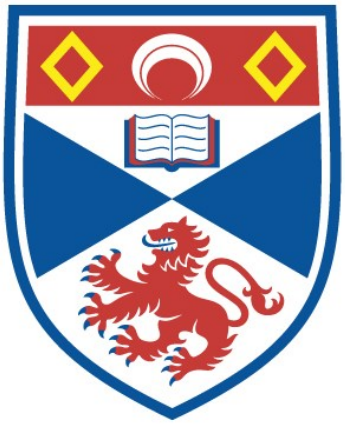
STUDIES ON SPERMIOGENESIS AND THE SPERM OF A PROSOBRANCH GASTROPOD, NUCELLA LAPILLUS (L)

Muriel Helena Walker

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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STUDIES ON SPERMIOGENESIS AND THE SPERM OF A PROSOBRANCH GASTROPOD, *Nucella lapillus* (L).

A Thesis presented for the degree

of

Doctor of Philosophy

to

The University of St. Andrews

by

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I.

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DECLARATION

I hereby declare that this thesis is based upon the results of my own work and that, apart from Appendix I, it has been written by me without assistance. Dr. M.C. Macgregor and I are jointly responsible for Appendix I.

My thesis has not been submitted for any other degree.

MURIEL HELENA WALKER.

CERTIFICATE

I certify that Miss Muriel Helena Walker has spent nine terms at research work on spermatogenesis and the mature sperm of *Mucalia lapillus* (L.), that she has fulfilled the conditions of the Ordinance and regulations and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

27.8.1969.

H.G. CALLAN

UNIVERSITY CAREER AND RESEARCH EXPERIENCE

I entered the University of St. Andrews in October 1962 and was awarded a II (a) Honours B.Sc. in Zoology in June of 1966. During my last year as an undergraduate student I began my work on the spermiogenesis of Mucella lapillus (L), and at the end of that year I wrote a thesis entitled A Study of Spermatogenesis and the structure of the mature sperm of Mucella (Purpura) lapillus (L).

In September 1966 I was admitted as a research student in the University of St. Andrews under Ordinance General No. 12 and as a candidate for the degree of Ph.D. under Ordinance No. 88. Since September 1966 I have completed my study on spermatogenesis in Mucella and have been occupied with two main problems. First, the coiling phenomenon shown by the mature sperm and, secondly, the arrangement of the nucleoprotein within the mature sperm. The up-to-date results of my researches are presented here as a thesis for the degree of Doctor of Philosophy.

ACKNOWLEDGMENTS

It is a great pleasure to express my most sincere thanks to all those people with whom I have discussed many aspects of my research. I am indebted to Professor H.G. Callan whose preliminary investigations into the coiling phenomenon of the mature sperm head of *Mucella* at the light microscope level in 1952 provided a starting point for my studies, also for his continued interest in my work during the past three years, to Dr. C. Muir (Zoology Department, St. Andrews) for his criticism and assistance with technical detail, to Dr. P.E. Lake (Poultry Research Centre, Edinburgh) for providing me with fertile cockerels for the final stages of this work, the technical staff of the Zoology Department, St. Andrews for all their assistance, Miss M. Monserieff for typing the text and to Mr. J.B. Mackie (Zoology Department, St. Andrews) who has, together with Dr. H.C. Macgregor trained me as an electron microscopist and whose technical assistance has been invaluable. I should also like to thank Mr. Mackie for his permission to include Figs. 124-129.

Above all, I wish to express my most sincere and warmest thanks to Dr. H.C. Macgregor for introducing me to a fascinating and rewarding line of research. I should like to thank Dr. Macgregor for his continued interest in my work, his criticism and generous guidance, and above all, for setting me the example of his own high standard of research which has been a constant source of inspiration.

It has been my very great pleasure to have been a research student of Dr. Macgregor and I feel that his training and example will prove invaluable to me in the future.

I N D E X

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INTRODUCTION

The work in this thesis covers three aspects of a molluscan sperm. The animal used throughout for these investigations was a prosobranch gastropod, the dog whelk, Nucella (Purpura) lapillus L.

The initial reason for my involvement with the sperm of Nucella was curiosity about the "Zentral-faden" described by Retzius (1912), which runs through the head of the mature sperm. Retzius described how, under certain conditions, the "Zentral-faden" became twisted into a spiral. I thought that the "Zentral-faden" running right through the sperm head indicated that spermatogenesis in this mollusc might show some unusual features. As a research project in my final (Honours) year for the degree of B.Sc. I began a study of spermatogenesis in Nucella using histological techniques at the light microscope level followed by a study of fine structure with the electron microscope. During my first year as a post-graduate student I continued this study and wrote up part of the work which was subsequently published; Walker and Macgregor (1968). (A typescript of this paper is included as Appendix I.).

Unpublished observations on the behaviour of the mature sperm of Nucella were made by Callan (1952). He described changes seen under phase contrast in sperm immersed in different media. He remarked upon the fact that in x 2 concentrated sea water the heads

of the living sperm show a tendency to become attached to the surface of a slide, and that when they do the nucleoprotein of the sperm head disperses and the head filament springs back to form a more or less tight spiral consisting of 6 or 7 turns in a clockwise direction from the front end. These observations made by Callan and also those made by Retzius were the basis for the second part of this study, an investigation into the coiling phenomenon shown in the mature sperm head of Nucella. I carried out various experiments to see by what means coiling of the head filament could be induced, and then looked at the state of the nucleoprotein and the head filament of coiled sperm heads with the electron microscope to try and find an explanation of the coiling phenomenon.

From observations on the state of the nucleoprotein during the experimental treatments of sperm in the second phase of this study, I decided that this could be a means of obtaining some knowledge of the arrangement of the nucleoprotein within the mature sperm head. It was exploitation of these observations which was the starting point for the final phase of this work, a study of the arrangement of the nucleoprotein in the sperm head of Nucella.

From these studies several striking features of Nucella sperm have emerged. The centriole penetrates to the anterior end of the nucleus with the flagellar shaft ("Zentral-faden" of Retzius) running through the head and extending backwards to form the tail.

The condensation of the nucleoprotein during spermiogenesis shows a well defined lamellar pattern. Microtubules are present during the final stages of spermiogenesis in the testis and are subsequently absent from the mature sperm in the testicular duct. The flagellar shaft in the head of the mature sperm shows the coiling phenomenon described by Retzius (1912) and Callan (1952). I felt that exploitation of these more striking features could possibly lead to a better understanding of microtubules, nucleoprotein condensation, the mechanism of nuclear elongation and the arrangement of nucleoprotein in the mature sperm head. It is these features that will be the main points for discussion. As an introduction to these topics, I feel that it is pertinent to review the information at present available concerning microtubules and the condensation and arrangement of nucleoprotein in sperm heads.

Review of observations on microtubules

The present interest in microtubules comes mainly from the apparent almost ubiquitous occurrence of such intracellular structures in plant and animal cells. Prior to 1963 the usual fixative for material for electron microscopy was a 1% buffered solution of osmium tetroxide, usually used at 4° C. With this fixation there were a few reports of microtubules, but their fixation on the whole was poor, and scant attention was paid to their significance or possible function. The introduction of glutaraldehyde as a fixative

(Sabatini and others, 1963) has led to the realisation that microtubules are uniformly encountered in many different types of cell. It was recognised that cytoplasmic microtubules are a particularly labile cell component and tend to be destroyed by osmium alone but not by glutaraldehyde followed by osmium tetroxide.

Microtubules are at least several microns in length, they are straight and uniform along their length usually showing no evidence of branching or blebbing. Instances of branched microtubules have been reported by Sandborn and others (1964). Microtubules vary in outer diameter between 150-300 Å, and have a dense wall ranging from 40-70 Å in thickness and a center of low density, which gives them a hollow appearance. There are many reports of configurations of microtubules closely packed in parallel arrays. The microtubules of such arrays according to Ledbetter and Porter (1963) are usually separated by a particle free zone. Cytoplasmic microtubules, mitotic spindle fibers and flagellar fibers are considered to be homologous structures (Porter and others, 1964).

Studies on flagellar fibers (Pease, 1963; André and Thiéry, 1963; Grimstone and Klug, 1966), microtubules (Gall, 1966a; Ledbetter and Porter, 1964) and mitotic spindle fibers (Harnicot, 1966) have shown that all these structures are composed of similar subunits. Each fiber of the 9 + 2 of the flagellar shaft was described by Pease (1963) to be a cylinder with a hollow core. He stated that

each of these cylinders was made up of 10 longitudinally oriented filaments. These filaments had a marked beaded appearance, with a repeating period of 88 Å. The filament thickness (bead width) was 35–40 Å. Beads of neighbouring filaments were in register with each other so that cross linking between the filaments completed the wall structure of each fibril. The centre-to-centre spacing of adjacent filaments was 55–60 Å. Grimstone and Klug (1966) describe the longtudinal repeating unit to be 40 Å, and suggest that there are at least 12 longitudinal subunits present in the flagellar fiber. Ledbetter and Porter (1964) and Gall (1966a) have shown that the microtubule is composed of subunits which, in cross section, are about 13 in number and have centre-to-centre spacings of about 45 Å. Moor (1967) has put forward a model for the substructure of microtubules. He suggests that the globular monomers are arranged as protofibrils when viewed longitudinally and as a helical chain when viewed transversely.

The tail sheath of the T2 bacteriophage virus is contractile and consists essentially of a hollow cylinder. Electron microscopy of negative-stained tail sheaths from these viruses (Brenner and others, 1959) has shown that the contracted sheath has a length of 350 Å, a diameter of 250 Å and central hole 120 Å in diameter. In the relaxed state, the same sheath has a length of about 800 Å, a diameter of 165 Å, and a central hole of about 70 Å diameter. The

structure of this sheath, as seen with negative staining, is based on approximately 144 spherical subunits about 30-40 Å in diameter and arranged in a helical chain. Each tail sheath contains 42 molecules of ATP, plus about 100 molecules of UTP, GTP and deoxy-ATP, as well as 160 calcium ions (Wahl and Kosloff, 1962). This corresponds closely to one calcium ion and one high energy phosphoryl per protein subunit.

In view of the similarities of substructure between the T2 bacteriophage virus tail sheath and cytoplasmic microtubules, it is significant that this type of structure in the virus carries out an active contraction coupled to the hydrolysis of ATP. There is much evidence that the movements of flagellae and cilia are ATP-dependent (Hoffmann-Berling, 1958; Child, 1961; Gibbons, 1963). It has been suggested that microtubules may perform "undulatory movements" in the cytoplasm, but there has been only one report of ATP being involved. Moses (1966) demonstrated ATPase activity associated with singlet microtubules in the aflagellate sperm of Stentorconcha tuberculatum.

Several different functions have been ascribed to microtubules according to their arrangement, situation, and type of cell in which they are found. The coincidence in distribution and orientation of microtubules in the cortical cytoplasm of plant cells with, respectively, the distribution and direction of streaming cytoplasm has

suggested that the tubules may provide direction and motive force for the streaming (Porter, 1966). The mechanism by which the tubules might provide this force is obscure, although it has been suggested that this might be achieved if the microtubules undulated in a cilia-like motion within the cytoplasm. Sabnis and Jacobs (1967) suggest that the microtubules in the coenocytic marine alga, Caulerpa prolifera, may serve a cytoskeletal function in cellular differentiation and serve to provide either the actual framework or to delimit areas of cytoplasmic substrate upon which the motive force responsible for streaming is generated. They do, however, add that evidence for this is only indirect.

The involvement of microtubules in cytoplasmic streaming in animals has also been suggested. The occurrence of varying sizes of microfibrils or microtubules together with evidence for their involvement in amoeboid movement has been reported (Danneel, 1964; Dugas and Bath, 1962; Komnick and Wohlfarth-Bottermann, 1965). DuPraw (1968) has suggested, with respect to pseudopod formation, that net locomotion in cells showing amoeboid movement may depend on the assembly of new cytoplasmic microtubules in the anterior part of the cell, combined with disassembly of old microtubules in the posterior regions. In this "assembly-disassembly hypothesis" of amoeboid movement the hyaline cap could be considered as a "microtubule precursor solution" forming the zone of assembly.

The possible involvement of microtubules in cell motility is not restricted to amoeboid movement. The axostyle of Saccinobaculus, an endoparasitic flagellate in termites, propagates undulatory waves along its length (Grimstone, 1966). It resembles a flagellum in its movements but does not show the characteristic 9 + 2 pattern of flagellar fibrils. The axostyle is made up of only one type of fiber or microtubule although fine cross connections are present between the tubules. Macgregor and Thomasson (1965) describe a different system where microtubules are involved in cell motility. In the Archigregarines, Selenidium fallax and Ditrypanocystis cirratuli, the pellicle is thrown into a series of folds which run parallel to the long axis of the body. Beneath the cytoplasmic membrane are one or two rows of "sub-pellicular fibrils" which run lengthwise through the pellicular folds. The Archigregarines are active creatures and perform random writhing and bending movements, rhythmically coiling and uncoiling their bodies. Macgregor and Thomasson suggest that the pellicle and "sub-pellicular fibrils" or microtubules together form the mechanism by which these animals move. Other instances where microtubules are present in motile systems include the auxiliary tubules found associated with the peripheral fibers of some flagellae (Phillips, 1969), also the manchettes of microtubules found round the tails of sperm in animals such as the cat (Burgos and Fawcett, 1955) and the domestic fowl (McIntosh and Porter, 1967).

Microtubules have been found to be a regular feature of cells which undergo changes in shape to attain an asymmetric form. Porter and others (1964) feel that microtubules represent cytoskeletal structures associated with the development of asymmetric cell shapes. There are many reports of microtubules present at some stage of the elongation of spermatid nuclei during spermiogenesis (Yasuzumi and Tanaka, 1958; Rebhun, 1957; Kessel, 1966, 1967; McIntosh and Porter, 1967; Hoage and Kessel, 1968). Mucalla is another example of sperm where microtubules are present during spermiogenesis. The actual function of microtubules in this situation is open to much speculation.

Reports of microtubules in sperm are not restricted to the tail manchettes and elongating spermatid nuclei. The mid-piece of Helix which, in the mature sperm, is about 200μ in length is made up of radially arrayed microtubules (Grassé and others, 1956). A similar arrangement has been described in Tentacella heliotidae Drap (André, 1962). In the water strider, Gerris remigis, the acrosome is about 2.5 mm long, which constitutes about half the length of the mature sperm (Tandler and Moriber, 1966). The acrosome is packed with a system of microtubules arranged parallel to its long axis. There is a second system of microtubules in the cytoplasm which lie either parallel to the long axis of the acrosome or are wound round the acrosome in a helical fashion, and which extend backwards to end level with the centriole at the base of the nucleus.

Tandler and Moriber suggest that the tubules within the acrosome give rigidity to the structure and those which surround it add further rigidity to the whole sperm head keeping the nucleus and acrosome in alignment. In addition to mechanical support, they suggest that the cytoplasmic microtubules may act as an intracellular transport system. Such a function for microtubules has been suggested for other cell types by Sandborn and others (1964) and Slautterback (1963). In changing from a spherical to an elongated form the volume of the acrosome in Gerris increases from two to five times. At the same time there is a significant increase in the total protein and polysaccharide content of the acrosome. Since the small rim of cytoplasm which surrounds the acrosome shows no morphological evidence of secretory activity, it is probable that protein and polysaccharide incorporated into the elongating acrosome is present in the cytoplasmic "remnants" in the posterior part of the cell, and is carried along the microtubules to sites of incorporation into the acrosome.

An experimental approach to the possible functions of cytoplasmic microtubules has recently been made by Tilney and others (Tilney and others, 1966; Tilney and Porter, 1965, 1967; Tilney, 1968). They have carried out their experiments on the Heliozoan Actinosphaerium. Actinosphaerium possesses numerous axopodia which radiate from the surface of the cell. Each axopodium has a birefringent core or axoneme, which consists of an ordered array of microtubules. Tilney and Porter have shown that when Actinosphaerium is

subjected to cold treatment, that is, kept at a temperature of 4° C, the axopodia retract and after 2½ hours no axopodia remain. On returning to room temperature the axopodia start to reform with the axonemes within them. Reforming axopodia without axonemes are never found. Sections of material fixed at various stages during cold treatment and recovery show that the retraction of the axopodia is due to the disaggregation of the microtubules of the axonemes. Similar results can be obtained using other anti-mitotic agents such as colchicine or high pressure. During recovery of the axopodia the microtubules reform from the medullary region of the cell in a pattern identical to that of untreated cells. It is evident that the cell membrane alone is not sufficient in itself to support such long cytoplasmic extensions as the axopodia and it is therefore fairly evident that the microtubules of the axonemes give skeletal support to the axopodia. Growth of the microtubules and elongation of the axopodia occur at the same time and it is thought that it is the growth of the microtubules that is responsible for the elongation of the axopodia.

Review of the condensation and arrangement of nuclear protein in sperm heads

There has been a tendency in recent years to pay little attention to the process of nuclear condensation which occurs during spermiogenesis in the majority of sperm. Since the introduction of glutaraldehyde and the apparent frequency of occurrence of microtubules in

spermiogenesis, most reports deal with "microtubules and spermiogenesis" and there is little emphasis on events taking place within the nucleus. Prior to 1963 there are several reports of studies on nuclear changes during spermiogenesis in a number of species. These studies in the invertebrates were carried out mainly on molluscs and insects where there is a considerable change in shape from the spherical early spermatid nucleus, in which the nucleoprotein is granular in appearance, to the elongate mature sperm nucleus which appears homogeneous, in that no fine structure can be seen within it.

There are what I consider to be three basic patterns of nuclear condensation described in these reports. The first pattern I have called the "fibrous type". "Fibrous" condensation is seen in the snails Cipangopaludina (Yasumasu and Tanaka, 1958) and Viviparus (Gall, 1961). In Cipangopaludina the early spermatid nucleus is spherical with a diameter of about 2 μ., and the nucleoprotein has a fine granular appearance. As the nucleus increases in length and decreases in diameter the nuclear contents aggregate into dense fibers or filaments varying in diameter from 100-160 Å. As elongation continues these fibers become twisted and appear to be arranged in a loose helix. The fibers aggregate into thicker structures and their twisting results in the twisting of the whole head. Eventually the fibers fuse together to give a helicoidal head about 15μ in length and homogeneous in appearance.

The second pattern of condensation I have called the "lamellar type". This has been described in the pulmonate snail Otala lactea (Rebhun, 1957). In this instance there is a change in nuclear shape from spherical to flame shaped. The early spermatid nucleus is again granular in appearance, but the nucleprotein aggregates into sheets or lamellae as elongation of the nucleus commences. These plates of nucleoprotein are about 60 Å thick, 100 Å - 1μ wide and several microns in length. The lamellae are orientated with their long axes parallel to the long axis of the sperm head. They are arranged in a regular fashion running radially at the base of the nucleus where the centriole is embedded in the nucleus, or else in hairpin-like loops fusing with the nuclear membrane. As elongation proceeds the nucleus twists, axial symmetry is lost, and the lammellae fuse to give the final homogeneous-appearing nucleus. Other examples of the lamellar type of condensation include the grasshoppers Dissosteira and Melanoplus (Gall and Bjork, 1958), and the locust Locusta migratoria (Gibbons and Bradfield, 1957).

A third pattern of condensation, which I have called the "granular type" is seen in the vertebrates. Examples of this type of condensation include the cat Felis domestica (Burgos and Fawcett, 1955) and the domestic fowl (McIntosh and Porter, 1967). Throughout nuclear elongation the nucleoprotein has a granular appearance and only when the nucleus has achieved its final length does isotropic condensation occur to give the mature nucleus its homogeneous appearance.

The question of how chromosomes are arranged within mature sperm heads where the nuclear material undergoes condensation during spermiogenesis remains unresolved. It is a unique property of DNA-protein fibers to exhibit negative birefringence relative to the long axis of the DNA. In consequence the fact that elongate, thread-like sperm heads are negatively birefringent relative to the sperm axis provides evidence that the DNA is orientated longitudinally and in parallel within the sperm head. X-ray diffraction patterns of squid sperm (which are birefringent) also indicate that the DNA-protein units lie parallel to the axis of the sperm (Wilkins and Randall, 1953). Schmidt (1941) found that the sperm of the snail Paludina showed a clear spiral pattern of negative birefringence, so that in polarised light the sperm head has a striped appearance. This would suggest that the nucleoprotein molecules although parallel, are loosely coiled into a secondary helix. More recently Inoué and Sato (1952, 1966) have invoked a super coiling model for DNA packing in the living sperm of the cave cricket. They have used a technique which is based on the loss of birefringence following polarised UV irradiation. As well as suggesting a super coiling model they also suggest that the chromosomes are arranged in a tandem fashion along the length of the mature sperm. Another study of mature sperm from several Orthoptera has been carried out by MacInnes and Uretz (1968). They have looked at the DNA organisation within the mature sperm head by the method of polarised fluorescence microscopy.

This technique utilizes the property of the fluorescent dye, acridine orange, to bind to native DNA in such a way that the flat planes of the dye molecules are rigidly held perpendicular to the DNA helix axis for low DNA to dye concentrations. From their results MacInnes and Ureta suggest, contrary to Inoué and Sato, that the DNA lies in an unsupercoiled array predominantly parallel to the long axis of the sperm head.

The most convincing evidence that the chromosomes in elongate sperm heads are arranged in a tandem fashion along the length of the sperm head comes from work by Taylor (1964) on grasshopper sperm. Taylor used thymidine-^{3}H to label spermatocytes and sperm. It was found that in the spermatocytes the heterochromatic X-chromosome replicates asynchronously with the autosomes so that some spermatocytes are labelled only in the X-chromosome and others only contain label in the autosomes. Correspondingly, in the sperm, some cells were found where only a short segment was labelled and in others only one short segment was unlabelled. As the diameter of the sperm head is about the same as that of a metaphase chromatid (0.5 μm) Taylor concluded that the chromosomes lie in tandem but in a random order along the length of the sperm head.

There is, however, one situation where the chromosomes are known to be arranged in a tandem fashion along the length of the sperm head. In the iceryine coccids Steatococcus tuberculatus, Echinicerya anomala and Icerya purchasi (Hughes-Schrader, 1946), the

sperm are formed by the invasion of the cytoplasmic anlage of the spermatid tail by a nuclear diverticulum containing the chromosomes. The rest of the cell, including the nucleus and cytoplasmic constituents, is discarded. The tail develops directly into the sperm. The chromosomes of the sperm are arranged in a linear order and are attached end to end. There are only two chromosomes and it is the shorter one of these which leads the way into the tail. During spermiogenesis there is a complex sequence of spiralisation and despiralisation. In the mature sperm, which are arranged in bundles, the chromosomes are arranged in a tandem pattern and are in a relatively extended condition.

A further approach to the study of chromosomes within sperm heads is the use of various spreading techniques. Gall (1966) has examined unsectioned spermatid nuclei from the grasshopper Melanoplus after spreading on water and drying by the critical point method. He found that the younger spermatids spread reasonably well and the bulk of the preserved nuclear material was fibrous. Closer examination showed the fibers to vary considerably in diameter and frequently to be associated into narrow sheets. He found the tendency to form sheets more pronounced in older, thinner spermatids but was not able to obtain good enough spreading for detailed examination. The nuclear condensation pattern in Melanoplus is of the lamellar type (Gibbons and Bradfield, 1957).

