY, P. 1968. in: "Radiation, radioisotopes and rearing methods in the control of insect pests", 355–363, International Atomic Energy Agency, Wenen.

IV

Beoordeeld naar histopathologische criteria zijn germinale cellen bij de uievlieg niet altijd gevoeliger voor ioniserende straling dan somatische cellen. Dit in tegenstelling tot de strekking van de 'wet' van Bergonié en Tribondeau, die de basis is van de radiotherapie.

BERGONIÉ, J. en TRIBONDEAU, L. 1906. C. r. Acad. Sci., Paris, **143**, 983,

V

Onderzoek naar criteria voor 'kwaliteit' van gekweekte en/of behandelde insecten voor gebruik in genetische bestrijdingsmethoden dient een hoge prioriteit te krijgen.

CHAMBERS, D. L. 1975. in: "Controlling fruit flies by the sterile-insect technique", 19–32, International Atomic Energy Agency, Wenen.

VI

Het aantal bladluizen van de soort *Myzus persicae* SULZ. in pootaardappelen is geen correct criterium voor het doen beëindigen van de groeiperiode van dit gewas.

J. A. B. M. THEUNISSEN
Wageningen, 21 Mei, 1976.

VII

Niet alleen insecten kunnen door Baculovirussen worden geïnfecteerd.

REED, D. K. en HALL, I. M. 1972. J. Invertebr. Pathol., 20, 272–278.

VIII

Stoffen die nematoden activeren tot wortelpenetratie, zoals 'hatching agents', behoeven niet noodzakelijkerwijze afgescheiden te worden in wortelexudaten.

CARROLL. K. K. 1958. Nematologica 3, 197–204.

IX

De daling van het aantal zeehonden in de Waddenzee kan niet worden toegeschreven aan de opname van kwik via voedselketens.

X

Het vanuit onze samenleving willen bepalen welke graad van technologische ontwikkeling voor ontwikkelingslanden passend zou zijn, getuigt van elitair denken en een paternalistische houding ten opzichte van deze landen.

XI

Als de zwijgende meerderheid gaat spreken, leert de sprekende minderheid zwijgen.

CONTENTS

1. INTRODUCTION

As the occurrence of large numbers is a major characteristic of insect pests, insect reproduction is a key topic in the biological background of insect control. The complexity of this function is reflected in the multitude of studies on insect reproduction in its various aspects. One of these aspects is the development of the reproductive cells in the male and female gonads: gametogenesis.

In The Netherlands possibilities have been investigated to use the Sterile Insect Method to control the onion fly, *Hylemya antiqua* (Meigen). The use of this genetic control method, which has been applied successfully in a series of small scale field trials (THEUNISSEN et al., 1973, 1975; TICHELER et al., 1974), involves irradiation and release of flies sterilized by hard X or gamma rays. Irradiation at the wrong stage of gametogenesis may cause adverse effects on the quantity of available sperm cells, the sexual competitiveness and physiological fitness of the released flies. Use of the correct dose is imperative to avoid unwanted fertility or unnecessary impairment of physiological performance in the irradiated individuals. In order to obtain an optimal combination of dose, age and developmental stage at the time of irradiation, a study has been made of histopathological irradiation effects on male and female reproductive organs and their constituting cells and cell populations (THEUNISSEN, 1976). The basis of this study is an investigation of spermatogenesis and oogenesis of *H. antiqua* from a histological point of view. The histo- and biochemical and genetical aspects of gametogenesis have been omitted deliberately, due to the vastness of this field.

Following some remarks on the gross anatomy of the reproductive organs and the early post-embryonic gonad development, spermatogenesis and oogenesis are described separately. Spermatogenesis is treated more comprehensively as compared to oogenesis because the latter, and especially the vitellogenesis, has been studied far more frequently in great detail in other insects. After the identification of the germinal and somatic cell types involved in gametogenesis, the development of the gonads during larval, pupal and adult life has been described. A preliminary comparative description of spermatogenic cell types and structures relates the present work on *H. antiqua* to other insect species.

2. MATERIAL AND METHODS

2.1. Insect Material

2.1.1. *Bionomics of the onion fly*

The onion fly, *Hylemya antiqua* Meigen, belongs to the family Anthomyiidae, the superfamily Schizophora and the suborder Cyclorrapha of the order Diptera. Köppen (1972) and Stork (1936) described respectively the larval and pupal characteristics of the family Anthomyiidae. Various aspects of the biology, rearing and control of *H. antiqua* are referred to in a bibliography of Scott (1970). Additional information is found in Anonymous (1965–1975).

Life cycle

The flies emerge in the spring from pupae which have passed the winter in a state of diapause. The date and the length of the emergence period are very much dependent on a number of factors including the depth in the soil at which the pupae hibernate, the course of the temperature profile of the relevant soil levels and the coverage of the soil by a new crop. The day following emergence the flies are very vulnerable to predation because of their low rate of reactivity and motion. They walk around while their cuticle hardens. During this period they remain in the area where they emerged. The next day they show flight activity and start to move to other areas.

The flies concentrate around onion fields where it takes some days for them to mature sexually. The males start to mate when they are about 5 days old, the females at about a week. In the meantime the gonads are ready for reproduction. Depending on the temperature the pre-oviposition period is about 10 days.

Eggs are laid on a suitable hostplant in the axle of the leaf sheaths and against or around the stem on the soil surface. At a temperature of 20 °C the larvae hatch after 3 days. Embryonic development is retarded considerably by low temperatures. Eggs can be stored at 4 °C for 1 week without serious embryonic mortality. The young larvae move towards and into the host plant and they begin to feed on the roots of the young plant till they reach the soil surface. Soon the plants wither, showing the characteristic symptoms of onion fly damage. When the hostplant is too small to nourish one or more larvae, they leave the plant at the level of the soil surface to crawl during the night to the next plant. In this way a row of plants can be destroyed. In cases of food shortage the leaves are also eaten.

The larvae are mature after about 14 days at 20 °C. They crawl into the soil to pupate. The depth at which the pupae are formed differs, but most pupae are found in the upper 15 cm. Ploughing increases the variation in depth considerably.

Diapause is induced in mature larvae and young pupae by light and tempera-

ture conditions. Pupae can stay in diapause for at least one year but sometimes also longer. When no diapause is induced the pupal stage lasts about 14 days at 20°C.

A second generation of flies emerges during July-August. In long hot summers a small third generation may appear in September but usually most larvae of the second generation are induced to enter diapause. Adult flies may live for over 40 days in laboratory cages. Their average life span in the field may be considerably shorter. They seem to hide and to feed in borders of fields and banks of ditches near the onion fields which are their oviposition and also possibly their mating sites.

External features in larval development

According to MAAN (1945) the larval stages can be distinguished by the number of abdominal stigmata, the presence of prothoracal stigmata and the appearance of parts of the cephalopharyngeal skeleton: the pharyngeal and mandibular sclerites. This is also valid in other Anthomyiidae.
Schematically:

Larval stage	Abdominal stigmata	Prothoracal stigmata
L1	2 pairs	absent
L2	2 pairs	present
L3	3 pairs	present

External features in pupal development

Gonad development of pupae has been studied. In particular during the first days the pupae have been examined closely. In order to contribute to a larger uniformity in the use of defined terms, the terminology of FRAENKEL and BHASKARAN (1973) concerning pupation in Diptera is used.

To reduce the variability as much as possible specimens were selected from a large group of post-feeding larvae, which had been reared at 20–21°C on a meridic diet (TICHELER, 1971) as a 4th generation laboratory strain of onion fly larvae. The post-feeding larvae were allowed to develop in vermiculite at 20–21°C. A first sample was fixed when the post-feeding larvae were converted into white prepupae. That moment was referred to as 0 hours.

0 hours: The prepupae show the following external features:

1. the larval cuticle is white, firm, stretched and tough but not showing plasticity like that from a larva.
2. in the larval cuticle and epidermis no movements are visible.
3. at gross examination the internal tissues are identical with those of the larvae: a loose, yellowish-white mass of adipose tissue, and among other tissues an intestinal tract with sometimes orange coloured contents.
4. there is no differentiation of head, thorax and abdomen.

12 hours: the larval cuticle shows a light brown colour and is somewhat brittle. The larval-pupal apolysis has not yet taken place.

24 hours: the former larval cuticle, the puparium, has reached its final, full brown colour. It is brittle and is nearly completely separated from the pupal cuticle. At the anterior and posterior poles of the puparium both cuticles are attached by means of thin, membranous filaments. A beginning of differentiation of the compound eyes is visible.

36 hours: apart from the two described filaments the larval-pupal apolysis has been completed. Perhaps the filaments are remnants of the larval cuticle as they are very delicate and easily torn. The pupa is a cryptocephalic pupa. It can be easily taken out of the puparium. External examination reveals a distinct differentiation of the compound eyes and the imaginal discs of legs and other extremities. The appearance of the pupa is glassy yellowish-white.

48 hours: the pupa has become a phanerocephalic pupa, resulting in a clear external segmentation and differentiation in head, thorax and abdomen.

72 hours: the external differentiation is advanced. Legs and wings have already been differentiated in a rather detailed manner, at least externally. Internally no differentiation or organization is visible.

96 hours: no progress in external and internal differentiation can be observed at gross examination.

Pupae in *diapause* are phanerocephalic. Their state of differentiation is like that of a non-diapause pupa of 72 hours.

Rearing

Although *H. antiqua* is an oligophagous species, it is reared on a meridic diet in the laboratory (TICHELER, 1971). The laboratory rearing has been developed to a modest mass-rearing (NOORLANDER, unpublished data) with a maximal capacity of about 0,5 million pupae/month.

Eggs are stored at a temperature of 4 °C for maximally a week to be transferred to trays with larval medium. At 20 °C fresh eggs hatch within 3 days. The larvae crawl into the medium and disperse while feeding. Towards the end of the larval stage they concentrate to some clusters of large numbers of larvae. At 20 °C the larval stage takes about 14 days. The mature larvae are washed out of the medium when the first pupae are observed. They are allowed to pupate in vermiculite from which they are separated by sieving at the appropriate moment of pupal development to be stored at 3 °C. When flies are required the requisite number of pupae is taken out of storage, and allowed to resume their development at 22 °C for the remaining 10 days. When the pupae are to be irradiated, this is done 2 days before the expected emergence of the flies. In the meantime the pupae may be transferred to the field if necessary.

In general, flies of the 3rd or 4th laboratory reared generation are used for laboratory or field experiments. In order to reduce inbreeding and selection, each year wild flies are collected, and subsequently reared in the laboratory for 1–5 generations. Inbred populations did not show unfavourable results of selection and rearing on artificial medium for 16 consecutive generations. However, as the reared flies are to be used in control measures, populations for release are only reared in the laboratory for 3–5 generations.

2.1.2. *Other insect species*

Insects of the following species have been used in comparative investigations: *Musca domestica* L., *Sarcophaga* spp., *Ceratitis capitata* Wied, *Adoxophyes orana* F. v. R., *Leptinotarsa decemlineata* Say, *Periplaneta americana* (L.), *Locusta migratoria migratorioides* R. & F,, *Pyrrhocoris apterus* L., *Apis mellifica* L. The insects were obtained from the insectary of the Laboratory for Entomology of the Agricultural University. From these species males were selected 24 hours or less after emergence or adult moult. The age of the young *Sarcophaga* males was not exactly known but less than 3 days. *Leptinotarsa* males were short day beetles, just emerged from the soil. The males of *Locusta*, *Apis* and *Pyrrhocoris* did not yet show their adult pigmentation. The testes of a number of individuals of each species were fixed separately per species but in the same fluid and under identical conditions. They were processed according to the standard histological procedure (paragraph 2.2.3.).

2.2. Methods

2.2.1. *Cytological methods*

As a source of mitotic chromosomes of *H. antiqua* testes of newly emerged adults were predominantly used, however preparations from mature larval brains could also be used. Meiotic chromosomes were found in young adult testes.

The testes or brains were dissected, and kept in distilled water for 10–15 minutes in order to promote the spread of the chromosomes in a metaphase arrangement by anisotropic swelling of the spindle fibers.

To make a squash-preparation the tissue is transferred from the distilled water to a clean slide with a drop of a suitable fixing-staining mixture (Theunissen, 1971). The tissues are fixed and stained in this solution during 3 minutes. A coverglass is carefully placed over the tissue and covered with a piece of blotting paper. While the coverglass is kept in its place and the excess fluid is taken up by the paper the coverglass is pressed carefully but firmly, making circular movements with the thumb. The fresh squash-preparation can be protected from evaporation by lining the edges of the coverglass with a fluid adhesive which can be removed after drying (Theunissen, 1971). The staining intensity of the tissues in this semi-permanent preparation increases slightly during the next few days. The preparations may be made permanent by mounting in Euparal (Theunissen, 1971).

2.2.2. *Phase contrast microscopic methods*

Living testicular cells were observed by means of phase contrast optics fitted to the Wild M 20 microscope.

Freshly dissected testes of a young male fly were transferred to a drop of Levy physiological saline on a slide and covered carefully with a clean coverslip. The increasing capillary pressure of the coverglass causes the testis to flatten till

the testicular sheath is torn at some point and the contents flow into the saline. After some time the cells have spread optimally and in a relatively large area a layer, one cell thick, is formed. Such unicellular layers are suitable for observation. Levy physiological saline consists of 9.000 g NaCl, 0.708 g KCl and 0.458 g $CaCl_2$, dissolved in 1 l of double distilled water.

To prevent evaporation of the water the preparation may be sealed by ringing with a quick drying glue of soluble rubber (Lero photographic glue). Although the oxygen supply of the cells is also cut off, the temporary preparations can be observed somewhat longer.

As an aid to the identification of the germinal cell types intermediate stages between living cells and cells in histological preparations were needed. Therefore, living cells were fixed and stained in such a way as to prevent as much as possible the formation of artefacts. Preparations of living cells in Levy saline were made as already has been described. For this special purpose coverslips were used which were made slightly greasy by contact with human skin. At the moment of optimal dispersion of the germinal cells Bouin fixation fluid was introduced at one edge of the coverslip, replacing the saline. The replacement was enhanced by using filterpaper to absorb the saline at the other edge of the coverslip. The fixation of the cells takes place quickly as is observed by the sudden dramatic change in appearance of glossy, brilliant living cells into dull, dead and distorted clumps of coagulated protein. After fixation during about 30 minutes at room temperature the slide is placed obliquely in a 70% ethanol solution. As the greasy layer is dissolved, the coverglass becomes detached leaving the material adhering to the slide. This is further treated as a normal histological preparation, stained with hematoxylin-eosin and mounted in Dammar resin. This kind of preparation has been called *semi-squash preparation* because it is neither a real squash nor a histological preparation.

2.2.3. *Histological methods*

Standard histological processing methods which we adopted have been described earlier for young female and male adults (THEUNISSEN, 1971) and for females older than 5 days (THEUNISSEN, 1973a). These methods were also used for larvae and pupae.

Bouin fixative was chosen because it permitted a good and reproducible distinction of the germinal cell types in gonads of all developmental stages of the onion fly. It is not critical as for the duration of the fixation and therefore easy to incorporate into a standard processing procedure. Additional observations were made on gonads fixed with the following fluids:

Fluid	Reference
Allen B3 and B15	GRAY (1958)
Carnoy-Lebrun	GRAY (1958)
Gilson	GRAY (1958)
Helly	GRAY (1958)

Zenker	GRAY (1959)
Carnoy	CARLETON and DRURY (1957
San Felice	CARLETON and DRURY (1957)
Susa	CARLETON and DRURY (1957)
Flemming strong[1]	LILLIE (1953)
Flemming[1]	LILLIE (1953)
Tellyesniczky	LILLIE (1953)
Glutaraldehyde[2]	
Glutaraldehyde (Bouin)[3]	
Bouin (Glutaraldehyde)[4]	
Formalin (neutral)[5]	

[1] Both fixatives were prepared with either distilled water or Levy solution as a solvent. In Flemming the acetic acid has been omitted.

[2] Glutaraldehyde 5% in Sörensen buffer pH 7.4 during 3–4 hours, washing in Sörensen buffer pH 7.0 overnight, post-fixation Allen B15 24 hours.

[3] Like 2 and followed by post-fixation with Bouin overnight.

[4] Mixture consisting of:

picric acid, saturated in water	15 parts
glutaraldehyde, 25% in water	1 part
glacial acetic acid	1 part

[5] Solution of 40% formaldehyde in water, shaken with $CaCO_3$ to eliminate formic acid. Fixation during 3 hours.

Comments on the list of fixatives pertain to special features or qualities of certain fluids:

- Carnoy fixatives in all variations and San Felice are penetrating rapidly and preserve chromosomes well but cause an unacceptable uniformity of cell morphology.
- Although Helly and Zenker are good general fixatives for micro-anatomical purposes, they did not allow a good distinction of the germinal cell types in *H. antiqua*.
- Glutaraldehyde fixative sometimes gives good results as a general fixative. It is, however, unreliable as to the penetration of paraplast and the staining properties, due to the gelating character of the fixation.
- Tellyesniczky was found to be an excellent fixative both for micro-anatomical purposes and for cell type distinction. It is just slightly inferior to Bouin in the latter aspect.
- Flemming with acetic acid and Levy solution as a solvent showed to be an excellent fixative for the distinction of testicular cell types. All cell types stain in a very characteristic and clear manner but sometimes staining difficulties are met. The use of distilled water resulted in a decreased cell type characterization, like when the acetic acid was omitted in the mixture with Levy. Fixation in the fluid without acetic acid and Levy gave poor results.

As a standard staining method Ehrlich's hematoxylin-eosin was used (CARLETON and DRURY, 1957). Other methods used were Mallory's triple stain and the Feulgen stain, but they proved to be inferior when compared to the hematoxylin stain. The observation of DEMALSY and CALLEBAUT (1967) concerning the

redundancy of sulfurous acid in the Feulgen method was confirmed in this study.

Dissection of larvae

The gonads of the larvae are hard to find in the larval fat body. Therefore, the entire part of the body in which the gonads are present is fixed. The larva is kept immersed in a watch glass with cold Bouin by a pair of forceps and the body is transversely cut into two halves. The protruding tissues are immediately fixed, preventing the total dislocation of the tissues. The caudal half of the body is cut again in two halves and the hind quarter is discarded. The gonads are present in the remaining quarter with tissues bulging out at both sides. Fixation takes place according to the standard method and is usually good.

Dissection of pupae

Pupae are taken out of the puparium by careful cracking of the brittle former larval cuticle with the points of a pair of forceps. The puparium breaks along the former intersegmental grooves and subsequently the caudal half of the puparium can be peeled off carefully while holding the other half with very flexible forceps. The glassy, whitish pupa can be taken out of the puparium. In the Bouin fixative the abdomen is isolated by separating it from the thorax with a fine pair of scissors. The protruding tissues are fixed immediately into a spongy, coagulated mass. The abdomen can be opened as far as is necessary to ensure proper penetration of the fixative and other fluids into the tissues. The isolated abdomen is fixed at least overnight in Bouin at 4 °C according to the described standard method. Care is taken to enhance the stability of the pupal tissues by keeping them long enough in MBC (methyl benzoate-celloidin) in order to compensate for the lack of connective tissue and for normal coherence of these tissues.

2.2.4. *Autoradiographic methods*

In autoradiographic experiments ^{3}H-thymidine (aqueous sol., Amersham, UK) was used as a DNA-precursor. During these experiments ^{3}H-thymidine was used with a specific activity of 5 Ci/mM and at a concentration of 4.10^{-3} mCi/ml.

In preliminary tests young adult gonads were placed in a mixture of the isotope solution and Levy physiological saline to obtain the required concentration. The freshly dissected tissues were washed in Levy saline, submerged and incubated in the isotope solution at room temperature (about 20–21 °C) during a variable period of time, not exceeding 24 hours, and subsequently washed in several baths of fresh Levy saline. The tissues were fixed and prepared according to the described histological method.

In most experiments adult flies were injected with 1 µl ^{3}H-thymidine solution/individual by means of a fine Hamilton syringe with dispenser. One day old flies were preferred because at that time the cuticle is hard and brittle, thus facilitating the penetration of the needle. The cuticle of younger flies is more

flexible and resists penetration of the needle, thus causing a more inaccurate and damaging injection. In one day old flies gonad development is about at its peak activity. The flies were injected dorso-laterally in the wing muscles and remained attached to the needle during 60 seconds in order to standardize the injection procedure. After the injection the flies were transferred to cages where they were provided with food and water. Samples were taken out of the cages to be sacrificed after certain periods of time and prepared according to the described histological method.

The slides were covered by Kodak AR 10 stripping film, exposed during 10 days, developed in Kodak DX 80 and fixed in a normal photographic fixer. The tissues under the film were stained by an Ehrlich hematoxylin stain which was somewhat adapted to this kind of preparation. For instance, the preparations were quickly transferred through the series of alcohols after staining in order to prevent CO_2 formation under the film following the immersion in Scott's solution. Eosin counterstaining was not applied due to the affinity of the film for this stain. The stained preparations were mounted in dammar resin.

2.2.5. *Electronmicroscopic methods*

When necessary testes and other tissues were dissected, cleaned and washed in the fixing fluid, but mostly the testes could be fixed immediately.

Freshly dissected testes were fixed in 2% glutaraldehyde in 0,06 M phosphate buffer pH 7.0 at 4°C for 2 hours. The tissues were washed in two changes of the buffer solution with 0.34 M sucrose at 4°C for 10 minutes each. They were kept overnight in phosphate buffer with sucrose at the same temperature. The next fixation took place in 1% OsO_4 solution in phosphate buffer with sucrose for 2 hours at 4°C. The fixative was washed out in double distilled water at room temperature for 30 minutes, after which staining (or contrasting) with 1% uranyl acetate took place by immersion for 30 minutes. The tissues were transferred through a series of ethanol of 70%, 80%, 90% and 3 changes of 100% for 10 minutes each and to propylene oxide for 30 minutes. Infiltration with epon-araldite took place by a series of mixtures of propylene oxide and epon-araldite in the ratios 3: 1, 1: 1 and 1:3 respectively, each lasting 30 minutes, followed by storage in epon-araldite overnight at room temperature and subsequent embedding at increasing temperatures: 35°, 45° and 65°C each lasting 24 hours.

Tissues were embedded and stored in dried gelatin capsules and cut by means of a LKB-ultramicrotome. Silver, pale gold and gold sections were picked up on a formvar coated grid. The sections were contrasted with a saturated solution of uranyl acetate by laying the grids upside down on a drop of the solution on parafilm for 30 minutes. The grids were transferred to a drop of water and washed by dipping in double distilled water for 1 minute, followed by the same procedure in a 0.01 N NaOH-solution. Contrasting with lead citrate followed for 30 minutes in a nitrogen atmosphere in a petri dish with KOH grains to exclude CO_2. The grids were transferred to a drop of a 0.01 N NaOH-solution and subsequently washed in 2 changes of freshly distilled water by

dipping them for 1 minute each. The grids were stored in a gridbox.

The sections were observed and photographed by means of a Philips EM 300 electron microscope.

2.2.6. *Illustrations*

The preparations were studied and photographed by means of a Wild M 20 microscope with photographic equipment. The drawings were made using a Wild drawing tube. Magnification is indicated by a bar which represents 10 μ unless indicated otherwise.

2.2.7. *Sampling.*

When possible each group of comparable larvae, pupae or adult flies which was used in experiments consisted of at least 25 individuals. If necessary a smaller number was studied but no statement was made based on the examination of less than 10 individuals, unless explicitely mentioned.

Usually random samples were made of larvae, pupae and adults which were available in abundance.

3. GAMETOGENESIS

3.1. INTRODUCTION

Our study of gametogenesis of *H. antiqua* which has been carried out to create a basis for radiobiological experiments, is necessarily of a limited scope. In general, insect oogenesis has been studied more extensively when compared to insect spermatogenesis. Therefore, in the present work spermatogenesis in *H. antiqua* will be treated more in detail than oogenesis, the latter being very similar to that in other dipterous species, e.g. *Musca domestica* and *Drosophila melanogaster.*

The anatomy of both the male and the female reproductive system is briefly described and discussed in the next paragraph. Some brief remarks on the chromosomes and cytogenetics of *H. antiqua* are made in a subsequent paragraph, followed by a description of young larval gonads at a stage of development at which they can not be distinguished as being a testis or an ovary. Paragraphs on spermatogenesis and oogenesis conclude this chapter.

3.2. GROSS ANATOMY OF THE REPRODUCTIVE ORGANS

Male reproductive system

The reproductive system of the young adult male consists of two ovoid or pear-shaped, pale orange coloured *testes,* each with a *ductus deferens.* Both of these unite with the orifices of the *accessory glands* to form the *ductus ejaculatorius.*

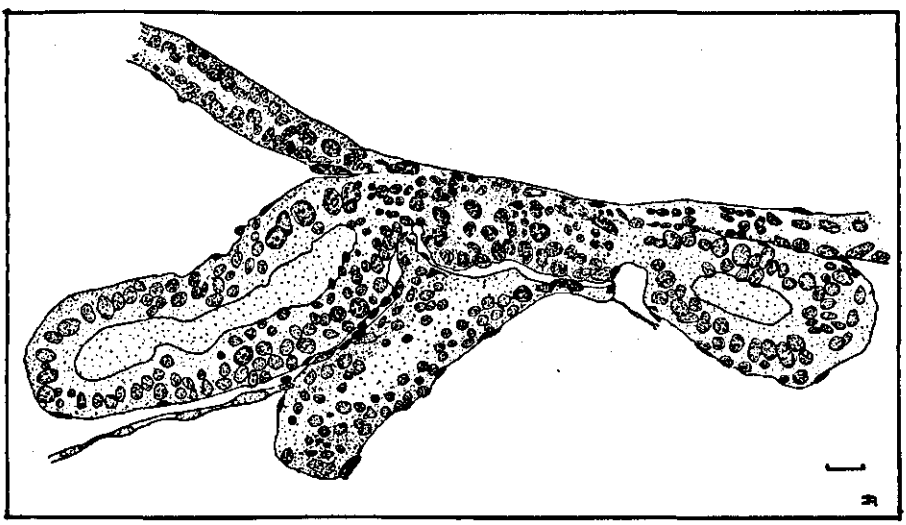


FIG. 1. Developing male accessory glands, ductus ejaculatorius (below, centre) and ductuli deferentia in pupa, 2 days before emergence (Carnoy fixation, HE staining). Bar = 10 μ.

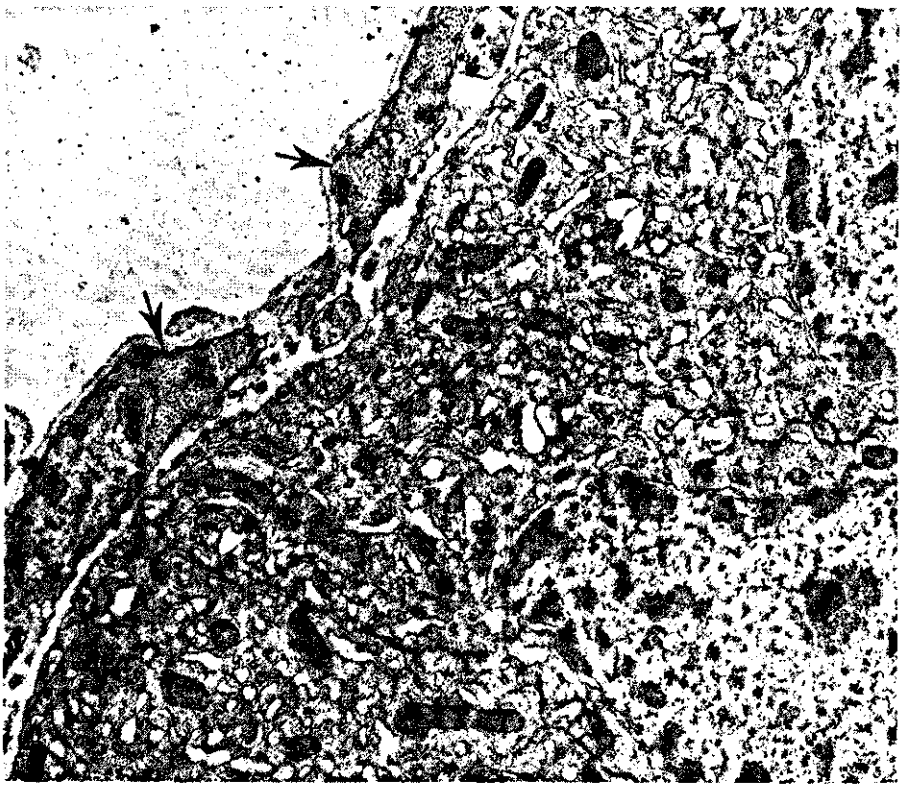


FIG. 2. Male accessory gland. Glandular epithelium with vesiculate cytoplasm at basal side of the cells. Note the covering squamous epithelium with bundles of myofibrils (arrows). 12.686 x.

The testes will be discussed in some detail later (3.5.2.1.). They are found in the 4th abdominal segment, dorsolateral of the intestinal tract.

The *ductus deferens* exhibits a layer of cubical epithelium covered by squamous epithelium. Contractile elements are evidently present. The two *accessory glands* are colourless, flat, oval structures which consist of cubical/columnar glandular epithelium (fig. 1.) and outside its basement membrane an outer covering of squamous epithelium with contractile elements is observed. At the ultrastructural level the cytoplasm of the squamous epithelium contains bundles of myofibrillae which are responsible for the contraction of the glands (fig. 2). Between this covering epithelium and the glandular epithelium the basement membrane is present. The secretory function of the glandular cells is suggested by the presence of a multitude of vesicles (fig. 2), small vacuoles and well developed Golgi-complexes in their cytoplasm. The rough ER is irregularly wound to form numerous vesicles. Microtubuli and small mitochondria are also seen. The Golgi-complexes mostly lie at the basal side of the cell. The often elongated nuclei are situated in the centre of the cells which are separated in certain places by septate desmosomes. These constitute a part of the highly

FIG. 3. Male accessory gland. Glandular epithelium with elongated nuclei and septate desmosomes. Narrow canals end in the lumen of the gland (top). 12,686 x.

irregular cell membranes. At the apical part of the cell, long and narrow canals seem to end in the lumen of the gland (fig. 3). The contents of the glands are a translucent fluid which is probably used as seminal fluid. The glands open in the somewhat dilatated junction of both ductuli deferentia, ventrally of the intestine at about the site where the Malpighian tubules join the gut. Here the *ductus ejaculatorius* begins. This duct leads towards a structure which lies against the rectum (dorsally) and clearly acts as a sperm pump, the piston and cylinder being formed of chitinous material secreted by glandular epithelium. Muscles are attached to the movable part. The ejaculatory duct passes to the chitinous intromittent organ which is a part of the external copulatory structures of the male fly.

Discussion

The findings of BAWA (1974) on the ultrastructure of the glandular epithelial cells of the ductus deferens of *Thermobia domestica* also apply very much to the accessory glands of *H. antiqua*. The vesiculate cytoplasm, rich in Golgi-

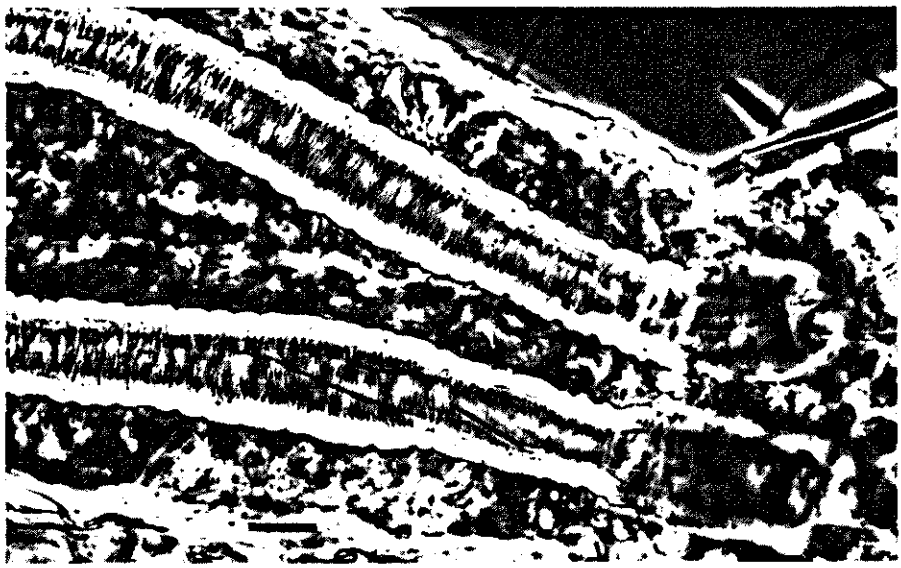


FIG. 4. Proximal part of paired ducts of spermathecae. Note insertion of ducts in bursa copulatrix and the presence of sperm cells in the lower duct. (Living material, phase contrast) Bar = 10 μ.

complexes, separated by convoluted cell walls with septate desmosomes presents in both species the same picture in different parts of the reproductive system. DAPPLES et al. (1974) studied the ultrastructure of the accessory glands of *Aedes aegypti*, their secretions and depletion phenomena after repeated matings. Depletion has never been observed in *H. antiqua*.

Female reproductive system

The position of the ovaries in the abdomen is largely dependent on the degree of egg chamber development. In a gravid female the ovaries may nearly fill the abdomen. In a young adult female they are located dorso-laterally of the intestinal tract. The *ovaries* are connected to the *common oviduct* by *lateral oviducts*. Where the common oviduct joins the *bursa copulatrix*, the *spermathecal ducts* and those of both *accessory glands* are inserted (fig. 4).

The *spermathecae* are chitinous spheroidal structures surrounded by a cubical glandular epithelium which is continuous with the epithelium of the ducts. The chitinous capsule shows many small nozzles pointing inward which probably serve to admit secretions of the glandular epithelium into the spermathecal lumen. Sometimes vacuoles are seen in these glandular cells. Usually three spermathecae are present: two with paired ducts, one single. In 1109 checked females 10 showed four spermathecae i.e. 0.9 %. In these cases the ducts of the 3rd and 4th spermatheca join to form a common duct. The spermatheca are attached to the common oviduct with connective tissue fibres.

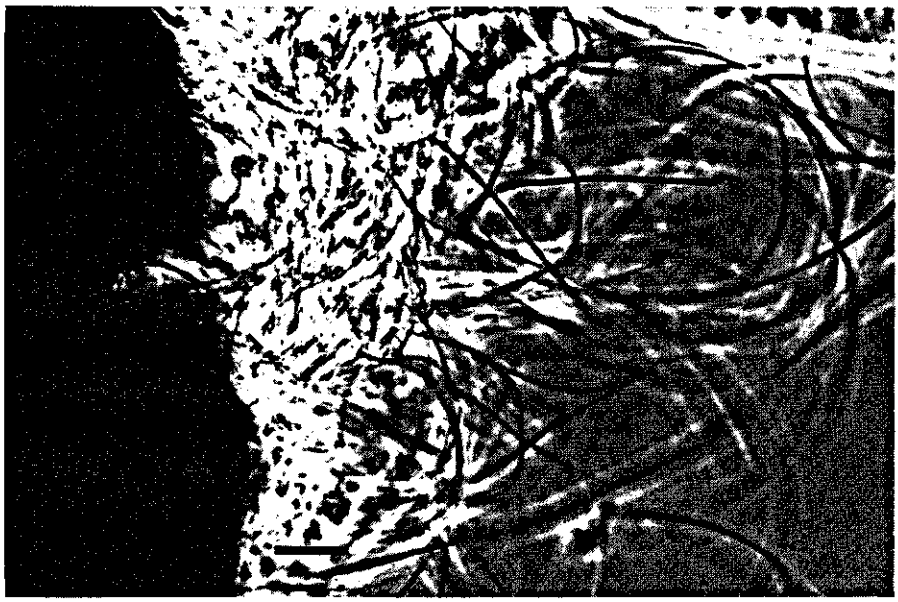


FIG. 5. Living sperm cells flowing from crushed spermatheca. Bar = 10 μ.

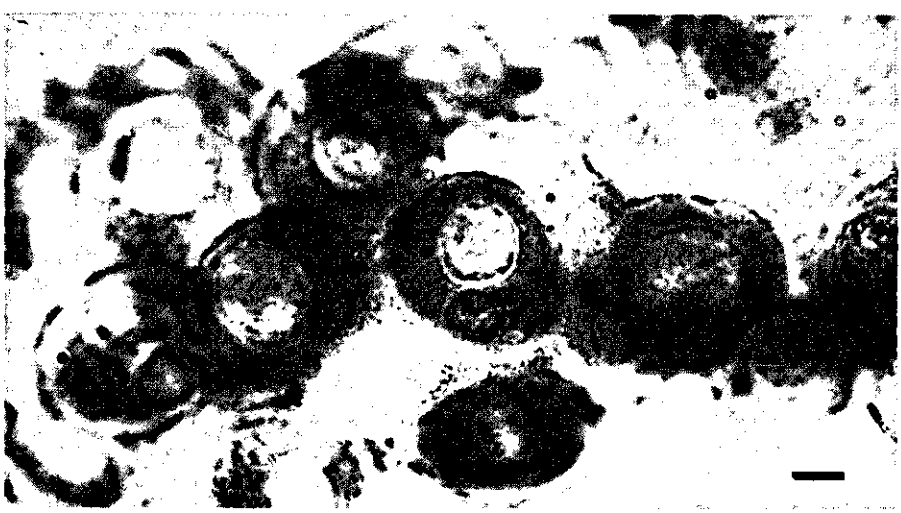


FIG. 6. Female accessory gland. Living spheroidal cells with large round vacuoles and dark nuclei. Bar = 10 μ.

The paired *accessory glands* consist of glandular cells arranged around two long central ducts which are distally connected to the lateral oviducts by means of connective tissue. The ducts open at the junction of the common oviduct and bursa copulatrix. Near the proximal end of the duct no glandular cells are found. The glandular cells contain a relatively large vacuole (fig. 6) which is

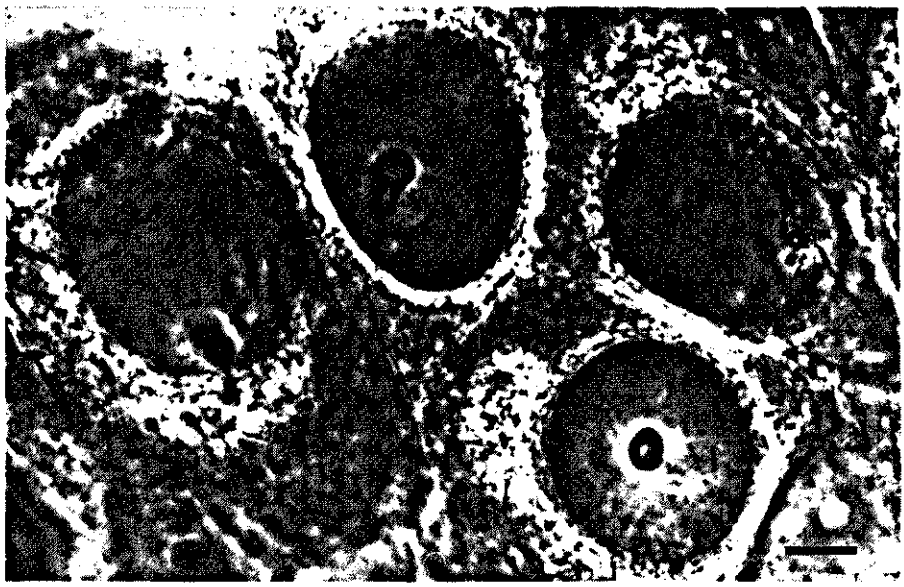


FIG. 7. Living cells of female accessory gland. The vacuole contains a dark structure which is the orifice of the canal which leads to the central duct. Bar = 10 μ.

connected to the central duct by a small, narrow canal (fig. 7).

Discussion

The structure and functioning of insect spermathecae has been well investigated in *Apis mellifera* (POOLE, 1970). A more complicated fine structure of the spermathecae was found in *Sitophilus granarius* (TOMBES and ROPPEL, 1972) in which a relatively large gland is attached to the spermathecae. In *Tenebrio molitor* the spermathecal ultrastructure was studied by HAPP and HAPP (1970), in *Periplaneta americana* by GUPTA and SMITH (1969) and in *Aedes aegypti* by CLEMENTS and POTTER (1967). Spermathecae and their ducts in Pyrrhocoridae were described by PLUOT (1970). JORDAN (1972) described spermathecal structures in *Glossina* spp.

3.3. CYTOLOGICAL OBSERVATIONS

Squash-preparations of young adult testes and occasionally other tissues have been made. Onion fly chromosomes stain well in a suitable lacto-aceto-orcein fixing and staining mixture (THEUNISSEN, 1971). Other methods were found to be inferior. Mitotic and meiotic chromosomes in the various stages of division were found abundantly. Polytene chromosomes of the larval salivary glands were present but appeared to be unsuitable for routine examination.

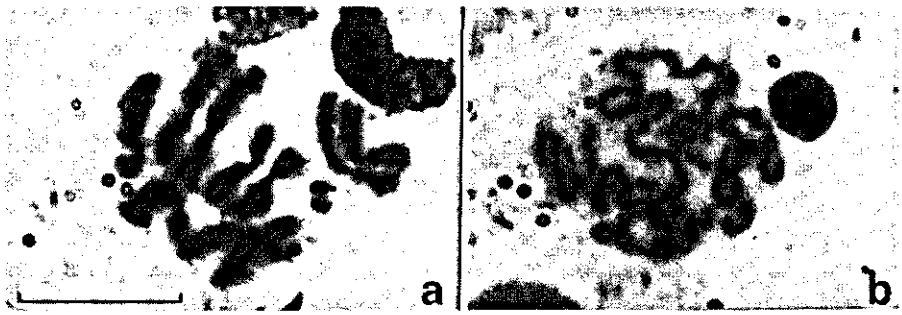


FIG. 8. a. Somatic pairing in dividing spermatogonium. b. Meiotic prophase I chromosomes of *H. antiqua*. Bar = 10 μ.

The karyotype of *H. antiqua* shows 5 pairs of sub-metacentric autosomes and 2–3 small sex-chromosomes. In mitotic metaphases somatic pairing is observed (fig. 8a). The mitotic activity in the young adult testis is very variable. Without any apparent reason the number of dividing nuclei in comparable testes may vary from a few (< 10) to very many. The same applies to the oogonia in the germarium of young females. During meiotic divisions of primary spermatocytes it appears that early prophase stages like leptotene and zygotene are lacking. Pachytene chromosomes may have been observed (fig. 8b) and diplotene chromosomes are present.

Discussion

The chromosomes of *H. antiqua* and other Anthomyiidae have been described by BOYES (1954), BOYES and SLATIS (1954) and BOYES and VAN BRINK (1965). The present observations confirm their findings. WIJNANDS-STÄB and VAN HEEMERT (1974) showed the sex-chromosomes to be two acrocentric X-chromosomes. A third small chromosome which was variably present was considered to be a B-chromosome (VAN HEEMERT, 1974). In a review BOYES (1967) gives an outline of the cytology of muscoid flies.

The somatic pairing already noticed by STEVENS (1908) and characteristic for Diptera has also been observed in *H. antiqua*. DOBZHANSKY (1934) reported the absence of early meiotic prophase stages in *Drosophila pseudoobscura* and considered this a general phenomenon in all Brachycera studied at that time. Earlier, METZ and NONIDEZ (1923) had observed the lack of a leptotene stage in *Asilus notatus*. In *H. antiqua* early meiotic prophase stages are very rare if not totally absent. The relatively short first meiotic prophase in *H. antiqua* may be related to the observed absence of DNA synthesis in primary spermatocytes, which seems to occur already in secondary spermatogonia. During primary spermatocyte interphase and early prophase, when usually DNA replication takes place (WHITE, 1973), this function may have been lost and shifted to the preceding germinal cell type. Concentration of chromatin material in the nucleus does not impair DNA synthesis (HENDERSON, 1964), contrary to RNA synthesis.

In squash-preparations only nuclei and nuclear elements are stained. Apart from the readily recognized chromosomes, interphase nuclei can be divided into the categories: somatic and germinal cell nuclei. In general somatic cell nuclei show more clear outlines as if their nuclear membranes are more heavily stained. Exceptions are the vaguely delimited nuclei of central cavity cells and the clearly outlined spermatids. Pre-meiotic germinal cell nuclei in interphase are very difficult to identify. Secondary spermatocytes are relatively rare while spermatids are easy to classify. Somatic nuclei like those of the apical cell may be recognized if their spatial arrangement has not been distorted too much. Central cavity cells are recognized as such because of their appearance and position amidst spermatids and sperm cells.

Concluding, the use of squash-preparations for a quick survey of both germinal and somatic testicular cell types to assess changes in those cell populations is hardly feasible. An exception to this might be the special case of cell populations which can be easily recognized.

3.4. Early post-embryonic development

Determination of the sex of young larval gonads is only possible after development of certain somatic cell structures at an age of 6–7 days. These structures, the apical cell and the apical somatic tissue in testes and ovaries respectively, originate from very simple gonads in newly hatched larvae.

The gonads of newly hatched larvae comprise a varying number of germinal cells, covered by an 'a-type' (Theunissen, 1973a) squamous epithelium. The germinal cells which show large and light nuclei with a regular chromatin dispersion, form an ovoid or pear-shaped cluster of cells (fig. 9). At the periphery of this small group of cells somatic cell nuclei are found, which constitute the covering epithelium. Somatic cells are also observed among the germinal cells. Mitotic activity in both cell types is low. Nevertheless, the number of both germinal and somatic cells increase. The latter tend to form a cubical epithelium around the germinal cells, locally even slightly stratified or packed in an irregular conglomerate of somatic cells. This situation can be observed in gonads of 3 day old larvae. The accumulation of somatic cells at the periphery of the gonads continues during the following days. The future oviduct or ductus deferens is seen as a massive strand of epithelial cells, which has no connection with the young gonad. At 5 days the mitotic activity seems to increase and the somatic cells continue to penetrate the population of germinal cells. Sometimes a row of somatic cells is observed infiltrating the intercellular spaces among the germinal cells. This observation does not necessarily mean that the gonad concerned is a testis because this does not always lead to the formation of an apical cell. Thus, the gonad cannot be sexed properly until a distinct apical cell or the beginning of the female apical somatic tissue is being formed. This happens usually in 6–7 days old larvae. Gonads of larvae of that age will be included in the descriptions of larval development of, respectively, the testis and the ovary.

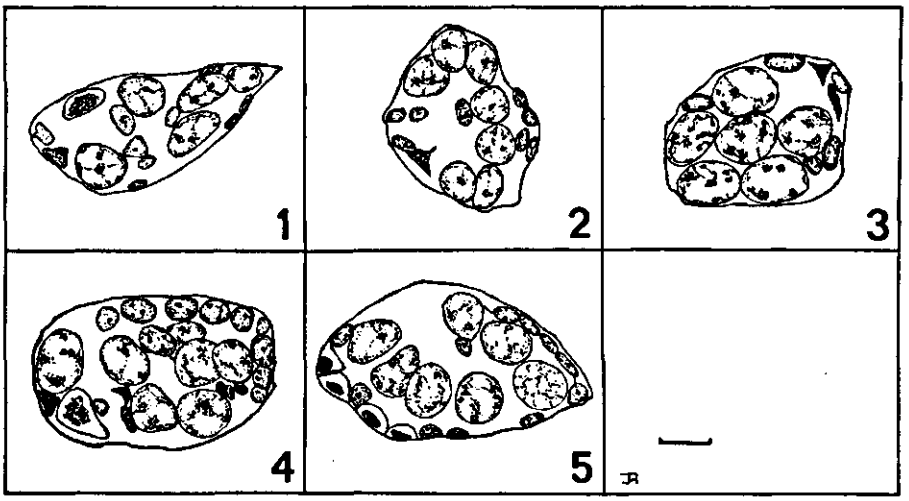


FIG. 9. Young larval gonads. The numbers indicate the age in days of the larvae from which the gonads were taken. Bar = 10 μ.

Discussion

The rate of the developmental processes from hatching through the larval or other immature stages varies considerably in different species and orders. For instance, in *Bombyx mori* SADO (1963) mentions that 3 days after hatching spermatogonia have been differentiated. The same has been reported for *Trichoplusia ni* (HOLT and NORTH, 1970). In many insect species the sex of the gonads can be recognized early in post-embryonic development, e.g. in the larva of *Drosophila melanogaster* in which one day after hatching the apical cell can be recognized (ABOIM, 1945; COOPER, 1950).

3.5. SPERMATOGENESIS

3.5.1. *Introduction*

Aspects of insect spermatogenesis provide a fascinating variation of biological problems. After the establishment of the general principles of spermatogenesis during the last part of the nineteenth century and the discovery of cell organelles and other cellular structures a widely adopted terminology denoting the germinal cell types involved was introduced by LA VALETTE ST. GEORGE (1876). Later, the attraction of geneticists was focussed on insect spermatogenesis. At first the morphology and the behaviour of the chromosomes was studied, but following the pioneering work of Muller, Bridges and Morgan with *Drosophila* spp. interest was attracted to these species, especially to *D. melanogaster*, leading to more basic genetic studies. Until recent times these studies often resulted in contributions to the knowledge of insect spermatogenesis, frequently as a mere by-product.

Reviews on aspects of the animal spermatogenesis have been published by DAN (1956), NATH (1956), HANNAH-ALAVA (1965), MANN (1969), BURGOS et al. (1970), COUROT et al. (1970), PARKER (1970) and CLERMONT (1972). Reviews which are more specialized on insects are those by: DEPDOLLA (1928), DAVEY (1965), BLUM (1970), ENGELMANN (1970) and DE WILDE and DE LOOF (1973).

The present study of the spermatogenesis of *H. antiqua* deals with the identification of somatic and germinal cell types and structures including a study of living cells and ultrastructural elements. The testicular ontogenic development, the dynamics of spermatogenesis and some aspects of comparative spermatogenesis have also been treated and discussed in this order.

3.5.2. *Identification of testicular cell types*

When studying any aspect of insect spermatogenesis it is imperative to be able to identify the cell types involved to establish a frame of reference.

The technical basis for the identification of cell types has already been discussed. These cell types will now be described in order to define the properties and limits of each of the cell groups concerned.

A general distinction can be made according to the origin of the cells. A minority of the cells are descendants of the primordial germ cells of the embryo. These cells originate from the pole cells, at least in *Drosophila melanogaster* (HUETTNER, 1923; ABOIM, 1945). The pole cells were discovered and named by WEISMANN (1863). During the first part of egg development the pole cells are set apart from the developing embryo to be incorporated at a later stage and to form the primordial germ cells (HUETTNER, 1940). An extensive account of insect embryogenesis has been given by COUNCE (1973). The group of cells derived from the primordial germ cells are named the *germinal cells*. They take a main part in the functioning of the gonads by becoming spermatogonia, oogonia and trophocytes. All other cells in these organs are *somatic cells*, which are the descendants of the other embryonic cells. They play an important role in the functioning of the gonads by forming specialized structures which protect and maintain the developing germinal cells.

Together the germinal and somatic cells are integrated in the functioning gonad, each in its own position and prospective role. Therefore, a short outline will be given of the structural organization of the young adult testis before the germinal and somatic elements are defined.

3.5.2.1. Structural organization of the young adult testis

In the testis of a newly emerged adult, an internal structural organization can be discerned. This organization is related to the spatial arrangement of the somatic and germinal elements of the testicular cell populations. Although the somatic and germinal cells are distinguished in a way by the requirements of their respective functions, they are mutually adapted to form the unity of the functioning organ. In this sense the germinal elements constitute the contents of the organ and the somatic elements the feeding, protecting and covering envelope. Both elements depend on each other to assure a normal development.

In agametic testes the whole somatic structure is present but highly abnormal in shape, rate of development and size.

In the most apical part of the young adult testis (fig. 10) the *apical cell* is found, surrounded by a population of *spermatogonia*. These spermatogonia do not seem to be enclosed by membranes. This population is bordered by an usually incomplete narrow zone of cavities, which sometimes contain single spermatogonia or multinucleate cells. These cavities are named: *apical cavities* (fig. 10q). Basally of the apical cavities another population of spermatogonia is observed which is organized in groups of cells: the *cysts*. All cells within a particular cyst are supposed to descend from a single spermatogonium. *H. antiqua* shows a weak development of the cyst epithelia, which can impair the recognition of cells as belonging to a particular cyst as well as the definition of the spatial boundaries of the population of spermatogonia and their derivatives, the *primary spermatocytes*. The primary spermatocytes are found basally to the spermatogonia and laterally against the testicular sheath. They enclose the most apical part of a cavity which is situated in the centre of the basal half of the testis. This cavity is named: the *central cavity* (fig. 10r). Groups of dividing primary spermatocytes and their descendants: *secondary spermatocytes*, *spermatids* and *sperm cells* surround the central cavity. The centre or core of the central cavity is occupied by a more or less globular mass of cell debris, cytoplasmic droplets and undefinable cellular remnants and substances. Round this core normal somatic and germinal cells and cell populations occur and develop.

In the basal part of the testis the groups of spermatids and sperm cells lose their cyst membranes which gradually become thinner and ultimately seem to dissolve. The sperm cells lie in bundles of varying size and density, both in the most basal part of the testis and in the central cavity.

In the young adult testis of *H. antiqua* a zonation of the germinal cell populations can be roughly observed, although this is far less distinct when compared to testes with tubular follicles such as are found in Orthopterans.

3.5.2.2. Germinal cell types

In the young adult testis a number of germinal cell types can be observed. Their proper identification is a key factor in studies of spermatogenesis of *H. antiqua*, which is also true in a more general sense. These cell types are: primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, early spermatids, intermediate spermatids, transformation spermatids and sperm cells (fig. 10, a–j). They will be described in this sequence, as this is the way they descend from each other.

To establish the identity of germinal cell types various approaches have been used. Cell types in histological preparations have been compared to those in semi-squash preparations and to living cells in vitro. Ultrastructural aspects of the cells have been investigated to verify their histological and morphological appearance.

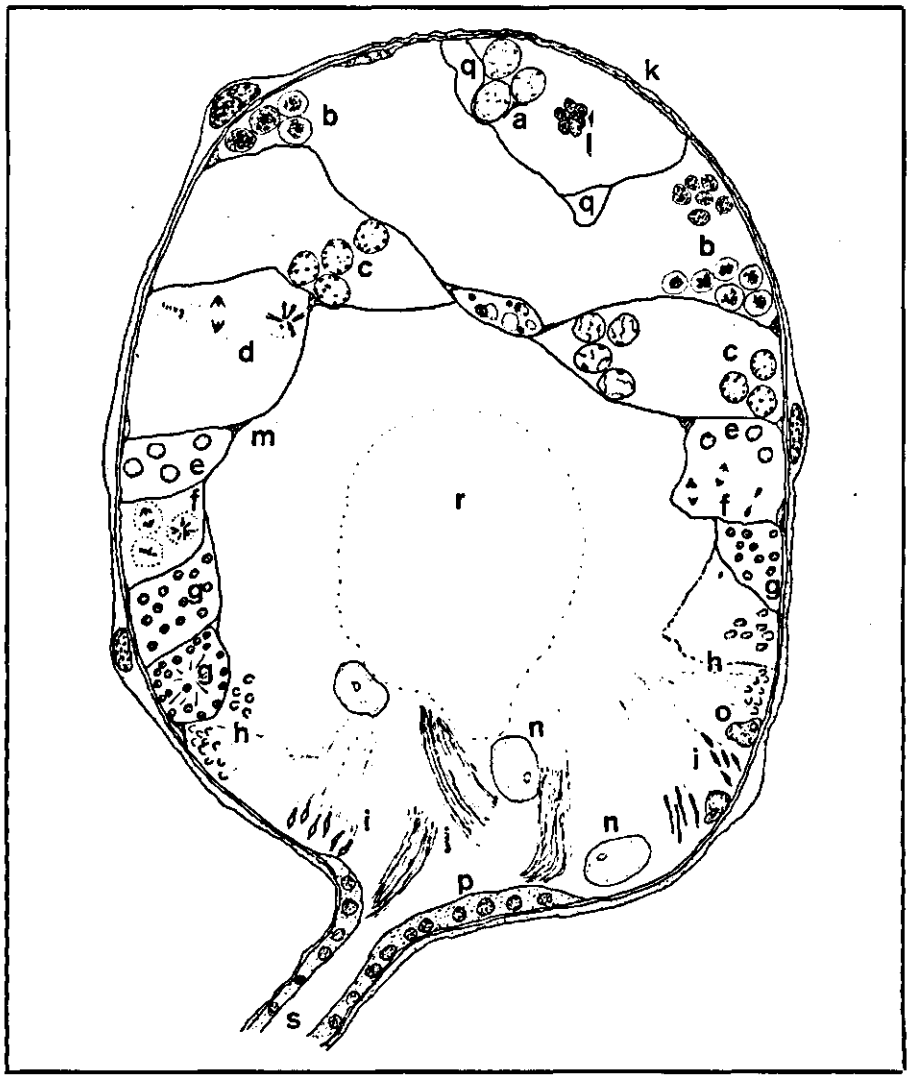


FIG. 10. Semi-schematic drawing of the young adult testis depicting all important types of germinal and somatic cell nuclei and other structures.

Germinal elements:
a. primary spermatogonia
b. secondary spermatogonia
c. primary spermatocytes
d. first meiotic divisions
e. secondary spermatocytes
f. second meiotic division
g. early spermatids
h. intermediate spermatids
. transformation spermatids
j. sperm cells

Somatic elements:
k. testicular sheath
l. apical cell
m. cyst cells
n. central cavity cells
o. basal cells
p. terminal epithelium
q. apical cavities
r. central cavity
s. ductus deferens

3.5.2.2.1. Primary Spermatogonia

The primary spermatogonia are cells which are situated in the most apical part of the testis around the apical cell. They can be characterized as follows. In the cells the nuclei are very dominant, the cytoplasm being a thin border around the ovoid or pear-shaped nuclei. The sizes of the nuclei range from 9.5–12.7 μ, with an average of 10.5 μ. The chromatin is regularly dispersed in loose, well stained floccules of varying sizes which are sometimes connected by fine filaments giving the nucleus a very light appearance in contrast with the surrounding cytoplasm. Finely granular and filamentous chromatin elements are observed also but they are small and inconspicuous.

The foregoing description fits the 'diffuse' type of primary spermatogonia which form the majority during some developmental stages of the testis.

The dispersion and organization of the chromatin in the nuclei can, however, also be of a more coarse nature. In that case the chromatin is organized in small, rounded concretions which lie against each other or are mutually connected by filaments. The units of connected chromatin concretions tend to lie round the centre of the nucleus (fig. 11), often roughly in a circle.

A sometimes considerable number of these spermatogonia show nuclei in which the chromatin is concentrated in a part of the nuclear lumen, leaving the remainder of it optically empty. These cells represent the 'concentrated' type of primary spermatogonia, whereas the preceding description referred to an intermediate type between 'diffuse' and 'concentrated' spermatogonia (fig. 11). All types of primary spermatogonia may show signs of mitotic activity. They divide individually but occasionally also in small groups of variable numbers. Formation of cyst membranes is hard to establish but has never been observed.

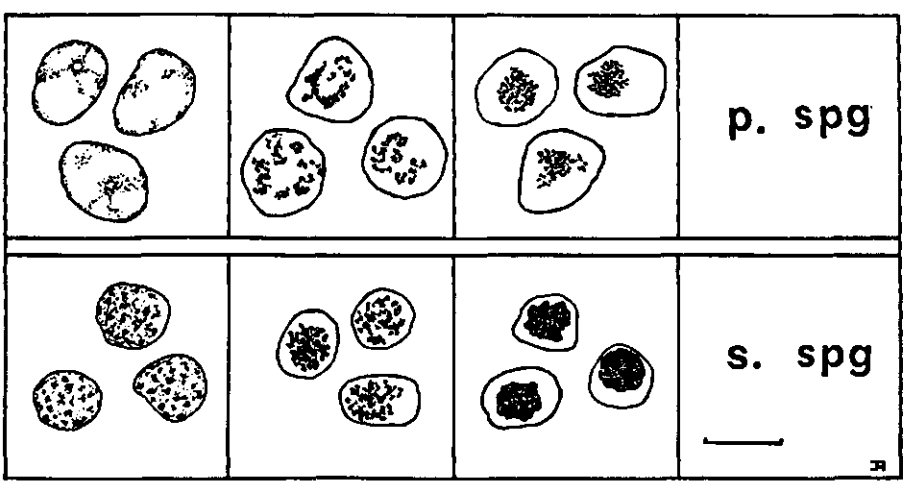
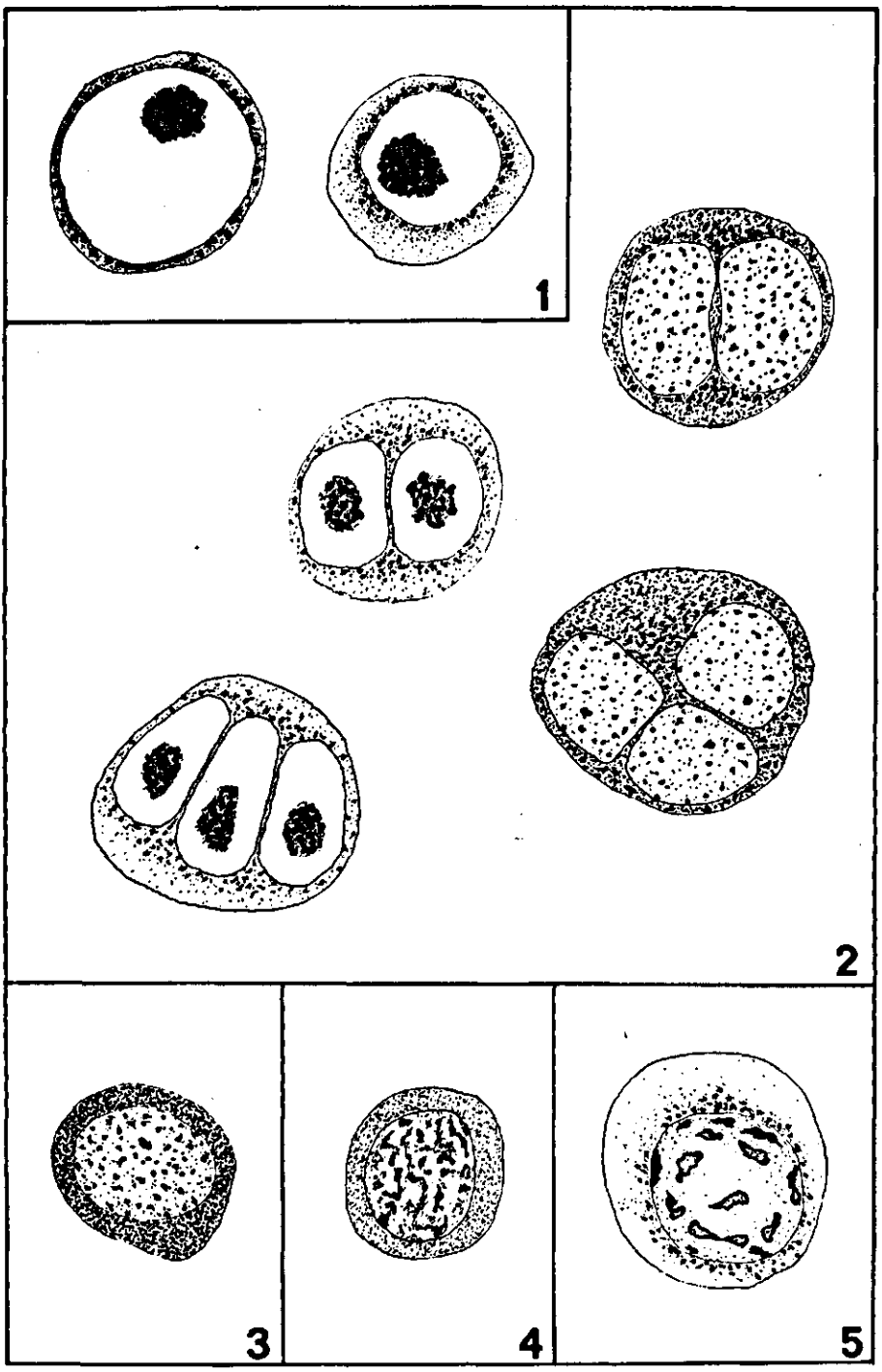


FIG. 11. Some types of nuclei within the range of variation of both the primary spermatogonia and the secondary spermatogonia. Bar = 10 μ.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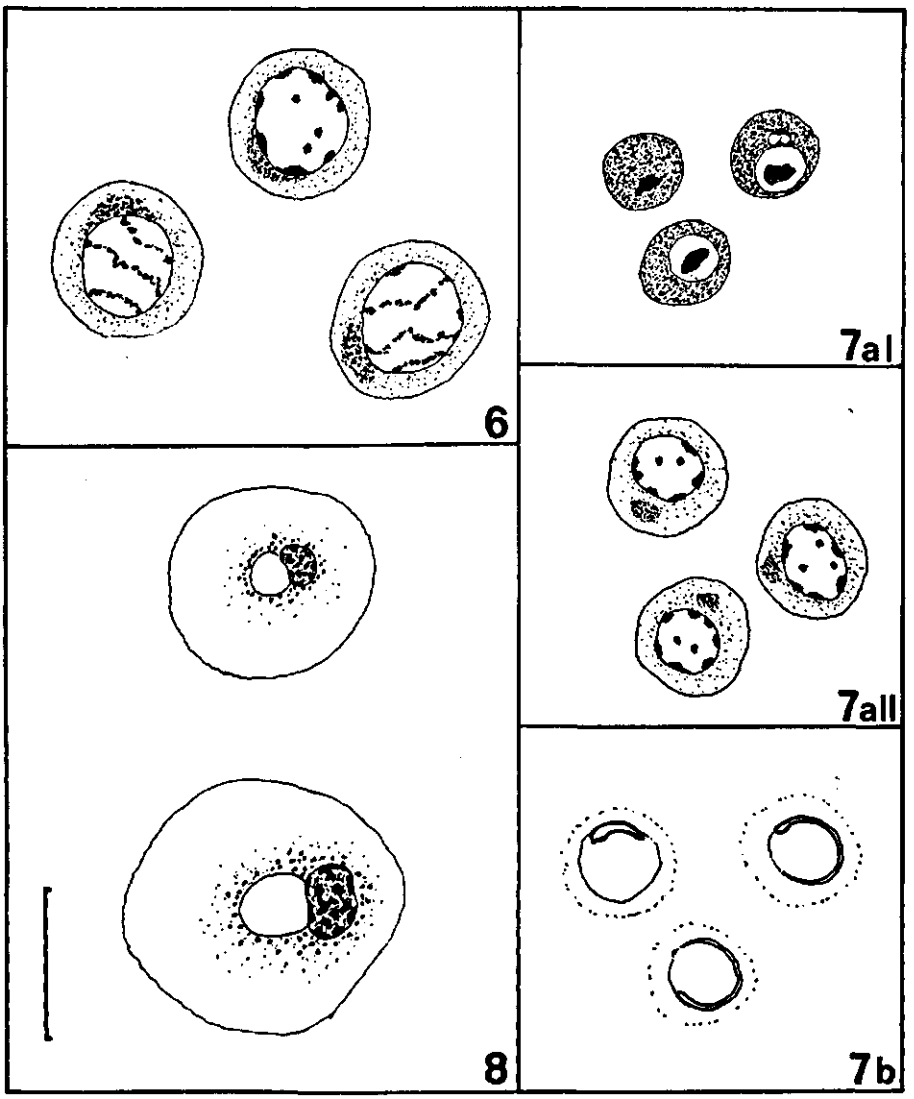


FIG. 12. Drawings of living testicular cells as seen by phase contrast optics. Description of cell types in text.

1. 'concentrated' *spermatogonia*
2. bi-nucleate and tri-nucleate cells of 'concentrated' and 'diffuse' type
3. interphase primary spermatocyte
4, 5. spermatocytes in meiotic prophase
6. secondary spermatocytes
7. spermatids
   a. early spermatids
      I. vere early
      II. more advanced
   b. intermediate spermatids
8. unidentified cell type (cf. 3.5.2.3.8.).

Note: The size of the observed living cells will vary due to a.o. the pressure of the coverslip on the cells.

Primary spermatogonia seem to be present as single cells, although bi-nucleate and multinucleate cells are found in their population.

The cytoplasm of all primary spermatogonia is mostly difficult to discern, but is sometimes visible as a dark margin which partially or totally surrounds the nucleus. In the nuclei, nucleoli are difficult to observe. The chromatin dispersion in the nuclei is quite variable, tending to avoid the nuclear periphery.

The primary spermatogonia generally form a relatively compact mass of cells as compared to other germinal cell populations. The number of cells involved depends on stage of development and age. The primary spermatogonia as a category can always be recognized by being lighter in appearance and more tightly packed. The apical cell is surrounded by the mass of gonial cells without apparent structural and functional relationships between the two cell types. The spermatogonia are mixed with small triangular or flattened somatic nuclei and often an association between a spermatogonium and one or more somatic cells seems to exist when both cell types form a small cluster (fig. 20a).

Viewed at moderate magnification the spermatogonia are recognized very easily by:

1. lightly stained and large nuclei
2. compactness
3. position in the testis.

When identifying germinal cells such as primary spermatogonia one should take into account these three criteria.

Among the living germinal cells in the testis primary spermatogonia as such are not recognized unambiguously. 'Concentrated' spermatogonia i.e. those with concentrated chromatin in the nuclei are frequently observed. They conform to the following description (fig. 12, 1):

Cells with large nuclei in which the chromatin is more or less concentrated in a part of the nucleus. The degree of concentration may vary but in many cells the chromatin is extremely concentrated. The nucleus is surrounded by an often narrow brim of cytoplasm in which the concentration of mitochondria is largest close to the nuclear membrane. Cells from this group may also exhibit a variation of size and nucleus/cytoplasm-ratio.

This cell type exactly fits the descriptions of most secondary spermatogonia and 'concentrated' primary spermatogonia. The varying degree of chromatin concentration in fresh preparations of living cells show the later described secondary spermatogonia not to be artefacts. Therefore, this type is classified as 'concentrated' spermatogonia.

Basally the mass of primary spermatogonia is bordered by apical cavities of various size and shape which, however, can be absent at large parts of the border between primary spermatogonia and other germinal cells. Many of the apical cavities are empty, but a relatively large number of them contain individual primary spermatogonia and gonial cells which for some reason exhibit pathological features, e.g. chromatolysis or hyperchromatosis. Multinucleate cells are also frequently observed in apical cavities.

In living testes multinucleate cells are abundantly found, especially bi-nucleate

cells. The large nuclei of the bi-nucleate cells fit in the cytoplasm with a slightly concave inner side and a convex outer side (fig. 12. 2). The chromatin in the nuclei may be more or less concentrated or evenly dispersed in finely granular or filamentous units. Sometimes nucleoli can be observed. The nuclei with concentrated chromatin tend to be somewhat smaller when compared to those of other multinucleate cells. Also the cytoplasm is more transparent in cells showing the bright nuclei with concentrated chromatin. The latter nuclear type is more frequently observed. Tri-nucleate cells are often found and less frequently cells containing five or seven nuclei.

Multinucleate cells are seen in very fresh preparations of living testicular cells and in histological preparations of both *H. antiqua* and *M. domestica*. Their frequent presence in relatively large numbers indicate an essential function in the spermatogenic processes. The always identical morphological appearance of the nuclei in these cells show the same range of 'concentrated' and 'diffuse' types as do the primary spermatogonia. In relatively undisturbed testes the multinucleate cells are found in or near the apical cavities in spermatogonial cell populations. These observations result in the classification of the multinucleate cells as being spermatogonia.

Although the primary spermatogonia as a cell population are readily identified, individual cells may cause difficulties in this respect because of the infinite

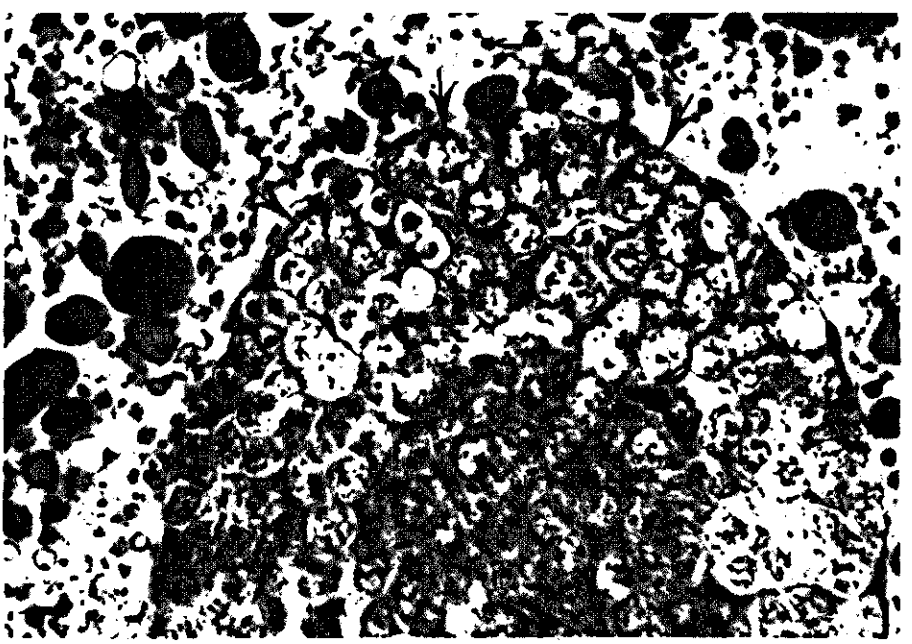


FIG. 13. Apical part of testis of a newly emerged fly, dominated by 'intermediate' primary spermatogonia (arrows). A small number of 'concentrated' primary spermatogonia is also observed. Apical cavities are absent here. Bar = 10 μ.

number of variations in chromatin structure and dispersion. In particular the 'concentrated' type of primary spermatogonia is morphologically completely identical to the 'concentrated' type of the next spermatogonial cell type.

Discussion

The most apically situated population of spermatogonia, which is referred to here as primary spermatogonia, is characterized by its variable nuclear morphology i.e. pattern of chromatin appearance and dispersion, its density and position in the testis (fig. 13) and its being composed of single and multinucleate cells. Other properties which distinguish this population of spermatogonia from another one can not be derived from the description of a static situation but emerge during the study of testicular development and reactions on external factors like e.g. irradiation.

The variable pattern of chromatin appearance and dispersion is not exclusively found in primary spermatogonia but these cells provide an example of striking differences in this respect. The type which is referred to as 'diffuse' is already found in young larval testes and will remain present continuously till in the senescent adult. It represents the basic type of germinal cell. Cells with strongly concentrated chromatin are named the 'concentrated' type (fig. 14), not to be confused with cells in some stage of division or with pathologic cells.

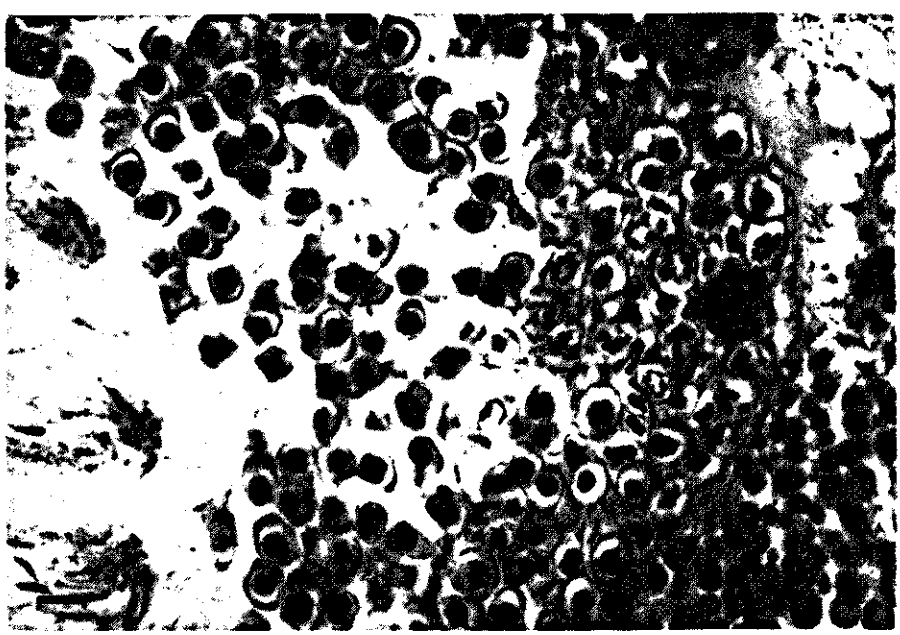


FIG. 14. Apical part of testis of a newly emerged adult. Apical cell is distinct and surrounded by both 'intermediate' and 'concentrated' primary spermatogonia. More basally 'concentrated' secondary spermatogonia are present in large numbers. They are less densely packed. Bar = 10 μ.

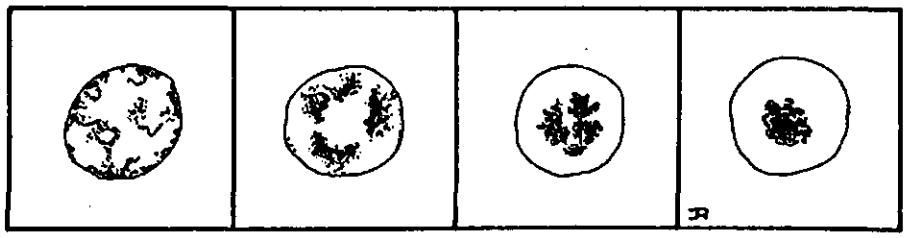


FIG. 15. 'Diffuse' and 'concentrated' primary spermatogonia (left and right respectively) and transitional nuclear types.

The structure and dispersion of the chromatin are important elements in the nuclear morphology of cells, apart from size and shape. These characteristics of the chromatin may reflect the state of activity of the cell, not only in relatively clear cases like mitotic or meiotic divisions, endomitotic processes and polyteny of chromosomes but also in cases which represent less well defined processes. The occurrence of cells showing chromatin concentration is well synchronized with relatively active periods during testicular development, from the early larval stage until the spermatogenic processes are completed in the mature adult. In such periods the proportion of 'concentrated' spermatogonia in the entire population increases sharply sometimes up to about 100%, followed later by a decrease. This type of interphase cell is morphologically similar to some other forms of cells e.g. the bouquet-stage which is a meiotic prophase stage which occurs in some animals or pycnotic cells in which the structure of the chromatin is lost. 'Concentrated' spermatogonia always show more or less densely packed but structured chromatin. It has been established that a transformation of 'diffuse' into 'concentrated' spermatogonia and vice versa takes place, resulting in an infinite number of intermediate forms (fig. 15).

As to the significance of this chromatin transformation nothing is known with certainty. However, it can be established that these types are not prophase primary spermatocytes, cells in degeneration or technical artefacts. They are observed in spermatogonial populations in perfectly normal and obviously healthy specimens and they occur in large numbers in fresh and living spermatogonial populations. It is supposed that the 'concentrated' appearance of the nucleus represents an expression of a physiological condition which differs from that resulting in the appearance of a 'diffuse' spermatogonial cell type. Both the 'diffuse' and 'concentrated' types are considered to be interphases which may proceed towards a mitotic or meiotic division. There is no evidence that the 'concentrated' type represents some mitotic or meiotic stage, although the relative presence of 'concentrated' types and mitotic activity may increase simultaneously. This is often observed, possibly as an indication of an increasing activity in the cell population concerned.

Living cells from the young adult testis of *H. antiqua* and, sometimes, by way of comparison, of *Musca domestica* have been examined by means of phase contrast optics in order to establish the presence or absence of cell types or

structures seen in histological preparations and to complement histological identification criteria. Another reason was to investigate the possibilities to assess radiation and other effects on living cells.

The populations of living testicular cells present a striking picture of beauty, brilliance and variability in appearance. Because of the latter property many cells can not be considered to belong to a particular group of cells, which can be described as such. Some categories of cells, however, can be recognized and have been described.

The presence of multinucleate cells both in fresh and in fixed preparations excludes the possibility of them being artefacts. Living germinal cells (fig. 16) show a tendency to fuse when exposed to stress. Fusion of individual cells of several cell types have been observed to take place. This can happen easily but occurs strictly with cells of the same type, suggesting a form of mutual recognition of the cells. Multinucleate cells of the same type may also fuse to a larger complex. When two similar cells are pressed against each other by crowding of other cells and by applying pressure to a group of cells under a coverslip the common border shows a bright, strongly refractory lining. At increasing pressure the borderline becomes longer till it suddenly disappears. Concomitantly the cell membranes of the two cells unite at both ends of the line and the nuclei are within a split second in the same cytoplasm. When the pressure is relieved before fusion has taken place the common border decreases till the cells are free. The fusion process has been observed to take place in a quick succession resulting in the formation of large multinucleate cell complexes (fig. 19).

An attempt to explain the fusion process may be made in assuming altered properties of the cell membrane and/or intercellular bridges by the stress situation, in this particular case the combined effects of anoxia and mechanical pressure in the preparations.

It must be pointed out that the fusion process does not represent an essential change in the intercellular relationships of cells of a particular cyst because they already constitute a syncytium by their intercellular bridges. The mutual 'recognition' system evidently prevents fusion of cells not belonging to the same group, family or cyst.

Induction of cell fusion caused by the introduction of virus particles has been observed in cultured mammalian cells (KOHN, 1965; OKADA, 1962; OKADA and MURAYAMA, 1968). Other fusion inducing factors have also been reported (AMAROSE and CZAJKA, 1962). In the mouse multinucleate spermatids are found as a normal feature (BRYAN and WOLOSEWICK, 1973; JOHNSON, 1974), whereas in insects the presence of multinucleate cells of various germinal types has been recorded in *Drosophila melanogaster* (ÅBRO, 1964). Apart from *H. antiqua*, the presence of multinucleate cells and the formation of large multinucleate complexes has also been found by me in *M. domestica*.

As for the presumed manner of spermatogonial multiplication a number of possibilities can be mentioned. Multiplication of the spermatogonia according to the 2^n system is though to take place by association of primary spermatogonia with a cyst cell, whether or not preceded by a bi-nucleate cell stage. At the same

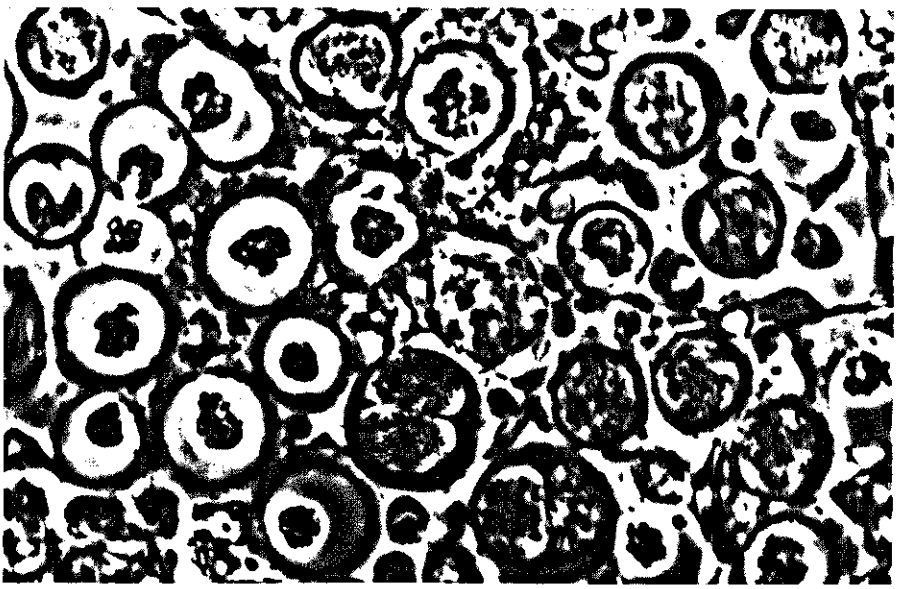


FIG. 16. Living testicular germinal cells, mainly spermatogonia (Fig. 12,1) and bi-nucleate cells (Fig. 12, 2).

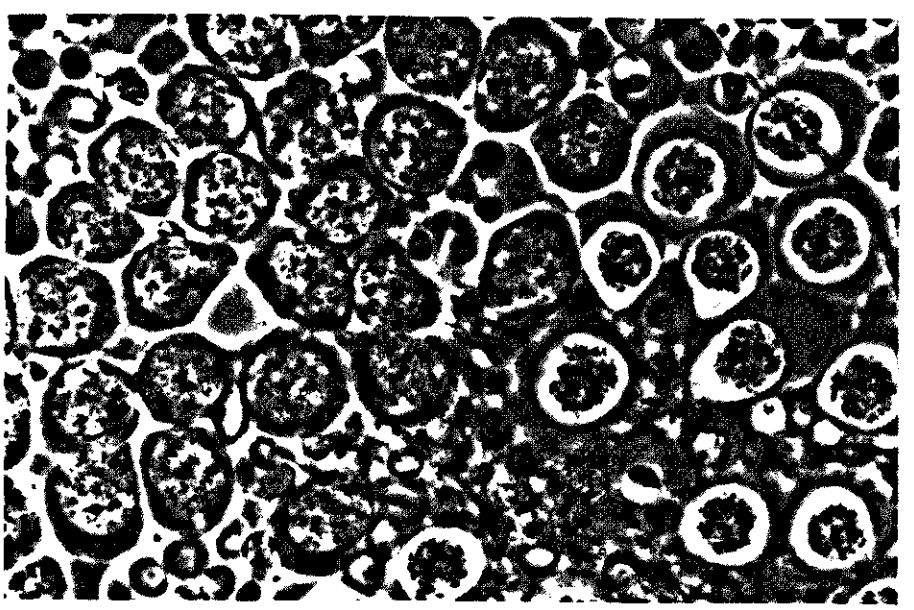


FIG. 17. Living testicular germinal cells, partly spermatogonia. (Fig. 12. 1). partly primary spermatocytes (Fig. 12, 3).

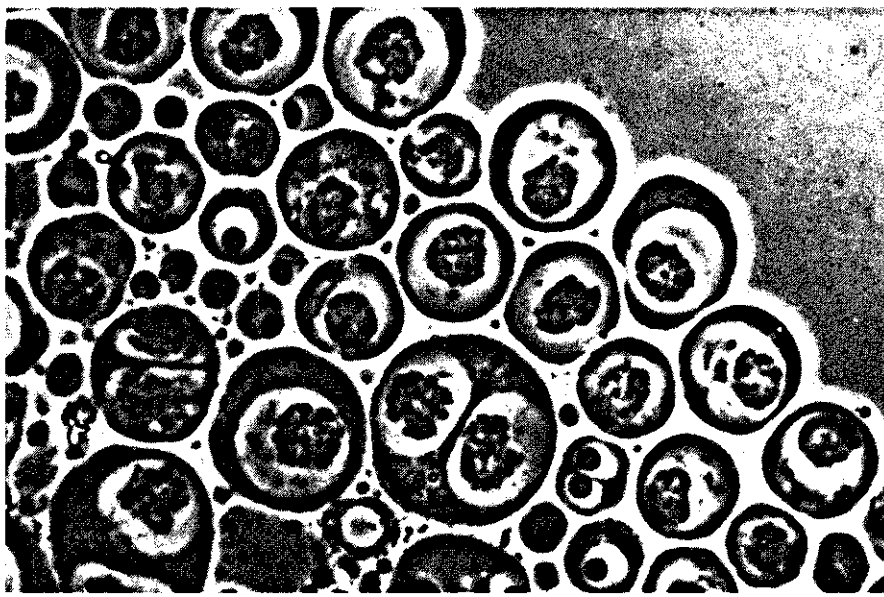


FIG. 18. Living spermatogonia as single or bi-nucleate cells.

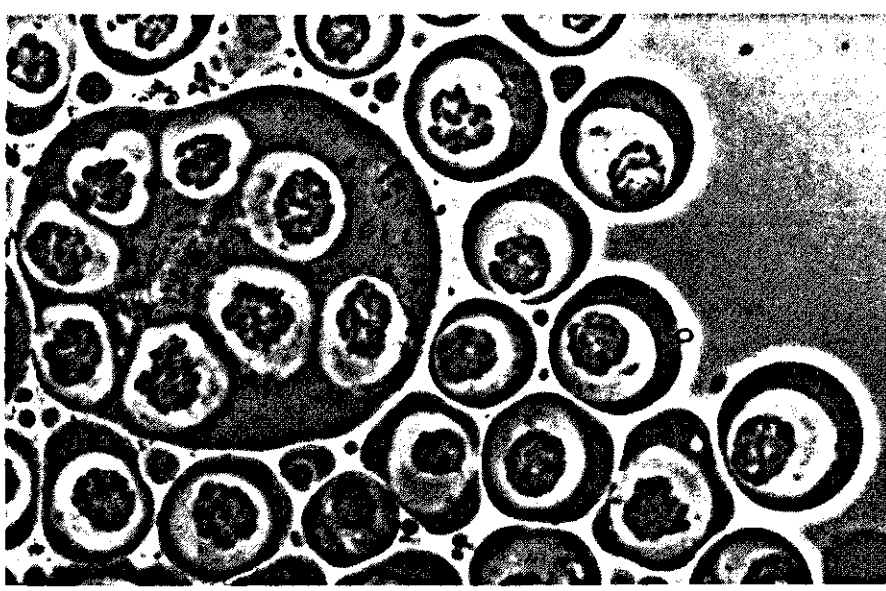


FIG. 19. Living spermatogonia as single or multinucleate cells. Figs. 16–19 same magnification as Fig. 4.

time other multiplication systems may be used e.g. 3.2^n or 7.2^n, in which the apical cavities and multinucleate cells play a role. This matter is discussed in some detail in paragraph 3.5.4.2.

3.5.2.2.2. Secondary Spermatogonia

This germinal cell type can be defined as comprising all spermatogonia except the primary spermatogonia. It is realized that this definition is quite unsatisfactory without additional comment.

In a young adult testis the secondary spermatogonia occupy roughly the greater part of the testicular apical half, between the apical cavities and the population of primary spermatogonia and the primary spermatocytes (fig. 10). The cells have ovoid nuclei, surrounded by a sometimes dark but mostly indistinct narrow zone of cytoplasm. In the nuclei the chromatin which is usually rather coarse and granular, sometimes containing strands and larger concretions, is mostly concentrated in a part of the nucleus. This concentration occurs in various degrees (fig. 20b). Because of the variable concentration of the chromatin, the population of secondary spermatogonia may have an irregular and sometimes seemingly chaotic appearance (fig. 21). The typical 'concentrated' cells are at superficial observation easily mistaken for pycnotic cells.

A type of secondary spermatogonia shows an even dispersion in the nucleus of moderately coarse chromatin particles of varying size (fig. 13, bottom). The size of the nuclei is similar to that of the 'concentrated' type nuclei of secondary spermatogonia.

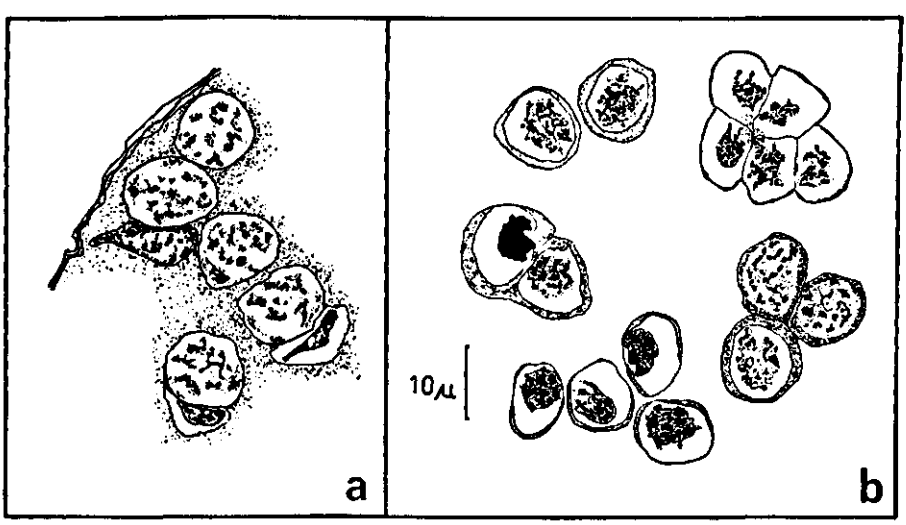


FIG. 20. Cells of primary spermatogonia (a) and secondary spermatogonia (b). The primary spermatogonia often show a kind of association with somatic cells which are mostly of 'type b'. The secondary spermatogonia may have a very varied appearance of which some examples are depicted.

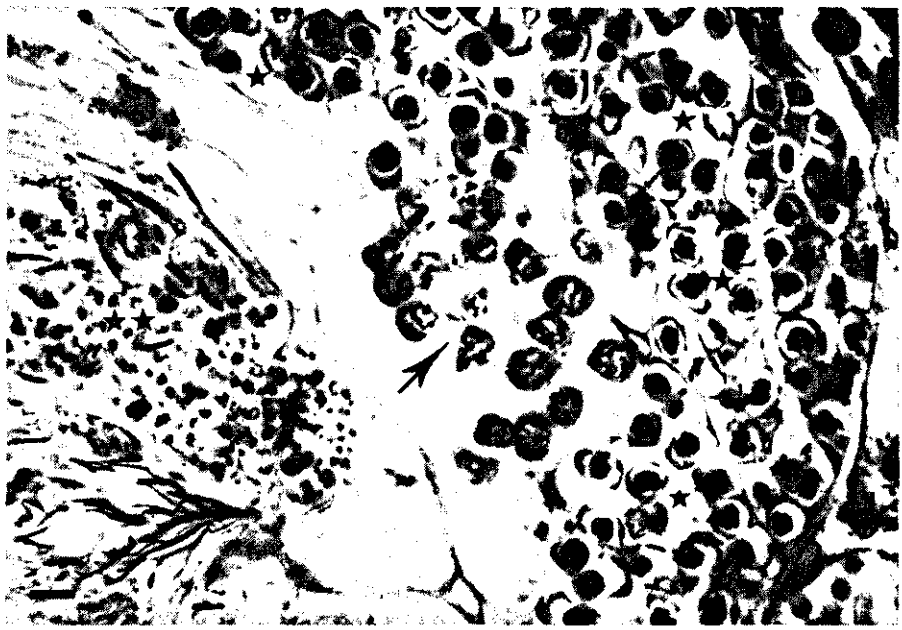


FIG. 21. Testis of newly emerged fly showing secondary spermatogonia (*) and a cyst of primary spermatocytes (arrow). Note a part of the core of the central cavity with waste products (**). Bar = 10 μ).

Between the described forms of secondary spermatogonia intermediates occur (fig. 20b). The 'diffuse' type of secondary spermatogonia has been observed in irradiated testes but is absent in normal, untreated ones. In general the nuclear size of the secondary spermatogonia seems to be somewhat reduced as compared to the primary spermatogonia but the difference is small.

The secondary spermatogonia are situated in cysts. Between the cysts of these spermatogonia small cavities or empty spaces are usually found. Within the cysts the cells do not always occupy all the available space. The liberal use of available space in this region of the testis gives the impression of the secondary spermatogonial populations as loosely packed cell families (fig. 14). In this respect a considerable variation exists. For instance, tightly pressed nuclei of secondary spermatogonia may constitute a large, dark mass of spermatogonial cells in the upper part of the testis (fig. 13) but these situations are exceptions to the general rule of a more spacious arrangement. The cysts are surrounded by one or more cyst cells and are interspersed with small somatic nuclei.

When observed at a moderate magnification, the secondary spermatogonia as a cell population are readily identified by:

1. relatively dark staining nuclei with more or less concentrated chromatin
2. relatively loose packing
3. position in the testis.

Mitotic divisions take place synchronously per cyst. No indications have been found pointing towards any synchronized mitotic activity between cysts. Mitotic activity in secondary spermatogonia is the highest in the testis when compared to other germinal cell types, with the possible exception of the secondary spermatocytes.

Living secondary spermatogonia can not be distinguished as such. 'Concentrated' types of spermatogonia have already been described and depicted (fig. 12, 1; figs. 16, 17, 18 and 19). Since living secondary spermatogonia in the normal testis are represented by cell types with concentrated chromatin ('concentrated' types) or evenly dispersed more or less coarse chromatin particles they are indistinguishable as such from 'concentrated' primary spermatogonia and primary spermatocytes respectively. Living cells which may represent a type of secondary spermatogonia but are more likely primary spermatocytes will be described in the next paragraph (cf. fig. 12, 3 and 4).

The ultrastructure of the spermatogonia of *H. antiqua* is characterized by the following features.

The nuclei are irregular in shape with many projections into and invaginations from the surrounding cytoplasm. The chromatin is found in concretions of varying but limited size and is regularly dispersed in the nucleus, causing

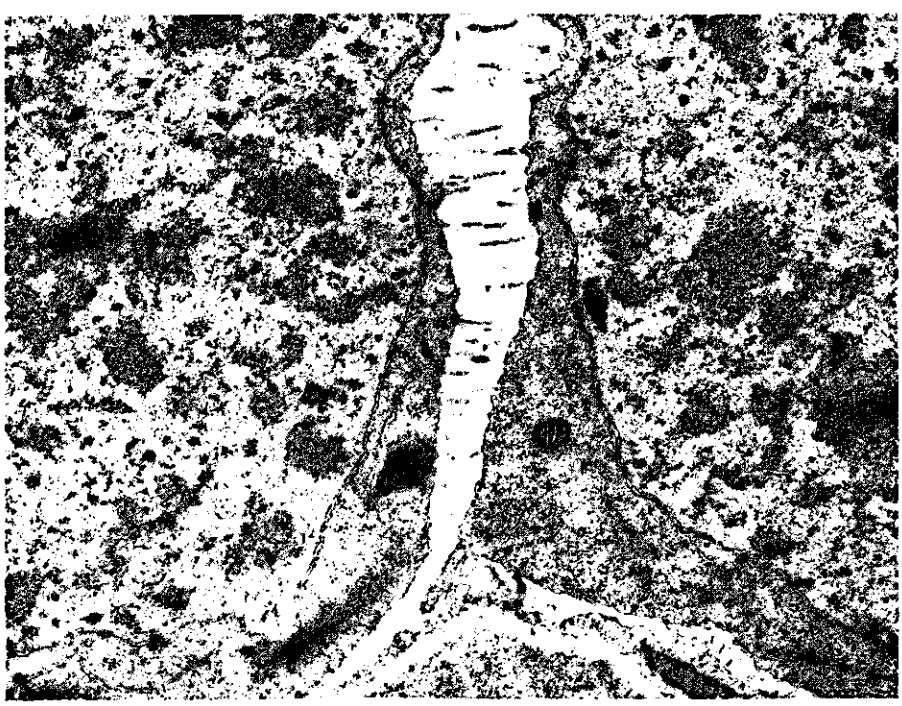


FIG. 22. Projections of the cell wall of spermatogonia in the intercellular space. Note the regular dispersion of the chromatin and the absence of cytoplasmic structures, 22,079 x.

a relatively light contrast. The cytoplasm is more compact as compared with the somatic cell cytoplasm but poor in structures. The cytoplasm seems to consist of a fine ground substance in which large numbers of minute granules can be observed, presumably representing single and free ribosomes. The endoplasmic reticulum (ER) is weakly developed, occasionally showing some membranes and membranous structures. The mitochondria are ovoid-elongated and small (about 225 × 150 nm). They seem to be randomly distributed in the cytoplasm. Golgi-complexes are indistinct and scarce. The plasmalemma is irregular and extensions of the cells are seen as thin layers between other cells. The plasmalemma forms often finger-like projections into the intercellular space (fig. 22). This is a form of surface extension of the plasmalemma, possibly related to nutrition or other exchange of matter. Intercellular bridges connect the spermatogonia. The darkly contrasting margins of these structures are folded, thus permitting by stretching a certain flexibility of the cluster of cells. Both in the nucleus and in the cytoplasm structures of more or less concentric convoluted single membranes are occasionally found, as well as between cells in the intercellular space.

Discussion

In the young adult testis the secondary spermatogonia constitute by far the majority of the spermatogonia. They show a relatively high mitotic activity and are only represented by 'concentrated' and intermediate interphase types.

In her excellent review on the premeiotic germinal cells in spermatogenesis HANNAH-ALAVA (1965) discussed the huge confusion in the terminology and identification of the spermatogonial cell types. The cause of the lack of knowledge on spermatogonia can be attributed, according to this author, to a number of circumstances:

- the general assumption that spermatogonial multiplication always takes place dichotomously.
- the omission of a clear identification of the spermatogonial types with a different cytomorphological appearance, and
- consequently the failure of properly defining and using a standard terminology for spermatogonial cell types.
- the focussing of the interest on meiotic processes by geneticists, disregarding the role of the spermatogonia.

In the present study the choice of criteria to identify spermatogonial cell types has been influenced by the existing confusion in the currently used terminology.

The use of the terms 'primary' and 'secondary' spermatogonia by different authors is based upon:

- whether or not the cells were enveloped by cyst epithelium, e.g. SUTTON (1900), DAVIS (1908), COOPER (1950), SADO (1963).
- the spatial relationship between the germinal cells and the apical cell, e.g. GERARD (1909), NELSEN (1931), MASNER (1965), MERLE (1969).

- the number of cell generations which had already been formed, e.g. MUNSON (1906), ROBERTSON (1931).
- being identical with predefinitive, indefinitive or definitive spermatogonia, e.g. CHEN and GRAVES (1970), HEMING (1970).

The routine use of these terms by many authors without any definition or specification, gives rise to further confusion.

The designation of spermatogonia as predefinitive, indefinitive and definitive has been based on the stem-cell mechanism of the species concerned and the use of these terms presupposes knowledge of the method of spermatogonial multiplication. Moreover, this terminology refers to a physiological-developmental distinction of the spermatogonia. But these criteria do not necessarily have a counterpart in the observed cytomorphological characteristics, unless the relation between both kinds of criteria is proven. Therefore, this terminology is considered to be ineffective and unsuitable for a direct identification of observed gonial cell types. Nevertheless, these terms will be used in accordance with the definitions of TIHEN (1946) and HANNAH-ALAVA (1965), when the stem-cell mechanism and related subjects are discussed (paragraph 3.5.4.1.).

In order to present a clear picture of the observed spermatogonial cells a distinction has been based on criteria referring to the nuclear morphology of the cells, the density of both spermatogonial populations and their respective positions in the testis.

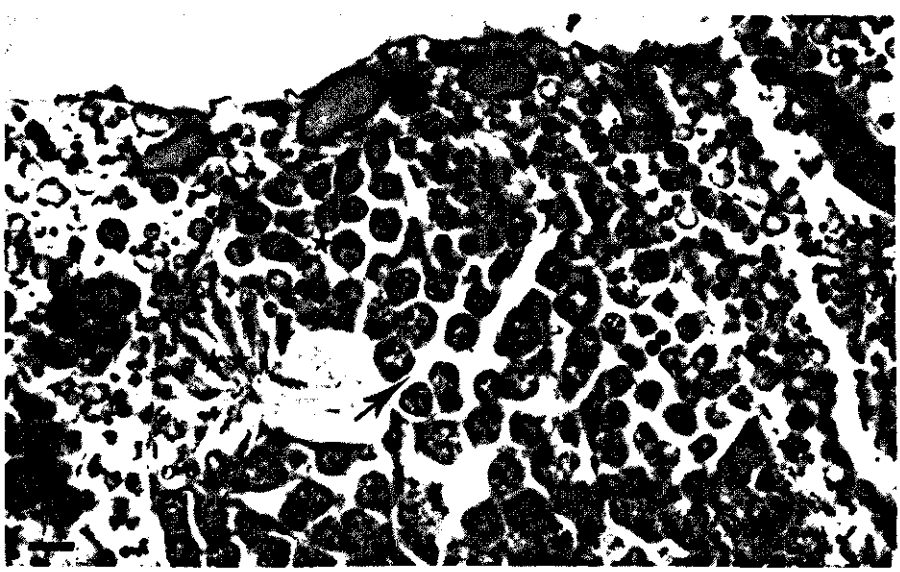


FIG. 23. Part of testis of newly emerged fly showing primary spermatocytes (arrow), secondary spermatocytes (*), early spermatids (**) and a mixed cyst of the latter two types, Bar = 10 μ.

3.5.2.2.3. Primary spermatocytes

The primary spermatocytes are situated in cysts which surround the upper part of the central cavity (fig. 10). Within a cyst the cells are very uniform in appearance (fig. 23). Often the cells seem to be rather spaciously arranged in the cysts when compared to the secondary spermatogonia (fig. 21). The nuclei are spheroidal-ovoid, and 7–10 μ in the longest diameter (fig. 24). The chromatin is organized in granula or small concretions which tend to prefer the periphery of the nucleus. The nuclei have in general a light appearance, accentuated by a clear nuclear membrane and a more distinct demarcation of the cytoplasm.

Numerous cells are observed in meiotic division, which takes place synchronously per cyst. When the meiotic division approaches, the concretions of chromatin seem to become rounder and filaments, which become gradually more distinct, connect these chromatin granula. Long and thin chromosomes shorten and become thicker and better stainable. They stain red with eosin in metaphase.

Among the germinal cells of the testis in vivo some types could represent primary spermatocytes:

a. Cells with a spheroidal-ovoid nucleus containing evenly dispersed very finely granular chromatin. A nucleolus is distinct. The cytoplasm surrounds the nucleus as a dark ring in which no organelles can be observed. The cells are often mutually connected by means of intercellular bridges. They are usually present in groups, often associated with groups of cells in meiotic division and large when compared to most other cell types (fig. 12, 3).

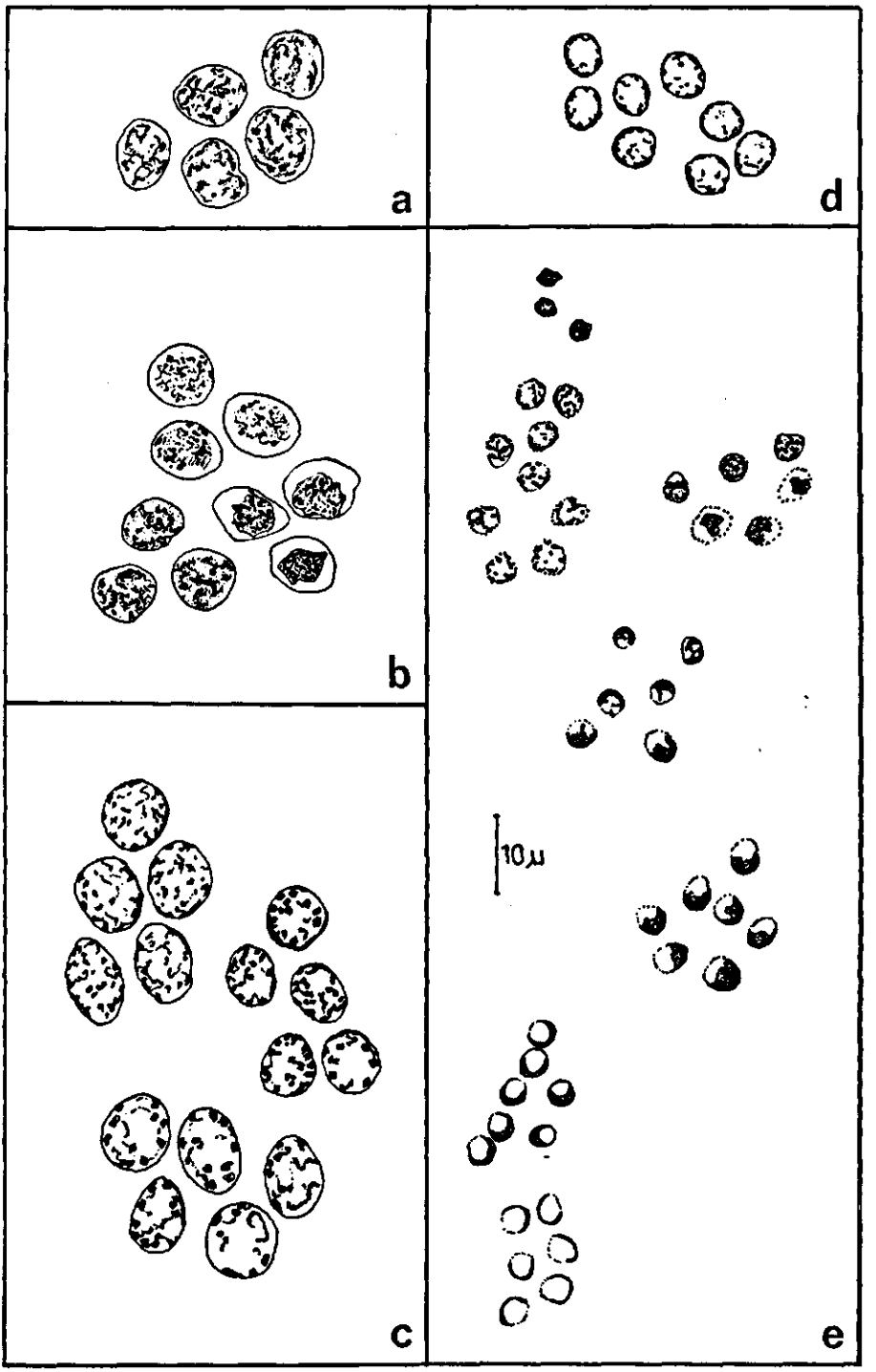
The characteristic chromatin dispersion in these living cells has no match in any cell type in histological preparations. Other features like the size and presence in large groups of the cells, their connection by intercellular bridges and the frequent association with cells in first meiotic division, however, classify them as primary spermatocytes in interphase or very early meiotic prophase.

b. Cells with a relatively large nucleus, with regularly dispersed chromatin in granules, concretions of varying size and filamentous structures which may be locally thickened. The resulting appearance is somewhat tangled. The cytoplasm is slightly transparent (fig. 12, 4).