Lung, (1968) has examined the heads of bull and human sperm spread after treatment with thioglycolate. He has shown that the chromatin in these two types of sperm has a fibrous appearance. Thin sectioning of mammalian sperm at different stages of spermatogenesis would indicate that the chromatin is in the form of "granules" aggregating into larger units (Burgos and Fawcett, 1955; Fawcett, 1958; Horstmann, 1961). The granules condense gradually and appear homogeneous in thin sections of the mature sperm. Contrary to this, Koehler (1966) reports the presence of sheets or lamellae in freeze etched bull sperm. Lung suggests that if the plates or lamellae of Koehler do exist, the treatment with thioglycolate may have resulted in their breakdown to finer substructures.

Solari (1968a) has examined the effect of different spreading conditions on sperm from the sea urchin Strongylocentrotus purpuratus. Solari (1968b) found that regardless of the degree of spreading the peripheral pattern of the chromatin fibers was the same with loops extending from the main mass of material. Where the sperm heads have been well spread the loops were of varying diameter from 30-100 Å.

The detailed examination of the structure of sperm heads by spreading techniques is a relatively new approach to the problem of ascertaining the arrangement of the nucleoprotein within the sperm head. It is however, very evident that there is much discrepancy between the results of this type of procedure and the results of thin sectioning techniques, so that great care must be taken in the interpretation of results.

OBSERVATIONS

Investigations were carried out along three main lines:

I. A study of spermatogenesis and the fine structure of the mature sperm. (Part of this work has been published and a typescript is included as Appendix I.)

II. A study of the coiling phenomenon of the flagellar shaft within the mature sperm head. (This work has been published and a typescript is included as Appendix II.)

III. A study of the arrangement of the nucleoprotein of the mature sperm head.

The methods and results of each section are considered separately.

MATERIALS

All whelks used in this study were collected from the Castle rocks and Kinkell Rocks regions of St. Andrews Bay. Nucella lapillus (L.) occurs in great abundance in the intertidal zones of the Kinkell and Castle regions of St. Andrews Bay. During the summer months they are distributed over the rock surfaces, but in the winter and during the breeding season they gather together in large groups, deep in the rock crevices. Sperm can be obtained from these animals throughout the year. On the whole, them Nucella which I collected in St. Andrews were in good condition, although in a few specimens the testis was

infected with Paracrobia acanthus. Infected specimens were not used in this study.

MICROSCOPY AND PHOTOGRAPHY

Throughout this study all light microscope observations were made using a Carl Zeiss Photomicroscope and a Carl Zeiss G.F.L. microscope fitted with Planachromatic objectives. Phase contrast observations were made with the same microscopes fitted with Neofluar objectives. Light micrographs were taken on Ilford Pan F or Micro-Neg Pan 35 mm film using the photomicroscope, or the G.F.L. fitted with a 35 mm camera and a Carl Zeiss electronic flash attachment.

All electron microscope observations were made on a Siemens Elmiskop I (80 Kv), fitted with a decontamination device consisting of a cooling finger and liquid nitrogen trap. All electron micrographs were taken on Ilford "Special" Lantern Contrast 2" x 3" plates. The plates were developed with Ilford P.Q. Universal developer for 2½ minutes.

I. A study of spermatogenesis and the fine structure of the mature sperm.

This section is divided into three parts:

(a) A study of the anatomy and histology of the gonad.

(b) Observations on sperm in the living condition and after fixation and staining.

(c) An electron microscope study of the fine structure of the stages of spermatogenesis and the mature sperm.

Ia. Study of the anatomy and histology of the gonad.

The following description of the gross anatomy of the male genital system in Nucella (after Fretter and Graham, 1962) is given to serve as a general introduction.

In the mature whelk the testis lies to one side of the upper region of the visceral mass (Fig. 1). It is pale yellow in young whelks, the colour changing to a deep orange in older specimens. The testis is spread over the lobes of the digestive gland. It consists of numerous tubules all directed inwards. The tubules join one another to form a single white testicular duct which passes along the surface of the digestive gland on the columellar side. This duct acts as a vesicula seminalis. At its anterior end a sphincter closes the entrance to a ciliated duct which runs beneath the intestine and pericardium to the prostate gland. From the anterior end of the prostate gland a narrow vas deferens passes along the right side of the head to the penis, which lies behind the right cephalic tentacle. Fertilisation is internal.

Method. The testis, testicular duct and parts of the attached digestive gland were cut from freshly opened whelks and fixed in sea water Bouin:

165 cc sea water saturated with picric acid
55 cc formalin
11 cc glacial acetic acid

The material was fixed for 12 hours prior to dehydration through an alcohol series and embedding in wax (m.p. 52-54° C). Sections, 4 μ in thickness, were cut and mounted on slides. The sections were dewaxed, mordanted for 1 hr in 4% ferric alum, stained for 1 hr in Heidenhains Haematoxylin and differentiated in a 3% solution of ferric alum until only the nuclear materials of the cells were darkly stained. The sections were counter stained with a 1% aqueous solution of Eosin.

The stained sections show tubules containing spermatogonia, spermatocytes, spermatids, mature sperm and branches of the testicular duct packed with mature sperm (Fig. 2). In the tubules the spermatogonia are arranged in groups round the periphery and the mature sperm are seen in groups round the inside of the tubule, with their tails directed towards the lumen (Fig. 3). The spermatocytes and spermatids are found in patches scattered throughout the tubules. The spermatogonia have irregularly shaped nuclei (Figs. 4 and 5). Some spermatogonial mitoses are usually evident. Cells in all stages of the first meiotic division can be identified (Fig. 6). In synaptic and post-synaptic nuclei a bouquet arrangement of the chromosomes is often clearly visible (Fig. 5). Second meiotic metaphases are rare.

Spermatid nuclei look like signet-rings in the early stages and like doughnuts in later stages (Fig. 7). In longitudinal sections the flagellar shaft is visible bisecting the nucleus as it elongates. The mature sperm are most abundant in the testicular duct. The tubules contain large bunches of the darkly staining sperm heads.

Ib. Observations on sperm in the living condition and after fixation and staining.

A. The living sperm.

> Small pieces of testis were macerated in sea water and a drop of this suspension was mounted on a slide. The contents of the testicular duct were diluted about 100-fold with filtered sea water. A drop of this suspension was mounted on a slide and covered with a coverslip. These preparations were examined under phase contrast.

Spermatids at different stages of maturation and mature sperm can be seen in material from the macerated testis. The early spermatid nucleus is about 3μ in length and is bisected by the flagellar shaft which is already its final length of about 86μ (Figs. 8 and 10). The developing acrosome can be seen at the anterior end, and the mitochondrial nebenkerne at the base of the nucleus. The nucleus is surrounded by a layer of cytoplasm. Frequently several spermatid nuclei are seen within the same cell membrane (Fig. 9). As many as 8 spermatids were seen together, but they were

also observed in twos and fours. The nucleus can be seen at various stages of elongation (Figs. 11, 12, 13 and 14) and the amount of cytoplasm decreases until no cytoplasm is distinguishable in the mature sperm seen under phase contrast.

Mature living sperm are all alike. They are about 86 ± 2μ in length and are of even diameter along their length, apart from a pointed acrosome and a tapering tail (Fig. 15). The sperm are motile throughout their length, waves of movement being initiated from both head and tail. The undulations of the head have a lower frequency from those of the tail. The living and freely moving sperm appear with uniform contrast along their length. As they die the tails show a slight increase in diameter with a corresponding decrease in contrast.

B. Studies on sperm after fixation and staining.

Four histochemical techniques were applied, the Feulgen reaction, Altmann's Acid Fuchsin/Picric acid technique for mitochondria, the Periodic acid Schiff reaction, and the fast green technique of Alfert and Geschwind.

Feulgen reaction.

Thin smears of sperm were prepared on glass slides and placed for 30 mins above a filter paper soaked with 40% formaldehyde. The slides were then immersed in 4% formaldehyde for 1 hr. The smears were rinsed with distilled water, hydrolysed in N HCl at 60° C for 14 mins and stained for 1 hr in Feulgen reagent. The latter was prepared according to Swift (1955). The slides were then rinsed with three changes of bisulphite rinse,

10 ml N HCl
10 ml 5% potassium metabisulphite
180 ml water,

dehydrated and mounted in Canada Balsam.

The sperm heads are stained a deep pink (Fig. 16). The remainder of the sperm is unstained. The sperm heads measure 40 ± 1μ in length.

Acid Fuchsin/Picric acid (Altmann, 1890)

Smears were fixed as described above. They were subsequently post-chromed in saturated potassium dichromate at 38° C for two days, washed in running water for two hours and stained with hot 4% acid fuchsin in aniline water. After cooling, the slides were rinsed in distilled water and then placed for 3-5 mins in saturated picric acid. This was following by rapid dehydration, clearing and mounting.

The mid-pieces of the sperm stained intensely by this reaction (Fig. 17). The remainder of the sperm appear faintly pink. The mid-piece lies immediately behind the head and is 7-9μ in length.

Periodic Acid Schiff reaction (after the method of Hotchkiss in Glick, 1949).

Smears of sperm were prepared as previously described, washed in running water and immersed for 15 mins in a solution consisting of 500 mg periodic acid, 45 ml water and 5 ml N/5 sodium acetate. The smears were then stained with Schiff's reagent for 1 hr before dehydration and mounting.

The Periodic Acid-Schiff reaction (PAS) is for the detection of polysaccharides. The sperm are completely unstained by this reaction indicating the absence of polysaccharide.

Fast Green (Alfert and Geschwind, 1953).

This technique was developed by Alfert and Geschwind for the detection of basic proteins in cell nuclei.

Sections were prepared as described in section Ia. The sections were dewaxed and rehydrated. Some were immersed in boiling TCA for 30 mins to remove nucleic acids. All slides were then washed in 70% alcohol, rinsed in water and stained for 30 mins on 0.1% solution of fast green FCF in 0.2 M Na_2HPO_4 at pH 8.2.

The preparations were subsequently washed in buffer at pH 8.2, transferred directly to 95% alcohol, dehydrated, cleared and mounted in Canada Balsam.

To determine the specific nature of histones with respect to lysine or arginine content, the deamination procedure of Van Slyke (1911) as described by Monné and Slautterback (1950) was used. Sections were treated with Van Slyke reagent for 1½ hours.

2 parts concentrated sodium nitrite solution
1 part glacial acetic acid
5 parts distilled water.

The sections were then stained with Fast Green. This procedure affects primarily the amino groups of lysine and not the guanidine groups of arginine (Deitch, 1955; Olcott and Fraenkel-Conrat, 1947). Consequently histones rich in arginine stain with alkaline fast green; those rich lysine do not stain.

Control slides which had not been treated with TCA remained unstained. The slides treated with TCA alone prior to staining showed intense staining of all the nuclei in the testis (Figs. 18 and 20). In those slides which had been treated with TCA and then the Van Slyke reagent only the mature sperm in the tubules and testicular duct and the late doughnut spermatids were distinctly green, the rest of the nuclei were unstained (Figs. 19 and 21).

Ic. An electron microscope study of the fine structure of the stages of spermatogenesis and the mature sperm

Fixation of material for electron microscopy.

Owing to the well known difficulty of fixation of marine material for electron microscopy, I carried out some experiments with different fixation techniques to find which would give the best results with Nucella.

The following fixation techniques were tried:

1. Sea water osmium. This consisted of:-

   12.5 ml 2% osmium in sea water
   5 ml 0.1 N HCl
   5 ml veronal acetate buffer
   2.5 ml water.

   The pH of this solution was 7.4. Fixation was carried out on ice for 1 hr.

2. Palade osmium. 1% osmium tetroxide buffered to pH 7.4 with a veronal acetate buffer (Palade, 1957).

   This solution consisted of:-

   12.5 ml 2% osmium
   5 ml 0.1 N HCl
   5 ml veronal acetate buffer
   2.5 ml water.

   Material was fixed on ice for periods of up to 1½ hrs.

3. Formaldehyde and glutaraldehyde fixation followed by post-fixation with osmium (Pease, 1964).

4 gms of paraformaldehyde were added to 10 ml water and 2 ml NaOH (2.5%) and warmed under a hot tap until the solution was clear. This solution was then added to 15 ml NaOH (2.5%), 83 ml NaH_2PO_4 (1.3%) and 2 ml glutaraldehyde (25%). Material was fixed in the resulting solution for 15 mins at room temperature, washed in several changes of distilled water over a period of 15 mins and post-fixed in Palade osmium for 1 hr.

4. Glutaraldehyde buffered with sodium cacodylate (Sabatini, Bensch and Barnett, 1963).

Material was fixed at room temperature in a 3% solution of glutaraldehyde buffered to pH 7.5 with 0.1 M sodium cacodylate for 2 hrs, rinsed in buffer for 2 hrs and post-fixed in Palade osmium for 1½ hrs.

5. Glutaraldehyde buffered with a phosphate buffer.

Material was fixed for 10 mins in a 10% solution of glutaraldehyde buffered to pH 7.38 with phosphate buffer (Sørensen) at room temperature. The material was subsequently washed in two changes, about 2 mins each, of phosphate buffer and post-fixed in Palade osmium.

I decided that fixation was poor where cells appeared greatly vacuolated, the nuclei comparatively empty, the cell membranes swollen and damaged and the mitochondria swollen. Applying these criteria,

the best overall fixation of all cell types was achieved with Palade osmium although with this fixation, microtubules were on the whole poorly preserved. Excellent fixation of microtubules and flagellar fibers as well as reasonable overall fixation was achieved with 10% glutaraldehyde followed by post-fixation with Palade osmium. I therefore chose these two methods of fixation to use in this study.

After fixation the material was rinsed, dehydrated and embedded as follows:-

70% acetone	15-30 mins
95% acetone	1 hr
dry acetone, several changes	2 hrs
25% Vestopal W in acetone	1 hr
50% Vestopal W in acetone	1 hr
75% Vestopal W in acetone	1 hr
Vestopal + initiator and activator 2 changes	4 hrs

After impregnation with Vestopal W the material was transferred to gelatin capsules in fresh Vestopal with initiator and activator and placed in an oven at 60° C for several hours until it was hard.

Sections, 250-500 Å in thickness were cut on a Cambridge Ultramicrotome (A.F. Huxley pattern) with freshly broken glass knives. The sections were mounted on Athene 483 grids without supporting films.

Staining for electron microscopy.

All thin sections cut for electron microscopy were stained with a 2% (W/v) solution of uranyl acetate followed by lead citrate staining (Reynolds, 1963).

At the start of this work the method of staining was as follows:-

> The grids were floated, face downwards on the surface of a 2% solution of uranyl acetate in double distilled water, in a small solid watch glass for 5 mins. The grids were then transferred to a second watch glass containing double distilled water, and rinsed for about 30 secs and then dried. The grids were next stained with lead citrate. A watch glass containing lead citrate was placed in a perspex chamber containing a dish of solid NaOH. The grids were floated on the lead citrate for 2 mins and then rapidly transferred to a 0.02 N NaOH solution for about 1 min prior to rinsing in double distilled water and drying.

This method gave reasonable results if extreme care was taken. However, even with carefully filtered solutions there was a tendency for some contamination from the stain. During the course of this work an improved method of staining was developed.

Filter papers were placed on the bottoms of three petri dishes. The filter paper in one dish was soaked with double distilled water, that in another with 2% NaOH, and the third one was left dry. 5 microscope slides were coated with embedding wax by dipping them into a beaker of molten wax. 2 of these slides were placed in the "water" petri dish, 2 in the "NaOH" petri dish and one in the "dry" petri dish. A clean 5 ml hypodermic syringe was filled with a 2% aqueous solution of uranyl acetate. A "MILLIPORE" swinney head loaded with a 13 mm MILLIPORE filter (pore size 0.45μ) was attached to the syringe. Drops of uranyl acetate were discharged from this syringe onto one of the slides in the "water" petri dish. Similarly, again using a MILLIPORE filter, drops of distilled water were placed on the other slide in the same dish. Immediately the top of the petri dish was replaced.

Using a clean syringe and MILLIPORE filter drops of 0.02 N NaOH were added to one of the slides in the "NaOH" petri dish. The syringe was emptied, and, without washing it or changing the filter, it was recharged with lead citrate. Drops of lead citrate were added to the other slide in the dish and dish covered immediately.

Finally drops of distilled water were placed on the slide in the "dry" petri dish.

To stain, the grids were placed face downwards on the drops of uranyl acetate for 5 mins. The excess uranyl acetate was drained off on the edge of a clean filter paper and the grids placed on the drops of distilled water for one minute. The grids were then drained and dried. Next the grids were placed on the drops of lead

citrate for 2 mins and then put directly, without draining, onto the drops of NaOH and left for about 2 mins. The grids were finally passed to the drops of water in the third petri dish, for 1 min and then drained and dried.

I found that if this procedure was carefully and rigidly adhered to the results were consistently of a high standard. There was no contamination from the uranyl acetate and the tendency for lead to precipitate was overcome.

Fixation of the testis and testicular duct of Buccalia.

Small pieces of testis were cut from a freshly opened whelk and placed directly in Palade osmium, fixed for 1½ hrs and dehydrated and embedded as described previously.

Sperm from the testis and testicular duct look the same in phase contrast. I thought it necessary however, to see if they differed in ultrastructure.

Small pieces of testis and testicular duct from the same whelk were fixed in 10% glutaraldehyde and post-fixed in Palade osmium prior to dehydration and embedding.

Electron microscope observations.

Spermatogonia are about 4μ wide (Fig. 22). Their nuclei are large and lobed. The nuclear envelope is double and perforated by pores. The chromatin is unevenly distributed, giving the nucleus a patchy appearance. Nucleoli are visible in some sections as circular or oval bodies of more uniform texture surrounded by chromatin (Fig. 23). The cytoplasm contains a few mitochondria of various sizes, and numerous small granules. There are several Golgi complexes, each made up of a stack of parallel lamellae and a number of small round vesicles. Close to one of the Golgi complexes is a pair of centrioles, lying at right angles to one another. The larger of the two centrioles measures 300 mμ long and 150 mμ wide. In some spermatogonia four centrioles were observed. Such cells were probably about to divide mitotically.

The primary spermatocytes are 3-4μ wide (Fig. 24). The nucleus nearly fills the cell. The chromatin is patchy and synaptinemal complexes (chromosome cores) are visible in most sections. The cytoplasm contains a few round mitochondria and several Golgi complexes.

Early spermatids are irregularly shaped cells 3-4μ in width (Fig. 25). The cytoplasm of these cells contains numerous, usually spherical, mitochondria clustered together at one side of the nucleus. Golgi complexes are evident in some sections but have fewer associated

vesicles than those of earlier stages. In some sections a Golgi complex and associated pro-acrosome granule may be seen in the cytoplasm. As development proceeds, the pro-acrosome granule and associated Golgi migrate to the opposite side of the nucleus from the mitochondrial cluster (Fig. 26). The nuclear material is evenly distributed throughout the nucleus in the form of a granular reticulum. The cytoplasm of adjacent spermatids is often continuous (Figs. 25 and 27). From phase contrast observations, up to 8 early spermatid nuclei are seen to be enclosed within the same cytoplasm, probably due to the cell membrane being incompletely reformed after cell division.

At the start of spermiogenesis the centriole is situated at the ultimate base of the nucleus. From it the flagellar shaft extends out from the cytoplasm surrounding the nucleus. The centriole then bores its way through the nucleus until it reaches and anterior end. The resulting spermatid nucleus is penetrated by a blind ended tube about 600 mμ in diameter, which is lined throughout by the nuclear membrane and accommodates a straight portion of the flagellar shaft. The flagellar shaft extends backwards from the nucleus as the developing tail. The flagellar shaft by this stage has already grown to its final length of about 86μ. (Phase contrast observations).

As the spermatid elongates the nuclear material condenses. At first it consists of numerous interlocking strands, each about 100 Å in diameter, arranged in a loose helix along the prospective

long axis of the sperm (Fig. 28). The spermatid nucleus at this stage measures about 3.5 by 1.75μ. The flagellar tube extends from end to end of the nucleus and is straight, but the portion of the flagellar shaft which it accommodates is loosely coiled within the tube (Fig. 29). As condensation proceeds the nucleoprotein strands fuse (Fig. 30) together into lamellae which are about 110 Å in width. In transverse section the lamellae are often seen to be radially arranged with respect to the flagellar shaft, with one or both edges of the lamella closely applied to the nuclear membrane (Figs. 34 and 35). At this stage the whole nucleus appears twisted and contorted. The lamellae are at first widely spaced but gradually fuse (Fig. 31) as the nucleus becomes longer and narrower and straightens. Eventually only 12-15 apparently concentric, closely packed lamellae are discernible in transverse section (Figs. 32, 36 and 37). There is a thin layer of cytoplasm between the nuclear membrane and the flagellar shaft and the diameter of the nucleus at this stage is about 1μ. During the later stages of lamellar fusion the edge of the nucleus has a fussy appearance. In a few transverse sections one or two microtubules are present, separated from the nuclear envelope by the fussy material. Further condensation of the nucleus occurs until, in the mature sperm, it presents a completely homogeneous appearance and the nuclear membrane is closely applied to the flagellar shaft (Figs. 33 and 37).

The mature sperm head consists of a cylinder of nuclear material which encloses the anterior portion of the flagellar shaft (Fig. 33). The diameter of this cylinder is about 400 mμ, tapering to about 250 mμ at its anterior end. The sperm nucleus is an elongate tube closed at its anterior end by a double layer of nuclear envelope. The walls of the tubular nucleus are about 100 mμ wide in the rear half of the sperm head but they narrow to about 50 mμ at the front of the head. Behind the double nuclear envelope at the anterior end of the nuclear tube is a centriole. This shows the typical arrangement of 9 triplet elements, and from it the flagellar shaft extends backwards. In mature sperm within the testis, outside the nuclear membrane, in a thin layer of cytoplasm which surrounds the nucleus, is a row of microtubules which stretch backwards from the acrosome (Figs. 38 and 39). These microtubules never surround the nucleus completely but appear either as a single row down one side of the nucleus or in two rows at opposite sides of the nucleus. The microtubules lie parallel to a flat membrane which is distinct from the nuclear membrane. This membrane is an extension of a "ragged membrane" which surrounds the acrosome.

Within the testis the sperm heads are embedded in material similar in appearance to the nutritive cells in Cipangopaludina (Yasuzumi and others, 1960) (Fig. 40). The sperm are arranged in batches, and all the sperm within a batch are orientated in the same direction.

The small mitochondria that aggregate at the base of the nucleus in the early spermatid, fuse to give four or five large Nebenkerne which are grouped round the flagellar shaft (Fig. 41). As spermatid development progresses the Nebenkerne can be seen in longitudinal section to be arranged in a loose spiral round the flagellar shaft (Fig. 42). In transverse section their cristae are arranged radially. The outer membranes between the mitochondria break down and finally the whole Nebenkerne appears to be surrounded by a continuous membrane (Fig. 43). The length of the mitochondrial sheath at this stage is about 3μ. Occasional microtubules have been observed in the cytoplasm surrounding the mid-piece at this stage.

The mid-piece of the mature sperm lies immediately behind the nucleus and is about 8μ in length. The mitochondria are fused together to form a sheath of outer diameter about 0.4μ and inner diameter about 0.22μ. The outer membranes are fused to form a sack which contains the mitochondrial elements bounded by their inner membranes alone. In transverse section these elements are radially arranged round the flagellar shaft (Fig. 44). In longitudinal section the mitochondrial elements appear to be arranged in a helical fashion (Fig. 45). The patterns seen in section are due to the inner mitochondrial membranes alone, the outer membranes between the coiled elements having broken down to form the continuous sack round the outside of the sheath. The mitochondrial sheath is separated from the flagellar shaft by a layer of cytoplasm (Fig. 45) in contrast to the situation in the head where the nuclear

membrane is closely applied to the flagellar shaft. No microtubules have been observed associated with the mid-piece of the mature sperm.

The acrosome develops alongside a large Golgi complex. The latter consists of a stack of lamellae in the characteristic horse-shoe arrangement, and many associated vesicles. Acrosome development starts at the early spermatid stage when the flagellar shaft has penetrated the nucleus, but before nuclear elongation has begun. A pro-acrosome granule is formed from the associated Golgi complex. Both Golgi and pro-acrosome lie to one side of the nucleus or at its anterior end. The pro-acrosome granule forms into a cylinder surrounded by a membrane, with a slight indentation in its base. The Cylinder elongates and becomes tapered, and the indentation in its base deepens. Some of the membranes of the Golgi complex are often continuous with the membrane which surrounds the pro-acrosome granule (Fig. 46). The pro-acrosome migrates to the anterior end of the nucleus and takes up a position directly over the centriole. There is some diffuse material at the base of the pro-acrosome which forms a plate, the "interstitial membrane" (Kaye, 1962) between the developing acrosome and the nucleus. The invagination in the base of the pro-acrosome deepens further until the latter has the form of a cone surrounded by a double membrane. Inside the cone a series of longitudinally directed microtubules, 100 Å in diameter, are formed. Within the invagination an acrosome granule appears.

The acrosome of the mature sperm is terminal and pointed. It is about 1.2μ long (Fig. 47). Its main component is an acrosome cone, about 1μ long which consists of a bounding membrane within which is a ring of longitudinally arranged tubules (Figs. 50, 51, 52). These merge at their anterior ends and consequently cannot be resolved in transverse sections through the tip of the cone (Fig. 48). At its base the cone widens slightly to form an inwardly directed lip. Within the cone, but outside the cone membrane are five rods which appear in transverse section as five dark patches arranged in a circle and embedded in material of a lighter shade (Figs. 49 and 50). These rods and the matrix in which they are embedded probably correspond to the acrosome "granule" described in Achaeta domestica (Kaye, 1962). The acrosome is separated from the cell membrane by a thin layer of cytoplasm, within which, and close to the acrosome, lies a row of microtubules. Each tubule is about 200 Å diameter (Figs. 47 and 50). The tubules completely surround the acrosome (Fig. 38) and extend longitudinally from near the tip of the acrosome backwards along about 3/4 of the length of the sperm head in one or two rows as previously described (Figs. 33 and 38). Between the microtubules and the acrosome cone is a discontinuous "ragged membrane" (Figs. 33 and 38). The latter fuses with the outer cone membrane near the apex of the cone where a conspicuous thickening of the cone membrane is evident (Figs. 50 and 51). The tip of the acrosome cone consists of a vesicle bounded on the inside by the outer cone membrane and on the

outside by a continuation of the "ragged membrane" (Figs. 47, 50 and 51). Remnants of the interstitial membrane lie between the acrosome and the nucleus (Fig. 50).

Behind the mid-piece the flagellar shaft extends backwards to form the tail. Within the cell membrane and outside each of the pairs of peripheral flagellar fibers there is a group of coarse fibers. In each group the fibers are packed together and twisted into a coil as in an electrical flex. In transverse section each coil appears compressed into a triangular shape. The apex of the triangle points inwards towards the adjacent pair of flagellar fibers. There are about 12 fibers in each group at the anterior end of the tail, the number decreasing gradually towards the tail tip (Figs. 53 and 54).

There is no trace of a second centriole or a derivative thereof anywhere in the sperm.

Comparison of mature sperm from the testis with those from the testicular duct from the same whelk shows that there is one conspicuous difference between the two. In sperm from the testicular duct there are no microtubules present in the cytoplasm. The microtubules which surround the acrosome and extend backwards along one side of the nucleus in sperm from the testis are absent in sperm from the testicular duct (Figs. 55, 56 and 57).

II. A study of the coiling phenomenon of the flagellar shaft within the mature sperm head.

The coiling of the flagellar shaft within the head of the mature sperm of Nucella was first observed by Retzius (1912). Retzius noted that when sperm were macerated in water the nuclear material swelled and the central fiber became twisted into a coil. He also observed this feature in sperm from Littorina and Buccinum.

Unpublished observations on the behaviour of the mature sperm of Nucella were made several years ago by Callan (1952). He observed living sperm and their movement as seen in phase contrast. He also observed changes in the sperm when they were immersed in different media. He remarked upon the fact that in x 2 concentrated sea water the heads of the living sperm show a tendency to become attached to the surface of the slide. When this happens the nucleoprotein of the sperm head disperses and the portion of the flagella shaft enclosed within the head (the head shaft) springs back into a coil.

In this study I have repeated some of Callan's experiments and attempted to induce the coiling phenomenon by various methods. I have carried out this investigation both at the light and electron microscope levels in an attempt to answer such questions as; does the coiling reflect some peculiar feature of the flagellar shaft in the head? What keeps the head straight in the living sperm? Why does the tail not coil?

For this study mature sperm were extracted from the testicular ducts of freshly opened whelks.

Concentration of sea water.

As concentrated sea water was required for part of this work, I attempted to concentrate sea water which had been filtered through a porcelain finger, by boiling under vacuum. With this method it was not possible to obtain consistent results.

I therefore decided to use artificial sea water throughout as a means of obtaining experimental consistency. Artificial sea water was prepared according to Barnes (1954).

Normal concentration sea water was made up of the following constituents in 1 litre of distilled water:

NaCl	23.991 gms		
KCl	0.742 gms		
$CaCl_2$	1.135 gms	($CaCl_2.6H_2O$	2.240 gms)
$MgCl_2$	5.102 gms	($MgCl_2.6H_2O$	10.893 gms)
Na_2SO_4	4.012 gms	($Na_2SO_4.10H_2O$	9.100 gms)
$NaHCO_3$	0.197 gms		
NaBr	0.085 gms	(NaBr. H_2O	0.115 gms)
$SrCl_2$	0.011 gms	($SrCl_2.6H_2O$	0.018 gms)
H_3BO_3	0.027 gms		

The chlorinity of this solution is calculated by Barnes to be 19‰ and the salinity 34.33‰.

A x 10 concentrated stock solution was prepared and filtered through a 47 mm MILLIPORE filter (pore size 5 μ) attached to a vacuum pump. The stock solution was stored in polythene bottles at 4° C and diluted as required.

I found that in x 1 concentrated artificial sea water the sperm showed signs of osmotic stress and tended to bend in the middle and stop moving. In x 1.5 concentrated artificial sea water the sperm behaved normally. x 1.5 concentrated artificial sea water was therefore used throughout as a substitute for normal sea water and will henceforth be referred to as normal sea water, and I considered x 3 concentrated artificial sea water to be equivalent to x 2 normal sea water. The stock solution was always refiltered before use.

As a start to these investigations I looked at preparations of sperm in normal sea water which were allowed to dry out, and also repeated Callan's procedure of treating sperm with x 2 concentrated sea water.

> Sperm were placed in normal sea water and a drop of this suspension was placed on a slide under a coverslip. The sperm were observed under phase contrast while the preparation was allowed to dry out causing an increase in the salt concentration.
>
> Callan's procedure of placing sperm in x 2 concentrated sea water was repeated. A very small amount of the contents of the testicular duct was added to a drop of x 2 concentrated sea water on a slide, covered with a coverglass and observed under phase contrast.

Observations.

The living sperm (Fig. 58) are motile throughout their length, moving with a vigorous bending and lashing motion. The movements of the head have a lower frequency than those of the tail. As a preparation of sperm in sea water dries out several changes occur in the sperm. The sperm become attached to the slide or coverglass. The attachment usually starts at the acrosome or the tip of the tail while the remainder of the sperm which is unattached continues to move vigorously. Gradually the attachment spreads along the length of the sperm. The tail frays into its individual fibers (Fig. 59). The nucleus swells gradually and the flagellar shaft within the head (head shaft) is thrown into a gentle spiral (Fig. 60). The nuclear material swells further and shortens and the head shaft is thrown into a tighter coil (Fig. 61). Finally the nuclear material may disperse completely and the head shaft forms a tight coil of 5-7 turns (Fig. 62). This process may take up to 15 mins to be completed. At the edges of the preparation where drying out occurs more rapidly and as a consequence the concentration of the sea water is increased, the coiling occurs more quickly and is more extreme. The nuclear material swells and disperses very quickly and the head shaft may be seen jumping back into a tight coil.

When sperm are placed in x 2 concentrated sea water the majority of the heads form tight coils immediately, the nuclear material is completely dispersed and the tail frays.

From these observations it appeared that to obtain coiling of the head shaft it was necessary for the nucleus to be swollen or dispersed. For the nucleus to swell or disperse it is probable that the cell and nuclear membranes are destroyed, probably when the sperm become attached to the slide. I decided to investigate the effect of several other factors on the sperm head to see if they would bring about breakdown of the nucleus and coiling of the head shaft.

Sperm were treated with a 0.01% (w/v) solution of sodium lauryl sulphate (SLS) in normal sea water. This was done in two ways; sperm were placed directly into the SLS solution, or the SLS solution was added to a preparation of sperm in normal sea water and drawn across the preparations by means of filter paper.

The effect of varying pH on the sperm was investigated. The pH of normal concentration sea water was altered over a range of 2.6-9.16 with Veronal-HCl (Michaelis) buffer. The pH was further increased from 9.16-12.0 by the addition of NaOH. Sperm were placed in sea water over the pH range 2.6-12.0 and observed.

The effect of distilled water upon the sperm was observed by both placing sperm directly into distilled water and by adding distilled water to one side of a preparation in sea water, and drawing the distilled water across the sperm by means of a filter paper.

The effect of the enzymes trypsin and pronase (Sigma) upon the sperm was investigated by placing the sperm in 50 µg/ml solutions of the enzymes in normal sea water at pH 8.0. Sperm that had been placed in the enzyme solutions were observed for periods of up to 3 hrs.

All preparations were examined under phase contrast. Finally I decided to make observations on the behaviour of sperm in the presence of ripe ova and in a homogenate of ovary to see if there was anything here that could affect the sperm in such a way as to bring about coiling of the head shaft.

Sperm were added to a solution of homogenised ovary of Eucalia. The ovaries were taken from 12 female whelks and placed in a homogeniser (glass pestle and tube) with about 30 ml normal sea water. The ovaries were then homogenised. A drop of the homogenate was placed on a slide and some sperm added. The preparation was observed under phase contrast over a period of 2 hrs.

Sperm were also placed in normal sea water in the chamber of a bored slide with one or two eggs from the proximal end of the ovary. The preparations were covered and examined over periods of up to two hours with an inverted phase microscope.

Observations.

Sperm placed in 0.01% SLS show almost instantaneous stripping of the nuclear material, tight coiling of the head shaft and fraying of the tail.

Alteration of the pH of sea water is not an effective means of inducing coiling of the flagellar shaft although it does affect the sperm. At pH 2.6 all sperm are non-motile, the nuclear material disperses and the head shaft coils. As the pH is increased the proportion of coiled sperm decreases and at pH 5.3 the majority of the sperm are motile. Over the pH range 5.5-5.8 the sperm behave normally. At pH 9.0 motility is gradually lost but only a few coils are formed. At pH 10.25 some sperm are still motile while others show untidy coiling. At pH 11.9 all the heads appear swollen and empty with the head shaft forming a zig-zag running through the centre of the head. Above this pH the sperm are completely destroyed.

When sperm are placed in distilled water, they show a tendency to swell in the middle and bend into a "hairpin" configuration. This bending is frequently more extreme and the sperm tie themselves into knots. When distilled water is drawn across a preparation of sperm in sea water, as soon as the salt concentration is decreased the sperm form a hairpin or tie themselves into a knot.

Treatment of sperm with 50 µg/ml trypsin and 50 µg/ml pronase at pH 8.0 have similar results. The head material disperses and the head shaft is thrown into a loose untidy coil. In some cases the flagellar shaft breaks.

A solution of homogenized ovary or proximity to intact eggs in sea water has no observable effect on the behaviour of the sperm.

The tight coiling phenomenon is consistent in that the coil is always in the same direction, clockwise from the anterior end, or right handed. The number of turns of the coil varies between 5 and 7. Not all the head shaft coils. The posterior ¼ remains uncoiled. The tight coil is only formed when the nuclear material is completely dispersed.

From these phase contrast observations it is evident that there are three conditions of coiling. First, complete and rapid coiling of the head shaft will occur when the nuclear material is stripped off as, for example, with SLS. Secondly, concentrated sea water in conjunction with the sperm coming into contact with the slide or coverslip may cause either an instantaneous or slower coiling. The slower coiling may take up to 15 mins to occur. The sperm become attached to the slide and the head swells gradually, the head shaft being thrown into a loose coil within the head. This is followed by the head shaft springing rapidly into a tight coil as the nuclear material disperses. Thirdly, with enzymes a loose coil is formed and the head material dispersed, either partially or completely.

For examination of coiled sperm heads with the electron microscope various methods were attempted.

Having observed, under phase contrast that sperm tended to coil when they became attached to a slide, I decided to utilise this factor to attach coiled heads to electron microscope grids.

Athene 483 grids with formvar/carbon supporting films were placed on clean glass slides with the formvar films facing upwards. Drops of sperm suspension in normal sea water were then placed over the grids and left for up to 10 mins to allow the sperm to settle. The slides were then placed in a petri-dish with a filter paper saturated with Palade osmium and the material allowed to fix for 20 mins. The grids were then removed from the slide and rinsed with distilled water prior to negative staining.
(the method used for negative staining is given on page 50)

This procedure on the whole tended to result in contaminated preparations, with sea water crystals and not much material remaining on the grid. Substitution of formaldehyde or buffered glutaraldehyde vapour for fixation of the sperm yielded similar unsatisfactory results. Some of the contamination in the preparations was removed by gentle centrifugation of the sperm solution and resuspension of the sperm in clean sea water.

A more satisfactory method for attaching sperm to the grids was to use a Langmuir trough.

The trough used was a porcelain dish, 4" x 4" and ½" in depth. The trough was filled to overflowing with normal sea water. The surface of the water was cleansed by sweeping the surface with waxed glass slides ½" x 6". The surface was considered clean when slight movement of one of the waxed slides caused no movement of a

small metal foil float placed on the surface. The best results were obtained when a drop of resuspended sperm suspension was placed on the surface and allowed to spread. Sperm were picked up from the surface on grids with formvar/carbon supporting films. The grids were drained on the edge of a filter paper, rinsed with distilled water and drained again prior to negative staining. Good clean preparations were also obtained when a small piece of testicular duct was placed directly onto the surface of the Langmuir trough and the sperm spreading out from this material picked up on the grids.

The Langmuir trough was then filled with x 2 concentrated sea water and the same procedure followed.

For all these preparations the same procedure for negative staining was used throughout.

The stain used was a 1% (W/v) solution of phosphotungstic acid (PTA). After rinsing the grids with distilled water the excess fluid was drained off with a filter paper and a drop of PTA placed on the grid and allowed to stand for about 10 secs. The PTA was added to the grids from a 5 ml syringe with a MILLIPORE swinney head loaded with a MILLIPORE filter (pore size 0.45 μ) attached. The PTA was then drained off from the side of the grid and the grid surface air dried.

The quality of the negative staining was found to be affected by the pH of the PTA. The best results were obtained using a freshly prepared solution of PTA adjusted over the pH range 6.4–6.6 with NaOH. Negative staining

was also improved if the formvar/carbon films on the grids were freshly prepared.

Negative stained preparations of sperm treated with enzymes were made by the following procedure. Small pieces of testicular duct were placed in 50 µg/ml solutions of trypsin or pronase in sea water at pH 8.0, for periods of up to 1½ hrs. The material was drawn in and out of a pipette at intervals to ensure thorough mixing of sperm in the enzyme solution. A drop of the digested sperm suspension was then placed on the surface of a Langmuir trough containing normal sea water and the sperm picked up and negatively stained as described previously.

A suspension of sperm in normal sea water was placed in a sonicator for 5-10 mins. A drop of the sonicated sperm suspension was allowed to settle on a grid, drained, rinsed and negatively stained.

Electron microscope observations.

Sperm spread on a Langmuir trough containing normal sea water have swollen heads and partially or completely frayed tails. The acrosome usually remains attached at the anterior end. The head increases in diameter from about 0.4 to 1.25µ. The nuclear material has a fine fibrous appearance although no structural detail is obvious (Fig. 63). Within the nucleus the head shaft may be thrown into a gentle spiral. The negative staining shows the fibers of the head shaft to be twisted like the wires of an electric flex (Figs

63 and 64). The mid-piece remains intact. The helical arrangement of the mitochondrial elements is conspicuous with the negative staining (Fig. 65). In a few cases there is a slight fraying of the mitochondrial elements but this is unusual (Fig. 66). The tail frays into the component fibers of the flagellar shaft. This fraying is not always complete and remnants of the fibrous material surrounding the flagellar shaft may be seen. In a few instances, immediately posterior to the mid-piece, the fibers of the tail appear twisted like those of the head shaft (Fig. 67).

The nuclear material is completely stripped from sperm spread on a Langmuir trough containing x 2 concentrated sea water. The majority of the head shafts are thrown into tight coils of between 5-7 turns. The coils are limited to the anterior 3/4 of the head shaft. The acrosome may remain attached at the anterior end of the head shaft (Figs. 68 and 74). The fibers of the head shaft are twisted (Figs. 69, 70, 71, 72 and 73). This twist is not so conspicuous in the tightly coiled regions. In the uncoiled regions there is a regular twist of the flagellar fibers (Fig. 74). The pitch of this twist is 2.5 $\pm$ 0.6 μ. The mid-piece remains intact, acting as a tie around the middle of the sperm. The tail is completely frayed. At their posterior ends the flagellar fibers are frayed into their component sub-units (Fig. 75). The coarse fibers seen in sectioned material are destroyed.

Treatment of sperm with pronase or trypsin results in the breakdown of the nuclear material into a meshwork of branching fibers of varying widths (Fig. 76). Alternatively, the nuclear material disaggregates into broad strips which run lengthwise with respect to the sperm axis (Fig. 77). The head shaft forms a loose coil and its fibers are irregularly twisted and in some cases may be broken or damaged.

Sonication of sperm has a similar effect to spreading sperm on a Langmuir trough containing normal sea water. The nuclear material has the same appearance after these two treatments. The head shaft is gently coiled and twisted within the nucleus. Sonication of the sperm usually causes the heads to break off from the tails and frequently the head itself is broken transversely into segments (Fig. 64).

From my observations the flagellar fibers of the head shaft, in sperm heads where the nucleoprotein is partially or completely dispersed, appear to be twisted. To see if this feature is a peculiarity of the head shaft of Mucella sperm, I also looked at negative stained preparations of sperm from the newt, Triturus cristatus, and the mouse, and also the flagellar shaft in Euglena. These preparations were made as described previously from suspensions of the material in amphibian saline, mammalian saline and water respectively.

Negative stained preparations of newt and mouse sperm showed that in these two cases the fibers of the flagellar shafts run straight (Figs. 78 and 79) and show no evidence of the twisting seen in the coiled head shaft of Eucalia. The presence of numerous mastigonemata on the flagellar shaft of Euglena made it impossible to examine the state of the flagellar fibers (Fig. 80).

III. A study of the arrangement of the nucleoprotein of the mature sperm head.

From observations of the breakdown of the sperm head into sheets or branching fibers after treatment of sperm with trypsin and pronase (see previous section), I thought that I might be able to approach the question of the arrangement of the nucleoprotein within the sperm head, by studying the pattern of breakdown of the nucleus under specified conditions. In this part of the work I have used various techniques to try and bring about a gradual breakdown of the nucleus. I have examined sperm after various treatments under phase contrast, with polarized light and with the electron microscope.

Treatment of sperm with pronase and trypsin.

Sperm from the testicular duct of a freshly opened whelk were placed in a solution of 50 µg/ml pronase (Sigma) in sea water at pH 8.0 at room temperature. Drops of this suspension were taken over periods of up to 3 hrs, placed on slides, covered with coverglasses and observed under phase contrast.

For examination with the electron microscope two methods were used.

1. A drop of the enzyme/sperm suspension was added to the surface of a Langmuir trough (see page 49) containing normal concentration sea water. Sperm were picked up by touching the surface of the sea water with Athene 200 grids with formvar/carbon supporting films.

2. Sperm were picked up directly by placing grids face downwards onto the surface of the sperm/enzyme suspension. To ensure that the sperm were distributed throughout the suspension, the suspension was stirred by pipetting.

The sperm were picked up for examination after periods of 30 mins, 1 hr and 3 hrs in the enzyme solution. The same procedure was followed using 50 µg/ml trypsin in sea water at pH 8.0.

After the sperm were collected on the grids, the excess fluid was rapidly drained off and the grids rinsed with distilled water prior to one of the following procedures:-

(1) The grids were negatively stained with 1% PTA (as described on page 50) and air dried.

(2) The grids were positively stained with 2% (W/v) uranyl acetate for 30 mins, rinsed in distilled water and air dried.

(3) The grids were dehydrated through an alcohol series and placed in amyl acetate prior to drying by the critical point method (Anderson, 1951). After drying, the grids were rotary shadowed with platinum/carbon at an angle of 30° using a Balzers BA3 high vacuum electron microscope specimen preparation unit. Evaporation was carried out at 10^{-5} Torr (mm/Hg). The apparatus was fitted with a cold trap for liquid nitrogen. Prior to evaporation the apparatus was always cooled with liquid nitrogen.

Sperm were also observed after double digestion with pronase. Grids with sperm treated with pronase for 30 mins were dried by the critical point method. Some grids were shadowed and observed with the electron microscope. Other grids were floated on the surface of a 50 µg/ml solution of pronase in sea water pH 8.0 for a further 30 mins prior to redrying by the critical point method and shadowing.

The expected effect of the enzymes is to remove the basic protein and thus allow progressive fragmentation of the nucleus. Other agents which can be used for the breakdown of basic protein are 2 M NaCl and 8 M urea, and I therefore decided to investigate the effect of these agents.

Treatment of sperm with 2 M NaCl.

Sperm from the testicular duct were placed in 2 M NaCl. A drop of the sperm suspension was placed on a slide, covered with a coverslip and examined under phase contrast.

This treatment has a similar effect to that of concentrated sea water on the sperm. The nuclear material disperses completely and instantaneously. The head shaft springs into a tight coil. As the dispersal of the nucleoprotein is so rapid by this method I decided to abandon it.

Treatment of sperm with urea.

Sperm from the testicular duct were placed in 7 M and 8 M solutions of urea. Drops of the sperm suspensions were added to glass slides, covered with coverglasses and examined under phase contrast.

Preparations of sperm treated with 8 M urea were also examined with polarised light using a Zeiss G.F.L. microscope fitted with a polariser and analyser. A mercury lamp was used as a light source.

For examination with the electron microscope, untreated sperm suspended in normal concentration sea water were allowed to settle on the surface of Athene 200 grids with formvar/carbon supporting films. The excess fluid was drained off the grids with a filter paper and the grids floated face downwards on 7 M and 8 M solutions of urea. After periods of 3 hrs, 6 hrs

and 9 hrs the grids were removed from the urea, rinsed thoroughly with distilled water, dehydrated through an alcohol series and dried by the critical point method. The grids were rotary shadowed at 30° with platinum carbon.

As a control for the results of previous experiments, I decided to treat some sperm with Desoxyribonuclease (DNase).

Treatment of sperm with DNase.