This cell type much resembles the previously described one, except for the structure and organization of the chromatin. These cells also occur in groups, but less frequently. It is possible that they also represent a form of primary spermatocytes.

c. Cells, conspicuous by irregular chromatin concretions in the large and

FIG. 24. Characteristic appearance of nuclei of germinal cell types in the testis.
a. primary spermatogonia
b. secondary spermatogonia
c. primary spermatocytes
d. secondary spermatocytes
e. early and intermediate spermatids.


a
d
b
10 µ
c
e

transparent nuclei, which contrast with the cytoplasm containing numerous mitochondria which are predominantly located near the nuclear membrane (fig. 12, 5).

These cells are occasionally seen. The large chromatin concretions may denote contracting chromosomes. The cells are often found near dividing cells of the same size and are supposed to be primary spermatocytes, which are probably already in meiotic prophase.

Ultrastructural features of primary spermatocytes are briefly described as follows:

The appearance of the nuclei is very similar to that of spermatogonia. The cytoplasm shows the same ground substance as do spermatogonia. Conspicuous differences are the occurrence in patches of clusters of ribosomes, the large and well developed Golgi-complexes (fig. 25), an increase in membrane fragments of the rough ER which are continuous in different cells by means of the cyto-

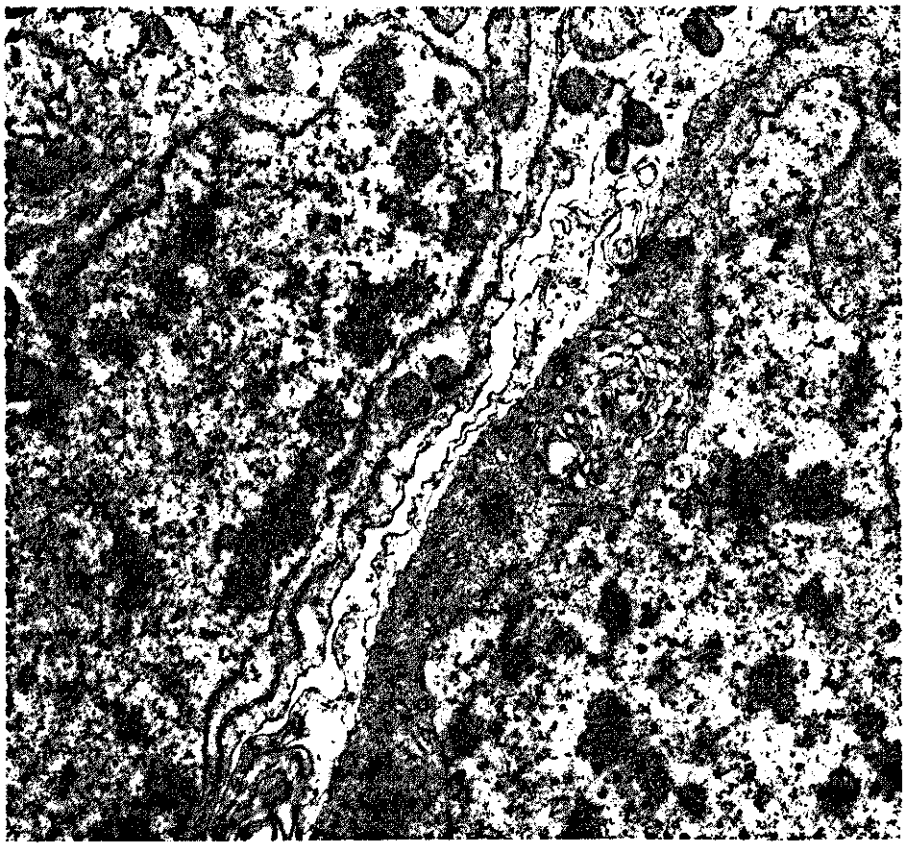


FIG. 25. Interphase primary spermatocytes with irregular nuclear boundaries and more developed cytoplasmic structures like mitochondria and Golgi-complex (centre). 22,079 x.

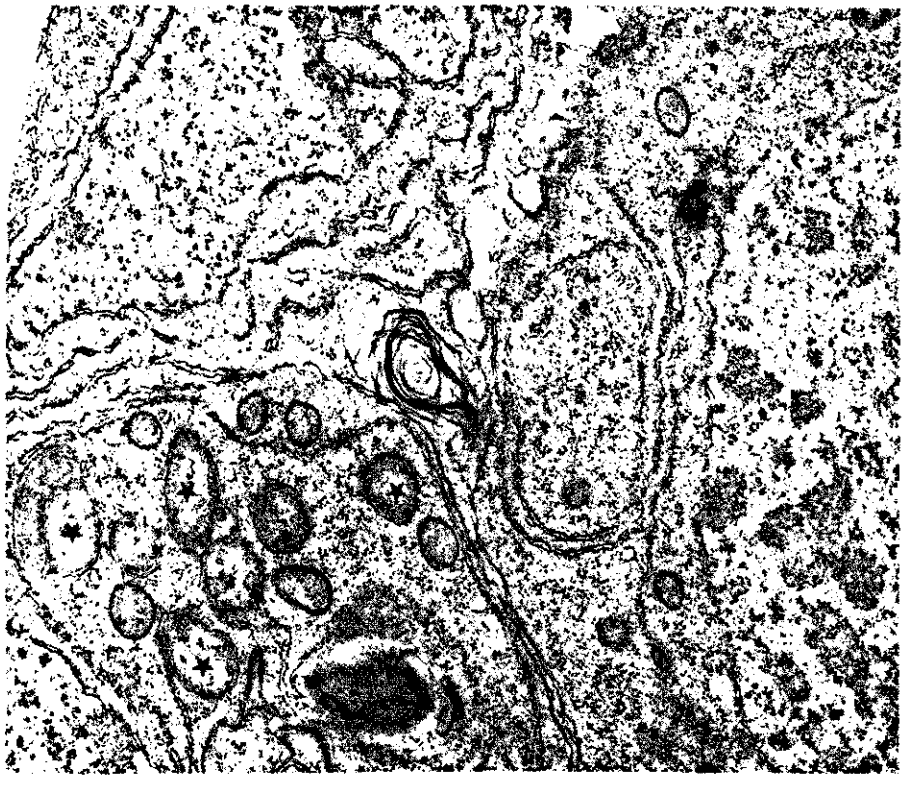


FIG. 26. Two types of mitochondria in primary spermatocytes: small, darkly delimited ones with homogeneous structureless contents and larger mitochondria surrounded by a double membrane and with patchy contents (*). The latter type is called the 'fusion' type. Note the membranous structures in the cytoplasm and the intracellular space. 22,079 x.

plasmic bridge cytoplasm and the appearance of electron dense structures shaped like a horse-shoe or a jar. The mitochondria show less cristae and often have evenly dispersed structureless contents. They are larger (about 290 × 190 nm) as compared with those in spermatogonia and mostly show clear, dark limits. Besides this type of mitochondria another type can be observed in some spermatocytes (fig. 26). These mitochondria are locally concentrated, swollen and their content is spread in patches. This type is named here the 'fusion' type because it is involved in fusion processes later in development. Sometimes in the cytoplasm several concentric double ER membranes from wide areas which may include mitochondria. In the intercellular space irregularly formed structures are frequently found which consists of spirally or concentrically arranged single membranes. These structures occur also in both cytoplasm and nuclei (fig. 27). They are also found in spermatogonia. The cell membrane does not seem to form finger-like extensions into the intercellular space.

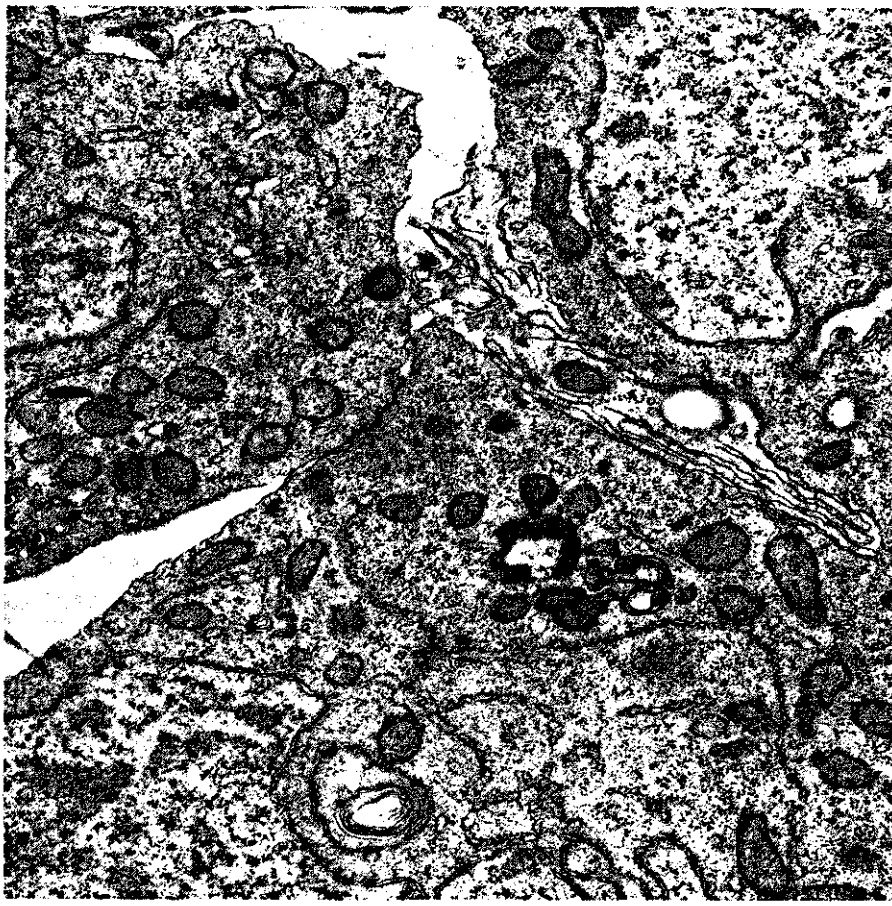


FIG. 27. Interphase primary spermatocytes. In the cytoplasm an increase of mitochondria, Golgi-complexes, membranous and other structures is found when compared to spermatogonia. 22,079 x.

Discussion

Continuing the terminology used to denote roughly the chromatin structure and dispersion in the nuclei of spermatogonia, the interphase primary spermatocytes always show the 'diffuse' type. 'Concentrated' types or intermediate ones have never been observed. Meiotic prophase stages are not very varied in appearance and types such as the bouquet-stage are absent in *H. antiqua*.

The features of the interphase primary spermatocytes of *H. antiqua* do not differ from those of numerous species, for instance *Periplaneta americana*, *Pyrrhocoris apterus*, *Sarcophaga spp.*, *Leptinotarsa decemlineata*, *Adoxophyes orana* and *Hylemya brassicae*. In several species bouquet-stages of the meiotic prophase, which have a certain resemblance with secondary spermatogonia, can be found. Bouquet-stages have been found in Odonata, Orthoptera (DAVIS,

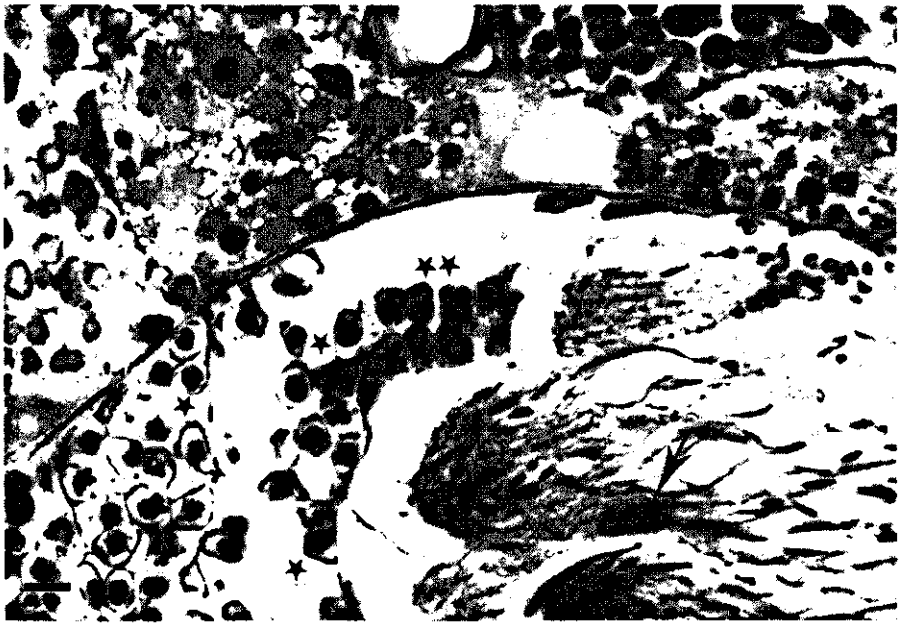


FIG. 28. Lateral part of testis of newly emerged fly. Note the secondary spermatogonia (*), the secondary spermatocytes (**), the intermediate spermatids, the transformation spermatids in the central cavity, and the central cavity cell (arrow). Bar = 10 μ.

1908), in *Periplaneta americana*, *Leptinotarsa decemlineata*, *Locusta migratoria* and *Tischeria angusticolella* (KNABEN, 1931).

MUNSON (1906) pointed to the spatial arrangement of the primary spermatocytes in *Papilio rutulus*, as did KNABEN (1931) for *Tischeria angusticolella*. In Lepidoptera the primary spermatocytes are arranged along the periphery of the cyst epithelium, leaving an empty space in the centre of the cyst (fig. 70). In some orders the cyst is well defined but completely filled with the cells. Diptera generally show a weak development of the cyst epithelia often resulting in a vague delimitation of a particular cyst. Compared to other dipterous species *H. antiqua* possesses this property in a generous measure, often impairing cell counts in densely populated areas.

3.5.2.2.4. Secondary spermatocytes

The secondary spermatocytes are relatively scarce because of the quick succession of the two meiotic divisions. The haploid cells have spheroidal nuclei with a diameter of 4.5–6 μ. The chromatin has the tendency to settle against the nuclear membrane in small concretions, but these concretions are also occasionally found more centrally in the nucleus (figs 23 and 24). The cysts with secondary spermatocytes are nearly always situated against the testicular sheath in the middle part of the testis (fig. 28). An indication of the short dura-

tion of the interphase stage is the occurrence of MI or MII divisions in the same cyst of interphase secondary spermatocyte nuclei.

Living secondary spermatocytes conform the following description:

Cells which are very similar to those of type II of early spermatids (see fig. 12, 7), but somewhat larger. The chromatin granules are predominantly peripherally located causing a very light appearance of the nuclei. Often the chromatin is organized in filaments of varying size which may be longer than the nuclear diameter. This condition of the chromatin probably reflects prophase phenomena. The transparent cytoplasm often shows more or less concentrated masses of mitochondria (fig. 12, 6).

These cells are infrequently found and in combination with the other characteristics they are easily classified as being secondary spermatocytes.

Probably due to the different fixation and subsequent processing of the tissue the ultrastructure of secondary spermatocytes differs from the picture in histological preparations and living cells.

Secondary spermatocytes have smaller nuclei as compared to primary spermatocytes. The chromatin is organized in numerous small concretions of varying size which are sharply delimited and are regularly dispersed in the nucleus. The chromatin in these concretions seems to have a finely granular, uniform structure as has been observed in nuclei of spermatocytes and spermatids of *Drosophila melanogaster* (RASMUSSEN, 1970) and in spermatids of *H. antiqua* (fig. 31). The cytoplasm contains both types of mitochondria, the 'fusion' type being locally concentrated, the other type randomly dispersed. The latter has a smaller and darker appearance when compared to the 'fusion' type. The cells are mutually connected by cytoplasmic bridges.

Discussion

Secondary spermatocytes are relatively rare in the testis, although during some periods of testicular development they are readily found. Their relative scarcity is due to the short existence in this stage between the two meiotic divisions which obviously succeed each other quickly. In many cysts of secondary spermatocytes a few primary spermatocytes and/or some early spermatids can still be observed.

Most authors agree that the secondary spermatocytes divide very quickly (e.g. MUNSON, 1906; MASNER, 1965; SADO, 1965; CHEN and GRAVES, 1970; HEMING, 1970; FYTIZAS, 1973). HEMING (1970) noted a ring-like appearance of the secondary spermatocytes in *Haplothrips verbasci* because of the peripherally precipitated chromatin. This appearance is quite similar to that of the secondary spermatocytes of *H. antiqua* in which most of the chromatin is located against the nuclear membrane. (fig. 33).

Both their intermediate size between primary spermatocytes and spermatids and their conspicuous nuclear morphology, which is otherwise very similar to a particular type of early spermatids (fig. 29, g), allow an easy identification.

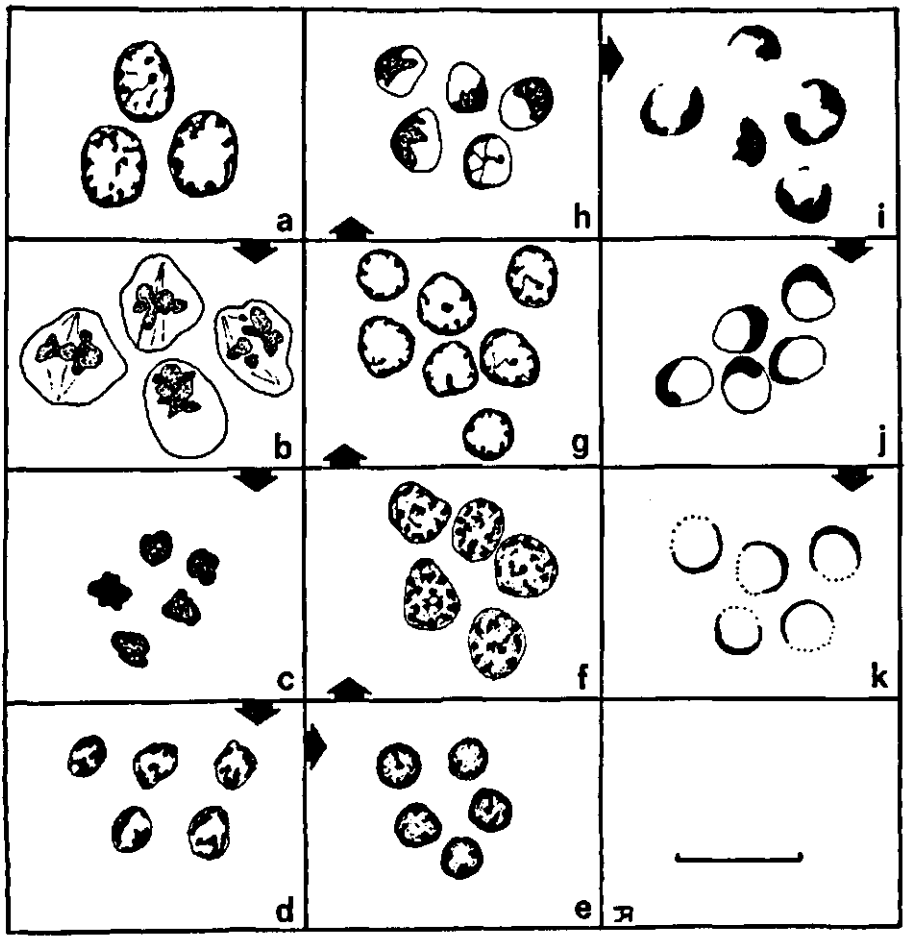


FIG. 29. Sequence of morphological sub-types of early (c-h) and intermediate (i-k) spermatids. Secondary spermatocytes (a) and second meiotic divisions (b) precede this development.

3.5.2.2.5. Early spermatids

The early spermatids are the direct descendants of the secondary spermatocytes. They are characterized by a spheroidal nucleus with a varying chromatin structure and dispersion according to their progress in development. The diameter of the nucleus is about 2.5–4 μ. The telophase of the MII-division results in the formation of small, darkly staining spermatid nuclei with irregularly clumped chromatin. Later the chromatin seems to expand and to break up in coarse concretions which gradually become smaller and regularly dispersed in the nucleus (fig. 29). During development the chromatin granules tend to accumulate at the nuclear membrane. They become smaller and finally precipitate against the nuclear membrane, assuming a ring-like structure. The tendency of the chromatin to accumulate causes a further collection of chromatin at

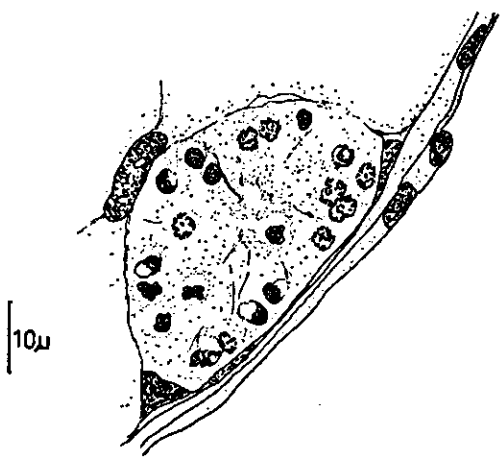


FIG. 30. Cyst of early spermatids showing rosette-like arrangement of the cells.

one side of the nucleus, the chromatin being converted from a number of small granules into a mass of homogeneous, darkly staining material. The chromatin mass is gradually flattened against a portion of the nuclear membrane. At that point the next spermatid stage continues its development. The cysts with early spermatids are situated basally of the primary and secondary spermatocytes and predominantly, if not almost exclusively, against the testicular sheath (fig. 10). The very early spermatids are distributed in a more or less random manner within the cyst. Later on they may take a more orderly position in a rosette-like arrangement (figs. 23 and 30) in which the nuclei are peripherally located in the more or less pyramidal cells. This situation disappears with progressing development and the cells are once more randomly dispersed within the cyst.

Living spermatids are easily recognized in phase contrast.

The early spermatids show two main types:

I. Small cells with a compact, small, irregularly shaped nuclear component which is often surrounded by a clear sphere. In the dark cytoplasm neither mitochondria nor a Nebenkern can be discerned. Sometimes, bright, small vesicles which are associated with dark material are present outside the nucleus (fig. 12, 7a I).

The reported vesicles may be acroblast-like structures such as were described by KNABEN (1931) in spermatids. Presumably the dark and irregular nuclear components are the telophase chromosomal structures of the preceding second meiotic division. Later the nucleus is formed as a light sphere around the still contracted chromatin of the very early spermatids.

II. Larger cells with lighter and larger nuclei in which the chromatin is organized in small concretions and granules which are mainly situated peripherally. The larger the nucleus, the more finely and evenly dispersed the chromatin is found to be. In the cytoplasm often seemingly fibrous Nebenkern structures are observed (fig. 12, 7a II).

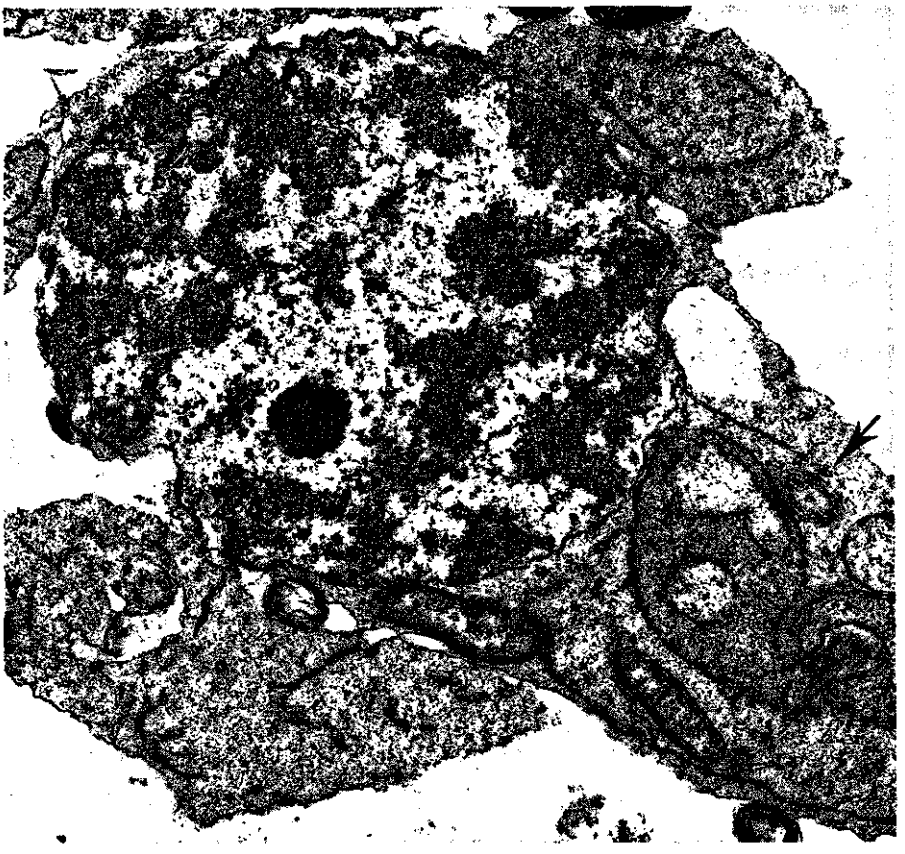


FIG. 31. Young early spermatid with regularly dispersed chromatin and distinct nucleolus. An axial filament is already present (arrow) and a Nebenkern structure with cytoplasmic enclaves (right). 'Fusion' type mitochondria are also present. 22,079 x.

This type represents more advanced early spermatids.

III. Many intermediate forms between types I and II are found, showing a considerable variation in size, chromatin appearance and dispersion, and transparency of the nucleus and cytoplasm. Owing to this variability a generalized description can not be given.

The ultrastructure of the differentiating spermatids reveals some of the programmed sequence of morphogenetic processes involved.

The very early spermatid has an approximately spheroidal nucleus with a diameter of about 2.5–3 μ with regularly dispersed coarse and indistinctly limited chromatin concretions (fig. 31). The contrast between the chromatin and the nucleoplasm decreases and the chromatin concretions desintegrate into smaller units (fig. 32) until an even dispersion of very fine chromatin elements has been achieved. In the cytoplasm, which is rich in clusters of ribosomes, few mitochondria are observed except those of the 'fusion' type which sometimes

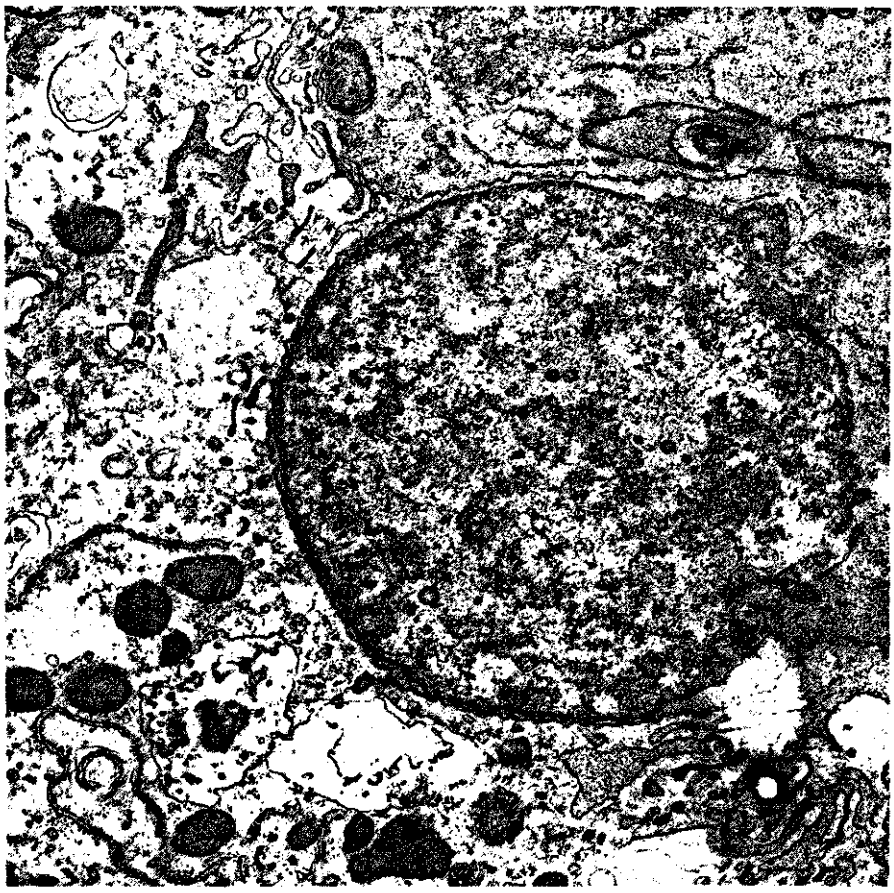


FIG. 32. *Early spermatid with evenly distributed chromatin.* Note the cyst cell cytoplasm (left and below) contrasting with the spermatid cytoplasm (right and top). 22,079 x.

partly seem to have fused to irregular structures delimited by a double membrane and showing cytoplasmic invaginations. Axial filaments are observed. The nucleus may be excentrically located in the cell, covered merely by a thin layer of cytoplasm.

Discussion

Early spermatids rarely present difficulties in identification. The sequence of their morphological nuclear differentiation has been depicted in fig. 29, although intermediate forms may show a slightly different appearance.

The described spermatid type has been named 'early' because it represents the first stage of the differentiation towards the sperm cells. It can be characterized by its chromatin appearance and behaviour in the same manner as has been done with preceding germinal cell types. The expansion of the chromatin

in the early stage after formation probably reflects the despiralization of the chromosomes after the MII telophase. Afterwards a reorganization of the chromosomal material seems to take place resulting in the chromatin transformations in the developing spermatid.

3.5.2.2.6. Intermediate spermatids

The intermediate spermatids are characterized by their spheroidal nucleus and a pattern in which the chromatin is precipitated against a part of the nuclear membrane to form a thin crescent while the remainder of the nuclear membrane is barely or not at all visible (figs. 24, 29 and 33). The diameter of the nucleus is about 2.5–3 μ. The cysts of intermediate spermatids are found among, and basally of cysts of early spermatids. In many cases the cells tend to aggregate at one side of the cyst (fig. 28). The cyst epithelia are often very thin or absent.

This well-recognizable type of spermatid is intermediate between the spermatids in which the chromatin shows a distinct variability and the spermatids which transform ultimately into the sperm cells and show a corresponding elongation of the nucleus.

Living intermediate spermatids are readily recognized. They have a very transparent cytoplasm which often impairs an accurate observation of the cell limits, in particular as the cells are usually present in groups (fig. 34). The nucleus is spheroidal, very transparent and the refractory chromatin is localized at the periphery along a variable part of the nuclear membrane. No other struc-

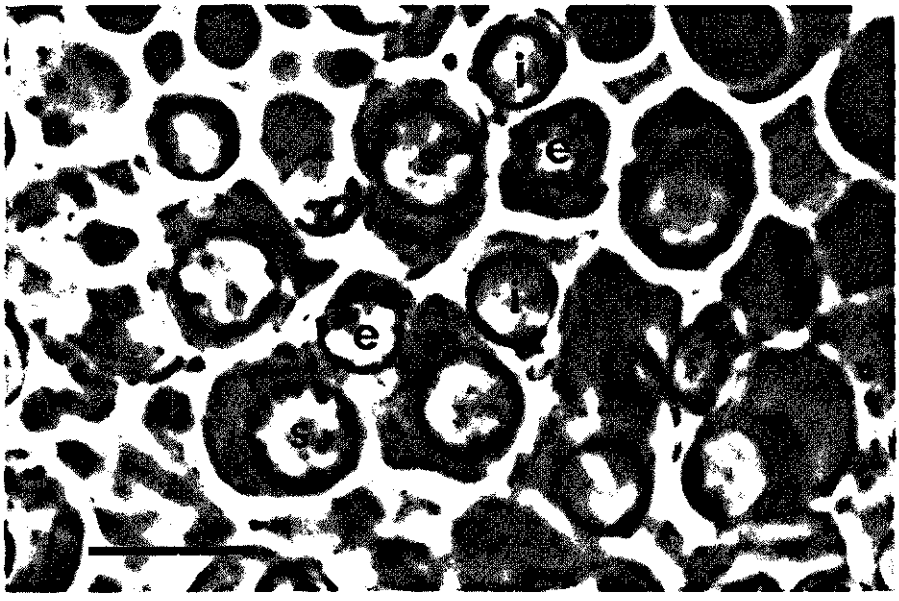


FIG. 33. Living secondary spermatocytes (s), early (e) and intermediate (i) spermatids. Bar = 10 μ.

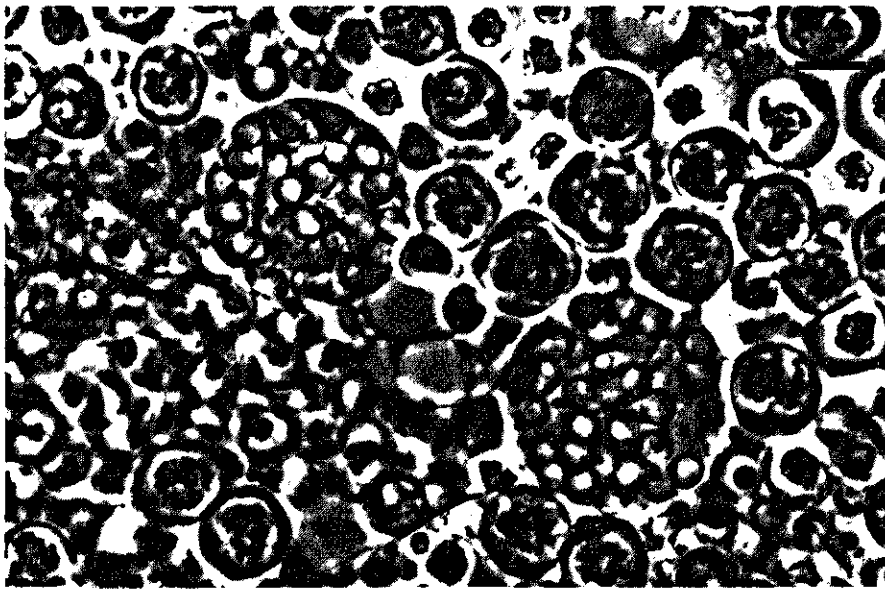


FIG. 34. Cysts with living intermediate spermatids among early spermatids and spermatogonia. Bar = 10 μ.

tures in the nucleus or cytoplasm are visible (fig. 12, 7b).

On the ultrastructural level the chromatin dispersion of the intermediate spermatids conforms to the observations in histological preparations and in living cells. The chromatin reappears as granular-fibrillar, irregularly shaped masses situated against the larger part of the nuclear membrane. The interior of the nucleus is lightly contrasted except for dark spots of a fairly uniform size (about 40 nm) and frequently present convoluted membranous structures which have been described earlier (fig. 35). No chromatin is present against the basal part of the nuclear membrane which shows numerous pores (fig. 35). The nuclei may be extremely excentrically located in the cells and be covered by only a very thin sheath of cytoplasm for the greater part of their surface. The cytoplasm shows remarkably few structures in this stage. Clusters of ribosomes are numerous, mitochondria low in numbers, the ER poorly developed and Golgi-complexes indistinct and scarce. 'Fusion' type mitochondria are locally present. Sometimes electron dense structures with extremely varying shapes are seen near the nucleus, perhaps representing the acroblast. All cytoplasmic structures keep a distance of about 150 nm from the basal nuclear pores.

Discussion

The name allocated here to this type of spermatids denotes its transitional nature. The intermediate spermatids remain in this stage for only a short time which explains their occasional scarcity. Owing to their morphological charac-

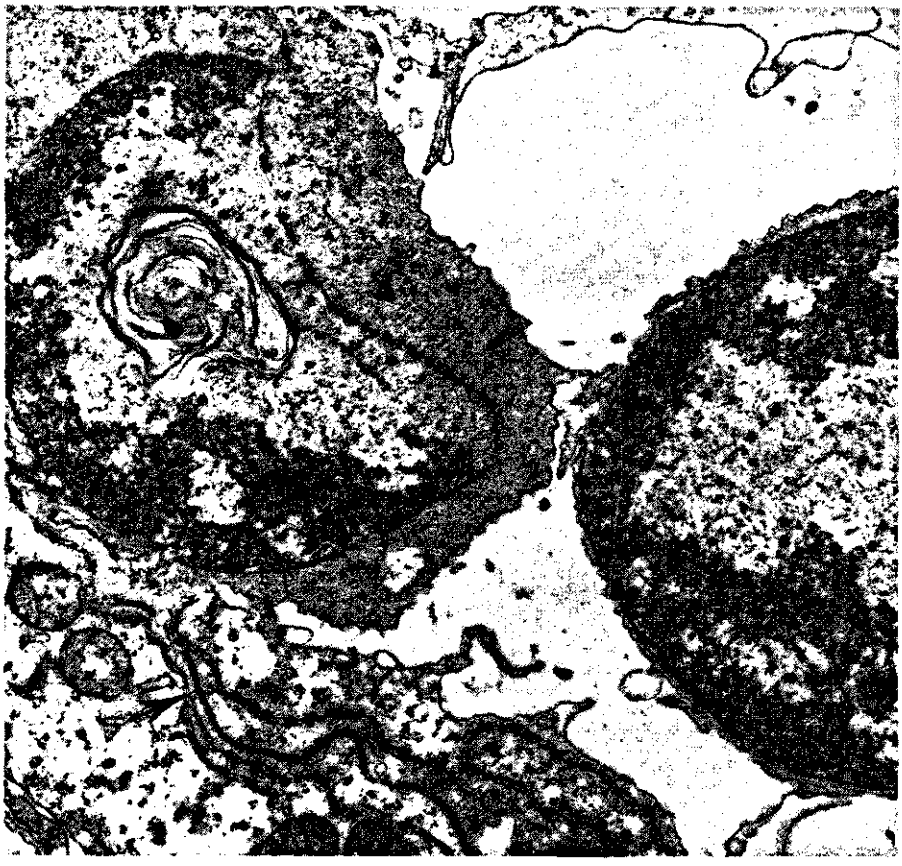


FIG. 35. Intermediate spermatids with peripherally located chromatin and nuclear pores at the basal part of the nucleus. Note the convoluted membranous structure in the nucleus and the desmosomes (arrow) between cells of the surrounding cyst epithelium. 22,079 x.

teristics their identification is simple. WOLFE (1972) denoted them as the 'shell' stage of spermatids.

3.5.2.2.7. Transformation spermatids

The transformation spermatids show more or less elongated nuclei with an increasing density of the chromatin. In early transformation spermatids, the chromatin is still precipitated against the more distinct nuclear membrane as a thin darkly stained layer. With progressing development the nucleus elongates and becomes more narrow until the chromatin fills the entire nucleus. The nucleus continues to elongate and ultimately takes a filamentous shape. The cells in the cysts show an increasing aggregation with progressing development till they lie in bundles like sperm cells. The cysts with transformation spermatids are observed in the basal part of the testis but may be found in the upper part

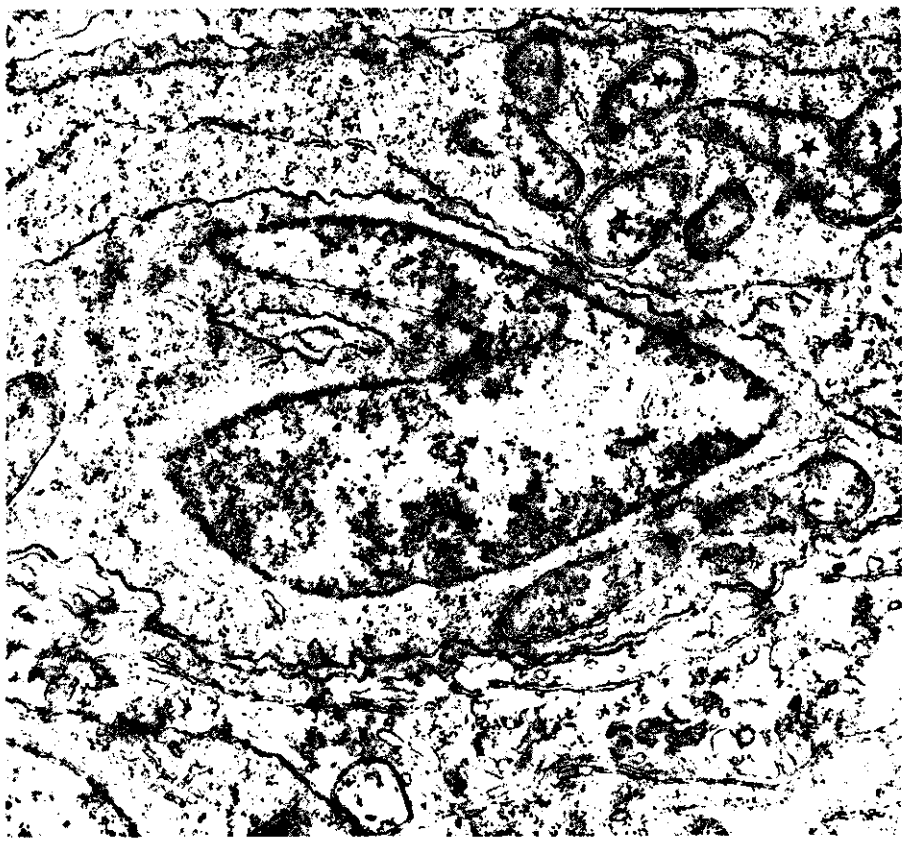


FIG. 36. Young transformation spermatid with regularly dispersed chromatin in the elongated nucleus. 'Fusion' type mitochondria are present (*). Note thin layer of cyst epithelium (below). 22,079 x.

of the central cavity as well. The cyst epithelium is mostly very indistinct.

Living transformation spermatids are present with a large variety of elongated nuclei from pear-shaped to filiform ones. The chromatin location ranges from peripheral to a dispersion throughout the entire nucleus. Far advanced filiform transformation spermatids show, outside the testis, a slow movement of the anterior part of the cells rotating in a conical plane.

Also on the ultrastructural level the transformation spermatids show an elongated nucleus. In young types the chromatin disperses evenly in the nucleus in small floccules of varying sizes. At this stage the nucleus still shows minor irregularities in shape (fig. 36). At the basal side the nuclear pores are less distinct. The cytoplasm is rich in clusters of ribosomes and membranes and Golgi-complexes become more developed and conspicuous. The 'fusion' type mitochondria aggregate to an elongated mass and partly fuse (fig. 37). Later

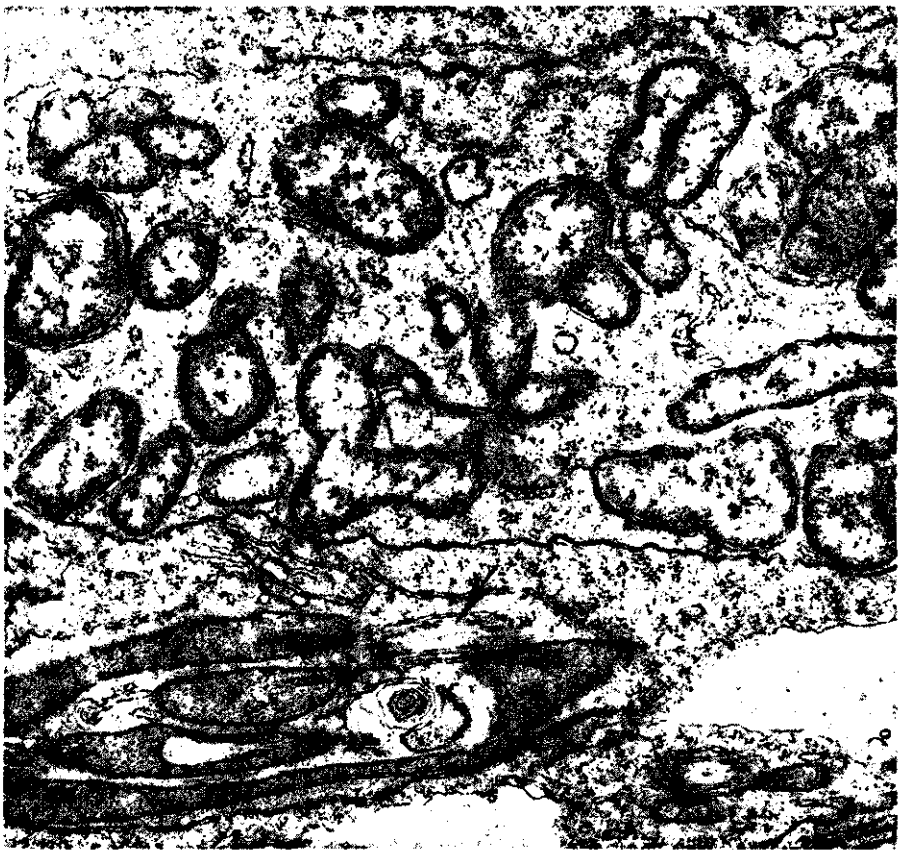


FIG. 37. 'Fusion' type mitochondria in transformation spermatid. The mitochondria have aggregated but are only partially fused. Against the axial filament (arrow) the darkly contrasted, irregularly shaped but elongated Nebenkern structure is visible. Cytoplasmic enclaves are commonly found in Nebenkern structures as are microtubules. 22,079 x.

the mitochondria form a more or less spheroidal structure of single, partly and entirely fused mitochondria mixed in a loose fashion within the limits of the structure. At a later stage the structure shrinks and the boundaries between the mitochondria become indistinct. Axial filaments are already present in early spermatids; in young transformation spermatids they are associated with a long and darkly contrasted structure which occasionally shows cristae. These structures are surrounded by microtubules. Thus, at this stage both 'fusion' type mitochondria in aggregation and partial fusion and the long structure of a presumably mitochondrial nature are present, flanking the axial filament. Apart from some cristae (fig. 38) the contents of the dark structure are finely granular. The shape is elongated, sometimes locally split into two parts and showing cytoplasmic enclaves. It is obscure which of these structures can be considered to be the 'Nebenkern' derivative, as is generally referred to the

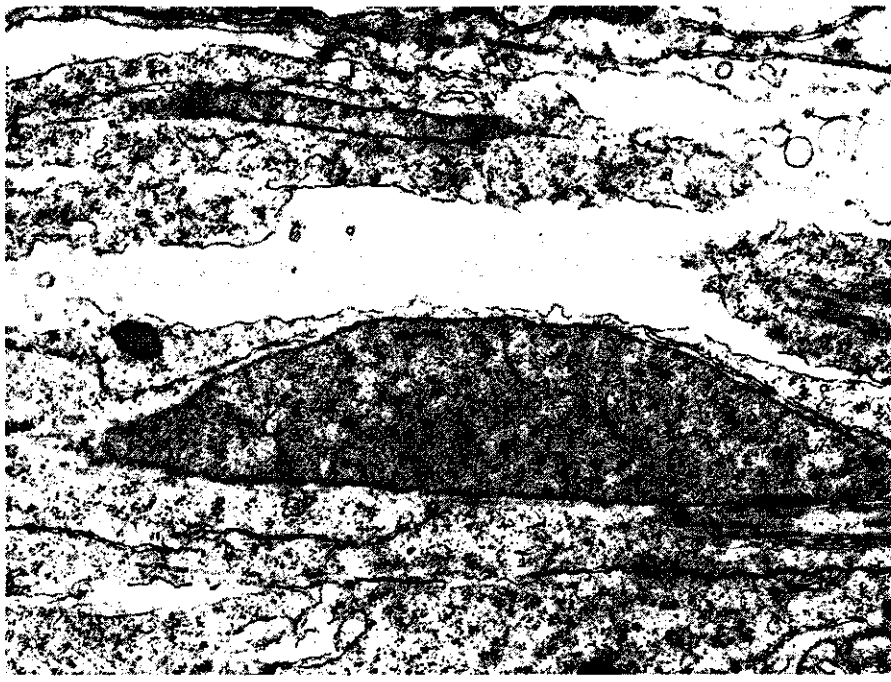


FIG. 38. Nebenkern structure in transformation spermatid closely against the axial filament still showing some fragments of cristae. Note the microtubules which are associated with the Nebenkern 22,079 x.

structure which results from the fused mitochondria in the spermatid. The dark structure seems to be more a 'Nebenkern' derivative than the aggregating and only partially mutually connected 'fusion' type mitochondria which are still observed as such in late transformation spermatids. In fact, it is doubtful whether these mitochondria contribute at all to the 'Nebenkern' derivatives which lie parallel to the axial filament in differentiating transformation spermatids. In early spermatids the axial filament is already present. 'Fusion' type mitochondria are found here as well as normal, smaller and darker mitochondria. The latter are sometimes observed aggregating near an axial filament, suggesting a possible increased aggregation and subsequent fusion. Both 'fusion' type and normal mitochondria in spermatid cytoplasm are clearly distinguishable. In transverse sections of developing transformation spermatids the 'Nebenkern' derivatives, mostly unequal in size, and 'fusion' type mitochondria may occur in the same cell. They differ considerably (fig. 39), demonstrating that the 'Nebenkern' structures are not derived from the 'fusion' type mitochondria. Therefore, it is difficult to designate the Nebenkern either as the never entirely fused mass of 'fusion' type mitochondria or as the fused 'normal' mitochondria which probably formed the dark structures along a part of the axial filament. For the time being the latter structures shall be designated here as 'Nebenkern' deri-

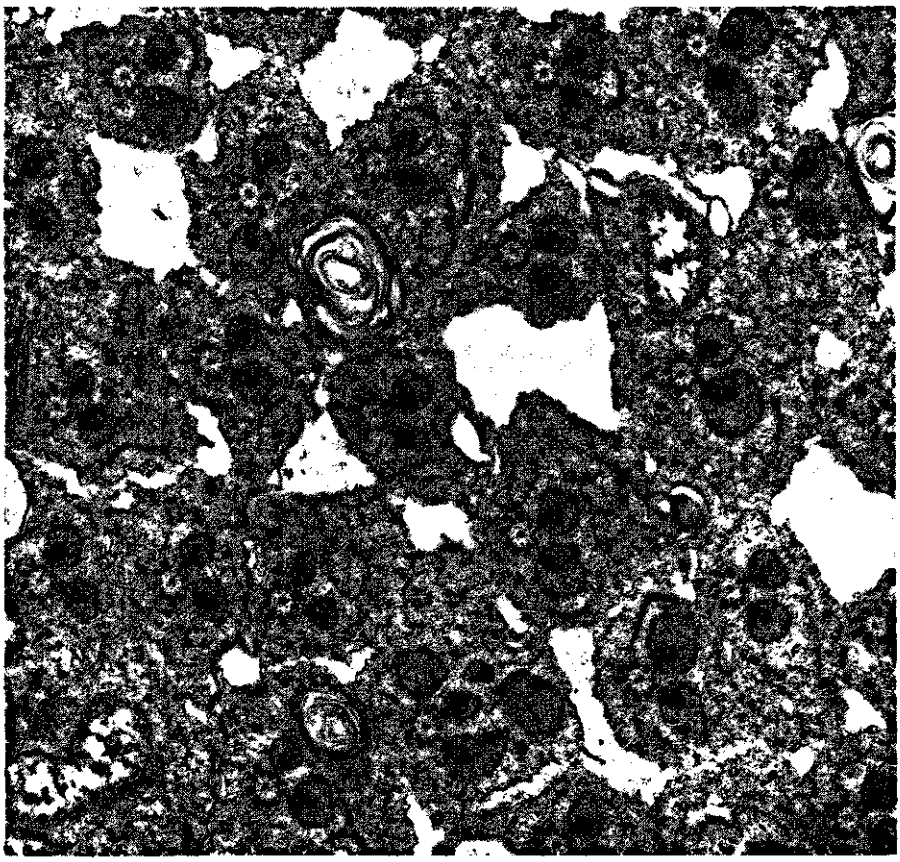


FIG. 39. Transformation spermatids in cross section. Both Nebenkern structures, which are often unequal of size, contain paracristalline material. Sometimes more of these structures are present due to an irregular shape. 'Fusion' type mitochondria are still present too (*). 22,079 x.

vatives. In these structures paracristalline material is often precipitated at the point nearest to the axial filament (fig. 39).

In the cytoplasm of elongated spermatids many Golgi-complexes are present near the 'Nebenkern' derivatives in the often ribosome studded cytoplasm. The complex of axial filament and 'Nebenkern' derivatives is surrounded by a single layer of microtubules. Bundles of microtubules have been found elsewhere in the cytoplasm as well as single and partially fused 'fusion' type mitochondria. These bundles appear in cross section as multivesicular bodies enveloped by a single membrane. Masses of single or partially fused 'fusion' type mitochondria are present in parts of the cytoplasm which also contain concentric membrane systems and vacuoles pointing to possible degenerative changes. In these regions bundles of microtubules and some normal mitochondria are frequently observed.

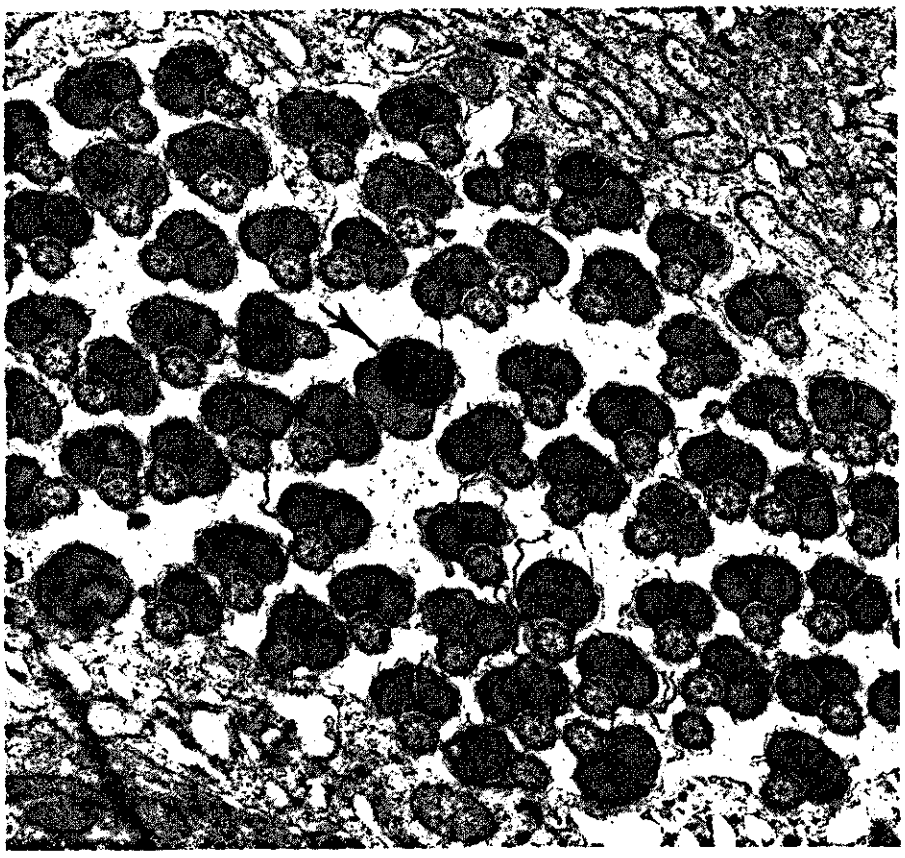


FIG. 40. Transformation spermatids in cross section. Both Nebenkern structures ultimately seem to fuse, beginning at the centriole adjunct (arrow). 22,079 x.