Sperm from a suspension in normal concentration sea water were allowed to settle on the surface of Athene 200 grids with formvar/carbon supporting films. The grids were floated face downwards on a 50 µg/ml solution of DNase (Koch-Light) in sea water at pH 7.8, and incubated at 38° C. Grids were removed from the enzyme, washed, dried by the critical point method and rotary shadowed after incubations of 1 hr and 3 hrs.

Phase contrast observations.

Treatment of sperm with 50 µg/ml trypsin and 50 µg/ml pronase at pH 8.0 have similar results. The head material is partially dispersed and the head shaft is thrown into a loose untidy coil. In some cases the flagellar shaft is seen to be broken.

Treatment with 7 M and 8 M urea has a considerable effect on the sperm. The first and most noticeable effect is a rapid disintegration of the tail. The tail develops swellings along its length.

These swellings merge and later the tail completely disappears. The mid-piece also disintegrates, but not so quickly as the tail. The head swells slightly and appears to break down into a sheet of material which has several twists along its length (Figs. 81 and 82).

Polarised light observations.

With the polariser and analyser perpendicular to one another and the sperm orientated at 45° to the crossed axes, the sperm head is strongly birefringent. In a fresh preparation of sperm suspended in sea water, before the sperm have become attached to the slide, the nucleus appears as a bright band bisected longitudinally by a straight dark band which represents the flagellar shaft (Fig. 83). After the sperm have become attached to the slide the nucleus still appears as a bright shaft, but the flagellar shaft is seen as a dark spiral (Fig. 84). The flagellar shaft is positively birefringent with respect to length and the nucleus is negatively birefringent. Sperm treated with urea and examined with polarised light still show bright birefringence. The twisting seen under phase contrast after urea treatment is even more evident under the polarising microscope (Fig. 85).

Electron microscope observations

Trypsin and pronase treated sperm

Some negatively stained preparations of sperm treated with trypsin or pronase showed the sperm head broken down into sheets of nucleoprotein and the flagellar shaft is twisted (Fig. 77). Other preparations showed the nucleus broken down to a branching network of fibers (Fig. 76), the flagellar shaft thrown into a loose coil and the flagellar fibers twisted. The fibers of nucleoprotein are of various diameters from 550 Å to 50 Å. Where the nuclear material has not disaggregated completely into a mass of branching fibers, the nucleoprotein appears to be wrapped round the flagellar shaft in a loose helix. At the base of sperm which are only partially damaged by this treatment, fibers of nucleoprotein can be seen looping back upon themselves (Figs. 86 and 87).

Preparations of sperm after 30 mins treatment with pronase and positively stained with uranyl acetate show sheets of material about 400 Å in width extending from the main axis of the nucleus. These sheets decrease in width and eventually appear to terminate in fine strands about 40 Å in thickness (Fig. 88). These fine strands loop out from the surface of the nucleus and appear to loop back to the main axis of the nucleus. They can be traced for lengths of up to 10μ. Some loops are seen to split into finer strands at the periphery of the spread zone, but it is impossible to determine splitting or

branching of the strands in the main mass of the material adjacent to the nuclear axis. However, with both negative and positive staining, the results which were obtained were not as consistent as I would have hoped for due to a great variability in staining. It proved very difficult to obtain uniform staining in different grids although the methods used were identical for each grid.

The results obtained from enzyme treated sperm which were dried by the critical point method and subsequently shadowed were readily reproducible. The extent to which different sperm on the same grid were digested was variable even though the sperm on each grid had been treated with enzyme for the same length of time. However, the pattern of breakdown was consistent whether the sperm head was only partially or almost completely digested.

Critical point dried preparations of sperm treated with pronase for 30 minutes showed the nucleus breaking down into sheets of material which were wound helically round the long axis of the sperm head in a clockwise direction from the posterior end of the head (Figs. 89, 90, 91 and 92). The sheets break up into thick strands which either remain helically wound, or spread out from the main axis of the head. In places where several strands are spread out from the nucleus they branch and fuse forming a meshwork around that part of the sperm head (Fig. 90). In other places these strands do not loop back to the main axis of the nucleus but branch to form finer strands. These strands branch again so that there is a series of strands of

decreasing diameter radiating from the main axis (Fig. 92). All the strands appear to be looped. No free ends were observed.

In preparations which have been treated with pronase for 30 minutes, dried and treated with enzyme for a further 30 minutes, the nuclear material is in the form of much finer loops than those seen after a single pronase treatment (Fig. 93). Again no free ends were observed.

Sperm treated with urea.

The breakdown pattern seen after urea treatment is essentially the same as that seen after pronase treatment. Here again, not all the sperm on each grid are at the same stage of breakdown. The flagellar shaft is usually almost completely destroyed after treatment with urea for 3 hours (Fig. 94). The mid-piece is only partially disrupted after this time, but is absent after prolonged treatment of up to 9 hours. As seen under phase contrast, the nucleus breaks down into a sheet of material twisted round the long axis of the head (Figs. 95 and 96). This sheet of material splits lengthwise into narrower strips. Material frays out from the head in a similar manner to that seen after enzyme treatment. After urea treatment the strands of nucleoprotein are smaller in diameter varying from about 170 Å where they leave the main axis of the head, down to about 40 Å (Figs. 97, 98 and 99). Again free ends are not seen and the threads of nucleoprotein

can be traced as loops. Lengths of up to 1μ of 40 Å diameter strands can be seen.

Sperm treated with DNase

After incubation of sperm in DNase for an hour, the nucleus is partially broken down. The flagellar shaft remains intact and the nuclear material is wound round it. There are a few fibers extending from the nucleus but these are fewer in number than after the previously described treatments. Also, these fibers are not so conspicuously looped. After 3 hours incubation with DNase most of the nuclear material is completely dispersed (Figs. 100 and 101). In some cases only the flagellar shaft remains with only a few small pieces of nuclear material still attached.

From the results obtained from Mucalla sperm after treatment with the enzymes trypsin and pronase, and with urea, it became apparent that there was a definite pattern in the breakdown of the nucleoprotein. As Mucalla also shows a definite pattern of nuclear condensation during spermiogenesis, I decided that it would be an interesting comparison to carry out similar experiments on sperm which showed a different pattern of nuclear condensation. The sperm which I chose for this study were those of the domestic fowl. The reasons for this choice were based on the following factors. First, the nuclear condensation pattern of the fowl has been shown to be of

the "granular" type and the microtubular systems associated with the nucleus during spermiogenesis have been described by McIntosh and Porter (1967). Secondly, sperm can be collected regularly from the fowl by the method described by Burrows and Quinn (1935). Thirdly, in an early study on the structure of sperm in the fowl, Grigg and Hodge (1949) treated sperm with trypsin. They used formalin fixed sperm for their experiments, but unfortunately do not indicate the concentration of enzyme which they used. After tryptic digestion their micrographs show the nucleus to have a granular appearance with faint fibers extending from the edges of the main mass of nucleoprotein.

I examined sperm from the domestic fowl with polarized light and carried out treatment with urea prior to examination with the electron microscope.

Materials:

The birds used were brown and white Leghorn cockerels obtained from Dr. P.E. Lake from the Agricultural Research Council, Poultry Research Centre, Edinburgh.

Method:

Semen was collected in a glass funnel from cockerels which were made to ejaculate by the method of Burrows and Quinn (1935). The semen was diluted about 50-fold with Tyrode saline.

Semen was also diluted using a saline suggested by Dr. Lake of the Poultry Research Centre, Edinburgh. This saline was made up from:-

sodium glutamate monohydrate	1.92 gms
magnesium acetate $4H_2O$	0.07 gms
sodium acetate, anhydrous	0.51 gms
glucose	0.6 gms
potassium acetate $1H_2O$	0.126 gms

dissolved in 100 ml distilled water.

This saline was stored at 4° C but allowed to reach room temperature prior to use. The semen was diluted three times with this saline.

Examination of sperm with polarized light.

Sperm were suspended in Tyrode saline. A drop of the suspension was placed on a glass slide and covered with a coverglass. The preparation was examined with a G.F.L. microscope fitted with a polarizer, an analyser and Planapochromatic objectives. A mercury vapour lamp was used as the light source.

Treatment of sperm with urea.

Sperm were attached to the surface of Athene 200 grids with formvar/carbon supporting films by allowing sperm to settle from a suspension in Tyrode saline. The excess fluid was drained off the grids and the grids were floated face downwards on the surface of an 8 M urea solution for periods of 3 and 6 hrs. The grids were rinsed thoroughly with distilled water, dehydrated through an alcohol series to amyl acetate and dried by the critical point method. The grids were rotary shadowed at 30° with carbon/platinum.

The same procedure was used for sperm which were suspended in Dr. Lake's saline and stored at 4° C for 4 hrs before treatment with urea.

To determine the dimensions of the nucleoprotein during spermiogenesis thin sections were cut for examination with the electron microscope.

A white Leghorn cockerel was killed by dislocation of the cervical vertebrae and the testes removed immediately. Small pieces of testis were fixed in 1% glutaraldehyde in phosphate buffer at pH 7.38 for 1 hr prior to a 20 min post-fixation in Palade osmium. The material was then dehydrated, embedded and sections cut and stained as described previously (page 31).

Polarized light observations.

With the polariser and analyser perpendicular to one another and the sperm orientated at 45° to the crossed axes the sperm showed no birefringence.

Electron microscope observations.

Urea treated sperm.

As in Mucella, the mid-piece and tail of the sperm are completely destroyed after prolonged treatment with urea. The acrosome

cap is also destroyed although the acrosome rod which extends the length of the acrosome and penetrates back into the anterior end of the nucleus, frequently remains (Figs. 102, 104 and 105). In the initial stages of nuclear breakdown the nucleus takes on a stippled appearance (Figs. 102 and 105). As the breakdown continues the nucleus swells slightly and the nuclear material appears to consist of numerous closely associated coarse "granules" about 350 Å in diameter (Figs. 103, 106, 107, 108 and 109). These "granules" separate and appear to be connected by short fibers about 100 Å in thickness (Figs. 106, 107 and 109). The nuclear material spreads further and appears as a mass of randomly arranged fibrous material of uneven diameter (Figs. 103, 108 and 109). The nucleus frequently fragments. There is no evidence of the fine loop structure seen in Mucella.

Sperm stored in Dr. Lake's saline at 4° C are affected in the same way by the urea as those in Tyrode saline. There is, however, an apparent tendency for the fibers to be of more even diameter along their length. Also longer lengths of fiber are apparent (Figs. 110 and 111).

Fine structure of the spermatid nucleus.

The early spermatid nucleus prior to its elongation is spherical. The nuclear material is in a diffuse state and is more

concentrated around the periphery of the nucleus. As elongation commences the nucleoprotein becomes evenly distributed throughout the nucleus (Figs. 112, 113 and 114). Paired centrioles, perpendicular to one another, are embedded in the base of the nucleus (Figs. 112 and 113). The nucleus becomes cylindrical in shape and is about 0.5μ in diameter. In longitudinal sections there is a row of microtubules, cut transversely, running down either side of the nucleus (Figs. 112 and 114). These microtubules are the two helices described by McIntosh and Porter (1967). There are a few places along the length of the nucleus where these microtubules overlap. When nuclear elongation is completed there is a change in the appearance of the nuclear material. Around the periphery of the nucleus the nucleoprotein aggregates into coarse "granules" about 350 Å in diameter (Figs. 115 and 120). As the number of these condensed 350 Å "granules" increases the arrangement of the microtubules alters. In longitudinal sections rows of microtubules appear to overlap (Figs. 115 and 117). The size of the "granules" increases and they become more evenly distributed throughout the nucleus (Figs. 116 and 118). The microtubules appear to increase in number and become organised into a manchette running straight along the length of the sperm head (Figs. 116 and 118). The "granules" increase in diameter and almost completely fill the nucleus (Figs. 119 and 121). At this stage the diameter of the nucleus is about 0.4μ. Finally, the nucleoprotein condenses to a uniform appearance (Figs. 122 and 123). The walls of the microtubules

become thicker and they no longer surround the nucleus completely (Figs. 122 and 123). Gradually the microtubules disperse and there are no microtubules associated with the mature sperm head.

DISCUSSION

Comparatively little work has been carried out upon the spermatogenesis and structure of the mature sperm in gastropod molluscs. The first work to draw attention to the process of spermatogenesis in this group was that of Von Siebold (1836). Von Siebold describes two types of spermatozoa in *Viviparus (Paludina) viviparus*. The first type are large and worm-like, one end being slightly thinner and pointed, and with many threads protruding from the other end. These threads have been shown to be numerous flagellae (Gall, 1961). The second type of sperm, which Von Siebold describes as being typical of the gastropods as a whole, are less motile, thin and hair-like with a thickened spiral head. Atypical sperm which show a loss in chromatin, similar to those described by Von Siebold, are found in many of the prosobranch molluscs but not in the Archaeogastropoda. The pond snail *Cipangopaludina malleata* (Yasusumi and Tanaka, 1958) has typical and atypical sperm similar to those of *Viviparus*. The atypical sperm of the marine snail *Janthina* are, however, very different in appearance and have been described by Ankel (1930) as the most bizarre in the animal kingdom. However, the pattern of development of atypical sperm is similar despite the variations in size and appearance amongst the different species.

Retzius (1912) describes only one type of sperm in Nucella. These sperm are elongate, threadlike structures with a central fiber, "Zentral-faden", penetrating the length of the head. He observed that when the sperm were macerated in water, the nuclear material swelled and the central fiber became twisted into a coil. He described ring-shaped "Zentral-körper" at both ends of the mid-piece. He also described a similar arrangement in sperm from Littorina, Rissoa, Conus, Neritina, Voluta, Murex, Fusus and Buccinum.

Portmann (1930) states that there are both typical and atypical sperm in Nucella. He states that the majority of sperm are atypical although indistinguishable from the typical mature sperm. According to Portmann the two types can only be distinguished at certain stages in their development. The atypical sperm are not oligopyrene since they do not show any loss of chromatin, but Portmann claims that they cannot be called eupyrene. He calls these atypical sperm dyspyrene, a term implying not only abnormal chromatin proportions but abnormal chromatin content.

In this study I have seen only one type of sperm, as described by Retzius (1912), with a single centriole giving rise to the flagellar shaft which runs the whole length of the sperm. At no stage in this study has there been any evidence of "atypical" spermatogenesis.

In Mucella the stages of spermatogenesis are similar to those in most animals. The spermatogonia divide mitotically to form primary spermatocytes. The latter contain the diploid number of chromosomes (1) and two centrioles. The two meiotic divisions follow in quick succession to give spermatids which contain a haploid chromosome set and a single centriole. It is evident from both light- and electron microscopy that the cells of each testis tubule are arranged in groups, and that the cells within a group pass through spermatogenesis in phase with one another.

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(1) In Mucella the number of chromosome pairs varies between 13 and 18 (Staiger, 1950). This variation is due to structural changes such as fusion and fragmentation, which affect the number of chromosomes.

Robertson (1916) first suggested that V- or J-shaped (or metacentric) chromosomes might have arisen by the apical or centric fusion of two rod-shaped (or acrocentric) chromosomes and, conversely, that a V or J might have fragmented into two rods. During his studies on insects, he had noted that many species had V- or J-shaped chromosomes and that other related species had fewer V's or J's but more rod-shaped chromosomes. If, instead of counting whole chromosomes, he counted the number of chromosomal arms, he obtained a nearly constant number in many groups. That is to say, a V or a J in one species might be equivalent to two rod-shaped, non-homologous chromosomes in a related species. This conception of fusion and its converse, fragmentation, is referred to as Robertson's law or Robertsonian variation.

The mature sperm of Mucalla resemble other molluscan sperm such as those of Cinnangopaludina (Yasusumi and Tanaka, 1958), Viviparus (Gall, 1961) and Otala lactea (Retzius, 1937), in that the centriole is buried in the head. Mucalla, however, shows this situation in the extreme in that the centriole is located immediately behind the acrosome and separated from it by only a double layer of nuclear membrane and a very thin layer of nuclear material.

I have stated that the spermatid and mature sperm of Mucalla have only one centriole. This conclusion is based on two considerations. First, there is absolutely nothing in the mature sperm which could be identified as a second centriole or a derivative thereof. Secondly, although I have not seen centrioles in early spermatids or spermatocytes of Mucalla, Gall (1961) states that there is only one centriole in the spermatid of Viviparus. It is probable that the same situation exists in Mucalla. In the spermatogonia two centrioles are clearly visible and it would seem likely that there is no further centriole replication after the first meiotic division. The centriole of the mature sperm is therefore probably one of those which were present in the primary spermatocyte. Alternatively, one must assume a loss of a centriole at some stage.

The fine structure and development of the acrosome in Mucalla is comparable to that described in the house cricket, Acheta domestica, by Kaye (1962). In both types the acrosome consists of two cones,

but the structure in Nucella would seem to be more complex in sperm in the testis than that of Achata, although the structure in Nucella is simplified in sperm from the testicular duct.

The only molluscan acrosome described in the literature is that of Cipangopaludina (Yasuzumi and Tanaka, 1958). In Cipangopaludina the acrosome is shaped like a harpoon barb and is contained in a head cap which consists of double membranes with a 50-100 mμ inter-space. Yasuzumi and Tanaka do not say anything about its formation apart from stating that it arises in association with the Golgi complex.

In Achata Kaye (1962) describes the development of the acrosome from a cup-shaped acroblast consisting of between 6 and 10 regularly spaced membranes. Along the outside border and filling the inside of the acroblast there are numerous vesicles similar to those associated with the Golgi apparatus in primary spermatocytes. In the early spermatid the acroblast and nebenkerne are close together near the base of the nucleus. In later spermatids there is a homogeneous pro-acrosome granule bounded by a double membrane situated within the cup of the acroblast. The acroblast rotates until its open end is facing the nucleus close to the developing axial filament (the latter is situated anteriorly). The pro-acrosome granule migrates towards the nucleus. It covers the anterior end of the nucleus and a sheet of membrane, the interstitial membrane,

appears between the pro-acrosome granule and the nuclear membrane. This membrane is continuous except for a space in the centre where there is an invagination into the granule. The granule now takes the form of a cone, the base having a darker appearance than the apical region. The invagination deepens and appears to contain a tapered structure of indistinct outline. This structure increases in size to form a hollow cone, the inner cone of the mature acrosome. In the mature sperm of Achata the acrosome consists of a cone within a cone. The whole structure is about 0.8 μ long. The outer cone is bounded by a double membrane. Although the inner cone is clearly outlined it possesses no membranes. Between the two cones there is a layer of diffuse material.

The acrosome of Hyalella sperm from the testis has several additional features to those described in Achata. The outer cone contains a series of longitudinally directed microtubules which appear to fuse at its anterior end. Within the cone, but outwith the cone membrane, are five rods which appear in transverse sections as five dark patches arranged in a circle and embedded in material of a lighter shade. These rods and the matrix in which they are embedded probably correspond to the acrosome "granule" in Achata. The acrosome in Hyalella is separated from the cell membrane by a thin layer of cytoplasm containing microtubules which partly surround the acrosome and extend backwards along the nucleus and associated "ragged" membrane. Over the anterior end of the cone there is a

vesicle. A further example of an acrosome with two systems of associated microtubules is found in Gerris remigis, the water strider (Tandler and Moriber, 1966). Tandler and Moriber suggest that the microtubules within the acrosome of Gerris are responsible for its rigidity and it is probable that the same applies to the microtubules within the cone of Hydrilla. The system of microtubules surrounding the cone in Hydrilla are only found in sperm still within the testis and are absent from sperm in the testicular duct. The acrosome of sperm from the testicular duct is a considerably simplified structure consisting of the two cones and capped by the vesicle. The possible function of the cytoplasmic microtubules will be discussed later.

Amongst the molluscs there is a great variation in the development and final form of the sperm mid-piece. In the cytoplasm of the spermatid of Viviparus (Gall, 1961) the mitochondria fuse into four nebenkerne which elongate and wrap spirally round the flagellum in a regular fashion. The mid-piece of Helix attains a final length of about 200μ and its development differs considerably from that seen in Viviparus. In Helix the mitochondria of the spermatogenia are described by Baccetti and Tahmisian (1954). They are between 0.2 - 0.3μ in diameter and in longitudinal section are seen to have a filamentous structure. There are 8 filaments of about 400 Å diameter. They are arranged parallel to one another. It is probable that these filaments are sections through longitudinal

coaxial lamellae continuous throughout the mitochondria. In transverse section they appear as four concentric lamellae about 400 Å in thickness. At the ends of the mitochondria the lamellae are continuous and form a dome. Grassé and others (1956) describe the development of the mitochondria in the spermatid. In the early spermatid the mitochondria are between 0.6 and 0.8μ in length, although there are a few instances where giant mitochondria of up to 2μ in length occur. At about the same time as acrosome formation begins, the structure of the mitochondria changes. The lamellae become squashed together around the periphery of each mitochondrion. The mitochondria become orientated around the flagellar shaft and fuse together. The lamellae fuse and form concentric rings around the flagellar shaft and fuse together. At this stage a layer of microtubules of about 200 Å diameter appears in the cytoplasm round the outer membrane of the mitochondria. These tubules are evenly spaced 200-300 Å apart. They do not extend the whole length of the mid-piece, being absent from the posterior region. The lamellae take on a granular appearance and, as the mitochondria elongate, the lamellae appear as rows of tubules arranged concentrically. As the mitochondria elongate still further the tubules become orientated radially and when elongation is complete the mitochondria are seen to be full of these tubules. A similar arrangement is described by André (1962) in Teatocella haliotidae Drap.

In Cinnagonoludina (Yasuzumi and Tanaka, 1958) the development of the mid-piece again differs. All the mitochondria of the early spermatid are located near the Golgi apparatus. The mitochondria have a limiting membrane and a system of cristae, which appear as double membranes, arranged radially. The cristae extend into the interior without reaching the other side, giving a doughnut appearance. As the development of the spermatid proceeds the mitochondria leave the Golgi complex, and migrate to the base of the cell where they aggregate into two nebenkerne. In each nebenkern the mitochondria fuse. Their limiting membranes are lost while the cristae persist at first tightly packed but randomly disposed. The cristae later appear radially arranged so that the nebenkerne are like the original mitochondria except they are larger. The flagellum extends out from a centriole situated in a pit in the base of the nucleus and the mitochondria increase in diameter and surround it. The mitochondria elongate until each nebenkerne derivative takes the form of an elongated body wrapped helically round the tail. Each of these bodies is limited by a double membrane and contains numerous tightly packed crista-like elements disposed parallel to one another and perpendicular to the long axis of the tail.

The development of the mid-piece in Nucella shows yet another variation. As in Helix the nebenkerne fuse so that the mid-piece isin the form of a torus surrounding the flagellum.

In Nucella the contents are not microtubular and only in one or two instances have occasional microtubules been seen in the cytoplasm around the developing mid-piece. In Nucella the early spermatid mitochondria fuse to form 4 or 5 nebenkerne. The nebenkerne elongate slightly and take up the form of a loose coil around the flagellar shaft. The cristae become arranged diagonally right across the mitochondria as seen in longitudinal sections. At this stage the outer mitochondrial membranes break down and fuse to form a sack surrounding all the mitochondria. The nebenkern elongates to about twice its original length, and the spiral becomes more compact. Between the cristae a series of small "secondary" cristae appear orientated at right angles to the "primary" cristae. These give the helically wound elements a ladder-like appearance.

The coarse fibers associated with the peripheral flagellar fibers in the tail of Nucella have been demonstrated to consist mainly of glycogen (Anderson and Personne, 1969). There is no manchette of microtubules associated with the tail as seen in birds and mammals (MacIntosh and Porter, 1967; Burgos and Fawcett, 1955). The coarse fibers of the sperm tail of Nucella are destroyed in isolated sperm where the tail is frayed.

The mature sperm nucleus of Nucella is a long slender cylinder about 40μ in length and of outer diameter of about 0.4μ.

The nucleoprotein of the mature sperm nucleus is homogeneous in appearance in that no fine structure can be seen within it. The early spermatid of Mucella is an irregularly shaped cell 3-4μ in width with a roughly spherical nucleus about 2.5μ in diameter. The nucleoprotein has a fine granular appearance at this stage. During the process of spermiogenesis there is a considerable change in nuclear shape and also a change in nuclear volume from about 8×10^{-12} cm^3 to about 4.5×10^{-12} cm^3, a decrease of about 50%. This change in shape of the nucleus and also the change in appearance of the nucleoprotein from fine and granular in the early spermatid to the homogeneous appearance of the mature sperm nucleus is a common feature in spermiogenesis of elongate sperm heads. However, the pattern of nuclear condensation may be one of the three main types which I have called "fibrous", "lamellar" and "granular". The nuclear condensation pattern in Mucella is a good example of the lamellar type of condensation.

The contents of the early spermatid nucleus of Mucella have a fine granular appearance, these granules being about 60 Å in diameter. The nucleus is about 2.5μ in diameter at this stage and is roughly spherical. The centriole penetrates through the nucleus until it comes to lie at the future anterior end, separated from the developing acrosome by a double layer of nuclear membrane and a thin layer of nucleoprotein alone. The nucleus elongates and by the time it is about 4μ in length, there is a change in the appearance

of the nuclear material. The nuclear material takes the form of a fibrous network made up of fibers of about 100 Å in diameter. They appear to be arranged in a gentle helical pitch round the nucleus. At this stage the flagellar shaft is twisted into a coil within a straight tube within the nucleus. The nucleus elongates further and the fibers fuse to form interlocking sheets. These sheets fuse to form lamellae 110 Å in width which are arranged either radially or looping back upon themselves and fusing with the nuclear membrane. These lamellae gradually become orientated around the outer and inner edges of the nucleus and eventually fuse together to give an apparent concentric pattern. The lamellae are not, however, concentric and there may be more lamellae on one side of the nucleus than on the other. The lamellae do not terminate at the ends of the nucleus. From some preparations of nuclei only partially broken down after pronase treatment, the nucleoprotein is seen to be folded back on itself at the base of the nucleus. The lamellae fuse further and when the nucleus has achieved its final length the nuclear material is homogeneous in appearance.

In my opinion the process of nuclear condensation and the arrangement of microtubules found associated with the spermatid nucleus during spermiogenesis must be considered together and not as separate topics for discussion. However, I think it is pertinent at this point to discuss the coiling phenomenon of Mucella prior to a full discussion of nuclear condensation and microtubules.

The flagellar shaft of Nucella sperm may, for the present discussion, be considered to consist of two portions; the head shaft which coils, and the tail shaft which does not. I consider that the potential for coiling of the head shaft is due to a condition which is imposed upon this part of the shaft at some stage in spermiogenesis.

The facts which have emerged from this study which, in my opinion, are relevant to the coiling problem are as follows. Disruption of the cell and nuclear membranes without dispersal of the nucleoprotein is sufficient to allow twisting of the head shaft and also to start a gentle coil. Tight coiling can only be obtained if all the nucleoprotein is removed. The coil is limited to the front 3/4 of the head, the shaft in the rear portion of the head remains uncoiled although it is twisted. When sperm are treated with detergent or concentrated sea water the tail frays whereas the head shaft twists and coils. In the early spermatid nucleus, which is about 3μ in length, the head shaft is thrown into a coil within the nucleus with the centriole at its anterior end, whereas in the mature sperm head, which is about 40μ in length, the head shaft is straight. Mature sperm in the testis have one or two banks of microtubules associated with a "ragged membrane" running down one or both sides of the nucleus. These microtubules extend over the anterior 3/4 of the head, and they are continuous with the microtubules which surround the acrosome. There are no such microtubules in mature sperm from the testicular duct.

On the basis of these observations I propose the following scheme to account for coiling of the head shaft. In the very early spermatid where the centriole and flagellar shaft have just penetrated the nucleus all the fibers of the flagellar shaft run straight. The length of the flagellar shaft within the nucleus increases without a corresponding increase in nuclear length, and so the head shaft is forced into a coil. In forming this coil the flagellar fibers become twisted and the coil remains imposed upon them. As the nuclear material condenses and the nucleus elongates to its final length of about 40μ the head shaft is untwisted and pulled out straight. This implies an active force which rotates one end of the flagellar shaft through about sixteen 360° turns. As the fibers are straight in the mature sperm but are able to become twisted again when the nucleus is dispersed, they must be held under tension and torsion in the sperm head.

If one end of the head shaft is allowed to rotate with respect to the other end without shortening then the head shaft will return to equilibrium in the twisted condition by relief of torsion. This is seen when sperm are spread on a Langmuir trough containing normal sea water. If the sperm head is completely and rapidly stripped the head shaft returns to equilibrium in a tightly coiled condition by relief of tension.

Several questions are raised by these suggestions. For example, what is the significance of the presence of microtubules during spermiogenesis? How and at what stage in spermiogenesis does untwisting of the flagellar shaft occur?

The microtubules only appear during the final stages of nuclear condensation. In the late concentric lamellar stage spermatid the outer edge of the nucleus has a fussy appearance due probably to the formation of microtubules. The microtubules are present during the last stages of spermiogenesis when the nucleus nearly doubles its length. The microtubules not only run along the side of the nucleus, but also surround the acrosome, and may at this stage act as a coupling device holding the nucleus and acrosome in alignment. The function of the microtubules is probably to assist in the pulling out of the nucleus to its final length and therefore also to extend and straighten the coiled flagellar shaft. In the mature sperm the microtubules disappear. The occurrence of microtubules associated with the nucleus during spermiogenesis and their subsequent absence in mature sperm has been described in several instances: for example, the domestic fowl, Gallus domesticus (McIntosh and Porter, 1967), the grasshopper, Melanoplus differentialis differentialis Thomas (Kessel, 1967), and the bee, Apis mellifera (Hoage and Kessel, 1968).

What then is responsible for maintaining the straight yet flexible form of the mature sperm head? Since the microtubules are absent they can not be involved. I consider that it is the nucleus itself which is responsible for maintaining the head shaft in a straight condition, that it is unable to do this until condensation of the nucleoprotein is complete, and that the microtubules of the immature sperm probably have a splint-like function in stretching and holding the shape of the sperm head while the nucleoprotein "sets" to a rigid state. In mature sperm the head shaft is "straight jacketed" in a close fitting and stiff nuclear tube.

How and at what stage in spermiogenesis untwisting of the head shaft occurs is uncertain. Elongation of the nucleus removes the coil from the head shaft but it is possible that the twist may be left still to be unwound by some further process. The coil runs clockwise from the anterior end and therefore one might expect the shaft to be twisted in an anti-clockwise direction. To attain the straight condition therefore one end of the shaft must be rotated with respect to the other end about 16 times in a clockwise direction from the anterior end. Subsequently, after unwinding is complete the straight shaft must be locked at both ends so that it can neither shorten nor rotate.

The twisting of the flagellar fibers was only seen in coiled sperm heads and there was no evidence of this twisting in the other

types of flagellæ which I examined. Recently Phillips (1969) has described twisted flagellar fibers in the sperm tails of the psocid Psocus, and the cat flea, Ctenocephalides. In longitudinal sections of the sperm tail of Psocus the flagellar tubules appear to form an angle of about 8° with the long axis of the cell. He describes other images where tubules on one side of the flagellum were transversely sectioned and those on the other side obliquely sectioned. This suggests that the flagellar fibers of Psocus describe a long pitched helix around a central rod. The triplet fibers of animal centrioles have also been reported to be disposed in a helix (André and Bernhard, 1964; Fawcett, 1966). The relationship between the possible helically disposed centriolar fibers is unclear, but, if the centriole is involved in the helical arrangement of flagellar fibers in Psocus, Phillips states that this involvement does not occur during flagellar formation as the tubules are initially parallel to the long axis of the cell. In Ctenocephalides, Phillips describes a slightly different situation. Here the peripheral flagellar fibers spiral round the central elements but it appears that the main axis of the flagellum also describes a helix and does not run parallel to the long axis of the cell. In this instance, the mitochondrial derivative is centrally located and the flagellum spirals round it. Phillips suggests that the several types of exceptional flagellae observed in insects have evolved independently

since there is a wide distribution of insect sperm with aberrant flagellae and in many of the orders which contain exceptional forms the majority of the species which he has examined possess typical flagellae.

Any evolutionary significance of the flagellar shaft passing through the nucleus in Mucalla is obscure. Indeed it seems unaccountable why an animal such as Mucalla where fertilisation is internal should produce enormous quantities of highly mobile sperm. Many questions with regard to fertilisation in Mucalla remain unanswered. Does the coiling of the flagellar shaft occur within the ovum after sperm penetration and the break up of the sperm head? Is the flagellar shaft cast off from the nucleus in some way, or does the whole sperm penetrate the egg?

When sperm from Mucalla are placed in distilled water, they all show a tendency to swell in the middle and bend into a hairpin configuration. A similar effect is described in mammalian sperm by Drevius and Eriksson (1966). They explain their results by an osmotic uptake of water followed by bending of the sperm. They state that the plane of bending is determined by the plane sectioning peripheral fiber pair 1 and passing between pairs 5 and 6. They found that if the salt concentration was raised to isotonicity, the swollen sperm lose water, straighten and regain normal flagellation.

To return to nuclear condensation patterns and associated microtubule patterns seen during spermiogenesis, a survey of the literature indicates a general preoccupation since 1963 with patterns of microtubules in spermiogenesis. This followed the introduction of glutaraldehyde as a fixative for electron microscopy (Sabatini and others, 1963). Prior to this time most studies on spermiogenesis included discussion of the changes seen in spermatid nuclei. I feel that these two topics should be dealt with in conjunction with one another. It is therefore important in reading and interpreting the various reports to note the fixation methods employed and also to note whether a report was made before or after 1963.

In normal flagellate sperm, that is sperm where in a general plan the various structures are aligned one after the other in the order acrosome, head, mid-piece and tail, the three types of condensation patterns which I have named are all seen.

Mucella shows a distinct lamellar condensation pattern. Microtubules are only found associated with the nucleus when the nucleoprotein has fully condensed and has a homogeneous appearance. However, the outer edge of the nucleus has a fussy appearance at the late concentric lamellar stage. This is probably due to the formation of microtubules. In Mucella therefore, microtubules are present from the late lamellar stage to the final condensed form, a period during which the nucleus more than doubles its length.

Lamellae similar in appearance to those seen in Mucalla have been described in Viviparus contectoides (Kaye, 1958). In Viviparus contectoides these lamellae do not have the apparent concentric arrangement in the late spermatid as seen in Mucalla, but become very compact as the head coils to its final helicoidal form. The apparent concentric formation found in Mucalla is probably due to the presence of the flagellar shaft running through the head. In Kaye's report there is no mention of microtubules.

There are other examples of lamellar condensation among the molluscs. Rebhun (1957) has described the changes in the nucleus during spermiogenesis in the pulmonate snail Otala lactea. In this animal the spermatid changes in shape from spherical to flame shaped. In the early spermatid the nuclear material is granular in appearance but aggregates into lamellae as elongation commences. These plates or lamellae of nucleoprotein are about 60 Å thick, 100 Å - 1μ wide and several microns in length. The lamellae are orientated in a regular fashion at the base of the nucleus and are radially arranged around the centriole which is embedded in the base of the nucleus. In the more anterior regions of the nucleus there is no apparent organisation of the lamellae when seen in transverse section. They may be parallel or perpendicular to the nuclear surface, occasionally they appear to be circular, some stop and start with no obvious connection to any other, some are relatively straight while others loop around. As elongation proceeds the nucleus twists, the axial

symmetry is lost, and the lamellae fuse to give the mature nucleus which is homogeneous in appearance. The material which Rebhun examined was taken from the ovotestis and fixed in osmium on ice. His micrographs of transverse sections of lamellar nuclei show faint microtubules around the nucleus. In a diagram of the lamellar nuclei he represents these tubules running in a helical fashion round the outside of the nucleus but does not make any further comment on their presence or function.

The lamellar nuclear condensation pattern during spermiogenesis is not limited to the molluscs. It is also found among the insects. Gibbons and Bradfield (1957) describe a lamellar pattern in the locust Locusta migratoria. In this instance the lamellae form a polygonal pattern before the final condensation. Gibbons and Bradfield fixed their material in osmium and found that in the cytoplasm surrounding the late spermatid there is some poorly fixed material which they describe as a "membrane". In view of more recent reports it is probable that this material could be microtubules which are poorly fixed due to their lability in osmium. Honeycomb patterns in late lamellar nuclei are described in the grasshoppers Dissosteira carolina and Melanoplus femur rubrum by Gall and Bjork (1958). Here again, fixation was with osmium and there is no mention of microtubules.

The fibrous type of nuclear condensation has been described in Cinanconaludina by Yasuzumi and Tanaka (1958). They examined material fixed in osmium taken from the testis. The early spermatid nucleus of Cinanconaludina is round, about 2μ in diameter, and the nucleoprotein has a fine granular appearance. As the nucleus increases in length and decreases in diameter the nuclear contents aggregate into dense fibers or filaments varying in diameter from 100-160 Å. As elongation continues these fibers become twisted and appear to be arranged in a loose helix. These fibers aggregate into thicker structures and their twisting results in the twisting of the whole head. Eventually the fibers fuse together to form a helicoidal head about 15μ in length and homogeneous in appearance. Finally, Yasuzumi and Tanaka describe a ring-like profile around the mature nucleus containing a number of dot-like profiles. In a diagram they represent these as fibers running in a helical fashion round the head and continuing anteriorly between the membranes of the head cap which surrounds the acrosome.

Kessel (1967) has described microtubules associated with an early spermatid nucleus where the nuclear material subsequently forms lamellae. In the grasshopper Melanoplus differentialis differentialis microtubules are fairly abundant in the early spermatid. Each early spermatid develops a concentration of dense granules against the outside of the nuclear envelope in a hemispherical

configuration, the centriolar adjunct. The proximal portion of the flagellum penetrates this region and terminates in a centriole which appears to be attached to the nuclear envelope. In association with the centriolar adjunct, numerous microtubules appear and extend radially from this region. These microtubules become closely associated with the early spermatid nucleus and Kessel suggests that they become arranged in a helical fashion around the elongating nuclei. During the course of elongation one to three concentric layers of microtubules surround the nucleus. As maturation of the nucleus proceeds the microtubules straighten and run parallel to the long axis of the head. When the sperm is fully developed the microtubules disaggregate and are absent from the mature sperm. The pattern of nuclear condensation in Melanoplus differentialis differentialis is essentially similar to that described in the locust (Gibbons and Bradfield, 1957).

A similar situation where there is a dense granular mass, or centriolar adjunct, surrounding the centriole, and microtubules associated with the spermatid nucleus throughout spermiogenesis is seen in the dragonfly Aeschna grandis (Kessel, 1966). Here again there are no microtubules associated with the mature sperm head.

In the honey bee Apis mellifera there is another variation. Hoage and Kessel (1968) describe the change in appearance of the chromatin in Apis from a dispersed state to coiled, fibrillar threads.

These threads are arranged perpendicular to the long axis of the sperm head unlike the usual parallel orientation seen in the examples described previously. Hoage and Kessel describe two or three layers of coiled microtubules which surround the nucleus. As the nucleus elongates these microtubules straighten and subsequently disappear in the mature sperm after nuclear condensation and elongation are complete.

The third type of condensation pattern, the "granular" type is seen in mammalian sperm (Burgos and Fawcett, 1955). The domestic fowl is also a good example of this type of condensation. In the fowl the early spermatid nucleus is spherical in shape and the chromatin is diffuse. As spermatid elongation commences the nuclear material becomes uniformly granular in appearance, the granules being about 30 Å in diameter. Throughout the elongation of the nucleus the chromatin remains in this state. Only when elongation of the head is complete do changes in the appearance of the nucleus occur. The nuclear material aggregates into larger "granules" about 350 Å in diameter around the edges of the nucleus. These granules increase in number and become more evenly distributed throughout the nucleus, although they are fewer in number in the central core of the nucleus. The granules increase in size and eventually fuse to give the mature nucleus its homogeneous appearance.

The microtubules associated with the nucleus of the fowl during spermiogenesis have been described by McIntosh and Porter (1967). The early spermatid nucleus is surrounded by microtubules arranged in a left handed double helix around the prospective long axis of the sperm. There are faint cross bridges connecting the consecutive turns of the helices. These microtubules are present throughout the elongation of the nucleus. When elongation has been completed the helically arranged microtubules disperse and are replaced by a manchette of microtubules which run straight along the length of the sperm head. These microtubules remain during the isotropic condensation of the nucleus. When condensation of the nucleus is complete the microtubules change in appearance and their walls become thicker in cross section. Eventually in the mature sperm they are completely dispersed.

Studies at present being carried out in the Zoology Department, St. Andrews, on sperm from the water beetle Agabus sulcatus (Mackie, 1969) indicate that in this animal there is a variation of the "granular" type of condensation. The early spermatid nucleus is spherical, and the nucleoprotein has a fine granular appearance and is evenly distributed throughout the nucleus. The periphery of the nucleus is completely surrounded by a jacket of microtubules. This study is still incomplete but it appears that the nucleus reaches its final shape, a highly asymmetrical ellipsoid about 9μ in length,

prior to any condensation of the nucleoprotein. The nucleoprotein condenses round the edge of the nucleus and in uneven clumps within the nucleus. There is no evidence of any structure in the condensed nucleoprotein (see Figs. 124-129). At this stage the microtubules still surround the nucleus and run parallel to its long axis. In the mature, fully condensed sperm head the nucleoprotein has a homogeneous appearance and the jacket of microtubules has disappeared.

Several functions have been postulated for microtubules. These include (1) the maintenance of the cytoarchitecture of cells or cytoskeletal function, (2) the intercellular migration of cytoplasmic components or cytoplasmic movements which may produce distinct cellular asymmetries and (3) cellular motility. All these different functions can be ascribed to the microtubules found during spermiogenesis and within the mature sperm.

Considering first the involvement of microtubules in sperm motility, it is generally accepted that flagellar fibers and microtubules are homologous structures and they have been grouped into different classes by Behnke and Forer (1967). As it is the flagellar region of most sperm that forms the motile part of the sperm the flagellar "microtubules" are evidently involved. In many insect sperm there are accessory microtubules associated with the flagellar shaft (Phillips, 1969) and this again implicates microtubules in the movement of cells. Amongst the birds and mammals some sperm

possess a manchette of numerous microtubules which extend back from the centriolar region into the cytoplasm surrounding the flagellar shaft (Burgos and Fawcett, 1955; McIntosh and Porter, 1967). Another situation where microtubules are implicated in sperm motility is in the sperm syncytium of the armored scale insect (Robison, 1966), and the sperm bundles of the mealybug (Ross and Robison, 1969).

The sperm of the mealybug Pseudococcus obscurus Essig are filamentous structures about 0.25μ in diameter and 300μ in length. Each sperm consists of a nuclear core 0.07-0.09μ in diameter surrounded by two and a half concentric rings of microtubules which are 200 Å in diameter. The microtubules are more numerous in the most actively motile region of the cell and fewer in comparatively less active regions. The sperm apparently possess no mitochondria, centrioles, typical flagellum or acrosome. The sperm are transmitted to the female in motile bundles, with approximately 16 sperm in each bundle. The motility of the bundle originates from the synchronous movements of its sperm which appear to be arranged in two concentric multi-stranded helices. This aflagellate sperm consists of little more than microtubules which Ross and Robison (1969) suggest might be the structural basis of its flagellar movement and undulations of constant amplitude. Also in P. obscurus sperm the microtubules appear to be the only structural components which could provide the rigidity required to maintain the shape of this extremely asymmetric cell. Another example of a motile aflagellate sperm is described by Christensen (1961). In the flatworm Plagiostomum

the sperm are motile but lack flagellae. They do, however, possess a prominent cortical system of microtubules running parallel to the long axis of the cell.

Porter and others (1964) have suggested that the microtubule in cell fine structure always accompanies cytoplasmic movements. Ledbetter and Porter (1963) observed that in plant cells the cortical cytoplasm contains numerous microtubules orientated in probable coincidence with the direction of cytoplasmic streaming. Similar observations have been made by Sabnis and Jacobs (1967) in Caulerpa. In another case, after lens induction in the chick eye the cells of the lens placode undergo an elongation, with no increase in volume. Byers and Porter (1964) concluded from their observations that the cortical system of microtubules, which develops in these cells just after induction, is responsible for these shape changes not only in the lens placode, but in the posterior lens epithelium as well. In both cases the microtubules subsequently disappear after cellular elongation is completed. Experimental studies on the axopod arms of the heliozoan Actinosphaerium (Tilney and others, 1966; Tilney and Porter, 1965, 1967; Tilney, 1968) indicate that an orientated system of microtubules is necessary for the formation and maintenance of the axopod arms in this protozoan. Taylor (1966) has shown that whenever a cultured cell forms an extension of itself, as in the formation of microvilli or microspikes, microtubules are found in this area.

Gibbins and others (1966, 1969) have studied the activity of the primary mesenchyme cells during gastrulation in sea urchin embryos. When the primary mesenchyme cells elongate and bulge into the blastocoel, microtubules appear from origins in the apical pole and run laterally to the nucleus and on to the bulge. Concomitantly these cells lose their cilia. Following their migration into the blastocoel they adopt a spherical form around a cytocentrum from which microtubules project in all directions. Later these cells develop slender, bristle-like pseudopodia containing microtubules associated in pairs. On the basis of these observations Gibbins and others suggest that the microtubules are a morphological expression of a framework which operates to shape cells and redistribute their contents. It has been postulated that the pseudopods of mesenchymal cells are essential in sea urchin gastrulation and that they are responsible for the migration and orientation of primary mesenchyme and the translocation of the archenteron by the secondary mesenchyme. Tilney and Gibbins (1966, 1969) investigated the effect of the antimitotic agents colchicine and hydrostatic pressure, and also the effect of heavy water on the structure and function of the microtubules in the pseudopods. Their results indicate that all these agents blocked gastrulation. The microtubules are dispersed after treatment with colchicine; heavy water, on the other hand, was found to cause stabilisation of the microtubules as it does in the mitotic spindle.

A further example of microtubules being implicated in intercellular cytoplasmic movement is described in Notonecta by Macgregor and Stebbings (1969). The trophic region of the ovariole of Notonecta is mainly in the form of a syncytium with many large nuclei arranged round a central cylinder of cytoplasm, the trophic core. From the posterior end of the trophic core, there extend backwards a number of cytoplasmic processes, the trophic tubes. These tubes are packed with numerous longitudinally directed microtubules. These tubes show strong birefringence when observed with polarised light. Macgregor and Stebbings have shown by labelling with ^{3}H-uridine that ribosomes move down the trophic tubes from the trophic core. They have treated ovarioles with colchicine and also cooled them to 2° C for 12 hours. The microtubules are destroyed by these treatments. After treatment with colchicine, the neck of the trophic region swells and its disorganisation results in the trophic nuclei invading the trophic core. The orderly arrangement of trophocytes, oocytes and the tubes in the prefollicular region disappears, the cells intermingle, and everything tends to flow backwards to fill the spaces originally occupied by the trophic tubes. They suggest that the microtubules preserve the trophic core and prevent large objects from entering and blocking the trophic tubes. They also state that in the ovarioles of Notonecta the microtubules facilitate a flow of ribosomes from

trophocytes to oocytes via the trophic core and trophic tubes by keeping these regions open. They suggest that the microtubules direct the flow along the trophic tubes although there is no direct evidence for this at present. Similar observations have been made by Hamon and Follet (1969).