The nuclei of the late transformation spermatids are long and lobed in transverse section. The chromatin is evenly spread in small, probably filamentous structures which give the nucleus a speckled appearance. The basal side of the nucleus is still concave and at this site the axial filament originates. Sometimes a dark structure is seen covering the basal end of the nucleus, possibly representing the centriole adjunct. The nucleus seems to be surrounded by microtubules.

At further differentiation the 'Nebenkern' derivatives tend to fuse towards one structure. The amount of cytoplasm decreases considerably, probably by sloughing off most of the excess material as cytoplasmic droplets. The tails in cross section lay in an orderly fashion arranged in the cyst (fig. 40) and may be counted.

Discussion

As for the morphological appearance of spermatids few investigators take the trouble to define what they consider to be spermatids. Some give indications (e.g. FYTIZAS, 1973) or even short descriptions (e.g. MASNER, 1965) of the morphological appearance of these cell types. In most older publications dealing with spermatogenesis or spermiogenesis attention has been focussed so much on the number, shape, behaviour and fate of the chromosomes that a description of the nuclear morphology of the cells containing them often has been omitted (e.g. BUDER, 1915). The numerous more recent investigations on spermatid and sperm ultrastructure and activity will be dealt with in the general discussion.

3.5.2.2.8. Sperm cells

The sperm cells are filamentous with a nucleus of about 57 μ long, the entire cell being 310 μ long. The cells are more or less united in bundles which are mostly very loose. Because of the absence of visible cyst epithelia the individual cells seem to be relatively free from each other, although the viscosity of their environment will prevent a complete mixing of the cells. The arrangement of the cells suggests the absence of any individual motility of the sperm cells within the testis. They are capable to move, however, which is shown when testes are slowly and carefully opened in Levy physiological saline by applying pressure. The outflowing spermatozoids immediately start to move in their characteristic, vibrating manner. The sperm cells leave the testis as individual cells. The nutrition of the cells does not seem to depend on a special cell type or structure but may be obtained from the contents of the central cavity.

Living sperm cells are identified by their filiform structure with dark, clearly distinguishable nuclei (fig. 5) and, only outside the testis, their typical vibrating movement. Along their flagellum they often show dark droplets of sometimes vacuolated cytoplasm. They rarely occur in defined bundles in living cell preparations, contrary to the living sperm cells of *M. domestica*.

Discussion

Cytoplasmic droplets have already been observed some decades ago in Lepidoptera (KNABEN, 1931) and have more recently been a subject of biochemical studies. They are supposed to be modified lysosomes (DOTT and DINGLE, 1968), like the acrosome (ALLISON and HARTREE, 1968).

3.5.2.2.9. General discussion on germinal cell types

A new element which we have introduced in the classification of male germinal cell types is the concept of 'concentrated' and 'diffuse' cell types and their intermediates. Therefore, it may be appropriate to test the validity of this concept by scanning other possible explanations for the observed phenomena and their presence in other insects as determined by descriptions and relevant illustrations in the literature.

Some authors have considered testicular germinal cells with concentrated

chromatin as primary spermatocytes in a stage of meiotic prophase. In a number of instances this was right. An example is provided by the descriptions of MEUSY (1963) of the primary spermatocytes in meiotic prophase of the Crustacean *Orchestia gammarella*. Examination of the relevant drawings reveal essential differences between the secondary spermatogonia in *H. antiqua* and the more or less similar primary spermatocytes of *O. gammarella*. The latter show a distinct bouquet-stage prophase, which is definitely absent in *H. antiqua*. MERLE (1969) described in *Pyrrhocoris apterus* a small population of spermatogonia around the apical cell. This population is surrounded by a much larger mass of cells with concentrated chromatin in the nuclei. The latter cell type is referred to as 'les spermatocytes d'ordre I' and is depicted on plate IV, Figs. 1, 2 and 3. As far as can be seen there, no bouquet-stage is present in these cells. The spermatogonia show a varied chromatin pattern similar to that described for primary spermatogonia of *H. antiqua*. The presumed primary spermatocytes in *Pyrrhocoris* may very well in fact represent a form of spermatogonia comparable to 'concentrated' secondary spermatogonia in *H. antiqua*. In histological sections of the testis of newly moulted *Pyrrhocoris apterus* adults identical pictures were found by us as were published by MERLE (1969). As for the nuclei with the concentrated chromatin, they were interpreted as secondary spermatogonia preceding the clearly different primary spermatocytes. In the following species such secondary spermatogonia have been observed by us (see also page 123): *Hylemya brassicae*, *Musca domestica*, *Sarcophaga* spp., *Ceratitis capitata* (Diptera), *Adoxophyes orana* (Lepidoptera) and *Pyrrhocoris apterus* (Hemiptera). They were absent in: *Locusta migratoria* (Orthoptera), *Leptinotarsa decemlineata* (Coleoptera) and *Periplaneta americana* (Dictyoptera). Thus, cells containing nuclei with concentrated chromatin in the spermatogonial area are not confined to the species *H. antiqua* or the order of the Diptera, but are found in the young adult testes of species of various orders. In these cases no meiotic prophases were involved. Cells with concentrated chromatin were often difficult to interpret for the earlier researchers who tried to explain the more or less concentrated chromatin in cytogenetic terms as being some part of the process of cell division. In this context the term 'synapsis' emerged, firstly used by MOORE (1895) to denote the concentration of the chromatin locally in the nucleus. Later this description has been given a wider, more cytogenetic, scope (WHITE, 1973) as a meiotic prophase phenomenon, probably because the chromatin concentration was not always observed in different animal species and this phenomenon could be fitted into the variable and often turbulent events during meiotic prophase. A complicating factor is the occasional presence of the 'bouquet' stage, discovered by MONTGOMERY (1900), as one of the possible meiotic prophase stages. However, a nucleus in 'bouquet' stage is definitely not identical to one with concentrated chromatin, although sometimes the difference in morphology may be difficult to establish. Consequently, synapsis in its original meaning is not necessarily related to any meiotic or mitotic prophase stage. It is supposed by me to be, on the contrary, more of an interphase stage dependent on the physiological condition of the

cell involved. This opinion has been expressed earlier by WILSON (1912) who considered spermatogonial cells with concentrated chromatin as a resting stage following the last diploid division. He described germinal nuclei with concentrated chromatin in Hemiptera as 'stage a'. The same description applies to DAVIS (1908) 'stage a' in Orthoptera and 'stage a' of *Asilus sericeus* (METZ and NONIDEZ, 1923). In *H. antiqua* these cells have been named 'concentrated' spermatogonia. METZ and NONIDEZ (1923) also found in *Asilus notatus* concentrated chromatin in spermatogonia and observed differences in time of existence of this cell type in both species. In the latter species the stage with concentrated chromatin lasts a very short time, contrary to *A. sericeus*. In other Diptera spermatogonia with concentrated chromatin have also been observed clearly e.g. by METZ (1926) in *Drosophila virilis*, by HUETTNER (1930) and WOSKRESSENSKY and SCHEREMETJEWA (1930) in *D. melanogaster*, by DOBZHANSKY (1934) in *D. pseudoobscura* and FYTIZAS (1973) in *Dacus oleae*. In insect species of various orders spermatogonia with concentrated chromatin have been observed: in Lepidoptera for instance by MUNSON (1906), KERNEWITZ (1915), BUDER (1915) and CHIPPENDALE and ALEXANDER (1973); by JUNKER (1923) in *Perla marginata* (Plecoptera); in Homoptera by KORNHAUSER (1914) and in Heteroptera by GROSS (1907) and MERLE (1969). So, 'concentrated' spermatogonia apparently have been observed in the testes of several insect species during many decades.

Objection to accepting the cell type with concentrated chromatin as a normal functioning form of interphase cells is the possibility that this type is an artefact. DOBZHANSKY (1934) used this argument to explain the concentration of chromatin in spermatogonial nuclei in *Drosophila pseudoobscura*. As far as *H. antiqua* is concerned, this possibility is not valid because fresh, living spermatogonial cells show the same picture of concentrated chromatin in an otherwise optically empty nucleus (fig. 12). HUETTNER (1930) went so far as to change his staining methods to avoid the presence of these cells in order to be able to ignore them.

Although cells with concentrated chromatin are found most frequently in the secondary spermatogonial populations, they are not limited to this cell type. It has already been mentioned that they are also observed among primary spermatogonia. Among other male germinal cell types they are totally absent, contrary to the female germinal cells. In ovaries of all developmental stages they are very common. Furthermore, these cells are not at all restricted to germinal cells. They occur in many tissues. In *H. antiqua* they have been observed in larval, pupal and adult tissues of various origin, for instance in imaginal discs, in larval ganglia, in rectal papillae, among hemocytes, in epithelia of the intestine and ovarioles.

The designations 'diffuse' and 'concentrated' have been used here only to refer to germinal cell types. Cells of somatic origin which also show a similar varying pattern of chromatin concentrations have been indicated earlier as 'a-type' and 'b-type' cells respectively (THEUNISSEN, 1973a). In somatic cells the 'a-type' represents the most common type, the 'b-type' the concentrated form. In all cases care must be taken in making a correct interpretation and a clear

distinction from dividing or pathological nuclei.

Although cells with concentrated chromatin are frequently observed in germinal and somatic cell populations, in a variety of tissues, in *H. antiqua* and other insect species, their physiological significance is still obscure.

When commenting on the observations on living cells it is admitted that the occurrence of living cell types in more or less concentrated groups is in itself no conclusive evidence for a common origin since essentially the original spatial relationships between cells and cell populations are lost when the testis is emptied into the saline. However, as the preparations are made very carefully, avoiding any undesirable mechanical shock or pressure on the cells leaving the testis, neighbouring cell populations often remain near each other.

The variability of appearance of testicular cells is, in spite of the recognition of some categories, too wide to establish a system for an easy and quick identification of these cells. The study of living testicular cells has contributed much to the formulation and establishment of such a system in histological preparations. As such it is an important method for the investigation of spermatogenesis.

The difficulties met in comparing living cells with those in histological preparations raised a need for fixed and stained cells which were influenced as little as possible by chemicals. Such cells have been obtained by means of semi-squash preparations. The cells show aspects of the appearance of both living cells and those in histological preparations, which often facilitates identification of the living cell type (fig. 41). In this way the previously described observations and classifications of living cell types could, for the major part, be verified. Cell types which could not be recognized in the living condition, could be identified in semi-squash preparations, for instance central cavity cells. Other features, for instance intercellular bridges, could be examined in relation

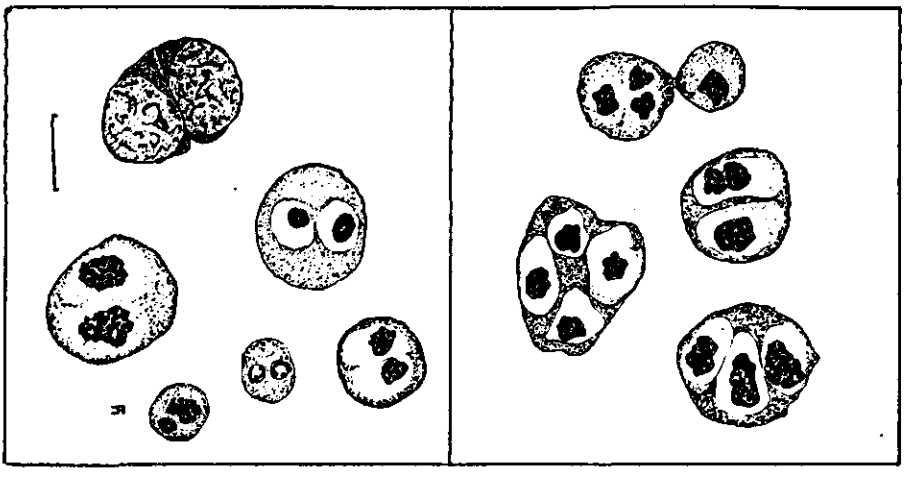


FIG. 41. Bi- and multinucleate male germinal cells in semi-squash preparations. Bar = 10 μ.

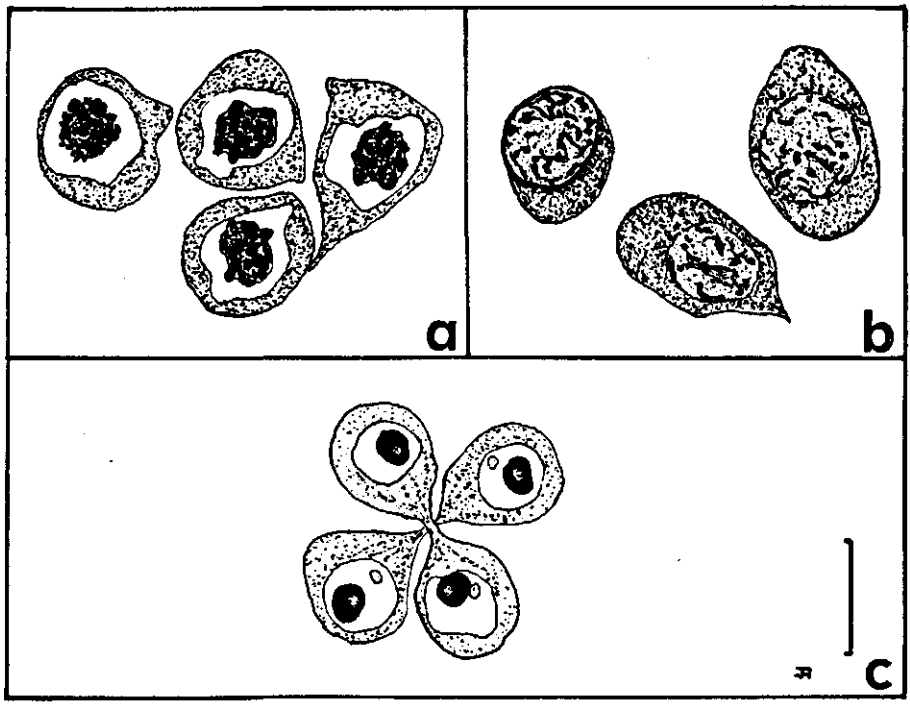


FIG. 42. Testicular germinal cell types as appearing in semi-squash preparations: a. secondary spermatogonia, b. primary spermatocytes, c. early spermatids with intercellular bridges. Bar = 10 μ.

to germinal cell types. The results are in accordance with the previously described properties of living cell types (fig. 42).

Studies on the ultrastructure of germinal cell types in Diptera are not numerous. Most of them focus on *Drosophila melanogaster*.

Very conspicuous elements in the ultrastructure of germinal cells are the intercellular bridges. These structures are very common in various animals (FAWCETT et al., 1959; MOENS and GO, 1972). MEYER (1961) who found intercellular connections between germinal cells in *D. melanogaster* called them 'Fusome'. Fusome differ from intercellular bridges in their potential to close the channel, contrary to intercellular bridges which always stay open (MEYER, 1961). Accepting this definition, it can be established that spermatogonia and spermatocytes of *H. antiqua* are mutually connected by intercellular bridges. Contrary to the findings of RASMUSSEN (1973) and TATES (1971) in *D. melanogaster* and MACKINNON and BASRUR (1970) in *Apis mellifera* the bridge cytoplasm in spermatogonia seems to be of the same composition, density and appearance as in the cell itself including continuous membrane systems, clusters of ribosomes etc. As in Drosophila the wall of the bridge is conspicuous due to precipitation of electron dense material.

The gradual formation of a second nuclear membrane in male germinal cells of *D. melanogaster* reported or depicted by MEYER (1961), TATES (1971) and RASMUSSEN (1973) cannot be confirmed for *H. antiqua*, in which each germinal cell type shows one nuclear double membrane.

The microtubuli play an important supporting role during spermiogenesis. They surround the individual nuclei in a cyst. In the cytoplasm they form bundles which may evidently act as a fixed point for membranes, not to mention the axial filament which shows a 9 + 9 + 2 pattern. This pattern is a very usual one (PHILLIPS, 1970; BAIRATI and PEROTTI, 1970), although there are dissimilarities in detail (TATES, 1971; SHAHANEY et al., 1972) or the entire spermatid or sperm cell (SHAY, 1972). According to RIEMANN and GASSNER (1973) many Lepidoptera seem to conform the 9 + 9 + 2 pattern.

An important difference seems to be the formation of the Nebenkern in spermatids. For many insects it is a well-established fact that the Nebenkern is formed from aggregating and fusing mitochondria which transform into the Nebenkern derivatives during spermiogenesis. In *D. melanogaster* this process has been described extensively by TATES (1971), for *Musca domestica* by GASSNER et al. (1972). In these species the spermatid mitochondria show beautiful and clear patterns of organization. In *H. antiqua* two types of mitochondria develop:

1. a type which is small and ovoid, shows a clear dark outline and dark, evenly spread and finely granular contents.
2. a type which has a swollen and light appearance with an irregular, rounded outline and a patchy pattern of contents. Between these mitochondria boundaries may be partly or entirely absent leading to fused aggregates of a limited number of units (fig. 37).

In spermatogonia and spermatocytes the first type is randomly distributed. The second type emerges locally in spermatocytes. It is still present in late transformation spermatids as separate units. During spermiogenesis these mitochondria aggregate to an elongated mass along the axial filament. In transformation spermatids a dark elongated structure, occasionally showing cristae, is present at the other side of the axial filament in the same cell as the aggregated and partially fused mass of mitochondria. Rarely an increased fusion of the latter has been observed, with a concomitant shrinking of the size of this structure which possessed more an appearance of a degenerative mass of cytoplasm than of a transforming and sharply outlined 'clew' or 'onion' stage (TATES, 1971) Nebenkern. As far as the observations permit, the preliminary conclusion is that the second type of mitochondria has partially or totally lost the normal Nebenkern function and destination. The dark structure is probably a derivative of the first type of mitochondria which also shows aggregation tendencies. This structure seems to represent a 'Nebenkern' derivative without apparently having passed the usual preceding elaborated structural changes of the Nebenkern as for instance have been described by TATES (1971) for *D. melanogaster*. According to FAVARD and ANDRÉ (1970) a function of these structural changes in Nebenkern derivatives is a redistribution of compounds.

The 'fusion' type of mitochondria might contribute to this process and subsequently degenerate in discarded cytoplasm, leaving other functions to the dark structure which seem to function as a Nebenkern derivative. More information on these aspects of spermiogenesis in *H. antiqua* is needed to arrive at solidly based conclusions.

The scarcity of truly comprehensive accounts of the ultrastructure of all germinal cell types in insect spermatogenesis indicates the vastness of the field covered. The only study on the ultrastructure of spermatogonia, spermatocytes, spermatids and sperm cells in a coherent description is the work of TATES (1971) with *Drosophila melanogaster*. Later RASMUSSEN (1973) published a similar study of a more limited scope.

The ultrastructure of insect spermatogonia has been investigated mostly as a by-product of other studies (e.g. MEYER, 1961; MENON, 1969; MACKINNON and BASRUR, 1969, 1970), which confirms the relatively little general interest in insect spermatogonia. Spermatocytes received more attention because of interesting phenomena connected with meiotic divisions. Synaptonemal complexes, which were described for the first time by MOSES (1956) and were subsequently found in a variety of organisms including insects (MAILLET and FOLLIOT, 1965; FOLLIOT and MAILLET, 1966; GASSNER, 1967a, b; FOLLIOT, 1968, MOSES, 1968; WHITE, 1973), seem to be lacking in male Diptera in which no crossing over takes place (MEYER, 1964). Centriole formation in relation to the spindle in dividing spermatocytes has been studied extensively (DIETZ, 1966; FRIEDLANDER and WAHRMAN, 1966, 1970; RATTNER, 1972). ERICKSON and ACTON (1969) reported in spermatocytes of a 'meiotic drive' strain of *D. melanogaster* the presence of granules which they assume to be rickettsiae. The ultrastructure of *D. melanogaster* spermatocytes has been described by MEYER (1960, 1964), MEYER et al., (1961), TATES (1971) and RASMUSSEN (1973), in *D. virilis* by ITO (1960).

The drastic morphological changes of the spermatogenic cells after the second meiotic division, the spermiogenesis, have attracted a considerable amount of attention. Spermiogenesis in *D. melanogaster* has been described by MEYER (1968), TOKUYASU et al. (1972a, b) and TATES (1971); of *Musca domestica* by GASSNER et al. (1971) and of *Sciara coprophila* by PHILLIPS (1966). The spermatid differentiation has been studied in Orthoptera: e.g. SCHIN (1965), KAYE (1967, 1970), KAYE and KAYE (1966) in *Acheta domesticus* and YASUZUMI et al. (1957, 1970a, b; 1971) in grasshoppers. In the bug *Murgantia histrionica* (PRATT, 1967) and the beetle *Cicindela flavopunctata* (BARKER et al., 1967) various aspects of spermiogenesis have been investigated, whereas comparative work has been carried out by BRELAND et al. (1966, 1968).

Probably because of their complicated and intrigueing ultrastructure sperm cells have been a favourite object. Reviews on insect sperm cells were published by PHILLIPS (1970) and BACCETTI (1970, 1972). ANDERSON and PERSONNE (1970) compared the localization of glycogen in sperm cells of a large number of animals including insects. BACCETTI et al. (1970, 1971) focussed their attention on the cell wall of sperm cells. Microtubuli were studied by ROSS (1968), SHAY

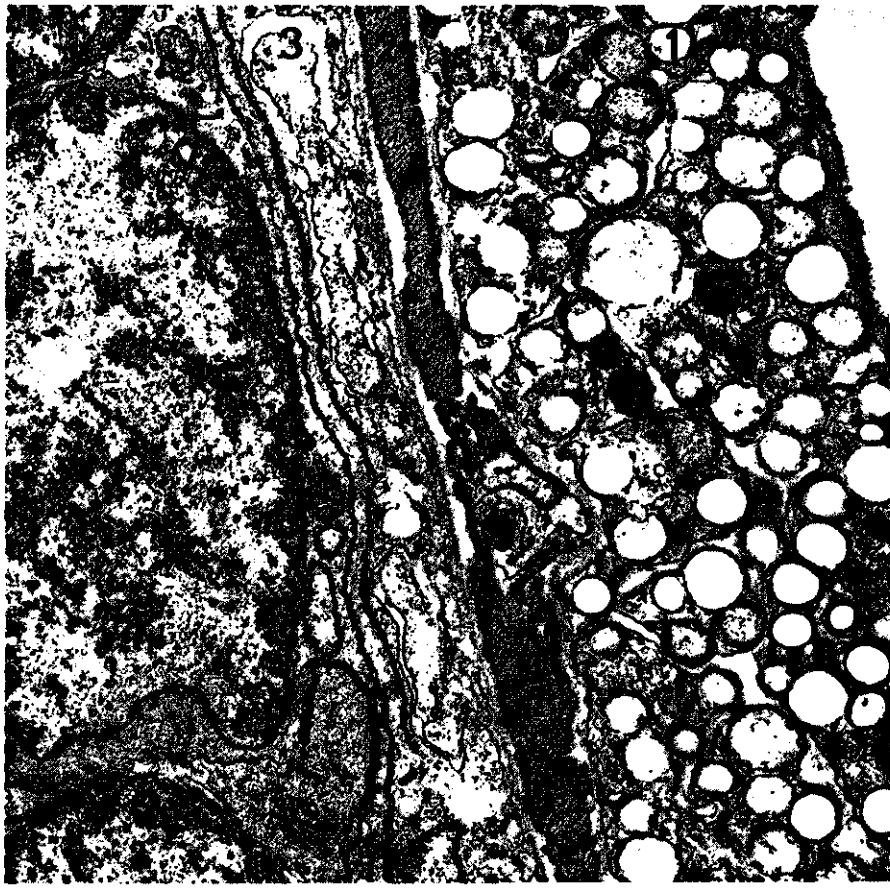


FIG. 43. The three layers of the testicular sheath:
1. the vacuolated and porous outer layer
2. the myofibrils containing middle layer
3. the squamous epithelium which covers the entire testis.
The boundaries of the germinal cells (left) with the covering epithelium are often indistinct. 22,079 x.

(1972) and others. Sperm cells of Lepidoptera have been investigated by RIEMANN and GASSNER (1971) and RIEMANN (1973), of the Dictyopteran *Pycnoscelus indicus* by SHAHANEY et al. (1971) and of the Mecopteran *Panorpa nuptialis* by GASSNER et al. (1972).

As for the way in which the germinal cells become organized in cysts and multiply, prior to the meiotic divisions, some data are given in paragraph 3.5.4. in which the dynamics of spermatogenesis is discussed.

3.5.2.3. Somatic cell types

In the young adult testis a number of cell types of somatic origin are found,

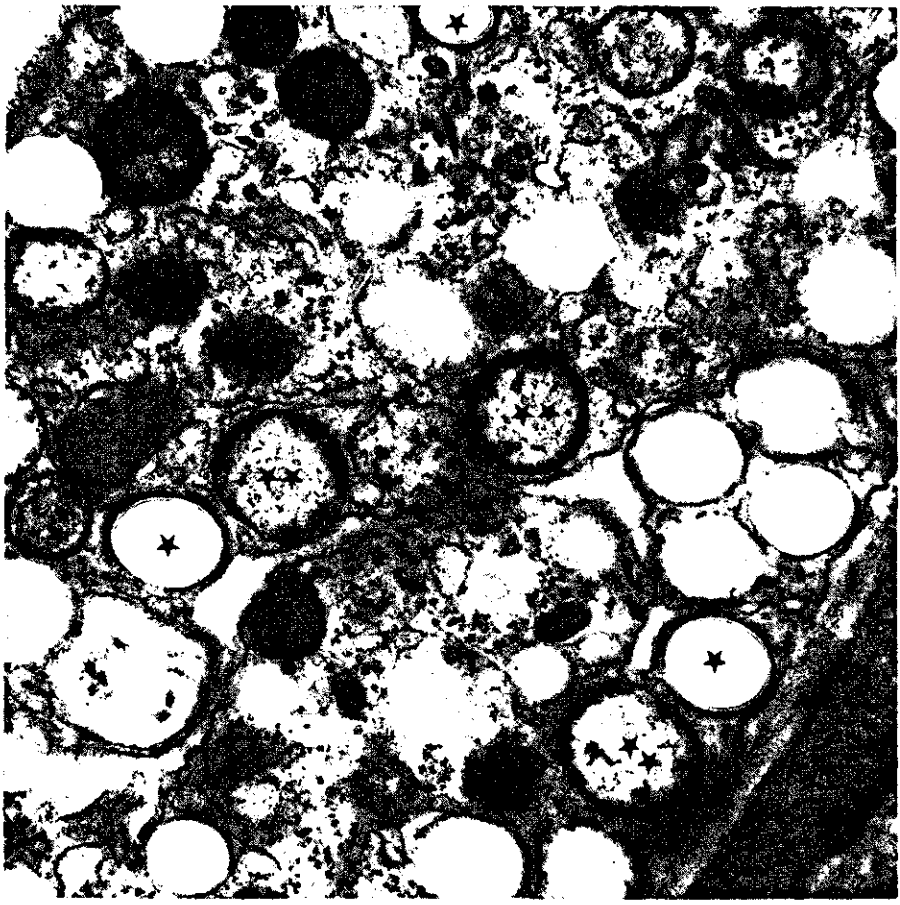


FIG. 44. Detail of outer layer. Microtubules, vesicles, clusters of ribosomes, vacuoles, membranous structures are visible. Some vacuoles show a sharply delimited brim (*), others precipitation of electron dense material against the wall of the vacuole (**). 59,613 x.

which form and shape the organ in order to enable a proper functioning of it. These cells comprise: those of the testicular sheath, the apical cell, the cyst cells, the central cavity cells, those of the terminal epithelium, and those which form the membrana basilaris.

3.5.2.3.1. Testicular sheath

The testis is covered by the *testicular sheath*. This structure is composed of three main layers separated by two non-cellular strata (fig. 43):

1. an *outer layer*, whose ultrastructure is very striking. The cytoplasm of the flat, stretched cells has a spongy appearance because of the numerous vacuoles of a fairly uniform size. These vacuoles, which are spheroidal and on average about 250 nm, are empty or show a varying degree of filling with more

or less electron dense material (fig. 44). Some vacuoles are nearly empty and only contain a sharply delimited rim (*), others show a precipitation of electron dense material against the wall of the vacuole, some of them containing less electron dense material in the vacuolar lumen (**). A number of more or less completely filled vacuoles show locally an orderly arrangement of electron dense granules of uniform size.

Apart from the vacuoles, the cytoplasm contains clusters of ribosomes, membranes, smooth endoplasmic reticulum, vesicles, single microtubules, relatively very few and small mitochondria and occasionally a Golgi-complex. The groups of ribosomes are numerous and very distinct. Membranes traverse the cytoplasm in a seemingly haphazard manner. Cisternae of smooth ER are locally present in restricted numbers as well as are groups of vesicles of somewhat dilated smooth ER in which material has been precipitated in varying degrees. Microtubules are not observed in bundles. The intracellular space between the organelles and other structural cellular components is filled with a cloudy substance. In addition to this in cells of this type covering the most apical region of the testis, large, seemingly empty and irregularly formed spaces are observed which are delimited by membranes. These spaces tend to occur preferentially at the innermost half of the thickened layer of cytoplasm.

This outer layer may be locally absent or deformed by the presence of a tracheal cell at the outside, but generally the thickness of it is in the range of 1.1–2.5 μ. The nuclei are ovoid and measure about 20 μ × 5 μ. Usually the outer layer covers the testis smoothly and the thickness is more or less constant. At the apical part of the testis, however, the layer undulates and exhibits the already described more irregular ultrastructure. The limits of the layer are fairly distinct both at the outer and the inner side. At the outer side the cell membrane is covered with a thin, even layer of amorphous material of about 70 nm. At the inner side of the outer layer a narrow non-cellular stratum is found, varying in thickness but nearly always present (fig. 45). It separates the outer layer from

2. the *middle layer*, which is formed by flattened cells which contain mainly myofibrils. Traces of other cytoplasmic components are seen at invaginations in the myofibrillar bundle of varying thickness which averages about 300–400 nm but may be locally absent. Nuclei are scarcely found. They are flat and measure about 6–7 μ × 1 μ. The myofibrillar layer can be clearly interrupted by means of transverse boundaries which may also be cell walls. Other cases show a certain overlap of thin cell margins. At places where the myofibrillar layer is interrupted, other cytoplasmic elements may be usually found: small parts of ground substance enclosed by membranes, small mitochondria, some microtubules, and small numbers of ribosomes. Of all cell organelles only mitochondria are seen in the myofibrillar layer itself.

There is some evidence that the middle layer at the most apical region of the testis is more irregular in presence and size than elsewhere.

The middle and inner layer are separated by a narrow, mostly clear zone (fig. 45) which varies in thickness but obviously is continuous. This is the second

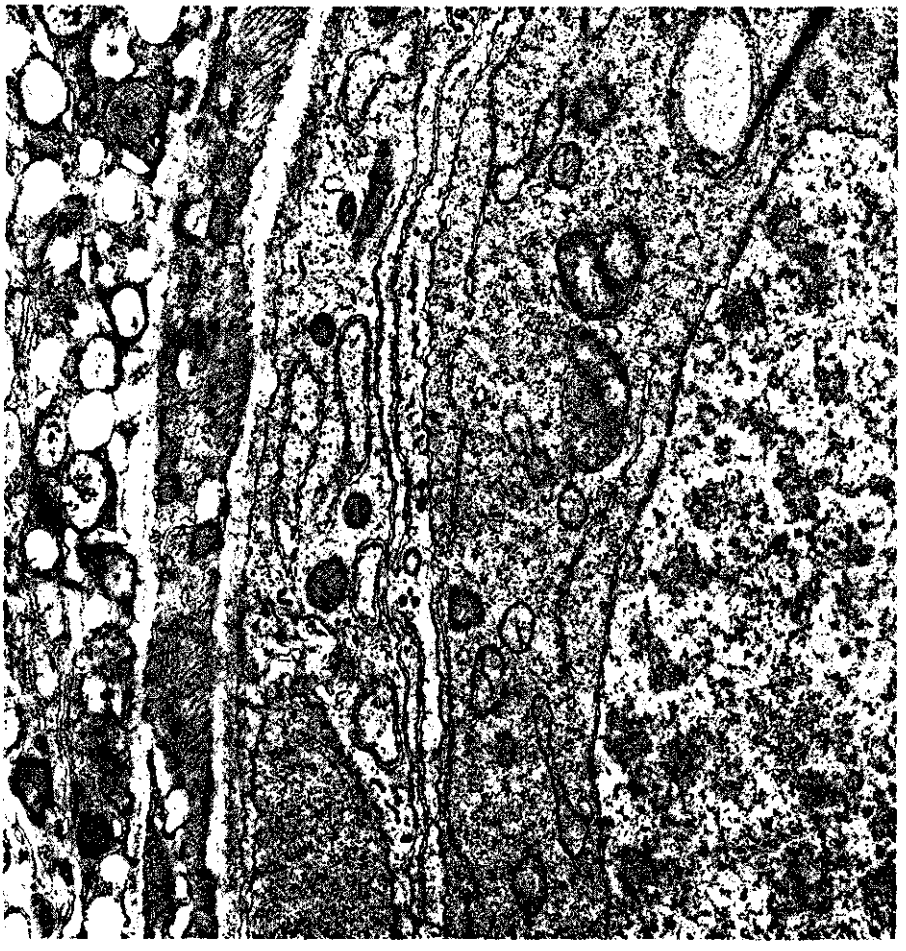


FIG. 45. The testicular sheath covering a large germinal cell with partly fused mitochondria. The difference in cytoplasmic structure of the germinal and epithelial cell is clear. The two non-cellular thin layers on both sides of the middle layer are very conspicuous. 22,079 x.

non-cellular layer of the testicular sheath. It covers

3. the *inner layer* which is a squamous epithelium with the typical ultrastructural appearance of a testicular epithelial cell (fig. 46). The cytoplasm is lightly contrasted with numerous clusters of ribosomes, large mitochondria mostly with clear cristae, a very irregular course of the cell walls and other membranous structures which are often covered with ribosomes. The nuclei are varying in shape and size with irregular dispersion of chromatin particles and concretions of widely differing sizes. As far as can be established this layer is always present and forms a continuous covering of the testis.

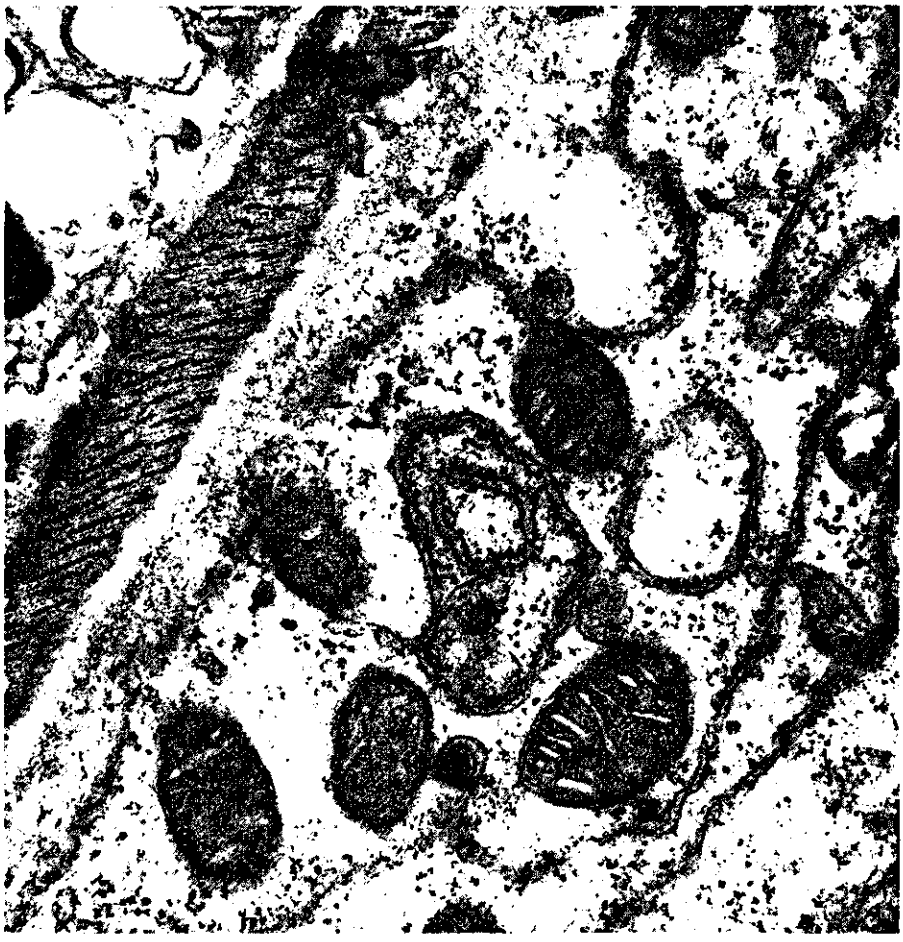


FIG. 46. Middle and inner layer of the testicular sheath. In the middle layer myofibrils are predominant. Cell limits in epithelium are very irregular and clusters of ribosomes and clear mitochondria are abundantly present. 59,613 x.

Discussion

The pigmentation of a young adult testis is yellow to pale orange and at close observation it appears that the pigmentation is not evenly dispersed over the testicular surface but that locally differences can occur in the intensity of the pigmentation. In testes of older males the pigmentation is considerably stronger. It increases with age to provide a dark brown colour. The structure of the outer layer of the testicular sheath strongly suggests that increase of the pigmentation might be correlated with an increased number of vacuoles filled with the electron dense substance. This substance might be responsable for the pigmentation and might consist of waste material derived from metabolic processes during the very active period of late pupal and adult testicular development.

The function of the undulations and the increase in thickness of the outer layer in the most apical region of the testis near the apical cell is not quite clear. Usually undulations represent a form of surface extension, such as in the intestinal tract, necessary to absorb or to release relatively large amounts of compounds. Perhaps the apical cell and/or the local population of spermatogonia require special compounds, which direct or influence spermatogonial differentiation. This could include the concept of a MF-like compound which activates spermatogonial instead of spermatocyte differentiation. The latter was observed by KAMBYSELLIS and WILLIAMS (1971 a, b) in *Samia cynthia, Hyalophora cecropia, Antheraea polyphemus* and *A. mylitta.* They concluded that a macromolecular factor (MF) is required for spermatocyte differentiation into spermatids and sperm cells. This factor is present in the hemolymph in varying concentrations but is itself unable to enter the testis. Ecdysone influences the permeability of the testicular sheath in such a way as to permit the MF to enter the testis and to cause spermatocyte differentiation. It is not known whether or not such a MF is active in Diptera. If so, the spongy structure of the outer layer of the testicular sheath of *H. antiqua* could account for storage and binding of ecdysone active compounds. In view of this structure the thickness of this layer does not seem to be an essential barrier to penetration by macromolecules. According to DUMSER and DAVEY (1974) a reduction of spermatocyte differentiation in *Rhodnius prolixus* is caused by application of a juvenile hormone analogue.

In a fresh, living testis movements along the surface can be observed. These movements are weak but clearly to discern. They appear to be caused by the middle layer of the testicular sheath which contains mainly myofibrils. The function of these movements is perhaps to stimulate the flow of the hemolymph along the testis in order to enhance the effectivity of the uptake of suitable compounds, comparable to the movements of the Malpighian tubules in the hemolymph. They might also be related to the basal transport of cysts in the testis.

The inner layer of squamous epithelium is the real covering of the testis because it is always present, contrary to the two other layers of the testicular sheath which may be sometimes locally absent. The epithelial cytoplasm is often continuous with that of the cyst cells.

The two non-cellular strata at both sides of the middle layer probably represent the basement membranes of both the cells of the outer layer and of the inner one.

HOLMGREN (1901) stated that the testicular sheath in *Staphylinus* is a syncytium and could give rise to spermatogonia as well as other testicular cell types. Discussing the pigmentation of the testis of *Melanoplus differentialis* NELSEN (1931) attributed the orange colour to fat cells covering the translucent testis follicles, the same conclusion was reached by CHASE and GILLILAND (1972) for *Heliothis virescens.* MCCLUNG (1938) reported a follicular covering consisting of a single layer of connective tissue cells in acridian testes. OMURA (1936) described the nature and development of the testis and testis follicles of *Bombyx*

mori. According to MASNER (1965) the testis wall of *Adelphocoris lineolatus* consists of two layers:

1. a peritoneal membrane, a pigmented membrane of connective tissue without nuclei, covering the whole testis.
2. a peritoneal epithelium, a syncytium with a few flat nuclei covering the individual testis follicles.

MENON (1969) described the testicular covering as consisting of a basement membrane and connective tissue sheath cells in *Tenebrio molitor*.

In general little attention has been paid to the testicular sheath, the structure of which seems to vary in accordance with the structure of the testis in the various insect species.

3.5.2.3.2. Apical cell

The apical cell is presumably a syncytium with a number of nuclei of somatic origin and is found in the most apical part of the testis among spermatogonia (fig. 10e). The number of nuclei is on an average 18.9 ± 3.5 (extremes are 10 and 25). They are basophilic and their chromatin is evenly dispersed in the nucleus. Their size and shape are fairly constant: 3–5 μ and ovoid or spherical. They are arranged in a more or less spheroidal group with a varying size of about 10–15 μ in diameter. Their mutual arrangement does not show any regularity and no cell membranes are ever seen, except at the outside of the group of nuclei where sometimes a small amount of cytoplasm is observed. Divisions have never been found in the apical cell.

The number of nuclei in the apical cell of both testes in an individual may vary as much as between individuals. They are present in the testis in an apparently unchanged condition from the early larval stage until the death of the fly. In *H. antiqua* the apical cell has no obvious structural or functional relationship with the testicular germinal cell populations.

Discussion

Considering the general features of the apical cell, which received its name 'Apikalzelle' from GRÜNBERG (1903), the apical cell of *H. antiqua* seems to be an exception in that

1. the structure consists of a syncytium of a large and variable number of nuclei surrounded by very little cytoplasm.
2. the apical cell is present in the testis from a very young larval stage until the death of the adult.
3. the spatial arrangement, size and other aspects of the appearance of the structure do hardly change with increasing age.

The apical cell is considered to be a juvenile structure because in most species it is present only in the larval stage, sometimes the pupal stage and relatively seldom in the adult stage (MENON, 1969). Usually, changes in appearance refer to regressive alterations in shape, size and stainability due to a gradual physiological degeneration, whether or not accompanied by ingrowth of tracheae (DEMOKIDOFF, 1902; CHOLODKOVSKY, 1905, MENON, 1969). Such phenomena

are not observed in *H. antiqua*. The variation in appearance of the syncytium between individuals in the larval and in the advanced adult stage is similar to that between individuals of the same age in every developmental stage. As far as the available information indicates, the variable presence of the apical cell in the developmental stages of the insects seems to coincide with the presence of spermatogonia in the apical part of the testis or testis follicle concerned. In many Lepidoptera the young adult testis does not anymore contain spermatogonia. In these cases the apical cell has also disappeared. For instance, in *Heliothis virescens* degeneration of the apical cell in the adult testis is reported to coincide with the degeneration of the last indefinitive spermatogonia and the termination of cell divisions in the testis (CHEN and GRAVES, 1970). In a young adult of *Adoxophyes orana*, the apical cell surrounded by spermatogonia is found. In *H. antiqua* both the apical cell and a population of primary spermatogonia are found at all stages of development.

Observations on the apical cell in insects of various orders including the Diptera, depict the apical cell as a structure rich in cytoplasm containing one or a small number of nuclei. Such an appearance may give rise to hypotheses regarding a nutritional function, both in terms of supply of nutrients or in formation of mitochondria (e.g. DAVIS, 1908; CARSON, 1945). Another concept implies that substances secreted by the apical cell may influence both degeneration and differentiation of germinal cells in the vicinity (e.g. NELSEN, 1931) leading towards a directing function in the formation and differentiation of germinal cells (ZICK, 1911) or in sex determination (JUNKER, 1923). Influence of secretion by the apical cell on the genetical information in the chromosomes of neighbouring germinal cells was proposed by MCCLUNG (1938). A more recent concept is the hormogenic activity of the apical cell in *Lampyris noctiluca* (NAISSE, 1965, 1966 a, b).

Despite large differences in appearance of the apical cell in different species it does not seem correct to assume essential different functions of the apical cell in these species because there is no experimental evidence to support such an assumption. A particular effect might be obtained by means of various pathways which may be more or less related.

In *H. antiqua* a possible function of the apical cell can hardly be imagined to depend on synthetic activities of the cytoplasm. More likely seems to be a function in which the nuclei play a major role.

The direction of the stem cell mechanism by means of secretions does not necessarily require large quantities of active compounds but could be accomplished by relatively minute amounts of nuclear origin. The general combination of apical cell with primary spermatogonia supports such a speculation. The local direct influence of the apical cell might be made effective in the whole of the spermatogenic processes by the regulation of the stem cell mechanism by which the number of spermatogonia in semi-dichotomous division is determined.

NAISSE (1965, 1966 a, b) proposed that the apical cell in *Lampyris noctiluca* could have an endocrine function in that it secretes an androgenic hormone:

'Cette masculinisation pourrait être attribueé au tissu apical qui serait la source de l'hormone androgène' (NAISSE 1966a). MENON (1969) tried to verify Naisse's results in *Tenebrio molitor* and *Zophobas rugipes*. She could not conclude to the presence of a hormogenic function of the apical cell in these species. Secretory activity of the apical cell in *Tenebrio molitor*, however, has been established. Although Naisse could be right, her conclusion that the apical cell seems to be the centre of androgenic activity may be considered premature because apart from the apical cell, other tissues of the male reproductive organs might be capable of secreting active substances. Examples could be the outer layer of the testicular sheath or the epithelium of the ductus deferens. The findings of DESTEPHANO et al. (1974) point in this direction.

In any case the conclusion derived from her work with *L. noctiluca* that the presence of the apical cell determines the sex of the gonad does not apply for *H. antiqua*. In the onion fly the sex of the young gonad can be discerned at an age of 6–7 days when cells of the surrounding epithelium either differentiate into an apical somatic tissue or into a number of individual somatic cells which migrate into the interior of the gonad and gradually form the apical cell (see page 81). In the former case the gonad is an ovary, in the latter a testis. Sex determination has taken place before the apical cell has been formed. Unless one presumes that its activity starts before the fusion of the individual epithelium cells into a possibly secreting syncytium takes place, the apical cell has no directing influence on the sex of the gonad.

3.5.2.3.3. Cyst cells

The *cyst cells* are squamous epithelial cells which separate the germinal cell families in '*cysts*'. The cyst cells originate from small somatic cells which lie among the spermatogonia in the apical part of the testis near the apical cell. They are already present in the young larval testis where they migrate from the surrounding epithelium into the interior of the gonad, as do the cells which form the apical cell.

In the top of the young adult testis the cysts cells are abundantly present as indicated by their small ($\leqslant$ 5–6 μ), darkly stained and variably shaped nuclei. The shape of the nuclei can be triangular, polygonal, spearhead-like, wedge-like or ovoid. Cyst cell nuclei which are situated against the testicular sheath tend to be triangular. The cyst cell cytoplasm is not commonly observed in histological preparations.

At a certain moment one or more cyst cells seem to become associated with a spermatogonium. When this spermatogonium divides the cyst cells may extend a thin epithelium around the daughter cells and a cyst has been formed. The cyst usually remains intact after repeated divisions until spermatid differentiation has proceeded half way towards sperm cells. At that moment the many times enlarged cyst tends to show vague and disturbed boundaries which are often disrupted. During the period of the repeating germinal cell divisions the cyst epithelium fits tightly to the growing group of germinal cells. After the first meiotic division the cyst epithelium tend to become more and more loosely

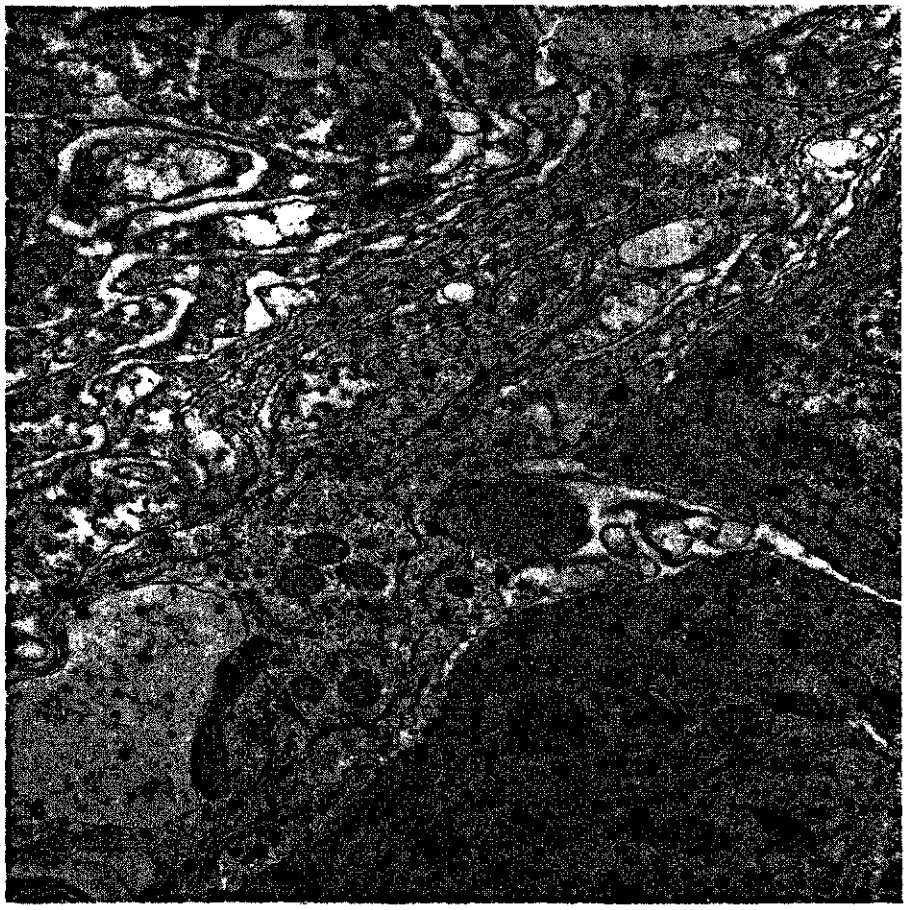


FIG. 47. Cytoplasm of cyst cells which cover germinal cells (below, right). The cytoplasm probably is a part of one or more cyst cells. The boundary between the cyst cells is very irregular and show occasionally septate desmosomes (arrow). 22,079 x.

fitting around the secondary spermatocytes and the various types of spermatids until the epithelium is torn and/or disappears.

The cyst cell nuclei which have migrated with the germinal cells towards a more central position in the testis become larger, more rounded or triangular in shape and whether less or more intensely stained with regularly dispersed relatively coarse chromatin.

On the ultrastructural level the cyst cells appear to be cells with a lightly contrasted loose cytoplasmic structure with little developed cytoplasmic organelles except very often elongated, mitochondria and with very irregular cellular limits (fig. 47). The cyst cells seem to be mutually connected partly by means of septate desmosomes (fig. 48). In the cytoplasm occasionally electron dense structures containing microtubules are observed (fig. 48).

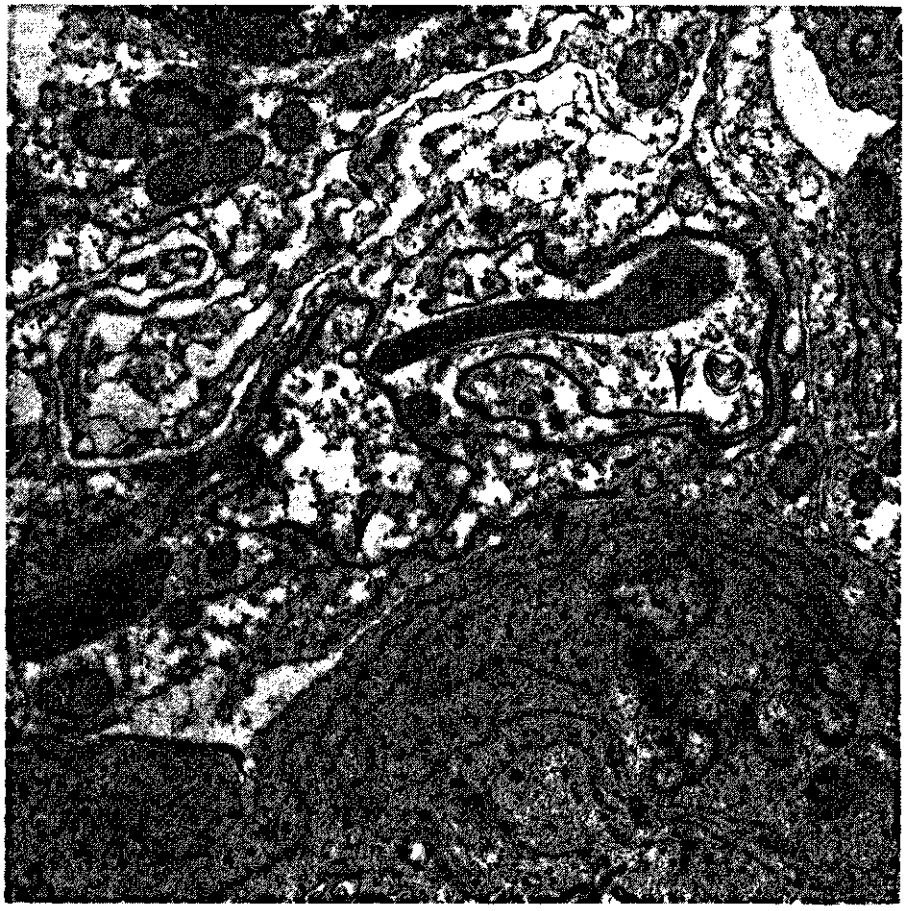


FIG. 48. Cyst cell epithelium between three cysts. The somatic cytoplasm is light and loose of structure. The boundaries between the cyst cells are very irregular and consist partly of septate desmosomes (arrows). 22,079 x.

Discussion

MASNER (1965) distinguished in *Adelphocoris lineolatus* two types of cyst cells according to their shape and location:

1. parietal cyst cells with flat nuclei as cyst epithelium 'sensu stricto'.
2. interstitial cyst cells with irregularly formed nuclei which take part in the inner architecture of the somatic elements in the testis.

In *H. antiqua* this distinction could be made as well but seems to be of a limited significance because most cyst cells are situated at junctions where the cysts touch each other and/or contact the central cavity (fig. 10 m). However, a minority of cyst cells correspond to the position and appearance as has been described for parietal cysts cells.