There are many examples in the literature where microtubules are assigned a cytoskeletal function. To quote only a few of these:- the microtubules in the acrosome of Gerris (Tandler and Moriber, 1966); the sperm of the mealybug Pseudococcus obscurus (Ross and Robison, 1969); the axonemes of Actinosphaerium (Tilney and Porter, 1965); the rods of the cytopharyngeal basket of the ciliate Nassula (Tucker, 1968).

It appears that in the majority of typical flagellate sperm microtubules are frequently found associated with the nucleus during spermiogenesis and are subsequently absent from the mature sperm. There are, of course, exceptions. The sperm of the flatworm Dugesia tigrina (Silveira and Porter, 1964) possess a row of cortical microtubules situated just within the cell membrane. These microtubules extend the length of the sperm but strictly speaking should not be considered to be associated with the nucleus. Silveira and Porter consider these microtubules to be involved in two functions, the undulations of the sperm and also in the maintenance of the elongate form of the cell.

There are two questions which must be answered. What part do the microtubules play in spermiogenesis? Is there any correlation between the patterns of nuclear condensation seen in spermiogenesis and the arrangement of associated microtubules?

During spermiogenesis there is a change in shape of the nucleus from a compact spherical shape to an elongate attenuated form. The occurrence of microtubules during this change in shape at once implicates them in this change. In the domestic fowl McIntosh and Porter (1967) suggest an explanation of how the microtubules bring about the nuclear elongation. They suggest that the cross links between the two helices of microtubules which are wound round the nucleus during its elongation act as modified Maxwellian demons. These demons push apart the consecutive turns of the helices. McIntosh and Porter support this suggestion with mathematical evidence of how a helical system of microtubules could give rise to forces which would both constrict the diameter of the nucleus and guide its elongation. They also noted that where there were irregularities in the helical system of microtubules, aberrant spermatid nuclei are developed which bulge at the points where the irregularities occur. They finally suggest that the manchette of parallel microtubules serves to maintain the long form of the nucleus as the isotropic condensation of the nucleus occurs. The inherent rigidity of tubular structures would enable the manchette to support

the extended and flexible nucleus until it had condensed into a sufficiently rigid state to maintain its shape without external reinforcement. Here again evidence comes from aberrant spermatids where the manchette has failed to develop, and, as a result, the final nucleus instead of being a gently arched structure becomes tightly curved.

In Nucella microtubules are only present during the final stages of elongation, from the late lamellar stage to the final mature nucleus. The nucleus changes its shape from spherical to cylindrical without microtubules being present. I consider that it is the nucleus itself which is responsible for the first elongation, and the microtubules are only involved during the final stages of maturation. I think that the condensation of the nucleoprotein in Nucella is the means whereby the nucleus changes from a sphere of about 2.5μ diameter to a cylinder of about 20μ in length and about 0.74μ diameter. I consider that the function of the microtubules is to assist in the final elongation of the nucleus and, as I have stated earlier, to act as a splint while the nucleoprotein sets to its final form and is able to maintain its own elongated form. The microtubules surround the acrosome and extend back along the nucleus. Here they are probably holding the nucleus and acrosome in alignment. It may be suggested that the presence of the flagellar shaft running through the nucleus gives support to the nucleus and could also

provide a mechanism for its elongation. I do not consider this to be the case. From observations on the coiling phenomenon it is evident that the flagellar shaft is held under tension and torsion in the mature sperm head. I consider that it is the nucleus which pulls the flagellar shaft out straight from its coiled condition in the early spermatid.

The early reports on spermiogenesis in Cipangopaludina (Yasuzumi and Tanaka, 1958) and Otala (Rebhun, 1957) indicate the "microtubules" to be arranged helically around the mature sperm nucleus. I think it is very probable that if they had examined sperm from the testicular duct there would be no microtubules. I consider, in general, that in typical flagellate sperm microtubules are associated with the nucleus during spermiogenesis and have a splint-like function until the nucleus is able to maintain its own shape. The microtubules are subsequently lost. As stated by McIntosh and Porter (1967) the inherent rigidity of tubular structures such as microtubules is sufficient to maintain an elongated and flexible form of the nucleus until it becomes sufficiently rigid to maintain its own shape.

It may be the case in Nucella that the microtubules are actively growing and are being assembled from a store of precursor monomer in the cytoplasm, and, as a result, are pulling or pushing the nucleus out to its final form. However, in the late spermatid there is little residual cytoplasm around the nucleus and it is hard

to visualise the site of the monomer. I consider a more feasible hypothesis to be that the nucleus itself, by means of the way it condenses, is responsible for the nuclear elongation. The lamellar spermatid nucleus of Nucella can be compared to a stubby cylinder of plasticine. If the plasticine is compressed evenly along its length the resulting form is a longer, thinner cylinder of plasticine. I consider that the function of the microtubules is primarily splint-like in holding the nucleus straight so that the tendency of the flagellar shaft to coil is overcome and the nucleoprotein can "set" in the straight form of the mature nucleus. I consider that this is the case in elongate sperm where the pattern of nuclear condensation is of the fibrous or lamellar type, and where there are no microtubules associated with the early stages of spermiogenesis, and the lamellae and microtubules of the later stages are finally orientated parallel to the long axis of the head. A variation of this is seen in the dragon-fly Aeschna grandis (Kessel, 1966) where sheets of nucleoprotein are arranged parallel to the axis of the nucleus and banks of microtubules run straight along the nucleus throughout condensation. In Melanoplus differentialis differentialis (Kessel, 1967) the nucleoprotein forms lamellae arranged parallel to the long axis of the sperm head but there is also a system of helically wound microtubules associated with the early spermatid nucleus. These straighten and in the final stages of maturation run parallel to the long axis of the head. In both Aeschna and

Melanoplus the microtubules in the early spermatid arise in association with the centriolar adjunct which surrounds the centriole.

In the domestic fowl where nuclear elongation is completed before condensation commences and in the bee Apis mellifera (Hoage and Kessel, 1968) where the lamellae are perpendicular to the long axis of the nucleus it is evident that the nucleus cannot be responsible for its own elongation as I have suggested in Nucella. It is therefore evident that the helically arranged microtubules are required to elongate and constrict the nucleus as described by McIntosh and Porter (1967).

From the evidence available I consider that where the nuclear condensation pattern is such that the nucleus is able to bring about its own elongation, microtubules are only required as supporting structures in the final stages of maturation. Where the condensation pattern is granular or similar to that seen in Apis the nucleus is unable to engineer its own elongation and the helical system of microtubules provides the mechanism for nuclear elongation. In the grasshopper Melanoplus differentialis differentialis it is probable that the nucleus could engineer its own elongation but the helical microtubules associated with the centriolar adjunct, the dense granular material round the centriole, provide an additional mechanism for elongation.

In the majority of sperm there is a change in the histone content during spermiogenesis from lysine-rich to arginine-rich histone or protamine such as that found in Salmo fario (Pollister and Mirsky, 1946). Lake (1966) has shown histochemically that arginine-rich histone is present in the sperm of the domestic fowl. This observation is consistent with the biochemical observations of Daly, Mirsky and Ris (1951). Other sperm in which arginine has been found include the bull (Porter, Shankman and Melampy, 1951), the boar (Dallam and others, 1950) and human sperm (van Duijn, 1954). From wax sections of Nucella testis stained with fast green both with and without treatment with Van Slyke reagent, it appears that there is a change from lysine-rich to arginine-rich histone at the doughnut spermatid stage. I consider this to be the early stage of lamellar formation. The arginine-rich histones may bind together the DNA of the individual fibrils seen in the very early spermatid to form the lamellae seen in later stages. Bloch and Hew (1960) have shown in Helix aspersa, using tritiated arginine, that the change in histones is brought about by the synthesis of arginine-rich histones although the exact timing of this change is uncertain. A change to arginine-rich histone is not, however, always related to formation of lamellae. Sperm from the domestic fowl are reported to have a high arginine content. Kaye and McMaster-Kaye (1966) report the loss of non-histone protein from spermatids in Acheta prior to the start of nuclear condensation.

Gall and Bjork (1958) suggest that the lamellae they describe in the grasshoppers Dissosteira carolina and Melanoplus femur-rubrum are formed by the lateral association of the fine threads seen during the early period of spermatid elongation, and that these fine threads are essentially similar to those found in many other types of nuclei. They present as evidence for this the following observations: (a) preceding the formation of lamellae there is a stage during which fibers are present and are roughly orientated into plate-like bundles in the long axis of the nucleus; (b) the fibrous condition is very marked at the centriole end of the nucleus, and a transitional zone between fibers and plates is sometimes found near the base of the nucleus; (c) the fiber diameter in the early spermatid is approximately equal to the lamellar thickness.

The fibers of spermatid nuclei are comparable with those seen in other nuclei but attempts to correlate the fibers and lamellar structures seen in the early stages of spermiogenesis with chromosome structure are generally hampered by poor understanding of chromosome structure and lack of information about the arrangement of chromosomes within mature sperm. There is no agreement in the reports of the dimensions of chromatin fibers seen in sectioned material. Diameters quoted vary between 500 Å - 100 Å and possibly decreasing to 25 - 30 Å, the diameter expected for a single Watson-Crick double helix (Beermann and Bahr, 1954; Gay, 1956; Kaufmann and McDonald, 1956; Nebel, 1957; Ris, 1956; Kaufmann, Gay and McDonald 1960; Moses, 1960). Wolfe and Grim (1967) suggest

100 Å for the typical fiber diameter present in nuclei which they examined from the blood of the salamander Triturus viridescens and the testis of the milkweed bug Oncopeltus fasciatus.

It would seem that 100 Å diameter "chromosomal" fibers are the first to become apparent in sperm nuclei where the condensation is of the fibrous or lamellar type. Gall and Bjork (1958) discuss the association of these fibers into lamellae in some detail. They suggest that the fibers align side by side rather like the fingers of a hand. This implies a bilateral symmetry of the fibers such that a limited number of pairing groups would be available for association with adjacent fibers. Otherwise Gall and Bjork state that they would expect to find fiber association leading to the formation of thick bundles rather than flat plates. Gall and Bjork also state:

> "Whatever the nature of the fiber association we see no *a priori* reason to equate it with the well known pairing of homologous chromosomes which occurs earlier in meiosis; or to be more general, with any scheme based on genetic similarity of the fibers (since the spermatid of course contains only a haploid set of chromosomes). To arrive at this conclusion, we have considered the two theoretical possibilities that (a) a chromosome consists of only a small number of long sub-microscopic strands, or (b) it is composed of a

large number (say 10's or 100's) of identical strands. In the first case, a lamella could only be formed by a complex folding of the few strands of one chromosome or by the association of regions from several chromosomes. This fact follows directly from observed widths of the lamella which are certainly of the order of microns in the middle stage. In either case non-homologous portions of strands would have to be associated. On the other hand, if a chromosome is multi-stranded and the association "gene for gene", then we might expect to find the number of lamellae in some fashion related to the number of chromosomes (11 or 12 in Discoglossus and Melanoplus)".

Gall and Bjork suggest that the chromosomes might be lined up end to end in the spermatid head, so that a cross section at any one level would pass through only a single chromosome and its constituent lamellae. Furthermore there is no suggestion of any chromosome boundaries in the later stages of condensation or in the final mature nucleus which appears homogeneous.

More recently Hsu (1968) has studied the very early spermatids of the ascidian Boltenia villosa. From observations on thin sections he states that the chromosomes exist in the early spermatids in the form of 120 - 180 Å fibers which he maintains are easily seen to be composed of two sub-units of about 50 - 70 Å each. He also sees

fibers of 30 - 40 Å again paired. The smaller diameter he considers to be due to loss of protein. He states that there is nothing present along the length of these fibers suggestive of chromomeres. Also, where the fibers are seen disposed more or less parallel to the long axis of the elongating nucleus there is no evidence of grouping into discernible separate bundles such as to suggest the existence of multi-stranded chromosomes. At the poles of the elongating nuclei the fibers appear to fold back on themselves. On the basis of his evidence Hsu concludes that the double stranded fibers in the young spermatids are segments of extremely uncoiled chromosomes, possibly arranged in tandem and folding back and forth within the nucleus. He suggests that his micrographs clearly demonstrate that the chromosomes of Boltonia villosa go into the sperm head as two stranded structures, each strand most probably containing a single double helix.

I feel that deductions made from thin sectioned material on the possible arrangement of chromosomes within sperm heads should be viewed most critically. There may be variations in apparent diameters of fibers according to fixation, staining, plane of sectioning and also to the short lengths of fiber that can be seen in any given section. More can probably be learnt about the arrangement of nucleoprotein by means of spreading techniques and examination of whole mounts. Here again this is a comparatively new approach and care must be taken with the interpretation of the results obtained.

Spreading techniques were first used by Kleinschmidt and Zahn (1959) to study DNA and RNA preparations. Gall (1963) in preliminary observations showed that the fibers from newt erythrocyte nuclei could be spread on an air-water interface from which they can be transferred to a specimen grid for examination with the electron microscope. Gall (1966b) made further observations on chromosome fibers from the interphase nuclei of Triturus erythrocytes, metaphase chromosomes of cultured human cells and grasshopper (Melanoplus) spermatocytes and spermatids spread on the surface of water in a simplified Langmuir trough and dried by the critical point method (Anderson, 1951). The erythrocyte nuclei spread fibers varied in diameter between 250 - 300 Å and those of the human culture cells had similar dimensions. Spermatids from the grasshopper spread in the same way showed the nuclear material to consist of fibers of varying dimensions which were starting to aggregate into sheets. Gall found that in later spermatids where the nuclear material was completely fused into lamellae he could not obtain satisfactory spreading of the nuclei. Gall suggests that the 250 - 300 Å fibers may result from the folding or coiling of thinner fibers having the approximate dimensions of the nucleohistone molecule. Gall also indicates that the fiber dimensions seen can be considerably altered by contamination from the electron beam. It is therefore essential, in this type of work, to photograph material before contamination has started to build up around the critical point dried fibers.

Since the application by Gall (1963) of the Langmuir trough-critical point method to the study of chromosomes isolated from higher organisms, it has been used by several investigators (DuPraw, 1965a, b, 1966; Ris, 1966; Wolfe, 1965a, b; Wolfe and John, 1965; Wolfe and Hewitt, 1966) to examine chromosome fine structure and the following important observations have been made. First, there is a striking constancy in average dimension of the chromosome fiber (approximately 250 Å) isolated from different species. Secondly, interphase nuclei isolated by this method show no structure other than loosely coiled or folded 250 Å fibers. Thirdly, the differences between the interphase and fully condensed condition of the chromosomes have been shown to depend simply upon the degree of coiling or folding of the basic 250 Å fibers (DuPraw, 1965a, b; Wolfe, 1965; Wolfe and John, 1965). Fourthly, no central backbone, from which side loops extend, often proposed as a model for chromosome structure, has been observed in isolated metaphase chromosomes.

The major difficulty in the interpretation of results obtained by this technique is encountered in relating the dimensions of the isolated 250 Å fibers with the thinner 100 Å fibers which are seen in thin sections. Wolfe and Grim (1967) have made a study of the relationship between isolated fibers and the fibers of the embedded nucleus. They have shown that the change in diameter of the chromosome fibers occurs early in the breakdown of the nucleus, whether

spread on a water surface or lysed in hypotonic solutions. The isolated fiber diameter is consistent regardless of the method of cell breakdown. Wolfe and Grim have shown that the change in diameter is not as a result of the spreading forces of the Langmuir trough, or as a result of the critical point method. They find it difficult to evaluate their results on present evidence but suggest that although it is doubtful that the 250 Å fiber is an entity to be found in the nucleus of the living cell, the arrangement of the 250 Å fibers within isolated interphase and dividing nuclei may reflect an exact correspondence to the arrangement of chromosome fibers of the living nucleus.

A number of possibilities can be invoked to account for the increase of fiber diameter from 100 Å to 250 Å. Ris (1966) and Ris and Chandler (1963) suggest that the 250 Å fiber is made up to two 100 Å sub-units, in turn made up of two Watson-Crick double helices with associated protein. DuPraw (1966) on the other hand has developed a model for fiber fine structure in which the 250 Å fiber is made up of a supercoiled Watson-Crick double helix, held in a 250 Å coil by a contractile protein in the sheath of the fiber. However, DuPraw's pictures of trypsin digested fibers, which show thinner areas along the lengths of the fibers, do not show any evidence of coiling or uncoiling in the digested areas.

The spreading technique has only had a limited application on sperm heads to date. Solari (1968a) has examined the effect of different spreading conditions on the ultrastructure of the chromatin fibers of sperm from the sea urchin Strongylocentrotus purpuratus. Solari's standard procedure for spreading chromatin includes a previous chelation of divalent cations with EDTA. He found that chromatin spread on 0.1 M NaCl and 0.7 M NaCl showed folding and aggregation of 30 Å fibrils. He states that the plastic behaviour of the chromatin fiber is in agreement with the existence of a continuous axis bearing negative charges in each chromatin fibril. In a second paper Solari (1968b) discusses the structure of the chromatin fibers seen in spread sea urchin sperm nuclei. The spread nuclei show a mass of fibers with loops extending from the periphery of the main nuclear mass. He states that these outer loops are made up from single fibrils about 30 Å wide and which are regularly folded and shown to aggregate with other fibrils. These fibrils are completely destroyed by the action of DNase, but not by trypsin. Extraction of these fibrils with 2 M NaCl results in continuous and convoluted strands that are similar to DNA molecules. Solari (1967) has examined native DNA from sea urchin sperm by spreading with chymotrypsin and EDTA. He found that the strands of DNA show no evidence of discontinuities even after staining or shadowing. Strands of up to 100μ in length and 21 - 25 Å height were unaffected by proteases but rapidly destroyed by DNase. Solari (1968b) suggests

a structural relationship between the 250 Å fiber of Wolfe and Grim (1967) and the 30 Å fibers of the chromatin loops from sea urchin sperm. He suggests that the 200 - 300 Å fiber is formed by the irregular folding of the 30 Å fibril. If a regular folding exists *in vivo*, the histones could have a role such as the one assumed by Zubay (1964).

Lung (1968) has examined whole mounts of bull and human sperm after treatment with alkaline thioglycolate and critical point drying. He found that the nuclei, in an unpacked state, are composed of a network of chromatin fibers. Single fibers range from 140 - 240 Å in diameter and exhibit possible cross fibers or branches. Lengths of measured fibers could be traced for at least 12µ.

The whole mounts of sperm described by Lung and Solari are all of sperm with comparatively small round heads and there appears to be no organized pattern to the breakdown they have described. *Mucella* is an example of an elongate sperm head. In *Mucella* the spreading of sperm on normal sea water only results in the slight swelling of the head. Spreading of the sperm on x 2 concentrated sea water proved to be an adequate method for demonstrating the coiling of the flagellar shaft but at the same time brought about more or less complete dispersion of the nucleus. Treatment of *Mucella* sperm with the enzymes pronase or trypsin, or with urea, have clearly demonstrated that the nucleoprotein is not a jumbled

mass of fibers but has a definite pattern. The nucleoprotein is in the form of a sheet which seems to be wrapped helically around the flagellar shaft. As breakdown of the nucleus proceeds this sheet breaks up into narrower stripe which again split into still narrower structures. This process continues down to 40 Å fiber seen in preparations after urea treatment. That these fibers probably contain DNA molecules is demonstrated by their absence after incubation with DNase.

From the results of fast green staining it would appear that there is a change in histone from lysine-rich to arginine-rich at the time when the lagellae are first formed. Presumably the binding of the nucleoprotein fibers to form the compact homogeneous nucleus of the mature sperm is due to histones and proteins. The action of trypsin and pronase results in the breakdown of this binding. 8 M urea is used biochemically to extract DNA. I think that the action of the urea on Mucella sperm heads is to dissociate the protein and the DNA. As this was carried out by floating whole sperm attached to grids with formvar/carbon films on the surface of the urea solution it is probable that the DNA is adsorbed from the urea solution on to the grid film. This is the principle described by Kleinschmidt (1968) in monolayer techniques for electron microscopic examination of nucleic acid molecules. Protein films are considered as monolayers and the DNA is adsorbed to the protein by basic side groups of amino

acid residues. Adsorption effectively brings the nucleic acid molecule from a three-dimensional position in an aqueous solution to a two-dimensional position adsorbed on to the protein monolayer.

In his observations on sperm from Nucella Callan (1952) noted that the birefringence of the sperm head was intrinsic and not form birefringence. The needle-shaped sperm heads found in a wide variety of organisms show a strong negative birefringence (Schmidt, 1941). The birefringence is intrinsic and has been attributed to a regular orientation of DNA (-protein) molecules aligned parallel to the length of the sperm head. Inoué and Sato (1962) have shown that the negative birefringence of various insect sperm heads can be abolished locally when a small spot of ultraviolet of wavelengths between 2500 and 3100 Å is shone on the sperm. They suggest that this loss of birefringence must reflect an *in situ* alteration and disorientation of the DNA molecules since no loss of refractility accompanies the change of birefringence. These observations were made on the mature sperm of the cave cricket *Centrophilus nigricans*, in which the axis of birefringence is only on the average parallel to the geometric axis of the sperm. With a high resolution rectified polarising microscope the optic axis is found to zigzag regularly within the smooth head as though the "crystalline" material were ordered into a microscopic helix (Inoué and Hyde, 1957). When these sperm are exposed to ultraviolet

polarised at 45° to the head, the zigs and zags each respond independently to the irradiation and lose their birefringence at different rates depending upon their azimuth orientations. From their observations Inoué and Sato suggest that there are at least two orders of coiling or zig zagging of DNA (or their bases) in the sperm head, with the average molecular axis orientated parallel to the long axis of the head. They postulated the following model for the packing arrangement of chromosomes and DNA in the cave cricket sperm head. The 20 - 30 Å thick DNA (-protein) molecules, presumably as a bundle several hundred angstroms in diameter, are wound to form a very long helix approximately 2000 Å thick. This in turn is wound into another elongated helix approximately 6000 Å in diameter, two such helices intertwined together with some matrix making up the sperm chromosomes. The microscopic zigzags would reflect the gyres of the intertwined chromonemata while the overall optical property of the sperm head reflects the alignment of those portions of the DNA molecules which within the coiled coil run roughly parallel to the sperm head.

In Mucalla, even after treatment with 8 M urea and the partial breakdown of the nucleus, where the nucleoprotein appears in the form of a sheet which seems to be helically wound round the long axis of the head, there is still strong birefringence. These observations indicate that the DNA is arranged predominantly parallel to

the long axis of the sperm. This is in agreement with the results of McInnes and Urets (1968) and also Inoué and Sato (1962) although they disagree as to whether the DNA is in a supercoiled or uncoiled condition. From the results of Inoué and Sato, McInnes and Urets and, most convincingly, Taylor (1964) it would appear that chromosomes are arranged in tandem along the length of elongate sperm heads. This no doubt is the case in Mucalla but it is not possible to say where the chromosome boundaries are situated, or to suggest how each chromosome is folded within the nucleus to give the patterns seen during nuclear elongation.

DNA is not arranged in parallel in all elongate sperm heads. Sperm from the domestic fowl are not birefringent. After treatment with urea these sperm do not show an organised pattern of fibers as seen in Mucalla, but are more reminiscent of isolated chromosomes as described by Wolfe (1965a), but without the long fibers extending from the main axis. There is, however, in the fowl, as in Mucalla, a reasonable correlation between the breakdown patterns seen after urea treatment and the pattern of nuclear condensation seen in spermiogenesis. I have stated that the arginine-rich histones are responsible for the binding of the nucleoprotein into sheets or lamellae. The significance and function of arginine histones in the fowl sperm is unknown but must be different to that in Mucalla.

Recently, Bloch (1969) has made a survey of basic proteins of sperm nuclei. The basic proteins vary widely among different organisms and for convenience are placed in five major classes; the monoprotamines (e.g. salmon); stable protamines or basic keratins (mouse); intermediate proteins (mussel); typical histones (frog); and relatively non-basic proteins (crab). Bloch has attempted to correlate protein type with sperm function and considers the following hypotheses. The highly basic proteins of sperm cause condensation of chromatin and consequent streamlining of the cell. The protein inhibits gene transcription. The protein erases the developmental history of the cell, providing a totipotent nucleus. The protein protects the chromosome from the adverse effects of the environments to which sperm are subjected. The protein reflects some requirements for subsequent function of the chromosomes during early ontogeny. Whatever the function, protein type reflects phylogenetic relationships. Bloch states that, in general, groups of species represent extremes in behaviour with regard to any one aspect of function also contain a range of histone types. No correlation is evident. Protein type seems to be of little consequence for sperm function. He proposes that the variability of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus. He suggests that the generally high arginine content of these proteins may be attributed to the fact that there are more triplets that code for arginine

than for lysine and histidine. Some histone genes may be freed from the constraints of selection in organisms in which sex determination has become chromosomally based and in which genes controlling sperm cell function have become localised on the sex chromosome. This hypothesis predicts that sperm of organisms that have never gone through this evolutionary sequence should contain typical histones and that histone variants should be less prevalent among the sperm of organisms in which secondary hermaphroditism or sex by differentiation had been established long ago. This hypothesis can not, however, be substantiated until all the functions of histones in sperm are understood.

Although present evidence is insufficient to give any definite suggestions as to the arrangement of chromosomes and protein within mature sperm heads, I feel that this work gives an indication of techniques, which if further applied could add considerably to an understanding of this particular unsolved problem.

SUMMARY

Investigations have been carried out along three main lines. Spermatogenesis and the fine structure of the mature sperm of *Mucella lapillus* (L) have been studied both by light- and electron-microscopy. The testis consists of numerous tubules, all directed inwards and joining to form a common testicular duct. In a single tubule the spermatogonia lie round the periphery. Mature sperm line the lumen of the tubule. Cells in the same stage of spermatogenesis are grouped together and all members of a group pass through spermatogenesis in phase.

Staining with fast green before and after treatment with Van Slyke reagent indicates a change from lysine-rich to arginine-rich histone in the maturing spermatid.

Sperm of *Mucella* are motile throughout their length. The sperm are threadlike and about 80μ long. The head is Feulgen-positive and about 40μ long. The mid-piece lies behind the head and is about 8μ long. The flagellum runs from the front end of the head to the tip of the tail; in the head it is completely surrounded by the nucleus.

The spermatogonia contain two centrioles situated near the nucleus and a conspicuous Golgi complex. There are synaptinemial complexes in spermatocyte nuclei in the synapsis stage. In the

early spermatid the centriole pushes a tube through the nucleus. This tube is lined throughout by nuclear membrane and is occupied by the anterior portion of the flagellar shaft. The nucleus elongates and the nucleoprotein condenses into strands arranged helically along the long axis of the nucleus. These strands fuse to form lamellae, which disappear in the mature sperm. Mitochondria aggregate at the base of the early spermatid nucleus and form a loose spiral around the flagellar shaft. The outer mitochondrial membranes fuse. The mid-piece of the mature sperm consists of a large tubular mitochondrion enclosing a portion of the flagellar shaft. At the early spermatid stage a pro-acrosomal granule is formed from a large Golgi complex. From this the acrosome develops; it consists of a cone and an acrosome granule. There are two sets of microtubules associated with the acrosome, one lying within the cone, the other outside the cone and separated from it by a "ragged membrane". The microtubules of the outer set extend backwards along the head for 3/4 of its length. These microtubules are only found associated with the sperm in the testis, they are absent from the fully mature sperm in the testicular duct. The centriole which gives rise to the flagellar shaft lies at the anterior end of the head and is separated from the acrosome by a thin layer of nucleoprotein and a double layer of nuclear envelope. There is no second centriole or derivative thereof in the mature sperm. In the tail groups of coiled fibers are associated with each pair of the peripheral flagellar fibers.

The coiling phenomenon exhibited by the flagellar shaft in the head region of the mature sperm has been studied in detail. This phenomenon was first mentioned by Retzius (1912) who showed that in sperm macerated in water, the "Zentral-faden", as he called it, was thrown into a coil. In Nucella the flagellar shaft runs from the acrosome at the front of the head to the tip of the tail. Its anterior region, the head shaft, is ensheathed by the nucleus. If a suspension of sperm in normal concentration sea water is observed under phase contrast, and allowed to dry out slowly, the nuclear material of sperm near the edge of the coverglass swells and the head shafts of these sperm are thrown into gentle spirals within the nuclei. In some sperm the nuclear material disperses completely, and the front three-quarters of the head shaft springs into a tight right-handed coil of 5-7 turns. Instantaneous coiling of the head shaft may also be induced by treatment of the sperm with x 2 concentrated sea water or 0.01% (w/v) solution of sodium lauryl sulphate in sea water. The enzymes pronase and trypsin at a concentration of 50 µg/ml in sea water at pH 8.0 cause dispersion of the head nucleoprotein and subsequently the head shaft forms a loose coil. The appearance and activity of the sperm do not change perceptibly over a pH range of 5.5 - 8.5.

Sperm spread on a Langmuir trough containing normal concentration sea water were negatively stained with phosphotungstic acid and examined with an electron microscope. After such treatment the

nuclei are partially spread and the fibrils of the head shafts appear twisted as the wires of an electrical flex. The twisted flagellar fibers are seen more clearly in negatively stained head shafts of sperm whose nuclei have been completely dispersed by spreading on a Langmuir trough containing x 2 concentrated sea water. Negatively stained preparations of sperm treated with enzymes show sperm with twisted and coiled head shafts. The nucleoprotein of these sperm is disaggregated into strips or sheets, or may appear as a mass of branching fibers. The flagellar fibers of the enzyme treated sperm are often bent or broken.

Longitudinal sections of mature sperm heads show that in the intact sperm the fibers of the head shaft are not twisted but run straight throughout the length of the head. As an explanation of this coiling phenomenon I have suggested that the head shaft as a consequence of some event in spermiogenesis, has an inherent tendency to twist and coil but in the mature sperm it is "strait-jacketted" by the sperm nucleus.

The arrangement of the nucleoprotein in the mature sperm head has been investigated by examining the nucleus in the process of breakdown after treatment with the enzymes pronase and trypsin and with 8 M urea. Material treated with these agents was dried by the critical point method and rotary shadowed with platinum/carbon, negatively stained or positively stained prior to examination with

the electron microscope. The nucleus of Mugilla sperm breaks up into a sheet of material which splits up further to form a branching network of fibers which show no evidence of free ends, but tend to form loops. There appears to be some correlation between the pattern of nuclear condensation seen during spermiogenesis and the pattern of breakdown of the nucleus after experimental treatment. As a comparison similar studies have been made on the sperm of the domestic fowl.

The main topics which are discussed are nucleoprotein condensation during spermiogenesis, the mechanism of nuclear elongation, microtubules and the arrangement of nucleoprotein in the mature sperm head.

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- A P P E N D I X I -

SPERMATOGENESIS AND THE STRUCTURE OF THE MATURE SPERM in *Nucella (Purpura) lapillus* (L.)

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INTRODUCTION

Gastropod sperm are usually long and thread-like. They conform to the typical plan of organisation of a flagellate animal sperm in that they have an acrosome, head, mid-piece, and tail. The head may be straight as in Bianca inconspicua (Fransén, 1955) or twisted as in Viviparus (Paludina) viviparus (Von Siebold, 1836). The acrosome is situated at the anterior tip of the sperm. The mid-piece lies immediately behind the head and consists of a mitochondrial sheath enclosing the flagellar shaft. The tail has the usual arrangement of flagellar fibrils. The proximal end of the flagellar shaft is often embedded in the base of the nucleus. In spite of a general conformity, however, there are peculiar features in the sperm of some molluscs. The sperm of Nucella (Purpura) lapillus is odd in that the whole sperm, from tip to tip, is capable of vigorous bending and lashing movement. It was this feature which prompted the present investigation into the genesis and structure of these sperm.

Retzius (1912) described in the sperm of Nucella a central fibre ("Zentralfaden") running from the acrosome to the tail tip. In the head region he noted that the central fibre was enshrouded by the sperm nucleus. When sperm were macerated in water the nuclear material swelled and the central fibre became twisted into a coil. Retzius saw these same features in sperm from Littorina.

Because the sperm head of Nucella has an axial shaft extending throughout its length and is motile we thought that the development and ultrastructure of such sperm might present some unusual features. We have therefore investigated spermatogenesis and sperm structure in Nucella by light and electron microscopy, and we have paid particular regard to the sperm head.

MATERIALS AND METHODS

All whelks used in this study were collected from the Kinkell Rocks region of St. Andrews Bay.

The testis, testicular duct, and parts of the adjacent digestive gland were cut from freshly opened whelks and fixed in sea water Bouin (165 ml sea water saturated with picric acid, 55 ml 40% formaldehyde, 11 ml glacial acetic acid) for 12 h, embedded in wax, and sectioned at 4μ. Sections were mordanted for 1 h in 4% ferric alum, stained for 1 h in Heidenhain's haematoxylin, differentiated in 3% ferric alum, counter stained with 1% aqueous eosin, dehydrated and mounted in balsam.

For detection of basic proteins by the method of Alfert and Geschwind (1953) some sections were stained for 30 m with a 0.1% solution of fast green FCF in phosphate buffer at pH 8.2. Other sections were treated with boiling TCA for 30 m, washed, and stained for 30 m in fast green at pH 8.2. The sections were subsequently

washed in buffer, rapidly dehydrated, and mounted in balsam.

To determine the specific nature of histones with respect to lysine or arginine content the deamination procedure of Van Slyke (1911), as described by Monné and Slautterback (1950), was applied. Sections were treated with boiling TCA, washed, treated with Van Slyke reagent for 1½ h at room temperature (65° F), and stained with fast green at pH 8.2. The Van Slyke reagent affects primarily the amino groups of lysine and not the guanidine groups of arginine (Deitch, 1955; Olcott and Fraenkel-Conrat, 1947). Consequently, histones rich in arginine stain with fast green; those rich in lysine do not stain.

For observation of living sperm the contents of the testicular duct were diluted about 100 fold with filtered sea water and a drop of this suspension was placed on a slide and covered with a coverglass.

For examination of fixed and stained sperm thin smears of sperm were prepared and placed for 30 m in a chamber saturated with formaldehyde vapour. The slides bearing the smears were then placed in 4% formaldehyde for 1 h. The smears were then stained by the Feulgen technique according to the method of Swift (1955). Other smears, fixed in formaldehyde as described above, were stained by the acid fuchsin - picric acid method of Altmann (1890). The latter is a simple and useful method for locating mitochondria and was used here to determine the position and limits of the mid-piece.

All light micrographs were made with a Carl Zeiss Photomicroscope on Ilford Pan F film. The microscope was fitted with planapochromatic objectives for examination and photography of stained specimens. Neofluar objectives and a Zeiss microflash were used for phase contrast micrography of living material.

For electron microscopy small pieces of testis and testicular duct were taken from freshly opened whelks and placed directly in 1% osmium tetroxide buffered to pH 7.4 with veronal acetate (Palade, 1952). They were fixed for 1½ h, dehydrated through an acetone series and embedded in Vestopal W. Sections 50 to 80 mμ in thickness were cut with glass knives on a Cambridge Ultra-microtome (A.F. Huxley pattern) and mounted on Athene 483 grids without supporting films. The sections were stained with 2% uranyl acetate for 8 m followed by 2 minutes in lead citrate (Reynolds, 1963). The sections were examined with a Siemens Elmiskop I (80 kv) at negative magnifications of 6,000 to 40,000.

OBSERVATIONS

In the mature male whelk the testis lies to one side of the upper region of the visceral mass. It is deep yellow in colour and is spread over the lobes of the digestive gland. It consists of numerous tubules, all directed inwards. The tubules join one another to form a single testicular duct which is white in colour and passes along the surface of the digestive gland on the columellar side.

This duct acts as a vesicula seminalis. At its anterior end a sphincter closes the entrance to a ciliated duct which runs beneath the intestine and pericardium to the prostate gland. From the anterior end of the prostate gland a narrow vas deferens passes along the right side of the head to the penis which lies behind the right cephalic tentacle.

Haematoxylin stained sections of the testis show tubules containing spermatogonia, spermatocytes, spermatids, and mature sperm, and branches of the testicular duct packed with mature sperm. In the tubules the spermatogonia are arranged in groups around the periphery and the mature sperm are clustered together round the inside of the tubule with their tails directed towards the lumen. Groups of spermatocytes and spermatids are scattered throughout the tubules. The spermatogonia have irregularly shaped nuclei. Some spermatogonial mitoses are usually evident. Cells in all stages of the first meiotic division can be identified. In synaptic and post-synaptic nuclei the chromosomes are usually arranged in an untidy bouquet. Cells in second meiotic metaphase are rare. Spermatid nuclei cut transversely look like signet rings, with chromatin massed to one side of the nucleus (Fig. 2), or, in later spermatids, like dough-nuts, with the chromatin formed into a ring. Longitudinal sections of the latter type of spermatid nucleus show the nucleus to be oval in shape and longitudinally bisected by a thin clear line

(Figs. 2, 3). Mature sperm are most abundant in the testicular duct. They are bunched together and their heads stain darkly with haematoxylin.

Sections of testis stained with fast green at pH 8.2 without prior extraction with TCA remained unstained. In sections which had been treated with boiling TCA and subsequently stained with fast green at pH 8.2 all nuclei stained intensely (Fig. 2). In sections which had been treated with TCA and the Van Slyke reagent and then stained with fast green only the mature sperm and the late "doughnut" spermatid nuclei were stained green (Fig. 3).

The living sperm of Mugella, when viewed in phase contrast are all alike. They are 80 ± 2μ long (Fig. 4). Apart from the pointed acrosome and a slight tapering of the tail they are of even diameter throughout their length. The sperm are motile throughout their length. They move with a vigorous bending and lashing motion. The movements of the head have a lower frequency than those of the tail. The living and freely swimming sperm appear with uniform contrast throughout their length.

In sperm smears stained with the Feulgen reagent after acid hydrolysis the sperm heads are stained a deep pink (Fig. 5). They measure 40 ± 2μ in length. Altman's acid fuchsin-picric acid technique stained only the mid-piece of the sperm. They lie immediately behind the head and are 7 to 9μ long (Fig. 6).

Electron microscopy

Spermatogonia are about 4μ wide (Fig. 7). Their nuclei are large and lobed. The nuclear envelope is double and perforated by pores. The chromatin is unevenly distributed giving the nucleus a patchy appearance. The cytoplasm contains a few mitochondria of varying sizes, and numerous small granules. There are several Golgi complexes each made up of a stack of parallel lamellae and a number of small round vesicles. Close to one of the Golgi complexes is a pair of centrioles. These are arranged at right angles to one another. The larger of the two centrioles measures 300 mμ long and 150 mμ wide. In some spermatogonia 4 centrioles were observed. Such cells were probably about to divide mitotically.

The primary spermatocytes are 3 to 4μ wide (Fig. 8). The nucleus nearly fills the cell. The chromatin is patchy and synaptinemial complexes (chromosome cores) are visible in most sections. The cytoplasm contains a few round mitochondria and several Golgi complexes.

Early spermatids are irregularly shaped cells 3 to 4μ wide (Fig. 9). The cytoplasm of these cells contains numerous small mitochondria which are clustered together at one side of the nucleus. Golgi complexes are evident in some sections but have fewer associated vesicles than those of earlier stages. In some sections a Golgi complex and pro-acrosome granule may be seen at the opposite side of

the nucleus from the mitochondrial cluster. The nuclear material is evenly distributed throughout the nucleus in the form of a granular reticulum. The cytoplasm of adjacent spermatids is often continuous. We think that in such cases the cell membrane was incompletely reformed after the second meiotic division.

The nucleus of an early spermatid is about 3μ long and is penetrated by a blind ended tube about 600 mμ in diameter which is lined throughout by nuclear membrane and which accommodates a portion of the flagellar shaft. The flagellar shaft also extends backwards from the nucleus as the developing sperm tail.

As the spermatid nucleus elongates the nuclear material condenses. At first it consists of numerous interlocking strands each about 450 Å in diameter arranged in a loose helix along the prospective long axis of the sperm (Fig. 10). As condensation proceeds the nucleoprotein strands fuse together into lamellae which, in transverse section, are often radially arranged with respect to the flagellar tube with one or both edges of the lamella closely applied to the nuclear membrane (Figs. 11, 12, 13). The spermatid nucleus at this stage measures about 3.5μ by 1.75μ. The flagellar tube extends from end to end of the nucleus and is straight; but the portion of the flagellar shaft which it accommodates is loosely coiled within the tube (Fig. 10). The nuclear lamellae are at first widely spaced but they gradually fuse as the nucleus becomes longer

and narrower until only 12 to 15 concentric, closely packed lamellae are discernible in transverse sections (Fig. 14). Further condensation of the nuclear material occurs until, in the mature sperm, it presents a completely homogeneous appearance (Fig.15).

The sperm head consists of a cylinder of nuclear material which encloses the anterior portion of the flagellar shaft. The outer diameter of this cylinder is about 400 mμ tapering to about 250 mμ at its anterior end. The sperm nucleus is an elongated tube closed at its anterior end by a double layer of nuclear envelope. The walls of the tubular nucleus are about 100 mμ wide in the rear half of the sperm head but they narrow to a width of about 50 mμ at the front of the head. Behind the double nuclear envelope at the anterior end of the nuclear tube is a centriole. This shows the typical catherine wheel arrangement of 9 triplet elements, and from it the flagellar shaft extends backwards. Outside the nuclear membrane in a thin layer of cytoplasm which surrounds the nucleus are the microtubules which stretch backwards from the acrosome. These never surround the nucleus completely but appear in banks, either as a single bank down one side of the nucleus or in 2 banks at opposite sides of the nucleus (Fig. 15). The microtubules lie parallel to a flat double membrane which is distinct from the nuclear membrane. This membrane is an extension of a "ragged membrane" which surrounds the acrosome.

The small mitochondria that aggregate at the base of the nucleus in the early spermatid fuse into four or five large nebenkerne which are grouped around the flagellar shaft (Figs. 10, 16). As the spermatid elongates these mitochondria stretch backwards along the flagellar shaft. The latter had already reached its final length of about 80μ (Fig. 16). They appear in longitudinal section arranged in a loose spiral round the flagellar shaft. The outer mitochondrial membranes between individual mitochondria break down and finally all the mitochondria become enclosed by a common outer membrane (Fig. 17). The length of the mitochondrial sheath at this stage is about 3μ.

The mid-piece of the mature sperm lies immediately behind the nucleus and consists of a flagellar shaft surrounded by a mitochondrial sheath. The outer diameter of the sheath is about 400 mμ. In longitudinal sections the mitochondrial elements appear to be arranged in a helical fashion (Fig. 18). The mitochondrial sheath is separated from the flagellar shaft by a layer of cytoplasm; this differs from the situation in the sperm head where the nuclear membrane is closely applied to the flagellar shaft.

The acrosome develops alongside a large Golgi apparatus. The later consists of a stack of lamellae in the characteristic horse-shoe arrangement, and many associated vesicles (Fig. 9). Acrosome development starts at the early spermatid stage when the flagellar shaft has penetrated the nucleus, but before nuclear elongation has begun. A

pro-acrosome granule is formed from the associated Golgi apparatus. Both Golgi and pro-acrosome lie to one side of the nucleus or at its anterior end. The pro-acrosome granule forms into a cylinder surrounded by membrane and with a slight indentation in its base (Fig. 19). The cylinder elongates and becomes tapered, and the indentation in its base deepens. Some of the membranes of the Golgi complex are often continuous with the membrane which surrounds the pro-acrosome granule (Fig. 19). The pro-acrosome migrates to the anterior end of the nucleus and takes up a position directly over the centriole. The diffuse material at the base of the pro-acrosome forms a plate, the "interstitial membrane" (Kaye, 1962), between the developing acrosome and the nucleus (Figs. 10, 19). The invagination in the base of the pro-acrosome deepens further until the latter has the form of a cone surrounded by a double membrane. Inside the cone a series of longitudinally directed microtubules are formed and within the invagination an acrosome granule appears.

The acrosome of the mature sperm is terminal and pointed. It is about 1.2μ long (Figs. 1, 20). Its main component is an acrosome cone which is about 1μ long (Figs. 1, 20). The cone consists of a bounding membrane within which is a ring of longitudinally arranged tubules (Figs. 21, 22). These tubules merge at their anterior ends and consequently cannot be resolved in transverse sections through the tip of the cone. The diameter of the base of the cone is about 0.3μ. At its base the cone widens slightly to form a

lip which is directed inwards. Within the cone, but outside the cone membrane, are 5 rods which appear in transverse sections as 5 dark patches arranged in a circle and embedded in material of a lighter shade (Fig. 21). We think that these rods and the matrix in which they are embedded correspond to the acrosome "granule" described in Achata domestica (Kaye, 1962). The acrosome is separated from the cell membrane by a thin layer of cytoplasm. Within this cytoplasm and close to the acrosome lies a bank of microtubules. Each tubule is about 100 Å in diameter (Fig. 21). The tubules partially surround the acrosome and extend longitudinally from near the tip of the acrosome backwards along about three quarters of the length of the sperm head (Fig. 15). Between the microtubules and the acrosome cone is a discontinuous "ragged membrane" (Fig. 15). The latter fuses with the outer cone membrane near the apex of the cone where a conspicuous thickening of the cone membrane is evident (Figs. 1, 20). The tip of the acrosome cone consists of a vesicle which is bounded on the inside by the outer cone membrane and on the outside by a continuation of the ragged membrane (Fig. 20). Remnants of the interstitial membrane lie between the acrosome and the nucleus.

Behind the mid-piece the flagellar shaft extends backwards into the tail. Within the cell membrane and outside each of the pairs of peripheral flagellar fibrils there is a group of coarse fibres (Fig. 23). In each group the fibres are packed together and

twisted into a coil as in an electrical flex. In transverse section each coil appears compressed into a triangular shape. The apex of the triangle points inwards towards the adjacent pair of flagellar fibrils (Fig. 23). The base of the triangle lies against the cell membrane. There are about 12 fibres in a coil at the anterior end of the tail; the number of fibres decreases gradually towards the tail tip (Fig. 23).

There is no trace of a second centriole or a derivative thereof anywhere in the sperm.

DISCUSSION

In Nucella the stages of spermatogenesis are similar to those in most animals. The spermatogonia divide mitotically to form primary spermatocytes. The latter contain the diploid number of chromosomes and 2 centrioles. The two meiotic divisions follow in quick succession to give spermatids which contain a haploid chromosome set and one centriole. It is evident from both light and electron microscope studies that the cells of each testis tubule are arranged in groups, and that all the cells within a group pass through spermatogenesis in phase with one another.