GRÜNBERG (1903) assumed a nutritive function of the cyst cells. According to

TOKUYASU et al. (1972) the cyst cells have a nutritive function for the developing sperm bundles in the basal part of the testis of *Drosophila melanogaster*. A nutritive function at a later stage of the spermatogenic process does not interfere principally with a supporting function in an earlier stage (MASNER, 1965).

As for *H. antiqua* the function of the cyst cells does not seem to be especially nutritive in view of the ultrastructural characteristics of the cytoplasm. The relative absence of endoplasmic reticulum, Golgi-complexes and ribosomes does not point to secretory activities. On the contrary, the dominance of occasionally large mitochondria and a very irregular and folded cell wall suggest more a function of selective and active transport, a passing through from the interior of the testis into the germinal cells and vice versa. The electron dense, microtubuli containing structures in the cytoplasm of the cyst cells (fig. 48) may contribute to a certain firmness of the cell but a pure supporting function does not seem probable. A nutritive function for the sperm cells, as found by TOKUYASU et al. (1972). in *D. melanogaster*, is not supported by the facts because the cyst epithelium tends to disintegrate during the stage of the intermediate spermatids. Few sperm cell bundles are observed still enclosed by cyst epithelium. Moreover, the ultrastructural characteristics of the cyst cell cytoplasm are retained up to and including the stage of the early spermatids. This precludes a gradual change of function of the cyst cell during the progress of the spermatogenic cycle.

3.5.2.3.4. Central cavity cells

Central cavity cells are cells of somatic origin which are found exclusively in and around the central cavity of the testis. They have long (10–16 μ) ovoid nuclei with finely granular and regularly dispersed chromatin and a brightly red staining nucleolus (fig. 10 n). These relatively weak staining large nuclei are never seen in large numbers. The cytoplasm of these cells is very indistinct and a clear cell membrane has never been observed. Their position in the central cavity is seldom in the core but nearly always among cysts of spermatids, individual sperm cells and sperm cells in bundles. When they lie near a sperm bundle the long axis of a nucleus is parallel to that of the bundle.

Discussion

The origin of the central cavity cells is not quite clear. They are most probably formed by the most apical parts of the terminal epithelium. The terminal epithelial cells cover internally the testicular sheath. Cells from the terminal epithelium seem to extend in an apical direction. The nuclei of these cells are generally larger than those in cells close to the ductus deferens and have an appearance very similar to that of the central cavity cells. Moreover, these cells are seen leaving their peripheral position and losing contact with the testicular sheath. Once free in the central cavity they can not be distinguished as former terminal epithelium cells. During the process of isolation from the main population and probable conversion into a central cavity cell the cytoplasm becomes increasingly indistinct till it is hardly or not at all visible.

In the literature cells like the central cavity cells in *H. antiqua* are very rarely described. CHOLODKOVSKY (1905) reported the presence of small numbers of large nuclei between sperm cells of a number of Diptera: *Culex annulatus, Calliphora erythrocephala, Xylota segnis, Empis tesselata, Thereva annulata* and *Leptis scolopacea.* VIRKKI (1953) described among other somatic testicular cell types of *Aphodius* spp. the so called 'Interstitialzellen'. They are characterized by a spheroidal-ellipsoidal or irregular nuclear shape and they vary in size. They are predominantly found in the most basal part of the testis but also between the cysts with germinal cells in meiotic division. These cells are considered to be related to the cyst cells and to have some nutritive function. It is quite possible that Virkki's 'Interstitialzellen' and the central cavity cells in *H. antiqua* have a similar function. However, the use of the term 'Interstitialzellen' for these cells is confusing as it is used to indicate cyst cells as well (e.g. MASNER, 1965).

The function of the central cavity cells in *H. antiqua* is far from clear. The position of these cells among the other elements in the central cavity suggests some involvement with the germinal cells, in particular the sperm cells. A nutritive involvement seems to be excluded because of the reduced development of the cytoplasm. A function by means of nuclear influence on the development and/or maintenance of the sperm cells might be assumed, thus accounting for the considerable nuclear dimensions. Against the nucleus or in the cytoplasm of central cavity cells sperm cells are never seen. The absence of a special spatial relationship between both cell types seem to exclude the possibility that central cavity cells are special cysts cells like the 'head cyst cell' in *Drosophila melanogaster* (TOKUYASU et al., 1972).

3.5.2.3.5. Basal cells

At the periphery of the basal part of the testis and sometimes in the central cavity, darkly staining and irregularly shaped nuclei can be observed. These cells with their very indistinct cytoplasm are named: *basal cells* because of their location (fig. 10 o). The nuclei are generally larger than those of cyst cells. The basal cells are found amidst cysts of developing spermatids and sometimes among sperm cells situated against the testicular sheath.

Discussion

As to the origin and function of basal cells nothing is known. They most probably represent intermediate stages between individualized cells of the terminal epithelium and central cavity cells when their location, appearance and nuclear size are taken into account.

No description or indication of basal cells is to be found in the literature.

3.5.2.3.6. Terminal epithelium

The *terminal epithelium* is found around the orifice of the testis. It is continuous with the epithelium of the ductus deferens. The cells of the terminal epithelium are cubical with prominent ovoid nuclei which show regularly

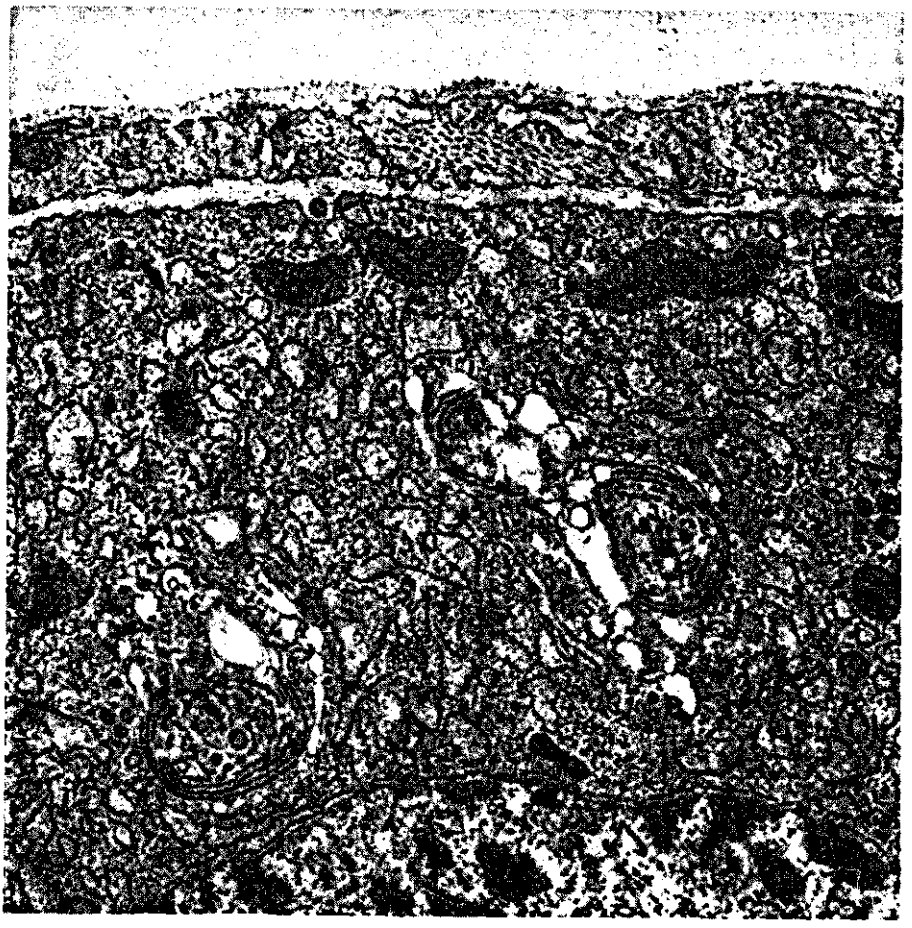


FIG. 49. Cytoplasm and nucleus (below) from cell of the terminal epithelium. The cytoplasm contains numerous vesicles and many Golgi-complexes. The external surface is covered with a squamous epithelium. This contains myofibrillar structures. 20,093 x.

dispersed chromatin. At the ultrastructural level the cytoplasm appears to be studded with smooth surfaced vesicles, Golgi-complexes and mitochondria (fig. 49). Near the Golgi-complexes vacuoles of varying size are found. The epithelium is covered by a basement membrane, a thin layer of squamous epithelium in which many bundles of myofibrils are seen and a very thin amorphous layer covering the outer layer of the testicular sheath.

Discussion

In *Aphodius* spp., VIRKKI (1953) reported various degrees of polyploidy in the large secretory cells of the very distal part of the ductus deferens near the testis. These cells were the largest in the testis. He is probably referring to

terminal epithelial cells as he mentions the cells of the ductus deferens as a separate somatic cell type, also showing polyploidy. Polyploidy generally indicates an increased metabolic activity. MASNER (1965) found no evidence for an active secretory function of the epithelium of the distal part of the ductus deferens in *Adelphocoris lineolatus*. This was described as the direct continuation of the peritoneal epithelium of the testis. No terminal epithelium was mentioned, contrary to DAVEY (1965) who reported the presence of special epithelium cells, in fact terminal epithelium, in the orifice of the testis of *Rhodnius prolixus*. A secretory epithelium has been found in the basal part of the testis of a Crustacean, *Orchestia gammarella* by MEUSY (1963). This 'couche bordante' seems to be similar with regard to its presumed function to the terminal epithelium in insects. Active secretory activity was observed in the epithelium of the upper ductus deferens, in *Choristoneura fumiferana* (OUTRAM, 1970). Large droplets of secretory material were seen in the lumen near the densely vacuolated cells, which are surrounded by a thin layer of circular muscle. Other indications of secretory activity in terminal epithelium cells have been found in the cockroaches *Byrsothria fumigata* and *Gromphadorhina portentosa*. In a histochemical study of the terminal epithelium, called 'neck cells', and the epithelium of the testis follicles LÜSIS et al (1970) identified several groups of compounds. In the terminal epithelium the presence of a distinct lipoprotein fraction, acid phosphatases and steroid dehydrogenases was established. In these cells well developed Golgi-complexes were observed pointing towards a secretory function. The problem of LÜSIS et al. (1970) of combining the secretory function of the terminal epithelium with the production of lytic enzymes such as acid phosphatases perhaps has been solved by TOKUYASU et al. (1972) in *Drosophila melanogaster*.

In *H. antiqua* no spatial or structural relationship has been observed between the terminal epithelium cells and individual or bundled sperm cells. Although many sperm cells and bundles may be located against or near the terminal epithelium, no evidence has been found to assume a special function in a mechanical sense, as described by TOKUYASU et al. (1972) for *D. melanogaster*. However, the abundance of Golgi-complexes and smooth surfaced vesicles strongly suggests a secretory function of these cells. A high acid phosphatase activity in the terminal epithelium cells of *D. melanogaster* has been related to a phagocytic function (TOKUYASU et al., 1972) necessary to dissolve cyst cells in order to liberate the sperm cells from the cysts. In *H. antiqua* this process does not seem to take place necessarily in the most basal part of the testis. Nevertheless, secretions of the terminal epithelium cells might contribute to the dissolution of waste material produced by the differentiation of the sperm cells.

3.5.2.3.7. Membrana basilaris

A '*membrana basilaris*', which prevents the sperm cells to leave the testis, is very weakly developed in *H. antiqua*. It is observed as a very thin membrane covering the orifice of the testis between the sperm cell mass and the terminal

epithelium during the first day after emergence of the fly. Its origin is not known, but its position close to the terminal epithelium suggests a possible formation of the membrane by this epithelium. The function of the membrana basilaris is more effectively performed by the terminal epithelium, which closes the testis during the first day after emergence, by a local swelling of a limited number of cells resulting in columnar epithelium cells. These columnar cells which oppose each other from all directions, form a plug in the orifice of the testis preventing sperm cells from leaving the testis. This is achieved in combination with the basilar membrane. During the second day after emergence the swelling of the cells diminishes causing an aperture which increases gradually in size. The membrana basilaris disappears. As soon as the entrance to the ductus deferens is opened individual sperm cells enter the duct.

Discussion

In *Rhodnius prolixus* a similar functioning of the terminal epithelium has been reported (DAVEY, 1965) but in *Heliothis virescens* a clear membrana basilaris is observed separating the lumina of the testis follicles and their ductuli deferentia (CHASE and GILLILAND, 1972). In *Bombyx mori* the basilar membrane prevents the exit of sperm cells until the late pupal stage (OMURA, 1936). VIRKKI (1956) reported the presence of a basilar membrane in Coleoptera.

3.5.2.3.8. General discussion on somatic cell types

Whereas the various somatic cell types could be identified in histological preparations, this was mostly impossible in the living condition. One conspicuous cell type conforms to the following description:

Cells with relatively small, compact and dark nuclei showing a regular-even dispersion of chromatin granules of varying size. The nuclei are excentrically situated in the cell against a distinct, round-oval vacuole of varying size which occupies the cell center. The vacuole is mostly about the same size or somewhat larger than the nucleus. Around the nuclear-vacuolar complex the cytoplasm contains numerous mitochondria (figs. 12, 8; 50).

The description of these living cells does not fit any of those in histological preparations. Especially the centrally located vacuole is nowhere seen in fixed testicular cells. Possibly this cell type represents a somatic type with a nutritive or secretory function like young central cavity cells.

Somatic cell types are generally distinguished from living germinal cell types by their sharp nuclear outline because of chromatin which is precipitated against the nuclear membrane (fig. 51). Differences between living somatic cell types are far less distinct as compared to those in squash and semi-squash preparations.

Specific testicular somatic cell types could not be identified. Nucleolar structures, like those described for species of *Drosophila* (STOCKERT and ESPONDA, 1971) were not found.

As for the occurrence of 'b-type' cells in the described cell types, the cells of the testicular sheath, the terminal epithelium and the cyst cells occasionally

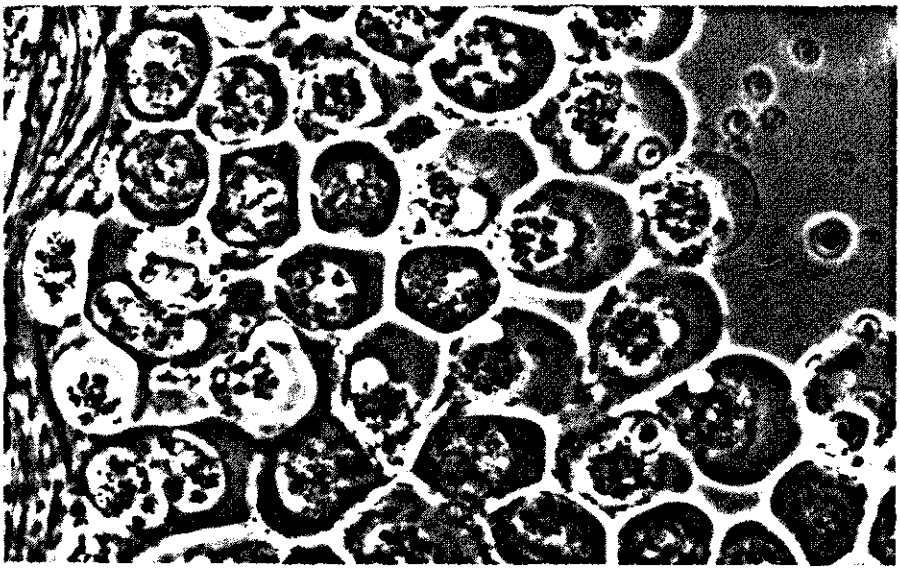


FIG. 50. Living cells of unidentified type (Fig. 12, 8) among primary spermatocytes (Fig. 12, 3).

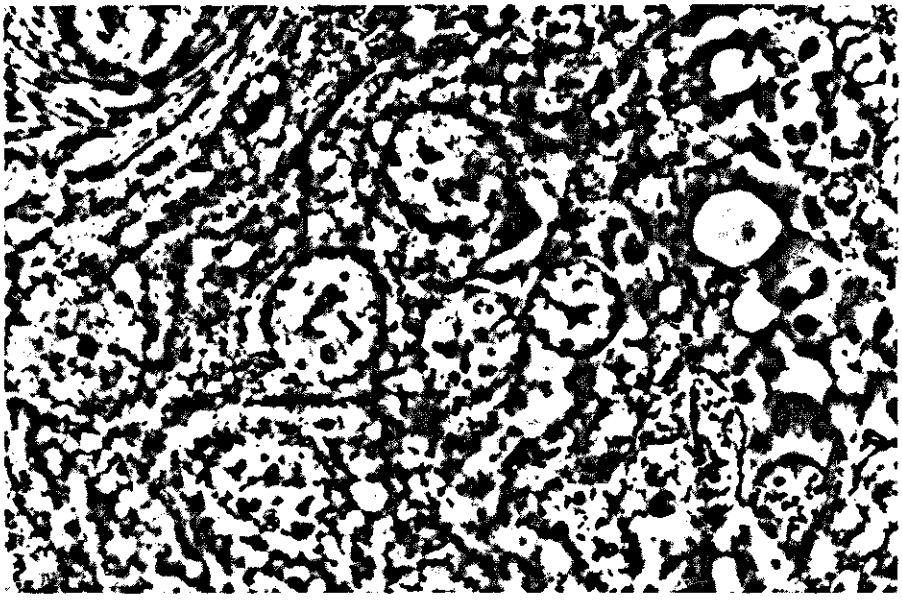


FIG. 51. Living testicular somatic cells. Figs. 50 and 51 same magnification as Fig. 4.

show nuclei with concentrated chromatin. Especially among cyst cells the proportion of 'b-type' cells may be very high at some moments during testicular development in particular in spermatogonial populations. In the apical cell, the central cavity cells and the basal cells, 'b-type' cell nuclei have never been observed.

3.5.3. *Testicular development*

The testis is hard to find in the living larva. It is smaller than an average fat cell, glassy opaque and hidden between the larval fat cells. The testes are situated in the third quarter of the body.

The pupal testes are situated in the caudal half of the abdomen, dorso-lateral and close to the epidermis. The apical part of the testis points caudad, the ductus deferens cephalad.

In the adult the testes can be found in the 4th–5th abdominal segments, dorso-lateral of the intestinal tract. They have rotated and their apical part now points cephalad. At emergence the testes are pale orange and become coloured from light to dark brown at increasing age.

The development of the testis during the larval, pupal and adult stage will now be discussed.

3.5.3.1. Larval development

In the course of larval development at the rearing temperature of 18 °C the following changes are observed in a chronological sequence:

6–7 days: The gonads can be usually recognized as male or female. The criterion for the male gonad is the formation of a distinct apical cell which penetrate the group of germinal cells. The apical cell is observed as a more or less spheroidal, but sometimes flattened or protracted, cluster of 'a-type' somatic nuclei, situated against the testicular epithelium. The nuclei are very similar in appearance and stainability when compared with the other somatic nuclei. This similarity may present difficulties in the identification of a very young apical cell.

8 days: The apical cell tends to separate from the covering epithelium. The somatic cells which cover the testis change from the 'a-type' into the 'b-type' (see page 59 and THEUNISSEN, 1973a) forming an epithelium wich is intermediate between a squamous and a cubical one. Basally of the testis the development of a *basal somatic tissue*, which later connects with the already present solid ductus deferens, is evident.

9 days: The separation of the apical cell from the testicular epithelium proceeds. Most somatic cells show the 'b-type', except the apical cell nuclei and those of the basal somatic tissue. The basal somatic tissue originates from an accumulation of somatic cells at the periphery of the young larval gonad and is formed at the basal part of the testis. At that position the somatic cells multiply to form the future terminal epithelium and a short connection which contacts at a later stage the independently developing ductus deferens. Sometimes 'a-type' somatic cells in the testis resemble very much those of the apical cell, thus suggesting a common origin. A general increase in mitotic activity is observed.

Externally a thin layer seems to be added to the 'b-type' cubical epithelium which covers the testis. Both layers will be indicated henceforth as testicular sheath. The primary spermatogonia show some 'concentrated' types.

10 days: The mitotic activity increases in the testes. In general, the apical cell is completely separated from the testicular sheath. It does not show any changes when compared to younger ones. The somatic cells are usually of the 'b-type', contrary to those of the basal somatic tissue. The primary spermatogonia show an increasing number of 'concentrated' types but the 'diffuse' type is still dominant in numbers (fig. 52, b).

14 days: The somatic cells have not changed in appearance. The mitotic activity is still increasing. The primary spermatogonia show predominantly 'concentrated' cell types.

17 days: The majority of the somatic cells show the 'b-type'. The nuclei of the apical cell are smaller in size and more darkly stained, giving the apical cell the appearance which is characteristic in the further testicular development in the pupal and adult stages. The density of the nuclei in the syncytium does not seem to have changed (table 1). Small apical cavities are observed basally in the testes. A considerable variety of 'concentrated' types of primary spermatogonia is observed. Like in the somatic cell populations the mitotic activity is variable but at a relatively high level.

21 days: Larvae of this age may be considered as 'mature'. The testis itself is still smaller than the average cell of the larval adipose tissue. The apical cell shows the appearance of a spheroidal structure of darkly stained, 'a-type' somatic nuclei among the primary spermatogonia. Somatic cells of both the 'a-type' and the 'b-type' are scattered in the testis. The testicular cells are tightly packed, except those situated against the basal somatic tissue where apical cavities are found, often containing bi-nucleate cells (fig. 52). The appearance of these cells mostly differs from that of the primary spermatogonia.

TABLE 1. Diameter of the apical cell in larval testes.

Age (days)	Diameter (μ)	Average (μ)
8	18, 27, 27	24.0
9	13, 14, 16, 20, 26	17.8
10	17, 19, 20, 20, 21, 22, 26,	20.7
14	21, 22, 25, 25, 26, 33	25.3
17	18, 21, 21, 25	21.2

The diameter of the apical cell does not seem to change significantly during the larval stage. Variations may be due to a number of causes, among others a varying number of nuclei in the apical cell.

Discussion

As in other Diptera, e.g. *Drosophila virilis* and *D. melanogaster* primary spermatocytes were found in testes of mature larvae (CLAYTON, 1957; KAUF-

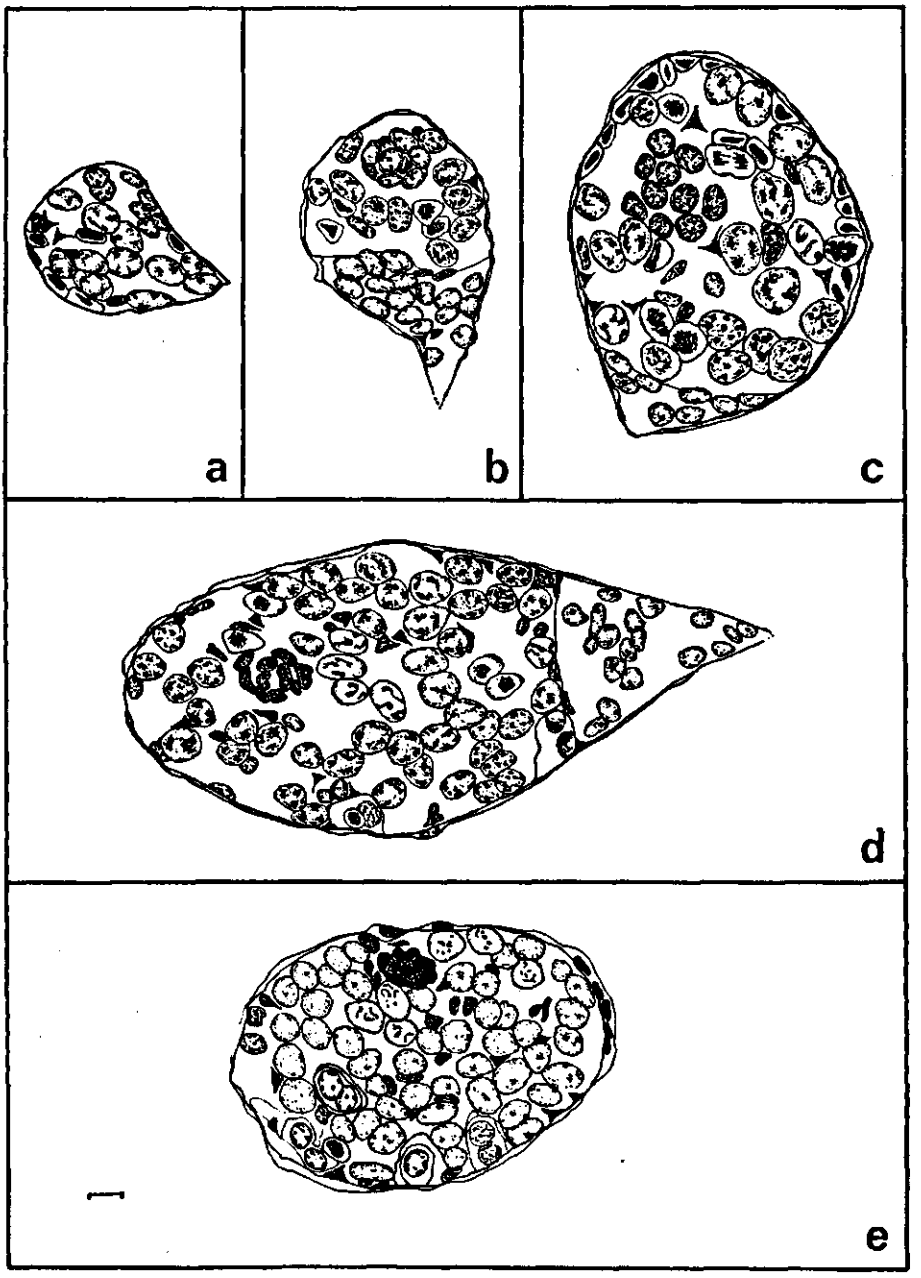


FIG. 52. Drawings of larval testes at various ages. Bar = 10 μ.
a. 7 days, L2
b. 10 days, L3
c. 14 days, L3
b. 17 days, L3
e. 21 days, L3.

MANN and GAY, 1963). The same situation exists in testes of mature larvae of *Dacus oleae* (FYTIZAS, 1973). In the prepupal stage spermatids were observed being the most advanced germinal cell type. HUETTNER (1940) found in mature larvae of *D. melanogaster* all germinal cell types, excluded mature sperm cells. FRENCH and HOOPINGARNER (1965) observed in the testes of final instar larvae of *Musca domestica* only spermatogonia, a similar situation like in *H. antiqua.*

The main criterion to distinguish a testis from an ovary in young caterpillars of *Deilephila euphorbiae* was the shape and position of the apical cell (BUDER, 1915). In *H. antiqua* the mere presence of an apical cell suffices because in ovaries an apical cell is absent, contrary to *D. euphorbiae* and other species (SCHNEIDER, 1915).

The somatic structures in larval testes of Diptera have been described very rarely. Most of these descriptions pertain to the apical cell. In *M. domestica* FRENCH and HOOPINGARNER (1965) described the basal somatic tissue to which they referred for obscure reasons as 'apical cap'.

3.5.3.2. Pupal development

A study has been made of gonad development in young pupal stages of *H. antiqua.*

The young pupal testis contains primary spermatogonia and apical cavities against or near the basal somatic tissue.

During the first hundred hours of the pupal stage at a rearing temperature of 20–21 °C, neither the shape nor the size of the testis change so much that they exceed the considerable individual variability. The shape of the testis is ovoid or flattened spheroidally.

When following the development of the testicular cell populations it appears that gradually the primary spermatogonia show changes of their nuclear morphology in increasing numbers. They change from the 'diffuse' type into the 'concentrated' type (fig. 15). These and other relevant changes will be given in a chronological order.

0 hours: 'Concentrated' primary spermatogonia are observed among the 'diffuse' type of primary spermatogonia. The mitotic activity of the germinal cells ranges from low to almost non-existent. Gradually during the next days the mitotic activity increases as does the number of 'concentrated' types of primary spermatogonia. The dispersion of 'concentrated' types in the testis is very homogeneous. The germinal cells divide individually although naturally two neighbouring cells may divide simultaneously. The apical cavities, in the late larval testis small and sometimes dispersed in the testis (fig. 52 e), remain small during the first 36 hours of pupal development.

12 hours: The apical cavities are also observed laterally and their dispersion seems to be less restricted to the zone next to the basal somatic tissue. The number of 'concentrated' type primary spermatogonia is still increasing. They are homogeneously dispersed. The mitotic activity also increases.

36 hours: The relative number of 'concentrated' primary spermatogonia still increases and their mixing with the now less numerous 'diffuse' primary sperma-

togonia is completely homogeneous and random. The apical cavities increase in size.

48 hours: A change in the distribution pattern of the 'diffuse' and 'concentrated' primary spermatogonia begins to appear. In the apical part of the testis, comprising a variable part of the apical half, the number of 'concentrated' types decreases sharply, whereas the number of these types still increases in the remainder of the testis although large groups of 'diffuse' primary spermatogonia are mixed with 'concentrated' types. The mitotic activity is also increasing. The apical cavities increase in size and number. They are restricted to the basal half of the testis.

72 hours: The tendency of 'diffuse' and 'concentrated' primary spermatogonia to separate, becomes more distinct. The apical cavities show a tendency to form a zone directly basal of the apical region of the testis containing the apical cell and the large population of nearly exclusive 'diffuse' primary spermatogonia. In this way the apical and basal populations of spermatogonia become separated by apical cavities, resulting in a division by zones in three parts. In the apical cavities, dividing primary spermatogonia are observed as are 'concentrated' types in interphase. Primary spermatogonia hardly ever show a 'diffuse' appearance when present in an apical cavity. The number of cells in such a cavity is limited to 2–4, rarely more. The mitotic activity is predominantly localized in the basal part of the testis. In the apical part it concerns individual divisions of primary spermatogonia. In the basal part the first groups of synchronously dividing germinal cells begin to appear outside, mostly basally, of the apical cavities. They are rounded although the surrounding cyst epithelium often can not be distinguished as such, contrary to the cyst cell nuclei.

96 hours: The distinction between the apical population of 'diffuse' primary spermatogonia and the more basal population of 'concentrated' primary spermatogonia and germinal cell families in cysts is very clear. The separation is accentuated by the zone of apical cavities which is decreased in size to form a better localized, wide border. In the cavities single germinal cells or small groups of cells are found. The mitotic activity is mainly restricted to the basal part in which both individual cells and groups of cells divide (fig. 53a). The mitotic activity in the apical part decreases relatively sharply and only occasionally individual primary spermatogonia divide.

6 days: Testes of these pupae show about the same shape. They may be a little more elongated. In most testes of this group the most advanced germinal cells are interphase primary spermatocytes (fig. 53b). However, due to the individual variability in speed of development, meiotic divisions and rarely even some early spermatids may be found. All cell types but the most advanced one are abundantly present. In the apical region 'diffuse' primary spermatogonia are mixed with darkly stained somatic cell nuclei. The apical cell has not changed. The number of primary spermatogonia appears to be decreased relatively when compared to the situation in 3 days old pupal testes. The apical cavities are empty or contain single cells, multinucleate cells, some very small groups of cells or degenerating cells. The latter ones can also be found united in entire

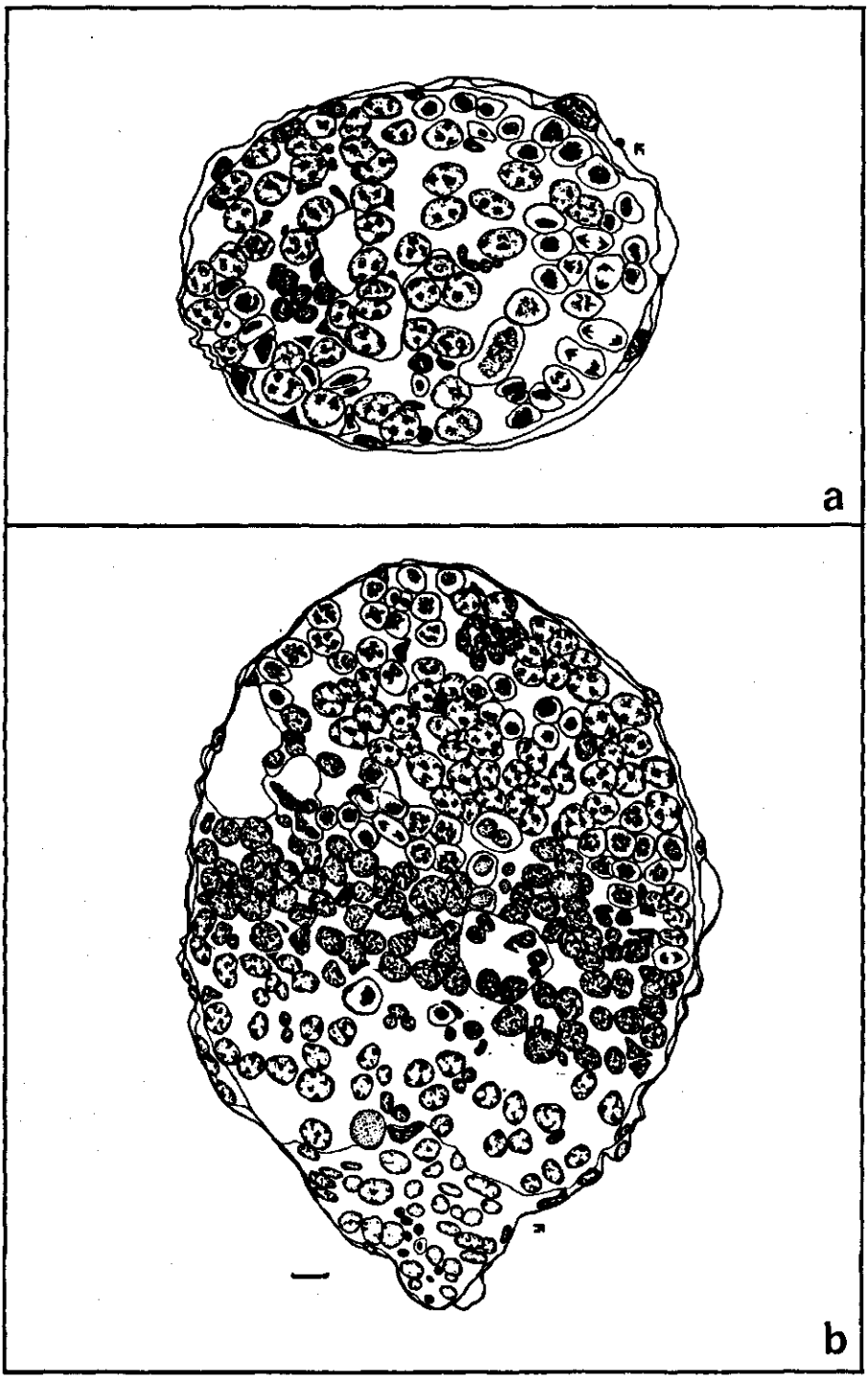


FIG. 53. Drawings of pupal testes at various ages. Bar = 10 μ. a. 96 hours or 3 days b. 6 days.

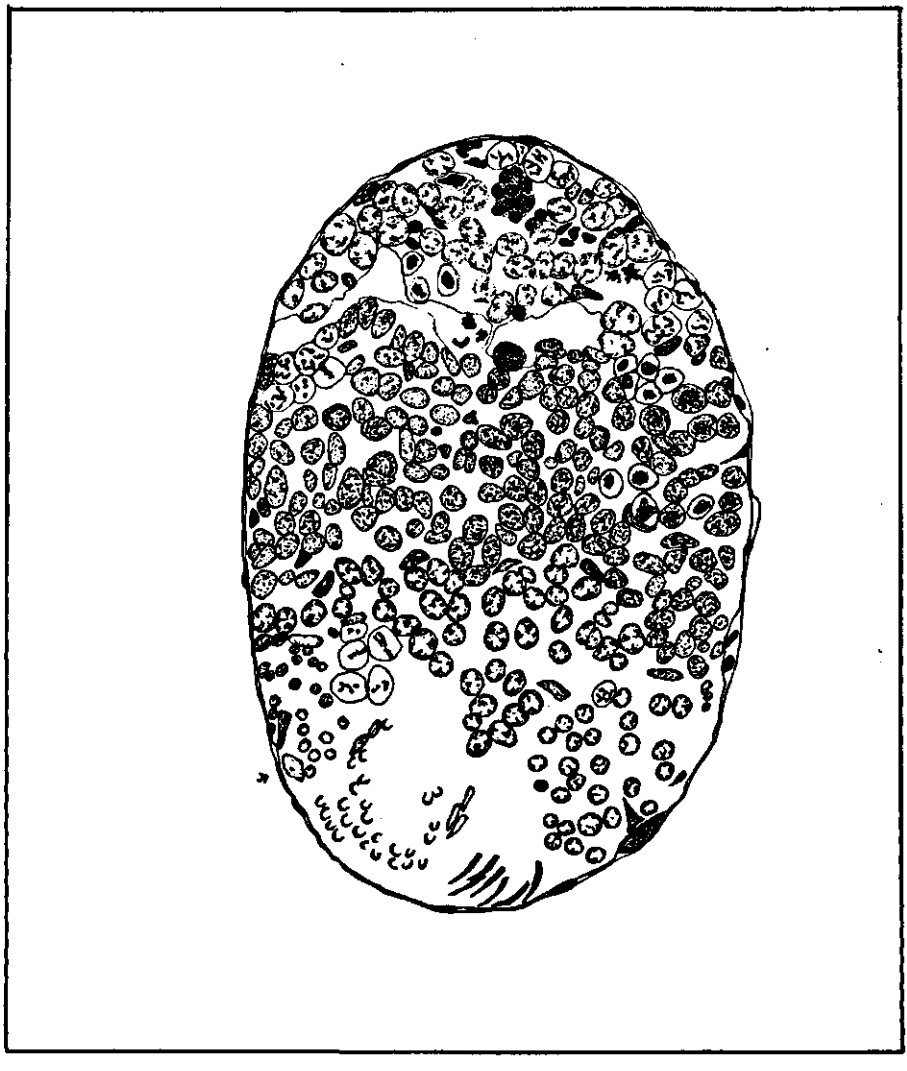


FIG. 54. Drawing of a pupal testis at an age of 11 days. Same magnification as Fig. 53.

cysts which apparently failed to develop. This is a very common phenomenon which occurs without an obvious cause. The secondary spermatogonia lie closely packed in groups of varying size basal to the apical cavities. In the cysts of primary spermatocytes the cells are more spaceously distributed. The sequence of the spermatogenic cell types is very clear because of the still undisturbed zonation. A central cavity has not yet been formed. The basal somatic tissue still encloses the extreme basal part of the testis and connects with the non-tubular strand of cells of the ductus deferens.

11 days: The testes are ovoid in shape. The most advanced germinal cell

type is the sperm cell. At this stage sperm is just being formed. The variation in the progress of development which was evident in the 6 days old pupae can be also found here. The relatively large number of secondary spermatocytes and cells in meiotic division is very conspicuous (fig. 54). Around the darkly stained apical cell the population of primary spermatogonia usually contrasts very clearly with the secondary spermatogonia which show a relatively high level of mitotic activity. Single cells but also entire cysts degenerate apparently spontaneously. Cysts of primary spermatocytes and relatively numerous secondary spermatocytes are situated basally of the secondary spermatogonia and laterally against the testicular sheath. The central cavity has been formed and may occupy a space comprising 2/3 of the entire testicular volume, but usually far less. All germinal cell types are present with the possible exception of sperm cells. The internal structure of the testis resembles that of a young adult testis, except for the absence or limited number of sperm cells. The basal somatic tissue has been differentiated into the terminal epithelium surrounding the orifice of the testis which connects with the tubular ductus deferens.

Discussion

During the first hundred hours of pupal testicular development an increasing number of 'diffuse' primary spermatogonia is transformed into 'concentrated' types of primary spermatogonia, followed by a certain regional specialization in the testis. This is expressed by the arrest and possible reversal of this chromatin transformation in the apical part of the testis, whereas more basally the transformation obviously proceeds. Concomitantly, the rate of mitotic activity increases gradually. During this development the apical cavities grow in size and number. They leave their basal position and for a short period they form a broad zone between the apical and basal part of the testis. This belt is reduced to a distinct border, separating increasingly different cell populations. With the clear establishment of this border the degree of mitotic activity has also been differentiated, being low in the apical part and high basally of the apical cavities. At this stage both the 'concentrated' spermatogonial types and the main mitotic activity are confined to the basal part of the testis. Here the first groups of dividing cells are observed. Cells among these groups never show the 'diffuse' type of primary spermatogonia but always have an 'concentrated' appearance. 'Diffuse' primary spermatogonia are dividing individually amidst the others or in the apical cavities. In spite of a relative increase of the mitotic activity in the population of primary spermatogonia, the number of divisional stages actually observed is rather limited. Prometaphase, metaphase and anaphase apparently proceed very quickly as they are very rarely observed, whereas prophases and telophases seem to last somewhat longer. Telophases of dividing primary spermatogonia are frequently found in apical cavities, as are other divisional stages. The number of cells involved is usually limited, mostly 2–4. Small somatic nuclei sometimes seem to be associated with primary spermatogonia in the apical cavities and occasionally more apically. In general the population of mixed 'a'- and 'b'-type somatic cells is more or less evenly distributed among

the germinal cells in the testis. Among the primary spermatogonia in the apical part some 'b type' somatic nuclei adhere to 'diffuse' primary spermatogonia (fig. 20a), while most 'diffuse' spermatogonia are free and most somatic cells are only mixed with the germinal cells, not associated. It is often difficult to determine whether or not a particular primary spermatogonium is associated with a somatic cell, because of the crowded space in the apical part of the testis and the difference in size. Groups of spermatogonia in the apical cavities are occasionally accompanied by one or more somatic cells. Presumably the transition of the primary spermatogonia into secondary spermatogonia takes partly place in the apical cavities. Here primary spermatogonia, whether or not associated with a somatic cell, divide and after a few divisions the group or cyst is expelled basally, to complete the spermatogonial multiplication as secondary spermatogonia. The change from primary spermatogonium into secondary spermatogonium is essentially characterized here by the irrevocable cessation of the appearance of 'diffuse' primary spermatogonium, which occurs in the apical cavities and perhaps elsewhere, in particular during the first phase of the spermatogenic processes in the young pupal testis. During this stage both 'diffuse' and 'concentrated' types of primary spermatogonia are still present in the basal part of the testis.

Summarizing the course of spermatogenesis, it can be concluded that the process really begins during the pupal stage. The sequence of cell types formed and their contribution to the total germinal cell population indicate the speed and the impetus of the processes during progressing development. Hence, an estimate has been made as to the relative volume of some germinal cell populations when compared to the total testicular volume. The results are summarized in table 2.

In Diptera as far as is known the main period of real spermatogenic activity i.e. the production of spermatozoa ultimately from spermatogonia, seems to coincide with the pupal stage. The peak activity of sperm production usually takes place at this stage. The preceding cell types have been produced in large quantities by that time. In young pupae (about 12 hours old) of *Musca domestica* FRENCH and HOOPINGARNER (1965) found spermatids to be the most advanced germinal cell type. Meiotic divisions were also observed. In these pupae about

TABLE 2. Estimated volumes of germinal cell populations as a percentage of total testis volume at different pupal developmental stages.

	Prim. spg	Sec. spg	Spermatocytes[a]	Rest
3[e]:	45–50	45[b]	–	10–5[c]
6:	15–25	50	35–25	–
11:	5–10	25	25	45–40[d]

a: including the meiotic dividing cells
b: the germinal cell population basally of the apical cavities
c: the apical cavities
d: including the central cavity
e: age in days.

50% of the available space is occupied by spermatogonia. In pupae of 1–2 day old (at 30°C) the spermatogonial share is reduced to about 25%. The meiotic divisions show a peak and these dividing cells take also about 25%. The spermatids occupy about 50% of the testicular volume. In pupae of 2.5–5 day old the number of spermatogonia, primary spermatocytes and meiotic divisions is declining, whereas the amount of sperm cells increases. All cell types are present from an age of 2.5 days onwards. In pupae of 5 days old the main cell type are sperm cells. The spermatogonia are strongly reduced in number as are the meiotic divisions. For *Drosophila melanogaster* KAUFMANN and GAY (1963) mentioned the progress of spermatogenic development. Very young pupae contain secondary spermatocytes as the most advanced germinal spermatids or sperm cells as the youngest types. According to CLAYTON (1957) all germinal cell types except sperm cells are present in the late pupal testis of *Drosophila virilis*. The first spermatids are formed about 50 hours after the beginning of the prepupal stage. The number of spermatogonia decreases considerably during the early pupal stage but stays at a constant low level afterwards. The primary spermatocytes show a peak during the first half of the pupal stage, later on they are somewhat reduced in numbers and remain on a constant but high level. The number of spermatids gradually increases, until a more or less constant number is reached towards the end of the pupal stage. In *Dacus oleae* FYTIZAS (1973) found all germinal cell types present in the young adult testis, as did LELOUP (1974) in *Calliphora erythrocephala*.

3.5.3.3. Adult development

In *H. antiqua* adult testicular development has been described with intervals of 5 days up till 30 days of age.

0 days: The emphasis of the functioning is clearly laid on the production of sperm cells. The testis is ovoid. The apical cell is surrounded by primary spermatogonia of which more than 50% show the 'concentrated' type. The local somatic cells are 'b type' cells. The cell populations are tightly packed. The apical cavities are small and generally well filled, thus causing them sometimes hard to find. The secondary spermatogonia show various 'concentrated' types, with a minority of cells with coarse chromatin concretions of about the same size in their nuclei (fig. 55a). The secondary spermatogonia are occasionally tightly packed more often not. In general the individual cyst epithelia are weakly developed and show very inconspicuous limits of cysts and groups of cells. The cysts with primary spermatocytes are more clearly defined, the cells being arranged more spaceously. Meiotic divisions are numerous. All types of spermatids and large numbers of sperm cells are present. The germinal cell types lie apically and laterally of the central cavity. In this cavity some central cavity cells, many sperm cells and groups of spermatids are observed. In the centre, the core, is a spheroidal-ovoid sometimes irregularly shaped mass of apparent waste products: cytoplasmic droplets, degenerated cells, failed cysts and undefinable debris. Most sperm cells are situated basally near the orifice of the testis, which is closed by the locally swollen cells of the terminal epithelium and an obviously

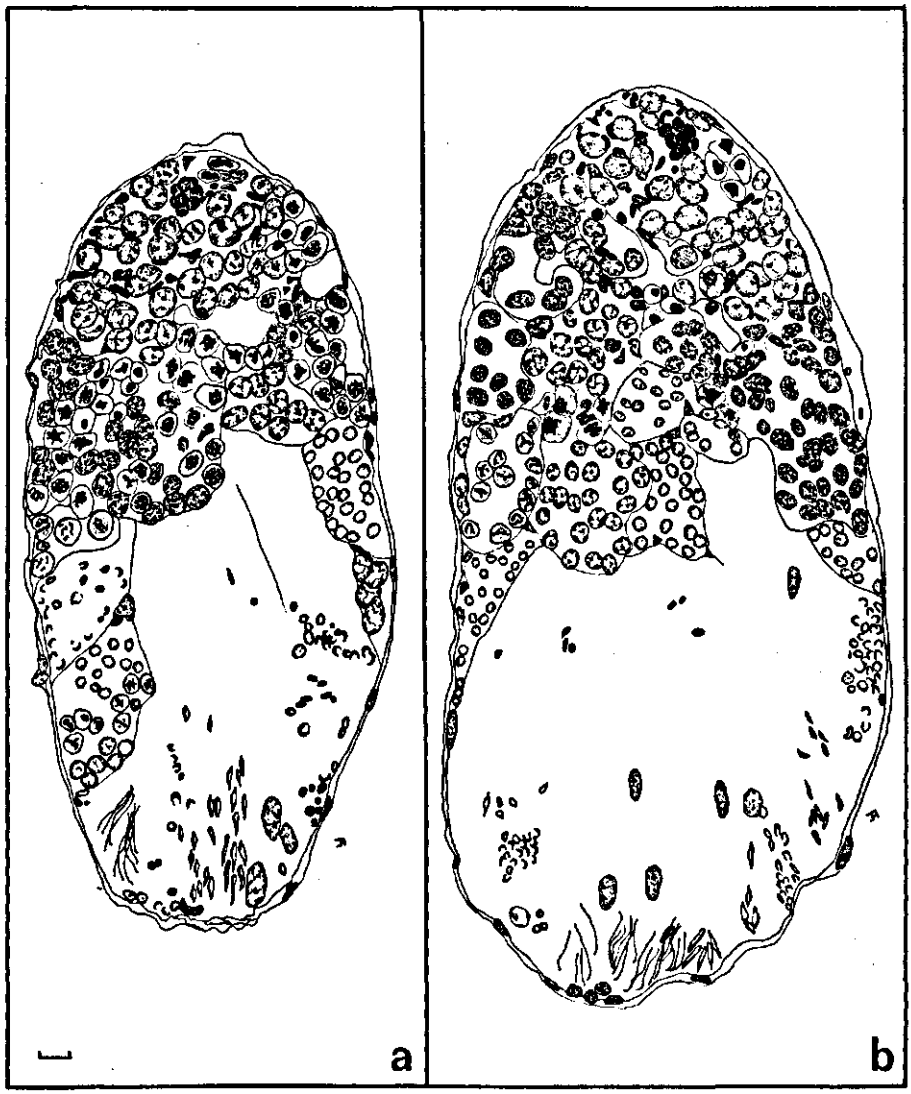


FIG. 55. Drawings of testes of a newly emerged (a) and a three days old (b) adult. Bar = 10 μ.

weak membrana basilaris. The overall impression of the testis at this age is characterized by activity and sperm production.

5 days: The testes show a longer ovoid appearance with a slight widening of the basal half. The most basal sixth part of the testis is clearly functioning as a vesicula seminalis and contains nearly exclusively sperm cells and some somatic cells. 50% or more of the primary spermatogonia are of the 'diffuse' type. The number of primary spermatogonia has decreased. They are tightly packed and easily recognizable as a population around the apical cell. The apical cavities

are mostly rather prominent and they border the primary spermatogonia basally. In the secondary spermatogonial population the number of cells with concentrated chromatin in the nucleus has decreased by 50–100%. The staining intensity also decreases partly as a consequence of the shift within the population of secondary spermatogonia towards less concentrated types with more homogeneously dispersed and less coarsely granular chromatin elements. The secondary spermatogonia are variably packed and occasionally show a considerable mitotic activity. The number of interphase primary spermatocytes seems to be smaller but many meiotic divisions can be found, suggesting that the total population of dividing and non-dividing spermatocytes has not appreciably decreased. All the spermatid cell types and sperm cells are abundantly present. In the ductus deferens individual sperm cells are found. The general impression of the testis at this age is one of somewhat decreasing activity in the spermatogonial region, of a stable production of sperm cells produced by a continued activity of the post-meiotic cell types and an initial shift towards a storage function.

10 days: The testes are long and narrow in shape. A clear shift in function from sperm production to sperm storage is obvious. The basal quart of the testis acts as a vesicula seminalis. The pigmentation and thickness of the testicular sheath increases in the basal half of the testis. This is an ageing symptom which can also be observed after irradiation. The germinal cell populations show a large individual variability in number and degree of activity. The primary spermatogonia are mostly all of the 'diffuse' type. The number of secondary spermatogonia differs per testis and they show a very variable pattern of 'concentrated' types. The mitotic activity decreases sharply in most cases, as does the number of primary and secondary spermatocytes and meiotic divisions. All types of spermatids and sperm cells are present. These cell types are also observed in the ductus deferens. In nearly every testis many groups of degenerating cells can be found possibly as a result of failing development. The general impression of testes of this age is one of sharply decreased spermatogenic activity and a distinct change towards a more important sperm storage function.

15 days: Testes of 15 days old individuals have a long and narrow shape with a somewhat bulging basal half, which represents the vesicula seminalis (fig. 57a). The population of primary spermatogonia around the apical cell seems to remain constant in number. Most of the cells show the 'diffuse' type but variations can occur. The number of secondary spermatogonia has decreased further and they show a very variable nuclear morphology. The primary spermatocytes are easily distinguished and are still present in some cysts. The mitotic and meiotic activity is low, as is the number of post-meiotic cell types, except sperm cells. All cell types can still be found but usually in low numbers. The general impression is of further decreased spermatogenic activity and storage of sperm cells.

20 days: In these testes (fig. 57b) a small but relatively large population of 'diffuse' primary spermatogonia can be observed around the apical cell. Even

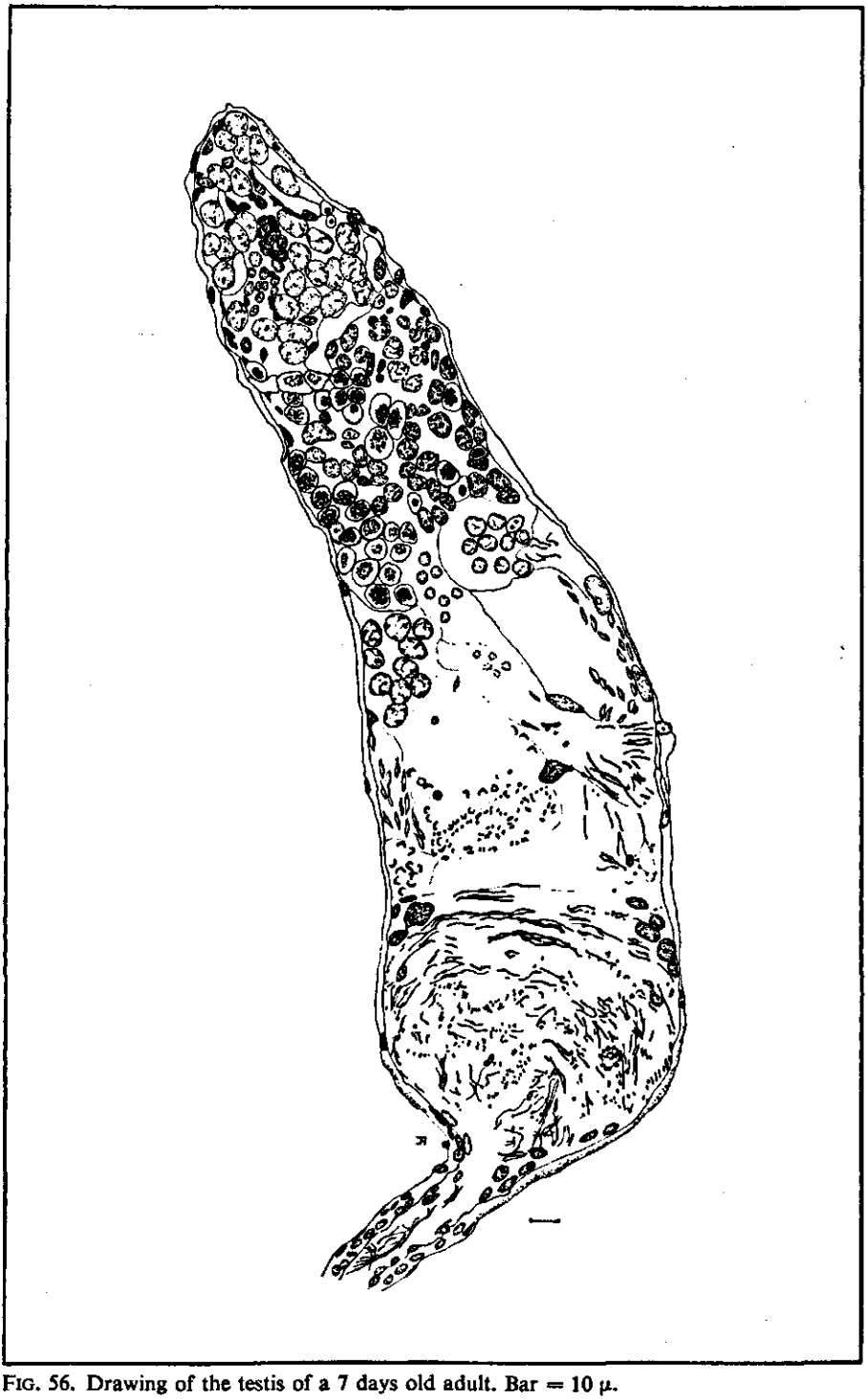


FIG. 56. Drawing of the testis of a 7 days old adult. Bar = 10 μ.

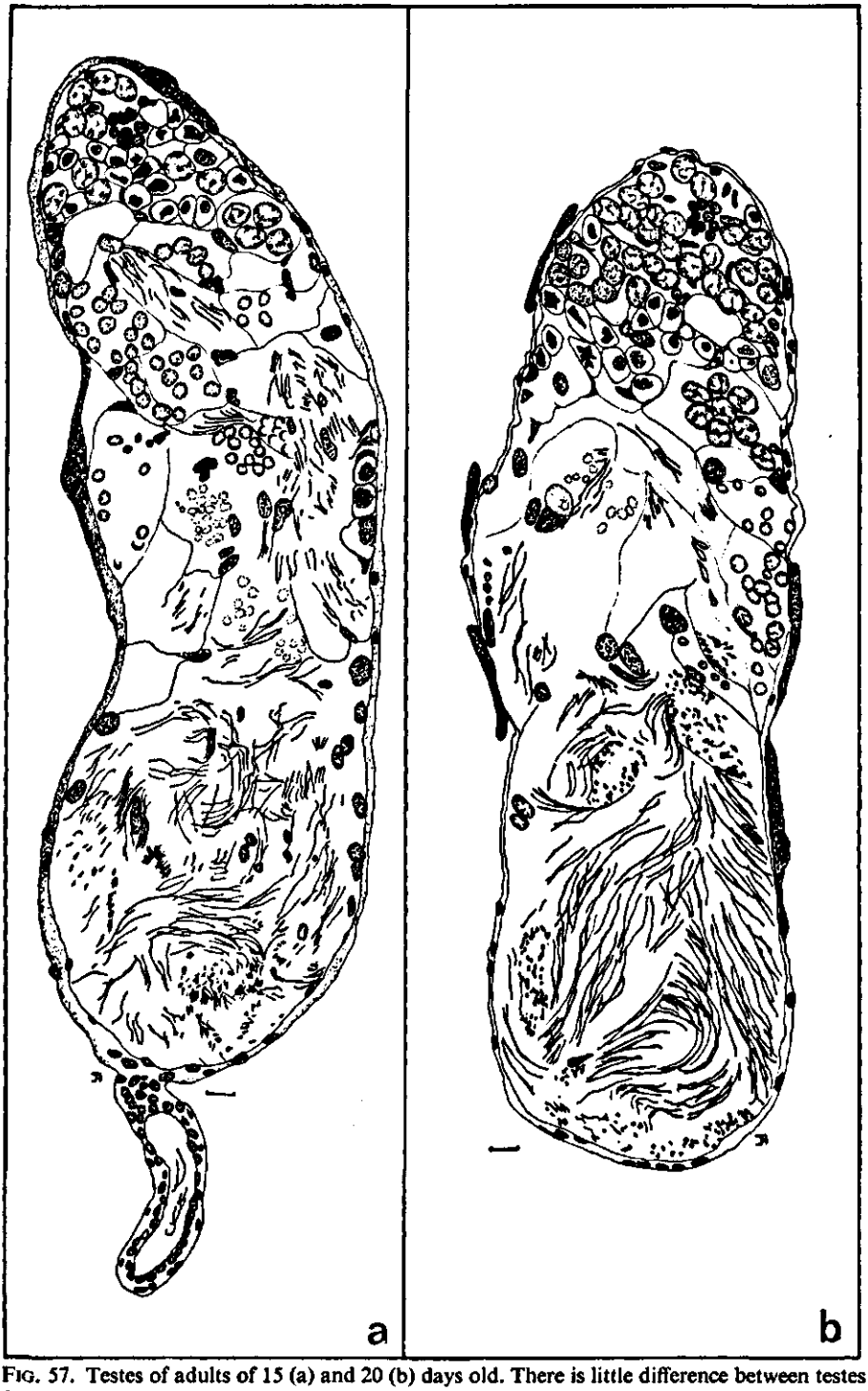


FIG. 57. Testes of adults of 15 (a) and 20 (b) days old. There is little difference between testes from both age groups. Bar = 10 μ.

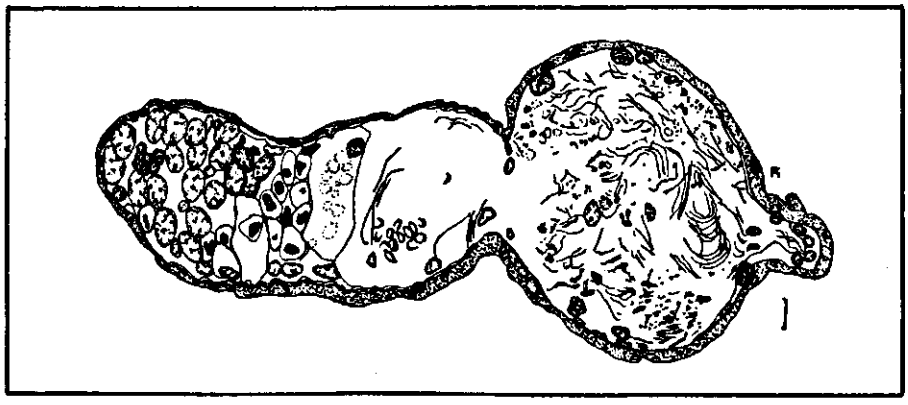


FIG. 58. Testis of adult of 30 days old. Bar = 10 μ.

mitotic divisions can be found here. The variability in number and nuclear morphology of secondary spermatogonia increases. Primary spermatocytes are still present in a few cysts. The mitotic and meiotic activity is variable but not less than in 15 day old flies. Spermatids are present in low numbers, sperm cells in high numbers. The basal half of the testis functions as a vesicula seminalis, the lower part of the apical half as a central cavity in which degenerating cells and unidentified cellular debris accumulate.

30 days: In these testes, which are reduced in size, small populations of 'diffuse' primary spermatogonia can still be seen around the unchanged apical cell. Populations of other germinal cell types may have disappeared or cannot be identified due to an abnormal or deviating nuclear morphology. There is still a considerable number of sperm cells in the vesicula seminalis i.e. the basal half of the testis. The testicular sheath is thickened and heavily pigmented (fig. 58).

Discussion

In the various successive age groups one observed that with increasing age:

- the function of the testis shifts from sperm cell production towards sperm cell storage.
- an increasing thickening and pigmentation of the testicular sheath.
- the shape of the testis changes from ovoid to a longer and narrower shape with an enlarged basal part.
- the germinal cell populations show a general decrease in number and activity.

In the population of primary spermatogonia a decrease of the number of 'concentrated' types in favour of the 'diffuse' type with increasing age is very distinct. The number of primary spermatogonia decreases gradually but seems to become more or less stabilized between 10 and 15 days, causing the presence of relatively large populations of 'diffuse' primary spermatogonia in very old testes. This characteristic ability indicates that the primary spermato-

gonia must indeed be considered as a separate cell type distinguished from the secondary spermatogonia. The apical cell, although individually somewhat variable in size and appearance definitely does not change with increasing age.

The number of secondary spermatogonia with concentrated chromatin in the nucleus decreases relatively in favour of less concentrated types with more dispersed chromatin particles. The rate of chromatin transformation is individually very variable at older ages. The absolute number of secondary spermatogonia decreases gradually but clearly and quickly with increasing age of the fly. In very old testes they may have disappeared entirely. In this respect they differ evidently from the primary spermatogonia. Their disappearance may partly be due to depletion after a sharp decrease of the mitotic activity in the secondary spermatogonial region with increasing age. The primary spermatogonia seem to be able to divide at a sufficient rate to maintain the population. Mitotic divisions among primary spermatogonia are indeed observed in very old testes. In cases where these divisions are dichotomous the primary spermatogonial population could compensate for losses of aged cells and maintain the population at relatively high numbers. Otherwise multiplication of secondary spermatogonia may start.

A distinct and continued decrease of the number of interphase primary spermatocytes is found to start at an age of about 5 days. At that time a large number of cells is seen in meiotic division. A stagnation of the formation of primary spermatocytes from secondary spermatogonia begins to take place at about 5 days of adult age. Between 5 and 10 days the number of spermatocytes and meiotic divisions starts to decrease sharply, indicating a continued stagnation in the supply of primary spermatocytes. A strong decrease of the spermatogonial mitotic activity is also established very clearly in 10 day old testes. Thus, both the multiplication of secondary spermatogonia and the formation and meiotic division of spermatocytes stagnate irrevocably during about the same period. The mechanisms regulating these concurrently acting stagnation symptoms remain obscure, but are perhaps hormonally influenced (DUMSER and DAVEY, 1974).

The depletion of the spermatids, especially between 10 and 15 days, indicates a continuing sperm cell production until at about 15 days when the sperm cells are the only numerous germinal cells left. The effective period of sperm cell formation lasts about 18 days: from the pupal age of 11 days until the adult age of 15 days.

Summarizing the course of testicular development and germinal cell formation, the optimal period of the spermatogenesis with respect to the abundant production of all germinal cell types involved may be considered the last 3 days of the pupal stage until an adult age of about 5 days (table 3).

During the larval stage individual multiplication of the primary spermatogonia takes place. At the end of the larval stage changes are observed in the structural organization of the larval testis. Apical cavities are formed in which single cells or pairs of cells occur (fig. 52e.) These changes anticipate those during the first days of the pupal stage when some stimulant causes the initiation

TABLE 3. Presence of germinal cell types in the testis during development.

	larva		pupa						adult				
	0**	21	4**	6	7	8	11	13=0	5**	10	15	20	30
prim. spg:	+	+	+	+	+	+	+	+	+/−	+/−	+/+	+/+	+/+
sec. spg:	−	−	+	+	+	+	+	+	+	+/−	+/−	+/−	−
p. spc:	−	−	−	+	+	+	+	+	+/−	+/−−	+/−	+/−	−
s. spc:	−	−	−	−	+	+	+	+	+	+/−−	+/−	+/−	−
e. spt:	−	−	−	−	+	+	+	+	+	+/−	+/−	+/−	−
i. spt:	−	−	−	−	−	+	+	+	+	+/−	+/−	+/−	−
t. spt:	−	−	−	−	−	+	+	+	+	+/−	+/−	+/−	−
sperm*:	−	−	−	−	−	−	+	+	+	+	+	+/+	+/+

prim. spg = primary spermatogonia; sec. spg = secondary spermatogonia; p. spc = primary spermatocytes; s. spc = secondary spermatocytes; e. spt = early spermatids; i. spt = intermediate spermatids; t. spt = transformation spermatids; sperm = sperm cells.

\+ = present and increasing
− = absent
+/− = present and decreasing
+/−− = present and sharply decreasing
+/+ = present and constant in number.

* The quantitative evaluation of the number of sperm cells is quite impossible. Therefore the increase or maintenance of this population has been deducted from the amount of transformation spermatids present.
** Age in days.

of the differentiation into the now well known sequence of germinal cell types. At this stage the spermatogenic production process is started. It proceeds at a peak capacity by the time the adult emerges. A few days later, however, the first signs of diminished production are observed, resulting in a gradual but quick and irrevocable decrease in activity and subsequent sperm cell production. This terminates largely between 15 and 20 days of adult life.

It may be noted that the only germinal cells which are continuously present with a morphologically unchanged appearance from the egg to the senescent adult are the 'diffuse' primary spermatogonia. They seem to represent the continuity in the entire spermatogenic development in *H. antiqua*.

3.5.4. *Dynamics of spermatogenesis*

3.5.4.1. Terminology

Little is known about the multiplication system of the germinal types in the insect testis during spermatogenesis. In particular, knowledge of spermatogonial multiplication is scarce. An extensive review on the possible mechanisms of spermatogonial multiplication and stem-cell renewal in insects and mammals and their genetic consequences has been given by HANNAH-ALAVA (1965).

TIHEN (1946) used the terms 'predefinitive' and 'definitive' spermatogonia, denoting the ability of the 'predefinitive' spermatogonia to produce both 'definitive' spermatogonia and cells like themselves by means of quasi-dicho-

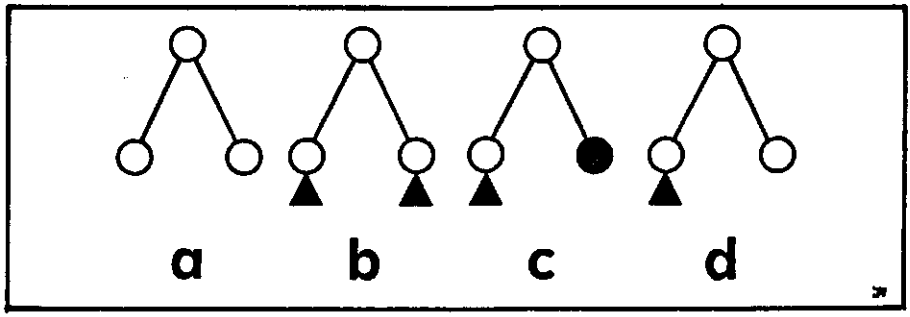


FIG. 59. Scheme of possible divisions of an 'indefinitive 'spermatogonium (according to and slightly modified from Hannah-Alava (1965)).
○ = 'indefinitive' spermatogonium
● = 'predefinitive' spermatogonium
♀ = 'definitive' spermatogonium.

tomous divisions. 'Definitive' spermatogonia divide dichotomously to form more spermatogonia, spermatocytes and ultimately sperm cells. HANNAH-ALAVA (1965) mentiones a third type: 'indefinitive' spermatogonia, being 'the generations of spermatogonia between the stem-cell division and the definitive divisions'. These terms are the only ones referring to the way in which the multiplication process operates. HANNAH-ALAVA (1965) further recognizes three morphological types of spermatogonia: primary spermatogonia, secondary spermatogonia and intermediate spermatogonia, without, however, giving relevant descriptions of the morphology of these cell types.

From the work of TIHEN (1946) and HANNAH-ALAVA (1965) a picture of the predefinitive, indefinitive and definitive spermatogonia emerges leading to the following descriptions.

Predefinitive spermatogonia may divide dichotomously to produce two predefinitive spermatogonia or quasi-dichotomously to form a predefinitive spermatogonium and a definitive spermatogonium, which is also called an initial definitive spermatogonium. The origin of the predefinitive spermatogonium is the primordial germ cell population during the formation of the testis. According to HANNAH-ALAVA (1965), however, a predefinitive spermatogonium may also originate from a definitive or an indefinitive spermatogonium. The main characteristic of a predefinitive spermatogonium is the ability to divide in a cambium-like manner by means of quasi-dichotomous divisions to maintain the population at a steady level.

Definitive spermatogonia divide dichotomously in most cases. There seems to be evidence that they may produce predefinitive spermatogonia. The definitive spermatogonia originate from predefinitive spermatogonia whether or not via indefinitive spermatogonia.

Indefinitive spermatogonia seem to be capable of dividing both dichotomously, producing indefinitive or definitive spermatogonia, and quasi-dichotomously

resulting in a definitive spermatogonium and a predefinitive or indefinitive spermatogonium (fig. 59).

When these possibilities are examined it appears that the indefinitive spermatogonium which divides dichotomously can be considered as a definitive one (a and b). A cell which gives rise to two initial definitive spermatogonia or two indefinite spermatogonia by a dichotomous division may be referred to as an initial definitive spermatogonium itself, irrespective of the time elapsing between the divisions. It only means that the series of dichotomous definitive divisions have started one or more cell generations earlier. The indefinitive spermatogonium which divides quasi-dichotomously may be referred to as a predefinitive spermatogonium in the case when daughter cells are a definitive and a predefinitive spermatogonium (c). When the daughter cells are a definitive and an indefinitive spermatogonium respectively (d), it depends on the nature and fate of the latter cell whether or not the division was a quasi-dichotomous or a dichotomous one, the parent indefinitive spermatogonium being a predefinitive or a definitive spermatogonium respectively. This means that the group of indefinitive spermatogonia as they are referred to by HANNAH-ALAVA (1965) is redundant.

When a spermatogonial cell is capable of dividing quasi-dichotomously it should be referred to as a predefinitive spermatogonium, regardless of its origin. A spermatogonial cell lacking this ability could be called definitive. This interpretation avoids inconsistencies when predefinitive spermatogonia are formed from definitive spermatogonia which are 'irrevocably destined through dichotomous division and differentiation, to become the spermatocytes...' (HANNAH-ALAVA, 1965).

In the case when a predefinitive spermatogonium is isolated from a group of definitive spermatogonia after a number of generations, a cell line of predefinitive spermatogonia is present up to this moment within the group of multiplying definitive spermatogonia. Because each division resulting in the formation of a definitive and a predefinitive spermatogonium is quasi-dichotomous the parent cell was a predefinitive spermatogonium. This parent cell on its turn may have been formed by a preceeding quasi-dichotomous division of a predefinitive spermatogonium. Some of these predefinitive spermatogonia will be able to act as stem-cells, others will not.

HANNAH-ALAVA (1965) defines stem-cell spermatogonia as 'the spermatogonia responsible for the maintenance of the germ line.' In her terminology this description includes both predefinitive and indefinitive spermatogonia, although in the legend of her fig. 1 stem-cells are restricted to predefinitive spermatogonia. Since indefinitive spermatogonia are redundant as a separate group, this restriction is justified. In order to link the stem-cell concept to the predefinitive spermatogonia the stem-cell could be considered as a single, individual predefinitive spermatogonium. A predefinitive spermatogonium which occurs in a group of definitive spermatogonia, e.g. prior to isolation, is a potential stem-cell until the moment of isolation. Such a cell is not morphologically distinguished from the other cells belonging to a group or a cyst of

spermatogonia. Hence, the conclusion of HANNAH-ALAVA (1965) 'that the time of origin of the new predefinitive (stem-cell) spermatogonium can be predicted on the basis of counts of the number of definitive spermatogonia, or spermatocytes, of common origin' is still valid.

3.5.4.2. Pattern of spermatogenic multiplication

In testes of young adults of *H. antiqua* of various origins, counts have been made of the number of cells per cyst. It has been mentioned before that the development of the squamous epithelia of the cysts is weak. Difficulties thereby often arose as to the borders of the cyst to be examined. Undoubtedly this sometimes resulted in counting two or even more cysts which were not recognizable separately but of which the total number of cells could be counted fairly accurately. The accuracy of counting is another problem to be solved. In general it is practically impossible to avoid double counting altogether. As more sections may contain the same cells, the latter tend to be counted twice or not at all unless strict regulations are made and applied. Even then, repeated counts of the same cyst may result in slightly different numbers of cells. However, this is not an essential problem because it is the approximation of the number of cells which may be as important as the actual number itself. In perfectly normal cysts one or more cells may spontaneously degenerate and disappear, thus causing an aberration from the ideal number derived from a power of two or whatever system of stem-cell renewing is being used. For this reason, often the actual number of cells in a normal cyst will be different from the theoretical number which is based on the system of multiplication. In this way even by accurate counts one may arrive at cell numbers differing from theoretical values.

The counts have been carefully made in testes, fixed with different fixatives. The age of the males was up to 6 days. Both non-irradiated and irradiated males have been used. In non-irradiated testes a number of 85 cysts have been counted, in irradiated testes 82 cysts. Cells of the following general cell types have been included: spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. The spermatogonia involve only secondary spermatogonia and bi- or multinucleate cells, as primary spermatogonia do not seem to divide in groups. Hence, the latter type can not be involved in the counting of numbers of cells per cyst. Primary spermatocytes in interphase have been distinguished from those in MI-division in order to check possible radiation effects on both the interphase and the dividing cells. As no divisions take place anymore in the group of spermatids it is irrelevant which morphological type has been counted.

Considering the possibilities of aberrations due to double-counting and the counting of two or even more cysts in reality instead of one, the results obtained (table 4) have been scored for the various categories of cell numbers formed by the powers of two and their sums. In this way much of the data were found to be close to these theoretical numbers. Based on this score a frequency distribution of the cell types has been plotted of the numbers of cells/cyst (table 5).

In this approach it is assumed that in *H. antiqua* the spermatogonial multipli-

TABLE 4. Number of cells/cyst/cell type in young adult testes.

Non-irradiated testes:	
spermatogonia:	2 (4)*, 4 (5), 6 (2), 7, 8, 10, 16 (2), 20 (2), 31, 32 (2), 33, 66.
prim. spc.:	8, 17, 23, 30 (3), 32 (3), 33 (4), 34, 40 (2), 45 (3), 49 (2), 72.
MI:	31, 32, 33, 38 (2), 40, 44 (4), 45 (3), 53, 64, 145 (2).
sec. spc.:	42, 46, 57, 67, 71, 72, 74, 96, 103, 105 (2), 185 (3), 192, 258.
spermatids:	57, 62, 102, 107, 130, 133, 135, 290.
Irradiated testes:	
spermatogonia:	8 (4), 9 (2), 12 (2), 14 (1), 16 (10), 17, 18 (2), 19, 20 (2), 22, 24, 25, 26, 27, 28, 29 (2), 30 (5), 31 (2), 32 (7), 33 (3), 46, 55, 60, 65.
prim. spc.:	17, 24, 28 (3), 30 (3), 32 (3), 33, 34 (3), 38, 41, 56.
MI:	15, 31, 32 (3), 44.
sec. spc.:	55, 56, 57.
spermatids:	104.

* The numbers in brackets denote the frequency of occurrence of the preceding number of cells/cyst.

TABLE 5. Frequency of numbers of cells/cyst of some germinal cell types in the young adult testis.

cell type	frequency of numbers of cells/cyst								
	2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9
Non-irradiated testes:									
spermatogonia:	6	9	6	2	1	–	–	–	–
prim. spc.:	–	–	2	7	21	2	–	–	–
MI:	–	–	–	10	18	3	–	–	–
sec. spc.:	–	–	–	4	5	13	5	1	–
spermatids:	–	–	–	1	3	4	3	1	–
Irradiated testes:									
spermatogonia:	–	5	13	10	11	1	–	–	–
prim. spc.:	–	–	10	16	26	1	–	–	–
MI:	–	–	–	2	5	–	–	–	–
sec. spc.:	–	–	3	3	3	–	–	–	–
spermatids:	–	–	–	1	1	1	–	–	–

The variations in the number of cells/cyst in these cell types are:

cell type	non-irradiated testes	irradiated testes
spermatogonia:	2– 32	4–64
prim. spc.:	8– 64	8–64
sec. spc.:	16–256	8–32
spermatids:	16–256	16–64

cation takes place according to the 2^n system, as is supposed for some orthopteran species (WHITE, 1955). However, the frequent observation of multinucleate cells, both alive and in histological preparations, containing various numbers of nuclei (2–7), and the often aberrant numbers of cells in cysts suggest a possible activity of other multiplication systems as well. The available evidence

on this aspect does not point to a particular system, as is shown by table 6 in which the counts have been scored for the 2^n, the 3.2^n, the 7.2^n and the 15.2^n systems, including the sums of two consecutive powers. These systems, except the 2^n, presume the isolation of a stem-cell after 1, 2 and 3 spermatogonial divisions respectively. The 2^n system is a multiplication by exclusively dichotomous divisions. The scoring has been based on the best fit on a number of cells/cyst in a particular system. When a number has an equal best fit to or is identical with figures emerging from another system, it is scored for both. These figures are obtained as is outlined below.

2^n	3.2^n	7.2^n	15.2^n
2^1 2	2^1 2	2^1 2	2^1 15
2^2 4	3.2^0 3	2^2 4	2^2 4
$2^1 + 2^2$ 6	$2^1 + 3.2^0$ 5	$2^1 + 2^2$ 6	$2^1 + 2^2$ 6
2^3 8	3.2^1 6	7.2^0 7	2^3 8
$2^2 + 2^3$ 12	$3.2^0 + 3.2^1$ 9	$2^2 + 7.2^0$ 11	$2^2 + 2^3$ 12
2^4 16	3.2^2 12	7.2^1 14	15.2^0 15
etc.	etc.	etc.	etc.

The frequency of number of cells/cyst fitting best into the mentioned systems is shown in table 6.

Discussion

The frequency distribution of the number of cells/cyst in the various multiplication systems suggests:

a. a distinct contribution of the 2^n system in both absolute numbers and percentage of exact scoring, the lower powers of 2 even not being included.
b. the possible existence of other multiplication systems contributing in different proportions.

It must be realized that the frequency distribution is based on the number of cells/cyst which may be determined very inaccurately for the various reasons

TABLE 6. Frequency of counted number of cells/cyst which is fitting best into the various multiplication systems and the percentage of exact fitting.

	non-irradiated testes			irradiated testes		
system	frequency	exact**	% exact	frequency	exact**	% exact
2^n *	31	11	35	43	27	63
3.2^n	19	6	32	18	8	44
7.2^n	16	2	12	17	4	23
15.2^n	20	10	50	18	10	55

* all powers of $2 \leqslant 8$ have not been taken into consideration here because they are aspecific for any of these systems.

** the number of instances in which the number of cells/cyst was identical with figures from one or more systems has been scored separately, apart from the ones approximating.

TABLE 7. Number of definitive spermatogonial divisions in some species of insects.

Species	Number	Reference
Diestrammena marmorata	4–8	SCHELLENBERG (1913)
Acrididae	8	MCCLUNG (1938)
Acrididae	4–9	WHITE (1955)
Drosophila melanogaster	4	PONTECORVO (1944)
Drosophila melanogaster	4	TIHEN (1946)
Drosophila melanogaster	4	ÅBRO (1964)
Drosophila pseudoobscura	5	DOBZHANSKY (1934)
Papilio rutulus	7–8	MUNSON (1906)
Tischeria angusticolella	5	KNABEN (1931)
Deilephila euphorbiae	5–6	BUDER (1915)
Heliothis virescens	8	CHEN and GRAVES (1970)
Bombyx mori	6	SADO (1963)

earlier discussed and that possibly other multiplication systems are used instead of or in addition to the already mentioned ones.

Cysts of spermatids in cross section in electron-microscopic preparations revealed the following numbers of cells: 64, 128, 256, 89 and 115. The first three are powers of 2, the next one shows a best fit in the 15.2^n system (90), the last one in the 7.2^n system (112). These data and also those of tables 5 and 6 suggest a diversity in multiplication systems and a variability in number of definitive spermatogonial divisions from 3–6 in the 2^n system. This results in cysts of primary spermatocytes of 8–64 cells. In other multiplication systems the number of definitive spermatogonial divisions may vary according to the moment of isolation of the predefinitive spermatogonium: 3.2^n: 1–4, 7.2^n: 0–3, 15.2^n: 0–2 definitive spermatogonial divisions. A reported number of definitive spermatogonial divisions in other insect species is summarized in table 7.

As more abundant and more accurate information is lacking on the aspects of the multiplication system in *H. antiqua* a link with the morphologically different spermatogonial cell types is based only on hypotheses. The primary spermatogonia exist as single cells as is shown by their division and ^{3}H-thymidine incorporation pattern, contrary to the secondary spermatogonia which occur in groups. The secondary spermatogonia arise in the young pupa, the spermatogenic processes being speeded up in the mature larva, whereas primary spermatogonia are present from the newly hatched larva to the senescent adult stage in a constantly existing population. When considering these properties of both cell types, it appears that those of the primary spermatogonia show parallels with the concept of the stem-cell spermatogonia, while the properties of the secondary spermatogonia resemble those of the definitive spermatogonia. The presence of multinucleate cells in the apical cavities, which rarely contain somatic cells, might suggest a possible role in the multiplication systems. Inside the apical cavities, cells are present as individual cells or, usually, as multinucleate cells with a varying number of nuclei, mostly 2–7. The multinucleate cells do not seem to represent cysts because a surrounding cyst cell is absent. Therefore,

it is tentatively supposed that the multinucleate cell might represent a cell family of common origin in which the predefinitive spermatogonium has not yet been isolated. The majority of multinucleate cells are bi-nucleate ones which occur in all systems before a 'choice' of the multiplication system to be used has been made. This 'choice' is determined at the moment a predefinitive spermatogonium, when present in the multinucleate cell, is stimulated to divide quasi-dichotomously. The predefinitive daughter cell is thought to be excluded from the group of remaining definitive daughter cells, which is converted to a cyst by being surrounded by one or more cyst cells. The predefinitive spermatogonium stays in the apical part of the testis as a newly formed stem-cell. This hypothetical procedure might explain the presence of multinucleate cells, which are not cysts, in the apical cavities and the varying number of nuclei involved. In case of a simple cambium-like quasi-dichotomous division of the predefinitive spermatogonium, the existence of the multinucleate cell ends after the bi-nucleate stage and a cyst of two definitive spermatogonia is produced at the next definitive division. The nature of the signals which induce a predefinitive spermatogonium to divide quasi-dichotomously and the cyst cells to enclose a particular multinucleate cell remains obscure. These signals could be the results of regulatory feedback mechanisms which govern the entire course of spermatogenesis.

3.5.4.3. Temporal pattern of young adult spermatogenesis

According to the methods described in 2.2.4., experiments have been carried out with ^{3}H-thymidine. Observations at increasing intervals after injection will be briefly described in a chronological order. More information will be provided in a subsequent publication (THEUNISSEN and NOORDINK, 1976).

2–2½ hours: Secondary spermatogonia were labeled in various intensities, mostly in groups, whereas primary spermatogonia were labeled as individual cells in the same variation of intensity. This ranged from absence of label to heavy labeling. The resulting impression is that the labeling in the primary spermatogonial population was more diffuse and quantitatively less than that in the secondary spermatogonial region. Other germinal cell types were not labeled. The central cavity cells show a moderate labeling of the nuclei.

2 days: Both the primary and the secondary spermatogonia were labeled in various intensities. In particular the secondary spermatogonia were often heavily labeled. No other germinal cells showed a label at this time. The central cavity cells contained labeled nuclei.

3 days: Labeled primary spermatogonia were virtually absent. Primary spermatocytes and secondary spermatogonia were labeled in groups or individually in variable intensities. No other germinal cells were labeled. Nuclei of central cavity cells were heavily labeled.

4 days: The secondary spermatogonia and primary spermatocytes were still labeled. Dividing primary spermatocytes and some secondary spermatocytes were weakly labeled. No primary spermatogonia or other germinal cells were found to be labeled. Central cavity cells were also still marked.

TABLE 8. Labeling of germinal cell types in testis after injection with ^{3}H-thymidine.

Cells	Days after injection						
	0[1]	2	3	4	6	8	9
prim. spg:	+	+	±	−	−	−	−
sec. spg:	+	+	+	+	−	−	−
prim. spc:			+	+	+	−	−
MI:				±	+	−	−
sec. spc:				±	+	−	−
MII:					+	−	−
e. spt:					+	±	±
i. spt:					±	±	±
t. spt:					±	+	±
sperm:							+

[1] fixation 2–2½ hours after injection
− no labeling
+ distinct labeling
± weak or rarely observed labeling.

6 days: Testes showed a distinct labeling of non-dividing and dividing primary spermatocytes, secondary spermatocytes and early spermatids with an intensity increasing in this sequence. No spermatogonia or sperm cells and almost no transformation spermatids were found labeled. The central cavity cells were labeled to a degree varying from weakly to heavily.

8 days: The transformation spermatids were clearly labeled and, also some weakly labeled early spermatids. No sperm cells or other germinal cell types were labeled. Central cavity cells were weakly labeled.

9 days: Sperm cells and sometimes some spermatids were labeled, contrary to other germinal cell types.

These observations are summarized in table 8.

Discussion

In testes fixed 2–2½ hours after injection, ^{3}H-thymidine was incorporated in some cysts of secondary spermatogonia and in individual primary spermatogonia (fig. 60). The labeled thymidine entered the succession of spermatogenic cells evidently only via the spermatogonia. It was found in primary spermatocytes 3 days later. So it took about 3 days for primary spermatocytes to develop from the most advanced, DNA-synthetizing secondary spermatogonia. It took one day more for the most advanced labeled primary spermatocytes to develop into secondary spermatocytes by the first meiotic division (fig. 61). The formation and division of secondary spermatocytes seemed to proceed very quickly because here no clear sequence in the labeling was found (fig. 62). This conclusion had already been drawn because of the relatively low number of secondary spermatocytes usually present in the testis. The differentiation from early spermatids to transformation spermatids takes 1–2 days, as does that from transformation spermatids to sperm cells.

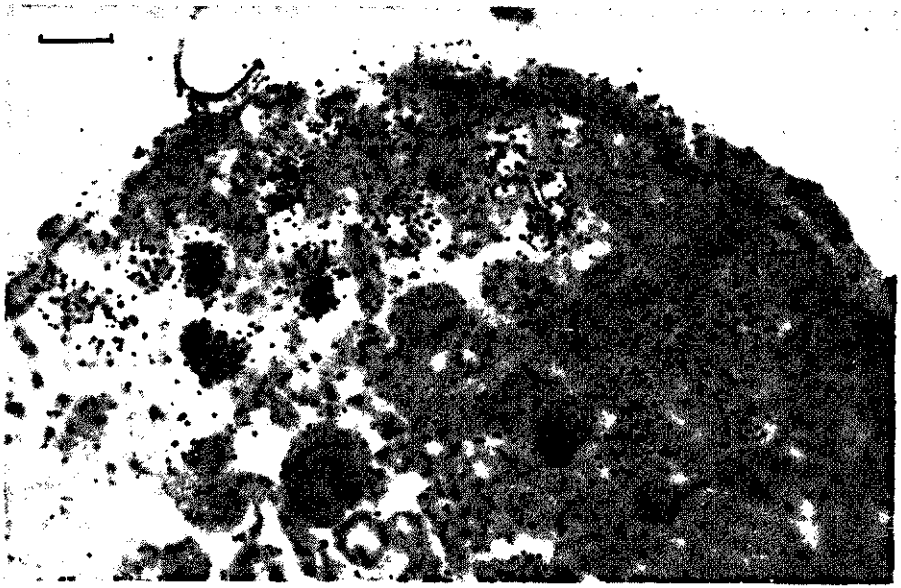
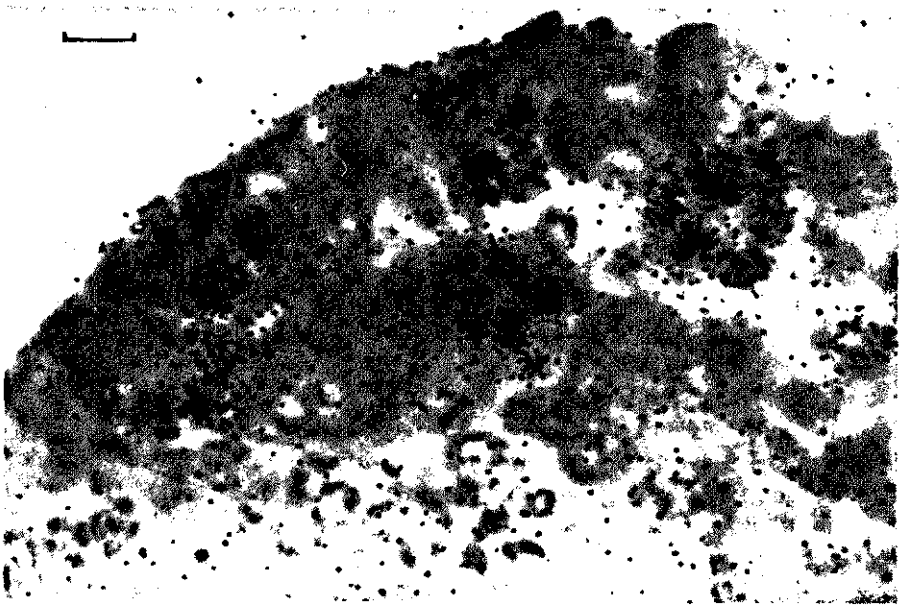


FIG. 60. a. Individual primary spermatogonia with a label of varying intensity.



b. Cysts of labeled secondary spermatogonia. Within a particular cyst the intensity of the labeling varies less as compared to that between individual primary spermatogonia. Bar = 10 μ.

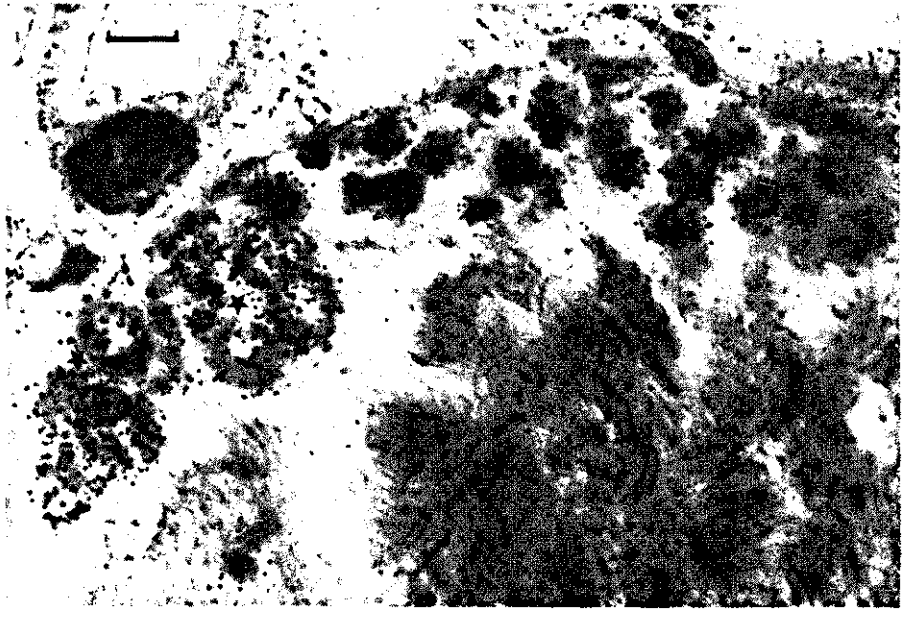


FIG. 61. Labeled primary spermatocytes (*) and MII-divisions in testis, 6 days after injection of ^{3}H-thymidine. Bar = 10 μ.

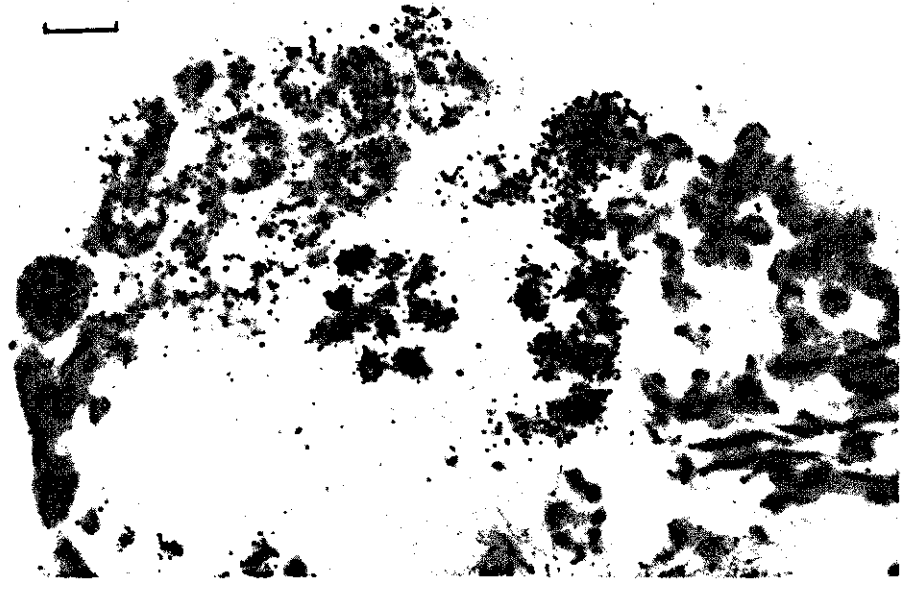


FIG. 62. Labeled primary spermatocytes (left), secondary spermatocytes (right) and early spermatids (centre) in testis, 6 days after injection of ^{3}H-thymidine. Bar = 10 μ.

TABLE 9. Estimate of the duration of cell type conversion in pupal and adult testes.

Cell types	Duration of development (days)	
	pupal	adult
prim. spg – sec. spg:	2	2
sec. spg – p. spc:	2	2–3
p. spc – e. spt:	1	2–3
e. spt – t. spt:	1	1
t. spt – sperm:	3	2
	9	9–11

By elaborating the labeling data from tabel 8 one may roughly estimate the duration of certain periods of the spermatogenic development. During the development of the pupal testis the consecutive cell types come into existence. From these data an estimate can be derived as to the duration of the cell type development in the pupal stage. Both estimates will be compared in table 9.

It appears that the duration of development from spermatogonium to sperm cell is more or less constant during both the pupal and adult stage.

In all preparations with labeled thymidine the following observations are made:

- the apical cell is never labeled.
- nuclei of individual epithelium cells of ductus deferens and accessory glands are found labeled in varying intensity in all post-injection groups (fig. 63).

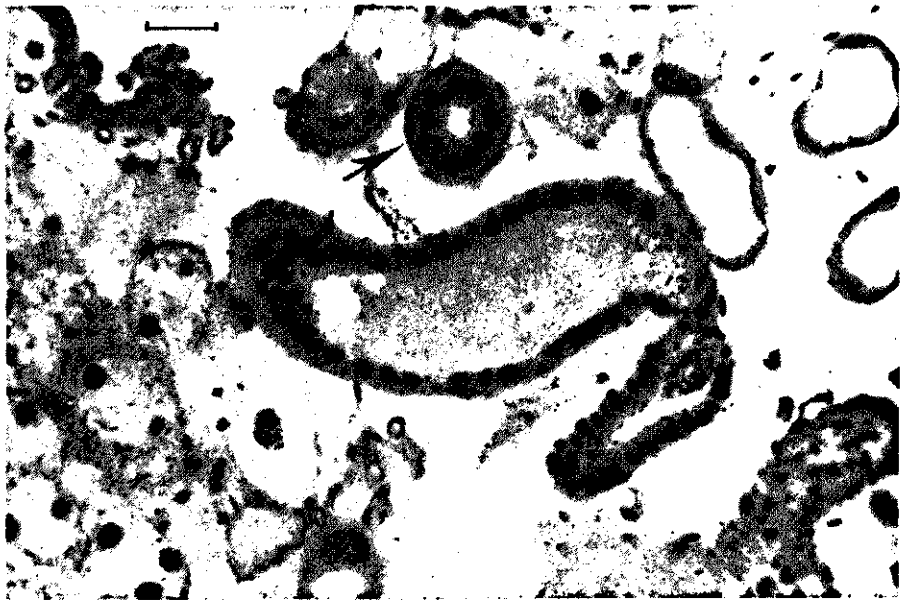


FIG. 63. Heavily labeled nuclei of the epithelium of the male accessory glands (centre) and the ductus deferens (arrow). Nuclei of fat cells are also labeled (left). Bar = 10 μ.

- no incorporation of ^{3}H-thymidine takes place before 90 minutes after injection.
- both 'diffuse' and 'concentrated' primary spermatogonia are labeled, but the 'concentrated' ones tend to show a heavier incorporation.
- the labeling of secondary spermatogonia is nearly always heavier than of primary spermatogonia in the same testis.
- at increasing time after injection the general intensity of the labeling decreases considerably. Hence, the designations 'weak' or 'heavy' are relative and only usable when cells within the same age group are compared.

As far as is known the period of time in *H. antiqua* necessary to develop from DNA synthetizing spermatogonia into sperm cells does not differ much from similar data relative to other Diptera. CHANDLEY and BATEMAN (1962) reported the presence of labeled sperm cells in *Drosophila melanogaster* testes 8 days after injection, which was confirmed by the results of OLIVIERI and OLIVIERI (1965). SHARMA et al. (1970) found a same period of 9 days in *Culex pipiens*. In the grasshopper *Melanoplus differentialis* the development from spermatogonia to sperm cells lasts 28 days (MUCKENTHALER, 1964). *Anthonomus grandis* requires 10 days according to CHANG and RIEMANN (1967).

CHANDLEY and BATEMAN (1962) established that ^{3}H-thymidine in *D. melanogaster* was incorporated exclusively in spermatogonia and primary spermatocytes. This is not in accordance with the findings in *H. antiqua* in which the isotope is incorporated only in spermatogonia. When taking into account the difference in opinion regarding the identification of the male pre-meiotic germinal cell types, however, similar observations could have been made. OLIVIERI and OLIVIERI (1965) commented on this problem by stating that they were unable to distinguish between very young primary spermatocytes and spermatogonia.

In accordance with observations of MUCKENTHALER (1964) on the steady percentage of labeled cysts in testes of *M. differentialis*, it was found that in *H. antiqua* only a limited number of cells or cysts of spermatogonia were initially labeled and never the entire population. At about 2 hours after injection an optimal labeling was reached. Short labeling times following injection of ^{3}H-thymidine have been observed before (MUCKENTHALER, 1964), suggesting a limited supply of precursor only to the cells in the S-phase at that time.

The apical cell in the adult testis apparently does not synthetize DNA. MUCKENTHALER (1964) found a distinct RNA synthesis in the apical cell of *M. differentialis* after injection of ^{3}H-uridine. This isotope has not been used in *H. antiqua*.

Occasionally nuclei of terminal epithelium cells and cells from the testicular sheath are heavily labeled. Very regularly nuclei of central cavity cells are labeled. About 2 hours post-injection they are distinctly-heavily labeled. This situation continues until at 6 days the intensity of marking becomes variable from weak to heavy. During the next days the intensity of labeling decreases quickly until labeled nuclei of central cavity cells are not observed anymore at 9 days post-injection. CHANG and RIEMANN (1967) mentioned the labeling of

cyst cells which were in close contact with bundles developing sperm cells in *Anthonomus grandis*. Labeled cyst cell nuclei were not observed in *H. antiqua*. The significance of the generally observed and active DNA synthesis in central cavity cells is obscure. Mitotic divisions of this cell type have never been observed. Therefore a possible explanation of the DNA synthesis without subsequent division could be the occurrence of one or more endomitotic divisions resulting in an increase of ploidy of the nucleus. The large size of the nucleus does not oppose to this assumption. However, the question concerning the function of these cells remains obscure.

The regularly observed but essentially incidental labeling of nuclei in the epithelium of the ductus deferens and the accessory glands points more towards a general, relatively high mitotic activity than an activity directly connected with secretory processes. It may be, however, that the secretory activity of these tissues cause the necessity for a quicker replacement of cells. Nothing is known of the length of the S-phase in these epithelial cells. The relatively large numbers of marked nuclei which are regularly found suggest besides perhaps a long S-phase a high number of cells in S-phase during the 30–60 minutes between the first labeling observed (90 minutes after injection) and the first fixations (120–150 minutes after injection). This implies a considerable DNA synthetic and mitotic activity per unit of time. In *Anthonomus grandis* ^{3}H-labeled nuclei of the ductus deferens have been reported (CHANG and RIEMANN, 1967).

3.5.5. *Comparative spermatogenesis*

In a preceding paragraph (3.5.2.2.) an identification system has been described for *H. antiqua* male germinal cells. However, the usefulness of this system would be considerably enhanced when similar results could be obtained when using the same standard methods on other insect species. Observations on *H. antiqua* would be comparable to those on other species. This possibility could be important when comparative research is to be carried out on distantly related species, for instance in insect radiobiology or insect reproduction. To evaluate the significance of the identification system of *H. antiqua* in this respect the standard histological processing and staining were applied to young male adults of a number of available, more or less generally studied insect species:

Diptera:	*Musca domestica* L.
	Sarcophaga sp.
	Ceratitis capitata Wied.
Lepidoptera:	*Adoxophyes orana* F.v.R.
Coleoptera:	*Leptinotarsa decemlineata* Say
Dictyoptera:	*Periplaneta americana* (L.)
Orthoptera:	*Locusta migratoria migratorioides* (R. & F.)
Hemiptera:	*Pyrrhocoris apterus* L.
Hymenoptera:	*Apis mellifica* L.

H. antiqua is used as a reference in these comparative descriptions and subsequent comments.

Musca domestica L.

The testicular size in *M. domestica* adults less than 24 hours old is clearly larger when compared with *H. antiqua*. Primary spermatogonia are not present, nor an apical cell. Remnants are perhaps a small group of flattened cells apical against the testicular sheath (fig. 64). Apical cavities are absent. The majority of premeiotic cells are secondary spermatogonia which are present in large numbers. The primary spermatocytes show a chromatin pattern which is distinctly different from the secondary spermatogonia. Both secondary spermatogonia and primary spermatocytes of *M. domestica* resemble the same cell types in *H. antiqua* closely and are readily identified. Cyst epithelium development is even weaker than in *H. antiqua*, resulting in a difficult identification of cysts, especially of cysts of secondary spermatogonia. Primary spermatocytes are situated as often elongated groups against the testicular sheath and as a little structured mass of cells between the secondary spermatogonia and the large central cavity. The chromatin pattern of primary spermatocytes often consists of 5–6 relatively large concretions. Later the concretions become longer and thinner, resulting in the appearance of meiotic chromosomes. The meiotic prophase seems to last a long time in comparison to other meiotic stages which are relatively rarely found, contrary to *H. antiqua* in which prophase I and other meiotic I stages are present in about equal numbers. Secondary spermatocytes are relatively scarce but show the same morphology as in *H. antiqua*, as do the various types of spermatids (fig. 65). Central cavity cells, which here obviously

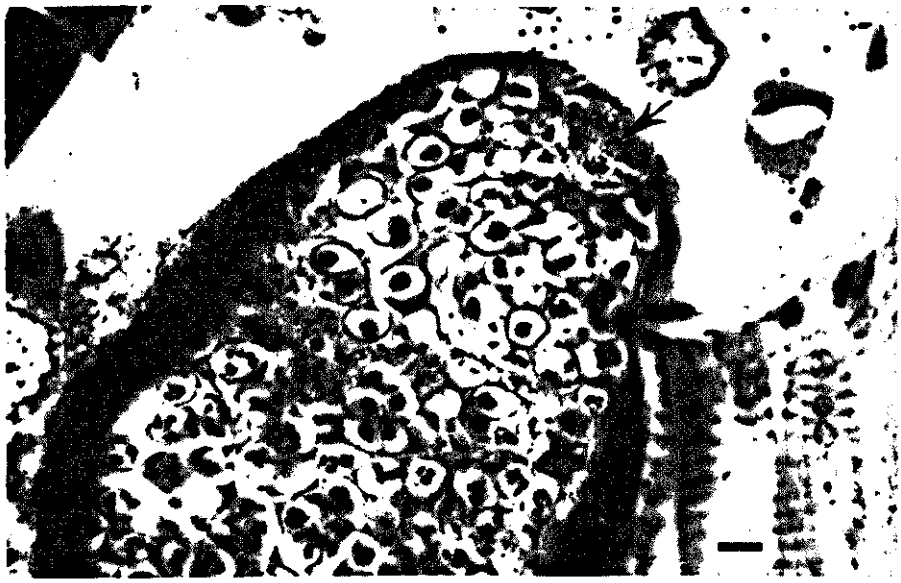


FIG. 64. *Musca domestica*. Top of testis of newly emerged fly. Only 'concentrated' types of spermatogonia present. Note possible remnant of apical cell (arrow). Bar = 10 μ. Figs. 65–73 same magnification.

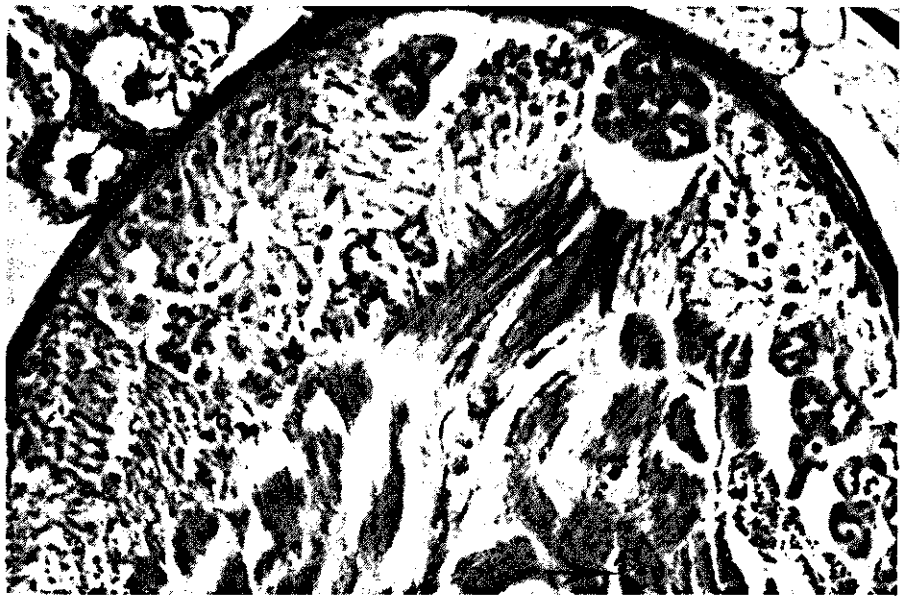


FIG. 65. *Musca domestica.* Primary spermatocytes, early and intermediate spermatids around the central cavity. Note sperm cells and central cavity cell (arrow) in central cavity. Newly emerged adult.

originate from the terminal epithelium, are present. The terminal epithelium seems to excrete a foamy substance which acts as a membrana basilaris closing the orifice of the testis. Sperm cells are organized more or less in bundles as they are in *H. antiqua.*

Sarcophaga spp.