The mature sperm of Nucella resemble other molluscan sperm in that the centriole is buried in the head. Nucella, however, shows this situation in the extreme in that the centriole is located

immediately behind the acrosome and is separated from the acrosome by a double layer of nuclear envelope alone.

According to Gall (1961) there is only one centriole in the spermatid of Viviparus. It seems that the same situation exists in Nucella. In the spermatogonia 2 centrioles are clearly visible, and in some which are presumably about to divide 2 pairs of centrioles have been seen. We suggest therefore that there is no further centriole replication after that which precedes the first meiotic division. The centriole of the mature sperm must therefore be one of those which were present in the primary spermatocyte.

The head of the mature sperm of Nucella consists of a tube about 40μ long which contains a portion of the flagellar shaft. The walls of this tube consist of compacted nucleoprotein surrounded by nuclear envelope. The condensation of the nuclear material is essentially similar to that described in some insects (Gibbons and Bradfield, 1957; Yasuzumi and Ishida, 1957; Gall and Bjork, 1958) and in other molluscs (Grassé et al. 1956; Yasuzumi and Tanaka, 1958; Kaye, 1958). Gall and Bjork (1958) suggest that the lamellae of late spermatid nuclei are formed by lateral association of the fine threads seen in early spermatid nuclei, and they suppose these threads to be essentially similar to the threads found in many other types of nuclei. They consider that the centriole may act as a centre

for the organisation of the nuclear material along the long axis of the sperm. The role of the centriole in organising the condensation of spermatid nuclear material in Mussella is unknown, though it undoubtedly plays an indirect role in this process in so far as the presence of a flagellar tube in the nucleus imposes certain restrictions upon the pattern of distribution of the nucleoprotein.

In the early spermatid the portion of the flagellar shaft which occupies the nuclear tube is straight, later it becomes coiled within the tube, and in the mature sperm it is once again straight. The nucleus itself, after its initial elongation, is straight. Later, when the nuclear material is in the loose lamellar form, the nucleus becomes twisted; yet in the mature sperm it is once again straight. The significance of these changes is not yet known.

In most sperm there is a change in the nature of the nuclear histones during maturation of the spermatid. Bloch and Hew (1960) have shown in Helix that the change is accompanied by a synthesis of

arginine-rich histone. The exact stage at which this change takes place is not yet known but in Mucella it seems likely that it occurs during or after the initial elongation of the spermatid nucleus, since only the elongated spermatid nuclei contain arginine-rich histones. We suggest that the change in histones coincides with the formation of the nuclear lamellae.

The fine structure and development of the acrosome in Mucella sperm is comparable with that of Aphasia (Kaye, 1962). In both types the acrosome consists of 2 cones, but the structure of each cone in Mucella would seem to be more complex than in its counterpart in Aphasia. In Mucella the acrosome has 2 sets of microtubules associated with it; one within the acrosomecone, and the other on the outside of the cone. A similar situation has been described by Tandler and Moriber (1966) in Gerris sperm. In these the acrosome is filled with a system of longitudinally arranged tubules each about 190 Å in diameter. As the acrosome elongates a membrane, the sleeve membrane develops in the cytoplasm between the acrosomal and cell membranes. In the space between the sleeve membrane and the acrosome a second system of tubules, each about 200 Å in diameter, appears. These tubules usually lie parallel to the long axis of the acrosome but may be wound around the acrosome in a loose coil. The sleeve membrane extends back to the distal end of the mitochondrion, whereas the microtubules end level with the centriole at the base of the

nucleus. Tandler and Moriber (1966) suggest that the tubules in the acrosome are responsible for its rigidity. This seems reasonable in view of the fact that in Carria the acrosome constitutes about half the total length of the sperm. They also suggest that the microtubules which surround the acrosome, the head, and the base of the tail may act as a coupling device which keeps these parts of the cell in strict alignment.

In Mucalla banks of microtubules lie outside what we have called the "ragged membrane". The latter probably corresponds to the sleeve membrane in Carria, although its orientation with respect to the microtubules is different in Mucalla. We think that, as in Carria, the tubules within the acrosome cone of Mucalla impart rigidity to the cone, whereas those outside the cone assist in this function and serve as a coupling device between acrosome and nucleus. There are however some other features of the microtubules which we wish to stress. First, in the late spermatid, where the nuclear material is in the lamellar form and the nucleus itself is twisted, there are no microtubules. Secondly, in the mature sperm the microtubules extend backwards over three quarters of the length of the head. Thirdly, the microtubules do not surround the nucleus but are arranged in one or two banks. On account of these observations we suggest that the microtubules play an active part in the elongation and straightening of the sperm head. In this connexion it is worth

noting that in Carria (Tandler and Moriber, 1966), Helix (Grassé et al., 1956), and Bucalla there are microtubules associated with acrosome, mid-piece, and head respectively. Each of these structures in the respective species undergoes immense elongation during the maturation of the sperm.

SUMMARY

The testis of Mucalla consists of numerous tubules, all directed inwards and joining to form a common testicular duct. In a single tubule the spermatogonia lie round the periphery. Mature sperm line the bore of the tubule. Cells in the same stage of spermatogenesis are grouped together and all members of a group pass through spermatogenesis in phase.

Sections of testis stained with fast green and stained after treatment with Van Slyke reagent indicate a change from lysine-rich to arginine-rich histone in the maturing spermatid.

Sperm of Mucalla are motile throughout their length. The sperm are thread-like and about 80μ long. The head is Feulgen-positive and about 40μ long. The mid-piece lies behind the head and is about 8μ long. The flagellum runs from the front end of the head to the tip of the tail; in the head it is completely surrounded by the nucleus.

The spermatogonia contain two centrioles situated near the nucleus and a conspicuous Golgi complex. These are synaptinemial complexes in spermatocyte nuclei in the synapsis stage. In the early spermatid the centriole pushes a tube through the nucleus. This tube is lined by nuclear membrane and is occupied by the anterior portion

of the flagellar shaft. The nucleus elongates and the nucleoprotein condenses into strands arranged helically along the long axis of the nucleus. These strands fuse to form lamellae. The lamellae disappear in the mature sperm. Mitochondria aggregate at the base of the early spermatid nucleus and form a loose spiral around the flagellar shaft. The outer mitochondrial membranes fuse. The mid-piece of the mature sperm consists of a large tubular mitochondrion enclosing a portion of the flagellar shaft. At the early spermatid stage a pro-acrosome granule is formed from a large Golgi complex. From this the acrosome develops. The acrosome consists of a cone and an acrosome granule. There are two sets of microtubules associated with the acrosome, one lying within the cone, the other outside the cone and separated from it by a "ragged membrane". The microtubules of the outer set extend backwards along the head for 2/3 of its length. The centriole which gives rise to the flagellar shaft lies at the anterior end of the head and is separated from the acrosome by a thin layer of nucleoprotein and a double layer of nuclear envelope. There is no second centriole or derivative thereof in the mature sperm. In the tail there are groups of coiled fibers associated with each pair of the peripheral flagellar fibrils.

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- A P P E N D I X II -

COILING OF THE FLAGELLAR SHAFT IN THE HEAD REGION OF THE MATURE SPERM OF *Nucella (Purpura) lapillus* (L.)

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INTRODUCTION

The sperm of Nucella lapillus are long and threadlike with a pointed acrosome and tapering tail. They are about 80μ in length. Retzius (1912) described in the sperm of Nucella a central fiber ("Zentralfaden") running from the acrosome to the tip of the tail. In the head region he noted that the central fiber was ensheathed by the sperm nucleus. When sperm were macerated in water the nuclear material swelled and the central fiber became twisted into a coil. Retzius saw these same features in sperm from Littorina and Buccinum.

Unpublished observations on the behaviour of the mature sperm of Nucella were made several years ago by Callan (1952). He observed living sperm and their movement as seen in phase contrast. He also observed changes in the sperm when they were immersed in different media. He remarked upon the fact that in x 2 concentrated sea water the heads of the living sperm show a tendency to become attached to the surface of the slide. When this happens the nucleoprotein of the sperm head disperses and the portion of the flagellar shaft enclosed within the head (the head shaft) springs back to form a coil.

From the observations of Retzius and of Callan it is evident that there are two unusual features about Nucella sperm. First, part of the flagellar shaft is enclosed in the head, and the sperm move throughout their length. Secondly, the head shaft coils up if the

nuclear material is dispersed. Several questions arise from this latter phenomenon. For example: does the coiling reflect some peculiar feature of the flagellar shaft in the head? What keeps the head straight in the living sperm? Why does the tail not coil?

The spermatogenesis and the structure of mature sperm in the testis of Hesalla lamillua have been described previously (Walker and Macgregor, 1968). The object of this present investigation was to examine more closely the phenomenon of head coiling by light and electron microscopy.

MATERIALS AND METHODS

All whelks used in this study were collected from the Kinkell Rocks region of St. Andrews Bay. Mature sperm were extracted from the testicular duct.

Artificial sea water was used throughout made up according to Barnes (1954). It was found that in x 1 concentrated artificial sea water the sperm showed signs of osmotic stress and tended to bend in the middle and stop moving. In x 1.5 concentrated artificial sea water the sperm behaved normally. x 1.5 concentrated artificial sea water was therefore used throughout as a substitute for normal sea water and will be henceforth be referred to as normal sea water and x 3 concentrated artificial sea water was considered to be equivalent to x 2 normal sea water.

Several factors were used to induce coiling of the flagellar shaft in the head region.

Callan's procedure of placing sperm in x 2 concentrated sea water was repeated. Sperm were also placed in normal sea water and a drop of the suspension placed on a slide under a coverslip. The preparation was then observed as it dried out. A consequence of drying out was a rise in the concentration of the sea water at the edge of the preparation. Sperm from the testicular duct were placed in 0.01% (w/v) solution of the detergent sodium lauryl sulphate (SLS) in normal sea water. A drop of this suspension was then placed on a slide under a coverslip and examined. The effect of varying the pH of sea water was examined. Sperm were placed in normal concentration sea water buffered with veronal : HCl (Michaelis) over a pH range of 2.6 to 9.16. The pH of the sea water was increased from 9.16 to 12.0 by the addition of NaOH. The effect of the enzymes trypsin and pronase were examined. The enzymes were made up at a concentration of 50 µg/ml in normal sea water, at pH 8.0. Sperm from the testicular duct were placed in the enzyme solutions and observed for periods of up to three hours.

Preparations of sperm treated in all the ways described above were observed with a Zeiss Photomicroscope fitted with Neofluar objectives. All light micrographs were made on Ilford Pan F film.

For examination with an electron microscope, sperm from the testicular duct were spread on a Langmuir trough containing normal sea water. Sperm spread on the surface were picked up on Athene 483 grids with formvar-carbon supporting films. The excess sea water was drained off and the grids were rinsed with distilled water prior to negative staining with 1% phosphotungstic acid (PTA) adjusted to pH 6.4 with NaOH. A drop of PTA was placed on each grid. After about 10 seconds the PTA was drained off the grid with a filter paper. The same process was repeated using x 2 concentrated sea water in the Langmuir trough. Sperm treated with enzymes were also examined with the electron microscope. A drop of the treated sperm suspension was placed on a grid and the excess fluid drained off. The preparation was then washed and negatively stained as described above. The grids were examined with a Siemens Elmiskop 1 at 80 Kv.

All previous observations on sectioned sperm from Nucella were made on sperm from the testis, whereas all the present observations were made on sperm from the testicular duct. Sperm from both these locations look the same in phase contrast. I thought it necessary, however, to see if they differed in ultrastructure. Small pieces of testicular duct and testis from the same whelk were fixed in 10% glutaraldehyde in phosphate buffer at pH 7.38 for 10 minutes, rinsed in buffer, post fixed in osmium (Palade, 1952), dehydrated through an acetone series and embedded in Vestopal W. Transverse sections of

sperm heads were cut and mounted on Athene 483 grids without supporting films, and stained with uranyl acetate and lead citrate (Reynolds, 1963) prior to examination with the electron microscope.

OBSERVATIONS

Light microscope observations

Living Mucalla sperm all look alike. They are about 86 ± 2μ in length and are of even diameter apart from a pointed acrosome and a tapering tail (Fig. 1). The sperm are motile throughout their length. The undulations of the head have a lower amplitude than those of the tail. The living and freely swimming sperm appear with uniform contrast along their length. As they die the tails show a slight increase in diameter with a corresponding decrease in contrast.

If a fresh preparation in normal sea water is observed for about ten minutes, many of the sperm tend to become attached to the slide or coverslip. This attachment usually starts at the acrosome and the tip of the tail, while the main body of the sperm continues to wriggle. Gradually the attachment spreads along the whole length of the sperm. As soon as the tail sticks to the slide its tip begins to fray into its individual fibrils (Fig. 2). At the same time the head increases in diameter and the head shaft within it is thrown into a loose coil (Fig. 3). The mid-piece remains unaffected throughout. At the edges of the preparation where drying out occurs and the

salt concentration is increasing, the coiling effect is faster and more extreme. The heads swell more quickly and as the nuclear material disperses the head shaft springs into a tight coil (Figs. 4 & 5). In the x 2 concentrated sea water the majority of the heads form tight coils almost instantaneously. In the presence of sodium lauryl sulphate the head material disperses and tight coiling occurs at once.

Alteration of the pH is not an effective means of inducing coiling although variation in pH does affect the sperm. At pH 2.6 all sperm are non-motile, the nuclear material disperses and the head shafts coil. As the pH is increased the proportion of coiled sperm decreases and at pH 5.3 the majority of the sperm are motile. Over the pH range 5.5 to 8.5 the sperm behave normally. At pH 9.0 motility is gradually lost but only a few loose coils are formed. At pH 10.25 some sperm are still motile while others show untidy coiling. At pH 11.9 all the heads appear swollen and empty with the head shaft forming a zig-zag running through the centre of the head. Above this pH the sperm are completely destroyed.

Treatment of sperm with 50 µg/ml trypsin and 50 µg/ml pronase at pH 8.0 have similar results. The head material is dispersed and the head shaft is thrown into a loose, untidy coil. In some cases the flagellar shaft breaks.

The tight coiling phenomenon is consistent in that the coil is always in the same direction, clockwise from the anterior end, or right handed. The number of turns of the coil varies between 5 and 7. Not all of the head shaft coils. The posterior ¼ remains uncoiled. The tight coil is only formed when the nuclear material is completely dispersed.

From the light microscope it is evident that there are three conditions of coiling. First, complete and rapid coiling of the head shaft will occur when the nuclear material is stripped off as, for example, with SLS. Secondly, concentrated sea water in conjunction with the sperm coming into contact with the slide or coverslip may cause either an instantaneous or slower coiling. The slower coiling may take several minutes to occur. The sperm become attached to the slide and the head swells gradually, the head shaft being thrown into a loose coil within it. This is followed by the head shaft springing rapidly into a tight coil as the head disperses. Thirdly, with enzymes a loose coil is formed and the head material dispersed, either partially or completely.

Electron microscope observations

Sperm spread on normal sea water on a Langmuir trough and negatively stained with PTA have swollen heads and frayed tails. The cell and nuclear membranes are dispersed. The nuclear material

appears more or less homogeneous (Fig. 6). The acrosome may remain attached at the anterior end. The head shaft is thrown into a gentle spiral. The fibers of the head shaft are twisted like the wires of an electrical flex (Fig. 6). The mid-piece remains intact. The helical arrangement of the mitochondria is shown by the negative staining (Fig. 7). The tail frays, starting at the posterior end. In a few sperm immediately posterior to the mid-piece the flagellar fibers sometimes appear twisted as they are in the head. In the majority of sperm the tail fibrils are untwisted.

The nuclear material of sperm spread on a Langmuir trough containing x 2 concentrated sea water is completely dispersed. The acrosome usually remains attached to the anterior end of the sperm (Fig. 9). Most of the head shafts are tightly coiled. I consider it significant that in tightly coiled regions the head shafts are not conspicuously twisted; whereas in uncoiled regions the head shafts show a regular twist with a pitch of $2.5 \pm 0.6\mu$. (Cf. Fig. 8 & 9). The mid-piece remains intact and acts as a tie around the "waist" of the sperm. The tail frays completely. At the tip of the tail the flagellar fibers fray into their component sub-units (Fig. 10).

In sperm treated with trypsin or pronase the nuclear material may be broken down to a meshwork of branching fibers of varying widths and the head shaft is thrown into a loose coil (Fig. 11). The fibers of the head shaft appear irregularly twisted and in some

places are broken or damaged. In other instances the nuclear material disaggregates into broad strips of material which run lengthwise with respect to the sperm axis. (Fig. 12).

Transverse sections of sperm in the testis show banks of microtubules associated with a ragged membrane in the cytoplasm between the sperm nucleus and the cell membrane (Fig. 13). No such microtubules or ragged membrane are present in sperm from the testicular duct (Fig. 14). Transverse sections of the acrosome in testis sperm show a complex sheath of microtubules surrounding the acrosome cone; but these microtubules are absent from the acrosome of sperm from the testicular duct. Apart from these features sperm from testis and testicular duct are identical.

The state of the head shaft in the straight mature sperm was examined by cutting serial longitudinal sections of sperm from the testicular duct. These sections showed that the fibers of the head shaft are not twisted but run straight through the head (Fig. 15).

DISCUSSION

The flagellar shaft of Nucella sperm may, for the present discussion, be considered to consist of two portions; the head shaft which coils, and the tail shaft which does not. I consider that the potential for coiling of the head shaft is due to a condition which is imposed upon this part of the shaft at some stage in spermiogenesis.

The facts which have emerged from this study which, in my opinion, are relevant to the coiling problem are as follows. Disruption of the cell and nuclear membranes without dispersal of the DNA is sufficient to allow twisting of the head shaft and also to start a gentle coil. Tight coiling can only be obtained if all the nucleoprotein is removed. The coil is limited to the front 3/4 of the head, the shaft in the rear portion of the head remains uncoiled although it is twisted. When sperm are treated with detergent or concentrated sea water the tail frays whereas the head shaft twists and coils. In the early spermatid nucleus, which is about 3μ in length, the head shaft is thrown into a coil within the nucleus with the centriole situated at its anterior end, whereas in the mature sperm, which is about 40μ in length, the head shaft is straight (Walker and Macgregor, 1968). Mature sperm in the testis have one or two banks of microtubules associated with a "ragged membrane" running down one or both sides of the nucleus. These microtubules extend over the anterior 3/4 of the head, and they are continuous with the microtubules which surround the acrosome (Walker and Macgregor, 1968). There are no such microtubules in mature sperm from the testicular duct.

On the basis of these observations I propose the following scheme to account for coiling of the head shaft. In the very early spermatid where the centriole and flagellar shaft have just penetrated the nucleus all the fibers of the flagellar shaft run straight. The

length of the flagellar shaft within the nucleus increases without a corresponding increase in nuclear length, and so the head shaft is forced into a coil. In forming this coil the flagellar fibrils become twisted and the twist remains imposed upon them. As the nuclear material condenses and the nucleus elongates to its final length of about 40μ the head shaft is untwisted and pulled out straight. This implies an active force which rotates one end of the flagellar shaft through about sixteen 360° turns. As the fibrils are straight in the mature sperm but are able to become twisted again when the nucleus is dispersed, they must be held under tension and torsion in the sperm head.

If one end of the head shaft is allowed to rotate with respect to the other end without shortening then the head shaft will return to equilibrium in the twisted condition by relief of torsion. This is seen when sperm are spread on a Langmuir trough containing normal sea water. If the sperm head is completely and rapidly stripped the head shaft returns to equilibrium in a tightly coiled condition by relief of tension.

Several questions are raised by these suggestions. For example, what is the significance of the presence of microtubules during spermiogenesis? How and at what stage in spermiogenesis does untwisting of the flagellar shaft occur?

The microtubules only appear during the final stages of nuclear condensation. In the late concentric lamellar stage spermatid the outer edge of the nucleus has a fuzzy appearance due probably to the formation of microtubules. The microtubules are present during the last stages of spermiogenesis when the nucleus nearly doubles its length. The microtubules not only run along the side of the nucleus in a bank, but also completely surround the acrosome, and may at this stage act as a coupling device holding the nucleus and acrosome in alignment. The function of the microtubules is probably to assist in the pulling out of the nucleus to its final length and therefore also to extend and straighten the coiled flagellar shaft. In the mature sperm the microtubules disappear. The occurrence of microtubules during spermiogenesis and subsequent absence in mature sperm has been described in the grasshopper Melanoplus differentialis differentialis (Thomas), (Kessel, 1967).

What then is responsible for maintaining the straight yet flexible form of the mature sperm head? Since the microtubules are absent they cannot be involved. I consider that it is the nucleus itself which is responsible for maintaining the head shaft in a straight condition, that it is unable to do this until condensation of the nucleoprotein is complete, and that the microtubules of the immature sperm probably have a spline-like function in stretching and holding the shape of the sperm head while the nucleoprotein "sets"

to a rigid state. In mature sperm the head shaft is "straight jacketed" in a close fitting and stiff nuclear tube.

How and at what stage in spermiogenesis untwisting of the head shaft occurs is uncertain. Elongation of the nucleus removes the coil from the head shaft but it is possible that the twist may be left still to be unwound by some further process. The coil runs clockwise from the anterior end and therefore one might expect the shaft to be twisted in an anti-clockwise direction. To attain the straight condition therefore one end of the shaft must be rotated with respect to the other end about 16 times in a clockwise direction from the anterior end. Subsequently, after unwinding is complete the straight shaft must be locked at both ends so that it can neither shorten nor rotate.

SUMMARY

The sperm of Mysella are long and threadlike. The flagellar shaft runs from the acrosome at the front of the head to the tip of the tail. Its anterior portion, the head shaft, is ensheathed by the nucleus. If a suspension of sperm in normal concentration sea water is observed under phase contrast, and allowed to dry out slowly, the nuclear material of sperm near the edge of the coverglass swells and the head shafts of these sperm are thrown into gentle spirals within the nuclei. In some sperm the nuclear material disperses completely, and the front 3/4 of the head shaft springs into a tight right handed coil of 5 to 7 turns. Instantaneous coiling of the head shaft may also be induced by treatment of the sperm with x 2 concentrated sea water or 0.01% w/v solution of sodium lauryl sulphate in sea water. The ensymes pronase and trypsin at a concentration of 50 µg/ml in sea water at pH 8.0 cause disperaion of the head nucleoprotein and subsequently the head shaft forms a loose coil. The appearance and activity of the sperm do not change perceptibly over a pH range of 5.5 to 8.5.

Sperm spread on a Langmuir trough containing normal concentration sea wated were negatively stained with phosphotungstic acid and examined with an electron microscope. After such treatment the nuclei are partially spread and the fibrils of the head shafts appear twisted as the wires of an electrical flex. The twisted

flagellar fibrils are seen more clearly in negatively stained head shafts of sperm whose nuclei have been completely dispersed by spreading on a Langmuir trough containing x 2 concentrated sea water. Negatively stained preparations of sperm treated with enzymes show sperm with twisted and coiled head shafts. The nucleoprotein of these sperm is disaggregated into strips or sheets, or may appear as a mass of branching fibers. The flagellar fibers of the enzyme-treated sperm are often bent or broken.

Longitudinal sections of mature sperm heads show that in the intact sperm the fibers of the head shaft are not twisted but run straight throughout the length of the head. Sections from the testis and testicular duct show that microtubules are present in sperm in the testis but absent from mature sperm in the testicular duct.

It is suggested that the head shaft, as a consequence of some event in spermiogenesis, has an inherent tendency to twist and coil but in the mature sperm it is "straight jacketed" by the sperm nucleus.

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