The young adult testis consists of one follicle, like in *H. antiqua* and *M. domestica,* but contrary to these two species it is tubular of shape. In the apical part of the testis generally no primary spermatogonia nor an apical cell and apical cavities are present. When present the primary spermatogonia show the characteristic appearance of the 'diffuse' type of primary spermatogonia in *H. antiqua.* Between these cells darkly stained somatic nuclei are seen spaceously arranged in a relatively large amount of cytoplasm. These nuclei might represent an apical cell. The secondary spermatogonia are very distinct and are observed in large numbers (fig. 66) at the apical end of the testis. The concentrated chromatin in their nuclei does definitely not represent a bouquet or other recognisable meiotic division stage. The primary spermatocytes originate from the secondary spermatogonia by the falling apart of the chromatin into smaller concretions which ultimately transform into meiotic chromosomes. The development of the cyst epithelia is weak. The cysts of secondary spermatogonia are almost impossible to determine as such, contrary to those of primary spermatocytes which are more widely dispersed in groups. Secondary sperma-

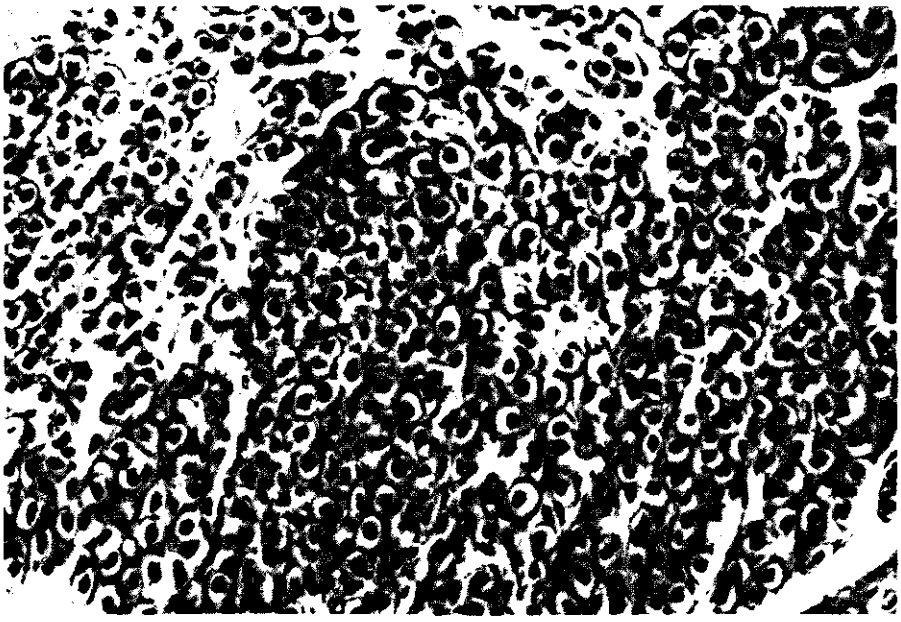


FIG. 66. *Sarcophaga* spp. 'Concentrated' spermatogonia dominate the testis. Newly emerged adult.

tocytes which are very similar to primary spermatocytes in their chromatin pattern are very scarce as are cells in meiotic divisions. These stages seem to be passed very quickly. The spermatids are very similar to those of *H. antiqua*. Early spermatids with dark nuclei strongly dominate in number, while the other types are rarely found, suggesting a very short duration of these stages. In very early spermatids large Nebenkerns are sometimes very conspicuous. The relatively wide and long central cavity is filled with bundles of sperm cells, except the long core which shows cell debris, cytoplasmic droplets and other waste material. Central cavity cells are scarce and are mostly found in the apical part of the central cavity near the secondary spermatogonia and primary spermatocytes. Other somatic cell types are also strikingly scarce. A terminal epithelium of columnar cells with an indistinct boundary forms a plug which closes the testicular orifice.

Ceratitis capitata Wied.

The young adult testis is spheroidal-ovoid as in *H. antiqua* and *M. domestica*. At the apical side a slight elevation contains the population of spermatogonia (fig. 67). A distinct apical cell, as is found in *H. antiqua*, has not been observed. However, in the most apical part a small group of lightly stained, inconspicuous somatic nuclei has been found amidst spermatogonia. These spermatogonia partly conform to the description of 'diffuse' primary spermatogonia in *H. antiqua*. Individual mitotic divisions take also place. Basally around this most

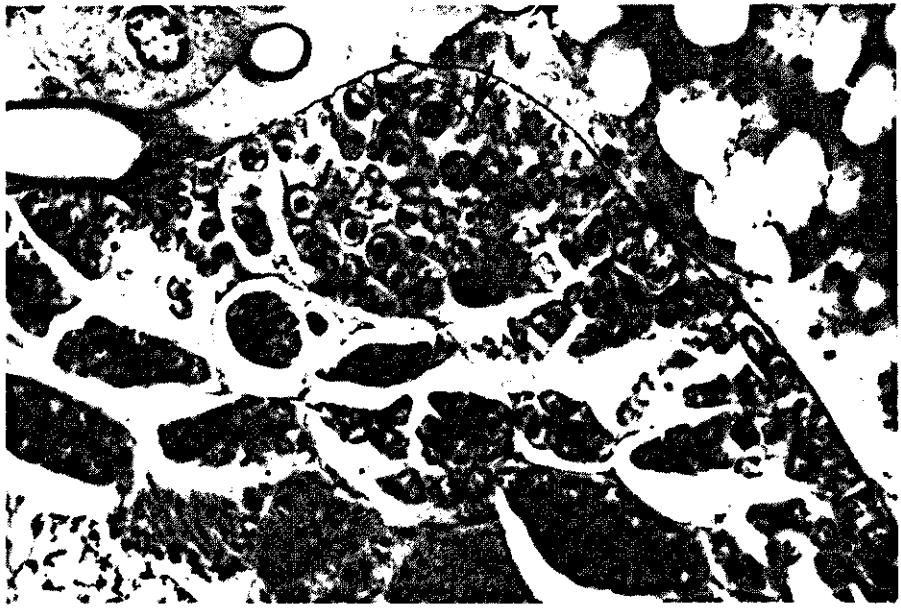


FIG. 67. *Ceratitis capitata*, newly emerged fly. In top of testis possible remnant of apical cell (arrow) surrounded by predominantly 'diffuse' spermatogonia. More basally also 'concentrated' spermatogonia and primary spermatocytes in groups.

apical cell population low numbers of cells are observed which are similar to primary and secondary spermatogonia, both in various 'concentrated' types (fig. 67) as in *H. antiqua*. Nucleoli are distinct in spermatogonial nuclei. Primary spermatogonia often contain two nucleoli. In apical cavities small groups of primary spermatogonia are frequently observed. Secondary spermatogonia show a somewhat different chromatin pattern. Concentration of the chromatin takes place both as has been described for *H. antiqua* and as an accumulation of more or less coarse particles against the nucleolus and the nuclear membrane. The latter way results in well defined nuclei with distinct nucleoli. The chromatin granules seem to become larger, coarser and united in concretions until the nuclei contain a limited number of relatively large chromatin concretions, which are generally periferally located, resulting in primary spermatocytes which conform to the appropriate description of this cell type in *H. antiqua*. The secondary spermatocytes (fig. 68) show the same appearance, but are smaller in size. The spermatids are similar to those in *H. antiqua*, the early spermatids dominating very much in numbers. The sperm cells in bundles are situated in the large central cavity. Central cavity cells are seen in low numbers. Basally a terminal epithelium lines the orifice of the testis.

Adoxophyes orana F.v.R.

The multifollicular adult testis is more or less spheroidal. The separation of

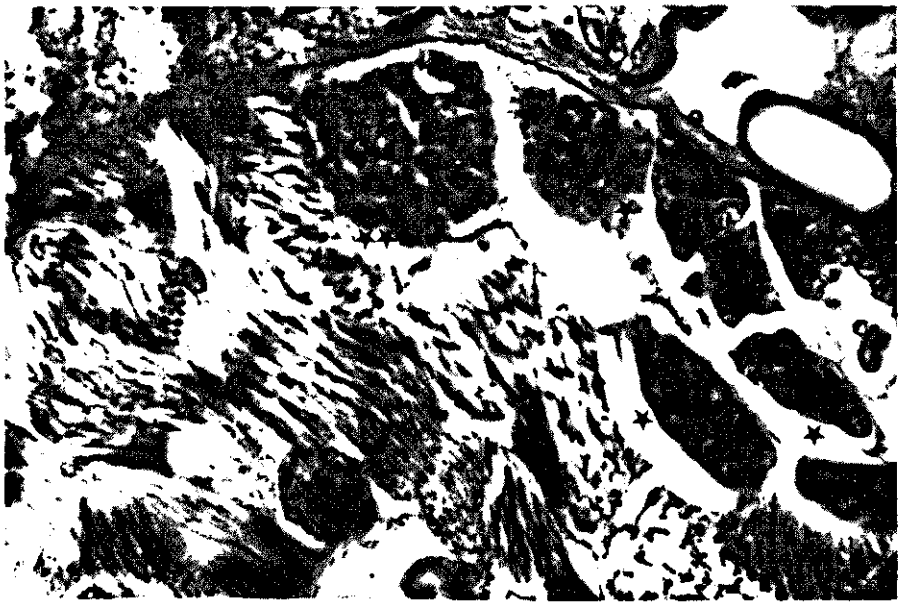


FIG. 68. *Ceratitis capitata*, newly emerged fly. Lateral part of testis with primary (*) and secondary (**) spermatocytes and early spermatids along the central cavity.

the follicles is very indistinct, if not locally virtually absent. In this way the testis seems to be one large central cavity with peripheral groups of premeiotic cells.

The primary spermatogonia are situated near and around the apical cell which consists of a large cell with one or two relatively weak staining nuclei (fig. 69). The primary spermatogonia are readily recognized by their large, light and characteristic nuclear morphology which is very much like of *H. antiqua* primary spermatogonia. Around the primary spermatogonia is a small zone of darkly stained secondary spermatogonia. The chromatin is coarser, denser and more heavily stained than the chromatin of the primary spermatogonia. Cells containing nuclei with concentrated chromatin are commonly present in groups. For these cells no evidence for degeneration can be found. Primary spermatocytes are cells with large, light and distinct chromatin granules containing nuclei. The chromatin is regularly dispersed, tending to a peripheral location. These cells conform to the appropriate description for the primary spermatocytes of *H. antiqua*. They are situated at the periphery of the cyst often leaving an open space in the centre. Small groups of cysts lay against the secondary spermatogonia or somewhat aside against the testicular sheath (fig. 70). Just as cells in meiotic divisions, secondary spermatocytes are scarce and are readily recognized as such because they are very similar to those of *H. antiqua* (fig. 70). The spermatids do not show such a variety of types, especially within the early spermatids, but they conform very well to the description of *H. antiqua* spermatids. Both apyrene and eupy-

FIG. 69. *Adoxophyes orana*, just emerged moth. Apical cell with 'diffuse' primary spermatogonia surrounded by smaller and darker secondary spermatogonia. Some primary spermatocytes, early spermatids and apyrene sperm cells are observed outside the dark mass of premeiotic cells.

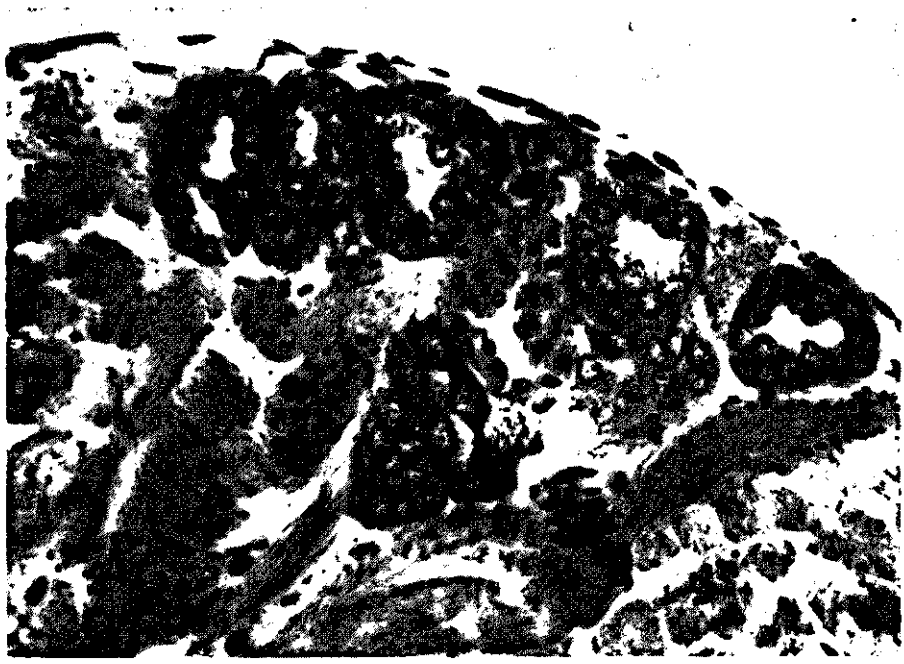


FIG. 70. *Adoxophyes orana*, newly emerged moth. Distinct cysts with primary spermatocytes, early spermatids and apyrene sperm cells are observed.

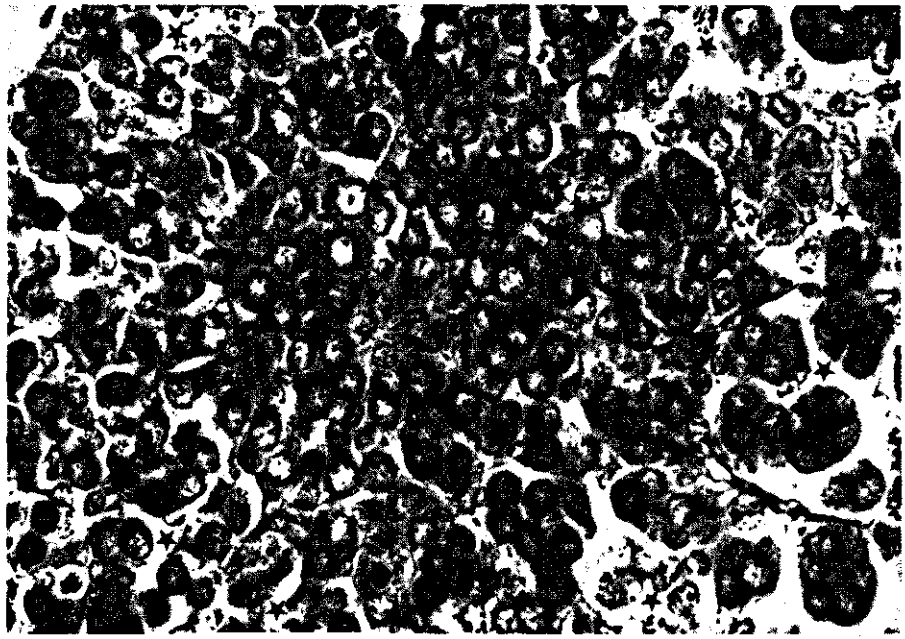


FIG. 71. *Leptinotarsa decemlineata*, young adult. Apical part of testis where follicles are still united. 'Diffuse' type of spermatogonia shows increasing tendency to become organized in groups when approaching the open apical part of the follicles (*).

rene sperm cell bundles are present, of which eupyrene are similar to the sperm cells of *H. antiqua*. The central cavity is large, apical cavities are absent as are central cavity cells. The terminal epithelium consists of columnar cells.

Leptinotarsa decemlineata Say.

The flattened spheroidal adult testis consists of a large number of follicles which are apically and basally connected. Apically the spermatogonia form one large population (fig. 71) and basally the vasa efferentia unite in the ductus deferens.

The spermatogonia show a large, light nucleus with distinct chromatin granules and filaments which are regularly dispersed. The cytoplasm is visible as a narrow, darkly stained ring around the nucleus. This cell type conforms well to the description of primary spermatogonia of *H. antiqua*. An apical cell is absent at this stage. In the centre of the testis where the apical parts of the testicular follicles unite, the spermatogonia are randomly mixed with some smaller and darker somatic nuclei. Approaching the periphery of the spermatogonial population the mitotic activity is relatively high and the spermatogonia become more and more arranged in groups without the formation of distinct cysts. The membranous walls, which separate the follicles, penetrate the mass of cells and when groups of spermatogonia are near these walls an association with

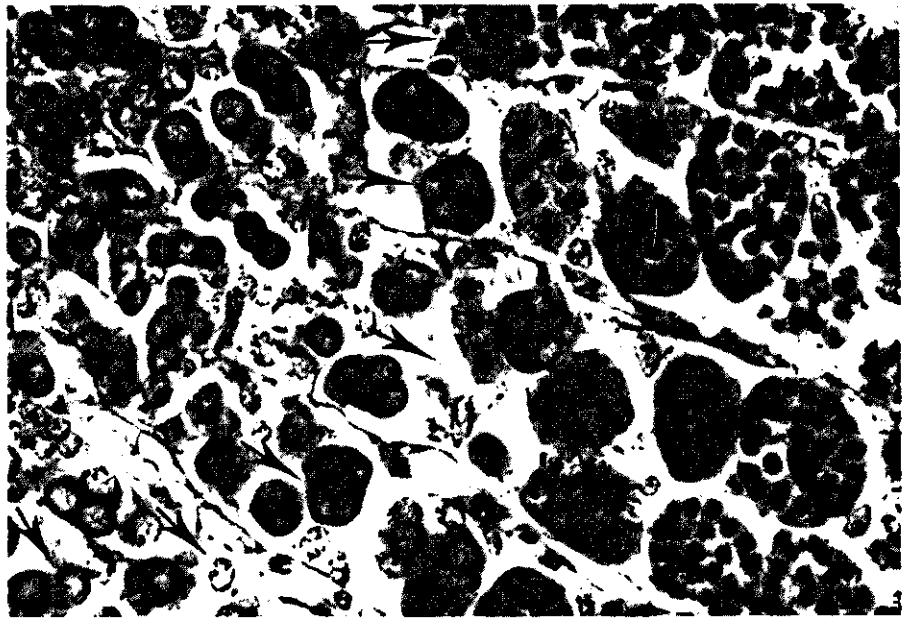


FIG. 72. *Leptinotarsa decemlineata*, young adult. Apical part of testis follicles showing multiplication of germinal cells in basal direction (arrows).

cyst cells seems to take place, resulting in the formation of cysts. In a more radial direction, cysts of spermatogonia are found (fig. 72). In cysts with many cells the nuclei are smaller when compared to cysts with a few cells, the chromatin is a little denser and coarser and the nucleus is more darkly stained. However, the basic nuclear morphology has not changed essentially and abruptly and these spermatogonia can not be designated as secondary spermatogonia on these grounds. The primary spermatocytes show a distinct bouquet stage during meiotic prophase resembling secondary spermatogonia. Previously they show a nuclear morphology which is similar to that of primary spermatocytes in *H. antiqua*. The cells fill the cysts completely. Cells in meiotic division and secondary spermatocytes are rarely found. The spermatids are very similar to those of *H. antiqua*. The sperm cells are firmly bundled and each bundle is accompanied by a cyst cell nucleus. The central cavity is filled with cysts of postmeiotic cell types, especially sperm cells. Central cavity cells are present in low numbers.

Periplaneta americana (L.)

The adult testis comprises of a large number of follicles and is dominated at this stage by large masses of primary spermatocytes.

Spermatogonia are observed in small numbers apically of the spermatocytes. The nuclei are ovoid, distinct and as darkly stained as those of the primary spermatocytes. The chromatin is finely granular and is regularly dispersed in

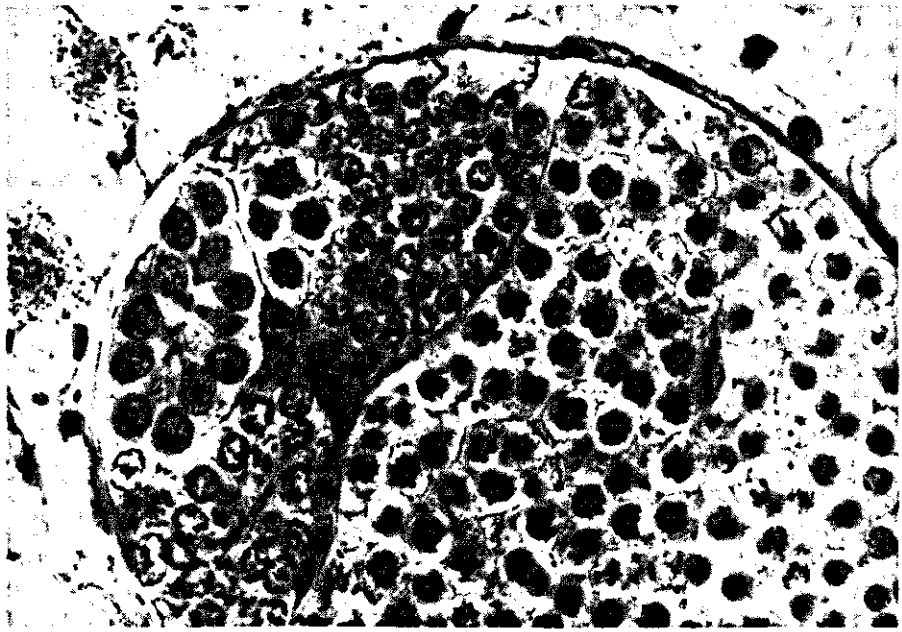


FIG. 73. *Periplaneta americana*, young adult. Apical part of testis showing 'diffuse' spermatogonia in a rosette surrounded by spermatogonia and interphase primary spermatocytes. More basally 'bouquet-stage' prophase primary spermatocytes and cell divisions are observed.

the nucleus. This nuclear morphology conforms to that of the primary spermatogonia of *H. antiqua*, apart from the dark staining. The cells seem to be arranged in rosette-like positions with peripherally located nuclei. They contain a relatively large amount of cytoplasm which causes a group of spermatogonia to be conspicuous among the densely packed primary spermatocytes (fig. 73). An apical cell is absent at this stage.

The primary spermatocytes show cells in a clear bouquet stage as well as interphase cells in large numbers. Within the group of interphase cells several variations on the size of the chromatin concretions and particles are observed which, however, are essentially similar to those of *H. antiqua.* Mitotic and meiotic divisions are very scarce as are secondary spermatocytes and intermediate spermatids. The latter conform to the descriptions for *H. antiqua* when present, just like the sperm cells and the early and transformation spermatids.

In cysts, large somatic nuclei are seen, probably cyst cells. At the periphery of the follicle another type of somatic cell can be found which is very similar to somewhat weakly stained basal cells of *H. antiqua.* They are numerous, contrary to the central cavity cells. The cubical terminal epithelium lines the vas efferens.

Locusta migratoria migratorioides (R. & F.)

The adult testis consists of many follicles which are tubular in shape.

In the follicles an apical cell is found, surrounded by spermatogonia which

show relatively coarse, darkly stained chromatin granules regularly dispersed in the nucleus. These nuclei are sometimes a little irregularly lobed and are surrounded by a relatively large amount of cytoplasm. Already close to the apical cell somatic cells seem to associate with spermatogonia to form well developed cysts. The size of chromatin granules and the stainability of the nuclei vary somewhat. There is no demarcation of spermatogonia and primary spermatocytes. The chromatin elements in primary spermatocytes become more filamentous and more variably granular as compared with those in spermatogonia where they are more uniform in size and shape. The primary spermatocytes show a more banded and often lighter chromatin pattern. A variety of prophase stages can be observed. Early spermatids are very abundant as are several meiotic stages. Secondary spermatocytes are very scarce, intermediate spermatids do not occur at all. Secondary spermatocytes show a pattern of dispersed chromatin granules not similar to secondary spermatocytes of *H. antiqua*. Early and transformation spermatids are very much alike those of *H. antiqua* just like the sperm cells.

Between the cysts degenerating cysts are often observed which in due course seem to become transferred to the core of the central cavity where waste material such as cell debris is stored and probably broken down. In the central cavity a small number of central cavity cells is present in and between cysts of spermatids and sperm cells. In the apical part of the follicle, about the sites where cysts are being formed, cavities are observed laterally which surround a part of the population of spermatogonia as a belt. These are apical cavities. Spermatogonia apically of these cavities are less densely packed when compared to those basally of the apical cavities. The terminal epithelium shows large nuclei with some coarse chromatin concretions.

Pyrrhocoris apterus L.

The adult testis is formed by a large number of follicles.

Most apically, an apical cell is surrounded by spermatogonia. These cells show the appearance which is characteristic for primary spermatogonia of *H. antiqua*, except the dark staining. At the outside of this population, spermatogonia with nuclei containing concentrated chromatin and somatic cells are found. These populations are separated from larger ones of typical secondary spermatogonia by apical cavities which occur in varying numbers. The secondary spermatogonia which are similar to those in *H. antiqua* show very variable chromatin patterns of 'concentrated' types, the one with concentrated chromatin being dominant in numbers. Basally of this rather narrow zone of secondary spermatogonia large cysts are found with larger cells: the primary spermatocytes. These cells also show a considerable variation of types. The interphase type as it also has been described for *H. antiqua* is present in large numbers. But all varieties of 'concentrated' types between the 'diffuse' interphase type, just mentioned, and a 'concentrated' type with heavily concentrated chromatin are present. The latter type does not seem to represent a bouquet stage. Cells in meiotic divisions are frequently found, contrary to secondary spermatocytes.

The spermatids conform generally to those of *H. antiqua*, although intermediate and transformation spermatids are scarce suggesting a quick transfer to sperm cells. The sperm is observed in bundles basally in the follicles. Cyst cell nuclei of cysts of spermatids are often swollen indicating some possible nutritive function. Central cavity cells with evenly spread finely granular chromatin in the nuclei are found in or between the cysts, predominantly basally in the follicle. Terminal epithelium is not found in the follicle itself but in the vasa efferentia.

Apis mellifica L.

The adult testis contains many tubular follicles.

The methods used do not seem to be suitable to study male germinal cells of *A. mellifica*. The Bouin fixation appears to fail. The germinal cells are very small, the somatic ones are larger and appear to be well fixed. Terminal epithelium is clearly present, as are central cavity cells basally between the bundles of sperm cells, which are distinctly smaller (fig. 74) than those in the already mentioned species of insects.

Discussion

Before discussing the results it must be mentioned that the time of sampling i.e. male adults younger than 24 hours, is an arbitrarily chosen moment. It is

FIG. 74. *Apis mellifica*, newly emerged drone. Very small bundles of sperm cells (arrows) in testis follicles. Note prominent nuclei of central cavity cells. Bar = 10 μ.

suitable for *H. antiqua* in which all germinal cell types are abundantly present and the testis shows all somatic structures at that time. But as has been shown before, in other insects this period of testicular development and activity may be reached earlier or later. Therefore it is quite possible that for this reason sometimes only low numbers of certain germinal cell types could be found. Between different species there is no synchronization in testicular development in relation to age. A point to be kept in mind also is the necessarily limited scope of this survey. The nuclear morphology of the germinal cell types and the occurrence of some somatic cell types and structures are involved, excluding other aspects for the moment.

When comparing the other dipterous species to *H. antiqua* (table 10) a clear similarity in the nuclear morphology of the various germinal cell types can be established. The only exception are the primary spermatogonia which are absent in *M. domestica* and partly absent in *Sarcophaga sp.* This cell type is probably only present in *M. domestica* during earlier stages in the testicular development. In *Sarcophaga sp.* they are occasionally observed, in that case conforming to the description for *H. antiqua.* In *C. capitata* a small population of primary spermatogonia seems to be able to maintain itself in the young adult stage. In all species of Diptera investigated here, corresponding somatic cells and structures are found, except those which seem to have some relationship with the primary spermatogonia. The apical cell is only present in *H. antiqua* and probably in *C. capitata* though in a morphologically different shape. It is absent in *M. domestica* and *Sarcophaga sp.*, as are the apical cavities. Concluding it can be said that if the dipterous species analysed reacted in a representative way, the identification method as defined for *H. antiqua* can be used for most Diptera.

As for the non-dipterous species, only *A. orana* and *P. apterus* show a clear distinction between two types of spermatogonia which can be obviously considered to be similar to the primary and secondary spermatogonia of *H. antiqua.* Only one type of spermatogonia has been found in *L. decemlineata, P. americana* and *L. migratoria.* When the nuclear morphology of these spermatogonia is compared to that of both spermatogonial types of *H. antiqua,* it is evident that in fact there is a clear similarity with the primary spermatogonia of *H. antiqua* with only minor differences like the staining intensity or the size of the chromatin granules. These differences also occur in primary spermatogonia of *P. apterus* which stain much more darkly when compared to those of *H. antiqua.* Only the primary spermatogonia of *A. orana* and *L. decemlineata* show a relatively light staining. However, as for the nuclear morphology these minor differences are not essential. Essential in this respect are the organization and the dispersion of the chromatin in the nuclei of the spermatogonia. When *A. mellifica* is excluded, it can be established that all the species examined exhibit primary spermatogonia at this stage of testicular development, except *M. domestica.* It appears that differences in the nuclear morphology of the primary spermatogonia within *H. antiqua* itself are more pronounced than those between the species presently in discussion. 'Concentrated' types of primary

TABLE 10. Presence of male germinal cell types and somatic cell types and structures in testes of some insect species.

	H.a[1]	M.d.	S.s.	C.c.	A.o.	L.d.	P.am.	L.m.	P.ap.	A.m.
prim. spg:	+	−	+/−	+	+	+	+	+	+	−
sec. spg:	+	+	+	+	+	−	−	−	+	−
p. spc[2]:	+	+	+	+	+	+	+	+	+	−
s. spc:	+	+	+	+	+	+	+	○	+	−
e. spt:	+	+	+	+	+	+	+	+	+	−
i. spt:	+	+	+	+	+	+	+	−	+	−
t. spt:	+	+	+	+	+	+	+	+	+	−
sperm:	+	+	+	+	+	+	+	+	+	+
ap. cell:	+	−	−	○	○	−	−	○	○	−
ap. cav.:	+	−	−	+	−	−	−	+	+	−
centr. cav.:	+	+	+	+	+	+	+	+	+	+
c. c. cells:	+	+	+	+	−	+	+	+	+	+
t. epith.:	+	+	+	+	+	+	+	+	−	+

\+ present and conforming essentially to the appropriate description for *H. antiqua.*
− absent
+/− sometimes present
○ present and not conforming essentially to the description for *H. antiqua.*
[1] conforming by definition
[2] interphase stage

H.a: *H. antiqua*
M.d: *M. domestica*
S.s: *S. sp.*
C.c: *C. capitata*
A.o: *A. orana*
L.d: *L. decemlineata*
P. am: *P. americana*
L.m: *L. migratoria*
P.ap: *P. apterus*
A.m: *A. mellifica*

spermatogonia have only been observed in *H. antiqua* and *C. capitata.*

The presence of 'concentrated' secondary spermatogonia seems to be a relative exception in non-dipterous species. The encysted definitive spermatogonia of *L. decemlineata* look like primary spermatogonia. It seems that the formation of secondary spermatogonia is species determined and is not a general feature of insect spermatogenesis as is the formation of primary spermatogonia, especially in ontogenic younger stages of spermatogenic development.

Primary spermatocytes are surprisingly uniform in their interphase nuclear morphology. Bouquet stages as in *L. decemlineata* and *P. americana* 'concentrated' types in *P. apterus* and a banded chromatin pattern in *L. migratoria* represent morphological differences with the main interphase type, which conforms very well to the description of primary spermatocytes in *H. antiqua.*

Secondary spermatocytes are generally rarely observed, suggesting a short duration of this stage. Another cause may be an unsuitable moment during the spermatogenic development occurring at the time of fixation. At certain moments in the spermatogenesis of *H. antiqua* secondary spermatocytes are not scarce at all, contrary to some days later. The same can be said for both meiotic divisions which seem to proceed rather quickly but are possibly more numerous during other moments of spermatogenesis. In some species

prophase stages are frequently found, possibly due to a relatively long duration of these stages and large numbers of cells available which increases the chance of observing cells in meiotic division, for instance in *L. migratoria*.

Usually early spermatids are dominant in numbers relative to the intermediate and transformation spermatids. The intermediate spermatids are generally scarce owing to a very short life time of this cell type and sometimes absent because of a slightly different spermatid differentiation, for instance in *L. migratoria*. The numbers of transformation spermatids are variable according to the species concerned. This cell type always conforms to the appropriate descriptions for *H. antiqua* spermatids.

Sperm cells which are more or less bundled in the various species, may be abundant in number or relatively few, depending on the rate of storage in the testis or elsewhere in the reproductive system. Except for the apyrene sperm of *A. orana* these cells are very similar to the *H. antiqua* sperm at this level of examination. At the ultrastructural level large differences might be observed. The sperm cells of *A. mellifera* are considerably smaller of size (fig. 74).

As for the somatic structures, a central cavity is always present in the testis or the follicle. Except in *A. orana* central cavity cells have been observed in all species in varying numbers, but essentially similar in appearance. They seem to play some essential role in the differentiation and/or maintenance of the postmeiotic cell types. Cyst cell nuclei may have the same appearance as central cavity cells in some species but are tied to the cysts, whereas central cavity cells seem to roam relatively free inside the entire central cavity. Observations like these raise questions pertaining to the origin of the central cavity cells. Apical cavities may or may not be present in the testis of the different species. Apical cells, generally considered to be juvenile structures, are still present in a number of species from various orders. Their presence seem to depend largely on the stage of spermatogenic development which is taken for observation, for instance in *M. domestica*. Terminal epithelium lining the most basal part of the follicle of the testis itself is very common. Usually it is continuous with the epithelium of the vas efferens or the ductus deferens respectively. The shape of the epithelial cells varies from cubical to elongated columnar, probably in relation to their function.

From this survey it appears that the dipterous species observed share the following features:

1. the presence of 'concentrated' secondary spermatogonia
2. a weak development of the cyst epithelium.

Generally, but not always present are central cavity cells and a terminal epithelium in the testis.

Concluding, it may be stated that the nuclear morphology of the male germinal cell types and the occurrence of somatic testicular structures as have been described for *H. antiqua* are also valid for a wide variety of insect species, provided the testes have been prepared according to the same standardized histological methods. This finding permits comparative research in normal and experimental spermatogenesis of insects from various orders.

3.5.6. *General discussion*

The identification of somatic and germinal testicular cell types of *H. antiqua* has been based mainly on nuclear morphology. In the study of insect spermatogenesis this is an unusual approach contrary to that followed in mammals. In 1901 Regaud provided the base for investigations of mammalian germinal cells by identifying these cells on their nuclear morphology i.e. the appearance, the organization and the dispersion of the chromatin in the nuclei concerned. This system has been developed and used since by many workers in this field.

Except for the morphology of the spermatogonial cell types, which have already been discussed, the interphase primary and secondary spermatocytes of *H. antiqua* show a general agreement with the relevant descriptions of these cell types in the literature. Apart from the sub-division of the spermatids in types, the morphology and the differentiation of the spermatids are also in agreement with existing descriptions.

Based on evidence discussed in a preceding paragraph the hypothesis has been brought forward that the primary spermatogonia as a morphologically defined cell type are identical to predefinitive spermatogonia in the physiological sense.

Since it has been established that spermatogonial multiplication in *H. antiqua* may take place according to various systems (paragraph 3.5.4.2.) a clear concept of this process is still lacking. The 2^n system obviously plays an important role. In this system primary spermatogonia could associate with a somatic cell within the population of primary spermatogonia. Associated primary spermatogonia (fig. 20a) are observed almost exclusively situated among other primary spermatogonia apically of the apical cavities. The associated initial definitive spermatogonia, which are daughter cells of a predefinitive spermatogonium, divide subsequently dichotomously, thus forming a cyst of secondary, definitive spermatogonia. Other possible multiplication systems presuppose the isolation of a stem cell after a few generations of spermatogonial multiplication. This might take place in the apical cavities. It is tentatively supposed that the multinucleate cells in these cavities might represent a number of spermatogonial cells prior to isolation of the predefinitive spermatogonium. After isolation the multinucleate cell might be converted into a cyst of definitive spermatogonia by association with cyst cells. These spermatogonia may or may not continue their multiplication by means of dichotomous divisions.

At hatching of the larva the gonad contains on an average 13 primary spermatogonia/testis. According to SONNENBLICK (1941) in *Drosophila melanogaster* this number is 36–38. The continuous presence of primary spermatogonia in *H. antiqua* is conspicuous, suggesting a presumed role as a small population of predefinitive spermatogonia until the end of adult life. The actual activity of the primary spermatogonia, especially during the later stages of adulthood, remains largely unknown. In *D. melanogaster* it has been estimated (LÜERS, 1956) that possibly only one spermatogonium/testis is actively dividing in old males. It is clear that little knowledge has been obtained on the period preceding the definitive spermatogonial divisions. Based on radiation studies with *D. mela-*

nogaster and *Pediculus corporis* PONTECORVO (1944) concluded that during the period of the definitive divisions and later no germinal selection in *D. melanogaster* takes place, contrary to the period prior to the definitive spermatogonial divisions. Although no cytoplasmic bridges had been recognized as such (FAWCETT et al., 1959), PONTECORVO (1944) observed that the cells within a cyst acted as a syncytium which in his opinion explained the absence of germinal selection. Intercellular bridges already had been observed e.g. by MUNSON (1906) and given names like 'Zwischenkörper' or 'intermediate bodies'. They were considered to be spindle remnants and the interpretation of PONTECORVO (1944) of the cells within a cyst as a syncytium opened essential new perspectives on the functioning of this cell family during spermatogenic processes. The synchronous development of the descendants of a single cell is used in the brood pattern analysis of *D. melanogaster* (PURO, 1964) to investigate the sensitivity of spermatogenic cell types to for instance ionizing radiation. A second example of the importance of the intercellular communication is the interesting matter of the genetic control of morphogenesis in the post-meiotic cell types (MULLER and SETTLES, 1927; LINDSLEY and GRELL, 1969). The latter workers discussed the question whether or not genes directly influence the formation of functional sperm cells and how the control mechanism could operate. They concluded that the normal differentiation of post-meiotic cell types is determined by the diploid genotype. The haploid genome does not influence the normal spermatid differentiation. They presume a continued synthesis of cellular compounds during spermiogenesis. This possibility might explain the continued sperm cell formation by differentiation of spermatids during 1–3 days after irradiation. Disappearance of spermatids may largely be due to depletion.

Protein synthesis during spermiogenesis has been observed in *D. melanogaster* (DAS et al., 1964; BRINK, 1968), in *Pales ferruginea* (PETZELT, 1970), in *Chortophaga viridifasciata* (BLOCH and BRACK, 1964), in *Schistocerca gregaria* (DAS et al., 1965) and in *Acheta domestica* (TESSIER and PALLOTTA, 1973). The synthesis of RNA in post-meiotic cell types has been investigated in *D. melanogaster* (CURGY and ANDERSON, 1972), in *Pales ferruginea* (PETZELT, 1970) and in *Schistocerca gregaria* (HENDERSON, 1964; DAS et al., 1965). The RNA synthesis generally decreases with increasing contraction of the chromosomal elements. According to DAS et al. (1965) the DNA synthesis is completed before meiosis and HENDERSON (1964) found that heavily contracted chromosomes were capable to synthetize DNA. This suggests that DNA synthesis may take place in secondary spermatogonia with even very concentrated chromatin, as has been confirmed by autoradiographic experiments with *H. antiqua*. The absence of tritiated thymidine incorporation into interphase primary spermatocytes leads to the assumption that possibly the necessary pre-meiotic DNA replication already takes place in the secondary spermatogonia and not in the primary spermatocytes themselves.

At a certain, presumably far advanced, stage of spermatid development (FAWCETT et al., 1959; ÅBRO, 1964) the connections between the cells are severed. The excess cytoplasm is stripped off as cytoplasmic droplets which

have been observed among the sperm cell bundles (CHOLODKOVSKY, 1905; MUNSON, 1906; KNABEN, 1931), individual sperm cells (SCHWARTZ, 1965) and in the core of the central cavity e.g. in *Schistocerca gregaria* (CANTACUZÈNE et al., 1972) and in *H. antiqua.*

The relevance of the morphological identification of male germinal cell types of *H. antiqua* applied to other insect species has already been discussed. The identification system for male germinal cells which is applicable to insects of various orders increases the significance of the results of the present study of *H. antiqua.*

3.6. OOGENESIS

3.6.1. *Introduction*

Comprehensive reviews on oogenesis are available both general (e.g. RAVEN, 1961; BIER, 1969) and more specifically dealing with insects (e.g. BONHAG, 1958; DAVEY, 1965; MAHOWALD, 1973; DE WILDE and DE LOOF, 1973a). Reviews and monographs on special aspects of oogenesis or a particular species also exist, for instance on hormonal control of oogenesis (DOANE, 1973; DE WILDE and DE LOOF, 1973b), on the ultrastructural aspects of vitellogenesis (TELFER nad SMITH, 1970), on the oocyte-trophocyte syncytium (TELFER, 1975), and on the oogenesis (KING, 1970a) and meiosis in oocytes (KING, 1970b) of *Drosophila melanogaster.*

As the present account has the limited purpose of providing sufficient knowledge on the structure and ontogenic development of the ovary of *H. antiqua,* which is to be used as a point of reference in radio-pathological studies, only some aspects of oogenesis are treated. The emphasis is laid here on the structure of the ovary, the germinal and somatic cell types involved and ovarian development during the larval, pupal and adult stages. Genetical, ultrastructural and physiological aspects have not been taken into consideration.

3.6.2. *Identification of ovarian cell types*

Owing to the relatively simple spatial relationships of germinal and somatic cell populations in the insect ovary whem compared to the testis the number of different cell types is small and the cell types are readily identified.

In earlier published accounts both germinal and somatic cells in the germarium and the developing egg chambers have been described (THEUNISSEN, 1973a, b). Effect of temperature on the rate of egg chamber formation has also been investigated (THEUNISSEN, 1974).

3.6.2.1. Structural organization of the young adult ovary

The young adult polytrophic ovary of *H. antiqua* contains a varying number of *ovarioles* (23.4 ± 3.9, $n = 293$). Each ovariole is connected to the *lateral oviduct* by the *pedicel.* At the time of emergence the first and second egg chambers are already in developmental stages S3 and S1 respectively (THEUNISSEN,

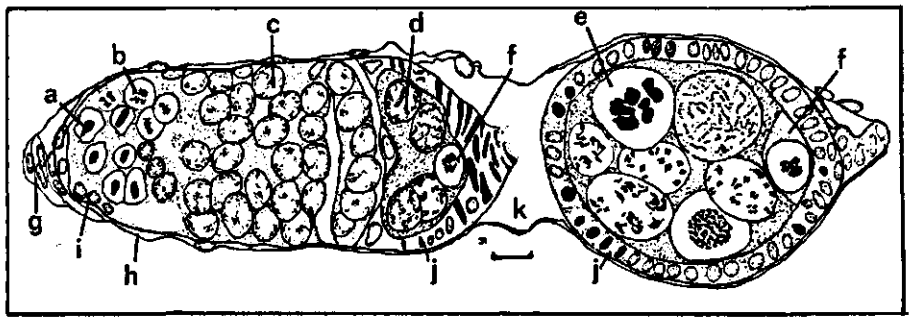


FIG. 75. Semi-schematic drawing of an ovariole of a just emerged female.

Germinal elements:
a. 'concentrated' oogonia
b. dividing oogonia
c. 'diffuse' oogonia
d. trophocytes in S1 egg chamber
e. trophocytes in S3 egg chamber
f. oocytes in S1 and S3 egg chamber

Somatic elements:
g. terminal filament
h. ovariole sheath
i. germarial epithelium
j. follicular epithelium
k. interfollicular space.

1973a, 1974, paragraph 3.6.3.3.) Hence each ovariole consists of a *germarium*, two *egg chambers* and the *covering somatic tissue*.

The germarium is an ovoid mass of cells of both germinal and somatic origin (fig. 75). Somatic cells are mainly situated at the periphery of the germinal cell populations. They form an enveloping structure for the germinal cells within and at the outside of the germarium. This structure shapes the germarium and is a covering of simple epithelium at its surface. The cells show relatively darkly staining, small nuclei of various shapes. Concentrations of somatic cells are absent in the germarium. They are present in the *terminal filament*, the *follicle epithelium*, the *interfollicular tissue* and the *pedicel*.

The germinal cells are situated in more or less distinct populations. These are usually more defined basally than apically in the germarium, because of the formation of very thin epithelia around the basal cell groups. The nuclei of the epithelial cells concerned are mostly situated laterally. A more detailed description of the germarium and the S1 and S3 egg chambers in the young adult ovary has been given earlier (THEUNISSEN, 1973 a, b).

The envelope of the ovariole consists of a squamous *epithelial sheath* with nuclei which are widely dispersed and a very thin outer layer of perhaps fibrillar material. This layer is rarely clearly distinguished and sometimes seems to contain nuclei.

The entire ovary is covered by a thin *peritoneal sheath* of squamous epithelium cells which contain bundles of myofibrils in their cytoplasm. Many tracheal cells are observed associated with the peritoneum.

Discussion

The general structure of the germarium of *H. antiqua* does not differ essentially from that of *Drosophila melanogaster* (KING, 1970a). As in *D. melanogaster*

a sub-division into three parts may be made when the S1 egg chamber, which is always present in the adult ovary (THEUNISSEN, 1973a), is included. Part 1 is the most apical part which contains 'diffuse' and 'concentrated' oogonia and shows the highest mitotic activity. Part 2 is the most basal part of the germarium which contains 'diffuse' oogonia. Part 3 is the S1 egg chamber. The distinction between germarium and egg chambers which is made in this account, is based on the presence of signs of differentiation into oocyte and trophocytes of cells of a particular cyst. The S1 egg chamber develops in the germarium as the most basally situated cyst of germinal cells which shows the indicated differentiation. At subsequent development the egg chamber is separated from the remaining germarial cell population by squamous epithelium and is finally pinched off from the germarium as an S2 egg chamber (THEUNISSEN, 1973a).

The observations on the covering somatic tissues of the ovary and ovarioles of *H. antiqua* are in general agreement with what has been described for *D. melanogaster* (KING, 1970a). The epithelial sheath which covers the ovariole of *H. antiqua* is observed to contain myofibrils and tracheae, as is the peritoneal sheath which covers the ovary. The presence of contractile elements is also indicated by contraction of living ovarioles in physiological saline.

The nature and exact location of the tunica propria is not quite clear. This term is used to denote the basement membrane of the covering (follicle) epithelium in *Drosophila melanogaster* (CUMMINGS, 1974) or a thin layer between the follicular epithelium and the interior basal lamella of the ovariole wall of *Galleria mellonella* (PRZELECKA and DUTKOWSKI, 1973). A similar observation was made by CRUICKSHANK (1973) who described the tunica propria in *Anagasta kühniella* as: 'the extracellular material found between the outer ovariole sheath and the follicle cells'. Structures as in Fig. 1 of. CRUICKSHANK (1973) have been observed in *H. antiqua* between the germarium and the covering epithelium as well as in the testicular sheath, and interpreted as the basement membranes of the adjacent epithelial cells. Apparently the tunica propria is generally identified with the basement membranes of one or more epithelial layers involved. In this case, therefore, there is no need to use the term tunica propria.

3.6.2.2. Germinal cell types

The number of germinal cell types in the ovary is far less when compared to the testis:

1. oogonia
2. oocytes
3. trophocytes.

3.6.2.2.1. Oogonia

Oogonia are already present in the young larval ovary and are found in the young adult ovary still showing the same appearance, which has been described earlier (THEUNISSEN, 1973a). This appearance is identical to that of 'diffuse' spermatogonia. Oogonia also show nuclei with more or less concentrated chromatin exactly as has been described for 'concentrated' primary spermato-

gonia and secondary spermatogonia. It is therefore proposed to name these types of oogonia 'diffuse' and 'concentrated' oogonia respectively, in order to express the morphological similarity between spermatogonial and oogonial cell types.

Within the germarium a clear spatial distribution of 'diffuse' and 'concentrated' oogonial types is found, which is most clear in late pupal ovaries. The 'diffuse' type is situated in groups of cells in roughly the basal half of the germarium. 'Concentrated' cell types are predominant in the apical part near the terminal filament (fig. 75). In this part of the germarium mitotic activity of germinal cells is observed. Both the presence of 'concentrated' cell types and the mitotic activity independently vary individually in a considerable measure.

The 'concentrated' oogonia have a more individual appearance. Mitotic divisions take place in individual cells, although some neighbouring cells may divide simultaneously. Absence of an epithelial enclosure determines the individuality of the cells. Between the regions of 'concentrated' and 'diffuse' oogonia cavities are sometimes found. Their significance is obscure although they might be analogous to the testicular apical cavities.

Discussion

Oogonia in groups were named 'cystocytes' by KING (1970a) because they are arranged in clusters of 2, 4, 8 or 16 cells. The 'cystoblast', one of both daughter cells of an initial quasi-dichotomous division, gives rise to the consecutive generations of 'cystocytes' till the number of 16 has been reached. This number varies according to the insect species involved (BONHAG, 1958; DE WILDE and DE LOOF, 1973). The term 'cystocytes' suggests the existence of a different germinal cell type instead of denoting only the mutual spatial arrangement caused by intercellular bridges. Moreover, the name 'cystocytes' has already been given to a type of hemocytes (JONES, 1962). As possible confusion in the terminology of gametogenesis should be avoided it seems to be appropriate to refer to the encysted oogonia as: 'definitive oogonia' to stress the basic similarity of the oogonial and spermatogonial multiplication processes. The 'cystoblast' of KING (1970a) is the 'initial definitive oogonium' and the result of the initial quasi-dichotomous division of a 'predefinitive oogonium' called 'stem cell' by KING (1970a) in *Drosophila*. Using the terms 'definitive' and 'predefinitive' oogonia in *H. antiqua* a parellel is drawn with analogous events during spermatogenesis. Apart from these physiological indications the morphological distinction is expressed by the terms 'diffuse' and 'concentrated' oogonia which show an identical appearance when compared to 'diffuse' and 'concentrated' spermatogonia respectively and which are morphological types of primary and secondary oogonia. These are characterized by their individuality and encystment respectively since they do not seem to differ in chromatin pattern and other features of nuclear morphology and may be sharing partially the same position in the apical part of the germarium. Hence, the designation primary/secondary oogonia is virtually identical with predefinitive/definitive oogonia. Preceding the period of maximal numbers of 'concentrated' gonial cell types morphologically

identical 'diffuse' gonial cells are observed in testis and ovary.

From these observations a picture emerges of morphologically identical gonial populations in testes and ovaries, which both produce temporarily 'concentrated' types during certain periods of multiplication to return to the 'diffuse' types when these periods have finished. In spermatocytes the multiplication period ends when primary spermatocytes have been formed, in oogenesis when the trophocytes and the oocyte have been enclosed and differentiated to form a young egg chamber. As the egg production obviously requires considerable efforts, special 'concentrated' forms of the trophocytes continue to appear due to endomitotic processes (THEUNISSEN, 1973b).

3.6.2.2.2. Oocytes

Oocytes are derived from definitive oogonia and are formed after the differential oogonial mitosis (VERHEIN, 1921; BONHAG, 1958) when from the resulting cells trophocytes and oocytes differentiate. In the young adult ovary the oocytes of the first and second egg chamber are readily identified by their appearance and position. Their nucleus, which at a later stage of yolk production is also named: 'germinal vesicle' (BONHAG, 1958), is ovoid-spheroidal with a largest diameter $\geqslant 10\,\mu$. The chromatin is organized in a number of flocculate-rounded concretions which are concentrated at one site in the nucleus (fig. 75). This appearance is essentially maintained during egg chamber development. The cytoplasm is more even and lighter stained when compared to the trophocytes. This difference in staining properties increases during egg chamber development when yolk is formed. The oocyte always takes the most basal position in the egg chamber (fig. 75).

3.6.2.2.3. Trophocytes

Trophocytes differentiate simultaneously with oocytes. In *H. antiqua* an egg chamber contains 15 trophocytes, sometimes called nurse cells, and one oocyte. The trophocytes show a clear sequence of chromatin transformations (THEUNISSEN, 1973b) indicating endomitotic processes; as they take place during egg chamber development they will be discussed and depicted elsewhere (3.6.3.3.). Trophocyte nuclei are ovoid-spheroidal with variable dimensions depending on the development of the egg chamber concerned. The cells are mutually connected by cytoplasmic bridges. In the egg chamber the most proximal trophocytes are connected with the oocyte in the same way.

Discussion

Intercellular connections have been observed by VERHEIN (1921) in muscoid flies. KING (1970a) and co-workers investigated this subject in detail in *Drosophila melanogaster*. The physiological and spatial relationships of trophocytes and oocyte have been discussed extensively by TELFER (1975). The presence of cytoplasmic bridges both in cysts of male germinal cells and in young egg chambers shows another similarity between spermatogenesis and oogenesis. In both cases a synchronous multiplication results in development of a cell family which

is in fact a syncytium. This cell family is in this study named 'cyst' until differentiation of the cells into oocyte and trophocytes becomes apparent. At that moment these cells and their enveloping follicle epithelium are referred to as an 'egg chamber".

3.6.2.3. Somatic cell types

All somatic cells in the young adult ovary originate from the mesodermal cells which envelop the young larval gonad. They may be distinguished according to their function:

1. cells of the ovariole sheath
2. germarial epithelium cells
3. follicular epithelium cells
4. border cells.

These cell types are rarely characterized by their appearance, but more frequently by their position in the ovariole. Their shape differs from a very flattened squamous epithelium to a cubical/columnar epithelium. The nuclei are all of about the same size but the shape may differ. In some types the pattern of chromatin appearance and dispersion may vary. The degree of mitotic activity is variable too, both between and within the cell types.

FIG. 76. Two S1 egg chambers surrounded by epithelia which mainly consist of 'type b' cells in a newly emerged fly. Parts of adjacent germaria illustrate the morphological contrast with S1 egg chambers. Note in trophocyte nucleus (arrow) of S3 egg chamber a chromatin pattern which conforms the one depicted in fig. 83, S3, 3. Bar = 10 μ.

3.6.2.3.1. Cells of the ovariole sheath

The epithelial sheath covering the germarium and egg chambers tightly fits around the germarium and the lateral sides of the egg chambers but leaves wide interfollicular spaces between adjacent egg chambers (figs. 75, 76). The ultrastructure of the thin squamous epithelium shows bundles of myofibrils dispersed in the cytoplasm and a structureless basement membrane. Flattened nuclei with regular chromatin dispersion are observed widely dispersed in the epithelium.

3.6.2.3.2. Germarial epithelium cells

These are the somatic cells which provide the internal structure of the germarium and the flow of future follicle epithelium cells. They are dispersed but usually found laterally in the germarium with nuclei of varying size and shape showing regularly dispersed chromatin. Cell limits are mostly indistinct. Some cells with often triangular nuclei seem to form thin membranous projections between and around cells and groups of cells, thus creating a kind of framework in which germinal cell populations develop. Prefollicular cells, being future follicle epithelium cells, are rarely distinguished as such. In *H. antiqua* they are not present in groups as in the ovaries of some insects (BONHAG, 1958). Occasionally prefollicular cells may be recognized because of their association with groups of 'diffuse' oogonia in the basal germarial region, but usually they are morphologically identical with other germarial somatic cells.

3.6.2.3.3. Follicular epithelium cells

The first definitive or initial definitive oogonial division is the division of the initial definitive oogonium, KING's (1970a) 'cystoblast', which results in a cyst of two definitive oogonia. At the time of the first definitive oogonial division the initial definitive oogonia become associated with somatic cells which surround these germinal cells with a thin squamous epithelium. After 'differential oogonial mitosis' (BONHAG, 1958) the differentiation of trophocytes and oocyte is accompanied by the growth of the epithelium around the young egg chamber. The egg chamber seems to be pinched off from the germarium by inward infiltration of cytoplasmic projections of the squamous epithelium cells, whose nuclei usually remain laterally located. At the basal side of the egg chamber the follicular epithelium becomes more cubical or the flattened cells are stacked in a characteristic way (figs. 75, 76). The chromatin in many follicle cell nuclei of young developing egg chambers may be concentrated to a varying degree instead of being regularly dispersed (fig. 76). The former type has been described earlier and named 'type b'. (THEUNISSEN, 1973a) contrary to 'type a' denoting somatic cell nuclei with a regular chromatin dispersion. 'Type b' epithelial cells are found in large numbers during the first stages of egg chamber development (THEUNISSEN, 1973a). 'Type b' follicle cells have already been observed by VERHEIN (1921). The follicle epithelium cells may be squamous, cubical or columnar in shape depending on their position in the follicle and the stage of development of the egg chamber.

Between egg chambers cells of follicle epithelium may be piled up in a strand of single cells connecting two adjacent egg chambers. This 'interfollicular tissue' is also named 'follicular stalk' (KING, 1970a). 'Lumen cells' (KING, 1970a) have not been found in *H. antiqua.*

3.6.2.3.4. Border cells

Although border cells are not yet present in the young adult ovary they might be mentioned here because of their follicular origin. They have been observed in the apical part of the egg chamber as epithelium cells infiltrating between trophocytes. They show both 'type a' and 'type b' cells and have been described and discussed earlier for *H. antiqua* (THEUNISSEN, 1973a).

An apical cell (GRÜNBERG, 1903) has not been observed in the ovary of *H. antiqua.*

3.6.3. *Ovarian development*

In particular in oogenesis the term 'development' is used to denote different processes. Ovarian development as understood in this paper refers to the ontogenetic development of the ovaries during the larval, pupal and adult stages of the life cycle. In addition to the building of structures the term 'development' is often also used to indicate activities of these structures which involve very considerable changes in volume and appearance. This confusion

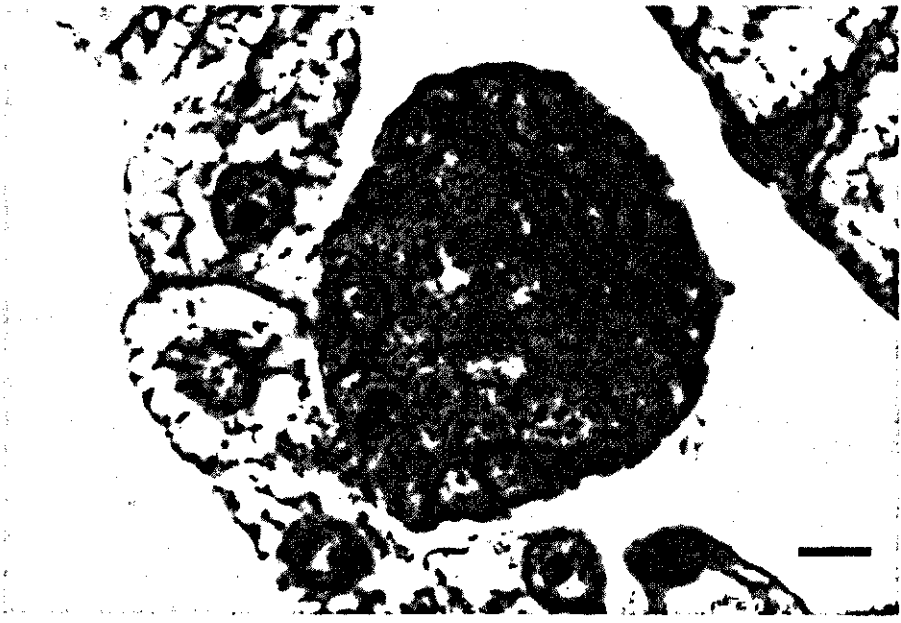


FIG. 77. Ovary of 7 days old L2 larva. Note the cap of developing apical somatic tissue and the 'diffuse', individually dividing oogonia. Bar = 10 μ.

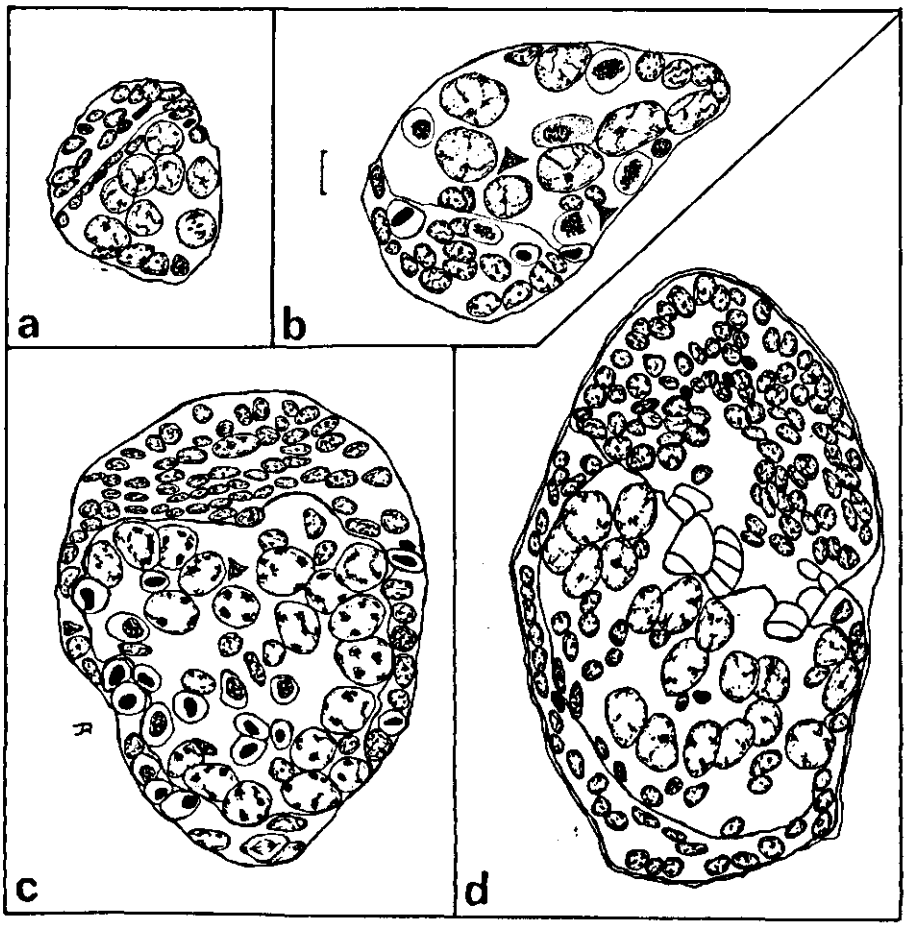


FIG. 78. Development of larval ovaries at different ages:
a. 7 days, b. 10 days, c. 17 days, d. 21 days.
Note the position of the oogonia and the development of the apical somatic tissue. Bar = 10 μ.

is most obvious in vitellogenesis, which is essentially an iterative process during which developmental information is stored rather than utilized.

3.6.3.1. Larval development

6–7 days: At this age when the larva has reached the L2 stage, larval ovaries can be identified as such. The criterium is the formation of a cap-like covering of somatic cells over the apical part of the gonad. This is the beginning of the '*apical somatic tissue*'. The ovary is spheroidal-ovoid and consists of two parts (figs. 77 and 78a):

1. the *apical somatic tissue*, which consists at this age of a more or less loose concentration of somatic cells at the periphery of the gonad. Gradually a

distinct border is formed between this mass of cells and the other part of the ovary by flattening of somatic cells along the borderline. The mitotic activity increases when compared to younger gonads and 'type b' nuclei appear among the predominant 'type a' cells.

2. a *mixed population* of 'diffuse' oogonia and somatic 'type a' cells. Both germinal and somatic cells show mitotic activity. This part of the ovary is less densely populated than the other one.

Both parts are surrounded by a layer of 'type a' somatic cells. The oviduct has already been formed but is not yet connected to the ovary.

10 days: Ovaries of L2/L3 larvae show a more pronounced composition of the two parts with a clear border of flattened cells between them. The apical somatic tissue contains many 'type b' cells as does the covering layer of cells. Among the 'diffuse' oogonia sometimes 'concentrated' oogonial types are observed (fig. 78b).

14 days: The ovaries begin to show a differentiation into three parts:

1. the *apical somatic tissue*, in which 'type b' cells are now often predominant. At the periphery cells are observed which are less flattened when compared to cells along the border with the mixed population. The mitotic activity has increased considerably.
2. a *mixed population* of oogonia and somatic cells. The number of 'concentrated' oogonia increases, as well as the number of dividing germinal and somatic cells. Gradually this region has been closed in at this time by two masses of somatic cells: the apical somatic tissue and
3. the *basal somatic tissue* consisting of a mass of somatic cells which has merged with the covering layer of cells, thus engulfing the mixed cell population. This basal somatic tissue originates from isolated, basally located, small concentrations of somatic cells in the mixed population which have proliferated and merged into one, quickly expanding population of somatic cells. As the somatic cells of both the mixed population and the basal somatic tissue are predominantly showing 'type b', the border between these parts may be locally indistinct.

17 days: In the ovaries a consolidation of the division into three parts takes place. The border of the apical somatic tissue and the mixed, and now centrally located, population is very distinct because of the mass of flattened somatic 'type a' cells which are crowding in the apical somatic tissue contrasting with the relatively loose spatial arrangement of the large oogonia mixed with smaller and darker somatic cells. The basal somatic tissue encloses the central, mixed population which does not seem to expand further, contrary to both the apical and basal somatic tissue. The oogonia show many 'concentrated' types. Both in germinal and somatic cells a moderate mitotic activity is observed. The number of 'type b' somatic cells decreases clearly relative to 'type a' cells (fig. 78a).

21 days: At a rearing temperature of 18°C the larvae are 'mature' i.e. able to enter the stage of 'post-feeding larva' (FRAENKEL and BHASKARAN, 1973) and subsequent stages leading towards pupation. The ovary at this stage of

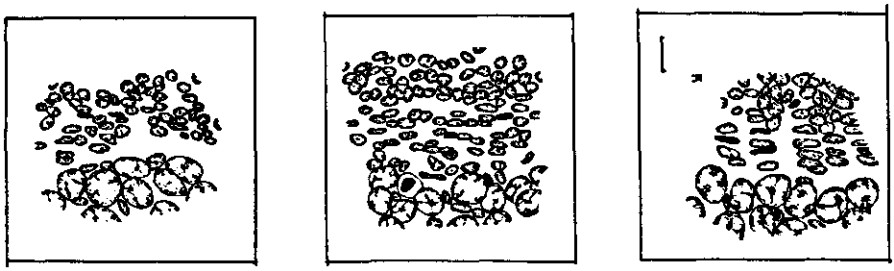


FIG. 79. Beginning of differentiation of terminal filaments in larval ovary. Apical somatic tissue (top) and mixed population of oogonia and somatic cells (below).
Explanation in the text.

development is clearly divided into the three described parts. A very conspicuous feature is the differentiation of the terminal filaments which will be described below. The 'diffuse' oogonia are again the dominant germinal cells, although some 'concentrated' types may be seen. The somatic cells in the entire ovary generally show 'type a' nuclei. A limited mitotic activity is observed in both germinal and somatic cells. The basal somatic tissue covers laterally the central mixed population with a slightly irregular cubical epithelium. This layer of somatic cells, which is still increasing in size, is basally connected with the oviduct. The border with the central, mixed population is often indistinct.

The differentiation of the terminal filaments takes place within a relatively short period of time because in comparable mature larvae many transitory stages are found from which the entire process can be reconstructed. It starts with the formation of a furrow which proceeds from the surface of the ovary inwards along the borderline of the apical somatic tissue till a certain depth has been reached (fig. 78d). Concomitantly, a rearrangement of the somatic cells at the remaining border takes place (fig. 79). The irregularly located flattened cells gradually pile up like coins and they become still more flattened. During this process nuclei seem to disappear from cells and an increasing number of cells is being involved in the rearrangements, resulting in the appearance of a broadening lighter band across the central region of the border of apical somatic tissue and the mixed population of large and light 'diffuse' oogonia (fig. 80a). In this band, neat piles of flat cells appear, some cells with nuclei others without, apparently already forming syncytia. These rows of cells already show the characteristic appearance of terminal filaments and they gradually increase in length, pushing back the border between these two parts of the ovary in a basal direction. The differentiation of the terminal filaments starts in the centre of the borderplane and proceeds laterally until where the furrow separates the apical somatic tissue from the central population containing the oogonia.

This description gives a general outline of the events in the mature larval ovary. Individual variations on this scheme may occur, for instance the borderplane at which the terminal filaments differentiate may not be located medially in the ovary but more laterally (fig. 80b) causing furrows of varying length.

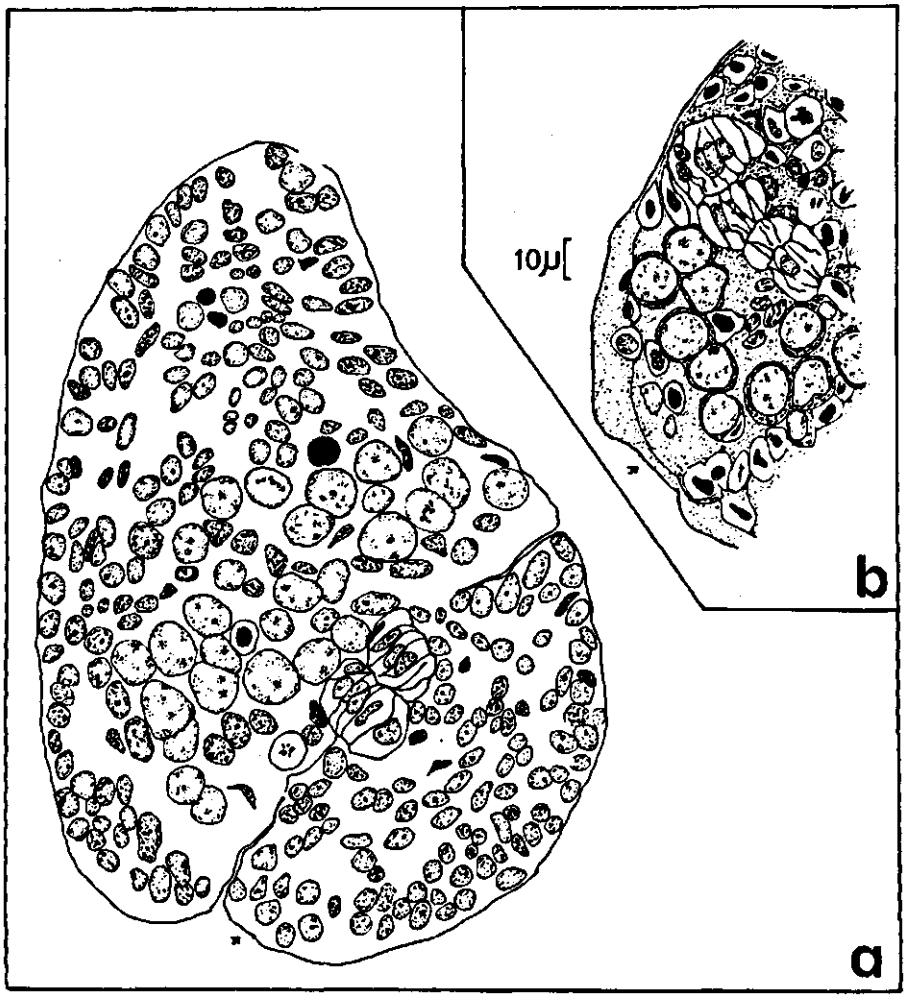


FIG. 80. Ovary of mature larva (a) showing the apical somatic tissue partly separated from the central, mixed population of large oogonia and somatic cells by means of a lateral furrow. Some differentiating terminal filaments are seen at the border of the two populations. The basal somatic tissue is seen at the other side of the mixed population. Detail (b) shows terminal filaments and the large, round oogonia.

Discussion

Very few investigations have been made on larval ovary development in Diptera. VERHEIN (1921) reported a spheroidal shape of the ovaries and the differentiation of terminal filaments, 'Endfaden', in larvae of *Musca vomitoria*. STRASBURGER (1933) described the ovary of nearly mature larvae of *Calliphora erythrocephala*. He recognized oogonia and 'kleine Zellen' and pointed to a discussion in the literature on the nature and origin of the last named category. Strasburger did not make a statement on this matter. A distinct division into

three parts was observed, the middle part containing the large oogonia. In mature larvae the differentiation of the terminal filaments was observed but mistaken for that of the pedicel. According to the accompanying drawings it is beyond doubt that here terminal filaments are going to differentiate from the characteristic flattened cells of apical somatic tissue. In the spheroidal larval ovary of *Mikiola fagi*, MATUSZEWSKI (1962) distinguished two parts: an apical part consisting of dark, small, densely packed somatic cells and a smaller basal part in which the oogonia are located, as did LELOUP (1974) in *Calliphora erythrocephala*. In larval ovaries of *Oscinella pusilla*, LAUGÉ and BORDON (1971) found a division into three parts: two populations of somatic cells basally and apically with an intermediate part consisting of a mixed population of oogonia and somatic cells.

The described larval ovarian development in *H. antiqua* fits well into the scarce literature data on other Diptera.

3.6.3.2. Pupal development

Ovarian development during the first 100 hours of pupal life has been closely examined.

The most conspicuous events in pupal ovarian development during the first days are the continued differentiation of the terminal filaments and the enclosure of oogonia into the ovarioles.

0 hours: The description of ovarian development which was given already for mature larvae also applies to pupal ovaries of this age.

12 hours: The only change is a slight increase in the mitotic activity of both germinal and somatic cells. In the ovaries formation of terminal filaments is apparently still taking place. 'Concentrated' types of oogonia are more frequently observed.

36 hours: Distinct changes begin to appear in the ovariole. At that time the terminal filaments are covered by a squamous epithelium, from which cells start to migrate basally into the mixed population of oogonia and somatic cells. They pass the border of this population and the apical somatic tissue from which they originate. The penetrating, 'type b' cells have a membrane-like cytoplasm which separates the cells of the central mixed population (fig. 81a). The infiltrated cells form a basement membrane and a new squamous epithelium from which new 'type b' cells proceed to penetrate the central mixed population until the oogonia are split up in a large number of populations enclosed in the basally extended ovarioles. The latter consist now of two parts:

1. the terminal filament, and
2. the germarium.

48 hours: These processes continue and are nearly completed in some ovaries at this age. Usually the remaining population of not yet enclosed oogonia is very small. Pathological cells which for some reason degenerate during cell division accumulate in cavities at the base of the ovarioles to disappear within a day. Oogonia more frequently show 'concentrated' types and somatic cells are more often of 'type b', but these phenomena are individually very variable.

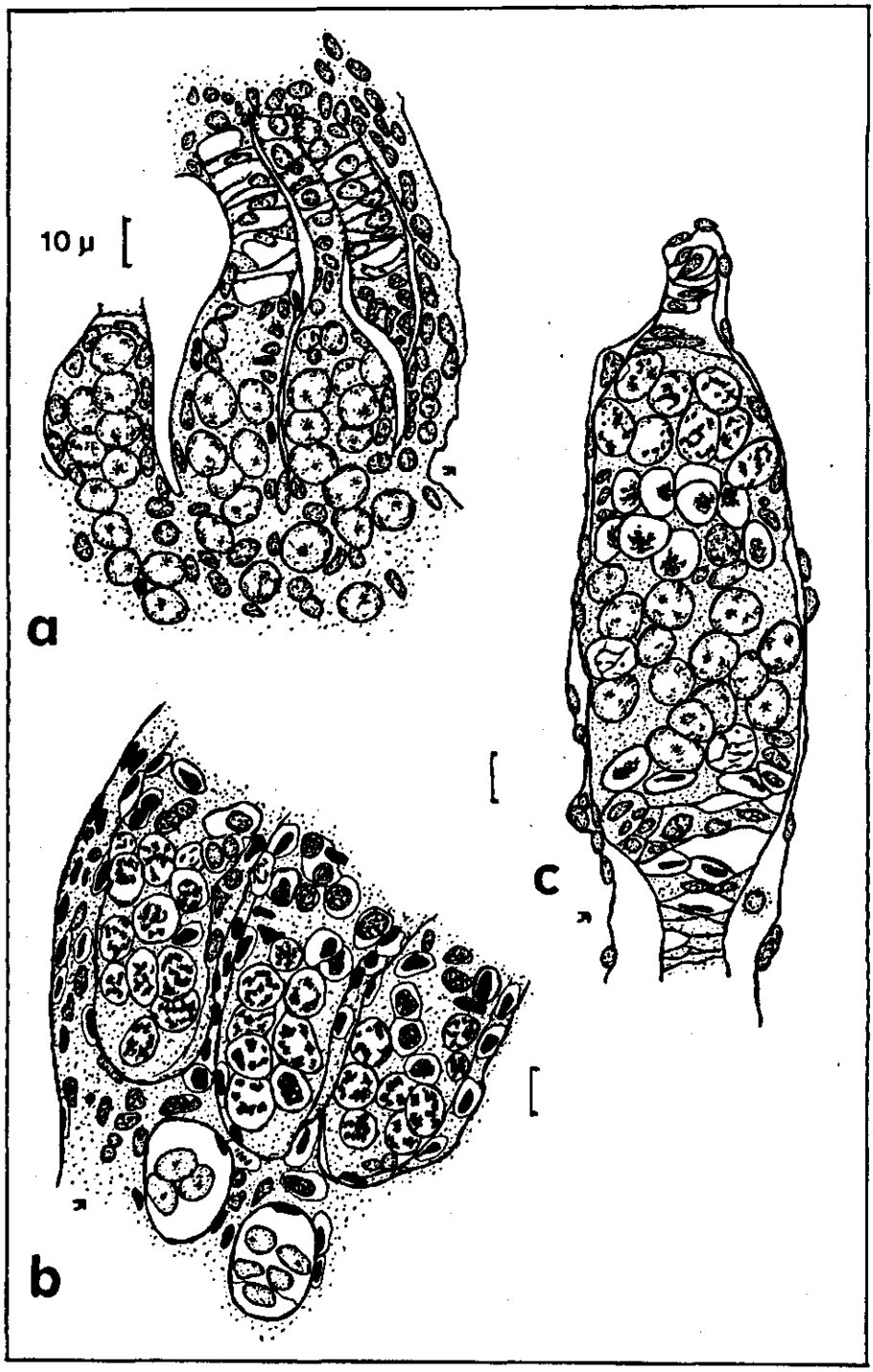


FIG. 81. Development of ovarioles in pupal ovaries of different age: a. 2 days, b. 6 days, c. 11 days.
Note the enclosure of the oogonia in the young pupal ovary and the 'concentrated' types of oogonia in the older ones.

72 hours: All oogonia have been enclosed in ovarioles and covered by epithelium. The number of 'concentrated' types in both oogonia and somatic cells is still slowly increasing. The mitotic activity of the oogonia increases sharply. During the process of enclosure the apical somatic tissue has been reduced to a rather thin covering of the ovary and groups of somatic cells between the ovarioles, in particular between the terminal filaments. The basal somatic tissue is still large. It covers and closes the basis of the ovarioles and forms the future connection with the still massive oviduct.

96 hours: The ovarioles comprise three parts:

1. the *terminal filament*
2. the *germarium*, formed by enclosed oogonia and the covering epithelium, and
3. the *pedicel*, which originates from cells of the basal somatic tissue and is formed by a double row of characteristically stacked cells.

During the described development the entire ovary is covered by a thin epithelial layer with cells of both the apical and basal somatic tissue.

6 days: The ovary is characterized by a considerable developmental activity. The apical and basal somatic tissue is reduced in size when compared to the ovarioles (fig. 82b). The remnants of the apical somatic tissue cover the apical part of the ovary as a thin cap of cells of 'type a', just like those of the terminal filaments. The cells of the basal somatic tissue and the covering epithelium of the ovarioles are frequently of 'type b'. The mitotic activity of the somatic cells is distinctly larger relative to the oogonia. The entire ovary is covered by a 1–3 cells thick layer of epithelium cells of 'type a' and 'type b'.

As for the germinal cells with their large, conspicuous nuclei, a different organization and dispersion of the chromatin is very clear. The appearance of the chromatin is generally much coarser and the dispersion variable from regular to concentrated (fig. 81b). In fact nearly all forms of chromatin organization and dispersion can be observed here which are also found later during S2 and S3 egg chamber development (3.6.3.3.) in trophocyte nuclei. These chromatin patterns strongly suggest endomitotic activity in the oogonia at this stage of development.

11 days: The germaria are well developed. The germinal cell population shows a differentiation in appearance. In the most apical part of the germarium often many oogonia are observed in mitotic division. Basally of these cells many 'concentrated' types of oogonia can usually be found with chromatin in various degrees of concentration (fig. 81c) as well as oogonia showing possible endomitotic activities as was observed in 6 days old pupal ovaries. Sometimes only 'concentrated' oogonia are seen in the apical part of the germarium. Pathological cells may show cytolysis here. In general, uniform 'diffuse' oogonia are found more basally, sometimes showing thin, long chromatin filaments. In some ovaries the first sign of egg chamber development already becomes evident by a coarser chromatin appearance of germinal cells in the most basal group, a stronger stainability of their cytoplasm and the formation of squamous epithelium which separates the group from other germarial cell

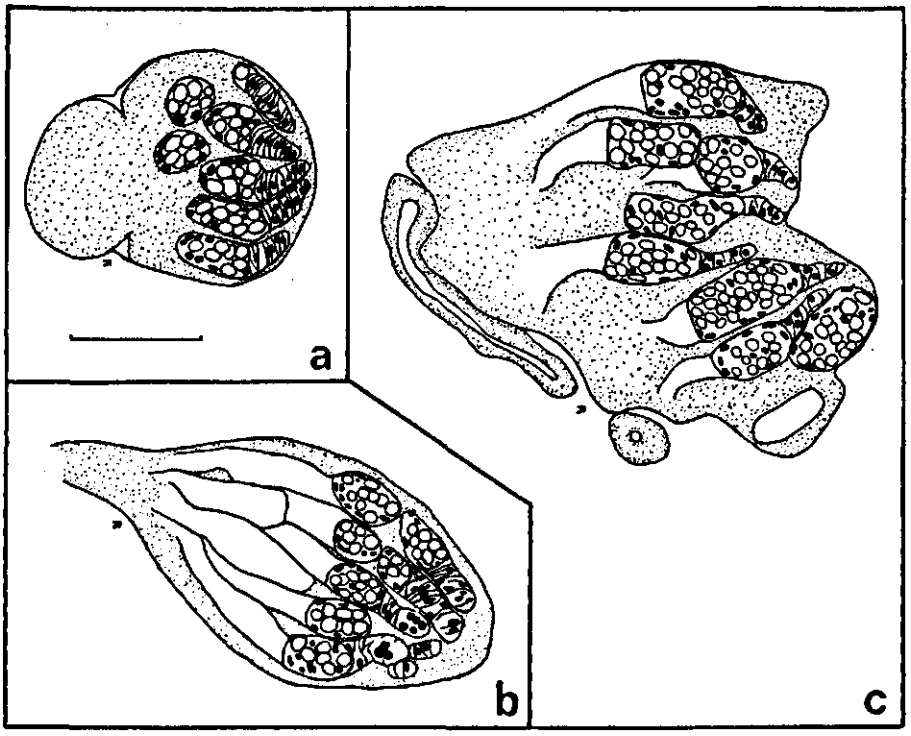


FIG. 82. Semi-schematic picture of relative size of ovarioles and their constituents in the pupal ovary. a: 3 days old ovary, b: 6 days old ovary, c: 11 days old ovary.
Stippled: somatic tissue outside ovarioles; blank space: pedicel, ovarioles; black: somatic nuclei; white: oogonial nuclei. Bar = 100 μ.

groups. The cells showing endomitotic activity are limited here to a certain region within the germarium, closely located near mitotically dividing oogonia. The differences in chromatin pattern presumably reflect differences in physiological activity during the oogonial multiplication and differentiation towards trophocytes and oocytes.

The pedicel is still a strand of flat, stacked epithelial cells connected to the oviduct which now has a tubular shape. The pedicel is about as long as the germarium. Divisions in the somatic cells are much more frequently observed than in germinal cells. Whether or not the relative mitotic activity differs is not known. The epithelium which covers the ovary consists of at least 2 layers of 'type a', 'type b' or both in varying ratios. The shape of the ovary as such is also rather variable.

Discussion

A similar course of pupal ovarian development as has been described presently for *H. antiqua* was already observed in general lines by VERHEIN (1921) in *Musca vomitoria*. KING (1970a) worked it out in detail for *Drosophila melanogaster*. He too recognized the described tripartition of the late larval and pupal

ovary and referred to the cells of the apical somatic tissue as: 'apical cells.' This term should, in our opinion, not be used in this context because it causes confusion with the much older 'apical cell' concept sensu VERSON (1891) and GRÜNBERG (1903), which applies to both ovary and testis. STRASBURGER (1933) reported a similar pupal ovarian development in *Calliphora erythrocephala*. He described and depicted (his Fig. 3b) 'concentrated' and 'diffuse' oogonia and perhaps oogonia with contracted polytene chromosomes. The pupal ovarian development of *Oscinella pusilla* shows the same pattern (LAUGÉ and BORDON, 1971).

An observation which strongly suggests a basic similarity of spermatogonial and oogonial differentiation processes is the striking morphological resemblance of spermatogonia and oogonia, both of 'diffuse' and 'concentrated' types. The apparent conversion of 'diffuse' into 'concentrated' gonial cells and vice versa adds to the impression of a certain unity of pattern in general gonial differentiation during larval, pupal and adult gametogenesis.

Already during larval ovarian development a period has been observed in which 'concentrated' oogonial types are relatively abundant. A similar picture is found during pupal ovarian development. 'Concentrated' oogonia seem to be most abundant about half way during the pupal stage. At that time the 'concentrated' types occupy the larger part of the germarium. During the same period many oogonia show chromatin transformations which are very similar to those found later during early egg chamber development (see 3.6.3.3.) and which are interpreted as being caused by endomitotic activities (THEUNISSEN, 1973b). A possible occurrence of endomitotic divisions in the oogonia during mid-pupal development involves some consequences:

1. Endopolyploidy or polyteny of these oogonia prior to the differential oogonial mitosis involves the necessity of reducing the number of chromosomes in future oocytes to the diploid number at some moment. A chromosome reduction, however, has never been observed in our observations, although such mechanisms are reportedly present in some insect species (WHITE, 1973).
2. Oogonial mitotic divisions after endomitotic chromosomal multiplication should reveal polyteny or polyploidy at metaphase. This has not been observed in dividing oogonia in pupal or adult germaria. Obviously, this matter needs more detailed investigation.

3.6.3.3. Adult development

Ovarian development in the adult stage involves in most insects the formation of eggs from oocytes by means of the nourishing and supporting elements of the ovary.

Egg chamber development in this context means the iterative event of the formation of eggs in adult ovaries. In this process a number of stages are recognized in *H. antiqua* (THEUNISSEN, 1973a). Their characteristics are summarized below (fig. 83):

- Germarium (G): no visible differentiation of oocyte and trophocytes.
- Stage 1 (S1): beginning of separation from the germarium and differentiation

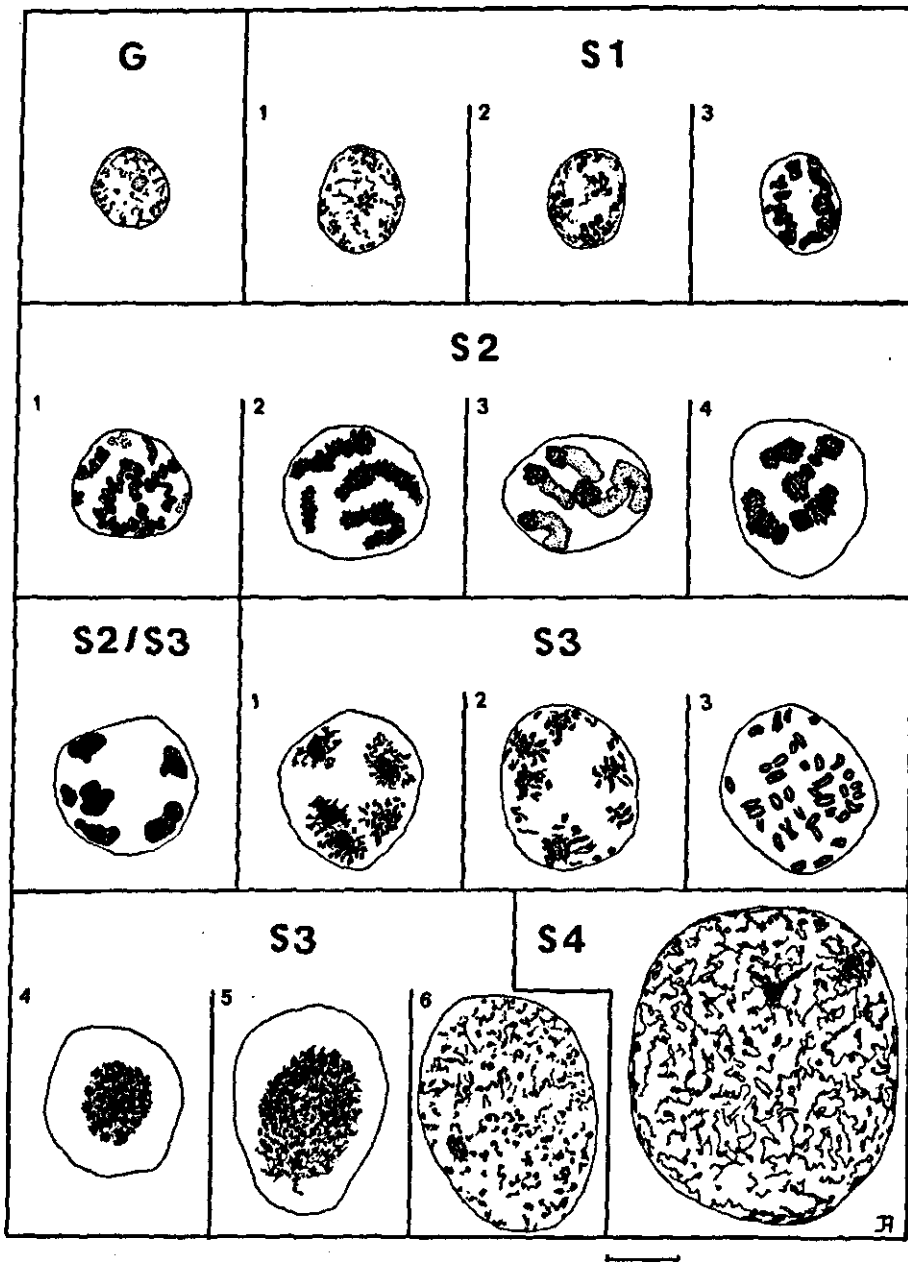


FIG. 83. Chromatin transformations in trophocyte nuclei during egg chamber formation. Changes in size and pattern of chromatin appearance, organization and dispersion are depicted in the sequence which starts in the germarium (G). During the first (S1) and subsequent stages distinct changes are observed concomitantly with other features of egg chamber development. In this schematic drawing the small numbers have been used when necessary to facilitate reference to a particular chromatin pattern.

of germinal cells as compared to germarial ones.
- Stage 2 (S2): young egg chamber separated from the germarium by epithelium; strand and ribbon-like chromatin structures in trophocyte nuclei.
- Stage 3 (S3): conspicuous chromatin transformations in trophocyte nuclei.
- Stage 4 (S4): increasing morphological uniformity of chromatin structures in trophocyte nuclei; beginning of yolk formation.
- Stage 5 (S5): egg chamber distinctly ellipsoidal; oocyte attains the same size as the average trophocyte.
- Stage 6 (S6): oocyte larger than the trophocytes.
- Stage 7 (S7): oocyte $\frac{1}{4}$–$\frac{1}{2}$ of the total egg chamber volume.
- Stage 8 (S8): oocyte $\frac{1}{2}$–$\frac{3}{4}$ of the total egg chamber volume.
- Stage 9 (S9): oocyte larger than $\frac{3}{4}$ of the total egg chamber volume.
- Stage 10 (S10): mature egg.

Chromatin transformations which reveal endomitotic processes have been described in trophocyte nuclei (THEUNISSEN, 1973b). The described chromatin transformations which are most conspicuous in young egg chambers, are depicted in fig. 83. The concentration of the chromatin (fig. 83, S3, 4) following maximal contraction of the polyploid number of chromosomes (figs 83, S3, 3;

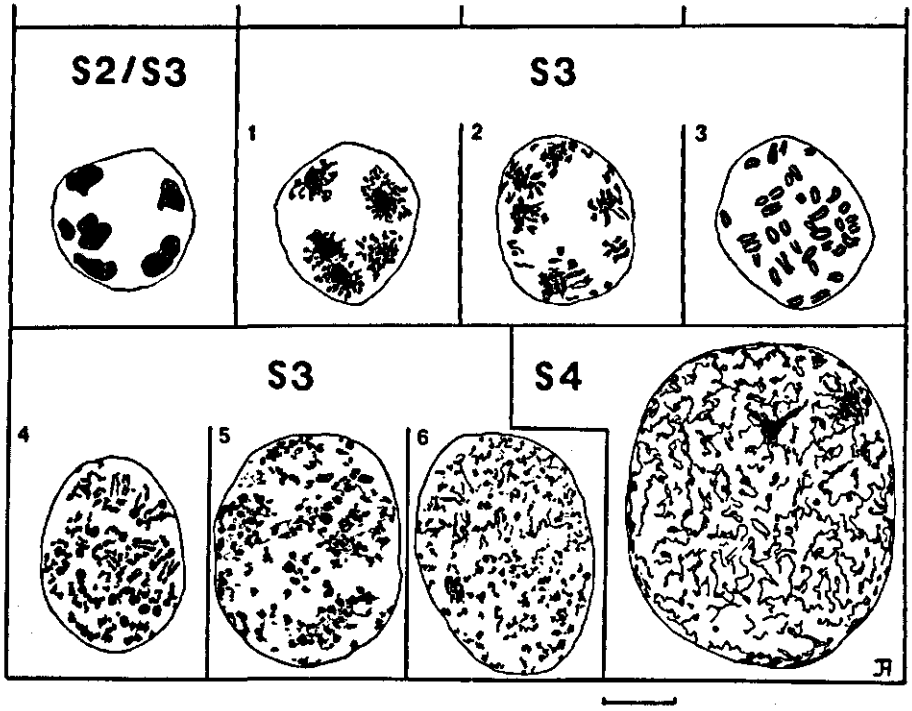


FIG. 84. Slightly different chromatin transformations in nuclei of trophocytes in S3 egg chambers.

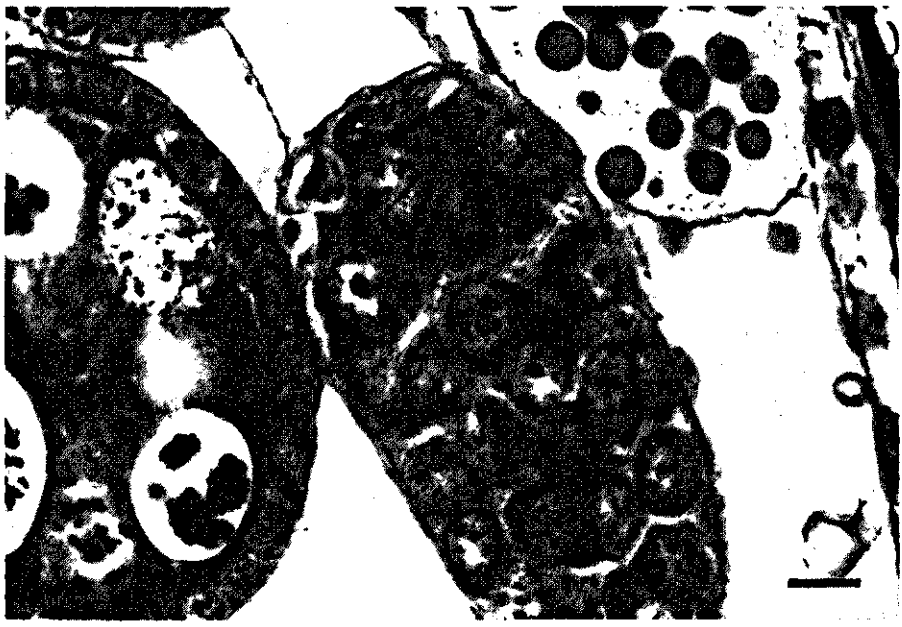


FIG. 85. Germarium. S1 egg chamber and part of S3 egg chamber in ovary of newly emerged fly. The germarium shows here only 'diffuse' oogonia which are separated from the S1 egg chamber by a light zone. Note the various chromatin patterns in the trophocyte nuclei of the S3 egg chamber. Bar = 10 μ.

85) does not always take place. In a very variable but sometimes considerable number of S3 egg chambers the contracted chromosomes seem to despiralize without prior concentration (fig. 84, S3, 4) towards the chromatin appearance which is characteristic for the S4 stage. In irradiated ovaries the latter way is predominant. The formation of consecutive egg chambers within one ovariole in *H. antiqua* has also been discussed including the effect of temperature (THEUNISSEN, 1973a, 1974). According to the limited purpose of this study of oogenesis, observations on other aspects of egg formation for instance chorion or vitelline membrane formation and the role of the follicle epithelium and trophocytes in yolk synthesis, have not been made. A detailed study of vitelline membrane and endochorion formation by the follicular epithelium in *Aedes aegypti* has been published by MATHEW and RAI (1975).

3.6.4. *General discussion*

The general course of oogenesis in *H. antiqua* appears to be rather similar as has been described in other more extensively studied dipteran species, for instance *Drosophila melanogaster*. Ontogenetic ovarian development differs between the various species in speed and possibly in details of ovarian structure. In *H. antiqua* ovarian development is being changed at adult emergence from building of structures into the iterative activity of egg chamber formation. At the

level of the germinal cell population this process is reflected in the signs of endomitotic activity in the trophocyte nuclei. The chromatin pattern of these cells indicates their special physiologic activity (cf. ASHBURGER, 1970).

According to KING (1970a) oogonia in *D. melanogaster* multiply during the larval stage to provide the newly formed germaria with 4–7 oogonia each, which henceforth act as oogonial stem cells. In *H. antiqua* a similar individual oogonial multiplication has been observed in the mixed population of the larval ovary prior to ovariole differentiation in the young pupal stage. In the young adult germarium of *D. melanogaster* (KING, 1970a) only a very small number of stem cells is supposed to be present, the other oogonia being incorporated in cysts. These cysts contain 2^n (maximal 16) oogonia which are mutually connected by intercellular bridges. These oogonia could be referred to as 'definitive oogonia' as opposed to the 'predefinitive oogonia', the individually quasi-dichotomously dividing oogonial stem cells. As far as the observations on *H. antiqua* permit, a similar situation might be found in young adult germaria. In the apical part dividing oogonia are observed among both 'diffuse' and 'concentrated' oogonia. As mitotic activity of germinal cells in the germarium is nearly exclusively found in this part it is supposed that multiplication of both predefinitive and definitive oogonia takes place here. The absence of mitotic activity of 'diffuse' oogonia in the basal part of the germarium might indicate that these cysts have already been completed i.e. contain the maximal number of 16 cells. The future oocyte in *H. antiqua* is not yet recognized and apparently the definitive oogonia within the cyst still react in the same way to stimuli. Since the oocyte is distinguished as such in S1 egg chambers the latter situation has been changed during the onset of differentiation of the cyst into an S1 egg chamber. The differential response of oocyte and trophocytes after this turning point is discussed by TELFER (1975). More detailed investigations of the germarium of *H. antiqua*, in particular its ultrastructure, may reveal the number and position of the predefinitive and definitive oogonia and their mutual physiological and spatial relationship.

When comparing oogenesis and spermatogenesis of *H. antiqua* a striking similarity of a number of features is observed. Morphological identical 'diffuse' and 'concentrated' gonial cells have already been mentioned. Both in testes and in ovaries 'diffuse' gonial cells show an apparent conversion into 'concentrated' gonia and vice versa during certain periods of ontogenetic gonad development in which one of both cell types may be dominant in number. Individually dividing gonia without an observed association with other germinal or somatic cells are found in testis and ovary. These gonia are supposed to represent the stem cells which act as a source of the large number of gametes which is to be produced during the reproductive period of the life cycle. In order to maintain this source, multiplication takes place by means of quasi-dichotomous divisions of gonia which characterize them as predefinitive gonia. These divisions are the first step in the multiplication process which mainly takes place in cysts. The encysted definitive gonia, which are mutually connected by intercellular bridges, behave in a synchronized way until the series of divisions ceases and

the cells prepare for meiosis. At that point differences between the premeiotic germinal cells emerge according to their sex, although speculations on the 'oocyte determinant' which are mentioned by TELFER (1975, pag. 255) suggest a possible extended similarity between spermatocytes and oocytes. If the initial destination of all definitive oogonia is to become oocytes, which is changed at a later moment, a clear similarity with definitive spermatogonia is added.

The above described parallelism in spermatogonial and oogonial processes tempts to compare the male and female germinal cell types as for their relevant properties. In larval gonads primary spermatogonia and oogonia multiply individually. No other germinal cell types are present although both 'diffuse' and 'concentrated' gonial types are observed in changing proportions during gonad development. In gonads of mature larvae and young pupae the first secondary spermatogonia are observed and the oogonia are enclosed in germaria. During pupal development multiplication of gonia and subsequent differentiation takes place. Both in testes and ovaries the populations of predefinitive gonia maintain themselves while producing large numbers of encysted definitive gonia, leading to sperm cell and egg chamber formation respectively at the end of this stage. The secondary definitive spermatogonia multiply in the apical part of the testis, basally of the apical cavities and the primary spermatogonia, which are thought to represent the predefinitive spermatogonia. In the germarium oogonial multiplication is supposed to take place in the apical part of the germarium by both quasi-dichotomous individual and dichotomous mostly synchronous cell divisions. More basally cysts of 'diffuse' oogonia may represent completed cysts of 16 definitive oogonia. Cysts of primary spermatocytes, which in interphase always show the 'diffuse' type, may contain a variable number of premeiotic germinal cells. Summarizing, the following premeiotic male and female germinal cell types show comparable positions in the gonial multiplication processes:

1. primary spermatogonia – primary oogonia, being individually dividing stem cells which, when acting as such by means of quasi-dichotomous divisions are classified as predefinitive gonia.
2. secondary spermatogonia – secondary oogonia, being synchronously dividing encysted gonia which are mutually connected by intercellular bridges and are classified as definitive gonia.
3. primary spermatocytes – completed cysts of secondary oogonia, being the last generation of premeiotic germinal cells.

As spermatogenesis and oogenesis show common features it seems to be appropriate to name the germinal cell types involved in such a way as to stress the observed similarities. In our opinion a terminology which differs for spermatogenetic and oogenetic cells and structures should be avoided as much as possible. A more uniform terminology, as has been used in this study, could be most advantageous in the study of gametogenesis.

4. SUMMARY

In the scope of a genetic control research project gametogenesis of the onion fly, *Hylemya antiqua* (Meigen), is studied as a base for investigations on radiation histopathology of the gonads.

Various cytological, histological, electronmicroscopical and autoradiographical methods, including investigation of living male germinal cells, are used.

The gross anatomy of the male and female reproductive systems is simple as compared to other insect species.

In newly hatched larvae the gonads contain on an average 13 germinal cells. Gonads in larvae which are less than 7 days old cannot be distinguished as being male or female. This distinction becomes possible after the apical cell and the apical somatic tissue respectively is formed in the young gonad.

Spermatogenesis is treated in a number of paragraphs dealing with the description and identification of germinal and somatic cell types, the ontogenetic development of the testis, the dynamics of spermatogenesis and aspects of comparative spermatogenesis.

A proper identification of the testicular cell types is considered to be imperative to any correct experimental approach of spermatogenesis. Morphological descriptions of the various germinal and somatic cell types are given accordingly. New elements are the observation of spermatogonial cells which show in their nucleus a varying degree of chromatin concentration. A similar phenomenon is observed in somatic cell types. Testicular cavities namely the apical cavities and the central cavity are described as are the somatic central cavity cells and the basal cells in the testis.

The ontogenetic development shows the structure and function of the testis during the larval, pupal and adult stages of the life cycle. At the end of the larval stage differentiation of primary spermatogonia starts, leading to sperm production which takes place in the late pupa and the young adult. During adulthood the testis function changes from sperm production towards sperm storage.

Based on counts of the number of cells per cyst of germinal cells it is supposed that various systems of spermatogonial multiplication operate in these populations. Primary spermatogonia may be denoted as being 'predefinitive' which indicates their presumed role in the multiplication processes. The number of definitive spermatogonial divisions may vary according to the acting multiplication system. The speed of formation of different spermatogenic cell types is revealed by incorporation of ^{3}H-thymidine in the spermatogonia. Development of a primary spermatogonium into sperm cells lasts about 10 days.

Comparison of mainly morphological features of male germinal cell types in a number of insect species, including *H. antiqua*, indicates possibilities for comparative research of spermatogenesis in various insect species. This could

provide a basis for comparative radiobiological or other experimental investigations on insect spermatogenesis.

Female germinal cell types and somatic cell types of the ovary are described. The ontogenetic development of the ovary shows ovariole differentiation in the young pupal ovary. In late pupal ovaries egg chamber formation begins its iterative course which is continued during adulthood. Egg chamber formation has been divided into ten stages.

Spermatogenesis and oogenesis share a number of features, which suggests a certain similarity of the processes involved.

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6. SAMENVATTING

Sinds 1965 wordt in Nederland onderzoek verricht naar de mogelijkheid de uievlieg, *Hylemya antiqua* (Meigen) te bestrijden volgens een der genetische bestrijdingsmethoden, de 'sterile-insect method'. Een van de vraagstukken die zich daarbij voordoen betreft het in voldoende mate induceren van dominant lethale factoren in het sperma, zonder ongewenste nadelige gevolgen voor het dier te veroorzaken. Toediening van te hoge doses ioniserende straling of van de juiste dosis op een ongeschikt moment in de ontwikkeling der gonaden kan nadelige gevolgen hebben voor de fysieke conditie van het bestraalde, in het veld los te laten insect. Daarom is de gametogenese van de uievlieg, inclusief de ontogenetische ontwikkeling van de geslachtsorganen, bestudeerd als basis voor een onderzoek van de histopathologische stralingseffecten in de gonaden.

Bij het onderzoek van de gametogenese is gebruik gemaakt van een aantal methoden waarvan de histologische en electronen-microscopische de belangrijkste zijn.

In het derde hoofdstuk wordt de gametogenese behandeld. In een aantal inleidende paragrafen worden achtereenvolgens de algemene bouw van het mannelijke en vrouwelijke geslachtsapparaat, het uiterlijke van de chromosomen en van de larvale gonaden in een vroeg stadium vóór sexuele differentiatie kort besproken. Onderzoek van de spermatogenese is alleen goed mogelijk als de daarbij betrokken celtypen correct kunnen worden geïdentificeerd. Daarom is aan de beschrijving van deze celtypen, germinale en somatische, veel aandacht besteed. Vervolgens is de ontwikkeling van de testis in de opeenvolgende stadia van de levenscyclus van het insect beschreven. In het larvale stadium wordt door celvermeerdering de basis gelegd voor het successievelijk ontstaan van de verschillende germinale celtypen gedurende het popstadium. Aan het einde hiervan en in de eerste twee weken van het volwassen stadium wordt de meerderheid van de spermacellen gevormd. Deze cellen worden in het basale deel van de testis, dat als vesicula seminalis functioneert, opgeslagen. In vliegen die ouder dan twee weken zijn, neemt de spermaproductie snel af.

Voor zover de kwantitatieve gegevens toelaten is aan de dynamiek van de spermatogenese aandacht besteed. Verschillende systemen van spermatogoniale vermeerdering lijken naast elkaar en gelijktijdig te kunnen functioneren. Het aantal opeenvolgende spermatogoniale delingen is variabel. Door het merken van spermatogoniën met het radioactieve isotoop tritium in het begin van hun vermeerderingsactiviteit kan de vermeerderingssnelheid van de achtereenvolgende germinale celtypen worden vastgesteld.

Teneinde na te gaan of de identiteit van de geslachtscellen in andere insectesoorten kan worden vastgesteld op basis van de voor de uievlieg ontwikkelde criteria, is een beperkt vergelijkend onderzoek verricht. Hieruit bleek dat de germinale celtypen van de meeste der onderzochte soorten zeer goed te identificeren zijn. Dit gegeven verschaft een fundament voor vergelijkend experimen-

teel onderzoek van de spermatogenese van verschillende insectesoorten.

De oogenese is minder uitgebreid behandeld omdat deze reeds in andere insecten beter en meer diepgaand is bestudeerd dan de spermatogenese. De geslachtscellen en de somatische celtypen worden beschreven, alsmede de ontwikkeling der ovariën in het larve-, pop- en volwassen stadium van de uievlieg. Ook in het vrouwelijke dier wordt in het larvale stadium door celvermeerdering de basis gelegd voor de latere, naar verhouding gigantische, prestaties tijdens de vorming van eieren in het volwassen stadium. Gedurende het popstadium worden de ovariolen gevormd, waardoor later een aantal eieren gelijktijdig tot ontwikkeling kan komen.

Een zekere mate van overeenkomst tussen de bij de spermatogenese en oogenese betrokken processen wordt waargenomen en besproken.

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CURRICULUM VITAE

J. THEUNISSEN
Geboren: 10 april 1937 te Den Haag.
HBS-B: Den Haag en Utrecht, militaire dienst.
Landbouwhogeschool Wageningen: september 1959–september 1966.
Studierichting: Planteziektenkunde.
Hoofdvak: Entomologie.
Keuzevakken: Virologie.
Organische Chemie.
Didaktiek en Pedagogiek.

Na het afstuderen in september 1966 werd de promovendus belast met onderzoek naar de gevolgen van ioniserende straling in de weefsels van de uievlieg, als deelproject van een onderzoek naar de mogelijkheden van genetische bestrijding van deze vlieg door het Instituut voor Plantenziektenkundig Onderzoek. Het histologisch en histopathologisch onderzoek werd voornamelijk uitgevoerd in het Laboratorium voor Entomologie van de Landbouwhogeschool. Andere taken van de promovendus binnen de uievlieg-groep werden in of vanuit het I.P.O. verricht.

Het onderzoek werd financieel mogelijk gemaakt door onderzoekcontracten met Euratom en het International Atomic Energy Agency. Na afsluiting van dit onderzoek is de promovendus begonnen aan onderzoek naar de mogelijkheden van geïntegreerde bestrijding van insecten in de vollegronds groenteteelt.